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Carbon based nanostructured bio-assemblies for bioelectrochemical applications

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Abstract

Carbon nanomaterial gained high interest for bioelectrochemical applications like biosensors and biofuel cells related to excellent conductivities, well established (bio) functionalization methods, and high surface area to immobilize a high number of biomolecules preserving accessibility for its substrate (analyte or fuel). Beside the huge varieties of different nanosized carbon structures, the principal materials used in this field are carbon nanotubes and graphene. However, within these carbon allotropes, there are tremendous differences in physical and chemical properties related to different production methods which affects processabilities and therefore, the appropriateness for an envisioned application. Here, the historical evolution of these carbon nanomaterials is summarized with focus on the different properties of carbon nanotubes and graphene materials. For bioelectrical applications, some of these carbon materials are more beneficial than others in terms of immobilization and wiring of bioreceptor units and biocatalysts. The chemical composition and specific domains of the biomolecules can establish efficient interactions with the carbon materials and can be improved with targeted functionalization strategies. The aim of this review is to provide a pedagogic summary of the different properties of carbon nanotubes and graphene materials from a chemical point of view citing most relevant reviews and discussing original strategies to form operational bioassemblies for analysis and biological energy conversion.

Keywords: Carbon nanotubes, Graphene, Biosensors, Biofuel cells, Functionalization, Immobilization of biomolecules, Enzyme wiring.

Statements and Declarations

The author declares no financial interests that are directly or indirectly related to the work submitted for publication. There are no competing interests to declare.

1. Introduction

Nanostructured carbon materials are known since many decades, but the scientific community did not address much attention until the fully characterization of the molecular C_{60} soccer ball by Kroto et al. in 1985 [1]. Few years later in 1991, Sumio Ijima reported the formation of “Helical microtubules of graphitic carbon” using arc-discharge [2]. Even when it is not the first description of multiwalled carbon nanotubes (MWCNTs) [3-4], it became the standard reference within the topic of carbon nanotube research. In fact, the simultaneous development of different synthesis methods in 1993 reported by Ijima[5] and Bethune[6] to produce single-walled carbon nanotubes (SWCNTs) conducted to an avalanche of research efforts focusing this material. SWCNTs have exceptional electronic and mechanic properties. Such seamlessly rolled-up graphene sheets provide semiconductor or metallic conductivity depending on the roll-up vector which determines the diameter and position of the hexagons in the tubes [7]. The induced bending of sp^2 carbon around the tube axis provoke further a real one-dimensional conductivity at room temperature. These firstly observed phenomena fascinated the scientific community and SWCNTs were rapidly envisioned for high sophisticated electronic application [8]. SWCNTs further provide extremely high tensile strengths and young’s moduli [9-10] and were even considered for utopic applications like cables for space elevators [11]. MWCNTs are to some extend less performing in terms of (metallic) conductivity[12] or tensile strengths but became the privileged material since the outer wall contain in general oxygenated defect functions which improve processability to form homogeneous conductive composites. [13]. These exceptional properties of carbon nanotubes (CNTs) and applications are reviewed in many articles [14-19]. One issue in carbon nanotube research was that single-walled, double walled, thin multiwalled and thick MWCNTs provide different properties when synthesized by arc discharge, laser ablation, or chemical vapour deposition (CVD) under different conditions (e.g. catalyst, temperature, carbon source) which made the comparison of the various studies difficult [20-21]. A great step forward was the development of processes for CNT mass-production and commercialization with reasonable costs ^[22] which enabled the availability of identic CNT samples for the scientific community.

The interest in carbon nanotube research clearly decreased with the first isolation and characterization of monolayer graphene by A. Geim and K; Novoselov in 2004 [23]. Similar to CNTs, graphene and related 2D carbon materials have been studied since several decades. H. P. Boehm firstly described the synthesis of monolayer flakes out of reduced graphite oxide in 1962 [24] and used the term “graphene”. The process for graphene oxide synthesis was developed by Hummers *et al.* [25] few years before and remained little noticed until the work of Geim and Novoselov. As for CNTs, there are also different types of graphene like monolayer graphene, bulk (few layer) graphene, graphene oxide, or reduced graphene oxide which are obtained by various methods [26]. Monolayer graphene can be produced by the scotch tape technique exfoliating one graphene layer from highly oriented pyrolytic graphite (HOPG) which Geim and Novoselov used for their first studies. The disadvantage is that only few graphene layers can be isolated and most of the deposited material are debris from HOPG. Almost defect free monolayer graphene can be obtained via CVD synthesis on copper foils [27]. The disadvantage here is that this graphene has to be transferred to other substrates before its use. In most cases, bulk graphene or related materials are obtained via top-down production methods from graphite via mechanical [28] or chemical exfoliation [29]. Bulk graphene materials are quite easily to prepare with standard laboratory equipment out of graphite. The issue is, as is was for the beginning of carbon nanotube research, that research teams produces its own graphene, each with their own protocol, forming graphene samples with different properties which impede comparison of the results within the different topics [30-33].

Beside CNTs and graphene materials, there exist further nanosized carbon allotropes such as carbon nanohorns [34], carbon quantum dot [35], helical or coiled CNTs [36], fishbone-type carbon nanofibers [37], nanodiamonds [38], or carbon (graphene) nanoribbons [39] (Fig. 1). All these materials are extensively studied for various applications like reinforced conductive composites,

energy storage and harvesting, biomedical applications, or (bio)analysis [40-42] but are little represented in the field of bioelectrochemistry.

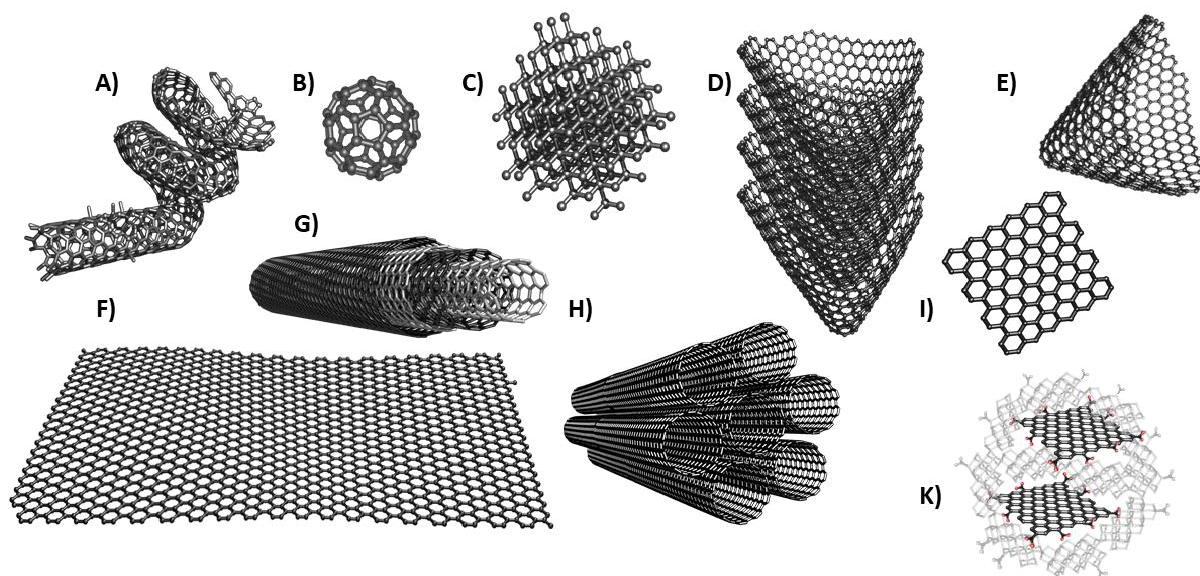


Fig. 1: Most studied carbon allotropes carbon allotropes. A) helical CNTs, B) fullerenes, C) diamond, D) fishbone-type carbon nanofibers, E) nanohorns, F) graphene, G) MWCNTs, H) SWCNTs (in bundle), I) graphene quantum dots, and K) carbon quantum dots.

For bioelectrochemical applications, CNT and bulk graphene materials are mostly used since simple deposits form highly porous extensions of a working electrode enabling improved interaction and immobilization of biomolecules. Further advantages of graphene materials and CNTs is that both can be shaped into free standing macroscopic electrodes while keeping enhanced porosities and conductivity [43-44]. The technological progresses of these carbon nanomaterials for (bio)electrochemical applications are nicely reviewed by Maduraiveeran *et al.* [45] and Kour *et al.* [46]. Bioelectrochemical applications principally concern the fields of biosensors and biofuel cells. Biosensors are defined by its receptor unit which is an entity of biological origin like enzymes, DNA strands, antibodies, antigens, or related proteins. Biomimetic models like aptamers, molecular imprinted polymers, or synthetic peptides are nowadays well accepted as sensing elements in the biosensor field [47]. Biofuel cells operate as classic fuel cells where the catalyst is an enzyme. For this technology, the challenge is not only to immobilize the biomolecule but also to establish electron transfers from and to the electrode material which consists nowadays of carbon nanomaterials. There are many review articles about carbon nanomaterials for biomedical applications [145-152] where many different techniques and for different analytes are discussed.

In this review, more the fundamental aspects of the beneficial properties of carbon nanomaterials in bioelectrochemistry are focused. Specific interaction of biomolecules with such nanostructures and efficient immobilization techniques are discussed with some original examples for promising biosensor and biofuel cell concepts.

2. Appropriateness of carbon nanomaterials in bioelectrochemistry

Conductive carbon is intensively used in electrochemical applications in form of glassy carbon, pencil graphite, HOPG. Carbon is electrochemically silent in a wide potential range which means that any electrochemical redox processes can be recorded without interferences of the electrode material. This potential range covers further all bioelectrochemical processes and is therefore a standard material in electrochemical biosensors and biofuel cells [48]. The nanosized sp^2 allotropes of carbon behave

electrochemically almost the same as macroscopic carbon electrodes [49] but the formation of porous structures provokes an extension of the electrode with drastically enhanced surface area. Interestingly, there are little quantitative studies about the effective enhancement of the electroactive surface using nanocarbon materials [50]. One reason might be the innumerable amount of different nanocarbons coupled with many different preparation methods such as drop casting, spin coating, screen printing, use of binders, or formation of composites. Graphenes and CNTs can also be shaped into free standing electrodes. The most famous examples are buckypapers which are generally formed by filtration of CNT or graphene dispersions [51]. Other macroscopic shapes like fibres or pellets can also be formed with these carbon materials [52-53]. Independent on processing or final shape of nanocarbon electrodes, as long as the nanosized structure is available for biomolecules, privileged interaction can be established related to their similar nanosized dimensions [54-56].

3. Biofunctionalization of carbon nanostructures

One great challenge in bioelectrochemistry is the immobilization of biological entities on electrode surfaces without altering their biological activity. Related to vast diversity of biomolecules, targeted immobilization methods, tailored for each type of compound, categorized in proteins (enzymes, immune systems) and nucleotides (DNA, RNA, aptamers), were developed [57]. It is not only a question of the accessibility of the active site to the analyte but also possible unfolding phenomena when attached to a solid surface. For instance, Hampitak et al, studied the interaction and conformation of bovine serum albumin (BSA) adsorbed on graphene oxide and reduced graphene oxide. He observed denaturation on hydrophobic reduced graphene oxide by flattening the globular protein during the adsorption process while BSA remained intact when adsorbed on graphene oxide [58]. Changes of these behaviours can certainly be expected when using other proteins or differently prepared graphene oxides. Besides the different proposed optimized Hummers methods [25], the origin of the graphitic carbon plays a crucial role for the properties of the obtained graphene oxide [59]. The same risks of reproducibility issues are present for graphene preparation independent on a top-down (exfoliation of graphite) or bottom-up (CVD synthesis) approach. There are several synthesis methods which all produces different kinds of graphenes in terms of layer amount and sizes, and quantity or nature of eventually introduced defects which needs adjustments of processing for each graphene source [60]. Carbon nanotube synthesis became industrial processes where homogeneous materials with similar properties are available even from different producers. Single walled, thin multi walled, thick multiwalled, or double/triple walled CNTs and the synthesis process, however, should still be taken into account [61]. Therefore, for each bioentity, an adjusted protocol has to be established for every carbon nanomaterial.

Nonetheless, carbon (nano)materials appear to be very suitable for the immobilization of bioentities. On the one hand, their hydrophobic character allows stable interaction with hydrophobic amino acid residues or nucleotides in aqueous media. On the other hand, there are many efficient functionalization methods available to graft any desired chemical functions on the carbon surface to establish an optimal anchoring of biomolecules. The different immobilization techniques for biomolecules on carbon electrodes can be divided into covalent, non-covalent, or via supramolecular interactions including coordination on metal organic compounds as summarized in Figure 2.

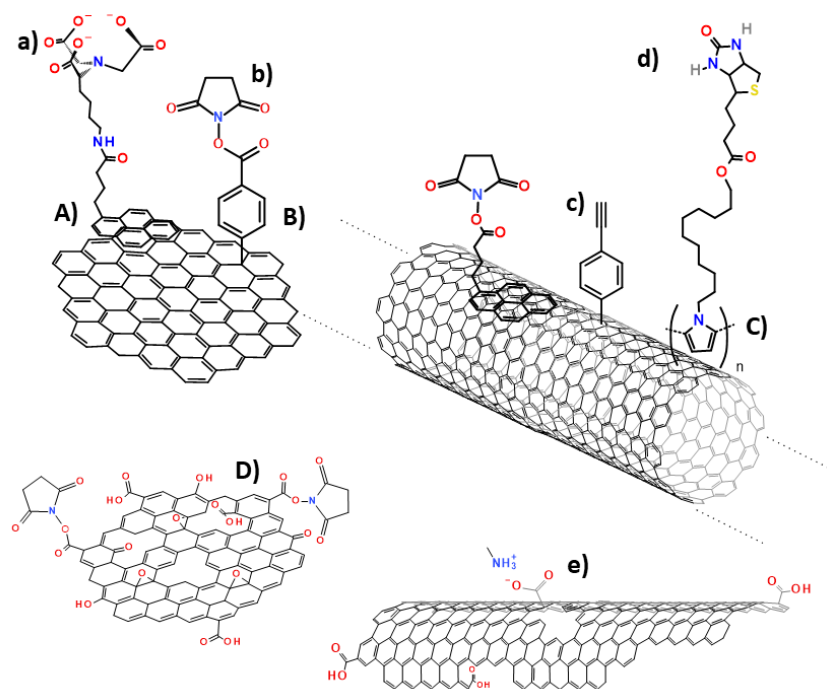


Figure 2 : Currently used functionalization methods for biomolecule immobilization. A) non-covalent functionalization with pyrenes, B) covalent functionalization via diazonium coupling, C) coating with polymers (here, electrogenerated poly pyrrolefilms), and D) oxidation of the carbon materials with subsequent activation of carboxylic acid defects via activated esters. These methods allow the attachment of various biomolecule linker functions like a) NTA, b) activated succinimidyl ester, c) alkynes for “click” coupling, d) biotin for the cross linking with avidin bridges, e) ionic interaction with defectcarboxylates

3.1. Hydrophobic interactions

Within the non-covalent functionalization methods, the hydrophobicity of carbon is a clear advantage for targeted interaction with biomolecules. An accumulation of hydrophobic amino acid sidechains in proteins can form domains which adsorb easily on carbon surfaces in aqueous media without the necessity of pre-treatments [62]. A very nice example is Laccase from *Trametes versicolor* which is intensively used for enzymatic degradation of phenolic lignin and more recently, as cathodic biocatalyst in enzymatic fuel cell applications [63]. This Laccase reduces oxygen to water at a trinuclear T2/T3 copper centre while oxidizing phenolic compounds at the separate mononuclear Type 1 copper centre. To capture these phenolic compounds, this T1 centre is close to a hydrophobic domain with an amino acid sequence containing aromatic and hydrophobic residues like phenylalanine, histidine, or leucine [64].(Figure 3) This domain therefore provokes oriented immobilization of this enzyme on hydrophobic surfaces that allows close contact of the T1 centre to the carbon electrode enabling direct electron transfers. This phenomenon could even be improved by attaching small hydrophobic or aromatic molecules onto the carbon surfaces [65-66].

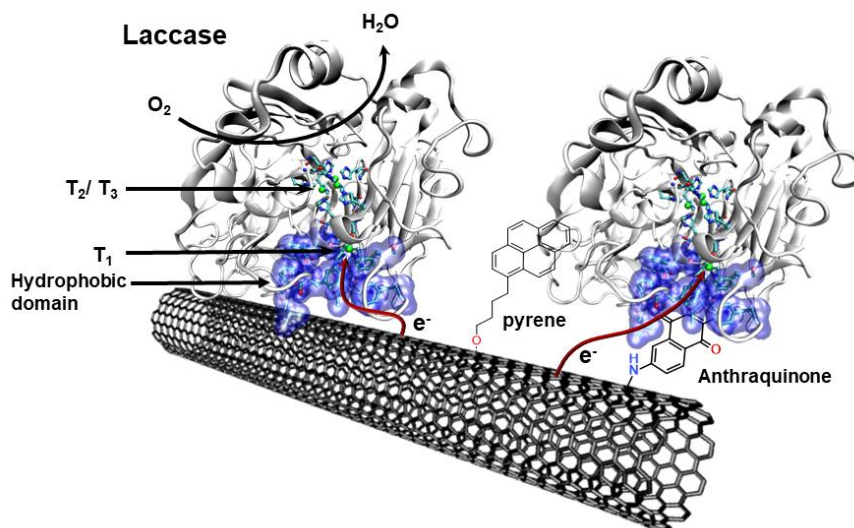


Figure 3: Wiring of laccase via hydrophobic interactions. Direct electron transfers can already be achieved by simple adsorption of laccase on the hydrophobic surface of nanocarbons. This effect can be improved with small molecules simulating phenols, the natural substrate of laccase.

The hydrophobic character of carbon nanomaterials represents also a challenge for the formation of bio-assemblies which needs to be done in aqueous media. This could firstly be demonstrated while using “high quality” SWCNTs with little surface defects [67]. A mixture of a hydrogenase and methylviologen pyrrole monomer was drop casted on a SWCNT modified electrode. The aim was to entrap the hydrogenase inside the electrogenerated polypyrrole matrix to obtain mediated electron transfer during bioelectrocatalytic H_2 oxidation. Under the same conditions, “low quality” MWCNTs, with many oxygenated surface defects showed clearly enhanced electrocatalytic currents ($300\mu A\text{ cm}^{-2}$) than the SWCNT bioelectrodes ($200\mu A\text{ cm}^{-2}$). Considering the enhanced conductivity of SWCNTs compared to MWCNTs, this result might appear surprising. The reason for improved electrocatalysis for “cheap” MWCNT bioelectrodes could be revealed with scanning electron microscopy (Figure 4). With the SWCNT deposits, the aqueous monomer-enzyme solution remained at the surfaces and formed a thick polymer layer after electropolymerization. The defective surface of MWCNTs allowed a better penetration of the aqueous solution in the deposits and a homogeneous polymer cover was formed around the tubes thus reducing the distances for electron transfers, leading to improved electrocatalysis.

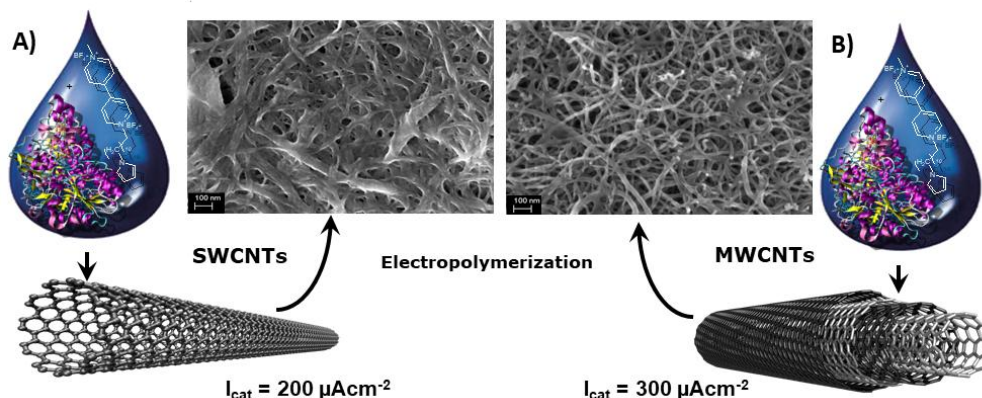


Figure 4: Electrochemical coating with viologen polypyrrole in presence of a hydrogenase on A) SWCNTs and B) MWCNTs.

More recently, the challenge to use superhydrophobic carbon nanotube forests for the formation of bioassemblies was faced by Singh et al. [68]. The forests were initially modified with the amphiphile pyrene-bis-anthraquinone in an organic solvent to improve the wettability. Pyrene-bis-anthraquinone has shown to be an excellent hydrophobic anchor for oriented immobilization of laccase [69]. Here, these molecules further allowed the enzyme coating of the forests in depth to form an efficient biocathode.

3.2. Pi-stacking

A further advantage of sp^2 nanocarbon materials is the possibility to employ π -stacking interactions with biological compounds. In this context, a multi-hemic nitrite reductase adsorbed spontaneously on carbon nanotube modified electrodes via its heme units which are iron-containing porphyrins. Such oriented immobilization enabled direct electron transfers during catalytic nitrite reduction [70]. This easily formed and performing biosensors lacked long-term stability which could be improved after electrogeneration of a protective cover using an amphiphilic pyrrole derivative mixed with clay. Similar approaches to modify CNT or graphene electrodes with heme-containing enzymes like horseradish peroxidase (HRP) or cytochrome c were reported for the fabrication of electrochemical H_2O_2 or nitrite biosensors [71-72]. HRP became even of interest as cathodic biofuel cell catalyst in combination with H_2O_2 producing enzymes like glucose oxidase [73-74]. This bienzymatic setup is particularly interesting for *in vivo* applications since these enzymes are less affected by interferants or inhibitors present in blood or extracellular liquids compared to the currently used multi-copper enzymes.

The ability of porphyrins to π -stack on CNTs was firstly studied by Roquelet et al. who used a micelle-swelling technique to form assemblies between water-insoluble porphyrins and CNTs in aqueous media for light harvesting [75]. Porphyrin-functionalized CNTs can also act for oriented immobilization of the enzyme bilirubin oxidase (BOx). This enzyme is also a member of the multi-copper enzyme family reducing oxygen to water at the T2/T3 centre by oxidizing bilirubin at the T1 centre, its natural substrate. Bilirubin is a linear tetra-pyrrole with two carboxylic acid functions at the inner pyrroles and is the metabolic breakdown product of heme. The Atanassov team firstly reported the oriented immobilization of BOx via functionalization of CNTs with bilirubin and/or synthetic analogues leading to a drastic enhancement of electrocatalytic currents via direct electron transfers [76]. The cyclic form of bilirubin, protoporphyrin IX, the iron-free form of heme, showed improved π -stacking interactions related to the enhanced conjugated π system compared to its linear form. Even greater catalytic current densities could be obtained with this cyclic form of bilirubin [77] and heme. This study also revealed the importance of carboxylic acid functions on the porphyrins. By having a detailed view on the protein structure around the T1 centre of BOx, an accumulation of basic amino acids (mainly histidine and arginine) can be observed [78]. Lalaoui et al. investigated the possibility of oriented immobilization of BOx only in the presence of carboxylic acids [79]. CNTs were covered via diazonium grafting with carboxy naphthalene which led to similar electrocatalytic performances as with protoporphyrin IX showing that such porphyrins are not essential for oriented immobilization of BOx. Interestingly, in the same manner, CNTs were functionalized with amino naphthalene and a different redox system at lower potential was observed after immobilization of BOx. Considering a higher density of aspartic and glutamic acid residues close to the T2/T3 centre, it could be concluded that the enzyme was oriented with this catalytic centre to the CNT surface (Figure 5). Detailed studies about the electrocatalytic behaviour and electron transfer kinetics of these two catalytic sites were realized with site-specific modified laccases with one pyrene function either close to the T1 centre or close to the T2/T3 centre [80].

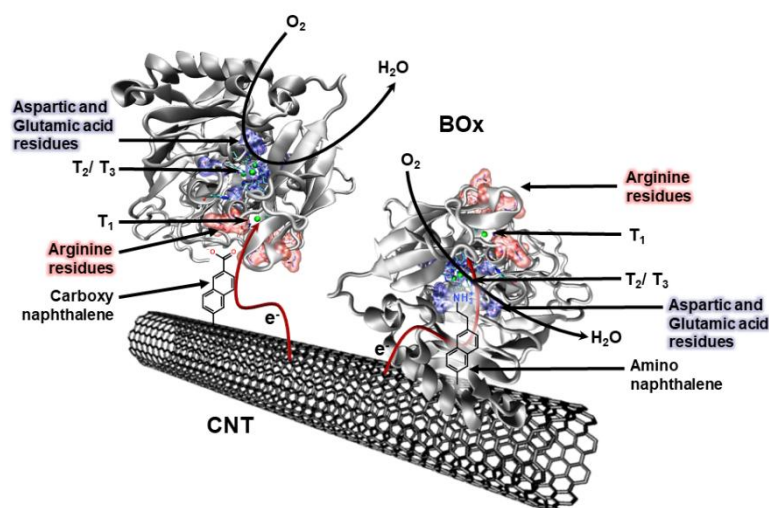


Figure 5: Controlled orientation of Box via targeted amine or carboxyl functionalization of CNTs wiring either the T1 centre or the T2/T3 centre.

3.3. Covalent binding of biomolecules

Pyrene derivatives became the entities of choice for non-covalent (bio) functionalization of carbon nanotubes and graphene via π -stacking interactions [81-83]. Chen et al. firstly grafted peptidic biomolecules onto SWCNTs using a standard amide coupling strategy with pyrene butyric acid N-hydroxy succinimidyl ester (pyrene-NHS) [84] modified CNTs. This covalent binding of biomolecules on non-covalently functionalized nanocarbon materials is easy to realise at room temperature via simple drop casting or incubations without the need of sophisticated synthesis equipment. This made this functionalization method the privileged strategy for the development of graphene or CNT based biosensors [85-86]. This strategy also commonly used with carboxylic acid functionalized carbon materials. Such acid function can be introduced by acidic oxidation of the carbon materials [87]. Another efficient method assuring the acid functionalization over the whole carbon nanomaterial surfaces is their attachment via diazonium coupling with 4-carboxyphenyl diazonium salts [88]. The common strategy is to activate these carboxylic acid functions with carbodiimides (dicyclohexyl carbodiimide, DCC, in organic solvents and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, EDC in aqueous media). In aqueous solutions, terminal amines of proteins or amine modified DCA strands can directly be attached to the carbon surface while when working in organic solvent, the NHS ester must be formed before transferring to the aqueous system. Another way for covalent grafting biomolecules is the use of the crosslinking capacity of glutar aldehyde (1,5-Pentanedial). Aldehyde can spontaneously form imines with the amines of enzymes and other proteins and link them to an amine modified surface [89]. Amine functions can efficiently be attached by coupling diamines with the carboxylic acid functions of oxidized carbon materials, by coating with aminated polymers, or by pyrene amines [90]. As a complementary covalent functionalization strategy to these common methods is the 1,3-Dipolar cycloaddition of azomethine ylides which allows a controlled grafting of multiple function for biomolecule immobilization and/or activation [91-92].

3.4. Non-covalent binding of biomolecules (Host-guest interactions, coordination)

The immobilization of the bioreceptor unit by covalent grafting, however, is little controlled and can affect its biological activity and thus lowering the performances. In this context, targeted labelling of bioentities with anchor functions for immobilization or transduction is a powerful tool for improved biosensor performances. The most famous example is the avidin-biotin strategy which has some notable advantages compared to NHS coupling. The extremely specific and high-affinity interactions between four biotins and the glycoprotein avidin (association constant $K_a=10^{15} \text{ L mol}^{-1}$) [93] allows to

strong associations similar to covalent bonds. The principle is based on the modification of both, the transducer surface and the bioreceptor with biotin where each component can be modified, characterized, and stored until use under the respective optimal conditions. Related to this, many different bioreceptors modified with a biotin function are now commercially available. The biosensor can then be constructed via formation of an avidin layer on the transducer followed by the immobilization of the receptor unit by simple incubation in the respective solutions. The intermittent avidin layer further serve for passivation of the surface preventing non-specific binding of interfering biomolecules. This passivation is quite beneficial for immune or DNA sensors. For electrochemical enzyme biosensing, this intermittent protein layer has more a detrimental effect since the enzymatic transformed redox probes has hindered diffusion to the electrode surface.

An alternative to this is to immobilize biotinylated bioreceptors on β -cyclodextrin[94] or nitrilotriacetic acid (NTA)[95] modified surfaces (Figure 6). NTA-copper or nickel complexes are generally used to immobilize histidine tagged molecules and found firstly applications for the purification of biomolecules. The chelating effect of NTA forms strong Ni(II) or Cu(II) complexes which provide two available coordination sites which are occupied by water molecules [96]. The imidazole function of histidine coordinates via its sp^2 nitrogen where this binding is strong enough for the immobilization of biological macromolecules but in presence of free imidazole, the competing interactions release the biomolecules and thus regenerate the surface. Biotin coordinates by its sulfur on copper or nickel NTA with approximately the same stability and this NTA/Cu²⁺/biotin system became a reliable immobilization strategy biosensing applications [95, 97-98].

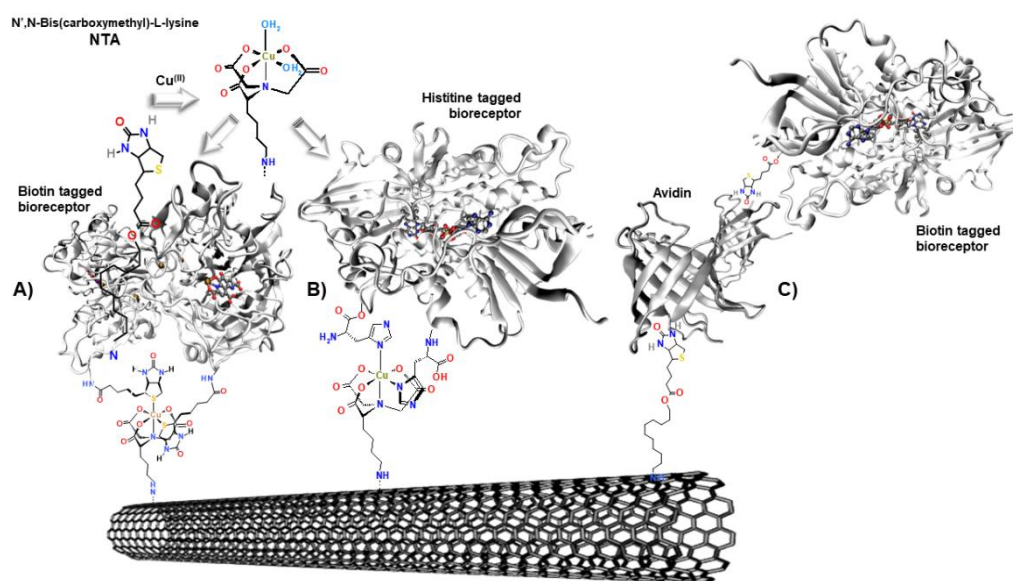


Figure 6: Immobilization of bioreceptor units by coordination of A) biotin or B) histitine tagged bioentities on NTA-Cu²⁺ modified nanocarbon based electrodes. C) Historical immobilization strategy using the biotin-avidin-biotin system.

Biotin and β -cyclodextrin form supramolecular inclusion complexes with an association constant of around 10^2 L mol^{-1} . This interaction is by far weaker than the biotin-avidin binding but strong enough to immobilize bioentities. Stronger and more common is the host-guest system β -cyclodextrin-adamantan [99-100] with an association constant of $K_a = 10^4 \text{ L mol}^{-1}$ [101]. However, β -cyclodextrin form inclusion complexes with a wide series of small molecules with sufficient hydrophobicity and appropriate sizes and even can act as surfactant for reduced graphene oxide [102]. Such non-specific behavior represents a disadvantage since proteins[103] and DNA[104] in complex fluids can interfere and form competitive inclusion complexes removing the initially immobilized bioreceptor unit.

Further immobilization methods were proposed combining affinity and host-guest interactions with coordination chemistry by immobilizing pyrene modified bipyridyl ligands on carbon nanotube electrodes. β -cyclodextrin modified enzymes could be immobilized via inclusion of pyrenes of a tris-bipyridyl iron complex [105]. A mixed ligand iron complex could be formed on CNTs with pyrene and biotin containing bipyridyl ligands [106]. Biotinylated glucose oxidase could then be immobilized via the standard avidin bridges.

Once the protein or nucleotide based bioreceptor is chosen and the most appropriated immobilization strategy for nanostructured has to be determined. The desired anchor functions can be attached to nanocarbon surfaces via various methods. As already mentioned, pyrene is the mostly used function for non-covalent functionalization. A large variety of derivative is available which allows its modification with any chemical function capable to immobilize bioentities. Pyrene has further beneficial properties for nanocarbon based bioelectrochemical applications which were reviewed recently [107].

Within the noncovalent functionalization strategy, electrosynthesis of conductive polymers became an attractive method since functions for biomolecule immobilization can be introduced during synthesis as it is for pyrene derivatives [108]. Within these polymers, polypyrrole is of particular interest since its monomers can be electropolymerized in aqueous media. This allows *in situ* entrapment of enzymes in the polymer matrix as already discussed with the example of hydrogenase immobilization on different CNT supports [67]. Beside this, this functionalization technique is quite efficient for homogeneous coating of porous nanostructures of CNTs and graphenes. Film thicknesses can easily be controlled by optimized charge transfer rates using chronoamperometry or chronopotentiometry. In the first case, the faradaic current is measured at a given potential until the desired charge is obtained. In the second case, the evolution of the potential is measured at given discharge current until the electropolymerization is completed. For optimal coverage of such polymer films on carbon nanostructures, organic solvents like acetonitrile or dimethyl formamide (DMF) allow better penetration and diffusion of the monomers to electropolymerize throughout the carbon electrode than aqueous media. The generated polymer film further allows improved wettability of the carbon nanostructures and represent an efficient platform for the immobilization of biomolecules.

4. Bioelectrochemical applications: Biosensors

Electrochemical transduction is an efficient tool for biosensing. Several techniques are available allowing a great choice of analytical methods [109] (Figure 7). For instance, amperometry is quite appropriate for enzymatic biosensing since redox enzymes transform or produce electroactive redox species which can directly be detected at the bioelectrode [110]. By applying the corresponding redox potential, the current intensity can be directly correlated to the concentration of the analyte. The evaluation of the performances of amperometric biosensors is represented in a so-called calibration curve which is a function of the recorded current towards the analyte concentration. Such curves are divided in a linear part and a maximum current part. At high analyte concentrations, the enzymatic is not capable to transform the excess of these species and indicates the amount of immobilized biocatalysts on the electrode when its activity is known. Here, carbon nanomaterials have shown to provide an efficient extension of the electrode material related to excellent conductivity and the formation of porous structures resulting in a clear enhancement of the specific surface and thus the enhancement of immobilized bioreceptors. The linear part represents all sensing kinetics concerning diffusion of the analyte or transduction of the recognition event (conversion of the generated redox probe at the electrode). This part is of interest for final sensor devices where the current intensity can directly be correlated to the analyte concentration. The higher the slope, the higher is the sensitivity of the sensors. For instance, the most applied example is enzymatic glucose sensing. Here, an enzyme like glucose oxidase, oxidised glucose into gluconic acid by reducing oxygen into hydrogen peroxide.

This hydrogen peroxide is redox active and can be re-oxidized to oxygen and water on platinum electrodes at around 0.7 V vs AgAg/Cl [111]. Platinum with its catalytic properties is unfortunately needed since it can separate and distinguish the electrochemical oxidation of H₂O₂ from water. In commercial point of care applications, platinum is generally avoided related to production costs where electron shuttles like ferrocene allows the transfer of the recovered electrons during oxidation from the FAD centre directly to the electrode and omit the need to detect the produced H₂O₂. In all cases, the measured currents are directly correlated to the glucose concentration in the linear part. There are further original and efficient transduction principles for glucose sensing which were nicely summarized by Pullano et al. [112]. Highly sensitive enzymatic amperometric biosensors can be produced for the detection of phenolic pollutants like phenol, cresols, or dimethylphenols. The enzyme polyphenol oxidase oxidises the compounds forming ortho and para quinones which can electrochemically be detected at reducing potentials around -0.2 V vs Ag/AgCl. The formed hydroquinones are re-oxidized by the enzyme resulting in an amplification of the signal and allows low detection limits[113].

More sophisticated setups are needed when amperometric transduction is chosen for the detection of immune systems or DNAs. Since the recognition event between antibody-antigen or corresponding DNA strands does not produce an electrochemical signal, a specific labelled secondary system (antigen, DNA strand) must be applied for signal capture. This more complex approach has the advantage of double recognition of two specific bioentities which reduces drastically interference and background effects and leads to more reliable signal capture. This principle of immunosensing is still the standard in clinical enzyme-linked immunosorbent assay (ELISA) tests [114]. The formation of carbon nanotube or graphene deposits on electrodes clearly enhance the specific surface area but the narrow pore sizes prevent the efficiency of such double recognition events in depth of the deposit [115]. Singh et al. faced this issue by forming layer by layer assemblies with CNTs and magnetic nanoparticles [116]. The particles served here as spacers for improved diffusion of the receptor antigen of cholera, the analyte, anti-cholera toxin, and the glucose oxidase labelled secondary antibody throughout the structure. Different particle sizes were used to optimize the performances. It was shown that the highest sensitivity of 88 $\mu\text{A } \mu\text{g}^{-1} \text{ mL cm}^{-2}$ was obtained with 500 nm sized particles which is still one of the best performing immunosensor with amperometric transduction.

Cyclic voltammetry (CV) is a powerful technique to modify and to characterize the resulting electrodes. For instance, by cycling within an appropriate potential range, functional monomers can be electropolymerized forming thin films with chemical functions for the immobilization or entrapment of the bioreceptors [57]. Such modified electrodes can then be characterized about diffusion and electron transfer kinetics through the formed films. For biosensing application, cyclic voltammetry is of interest when the product of an enzymatically formed product changes locally the pH value and thus the potential of the electrochemical probe. For immune or DNA sensing, the diffusion of an electrochemical probe is reduced after the recognition event since the receptor-analyte layer hinders the access to the electrode surface. One disadvantage of cyclic voltammetry is that not only redox events are recorded but also capacitive charging and discharging which results in low resolution of the current intensities. Carbon nanomaterials are high capacitive and to avoid a strong capacitive hysteresis, very slow scan rates have to be chosen.

An alternative solution is the technique of differential pulse voltammetry (DPV). Here, short voltage pulses are applied where two current intensities are recorded before and at the end of the pulse. The subtracted current intensities for the whole potential range allow to measure only the faradaic current without the disturbing capacity.

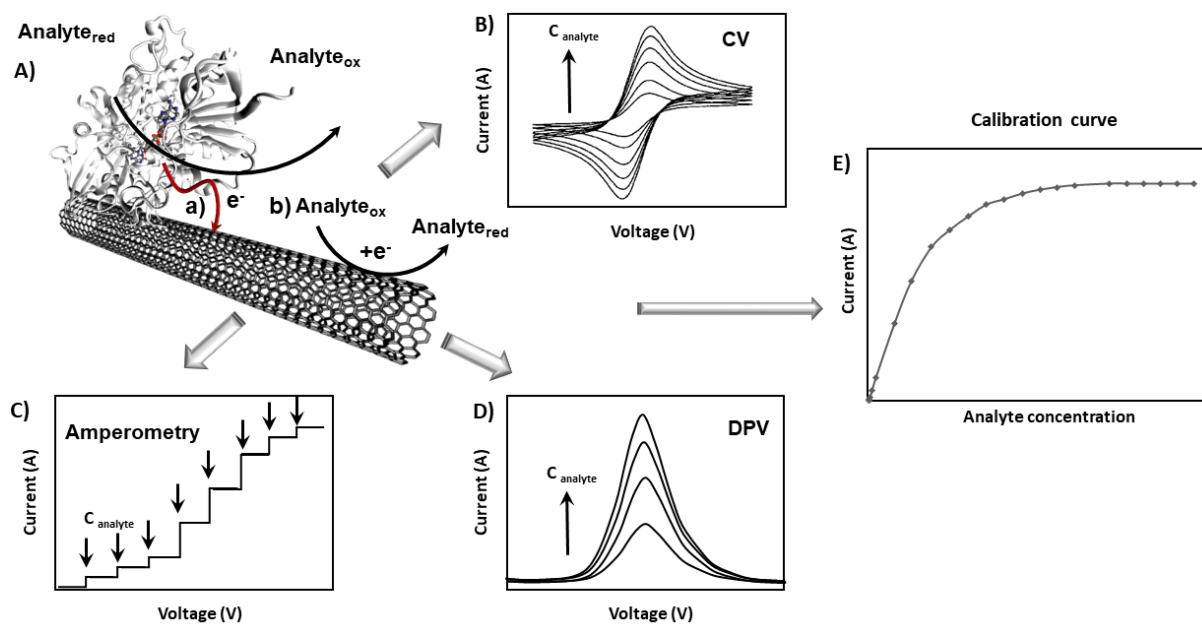


Figure 7: Common transduction techniques for electrochemical biosensing: A) example of the operational mode of enzymatic biosensors where the analyte is oxidized or reduced producing a redox active species which can be in some cases the analyte itself. Or, the involved electrons can be transferred to the electrode. The redox processes at the electrodes can then be transduced via e.g. B) Cyclic voltammetry, C) Amperometry, and D) Differential pulse voltammetry. E) The recorded current evolution allows to establish a calibration curve.

Electrochemical impedance spectroscopy (EIS) is more appropriate for immune- and DNA sensing since the signal of the recognition event can be recorded without such supplemental labelling steps [117]. EIS records capacitance and interfacial electron transfer resistance during the recognition event using an alternative current with small amplitudes around a redox probe potential at a wide range of frequencies. When the immobilized antibody or DNA strand recognizes its counterpart, and electrochemical probe is retained for its diffusion to the electrode surface by the formation of an increased layer thickness or conformational changes causing higher electron transfer resistances. This technique is extremely sensitive and allows detection limits down to the femtomolar range [118]. There are little examples reporting the use of nanostructured carbon materials for impedimetric biosensing. Most examples confine these materials in polymer matrices [119-122] which improves the interfacial electron transfer but omits to profit from the enhanced specific surface of this material.

The drawback is that deposits of CNTs or graphene form randomly nanostructures with slight differences of the final surface morphologies. The high sensitivity of EIS is strongly related to the surface morphology and even slight changes affect drastically the repeatability and reliability. To circumvent this effect, and to provide identical sensing elements, vertically aligned CNTs were used for impedimetric cancer cells [123]. EIS immunosensors were reported by Hafaiiedh et al. who used air brush techniques to form homogeneous films of oxidized CNTs to form an impedimetric immune sensor for Rabbit IgG [124]. Palomar et al. formed thin films by filtrating highly diluted aqueous CNT dispersion over cellulose ester filter membranes. These films can then be transferred to electrode supports by dissolution of the filter in acetone. The resulting EIS biosensor for anti-cholera toxin detection showed great reproducibility and provides a linear range over more than eight orders of magnitude [97].

5. Bioelectrochemical applications: Enzymatic Biofuel cells

Besides electrochemical enzyme biosensing, the possibility to use biocatalytic enzymes to oxidize a “fuel” like glucose or hydrogen, and to reduce an oxidizer like oxygen or hydrogen peroxide, to assemble these two “biosensors” to produce electric power, became a fascinating research field. Such biofuel cells can produce electric energy using non-toxic and non-polluting fuels and the biological

energy production can take place at neutral pH, at room temperature, and in complex media due to the high specificity of enzymes. ^[125]. This technology gained high interest when a glucose biofuel cell design could harvest few μW of power from a grape [126]. Since glucose and oxygen are both present in body fluids, the idea of powering implantable devices like pace makers or insulin pumps by such biological fuel cells but the strict requirements, especially operational life times, are still far to be met [127].

However, the main critics for enzymatic biofuel cells are their limited power output and operational life time. Katz et al., one main actor of enzymatic biofuel cell research over the last decades, recently published a very critical review article about this topic where the authors cannot imagine practical applications with industrial interest [128]. It is true that enzymatic fuel cell will never be capable to face the steady increasing energy need. However, this technology has realistic potential in the field of low power consuming devices for single or short time use before disposal like pregnancy, ovulation or other point of care tests [129]. Such disposable medical devices are often powered by coin or button cell batteries which is the most polluting component. Biofuel cells meet the power demand of these devices and can replace the coin cell batteries in the future for a series of devices.

The challenges in biofuel cell research are not only to immobilize the enzymes but also to establish an electron transfer with the electrode. Such electron transfers should be at low overpotentials since the power output is directly related to the current density and the voltage. In the optimal case, direct electron transfers can be established between the redox center of the enzyme and the electrode as mentioned with the examples of multicopper enzymes. Concerning direct electron transfers with flavin adenine dinucleotide (FAD) dependent enzymes like glucose oxidase, the Bartlett team clearly demonstrated that all reports about direct oxidoreduction of the FAD co-factor is in fact the redox signature of free, non-catalytic, FAD, commonly found in commercialized enzyme samples [130]. In fact, the catalytic active cofactor is deeply embedded inside the protein which is further covered with a carbon hydrate shell. The distance between the redox center and a conductive surface is therefore too high which makes direct electron transfers via tunneling more than improbable [131]. The only credible example of direct electron transfer with catalytic FAD of glucose oxidase from *Aspergillus niger* was shown by Courjean *et al.* [132]. Here, the oligo saccharide layer could completely be removed thus reducing the distance between the active site and the electrode and thus enabling direct electron transfers with this modified enzyme. For native glucose oxidase and other FAD dependent enzymes, however, so-called mediated electron transfer is necessary. Such mediation is achieved by using small redox active molecules which can shuttle electrons between the enzyme's redox center and the electrode. The redox potential of such electron shuttle has to be chosen carefully since a compromise between high overpotentials with high currents but loss of the cell voltage and low overpotential with low currents, but higher cell voltage has to be accepted (Figure 8). However, Tsuruoka et al. studied the mediated electron transfer rates with different organic redox mediators [133]. He concluded that the correlation between electrocatalytic currents and the redox potential is not evident since other factors like functional groups, size, or charges of such mediators also influence the transfer kinetics. The "best" mediator has to be experimentally determined for each enzyme even when it has the same co-factor.

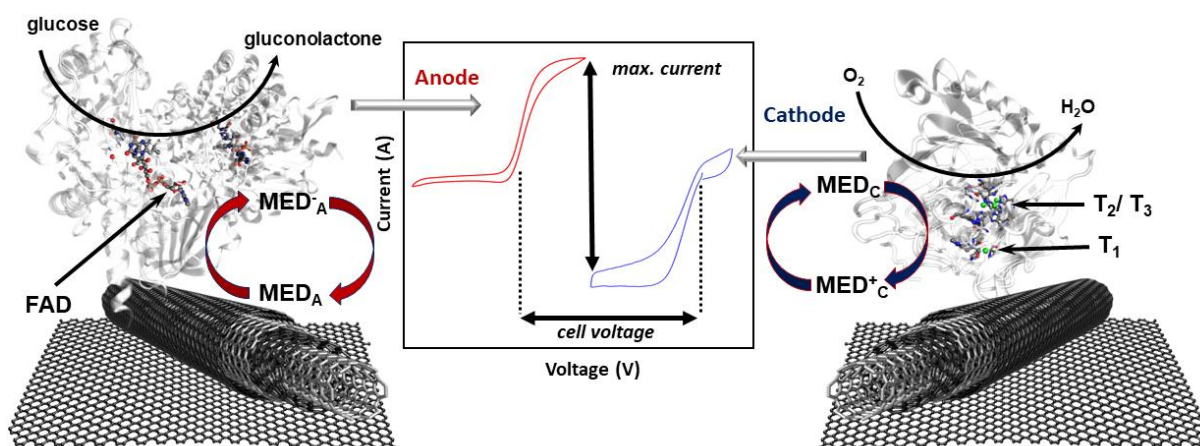


Figure 8: illustrations of an enzymatic bioanode and a biocathode with optional mediated electron transfers leading producing electrocatalytic current densities at distinguish voltages and consequently power.

Carbon nanomaterials play a crucial role for the performance of biofuel cells. After a first proof of concept using enzymes for the production of energy in 1964 [134], there were little activities in this research field until the late 1990's where great improvements were achieved in enzyme wiring [135] and the first use of CNTs [136]. It is well accepted that the tubular structure of CNTs is beneficial to enhanced access to the redox center of the enzymes and this material is still mostly used in this research topic. However, the toxicity of CNTs as aerosols is compared with asbestos fibers. Even when up to date only one class of stiff and long MWCNTs has been classified as a possible human carcinogen when inhaled [137-138] and a vast amount of different CNTs is still not classified yet, safety and security is of predominant importance for existing and future CNT based products where the release of CNTs as aerosols even after disposal must totally be excluded. Furthermore, compared to other carbon nanomaterials, the production costs are still relatively high despite well-established mass production methods. In this context, clear progresses were achieved using other nanostructured carbons like Ketjen/carbon black or graphene [139-140]. Carbon black in general is composed of amorphous carbon micro/nanoparticles with conductive and insulating domains. Within this category, Ketjen and Vulcan black provides improved conductivities and nanosized structures and are therefore prominent alternatives for CNTs for biofuel cell applications [141]. Targeted synthesis of carbon particles with controlled nanopore sizes were proposed for biofuel cell applications by the Tsujimura team [142]. These particles are formed by annealing of a carbon precursor, mostly polyvinyl alcohol, in presence of magnesium oxide particles with controlled size and shape. After the carbonization process these MgO particles can easily be removed by dissolution in diluted acids leaving identical pores in the particles which has been shown to improve the operational lifetime of enzymes combined with efficient electron transfer kinetics [143]. Beside the tremendous impact of graphene materials in bioelectrochemical applications, this carbon material shows less efficiency for biofuel cell technologies. Within the different graphene materials, reduced graphene oxide appears to be most promising, but the performances are generally far below compared to the other carbon nanomaterials as discussed above [144].

6. Conclusion

Considering the vast number of available carbon nanomaterials, immobilization techniques, and electrochemical transduction methods, the optimal biosensor setup might be hard to determine for an analyte of interest. However, there are some standard advices which facilitates the choice. For the electrochemical detection of small molecules, enzymatic biosensors or aptasensors are mostly used. Several immobilization techniques were discussed to for a reliable enzyme-based sensing element via simple adoption, forming electrostatic, or

hydrophobic interactions. The enzymatic reactions can form electroactive species which can be transduced via amperometry of cyclic voltammetry. When no electroactive species is formed, electron transfers between the enzyme and the electrode can be achieved by direct electron transfers or by using redox mediators. Cyclic voltammetry gives most information about the electrocatalysis but related to the high capacitance of carbon materials, slow scan rates must be used. For the detection of diseases, immune systems or DNA are the sensing element of choice. Here, more sophisticated immobilization is recommended to avoid inhibition or loss of biological activities. Electrochemical impedance spectroscopy is here a powerful transduction technique since it allows label free detection. Carbon nanomaterials by nature provide an extended electroactive surface area for enhanced immobilization of generally bulky bioreceptors. The choice of which nanomaterial is most appropriate for which receptor unit or electrochemical transduction remains, however, controversial.

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