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Review

Strategies of Nano-Manipulation for Application in Electrochemical Biosensors

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Manipulation techniques of materials at nanoscale level have been pointed in the last decades as a promising tool to develop a new class of modified electrodes applicable in biosensors. Also, the high control of material properties is crucial to detect single events at molecular and atomic level. For instance, nanostructured thin films obtained by Langmuir-Blodgett (LB) and Layer-by-layer (LbL) techniques has been reported as an interesting approach to obtain more selective and sensitive modified electrodes and in addition to nanostructured materials are promising strategies for the fabrication of electrochemical biodevices. Thus, our main focus in this review paper is to show an overview on recent trends in the utilization of manipulation techniques applied to the development of electrochemical biosensors. Emphasis will be given in the utilization of different techniques utilized on the modification of electrodes to enhance electrochemical biosensing performance of enzyme-, protein- and DNA-based biosensors and nano-based electrochemical biosensors for diagnosis.

Keywords: Nanomaterials, nanostructured films, nano-manipulation, biological molecules, electrochemistry, biosensor.

1. INTRODUCTION

In the last decades, nanostructured materials have received much attention due the possibility to develop new architectures applied in electrochemical sensing and biosensing devices [1-12]. An

important point besides the applicability of nanomaterials as recognition elements in electrochemical devices is to use manipulation techniques for their fabrication [13-16]. It is well known that materials, at the nanoscale level, present interesting properties [17-24]. In this regard, nanostructured materials with a large range of sizes and with a diversity of morphologies can be produced and utilized in biosensing devices [25-41].

Adsorption of organic molecules at solid conducting supports to produce thin nanostructured films are one of the most employed architectures and represents an important approach in the field of nano-manipulation. Langmuir-Blodgett (LB) technique promotes a high control of the physical and chemical properties of nanostructured organic films and plays an important role in the production of miniaturized devices applicable as platforms for enzyme immobilization [42]. Other pathways to prepare platforms based on nanomaterials, aiming the fabrication of electrochemical biosensors are dispersion in solvents, adsorption (e.g. Layer-by-layer), formation of covalent bonding and electropolymerization.

Also, the utilization of hybrid organic/inorganic thin films can, in a simple way, be employed in solid conductor electrodes. The possibility to incorporate hybrids containing nanostructured materials for enhance electrochemical properties makes these techniques much attractive in the field of bionanoelectrochemistry [43,44]. Biomolecules such as enzymes, proteins and DNA have been extensively utilized in conjunction with nanostructured materials for biosensing applications [45-47]. Regarding the use of biomolecules for biosensing, the main challenge is to obtain high signal responses without loss of structure and activity of these molecules [48,49]. Carbon nanomaterials and metallic nanoparticles are promising materials for charge transports studies at modified electrode and many efforts have been made to improve the electrochemical performance of modified electrodes containing these materials [50-52].

Regarding these materials, the main focus has been to improve the use these nanomaterials as recognition supports for miniaturized electrochemical devices [53]. Nowadays, the manufacturing and manipulation of nanostructures or biomolecules at a solid support open a new range of possibilities to construct different and specific arquitectures for biosensing.

Recent approaches have reported new routes for fabrication of such devices and many efforts have been made in order to control more accurately materials properties utilizing nano-manipulation techniques [54,55].

In this scenario, this review article shows an overview of nano-manipulation techniques applied in the development of electrochemical devices and the most recent works published in the last years in the field of bio-nano-electrochemistry that were concerned with the improvement of the performance of electrochemical systems.

Emphasis will be given to organic nanostructured platforms and hybrids of organic/inorganic materials applicable in modified electrodes for enzymatic biosensing, protein- and DNA-based biosensors, and fabrication of nanostructured biodevices by (LB) and (LBL) techniques and recent trends as well as perspectives in nanomanipulation techniques for future application in electrochemical biosensors.

2. LANGMUIR-BLODGETT ADSORPTION PROCESS FOR ELECTROCHEMICAL BIOSENSORS

2.1. Langmuir-Blodgett technique

The Langmuir-Blodgett (LB) technique is based on the transfer of insoluble monolayers supported on liquid-air interfaces to solid supports that intercept vertically the liquid surface. Based on the reports of a pioneering experiment by Langmuir [56], an organic solution of an amphiphilic material is firstly deposited on the top of the water surface, drop by drop. If the solvent evaporates quickly, and the material spreads uniformly along the interface, appropriate quantities of the material can form a monomolecular film at the liquid-air interface in such a way that hydrophobic moieties stand oriented towards the air and the hydrophilic ones towards the aqueous subphase. This film, usually called Langmuir film, or Langmuir monolayer, can be compressed through liquid barriers (Figure 1) in order to attain the desired surface density. The compression of the monolayer can be followed by measuring certain properties of the liquid surface, such as surface tension, surface potential. Additionally, microscopy and optical spectroscopy techniques can be also employed. The most classic characterization is the measurement of the decrease of the water surface tension owing to the introduction of the amphiphilic material, called surface pressure (π), defined as:

$$\pi = \gamma_o - \gamma \qquad (1)$$

Where, γ_o is the surface tension of the pure water, and γ is the surface tension of the water after the monolayer formation. The π–A (surface pressure – area) isotherms give information on the 2-D states of the mater, which is related to the compressibility of the monolayer.

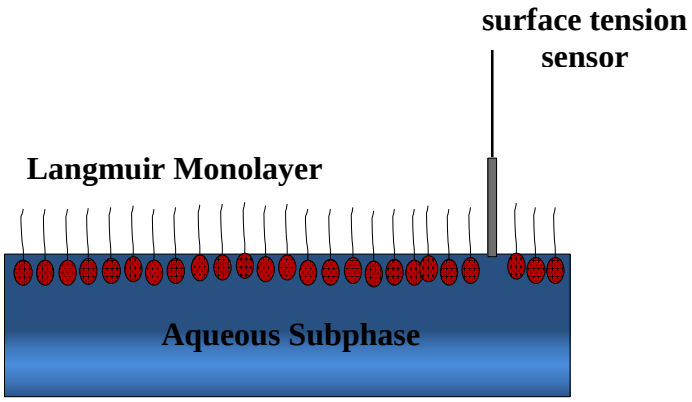


Figure 1. Schema for a Langmuir Monolayer formed on the top of the air-water interface.

In a pioneering experiment by Blodgett [57, 58], the monolayer was compressed until a certain surface pressure and successive layers were deposited by dipping and removing a solid support vertically from the aqueous subphase. The passage of the support by the interface, because the surface

tension gradient, will provide the transfer of the Langmuir monolayer from the liquid surface to the solid support. Each dip and removal of the plate must provide one molecular layer, whose quality is firstly attested by the transfer ratio, which is defined by the area swept by the barrier to keep the surface pressure constant, divided by the area of the solid support that was in contact with the interface during the deposition. A transfer ratio close to the unity attests the quality of the deposition. Transfer carried out with successive dips and removals results in Y-type LB films, which contains architecture allowing head-head and tail-tail interactions, which will determine the stability of the film. The most interesting fact in constructing this kind of film is the possibility in controlling rigorously the number of layers deposited and consequently the thickness of the material.

Therefore, it is possible to construct highly ordered materials, whose architecture is controlled at the molecular level, which is promising for the construction of miniaturized devices for optical and electrical purposes, and in a general speaking, for nanotechnology. The classical materials used to form Langmuir and LB films are lipids, such as fatty acids, glycerophospholipids and so on. These materials contain in their molecular structures one or more long hydrophobic tails, and a small hydrophilic head, which is ideal to form stable Langmuir monolayers at the air-water interface. However, it there is not a limitation for other materials to form stable films at the liquid interfaces, since nowadays we can find in the literature reports of Langmuir monolayers formed with ionic liquids [59], quantum dots [60], synthetic polymers [61], proteins [62], nucleic acids [63], and polysaccharides [64]. Even when the substance has gotten some solubility in water, studies on the air-water interface, considering the formation of a Gibbs monolayer [65], or the formation of hybrid materials [61] have been employed. Particularly, the interaction of soluble molecules coming from the aqueous subphase with the materials on the monolayer can be investigated by the Langmuir technique, which enables the investigation of these interactions in process of molecular recognition [66].

Particularly, it is of growing interest the immobilization of enzymes as Langmuir-Blodgett films. Enzymes do not form classical Langmuir monolayers, because their usual solubility in water and their macromolecular structure. To overcome this problem, some strategies have to be followed such as the use of high salt concentration in the subphase to enhance the surface excess of the enzyme [67]. Other strategies involve the employment of hybrid lipid-enzymes at the air-water interface. It is defended that the amphiphilic nature of the a lipid monolayer helps the enzyme preserve its molecular conformation, mainly if the enzyme comes from mild conditions in the aqueous subphase and adsorbs spontaneously on a pre-formed lipid monolayer [68] (Figure 2). When transferred to solid supports as LB film, the lipid-enzyme film can be further used to basic investigation of catalytic activity and also can be employed as a promising and sophisticated device able to detect several analytics of interest working therefore as a biosensor, whose detection can be done by several techniques including gravimmetry by quartz crystal microbalance [69], statistical impedance [70], optical spectrocoscopy [71] and by voltammetry and amperometry [72]. We will focus in the following topic on the so called electrochemical biosensors fabricated by using the Langmuir-Blodgett technique. The LB technique in all the papers reported so far have been shown as a suitable method for immobilization of enzymes because of its uniform well-ordered structure imposed to the ultrathin films and ability to control the amount of biocomponents immobilized by manipulating the number of deposited layers.

2.2. Langmuir-Blodgett based electrochemical biosensors

In a pioneering work by Schuhmann [73], an amperometric biosensor for glucose was constructed immobilizing glucose oxidase on Langmuir-Blodgett films via a membrane receptor (bilayer lipid membrane) and the device allowed the enzymatic oxidation of glucose and a amperometric sensor for glucose could be performed due to the formation of H_2O_2. Further progress was carried out with the enzyme immobilized from aqueous subphase and the hybrid enzyme-lipid monolayer transferred to solid supports by a Langmuir-Blodgett technique. By using this methodology, several conditions of aqueous subphase, especially pH, could be essayed, and until 10 layers of lipid-protein were deposited resulting in a glucose sensor able to detect a 0.5-5.0 mM of glucose.

The aim is to preserve the native enzyme molecular conformation and to arrange it in a suitable position for the molecular recognizing of an external molecule of a solution put on contact with the Langmuir-Blodgett device. As defended in a recent review by Girard-Egrot [68], the successful incorporation of enzymes on a pre-formed Langmuir monolayer depends strongly on the methodology employed. The most one commonly used is the adsorption of the enzyme from the subphase, avoiding direct adsorption of the macromolecule present at the water surface. If enzyme solution is used as subphase, adsorption of the enzyme before lipid interaction can occur, an event that can be overcame by injecting the enzyme after the lipid monolayer formation, which requires multipoint infections to avoid enzyme adsorption on badly distributed points along the lipid monolayer. This strategy was used to produce electrochemical sensors containing phytic acid [72], horseradish peroxidase [74], hemoblogin [75], glucose oxidase, [76], acetylcholinesferase [77] and urease [78, 79], tyrosinase [80], to detect a diversity of substances such as including phytic acid, hydrogen peroxide, glucose, choline, urea, and phenols.

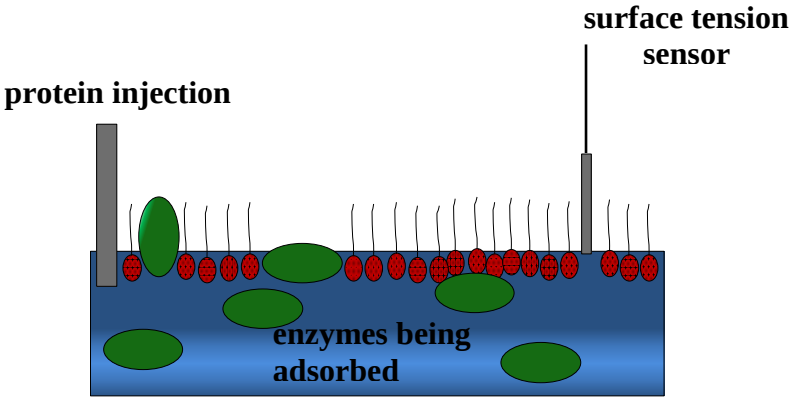


Figure 2. Schema for enzymes being adsorbed at Langmuir films.

The defense of taking advantage of the lipidic matrix and of the hydrophobic or hydrophilic lipidic environment was discussed on a recent paper within which choline oxidase activity was retained in Langmuir-Blodgett (LB) films prepared with behenic acid or with mixed dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidic acid (DPPA) [81]. The enzyme activity was detected through an electrochemical sensor whose platinum electrode was coated with the

protein-lipid LB films. It was shown that the hydrophilic environment provided by the phospholipid LB films was directly responsible for the inactivation of choline oxidase likely due to a competition with the choline moiety of DPPC. The environment provided by fatty acid multilayers allowed retention of the highest activity for the enzyme.

Also, it is interesting to mention that the use of submicron hydrophobic SiO_2 particles as Langmuir-Blodgett (LB) films is reported to help the incorporation of glucose oxidase (GOx) into the films [82]. After incorporation, GOD retains its enzymatic activity. In addition, GOD activity per area of membrane increased because of the stable adsorption of GOD on the SiO_2 particle surface. The lifetime of the GOD-LB film containing SiO_2 particles is much longer than the lifetime of GOD-LB film without SiO_2 particles. Another strategy recently employed was to fabricate amperometric lactose biosensor by immobilizing lactase and galactose oxidase in Langmuir-Blodgett (LB) films of poly(3-hexyl thiophene) (P3HT)/stearic acid for dosage of lactose in milk and its derivatives in specific products for people owing "lactose intolerance" [83]. The working electrode may be used for the estimation of lactose/galactose in food and biological fluids.

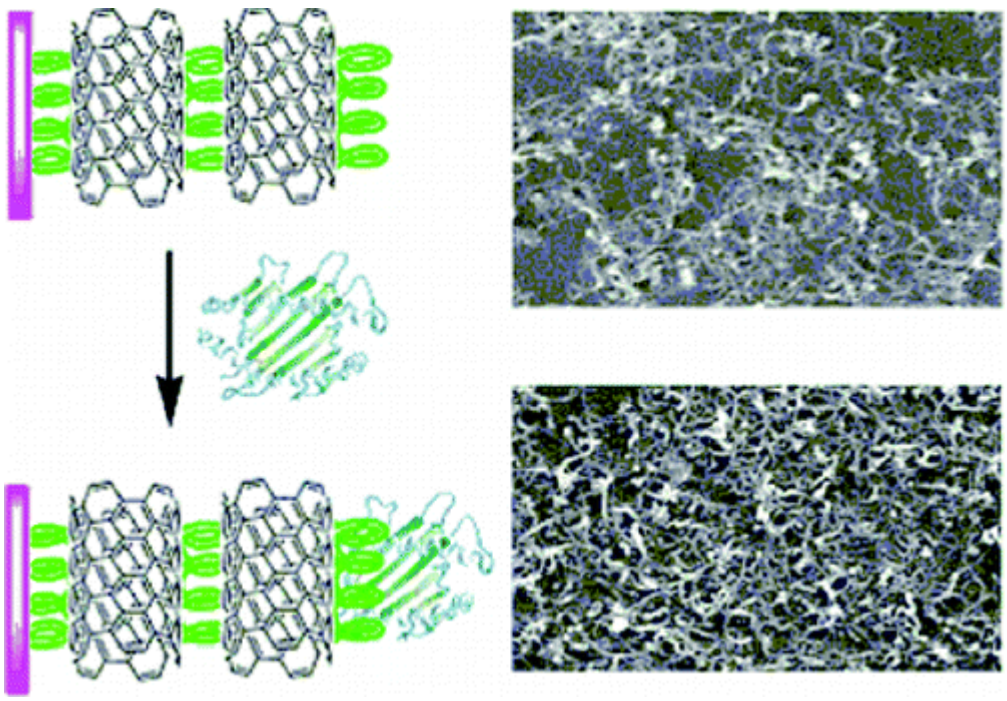


Figure 3. Schema for the immobilization of Hydrogenase on LB pyriduylthio-modified carbon nanotubes. Reprinted with permission from ref. 85. Copyright@2010 American Chemical Society.

However, enzyme spread directly on the water surface, without the presence of any protector lipid has also been shown as an alternative way to form stable biosensors. For instance, laccase was incorporated into an LB film to be used for the detection of phenolic compounds. The function of enzyme immobilization was carried out by adding glutaraldehyde to a film of stearic acid and laccase.

The sensor sensitization was achieved by an amphiphilic *N*-alkyl-bis(thiophene)diphenylamine admixed into the film. The interlaced diphenylamine derivative was in this case used to facilitate the electron transfer thereby enhancing the sensor sensitivity [84].

Nowadays other more sophisticated architectures have been developed in order to enhance the performance of LB-based enzyme electrochemical sensors. For instance, Sun et al. [85] used pyriduylthio-modified carbon nanotubes as Langmuir-Blodgett films to support hydrogenase added in a subsequent adsorption from solution (Figure 3). The bionanocomposite formed could be used as a heterogeneous biocatalyst to catalyze reversible reaction between protons and H_2, resulting in potential applications for biohydrogen evolution and H_2 biofuel cells. In this case, the Langmuir-Blodgett technique was used to modify the electrode for further adsorption of an enzyme by other methods, such as physical adsorption from solution. Another example is a uric acid biosensor fabricated by using the Langmuir-Blodgett (LB) technique to immobilize the uricase on chitosan/Prussian blue (CS/PB) prefunctionalized indium-tin oxide (ITO) electrode [86].

The electrochemical properties of the uricase - n-nonadecanoic acid (UOx/NA) LB film proved that CS/PB was a good electro-catalyst for the reduction of hydrogen peroxide produced by enzymatic reaction of UOx and protein molecules retained their natural electro-catalytic activity.Other interesting work [81] describes the production of a mediator free enzyme sensor by covalent immobilization of cholesterol oxidase (ChOx) onto 11-mercaptoundecanoic acid functionalized gold nanoparticles (MUDA-AuNPs) - octadecylamine (ODA) hybrid Langmuir-Blodgett film. The device showed good affinity for ChOx and provided favorable microenvironment for direct electron transfer between enzyme and electrode to detect cholesterol without interferences. In a similar way, glucose oxidase was immobilized in octadecyltrimethylammonium and Prussian blue (PB) nano-size clusters [87]. The successful preparation of glucose sensors operating at the very low potential indicated that the adsorbed PB clusters in the LB films act as an electrocatalyst for the electrochemical reduction of hydrogen peroxide, which is the final product of the enzymatic reaction mechanism. The observed low potential applicability is estimated to inhibit the responses of interferents such as ascorbic acid, uric acid, and acetaminophen.

Not only enzymes can be immobilized by this strategy, but also DNA molecules. In a recent work [88], DNA strands were immobilized onto a glassy carbon electrode surface with Langmuir-Blodgett technique to produce modified electrodes improving the sensitivity for 8-azaguanine. The proposed method could be applied successfully for the determination of 8-azaguanine in diluted human urine. Also, redox molecules such as ferrocene, ferrocene carboxylic acid tetramethylbenzidine have been commonly used as mediators for coenzymes of several dehydrogenases immobilized in monolayers of dipalmitoylphosphatidic acid (DPPA) by means of the cospreading technique at the water-air interface [89,90]. Although no specific sensing measurements have been essayed, information on the influence of the multilayer structure on the electrode current and on the redox activity of the immobilized mediators was obtained by cyclic voltammetry by means of the production of significant electrochemical signals.

3. LAYER-BY-LAYER SELF-ASSEMBLY PROCESS FOR PROTEINS AND DNA-BASED BIOSENSORS

3.1. LBL based electrochemical biosensor

The layer-by-layer technique (LBL) has been used as an interesting tool for manufacturing thin organic films by the self-arrangement of molecular chains on a solid substrate [91,92]. The LbL technique permits the design of different architectures for the production of thin films and constitutes a useful strategy to improve electrocatalytic activity of solid conductor electrodes. Therefore, the use of LbL technique opened a new pathway for development of miniaturized bioelectrochemical devices with high control of orientation and molecular order [93]. At pioneering works, Savig and co-workers [94] formerly utilized covalent adsorption of organic molecules to produce a well-organized structure on solid supports. However, the organic molecules utilized for this purpose are very limited referring to the specific functionality to achieve multilayers growing. Alternatively, a modern concept of self-assembly was introduced by Decher and co-workers [95,96] at 90 decade as a low-cost and simple method to obtain nanostructured thin films under controlled conditions (pH, temperature, polyelectrolyte concentration, ionic strength, etc…). For this purpose, a large variety of materials for electrochemical sensing and biosensing can be obtained [97-138]. Basically, the processes of film fabrication by LBL technique is governed by the adsorption of organic polyelectrolytes with opposite charges present on their molecular structure, in such a way that film roughness, thickness, porosity and morphology can be controlled at molecular level [139]. Important advantage in the use of LbL technique to construct biosensors is the possibility to incorporate organic/inorganic composite materials that contributes for the maximization of the biodevices electrochemical signal of [140]. Also, it is important to emphasize that most hybrids based on nanomaterials has been utilized to detect electrochemical signal from biochemical reactions.

It is well known that biomolecules can exhibit partial or total loss of the bioactivity when immobilized on solid surfaces due to drastic structural changes [141]. Therefore, emphasis has been given to explore new methodologies for immobilization of redox enzymes without loss of activity to develop more optimized nano-architectures for electrochemical biosensors [142-145]. Particularly, the use of nanostructured hybrid materials in the fabrication of LBL films has been largely applied for studying enzymatic mechanisms and most authors reported their utilization as favorable platforms for the immobilization of biological components such as enzymes, proteins and DNA, as described herein.

3.2. Proteins LbL-based electrochemical biosensors

The versatility of LBL technique and the use of mild experimental conditions allow the fabrication of nanostructured films by using a diversity of material types for biomolecules immobilization such as proteins and enzymes [146-149]. Proteins are well known as important elements in recognition processes of living organisms [150]. Therefore, several types of proteins have also been used to construct biosensors due to their specific properties in biological systems that are usefully reproduced in biodevices [151-154].

Concerned the development of nanostructured thin films, the utilization of proteins in alternating layers with organic polyelectrolytes has also been used as interesting approaches to construct bioactive films for biosensing applications [155, 156]. As mentioned above, the electron transfer via macromolecules is very slow due to their large structures. Considering this challenge, LBL technique is also used for the fabrication of a diversity of nanodevice architectures based on proteins that are able to improve electronic transport on the electrode surface. In a pioneer work, Lvov and co-workers [157] reported the utilization of self-organized multilayer of proteins and polyelectrolytes such as (PSS/Mb) and (GOx/PEI) under controlled pH as attractive biologically-active materials. Proteins have also been used in LBL method to construct alternate multilayer of ceramic nanotubes (halloysite), spherical particles leading to an array of new ordered nanoparticles-tubules, which were applied to load co-enzymes (NAD) for the development of enzymatic nanoreactors. Decher and co-workers [158] also reported the use of protein/polyelectrolyte hybrid films via specific recognition. One of the main challenges is to maintain the integrity of the native protein structure to promote their utilization for technological applications.

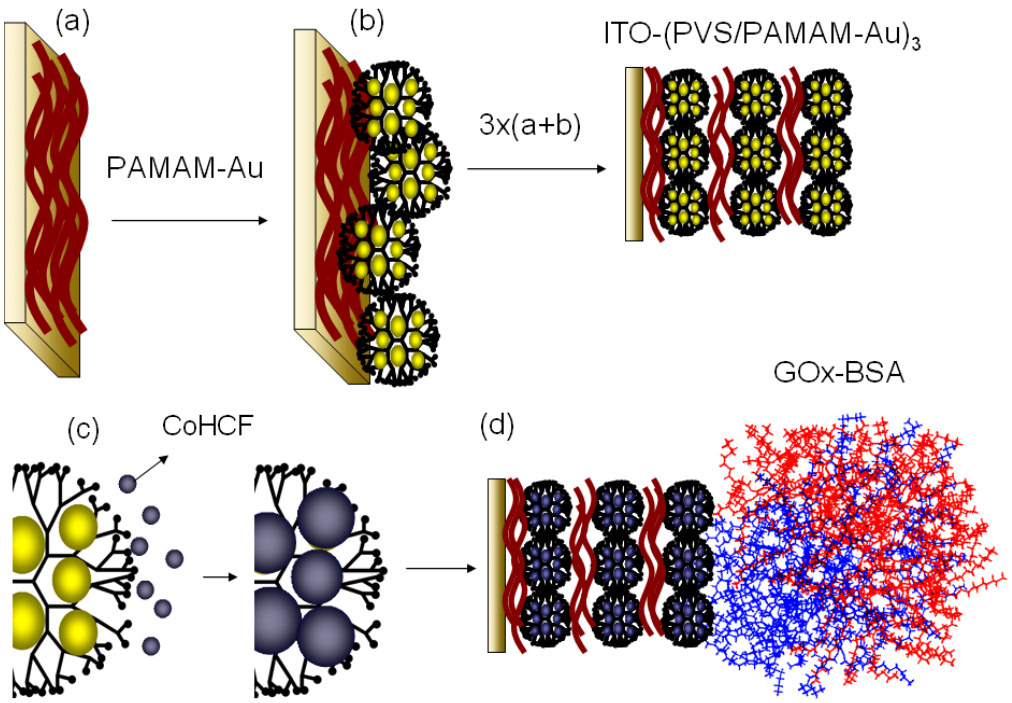
Regarding the use of biological materials for the development of new architectures for electrochemical biosensors, nanostructured thin films have been reported to maximize the electrochemical signal of immobilized biomolecules at electrode/electrolyte interface. Also, the use of organic matrices is currently utilized as a more favorable environment for enzyme immobilization and many efforts have been done with the aim to improve the electron transfer signal on modified electrodes [159].

The use of hybrids such as metal nanoparticles stabilized on organic matrices has been reported as an interesting approach to enhance electrochemical signal on electrode surfaces [160]. Noble metal nanoparticles such as platinum [161-164], palladium [165,166], silver [167-169], gold [170-173] and others has been focus of extensive studies due to their potential application as recognition elements on modified electrodes. As an example, colloidal gold nanoparticles (AuNPs) have been extensively studied, especially in the context of bioelectrochemical devices. Many studies reported the utilization of gold in the nanometer scale as a very stable metal material in a wide range of applications [174] and a diversity of methods have been developed to obtain gold nanostructures in stable aqueous solution [175]. The synthesis of gold nanoparticles by the chemical route is the most common and simple method utilized [176,177]. Sau and co-workers [178] reported the synthesis of gold nanostructures with different shapes in aqueous solution. Manipulation of size and morphology of the materials requires high experimental control of thermodynamic and kinetic parameters provided by the use of coadjuvants suchs as surfactant stabilizing agents [179]. In the field of bionanoelectrochemistry, metal nanoparticles associated to redox mediators has also been reported in the latter years as an important tool to enhance electrochemical signal originated by enzymatic reactions. As an example, the utilization of hybrids formed by gold nanoparticles (AuNPs) and dendrimers has been reported as efficient systems to improve charge transfer on electrode surfaces and created the concept of electroactive nanostructured membranes (ENM) [180]. This strategy involves the utilization of dendrimer polyamidoamine generation 4 (PAMAM G4) containing AuNPs and PVS as polyelectrolyte matrices for bilayers fabrication on ITO (indium tin oxide) electrodes. This modified electrode was

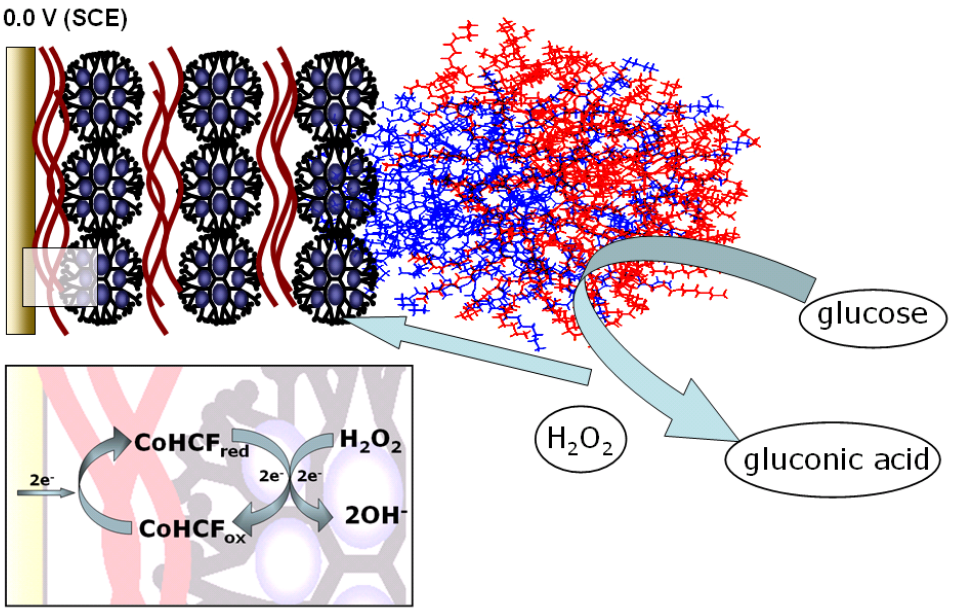
utilized with redox mediator (Me) around AuNPs to improve electrochemical performance on electrode/electrolyte (Figure 4).

Other strategies involve the utilization of carbon materials such as carbon nanotubes [181-195] and graphene nanosheets [196-210] to improve the charge transfer on solid electrodes as more powerful approaches to improve signal originated from biocatalysis. Since their discovery, carbon nanotubes have been extensively used as a promising material for electrochemical sensing and biosensing due to their unique electronic and electrochemical properties [211]. Several authors reported their utilization as a recognition element applied to glucose sensors biodevices [212]. As an example, Zhao and co-workers [213] reported the utilization of multiwall carbon nanotubes (MWCNTs) and enzyme glucose oxidase (GOx) on poly(dimethyldiallylammonium chloride) (PDDA) and poly(sodium 4-styrenesulfonate) (PSS) arranged as multilayers on a 3-mercapto-1-propanesulfonic-acid-modified Au electrode for amperometric biosensors. Other important work reported the utilization of graphene sheets due to their intrinsic electronic properties and recent researches have reported their utilization in electrochemical biodevices [214-224]. Kim and co-workers [214] show a novel and simple strategy to detect dopamine in the presence of ascorbic acid utilizing graphene sheets on modified electrodes in order to enhance electrochemical performance. Also, electrochemical impedance spectroscopy shows that the charge transfer on electrode surface was improved by graphene and can be an interesting tool for utilization as a biorecognition element and selective determination of dopamine on electrolyte media.

The utilization of LBL method to immobilize biological components such as proteins to simulate membrane cells also plays an important role in mechanistic mass transport studies of living organisms.



A)



B)

Figure 4. A) Schematic fabrication of LbL films comprising PVS and PAMAM-Au. The sequential deposition of LbL multilayers was carried out by immersing the substrates alternately into (a) PVS (a) and PAMAM-Au (b) solutions for 5 min per step. After deposition of 3 layers, an ITO-(PVS/PAMAM-Au)$_3$@CoHCF electrode was prepared by potential cycling (c). The enzyme immobilization to produce ITO-(PVS/PAMAM-Au)$_3$@CoHCF-GOx (d) was carried out in a solution containing BSA, glutaraldehyde and GOx. B) Schematic representation of reaction of glucose at ITO-(PVS/PAMAM-Au)$_3$@CoHCF-GOx electrode. Reproduced from ref. 180 with kind permission of Elsevier.

Ram and co-workers [225] reported the utilization of LBL technique to produce nanostructured films of poly(ethylene imine) (PEI) and poly(sodium polystyrene sulfonate) (PSS), cholesterol oxidase (COx) and cholesterol esterase (CE). The strong stability of multilayer films was also evaluated and contributes directly for the electrochemical properties of the film warranting the enzyme GOx immobilized on solid conductor supports remained active on the electrode surface.

Cytochrome P450 (cyt P450) and respiratory cytochrome c (cyt c) were also extensively studied as recognition element in the fabrication of electrochemical biosensors [226]. This class of enzymes is responsible for the catalysis of several chemical reactions and so, very attractive in the field of bioelectrochemistry for biosensing applications [227]. Wegerich and co-workers [228] reported the utilization of mutant cytochrome c (Cyt c) to study the kinetic of the reaction with superoxide radicals. Xiang and co-workers [229] reported the utilization of AuNPs/ionic liquid/CNTs nanocomposite utilizing LBL method for preparation of electrodes. In this case, DET of Cyt c was achieved and the electrocatalytic activity towards H_2O_2 showed a good response in a range of $5.0x10^{-5}$ to $1.15x10^{-3}$ M. However, many efforts have been made to produce more sensitive methods for substrate detections utilizing Cyt c as recognition element.

Another hemeprotein largely used for biosensing is *horseradish peroxidase* (HRP). This enzyme has been used as recognition elements on modified electrodes for biosensing [230]. Wu and co-workers [231] reported an interesting approach to obtain proteins on multilayer films of lipid membranes for biosensing. In this case, a glassy carbon electrode (GCE) was utilized as support for self-assembly of dimyristoylphosphatidy1choline lipid bilayers membranes (DMPG). Also, horseradish peroxidase (HRP) was utilized as a kind of mediator-free for H_2O_2 biosensing to produce a good signal response and electrochemical stability. On another approach, Cao and co-workers [232] utilized a mediator free H_2O_2 amperometric biosensor by immobilization of enzyme HRP on AuNPs modified platinum wire electrode. The results obtained evidenced an excellent electrocatalytic response range from 4.2 $x10^{-7}$ to 1.5$x10^{-3}$ mol L^{-1}. On the other hand, the determination of polyphenols has also been reported in literature utilizing HRP and DNA immobilized on silica-titanium substrates utilizing biological samples [233].

Polyphenols are a class of organic compounds that is very important in metabolic biological processes. This biosensor presented a good response that ranged from 1 to 50 μmol L^{-1} in an applicable potential of -50 mV vs. (Ag/AgCl) reference electrode. Many efforts have been made in order to improve the electrochemical response when biomolecules were immobilized on a solid substrate. Regards the development of enzymatic biosensors, the main challenge is to use new nanostructured arquitectures to shorten the distance for electron transfer and improve electrochemical signal on electrode surface.

3.4. DNA LbL-based electrochemical biosensors

DNA is commonly used on modified electrodes to fabricate the so called genosensors and has become a very interesting path for monitoring and diagnosis of diseases [234-241]. While, in cells DNA is responsible for the storage and transfer of genetic information, the peculiar chemical and structural properties of this molecule are useful as an engineering tool for electrochemical biosensors development [242,243]. In the last decades, DNA-based biosensors have been developed to recognize specific sequence detection [244]. DNA biosensors are based on the immobilization of a specific sequence ssDNA (single strand DNA) that act as a probe on the identification of molecular species or a specific analyte [245].

The immobilization of ssDNA can be performed onto electrode surface by different methods, such as physical adsorption, crosslink, encapsulation, covalent bonds and avidin-biotin complexation [246-251]. The recognition element present on the modified electrode surface converts hybridization with molecular structure sequence on electrical signal [252,253] brings several advantages in the utilization of DNA biosensors likes the possibility of fast diagnosis, low-cost, selectivity and easy of manipulation.

As an example, the utilization of modified electrodes with polymers has been reported as an interesting path for DNA immobilization [254-257]. Moreover, the presence of binding sites on electrode surface possibilities the development of a more stable electrode for biosensing applications [258,259]. For this purpose, several authors reported different electrochemical techniques such as

cyclic voltammetry [260,261], electrochemical impedance spectroscopy [262-266] and differential pulse voltammetry [267-270] for hybridization studies.

Also, nitrogenated bases adenine, thymine, cytosine and guanine can be identified by their redox processes on modified electrodes. On the other hand, DNA biosensors have been utilized to detect and monitor diseases [271]. Some works reported the development of DNA binding on solid modified electrodes for breast cancer detection [272], human immunodeficiency virus HIV-1 and HIV-2 biosensors [273], dengue virus detection [274], sexually transmitted diseases [275,276] and other infectious agents [277,278].

Concerned the development of more sensitive and selective biosensors-based on DNA immobilization, the use of LBL method has also showed a good improvement of electrochemical sensibility on modified nanostructured electrodes. In recent approaches, the utilization of DNA sequences attached on gold AuNPs and carbon nanotubes and AuNPs by LBL technique have been reported as able to improve the power of recognition of genosensors [279].

This strategy provides and efficient way for monitoring hybridization events throughout differential pulse voltammetry. On another recent approach, Du and co-workers [280] reported the utilization of LBL thin films of (PAH/PSS) coating with PbS NPs and combined with DNA for biosensing. The resulted DNA biosensor shows a good selectivity and sensibility with target DNA concentration on electrode surface ranging from $2.0x10^{-14}$ M to $1.0x10^{-12}$ M. These studies show us the high potentiality of substrate detections when DNA is immobilized on modified nanostructured electrodes.

4. RECENT ADVANCED BIO-NANOMANIPULATION TECHNIQUES FOR BIOSENSING

The nanomanipulation techniques have provided a very promising strategy in the field of nanoscience and nanotechnology to understand and improve the performance of biodevices [281]. Nowadays, the aim interest behind the manipulation at nanoscale science is to elucidate biological mechanisms detecting single events originated by manipulation of nanomaterials controlling their physical and chemical properties [282]. One important contribution for manipulation of nanostructures and biomolecules resulted from the development of microscopy instruments at the beginning of 80 decade [283]. The scanning electron microscopy (SEM) and atomic force microscopy (AFM) are the pioneering techniques used to obtain images from materials at nanoscale science and, recently, as important technologies to manipulate carbon nanotubes [284], nanoparticles [285], polymer nanoparticles [286] and others [287,288] at a solid surface. Although the utilization of microscopy technique became feasible the manipulation of biomolecules at nanoscale level, it is important to attempt to the fact that biomolecules such as enzyme required special manipulation techniques in order to preserve their structural integrity and function. Besides the nanomanipulation of nanostructured materials, literature data report the use of interesting techniques for nanomanipulation of living cells for application in therapeutic and technological devices [289-294]. Nishida and co-workers [289] used AFM technique and fluorescence microscope (TIRFM) to manipulate single cells for bio-nanotechnology applications. On the other hand, the manipulation using magnetic tips of biomolecules and cells at nanoscale level was also been reported such as selectivity for tissue artificial growth or

control of cells on substrate. Beck and co-workers [290] reported the successful detection and manipulation of individual bacterial cells at nanoscale level. In this case, cells of *Bacillus mycoides* with diameter of ~800 nm surrounded by a capsule consisted by polysaccharides were used to detect bridging of individual bacteria directly through changes in the electrical response. In another interesting approach, miniaturized devices were constructed for DNA and proteins immobilization on interdigited electrodes.

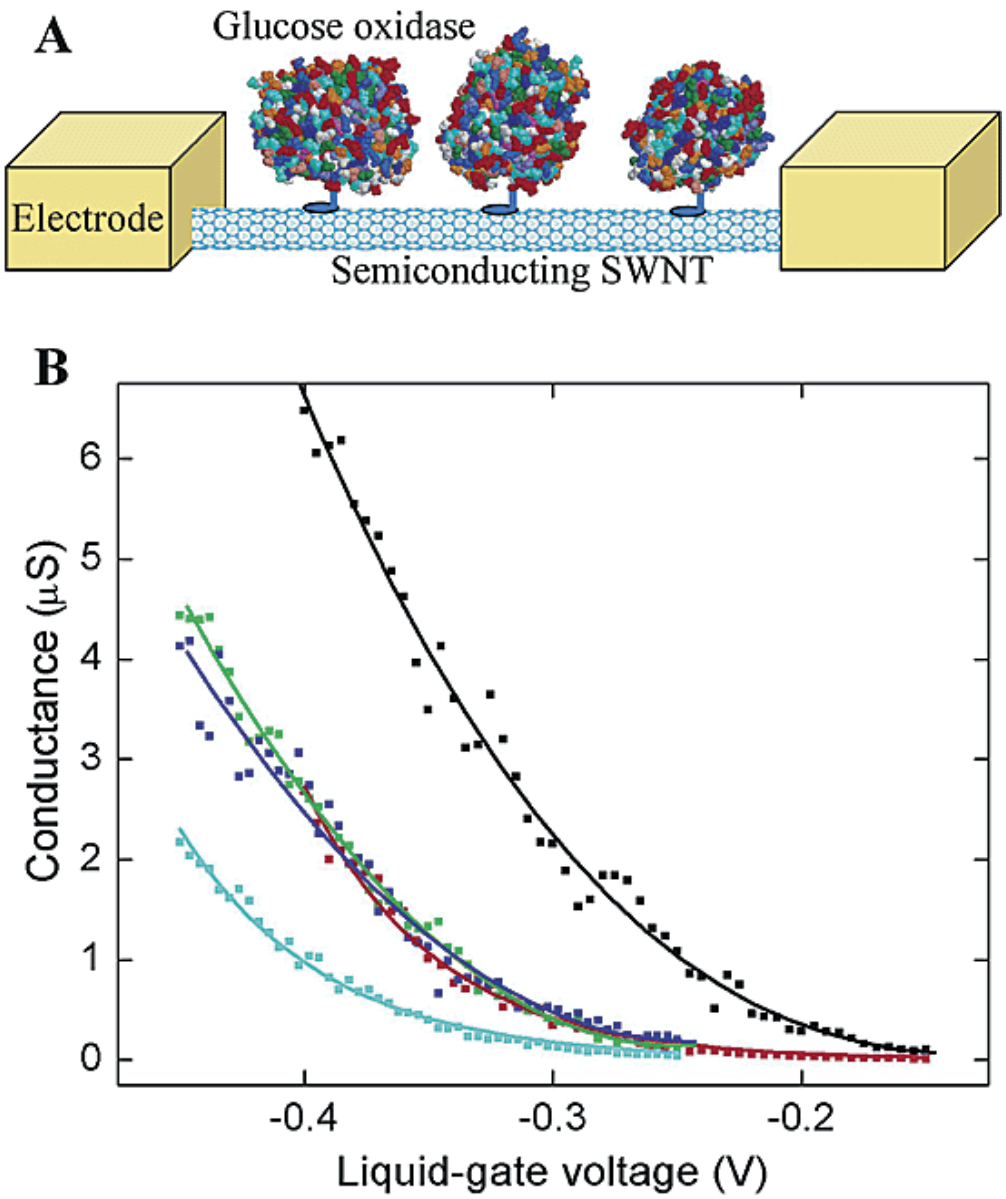


Figure 5. A) Schematic picture of two electrodes connecting a semiconducting SWNT with GOx enzymes immobilized on its surface. B) Conductance of a semiconducting SWNT as a function of the liquid-gate voltage in milli-Q water. Data are for the bare SWNT (black), after 2 h in DMF (red), after 4 h in DMF (green), after 2 h in DMF with the linking molecule (dark blue), and after GOx immobilization (light blue). Lines are guides to the eye. We observe that attachment of GOx significantly decreases $G(U_{lg})$. The source-drain voltage was kept constant at 9.1 mV for all measurements. Reproduced from ref. 291. Copyright@2003 American Chemical Society.

Other approaches have reported the manipulation techniques for single molecules detection utilizing nanomaterials to develop a more sensitive and selective systems applied to electrochemical devices [291-309]. In recent works, Besteman and co-workers [291] demonstrate the utilization of individual carbon nanotubes as semiconducting components as a versatile biosensor. The enzyme glucose oxidase was attached to the single-walled carbon nanotube (SWNT) to work as a real time pH sensor. This system was developed for the first time as a powerfull tool for enzymatic study by changes in conductance of GOx-coated semiconducting SWNTs upon addition of glucose. Also, these results indicate a promissing tool for the development of individual semiconducting sensor constructed at the single molecule level supported on a SWNT. Figure 5 shows the schematic representation of two electrodes connected by SWNT with GOx enzymes immobilized on its surface.

The lithography is an interesting technique for the development of interdigited circuits applied to electrochemistry and has been proposed as an interesting tool for obtention of miniaturized electrodes for electrochemical applications [310-320]. A new strategy was reported using ITO (indium tin oxide) semiconducting nanowires (ITO-NW) for bioelectrochemical studies of the enzyme GOx, in which the enzymatic activity was evaluated [310]. In this case, ITO-NW electrode was deposited on gold contacts on the top of a microchip with a protective polymer layer in which an aperture were achieve by photolithography technique to isolate metallic contacts. This approach opens a new tool for studying redox enzymes at the single molecule level, which can be used in bioelectrochemical devices. Other interesting approach reported a novel class of biochips for single molecule detection utilizing nanomaterials conjugated with enzymes. As an example, Hoeben and co-workers [321] utilized gold nanoelectrodes fabricated by lithography with dimensions down to 70x70 nm^2 to study a small quantity of redox enzymes on electrode surface. For this purpose, a SiO_2 Si_3N_4 /SiO_2 electrode was covered with PMMA and lithography technique was utilized to obtain a small electrode exposure area. Also, the development of nanoscale electrodes opens a new class of electrochemical biodevices applicable in recognition processes of biochemical reactions.

5. OTHER TECHNIQUES FOR NANOPLATFORMS-BASED ELECTROCHEMICAL BIOSENSORS

Many materials (graphenes, nanotubes, nanowires, nanoparticles, polymers, quantum dots, and others) have been used as platforms for incorporation of biomolecules (antibodies, enzymes, microorganisms, nucleic acids, organelles, tissues, etc), aiming the development of electrochemical biosensors for use in clinical chemistry, environmental and industrial monitoring, food safety, biological warfare agents detection, and others areas. The detection of target molecules is linked, among others factors, with the specificity of the probes and the unique optical, electronic, chemical and mechanical properties of these nanomaterials. Some characteristics of these nanomaterials are described below.

Graphene is a two-dimensional layer consisting of sp2 hybridized carbon atoms in hexagonal configuration. The properties of graphene are very interesting, including very large surface area (at 2630 m^2/g, twice of single-walled carbon nanotubes), high mechanical strength (200 times greater than steel), high elasticity and thermal conductivity [322].

Carbon nanotubes (CNTs) are hollow tubular structure formed by graphene sheets, metallic or semi-conducting properties [323, 324]. The main categories of CNTs are single-walled (single tube, SWNT) and multiwalled carbon nanotubes (multiple concentric tubes, MWNT). The ends of the tubes are usually closed off by a carbon cap. Others variations of CNTs include double-wall tube, bamboo and herringbone structures [325, 326]. CNTs have high electrical conductivity, extremely high mechanical strength and a large surface to volume ratio, resulting in surface phenomena predominating over the chemistry and physics that happen in the bulk [327, 328].Their unprecedented electrical properties have fostered their use in electrochemical biosensors [329]. Carbon nanotubes have been extensively studied as transducer element of biosensors that display high selectivity, sensitivity and real-time label-free detection [330-332].

Nanowires are metallic or semiconducting particles with cross-sectional diameters << 1 μm, and lengths as long as tens of microns. [333]. Nanowires can serve as electrodes or interconnects between micro- and nanoelectronic devices. Additionally their dimensions are on the same scale as biomolecules, possibiliting their interaction with biological species, such as cells, antibodies, DNA and proteins [334].Metallic nanoparticles have <1 μm in length, exhibiting unique optical response to light which allow them to resonantly scatter light when excited at their surface plasmon resonance frequency [332]. Metallic nanoparticles (gold, silver, platinum and others) provide highly conductive, high area surfaces for attachment of a high surface concentration for the capture of biomolecules and good biocompatibility [334].

Conducting polymers are polyconjugated polymers with electronic properties resembling those of metals, while retaining properties of conventional organic polymers. Conducting polymers have an electronic structure which is responsible for their electrical conductivity and low ionization potentials. Their intrinsic conductivity results from the formation of charge carriers upon oxidizing (*p*-doping) or reducing (*n*-doping) their conjugated backbone [335,336].

Quantum dots are colloidal fluorescent semiconductor nanocrystals, roughly spherical and typically composed of a combination of II–VI elements (CdS, CdSe, etc), or oxides, halides, tellurides and combinations of III–V elements, (InP and InAs). The size of a quantum dot is between 2 and 10nm in diameter. In larger sizes, the quantum confinement effect is lost, and fluorescence is not observed. [337].

5.2. Preparation of platforms based on nanomaterials

Other pathways to prepare platforms based on nanomaterials, aiming the fabrication of electrochemical biosensors are dispersion in solvents, adsorption, formation of covalent bonding and electropolymerization. These methods are described and illustrated below.

5.2.1. Dispersion

Dispersion of nanomaterials in solvents or interaction with dispersant agents is a method widely used for modification of electrodes. It is a simple and effective method and procedures as

prolonged heating, filtration and washing, which can damage the nanomaterials, generally are not required. The nanomaterials are simply dispersed in solution by sonication, resulting in a suspension. An aliquot is dropped on the electrode surface, followed by air drying, producing the modified electrode.

Several solvents can be used to dispersion of nanomaterials and production of electrochemical biosensors, for example dimethylformamide (detection of *Salmonella enterica serovar Typhimurium*) [338-340]. Other option is the use of dispersant agents to form suspensions of nanomaterials. Among these materials, one may cite chitosan, nafion, ciclodextrin and gelatin.

Chitosan is a polysaccharide with good biocompatibility, soluble in slightly acidic solution due to protonation of amino groups and presenting film-forming ability due to insolubility in solution with pH above pKa (6.3) [341]. Chen and col. described a nitrite biosensor prepared via immobilizing Cytochrome *c* onto glass carbon electrode modified with multi-walled carbon nanotubes–poly(amidoamine), using chitosan as dispersant [342].

Other interesting dispersant is nafion, a perfluorinated sulfonated cation exchanger, showing chemical inertness, thermal stability, mechanical strength, and antifouling property [343]. A modified electrode was prepared by dispersing grafene–CdS nanocomposite in ethanol with ultrasonic agitation. Nafion was mixed with ultrasonic agitation and the suspension was mixed with glucose oxidase solubilized in pH 7.4. The suspension was cast on the glassy carbon electrode and dried at 4 ◦C to form G–CdS–GOD modified electrode, producing a glucose biosensor [344]. Other example of nafion as dispersant is the fabrication of screen-printed electrodes (SPEs) modified with MWCNTs functionalized with carboxyl groups. The COOH-MWCNTs were dispersed in Nafion solution and dried in air. The resulting modified SPE was immersed in H_2SO_4 solution containing $HAuCl_4$ and Au nanoparticles were electrodeposited by cyclic voltammetry. This matrix was used for development of immunosensor for detection of Malaria [345]. β-Cyclodextrin is a cyclic oligosaccharide that consists of seven glucopyranose units. It has an electronic and hydrophobic interior microenvironment in its cavity structure, which allows hydrophobic molecules to be incorporated into its cavity [346] used carbon nanotubes dispersed in mixed solution of cyclodextrin/cyclodextrin prepolymer by ultrasonication to get black suspension, and used as modifiers to fabricate chemical modified electrode aiming the development of glucose biosensor.

Gelatin is the chief protein present in skin, bones and white connective tissues which is generally separated by the partial hydrolysis of collagen. It is non-toxic, non-irritant, biocompatible properties, and therefore has been widely utilized in the food and pharmaceutical industries [347]. Gelatin is composed of polypeptides of various sizes and it has a molecular weight distribution in the range of 15,000 to 250,000 prepared a MWCNT dispersion using gelatin as the dispersing agent. Glucose oxidase was immobilizated on glassy carbon electrode modified with this matrix to fabrication of amperometric glucose sensor [348].

5.2.2. Adsorption

Adsorption can be applied for incorporation of nanomaterials on electrode surfaces. This surface phenomen produces a film of ions, biomolecules or molecules of gas, liquid, or dissolved

solids to a surface. The bonding depends on the species involved, and the adsorption process can be classified as physisorption, chemisorption or electrostatic attraction

Tam and col. [349] applied the physisorption process to incorporate MWCNTs functionalized with carboxylic groups onto the electrode for production of genosensor for influenza virus. The functionalized MWCNTs were dispersed in ethylenediamine to form amine groups and, subsequently, the MWCNTs' dispersion was deposited at Pt interdigitated electrodes. The MWCNTs adhered to the electrodes by electrostatic force (van der Waals forces). DNA probe was connected by covalent bonds between the amine groups of the MWCNTs and the phosphate group of the DNA sequence.

Other methodology to immobilize biomolecules onto the chip surface makes use of the interaction between the thiol group and gold.

The technique is based on the fact the thiol groups are able to form a chemisorption or quasi-covalent bonding with gold [350, 351]. The model for these bonding is described by Tournier et al. [352] where gold nanoparticles were anchored to a gold electrode surface which had been previously modified with self-assembled monolayers of 1,6-hexanedithiol. The double-stranded DNA complex was chemi-absorbed to a gold electrode for produced an electrochemical biosensor for L-histidine (L-histidine-dependent DNAzymes) of according with Tournier *et al* [352] (Figure 6).

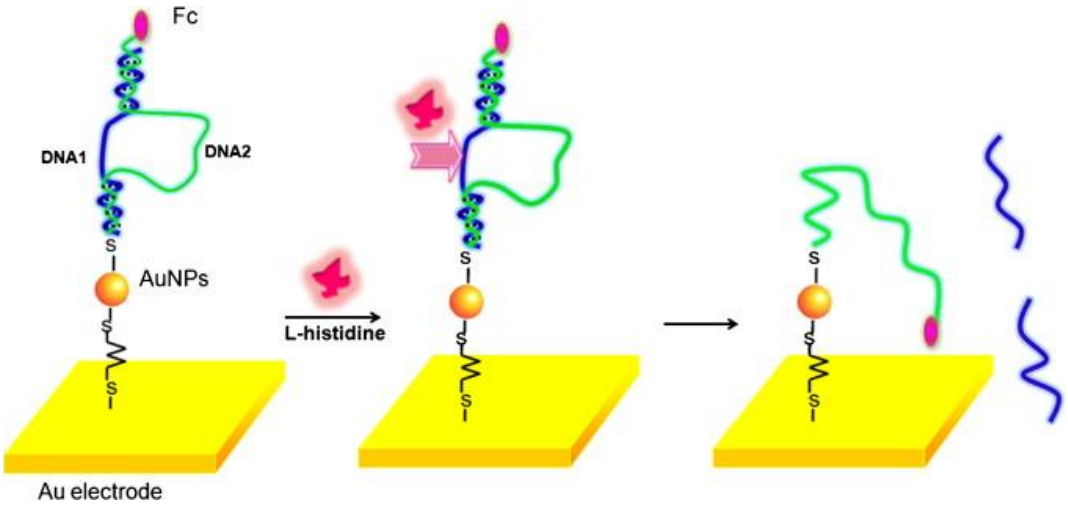


Figure 6. Schematic diagram of the sensing mechanism for the self-cleaving DNAzyme biosensor. Figure from ref. 352 with kind permission of The American Association of Immunologists.

Yang and co-workers [353] used electrostatic attraction to incorporate nanotubes on the glassy carbon surface. The process was based on MWNTs–chitosan suspension, prepared by ultrasonic agitation at pH 5.0. A glassy carbon electrode, dipped with MWNTs–chitosan solution, was polarized applying −3.0 V during 5 min. In this condition, H^+ in the solution is reduced to H_2 at the cathode, resulting in a pH gradient at the vicinity of the cathode. When the pH exceeds chitosan pKa, the natural polymer become insoluble and the electrodepositing MWNTs–chitosan onto glassy carbon electrode is completed. This modified electrode was used as the basis to develop apparatus for detection of human chorionic gonadotrophin.

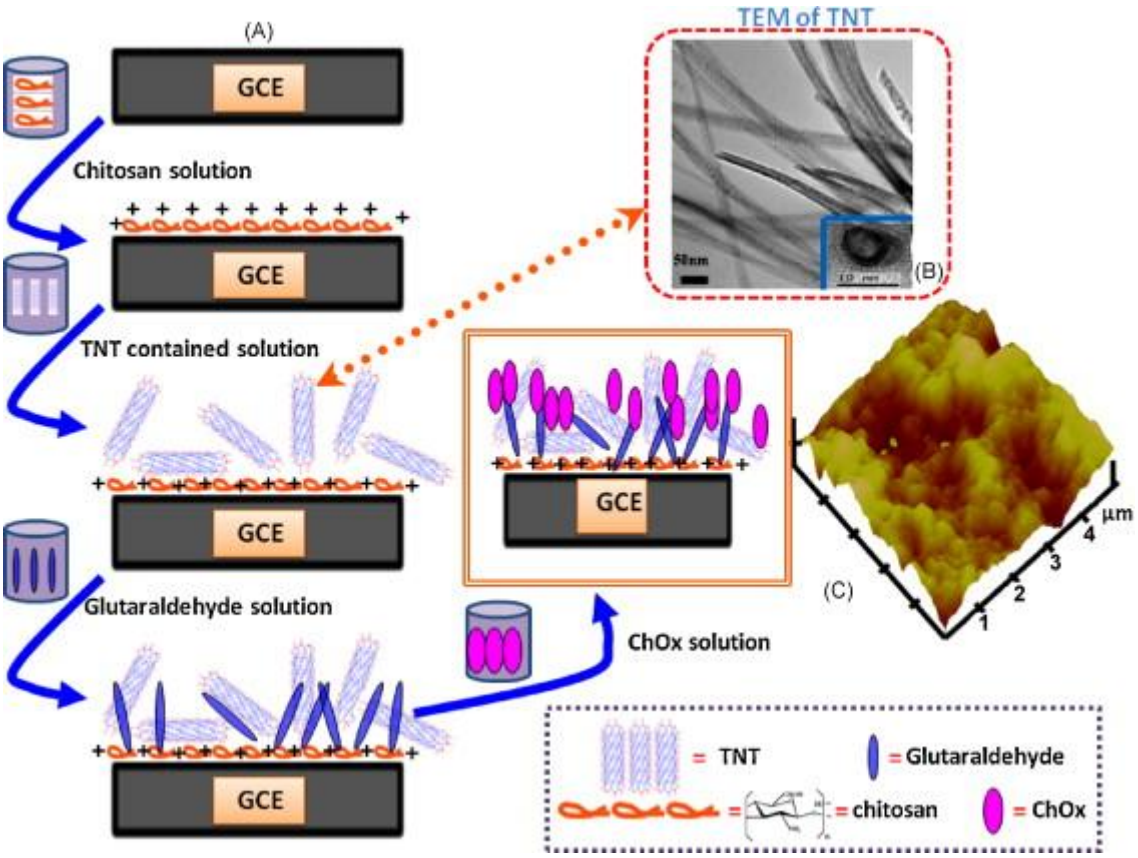


Figure 7. A schematic steps for fabrication of the ECL biosensor (A). Insets are TEM (B) and AFM (C) images of TNTs and ChOX/TNTs/CHIT film. Reproduced from ref. 354 with kind permission of Elsevier.

A variation of the method described previously was used for fabrication of biosensor based on enzyme/titanate nanotubes/chitosan composite film for the determination of choline in biological samples. Chitosan film modified electrode was prepared by coating chitosan solution on the surface of glass carbon electrode (GCE). The evaporation of water resulted in a thin chitosan film. The chitosan-modified GGC electrode was immersed into negatively charged titanate solution and washed with doubly distilled water. The choline oxidase was immobilized onto the modified electrode surface by cross-linking the enzyme with chitosan using glutaraldehyde (Figure 7) [354].

5.2.3. Covalent bonding

Modification of electrode surface by covalent bonding is advantageous, since it produces a more stable material [355]. Covalent functionalization of nanomaterials, such as carbon nanotubes, can be accomplished by either modification of the surface-bound carboxyl groups on the nanotubes or directly on the side walls of these materials. Yang and collaborators [356] described a label-free amperometric immunosensor for human chorionic gonadotrophin (hCG) based on multiwall carbon nanotubes–chitosan (CS) deposited on a glassy carbon electrode. In the following, thionine (Thi) was covalently bound onto the MWNTs–CS film with glutaraldehyde to obtain the Thi/MWNTs–CS film.

The free amino groups of the composite membrane were used to adsorb AuNPs–TiO2 hybrid for biosensor development.

In other studies, conventional-size glassy carbon electrodes and glassy carbon fiber microelectrodes have been covalently modified with of *p*-phenylacetic acid diazonium fluoroborate in acetonitrile. The covalent modification of carbon electrodes using a diazonium salt coupling procedure has been reported in the literature [357]. Gold nanoparticles functionalized with 4-aminothiophenol (4Atp) and modified with thionine (Thi-4Atp@GNPs) by covalent bonding and was dipped into nano-Au to obtain a well-aligned self-assembled GNP monolayer for the construction of a label-free immunosensor to detect the presence of an carcinoembryonic antigen [358].

5.2.4. Electropolymerization

The deposition of nanostructures onto the electrode surface can be carried out using chemical or electrochemical techniques. The most widely used technique is the oxidative coupling involving the oxidation of monomers to form a cation radical followed by coupling to form di-cations in successive repetitions leading the polymer production.

Polymers have gained a remarkable position in the biomedical field as materials for fabrication of various devices and for tissue engineering applications. Electropolymerization has received great attention in recent years as a good approach to prepare immobilization matrices for biosensors [359, 360]. Adjusting the electrochemical parameters it is possible control film thickness, permeation and charge transport characteristics. Besides this, polymer films have good stability, reproducibility, more active sites, homogeneity in electrochemical deposition and strong adherence to electrode surface [361, 362]. The modification of surfaces with polymeric films has been used in the development of biosensors to protect the surface of the electrodes from impurities, block interfering, incorporating mediators and provide biocompatibility [363].

Electrodeposition of aminophenols onto electrodes using cyclic voltammetry produces an interesting platform for biosensor construction. Monomer 4-aminophenol was electropolymerized on graphite electrode through continuous cycling of the potential aiming the bioelectrode production for *Mycobacterium leprae* [364]. The same platform shows efficiency for detection of purines bases (ATP and GTP) of DNA when compared with bare graphite surfaces [365].

Other polymer derived from aminophenol was electrodeposited onto Pt nanoclusters embedded polypyrrole nanowires. Platinum nanoclusters were electrochemically deposited by cyclic voltammetry. After synthesizing the polypyrrole nanowires and Pt nanoclusters on the electrode, the designed glucose sensor was fabricated by electrodeposition of o-aminophenol on the polypyrrole-Pt/GCE and glucose oxidase [366].

Graphite electrode modified with poly(4-hydroxyphenylacetic acid) electrodeposited by potential cycling was utilized for construction of genosensor for oligonucleotide and dengue serotype 1 (DENV-1) [367]. AFM images were used to demonstrate the efficiency of hybridization process (Figure 7). The same platform was utilized for construction of impedimetric immunosensor for *leishmania chagasi* [368,369].

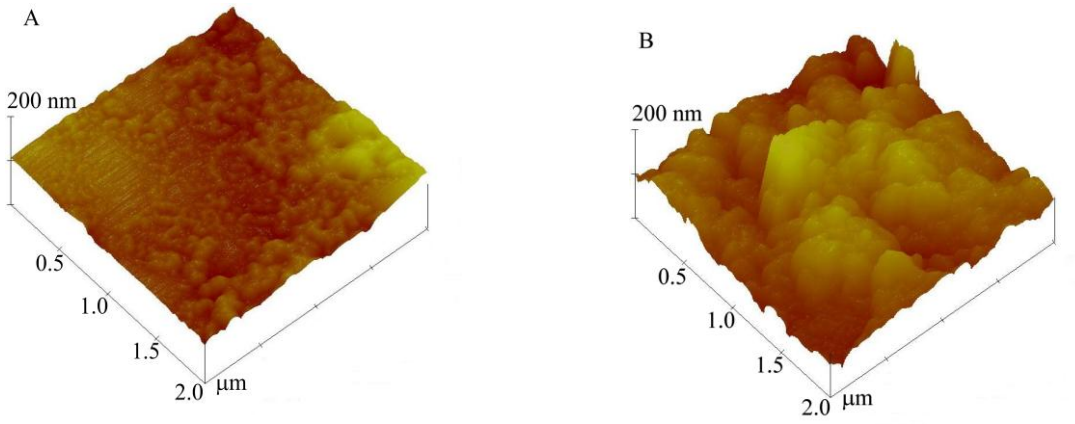


Figure 8. AFM topographical images of modified graphite electrode with poly(4-HPA): (A) before hybridization (B) after hybridization with the complementary target. Reproduced from ref. 368 with kind permission of Elsevier.

A glucose biosensor was developed by Labib *et al* [370] based on gold nanoparticles fixed on a gold electrode modified with polytyramine. Electropolymerization of tyramine was performed from solution of tyramine by cyclic voltammetry. Other glucose biosensor was prepared using polyaniline via oxidative graft polymerization by cyclic voltammetry [371]. A multilayer made by layer-by-layer assembly of single-walled carbon nanotubes modified with glucose oxidase and cationic polymer by electrodeposition of poly(1-vinylimidazole) on a screen-printed carbon electrode surface was developed [372].

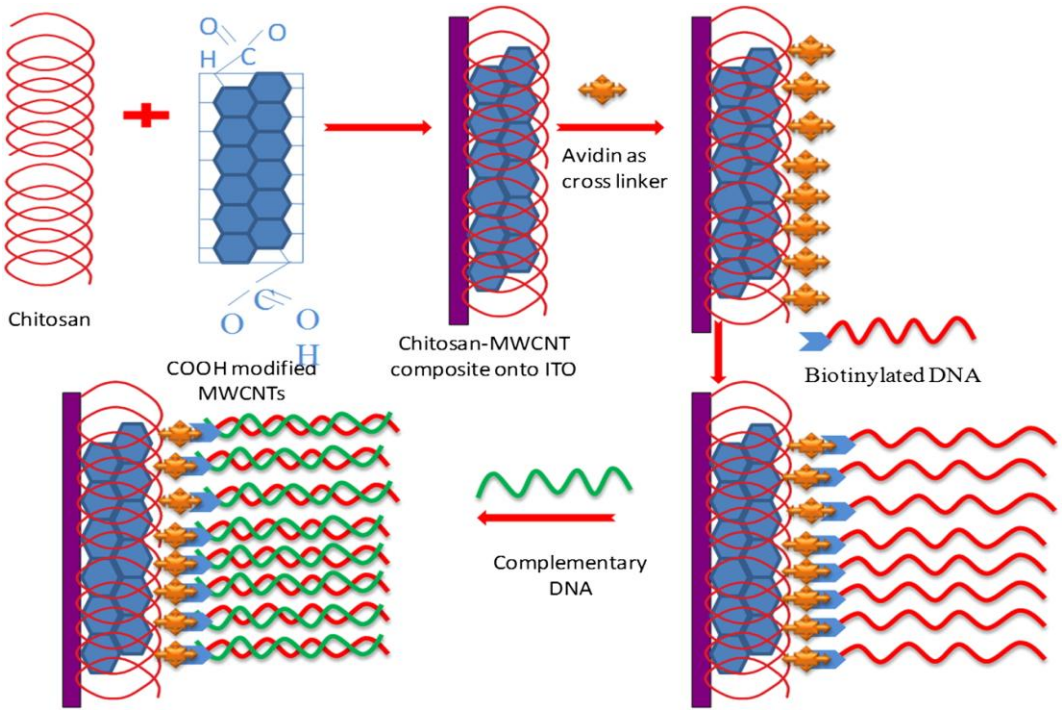


Figure 9. Schematic on preparation of BDNA–Avi–Chit–MWCNT/ITO electrode and DNA hybridization. Reproduced from ref. 374 with kind permission of Elsevier.

Direct electrochemistry of hemoglobin was obtained by immobilizing Hb–chitosan on a gold colloid nanoparticles (AuNPs)/3-aminopropyl triethylene silane (APS)/Prussian blue (PB) composite film-modified glassy carbon electrode. PB was deposited on glassy carbon electrode by applying a constant potential. In sequence, the modified electrode was immersed in APS to introduce the amine functional groups. The modified electrode was dipped into the AuNPs solution [373] and used to construction of hemoglobin biosensor.

Singh et al. deposited carbon nanotubes functionalized with carboxyl onto indium-tin-oxide (ITO) coated glass plate and modified with chitosan by electro chemical polymerization. The polymerization has been performed chronoamperometrically (Figure 9). These nanobiocomposite based in chitosan-MWCNT was utilized as platform for indirect electrochemical detection of gonorrhoe [374].

6. PERSPECTIVE AND FUTURE IN NANO-MANIPULATION FOR APPLICATION IN ELECTROCHEMICAL BIOSENSORS

The insertion of enzymes and other molecules with recognition properties in Langmuir-Blodgett films of substances presenting similar amphipathicity offers the possibility to diversify the architecture of ultrathin films controlling their molecular interactions with high precision. For many years, Langmuir technology has been used to investigate the interaction among lipids and other classical amphiphilies, and only in the last two decades, a more considering number of papers on the interaction of biomolecules, such as enzymes, DNAs, and antibodies at air-water interface has been reported. This strategy opens the way to construct new and sophisticated materials for sensors using hybrid materials, which have been employed to detect several kinds of substances with diverse techniques, including electrochemical. The utilization of LBL technique can be another interesting approach to produce very sensitive electrochemical biodevices utilizing different architectures on the fabrication of the nanostructured films with simplicity. Moreover, the possibility to integrate nanomaterials on films structures have attracted much attention due to the possibility on technological applications and more recently, the possibility to study mechanistic processes of biological molecules at molecular and atomic level. Also, the improvement of nano-manipulation techniques opens a new path for a more sensitive and selective electrochemical biodevices development utilizing enzymes, proteins and DNA as specific recognition elements. Furthermore, the major challenges for electrochemical biosensors using nanomaterials include optimization of the fabrication, aiming higher specificity, reproducibility, stability, miniaturization and integration of the devices. The validation using real samples is another important challenge in the point-of-care applications.

7. CONCLUSIONS

The utilization of nano-manipulation techniques has become an interesting approach to fabricate electrochemical devices with high specificity and molecular order. The utilization of LB and LBL methods has received special attention due to their relative simplicity and became an important

tool in the field of electrochemistry to produce new and more sensitive biodevices. In this review we presented an overview of recent advances in nano-manipulation techniques to produce platforms for electrochemical biosensing utilizing metal nanoparticles, carbon nanotubes and graphene sheets as interesting nanomaterials for biomolecules immobilization. Also, we described several interesting approaches reported in the last years as promising materials for technological applications, in which enzymes and DNA can be employed as recognition elements for electrochemical biosensing.

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