Review

Electrochemical nanobiosensors

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Abstract

This review discusses main techniques and methods which use nanoscale materials for construction of electrochemical biosensors. Described approaches include nanotube and nanoparticle-based electrodes relying on aligned nanotube arrays, direct electron transfer between biomolecule and electrode, novel binding materials and mass production technology; and nanoscale materials as biomolecule tracers, including gold nanoparticles, quantum dots for DNA and protein multiplexing, novel nanobiolabels as apoferritin, liposomes and enzyme tags loaded carbon nanotubes. Specific issues related to electrochemistry of nanoscale materials are discussed. Various applications for genomic and proteomic analysis are described.
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Keywords: Nanomaterials; Biosensors; DNA; Protein; Enzyme; Nanoparticle; Carbon nanotube; Review

Contents

1. Introduction

Nanomaterials are acquiring a big impact on development of electrochemical biosensors. Nanotechnology brings new possibilities for biosensors construction and for developing novel electrochemical bioassays. Nanoscale materials have been used to achieve direct wiring of enzymes to electrode surface, to promote electrochemical reaction, to impose nanobarcode for biomaterials and to amplify signal of biorecognition event. The electrochemical nanobiosensors were applied in areas of cancer diagnostics and detection of infectious organisms. This article reviews the important achievements in the field of amperometric and voltammetric electrochemical nanobiosensors.

The use of nanoscale materials for electrochemical biosensing have seen explosive growth in the past 5 years, since discovery of low-potential detection of NADH on carbon nanotube-modified electrode by Wang and co-workers [1] and the first use of gold nanoparticles as labels for electrochemical

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immunosensors by Limoges and co-workers [2]. Hundreds of research articles using nanomaterials for electrochemical bioassays have been published since then. There are several reviews available which partly deal with use of nanomaterials for amperometric or voltammetric electrochemical nanobiosensors [3–6], more detailed reviews on carbon nanotube-based sensors [7–11] and nanoparticles-based biosensing [12–16] can be found.

The aim of this paper is to review important achievements in the field of amperometric and voltammetric electrochemical nanobiosensors. The review will be divided to following two parts: nanoscale materials electrodes for bioassays and nanomaterials as biomolecule tracers.

2. Nanoscale materials electrodes

2.1. Enzymatic biosensors

The advantages of carbon nanotubes, such as high surface area, favorable electronic properties and electrocatalytic effect attracted very recently considerable attention for the construction of electrochemical enzyme biosensors. The first report of carbon nanotube-modified electrode for biosensing was based on dispersion of CNT and enzyme in Teflon binder [17]. Wang and Musameh [17] showed that the electrocatalytic activity of CNT toward hydrogen peroxide and NADH permitted effective low-potential amperometric biosensing of glucose and ethanol in connection with the incorporation of glucose oxidase and alcohol dehydrogenase/NAD⁺ within the three-dimensional CNT/Teflon matrix. The authors found that accelerated electron transfer was coupled with minimization of surface fouling. These advantages of CNT-based composite devices were illustrated from comparison to their graphite/Teflon counterparts, which clearly demonstrated higher sensitivity of CNT/Teflon biocomposite. Electrocatalytic effect of CNT was explained by Compton's group, which showed that the electrochemical behavior of open ends of CNT is similar to edge plane pyrolytic graphite and the electrochemical behavior of CNT walls is similar to basal plane of pyrolytic graphite [18,19].

CNT and glucose oxidase were also incorporated in paste electrodes using oil as binder for glucose biosensing [20] and later on the variety of CNT/paste incorporated enzymes was expanded toward lactate oxidase, polyphenol oxidase and alcohol dehydrogenase/NAD⁺ [21]. Construction of CNT/paste biosensors was extended toward incorporation electrocatalytic nanocrystals to CNT/paste matrix for even lower potential and higher sensitivity biosensing [22] (for explanation of role of nanoparticles in enhancing electrocatalytic activity of CNT, see [23,24]). Analogous type of biosensor was prepared incorporation laccase in CNT/chitosan matrix [25].

However, one of the main disadvantages of paste electrodes is their poor mechanical properties. More rigid carbon nanotube biosensors were constructed by incorporating carbon nanotubes and glucose oxidase in epoxy matrix [26,27]. Screen-printed CNT sensors, based on thick-film fabrication, are mechanically stable with good resistance to mechanical abrasion and they offer possibility of large-scale mass production of highly reproducible low-cost electrochemical biosensors [28]. CNT matrix also allows easy incorporation of enzyme in screen-printed electrode, as it was demonstrated recently on example of horseradish peroxidase in connection to MWCNT and polysulfone binder [29] (see Fig. 1). The apparent Michaelis–Menten constant K_M^{app} was calculated to be 0.71 mM. This K_M^{app} indicates that the enzyme immobilized in the carbon nanotube/polysulfone biocomposite keeps its activity with a very low diffusion barrier. This K_M^{app} value is smaller than others for H_2O_2 biosensors based on sol–gel (4.6 mM) [30], on siloxane homopolymer (2.5 mM) [31] and other composites (2.0 mM) [32]. This is a great advantage over other composites since the carbon nanotube/polysulfone membrane maintains the conducting properties of MWCNT and allows the easy and fast incorporation of the enzyme with a very low K_M^{app} and high sensitivity (0.12 μA/mM).
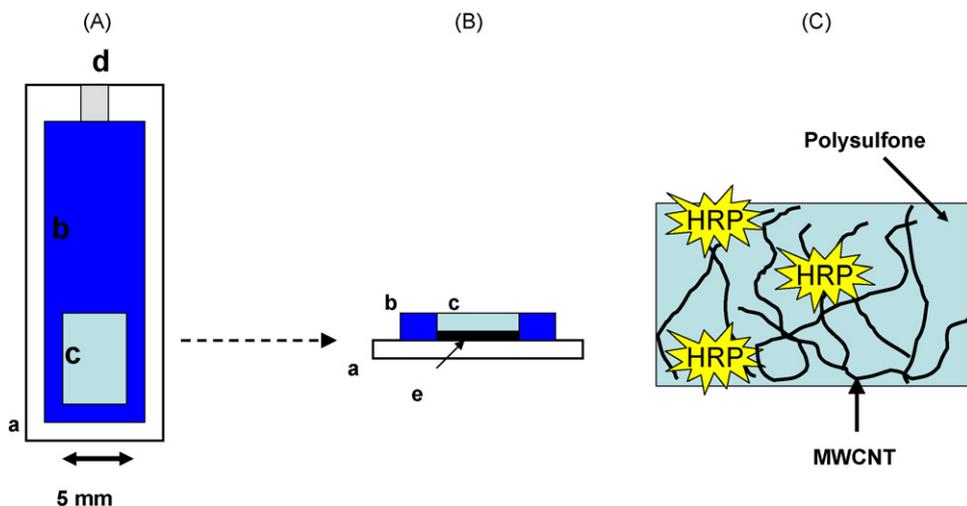
Fig. 1. (A) The enzyme/MWCNT/polysulfone screen-printed thick-film electrochemical detector, top view. (B) Cross-section of the detection area of enzyme/MWCNT/polysulfone screen-printed detector. (C) Schematic drawing of showing structure of HRP/MWCNT/PS composite. (a) Polycarbonate substrate, (b) insulator layer, (c) HRP/MWCNT/polysulfone conducting composite, (d) silver contact for the working electrode and (e) carbon ink contact layer. Modified from [29] with permission.
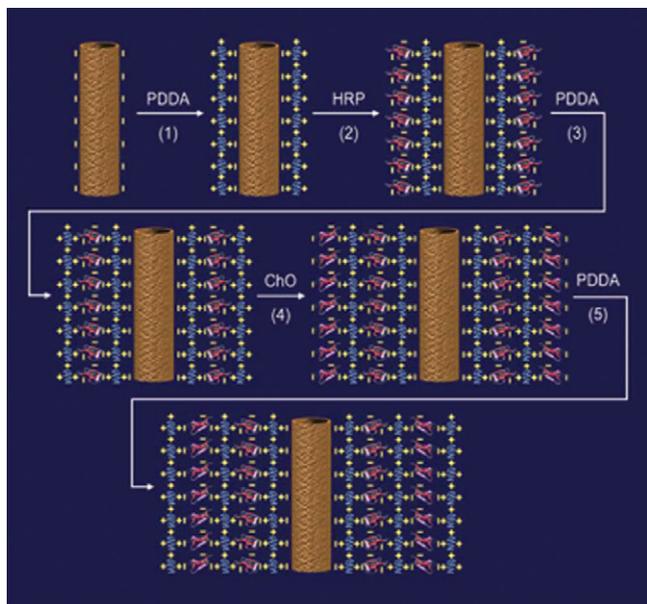
Fig. 2. Layer-by-layer coating of CNT with enzymes. Schematic illustration of the procedure for the attachment of choline oxidase (ChO) and horseradish peroxidase (HRP) on carbon nanotube surface through a layer-by-layer technique. Reproduced from [36] with permission.

Elegant way to prepare biosensor is to coat carbon nanotube with one or multiple layers of enzyme by layer-by-layer process [33,34]. Glucose oxidase (GOx) can be immobilized on the negatively charged carbon nanotube surface by alternatively assembling a cationic poly(diallyldimethylammonium)chloride (PDDA) layer and GOx layer. The sandwich-like layer structure (PDDA/GOx/PDDA/CNT) formed by self-assembly technique provided a microenvironment to keep the bioactivity of GOx and it prevented enzyme molecule leakage. The strong electrocatalytic activity toward hydrogen peroxide of the fabricated PDDA/GOx/PDDA/CNT electrode indicated that the polyelectrolyte–protein multilayer did not affect the electrocatalytic properties of CNT, enabling sensitive determination of glucose. For example, of coating process, see Fig. 2. Bi-enzyme CNT nerve agent biosensor was prepared by same group using this layer-by-layer assembling approach of PDDA, horseradish peroxidase (HRP) and choline oxidase (ChO). A bioactive nanocomposite film of PDDA/ChO/PDDA/HRP/PDDA/CNT (ChO/HRP/CNT) and PDDA/ChO/PDDA/CNT (ChO/CNT) was fabricated on the GC surface. Owing to the electrocatalytic effect of carbon nanotubes, the measurement of faradic responses resulting from enzymatic reactions was realized at low potential [35,36]. By using the layer-by-layer method, homogeneous and stable ChO/polyaniline/MWCNT biosensor for choline detection was also prepared [37]. Employing similar approach, glucose nanobiosensor was prepared by forming bilayer of the polyelectrolytes PDDA and poly(sodium 4-styrenesulfonate) (PSS) on a 3-mercapto-1-propanesulfonic acid-modified Au electrode and subsequent consecutive layer-by-layer addition of multiwall carbon nanotubes wrapped by positively charged PDDA and negatively charged glucose oxidase onto the PSS-terminated bilayer [38].

Single-wall carbon nanotubes were employed as long-range wires connecting surface of electrode with redox center of enzyme [39,40]. Yu et al. [39] attached enzymes covalently onto the ends of vertically oriented single-wall carbon nanotube (SWCNT) forest arrays, which were used as nanoelectrodes. Quasi-reversible Fe^{III}/Fe^{II} voltammetry was observed for the iron heme enzymes myoglobin and horseradish peroxidase coupled to carboxylated ends of the nanotube forests by amide linkages. Authors suggested that the "trees" in the nanotube forest behaved electrically similar to a metal, conducting electrons from the external circuit to the redox sites of the enzymes. In other work, SWCNT was covalently linked to gold electrode surface and to glucose oxidase redox center [40]. Authors estimated the turnover rate of electrons transferred via SWCNT to the electrode surface to be about $4100 s^{-1}$, which is about six-fold higher than the turnover rate of electrons from the active site of GOx to its natural O_2 electron acceptor ($700 s^{-1}$). Such fast electron transfer makes this glucose sensor oxygen independent. Even faster electron turnover rate (about $5000 s^{-1}$) was provided by gold nanoparticles covalently linked to electrode surface and to GOx redox center [41]. See Fig. 3 to elucidate covalent pluging GOx redox center to electrode surface via Au nanoparticle.

Fullerene C_{60} was used as an electron mediator for electrocatalyzed biotransformations [42]. A fullerene carboxylic derivative was covalently attached to a cystamine-monolayer-functionalized Au electrode. C_{60} provided electrical communication between the electrode and a soluble glucose oxidase (without covalent link between C_{60} and GOx redox center). CdS quantum dots were also used as an electron mediator for glucose oxidase (without covalent link between CdS and enzyme) [43]. The enzyme demonstrated significantly enhanced electron-transfer reactivity and glucose oxidase adsorbed on CdS nanoparticles maintained its bioactivity and structure.

2.2. Genosensors

Carbon nanotube and nanoparticle electrodes play important role in DNA electrochemical sensing for reasons similar to their role in enzyme biosensors: high surface area, fast heterogenous electron transfer and long-range electron transfer.

MWCNT electrode was used for label-free detection of DNA hybridization [44,45]. Enhanced guanine signal was attributed to a CNT-induced interfacial accumulation, rather than to an electrocatalytic reaction. Increase of guanine and adenine oxidation peaks (versus glassy carbon electrode) was also observed by other group and applied to label-free analysis of calf thymus DNA [46]. Having in mind that bamboo-like multiwall carbon nanotubes (BCNT) provide more edge planes of graphene than SWCNT, Gooding's group investigated their application to indicator-free DNA detection [47]. It was found that BCNT have superior electrochemical performance over SWCNT from following aspects: (a) peak separation of oxidation and reduction waves showed faster electron transfer on BCNT (versus SWCNT) and (b) greater number of electroactive sites along the walls of BCNT resulted in larger current signals and more broad dynamic range for oxidation of DNA bases (versus SWCNT).
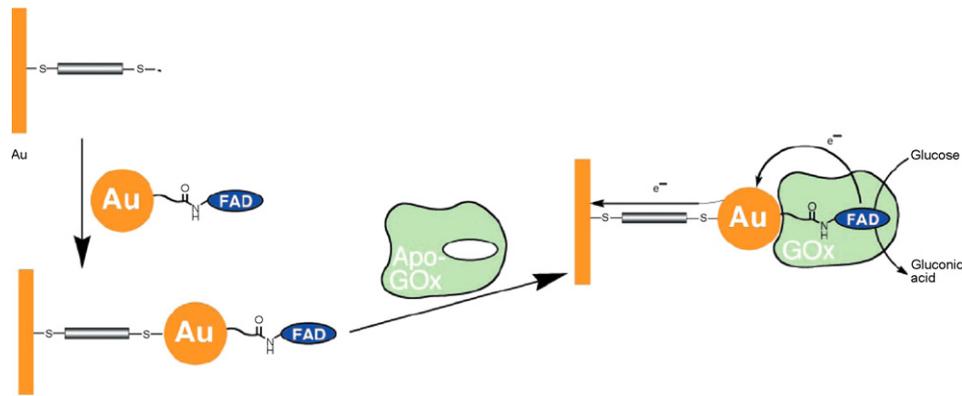
Fig. 3. Assembly of gold-nanoparticle-reconstituted GOx electrode by the adsorption of gold nanoparticles functionalized with FAD on the dithiol-modified Au electrode followed by the reconstitution of apo-GOx on the functional gold nanoparticles. Based on [41] with permission.

Carbon nanotubes can also promote electron transfer between the electroactive centers of biological cells and electrode [48]. Leukemia K562 cells exhibited well-defined anodic peak of guanine. This was used for monitoring of efficiency of anti-tumor drugs on the Leukemia K562 cells.

It is known that performance of electrodes with respect to speed and spatial resolution increases when electrode radius decreases. In this sense, the array of vertically aligned, SiO_2 insulated MWCNT electrodes was constructed and used for ultrasensitive detection of DNA [49]. The open ends of aligned,

insulated multiwall carbon nanotube electrodes were derivatized by carbodiimide chemistry and probe DNA was attached. $Ru(bpy)_3^{2+}$ was used as mediator of guanine base oxidation of target molecules (for scheme, see Fig. 4). The detection limit was found to be very low (few attomoles) and authors expect it to be lowered after assay optimization down to thousands DNA molecules.

CNT-modified transducers showed also amplification role in DNA hybridization detection using enzyme label [50]. Signal of enzymatic reaction product (α-naphtol) showed about 30-
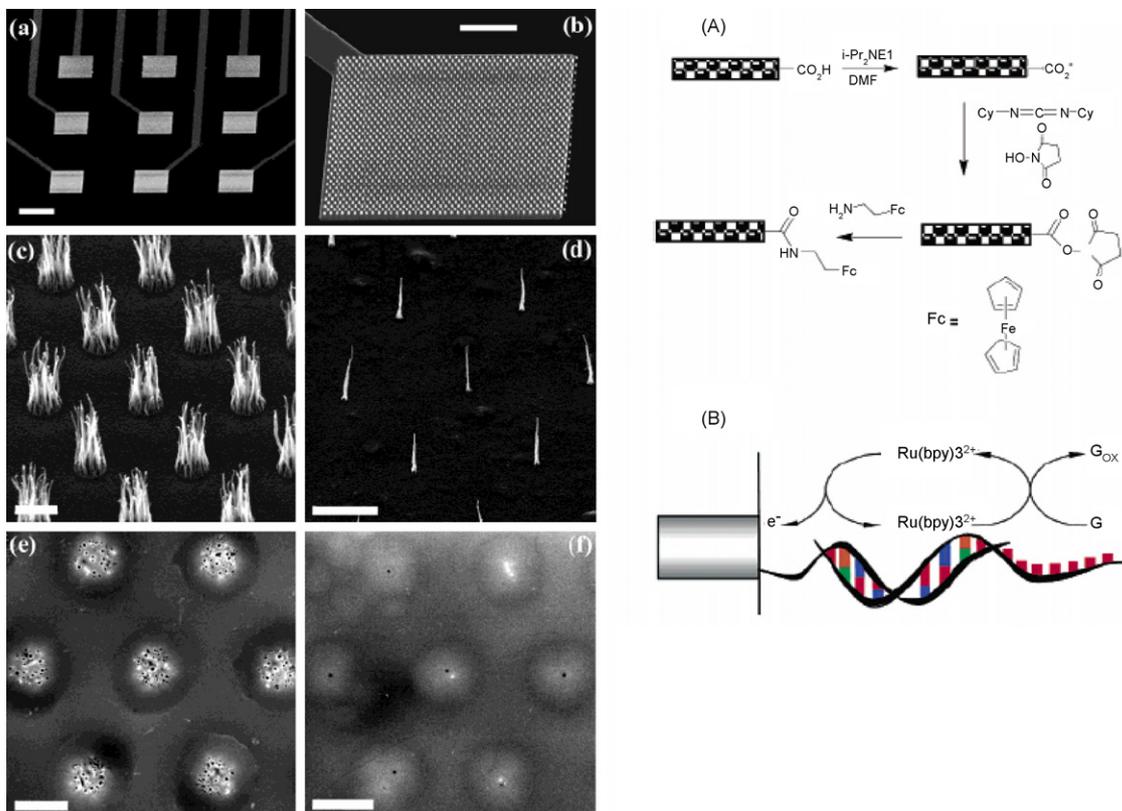
Fig. 4. MWCNT electrode array for DNA detection. SEM images of (a) 3×3 electrode array, (b) array of MWCNT bundles on one of the electrode pads, (c and d) array of MWCNTs at UV-lithography and e-beam patterned Ni spots, respectively, and (e and f) the surface of polished MWCNT array electrodes grown on $2\,\mu m$ and $200\,nm$ spots, respectively. Panels (a–d) are $45°$ perspective views and panels (e and f) are top views. The scale bars are 200, 50, 2, 5, 2 and $2\,\mu m$, respectively. (A) The functionalization process of the amine-terminated ferrocene derivative to CNT ends by carbodiimide chemistry. (B) The schematic mechanism of $Ru(bpy)_3^{2+}$-mediated guanine oxidation. Reproduced from [49] with permission.

fold increase versus glassy carbon electrode. This was attributed to strong adsorptive accumulation of α-naphtol on the CNT layer.

Platinum nanoparticles were used in combination with MWCNTs for improving detection limits of electrochemical DNA biosensor [51]. Multiwalled carbon nanotubes and platinum nanoparticles were dispersed in Nafion, which were used to fabricate the modification of the glassy carbon electrode (GC) surface. Oligonucleotides with amino groups at the 5′ end were covalently linked onto carboxylic groups of MWCNTs on the electrode. The hybridization events were monitored by differential pulse voltammetry (DPV) measurement of the intercalated daunomycin. Due to the ability of carbon nanotubes to promote electron-transfer reactions, the high catalytic activities of platinum nanoparticles for chemical reactions, the sensitivity of presented electrochemical DNA biosensors was high. The detection limit of the method for target DNA was 1×10^{-11} M.

Gold-nanoparticle-modified electrodes were used to enhance the amount of immobilized probe DNA [52]. Hybridization was induced by exposure of the ssDNA-containing gold electrode to ferrocenecarboxaldehyde-labeled complementary DNA in solution. The detection limit was 5×10^{-10} M of complementary DNA.

2.3. Immunosensors

CNT can act as both an electrode and an immobilization phase in an electrochemiluminescence (ECL)-based sensing device [53]. The poly(ethylene vinylacetate) (EVA) was used as binder to produce a carbon nanotube–EVA–antibody (specific to α-fetoprotein) composite sheets. The immunoassay was carried out in sandwich design by exposing CNT–EVA sheets to a sample containing α-fetoprotein (AFP) and anti-AFP antibodies conjugated with colloidal gold or Ru(bpy)$_3{}^{2+}$. The SEM observations and ECL measurements verified that sandwich immunoassays complexes formed on the surface of the nanotube–EVA composites and that the formation of these complexes was biospecific. The ECL signal was linearly dependent on the concentration of AFP up to AFP concentrations of 30 nM and limit of detection was about 0.1 nM.

A carbon nanotube thick-film composite screen-printed immunosensor was constructed using polysulfone (PS) as binder [54]. This matrix retained the RIgG antibody at the surface of screen-printed electrode. The combination of MWCNT, polysulfone and antibodies resulted into a novel composite material, consisting of an interconnected CNT–polymer network, and possessing mechanical flexibility, high toughness and high porosity. SEM proved the significant difference in porosity between MWCNT/PS and graphite/PS nanocomposites. The amperometric measurements showed a six times higher sensitivity for MWCNT biocomposite comparing with graphite biocomposite. MWCNT/PS biocomposite retains electrochemical behavior of MWCNT electrodes, the biocompatibility of PS binder and acts as integration matrix for all elements needed for the production of a complex biocomposite.

Amperometric immunosensor based on the adsorption of antibodies onto perpendicularly oriented assemblies of single-wall carbon nanotubes called SWCNT forests was developed [55]. The forests were self-assembled from oxidatively shortened SWCNTs onto Nafion/iron oxide-coated pyrolytic graphite electrodes. Anti-biotin antibody was strongly adsorbed to the SWCNT forests. In the presence of a soluble mediator, the detection limit for horseradish peroxidase-labeled biotin was 2.5 nM. Unlabeled biotin was detected in a competitive approach with a detection limit of 16 μM. Improved fabrication of SWCNT forests utilizing aged nanotube dispersions provided higher nanotube density and conductivity [56]. Unmediated sandwich immunosensors achieved a detection limit of 75 nM using HRP labels. However, mediation dramatically lowered the detection limit to 1 nM (for scheme of CNT forest-based immunoassay, see Fig. 5A). Authors concluded that the difference between mediated and unmediated assays is due to the fact that the average distance between HRP labels and nanotube ends is too large for efficient direct electron exchange, which can be overcome by electron mediation.

Electrochemical immunosensor for cholera toxin was developed based on poly(3,4-ethylenedioxythiophene)-coated carbon nanotubes [57]. The sensing interface consists of monoclonal antibody against the B subunit of cholera toxin that is linked to poly(3,4-ethylenedioxythiophene) coated on Nafion-supported multiwalled carbon nanotube casted film on a glassy carbon electrode. The cholera toxin (CT) was detected by a "sandwich-type" assay on the electronic transducers, where the toxin is first bound to the anti-CT antibody and then to the ganglioside-functionalized liposome loaded with potassium ferrocyanide for amplification. For schematic depiction of the assay, see Fig. 5B.

It was also demonstrated that CNT can play a dual role in recognition and transduction events, acting as carriers for enzymes and for accumulating the products released from the enzymatic reaction involved in antigen–antibody recognition, in analogous matter as in DNA recognition event [50,58].

The gold nanoparticles have been also used for construction of electrochemical immunosensors. Amperometric and potentiometric immunosensors based on gold nanoparticles/tris(2,2′-bipyridyl)cobalt(III) multilayer films for hepatitis B surface antigen determination were constructed [59]. Layer of plasma-polymerized Nafion film (PPF) was deposited on the platinum electrode surface, then positively charged tris(2,2-bipyridyl)cobalt(III) (Co(bpy)$_3{}^{3+}$) and negatively charged gold nanoparticles were assembled on the PPF-modified Pt electrode by layer-by-layer technique and hepatitis B surface antibody (HBsAb) was electrostatically adsorbed on the gold nanoparticles surface (for scheme of multilayer construction, see Fig. 6). The immunosensors were used to analyze HBsAg in human serum samples and the results were comparable to those obtained from standard ELISA method.

3. Nanomaterials as biomolecule tracers

Nanomaterials have been widely used as biomolecule tracers for electrochemical biosensing since year 2000, when Limoges and co-workers published their work on gold nanoparticle-labeled electrochemical immunoassay [2]. Nanoparticles are very stable (comparing to enzyme labels), they offer high sensi-
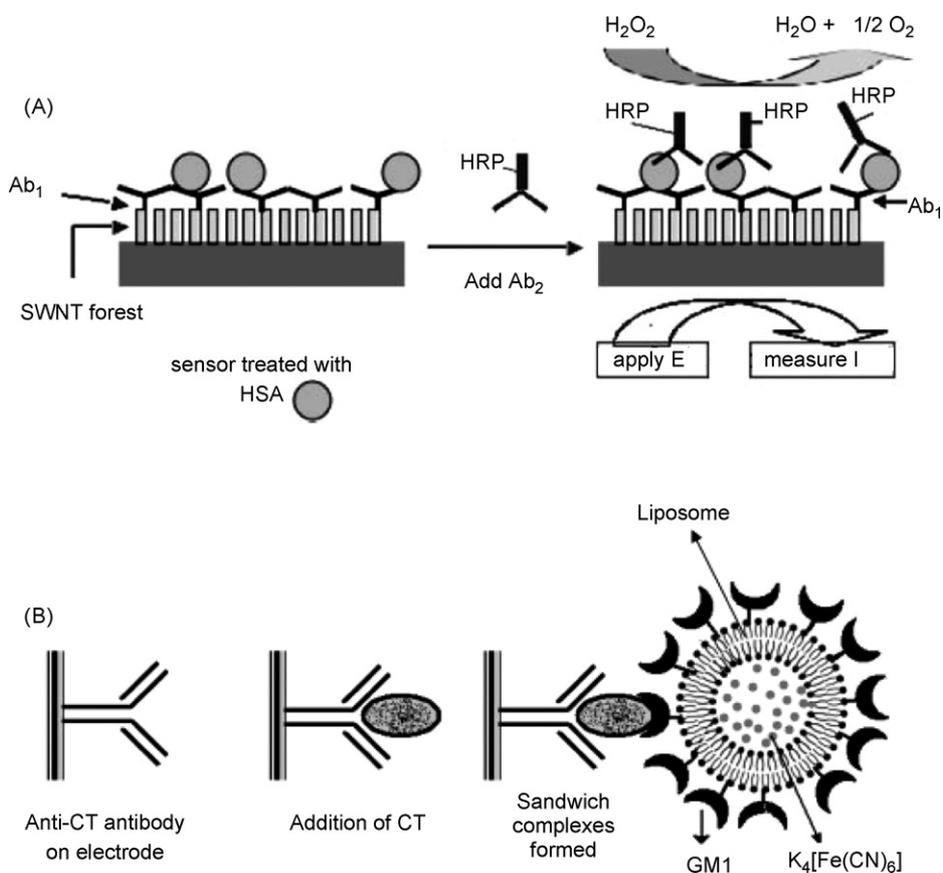
Fig. 5. (A) Schematic diagram of carbon nanotube forest sandwich immunoassay procedure. (B) Schematic outlines of electrochemical immunosensor for cholera toxin based on poly(3,4-ethylenedioxythiophene)-coated carbon nanotubes with liposome amplification. Reproduced from [56] (A) and [57] (B) with permission.

tivity (thousands of atoms can be released from one nanoparticle) and wide variety of nanoparticles opens doors for multiplexing. Nanoparticles are used nowadays as electrochemical label or as vehicles containing several hundreds or thousands of electroactive labels, pushing detection limits down to several hundreds of biomolecules.

3.1. Genosensors

Gold nanoparticles for DNA electrochemical sensing were introduced independently by Limoges' group [60] and Wang et al. [61] in year 2001. In Wang's protocol, hybridization of a target oligonucleotide to magnetic bead-linked oligonucleotide probes was followed by binding of the streptavidin-coated metal nanoparticles to the captured DNA, dissolution of the nanometer-sized gold tag by HBr/Br_2 solution, and potentiometric stripping measurements of the dissolved metal tag at single-use thick-film carbon electrodes [61]. Limoges and co-workers immobilized amplified 406-base pair human cytomegalovirus DNA sequence (HCMV DNA) to polystyrene microwell by passive adsorption. The assay relied on the hybridization of the single-stranded target HCMV DNA with an oligonucleotide-modified Au nanoparticle probe (using thiol-modified probe DNA), followed by the release of the gold metal atoms anchored on the hybrids by oxidative metal dissolution, and the indirect determination of the HBr solubilized Au^{III} ions

by anodic stripping voltammetry [60]. Similar assay using Ag nanoparticle was also developed, relying on hybridization of the target DNA with the silver nanoparticle–oligonucleotide DNA probe, followed by the release of the silver metal atoms anchored on the hybrids by oxidative (using nitric acid) metal dissolution and the indirect determination of the solubilized Ag^I ions by anodic stripping voltammetry (ASV) at a carbon fiber ultramicroelectrode [62]. However, the HBr/Br_2 or HNO_3 solution is highly toxic and therefore method based on direct electrochemical detection of nanoparticle tags, which would replace the chemical oxidation agent, was developed [63,64]. Probe DNA was immobilized on the paramagnetic beads surface via biotin–streptavidin interaction and target DNA was labeled with Au_{67} nanoparticle in ratio 1:1 preventing multiple DNA links between paramagnetic bead and nanoparticle (typical for above described Au nanoparticle-based assays), thus enhancing achievable detection limits. The hybridized paramagnetic beads were accumulated on the surface of a magnetic electrode and enabled the magnetically triggered direct electrochemical detection of gold quantum dot tracers without prior chemical dissolution of the Au quantum dots. For scheme of above described assay, see Fig. 7. Electrochemical magnetogenosensors for biomedical applications based on above described direct detection of gold nanoparticles was recently developed [65].

In need of further improvement of detection limits, methods based on the precipitation of silver on gold nanoparticle
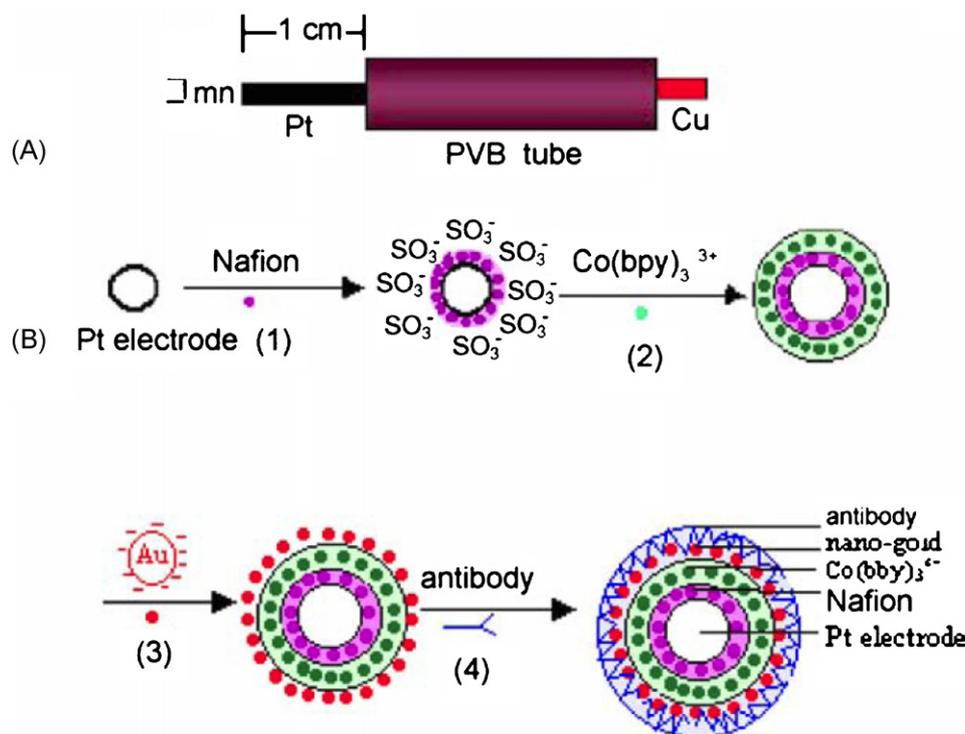
Fig. 6. Gold-nanoparticle-based immunosensor: schematic illustration of the assembly of antibody/{nanogold/$Co(bpy)_3^{3+}$}$_n$/Nafion multilayers on the platinum electrode surface to core–shell particles. The first stage involves the formation of a multiplayer film (antibody/{nanogold/$Co(bpy)_3^{3+}$}$_n$/Nafion/Pt), formed by the sequential adsorption of Nafion, $Co(bpy)_3^{3+}$ and Au nanoparticles under conditions where they are oppositely charged (step 2) and (step 3). The $Co(bpy)_3^{3+}$, positively charged, aids the adsorption of negatively charged Au nanoparticles and Nafion (step 2) and (step 3). Based on [59] with permission.

tags and a subsequent electrochemical stripping detection of the dissolved (by nitric acid) silver were developed [66–68]. Direct solid-state electrochemical detection of DNA hybridization based on direct voltammetric determination of precipitated silver on gold nanoparticles avoiding acid-based dissolution was also reported [69].

It is important for multiple DNA strand detection to have wide variety nanoparticle tags with different electrochemical properties. Addition to an arsenal nanoparticles for electrochemical detection is the use of core–shell Cu@Au (Cu core and thin Au shell) for DNA hybridization detection. Hybridization events between probe and target were monitored by the release of the copper metal atoms anchored on the hybrids by acidic oxidative metal dissolution and the indirect determination

of the solubilized Cu^{2+} ions by anodic stripping voltammetry [70]. CdS quantum dots are another addition to the spectrum of nanoparticle tags for DNA hybridization detection [71,72]. PbS nanoparticles also offer different voltammetric detection potential for following DNA hybridization [73].

Recent activity has focused on the development of hybridization assays that permit simultaneous determination of multiple DNA targets. Three encoding nanoparticles (zinc sulfide, cadmium sulfide and lead sulfide) have been used to differentiate the signals of three DNA targets in connection with a sandwich DNA hybridization assay and stripping voltammetry of the corresponding heavy metals [74]. The potential window over which heavy metals (principal constituents of QDs) are stripped is ~1.2 V, therefore five metals can be measured simultaneously
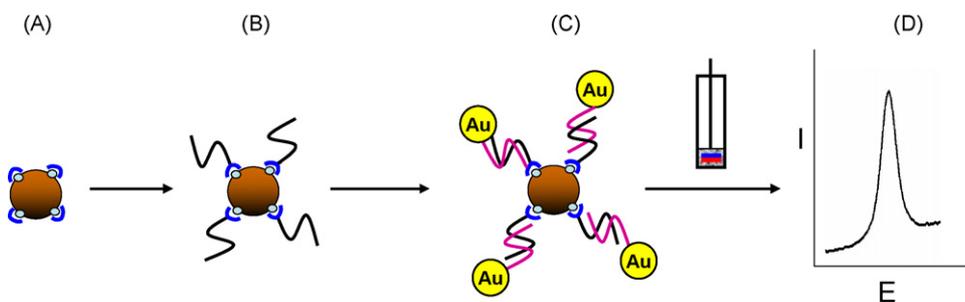
Fig. 7. Gold nanoparticles as labels for DNA sensing. Schematic representation of the analytical protocol (not in scale): (A) introduction of streptavidin-coated paramagnetic beads; (B) immobilization of the biotinylated probe onto the paramagnetic beads; (C) addition of the 1:1 Au_{67}–DNA target; (D) magnetically triggered direct DPV electrochemical detection of gold quantum dot tag in Au_{67}–DNA/DNA–paramagnetic bead conjugate. Based on [63] with permission.

with minimal peak overlap (theoretical peak widths of $75.5/n$ (mV), where n is number electrons transferred). Particularly attractive for future development (in addition to CdS, ZnS and PbS, developed previously [74]) are InAs and GaAs quantum dots.

Carbon nanotubes can play an important role as load carriers of electrochemical tags, offering the possibility of pushing down the limit of detection by several orders of magnitude. Anchoring of the monolayer-protected CdS quantum dots to the acetone-activated CNT was accomplished via hydrophobic interactions [75]. Such loaded CNT was attached to probe DNA and CdS QDs were determined by stripping voltammetry. SEM images showed that the nanocrystals were attached along the CNT sidewall, with a loading of around 500 particles per CNT. A substantial 500-fold lowering of the detection limit was therefore obtained compared to conventional single-particle stripping hybridization assays, reflecting the CdS loading on the CNT carrier. Further lowering of detection limits was achieved by loading CNT with enzymatic tag [50]. In such loading, up to 9600 alkaline phosphatase molecules were found to cover one CNT. Limit of detection was found to be 820 DNA copies. Further lowering of the detection limit to 80 DNA copies was achieved by increasing the ratio of loaded enzymes by electrostatic layer-by-layer techniques [76]. SWCNT was functionalized with carboxy groups and in basic buffer (to keep negative net charge of carboxy groups) coated with layer of poly(diallyldimethylammonium)chloride. After washing procedure, a layer of the negatively charged protein was adsorbed alternately with the positively charged PDDA polyelectrolyte. After repeating this procedure four times, layer of streptavidin was deposited on the CNT–multilayer–PDDA/alkaline phosphatase composite, allowing binding of biotinylated DNA with such CNT/enzyme-loaded marker.

3.2. Immunosensors

A metal nanoparticle-based electrochemical magnetic immunosensor was developed by using magnetic beads and gold nanoparticle labels [77]. Anti-IgG antibody-modified magnetic beads were attached to a carbon paste transducer surface by magnet that was fixed inside the sensor. Gold nanoparticle labels were capsulated to the surface of magnetic beads by sandwich immunoassay. Highly sensitive electrochemical stripping analysis offered a simple and fast method to quantify the captured gold nanoparticle tracers. The stripping signal of gold nanoparticles was found to be proportionally related to the concentration of target IgG in the sample solution.

Multiplexing capabilities of quantum dots were also demonstrated in connection to immunoassay. Electrochemical immunoassay protocol for the simultaneous measurements of proteins, based on the use of different inorganic nanocrystal tracers was described [78]. The multiprotein electrical detection capability was coupled to the amplification feature of electrochemical stripping transduction (to yield fmol detection limits) and with an efficient magnetic separation (to minimize non-specific adsorption effects). The multianalyte electrical sandwich immunoassay involved a dual binding event, based on

antibodies linked to the nanocrystal tags and magnetic beads. Carbamate linkage was used for conjugating the hydroxyl-terminated nanocrystals with the secondary antibodies. Each biorecognition event provided a distinct voltammetric peak, whose position and size reflected the identity and concentration of the corresponding antigen. The concept was demonstrated for a simultaneous immunoassay of β_2-microglobulin, IgG, bovine serum albumin and C-reactive protein in connection with ZnS, CdS, PbS and CuS colloidal crystals, respectively (for schematics of the protocol, see Fig. 8).

Effort toward ultrasensitive electrochemical immunosensing (analogous to ultrasensitive DNA sensing) using carbon nanotube loaded with enzyme was carried out with sensitivity down to 160 zmol of IgG [50] and 1 amol for prostate specific antigen [58]. Further lowering detection limits were achieved by multilayer coverage of SWCNT with alkaline phosphatase with limit of detection of 2000 protein molecules (3.3 zmol) [76].

Very recently, there have been substantial research activity in development nanoparticle labels which are easy to synthesize and easy to detect by electrochemical means. Liu et al. developed apoferritin-templated phosphate nanoparticle labels and used them for electrochemical immunoassay [79,80]. Apoferritin is a native protein composed of 24 polypeptide subunits that interact to form a hollow cage-like structure 12.5 nm in diameter; the interior cavity of apoferritin is about 8 nm in diameter and has an interior volume that can store several thousands of metal ions in form of phosphate salt. There is wide variability of metals which can be introduced in apoferriting cavity which paves the way for highly multiplexed assays. These novel nanobioparticles brings new possibilities for electrochemical sensing of proteins and potentially also for DNA biosensing.
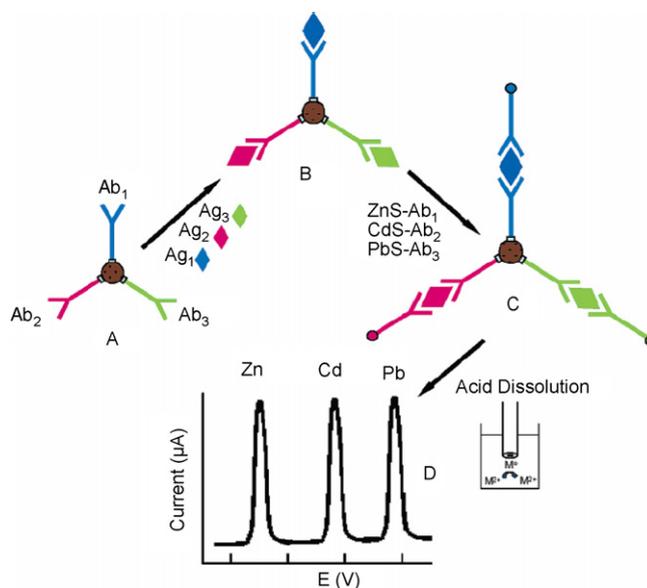
Fig. 8. Multiprotein electrical detection protocol based on different inorganic colloid nanocrystal tracers. (A) Introduction of antibody-modified magnetic beads. (B) Binding of the antigens to the antibodies on the magnetic beads. (C) Capture of the nanocrystal-labeled secondary antibodies. (D) Dissolution of nanocrystals and electrochemical stripping detection. Reproduced from [78] with permission.

4. Conclusion and outlook

Electrochemical nanobiosensors offer without doubts an important step toward development of selective, down to few target molecules sensitive biorecognition device for medical and security applications. In addition to described approaches, there are many other, yet unexplored, strategies. Very high amplification of signal could be reached, i.e. using high diameter carbon nanotubes filled with nanoparticles [81] and their following electrochemical stripping. Electrochemical nanobiosensors consisting from single carbon nanotube are another future path of biosensor development. These strategies are waiting to be explored. There is high expectation that such devices will develop toward reliable point-of-care diagnostics of cancer and other diseases, and as tools for intra-operation pathological testing, proteomics and systems biology.

Acknowledgement

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