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Review

Recent advances in carbon nanotube based electrochemical biosensors

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ABSTRACT

There is an increasing need for rapid, low cost, reusable, reliable and sensitive detection systems for diagnosing infectious diseases, metabolic disorders, rapidly advancing cancers and detecting the presence of environmental pollutants. Most traditional methods are invasive, slow, expensive and laborious, requiring highly specialized instruments. Introduction of biosensors with nanomaterials as transducers of signals have helped in removing the disadvantages associated with traditional detectors. The properties of high mechanical strength, better electrical conductivity and ability to serve as efficient signal transducers make carbon nanotubes (CNTs) ideal material for biosensor applications among the gamut of nanomaterials. Further, CNTs with their high surface areas, easily functionalizable surfaces for receptor immobilization are gaining importance in the construction of biosensors. The expanding field of CNTs bridges the physical sciences with biology, as chemical methods are employed to develop novel tools and platforms for understanding biological systems, in disease diagnosis and treatment. This review presents recent advances in surface functionalization of CNTs necessary for immobilization of enzymes and antibodies for biosensor applications and the methodologies used for the detection of a number of chemical and biological species. The review ends with a speculation on future prospects for CNTs in biology and medicine.

Contents

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1. Introduction

Carbon nanotubes originally described in 1991 by Sumio Iijima [1], have been found to be associated with many useful and unique properties [2]. CNTs are divided into two types namely, single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) as seen in Fig. 1. Rolling a graphene sheet into a cylinder results in SWCNT, on the other hand arrangement of concentric graphene cylinders with an interlayer space of 0.34 nm leads to formation of MWCNT. The properties of CNTs are a consequence of their structure. SWCNTs may be zig-zag, arm chair or chiral in their structure (Fig. 2) [3]. SWCNTs can be either metallic or semiconducting, a property determined by the atomic arrangement (chirality) and nanotube diameter [4]. The roll-up vectors (n, m) of the cylinder describe the electrical properties of SWCNTs [5,6]. Metallic SWCNTs have roll-up vectors like n-m = 3q, while semiconducting SWCNTs have n-m $\neq$ 3q (here q is any integer/zero). If m = 0, the nanotubes are called zigzag. If n = m, the nanotubes are called armchair and the rest are called chiral.

Single-walled carbon nanotubes (SWCNTs) show excellent chemical stability, good mechanical strength and a range of electrical conductivity properties. MWCNTs show metallic electronic properties similar to metallic SWCNTs [7], which in some respects makes them more suitable for electrochemical applications. Optical properties exhibited by MWCNTs are less striking than SWCNTs, so they are used as a delivery system for large biomolecules including plasmids (DNA) into cells. Both types of CNTs contain one-dimensional structure and exhibit excellent properties viz. good electrical conductivity, strong adsorptive ability and excellent bioconsistency. These properties enable CNTs to carry high currents with negligible heating [8]. CNT based electrochemical biosensors are explored as amperometric enzyme electrodes, immunosensors and nucleic acid sensing devices. Amperometric biosensors based on CNT-modified electrodes for numerous oxidase and dehydrogenase enzymes have been developed [9–17].

Enzyme-based electrochemical biosensors have been used widely in health care, food safety and environmental monitoring. Health care is the main area for biosensor applications, such as monitoring blood glucose levels especially for diabetics by glucose biosensors [10,11]. Besides, the reliable detection of urea in the blood of patients with renal disease has potential applications either at home or in hospital [18]. One of the driving forces in basic and applied science has been the search for biomarkers, such as proteins, peptides or metabolites, which are unique and representative of a particular cell type or disease state. Protein biomarkers are the most common type of biomarker used in medical diagnostics [19]. CNTs electrodes can also be used for routine clinical determination of hemoglobin in whole blood samples [20,21].

Presence of pathogenic bacteria in food and water is a major concern in the food industry because of its critical impact on public health and economy. Outbreaks of food borne illnesses continue to rise as a consequence of globalized food supply, large-scale food production, and a growing population of disease susceptible consumers [22]. Many techniques are currently used for detection of pathogenic microbes and various molecules produced by them. Usually, detection is performed by enzyme-linked immunosorbent assay (ELISA), RIA (radioimmunoassay), electrophoretic immunoassay and conventional culturing techniques as well as nucleic acid based polymerase chain reaction (PCR) technology or another optical based technology. Most of these techniques, tend to suffer from low sensitivity and require sophisticated instrumentation and time-consuming procedures, and most of the reagents employed in immunoassays such as antibodies, enzymes, and fluorescence labels are very expensive [23]. Therefore, there is a growing interest for the development of new, simple, sensitive, reliable, and cheaper diagnostic methods. Biosensing technology for early disease diagnosis and food safety monitoring is a promising alternative, owing to its potential for rapid, sensitive, simple, low cost and portable detection [24]. Industrial applications for biosensors include monitoring concentrations of glucose and end products in fermentation broths, during food processing procedures [12–14]. Sensitive detection of phenolic compounds is an important topic for environmental research because phenolic compounds often exist in the effluents of many industries, giving rise to environmental problems as many of them are very toxic [19].

This review focuses on the fabrication of enzyme based CNT biosensors, covalent and noncovalent approaches used for modification of CNTs and methods for immobilization of enzymes, antibody and DNA aptamer on CNTs. This is followed by discussion on different enzyme, antibody and DNA aptamer based electrochemical biosensors, i.e., (i) amperometric (oxidase or dehydrogenase) enzyme electrodes based on the accelerated oxidation of NADH or hydrogen peroxide, (ii) bioaffinity devices (particularly DNA aptamer based biosensors) based on the enhanced detection of the product of the enzyme label or of the target guanine (iii) detection of cancer biomarkers. And finally the review ends with future perspectives for biosensing applications.

2. Sensors and biosensors

Sensors are a class of devices that have found widespread use, ranging from the detection of gas molecules to the real time tracking of chemical signals in biological cells [22]. Sensors are small or miniaturized devices designed for the continuous monitoring of the physicochemical or biochemical properties of specific analytes so as to provide qualitative and/or quantitative analytical data.

Chemical sensors react to the presence and concentration of an analyte by responding selectively to analyte's electrical, optical, thermal or other property. Biosensors can be defined as devices with a thin layer serving as support to immobilized biomolecule that contains biorecognition sites. The biomolecules may be proteins, often enzymes, or other macromolecules (e.g., cell receptors, antibodies), oligo- or polynucleotides, microorganisms, or even whole biological tissues that display specific interactions with analyte species [25]. Biosensors were first reported in the 1960s [26], and these sensors were applied for monitoring biological processes or for the detection of biomolecules. Majority of biosensors developed to date are electrochemical and they are preferable due to their low-cost, relatively fast response times, ease of use, and small size. Electrochemical biosensors contain potentiometric, amperometric or conductimetric cells as detectors which generate signals from electrochemical reactions of the analytes or in response to the presence of particular chemical species related to them. A biosensor

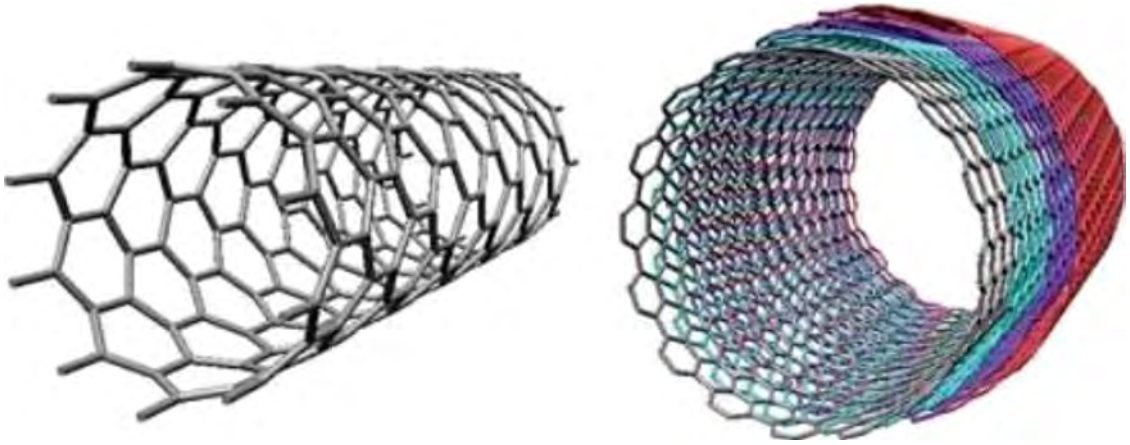

Fig. 1. The classification of the carbon nanotubes.

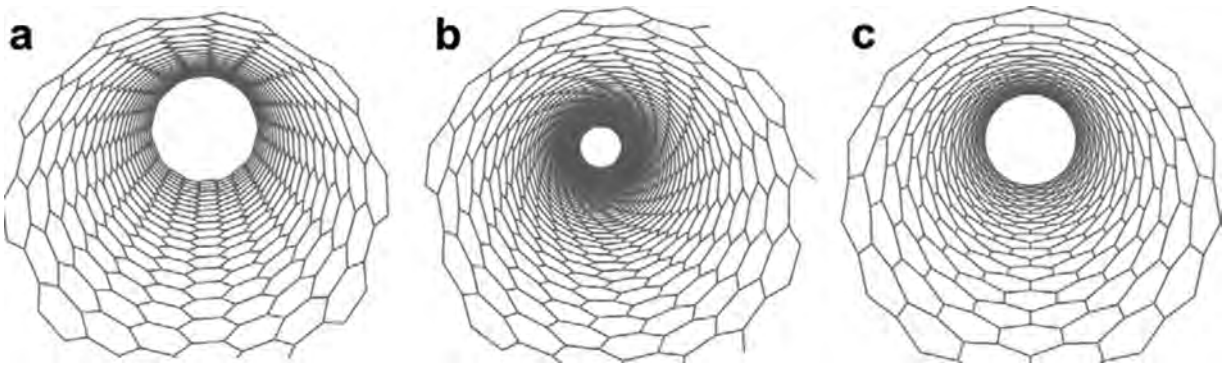

Fig. 2. Schematic representation of three typical types of SWCNTs (a) Armchair (10, 10), (b) Chiral (13, 6), and (c) Zigzag (14, 0). Reproduced from [3] Copyright (2012), with permission from Elsevier.

generally contains a reference electrode, a working electrode and a counter electrode. In Enzyme-coupled electrochemical biosensors, target analyte is recognized by enzymes immobilized on the working electrode and their catalytic activity may cause either electron transfer, thereby producing electric current or contribute to produce voltage [27].

2.1. CNTs as attractive material for biosensors

Among a variety of nanomaterials, CNTs often become the choice for fabrication of biosensors owing to their superior strength and remarkable physicochemical properties, as a result of their unique tubular nanostructure with unusually large length to diameter ratios, which may be as high as 132,000,000:1 [28]. Further, CNTs are associated with excellent conductivity, high sensitivity, good biocompatibility and outstanding chemical stability [29].

Moreover, the ends and sidewalls of CNTs can be easily modified by attaching almost any desired chemical species. CNTs can be excellent transducers in nanoscale sensors due to their significant sensitivity. From the literature it is known that CNTs can enhance the electrochemical reactivity of important biomolecules, and mediate fast electron transfer kinetics for a wide range of electroactive species [30]. The features of enhanced electrochemical reactivity of hydrogen peroxide and NADH, and easy detection of biomolecules (viz. cells, enzymes and other small molecules) make CNTs make a preferable nanomaterial for fabricating electrochemical biosensors ranging from amperometric enzyme biosensors to detectors of pathogenic microbes. Successful exploitation of these properties, in addition to use of right crosslinking agents for receptor enzymes and antibodies on the electrodes deliver sensitive, rapid, reliable, reusable and cost effective biosensors for the analytes.

Another attractive property of these carbon nanostructures is their exceptional photothermal response. Photothermal therapy is used to reduce the size of tumors or even to eliminate them. Near Infrared (NIR) laser irradiation has been used to generate heat which has been followed by SWCNTs internalization to destroy cancer cells [31,32]. The conjugation of various biomolecules to CNTs has been depicted in Fig. 3 [33]. The conjugates of carbohydrates (such as galactose, lactose, mannose etc.) and functionalized nanotubes are called as glyconanotubes. The great contribution of conjugation chemistry in the production of surface engineered CNTs is chiefly responsible for biomedical applications of CNTs including in biosensors. Surface engineered CNTs are amenable to modification with protein, aptamer, nucleic acid, antibody and biomarkers. Still there is much room for exploiting the potential of CNTs for the development of new products.

2.2. Functionalization of carbon nanotubes (CNTs)

Pristine CNTs are insoluble in aqueous solutions, polymer resins, and most solvents as they have hydrophobic surfaces. Functionalization is a process in which certain molecules or functional groups are physically or chemically attached to the smooth sidewalls of CNTs without downgrading their desirable properties. Functionalized CNTs are more easily dispersible in liquids, have better biocompatibility and low toxicity [34].

Functionalization of CNTs can be done by covalent or noncovalent approaches. Covalent functionalization is done by forming bonds with nanotube sidewalls, whereas noncovalent function-

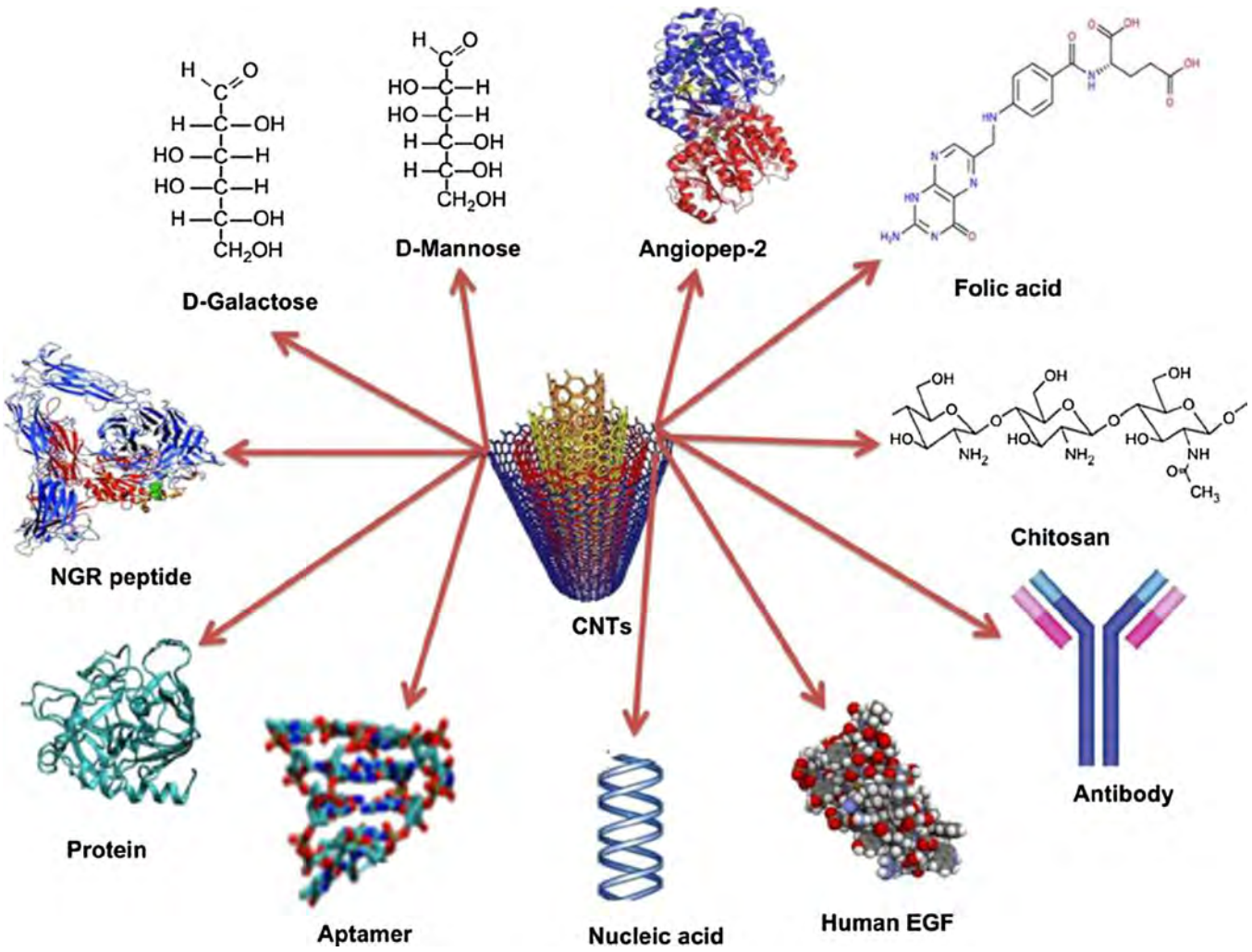

Fig. 3. Conjugation of various biomolecules to CNTs. Reproduced from [33], Copyright (2014), with permission from Elsevier.

alization occurs through interaction between the hydrophobic domain of an amphiphilic molecule and the CNT surface. The functionalized CNTs can effectively cross biological barriers and penetrate individual cells [35]. This feature and the mechanism of internalization and release of CNTs from the cells are of major interest for biological and in particular intracellular biosensing applications.

2.2.1. Covalent functionalization of CNTs

There are different covalent reactions formulated to functionalize CNTs. Oxidation is one of the commonly used methods of covalent functionalization. Nitric acid, or a mixture of concentrated nitric acid and sulfuric acid is used as oxidizing agent for the oxidation of CNTs [36–38].

Chemical modification of nanotubes through oxidation is followed by subsequent esterification or amidization of the carboxyl groups (Fig. 4 (i)) [39].

Oxidized CNTs have a disadvantage of aggregating in the presence of salts due to charge screening effects, making further modification necessary. Hydrophilic polymers viz. poly(ethylene glycol) is used for the modification [40–42]. The oxidative treatment of CNTs also leads to erosion of their structure [43–46]. It was observed that as the shortening and thinning of the CNT layers occurred, carbonaceous debris was produced [47].

In photochemical functionalization, photoirradiation has been used to generate reactive species such as nitrenes in the course of sidewall addition reactions (Fig. 4 (ii)) [48]. Alternatively, Bingel reaction is used for carbene generating compounds (Fig. 4 (iii)) [49,50]. Cylcoaddition reaction, which occurs on the aromatic sidewalls, instead of nanotube ends is another method to oxidize CNTs. Azides are applied for photochemical reaction of CNTs by cycloaddition reactions (Fig. 4 (vi)) [49–51]. Photochemical funtionalization by sidewall osmylation using osmium tetroxide (OsO_4) under UV-light irradiation has also been reported [52–54]. Lee et al. [55] used an approach to functionalize the carbon nanotube side walls in four steps, starting the reaction with a nitrobenzene diazonium salt, followed by reduction and reaction with a heterobifunctional spacer to introduce maleimide group that was then reacted with a 5-thiol-modified single-stranded DNA.

In electrochemical functionalization, potentiostatic or galvanostatic procedure is followed wherein a constant potential or a constant current is applied to a CNT electrode immersed in a solution containing a suitable reagent. The reagent generates a highly reactive (radical) species, as a consequence of electron transfer between the CNT and the reagent. Many organic radical species have a tendency to react with the starting reagent or to self-polymerize, resulting in a polymer coating on the tubes. By using this principle to aromatic diazonium salts, phenyl residues have been covalently grafted onto SWCNTs [56]. Fig. 5 shows the schemetic presentation of functionalization of single-walled carbon nanotubes by electropolymerization of adamantine pyrrole

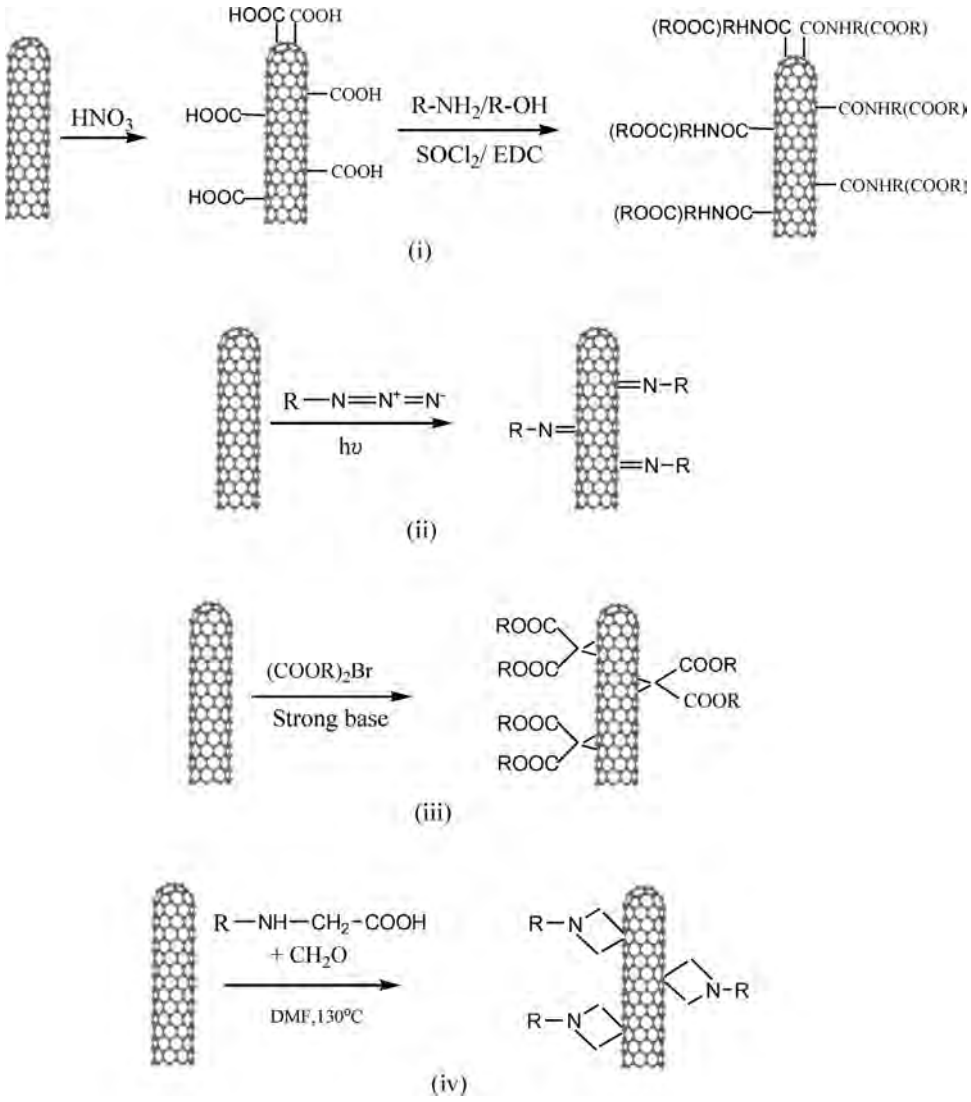

Fig. 4. Carbon nanotubes covalent functionalization: (i) CNTs are oxidized and then carboxyl groups are esterified or amidization take place (ii) photoinduced, addition of azide compounds with CNTs; (iii) CNTs have Bingel reaction (iv) 1,3-dipolar cylcoaddition done on CNTs.

and immobilization of beta cyclodextrin tagged glucose oxidase enzyme [57].

2.2.2. Noncovalent functionalization of carbon nanotubes

Noncovalent functionalization is carried out to modify CNTs as it has many advantages over covalent methods. They help mainly in the preservation of the structural and electrical properties of CNTs. The noncovalent approaches are based on interactions of the hydrophobic part of the adsorbed molecules with sidewalls of the nanotubes through van der Waals, p-p, CH-p, and other interactions. The hydrophilic part of the molecules gives solubility in aqueous solution [58]. The advantage of noncovalent methods is the attachment of various functional groups on the surface of CNTs, while keeping the p system of the graphene sheets intact. As a consequence, the physical properties and chemical structure of CNTs are not changed by noncovalent methods unlike the case with covalent chemical attachment. However, the length of CNTs is shortened in these methods [59].

Noncovalent functionalization can be achieved either through sonication or π–π interaction. The π, π stacking can be exploited for the binding of aromatic molecules viz., porphyrin derivatives, pyrene and its derivatives to the polyaromatic graphitic surface of carbon nanotubes [60–62]. Chen et al. have used an amine-reactive pyrene derivative [58], whereas Wu et al. exploited pyrene conjugated glycodendrimers to functionalize CNTs [63]. π, π stacking between aromatic DNA base units and surface of CNTs was also used in some of the methods [64,65]. Veerapan et al. exploited π, π stacking interactions between MWCNT and graphene oxide for the fabrication of glucose biosensor [66]. Hydrophobic effects and van der Waals forces play important role in the coating of CNTs with amphiphilic surfactant molecules or polymers viz. Tween-20, a pluronic triblock copolymer, sodium dodecyl sulfate (SDS) and Triton X-100 [67–70].

Steric repulsion caused due to crowding of higher negative surface charge as a result of interaction between CNTs and SDS through the hydrophobic segment improves the dispersion of CNTs [71]. However, simple alkyl chains of surfactants such as SDS, SDSA (sodium dodecyl sulfonate), DTAB (dodecyl trimethylammonium bromide) are believed to form nonspecific hydrophobic interactions with the nanotubes [72,73]. Polymers such as polyvinyl pyrrolidone (PVP), polystyrene sulfonate (PSS), and poly(2-ethyl-2-oxazoline) (PEOX) are reported to functionalize CNTs noncovalently [72–75].

The main advantage of using polymers instead of small molecular surfactants is that the polymers reduce the entropic penalty of micelle formation. An ideal non-covalent functionalization coat-

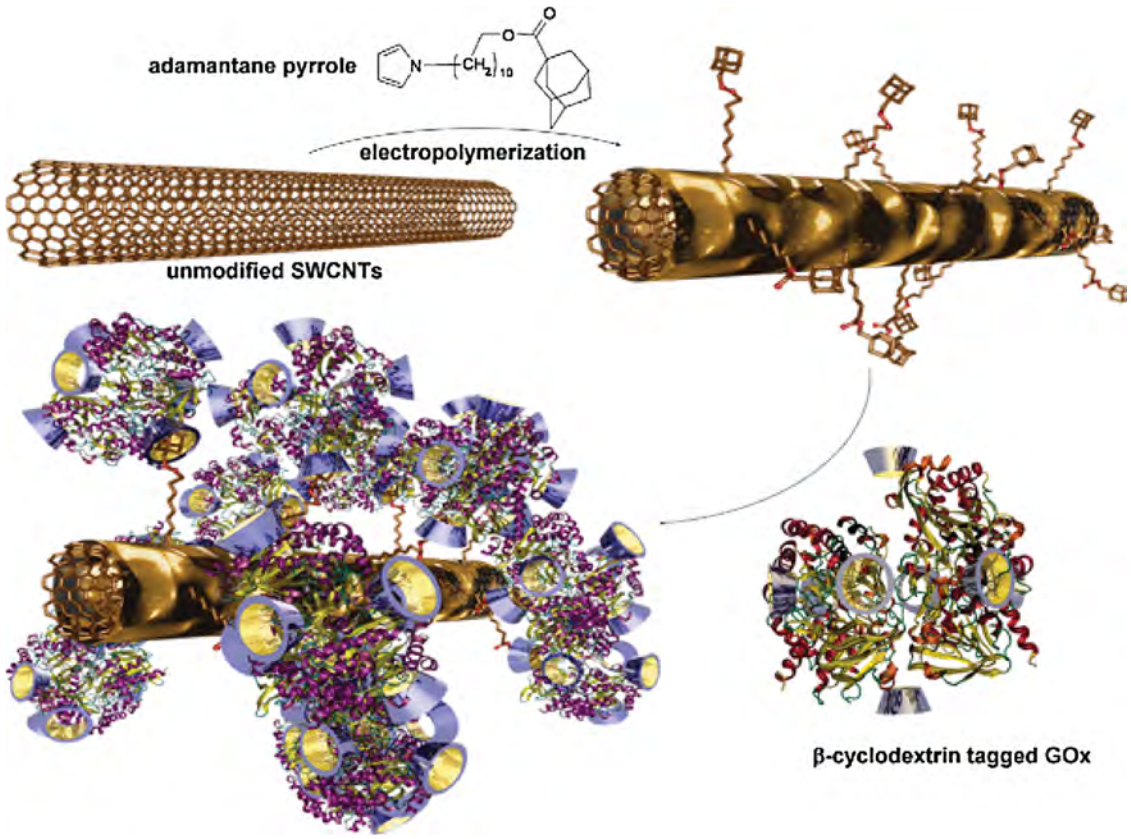

Fig. 5. Schemetic presentation of functionalization of single-walled carbon nanotubes by electropolymerization and glucose oxidase enzyme immobilization, Reprinted with permission from [57]. Copyright [2011] American Chemical Society.

ing on CNTs for biological applications should be (i) biocompatible and non-toxic, (ii) stable enough to avoid detachment from the nanotube's surface in biological solutions, and having very low critical micellar concentration values, so that it remains stable and (iii) have available functional groups for bioconjugation with antibodies or other molecules. Polyethyleneglycol (PEG) can be used to non-covalently coat CNTs and prevent non-specific protein absorption [76]. Non-covalent functionalization of SWCNTs by PEGylated phospholipids (PL-PEG), branched polyethylene-glycol (PEG) chains and fluorescein–polyethylene glycol (Fluor-PEG) [77–81] have also been used for biosensors. Perfluorinated polymer Nafion is also used extensively as a novel-solubilizing agent for the modification of CNTs. Nafion has an advantage that its coating does not impair the electrocatalytic properties of the CNTs, overcoming a major obstacle for creating CNT-based biosensing devices [82].

2.3. Immobilization of enzymes for biosensors

Immobilization of enzymes is the process of confinement or localization of an enzyme to a certain defined position (or support) with the retention of catalytic activities, leading to their repeated and continuous use [83]. Sufficient enzyme loading and optimal activity is required for a good biosensor. There is a need to treat enzymes softly, as they may lose their activity upon harsh chemical treatment or exposure to high temperature. Immobilization of enzymes on the surface of CNTs provides a platform for fabricating biosensors utilizing their unique electronic and optical properties for signal transduction.

The best strategy for successful enzyme biosensor fabrication is to devise a configuration by which electrons can directly transfer from the redox center of the enzyme to the underlying electrode. The strategy of physical adsorption or covalent immobilization of large biomolecules onto the surface of immobilized CNTs may well represent an efficient strategy through which direct electrical communication between electrodes and the active site of redox-active enzymes can be established. It is anticipated that in the near future the electrocatalytic properties of CNTs will find applications in the development of enzyme biosensors without mediators.

2.3.1. Enzyme immobilization methods

There are different methods of immobilization available in literature with their own advantages as well as disadvantages [84–89]. This article highlights the mthods for immobilization of enzymes and other molecules on CNT platforms. The designing of an efficient enzyme immobilized system is an art. The ideal enzyme immobilization method would 1) employ mild chemical conditions, 2) allow for large quantities of enzyme to be immobilized, 3) provide a large surface area for enzyme substrate contact within a small total volume, 4) minimize barriers to mass transport of substrate and product, and 5) provide a chemically and mechanically robust system. The selection of immobilization technique and support are dependent on different factors. The parameters that influence the above selection in case of biosensors are the nature of enzyme, the type of transducer, physicochemical properties of the analyte and the operating conditions. The methods of immobilization are commonly divided into three main categories: (i) physical methods, where weaker interactions are utilized (often by electrostatic or hydrophobic interactions), (ii) chemical methods, where covalent bonds are formed with between enzymes and support matrix and (iii) entrapment, where enzyme molecules are entrapped [90]. Different techniques for enzyme immobilization on CNT platforms are mentioned below (Fig. 6) [91].

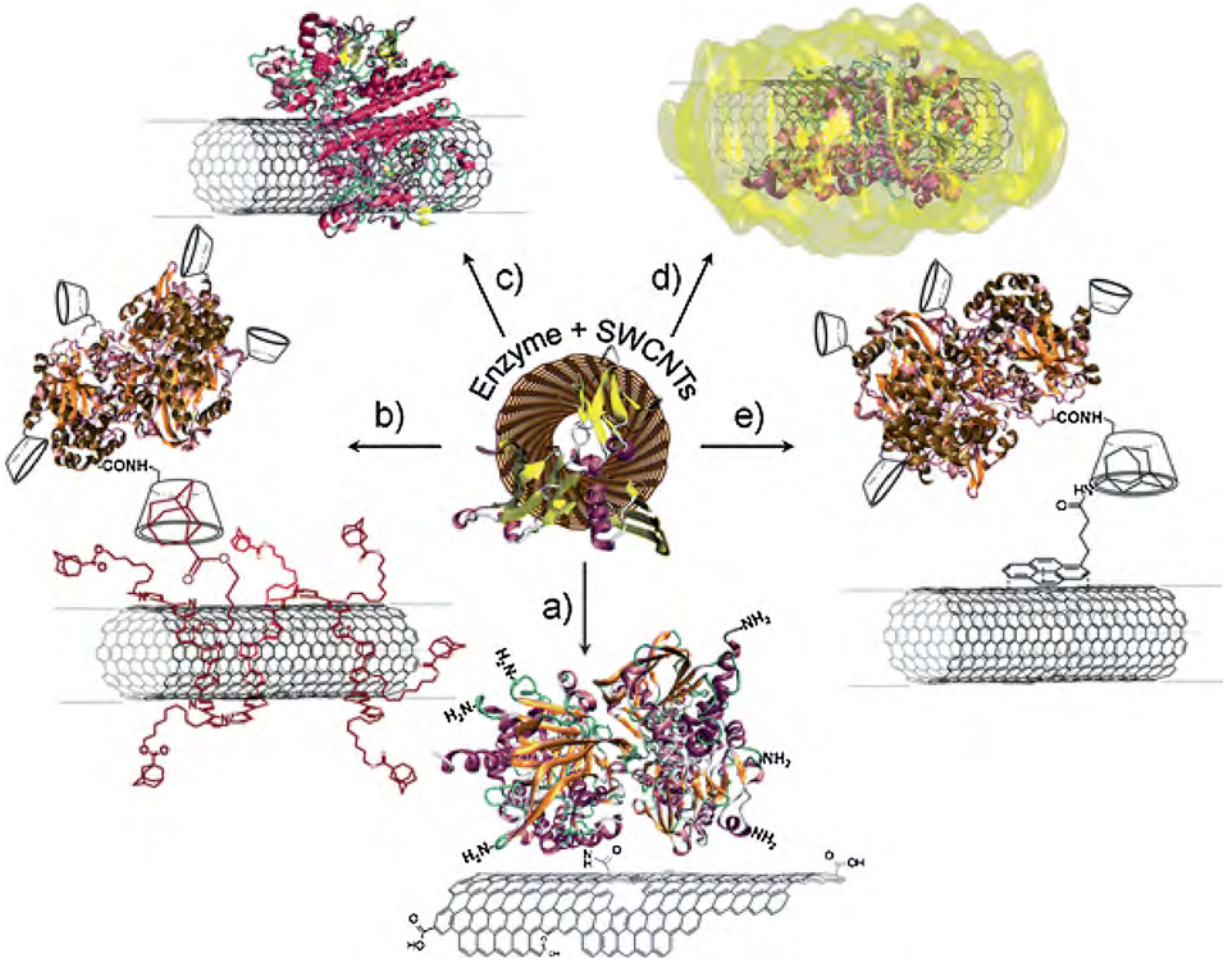

Fig. 6. Various strategies of enzymes immobilization on SWCNTs: (a) covalent binding via amide coupling with the carboxylic acid groups of oxidized nanotubes; (b) electrochemical coating of nanotubes with affinity partners and subsequent immobilization of affinity counter part modified enzymes; (c) adsorption of enzymes on SWCNTs via hydrophobic or electrostatic ineractions; (d) entrapment of enzymes in a polymer matrix formed around SWCNTs; and (e) immobilization via affinity interactions onto functionalized nanotubes. Reproduced from [91] with permission of The Royal Society of Chemistry.

2.3.1.1. Adsorption. Attachment of molecular probe on the CNT platform through hydrogen bonds, van der Waals, hydrophobic, hydrophilic or ionic interactions is termed as adsorption. Immobilization by adsorption is the easiest, economical and little time consuming technique for preparing biocatalytic systems. Further, enzyme activity is not damaged as immobilization by adsorption involves weak linkages between enzymes and supports, and the enzyme is not chemically manipulated. Besides many advantages, this method also has some drawbacks. In comparison with other methods, the risk of enzyme leaching from the support is higher due to the relatively weaker enzyme-support interactions. The CNT-modified electrode is first prepared by evaporating or casting CNT dispersion onto a GCE (Glassy carbon electrode), and then a Nafion-containing solution of the desired enzyme is dropped on top of this electrode and allowed to evaporate. In this manner, a tyrosinase-based amperometric sensor has been realized for the detection of phenolic compounds [19]. However, it is possible to reduce leaching of the enzyme and improve the stability of the biosensor by subsequently depositing a Nafion film onto the electrode [92]. Another method for enzyme adsorption involves a layer-by-layer technique wherein alternate layers of oppositely charged polyelectrolyte and enzyme are deposited onto the electrode [93].

2.3.1.2. Entrapment. In entrapment method enzymes are mixed with monomer solution and then polymerized to gel. During this process the enzyme molecules are entrapped in the pores of gel. Since, entrapment is not done using chemicals, the enzyme activity is not affected. It is a simple technique with a scope for high protein loading. Additionally, such sensors exhibit enhanced sensor response, due to an increase in the surface area as well as an improvement in the electrical communication between the redox centers of the hydrogel or the sol-gel derived matrix and the electrode. The gels commonly used include polyacrylamide, starch gels, nylon, silastic gels, conducting polymers and others. Wide range of enzymes has been successfully immobilized onto CNT-incorporated redox hydrogels to yield sensitive biosensors [94]. On the other hand, a glucose sensor has been obtained by encapsulating GOx and MWCNTs in Nafion matrix in appropriate amounts [95].

2.3.1.3. Electropolymerization. In electropolymerization methodology, the enzyme is mixed with a monomer, which is electropolymerized at a GCE or a metal electrode, where upon the enzyme becomes embedded into the polymer matrix. The incorporation of the enzyme into the matrix is often promoted through electrostatic interactions, for which many examples exist in literature [96,97]. The main advantages of this immobilization method are the simple one-step preparation, exclusion of electroactive and surface-active interferences, control of film thickness and localization of biocatalysts onto tiny electrode surfaces. In many cases conductive polypyrrole (PPy) has been used as a polymer matrix. This choice relates to the fact that pyrrole can be electropolymer-

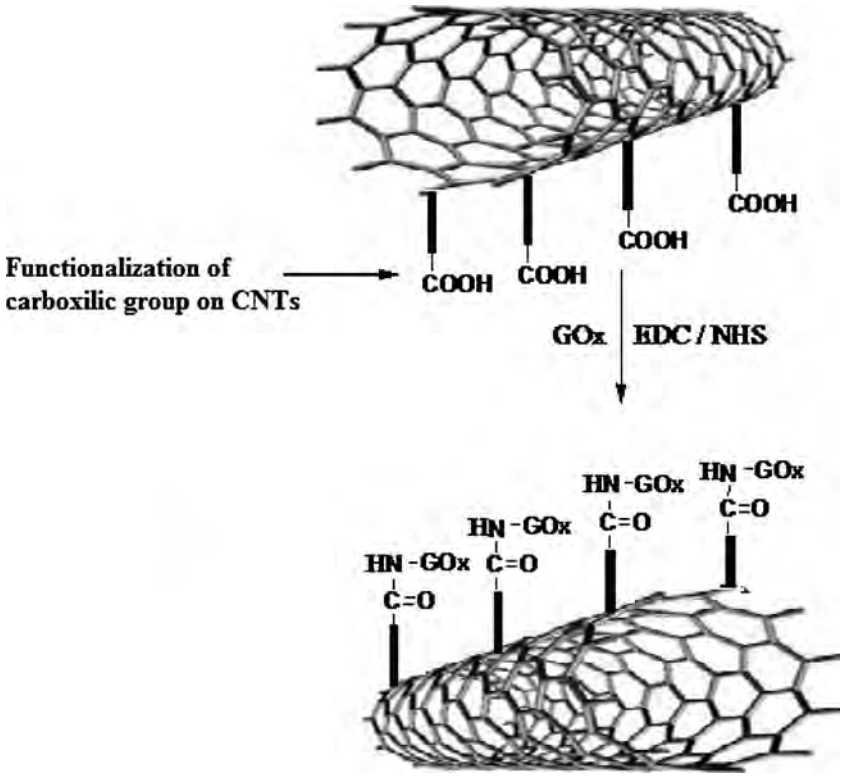

Fig. 7. Glucose oxidase covalently immobilized on CNT nanoelectrodes via carbodiimide.

ized at low oxidation potentials in aqueous solutions at neutral pH, which is compatible with a wide range of biological molecules. Polypyrrole has proven effective at electrically wiring the enzymes and CNTs to the underlying electrode.

Wang et al. reported a glucose electrochemical biosensor through the immobilization of GOx on oxidized MWCNTs and aligned carbon nanotube arrays grown on a quartz substrate through pyrrole electropolymerization [97,98]. Moreover, single-stranded DNA has been incorporated into a PPy matrix through electropolymerization on MWCNT-GCE for a DNA hybridization sensor [99].

2.3.1.4. Covalent bonding. Although the noncovalent methods for fabricating CNT-based electrochemical biosensors have some advantages like easy fabrication and low cost, poor stability and leaching of enzymes sometimes significantly influence the analytical performance of these biosensors. To overcome this limitation, great efforts have been made to covalently attach enzymes to the electrode surfaces via various methods and reagents. The attachment of enzyme on a support using a chemical reaction or on activated or monomer-grafted surfaces results in the strongest kind of enzyme-carrier bond. In this method, the bond occurs between amino acid residues present in the enzyme backbone and the functional group originally present in the support matrix or formed by modification. Further, in this method the functional groups, which are not essential for the enzyme's catalytic activity are only allowed for bonding. The most often used reactive groups on side chains of amino acids of enzymes are the amine of lysine or arginine, carboxyl from aspartic or glutamic acid, hydroxyl of serine or threonine and sulfydryl (or thiol) of cysteine [100] and terminal amino and carboxyl groups of the polypeptide chains. The main limitation of the method is possible loss of enzyme activity since covalent bonding is usually carried out using chemicals and at different pH ranges. Fig. 7 demonstrates covalently immobilized glucose oxidase on CNT nanoelectrodes using carbodiimide chemistry by forming amide linkages between their amine residues and carboxylic acid groups on the CNT tips [101].

Some Glucose biosensors are also reported by covalent attachment of GOx onto SWCNT modified Pt electrode and on a gold substrate [102–104].

2.3.1.5. Cross-linking. In the method of cross-linking usually biomaterial is chemically bonded to solid support or to material such as cross-linking agent to significantly increase the attachment. It is a useful method to stabilize adsorbed biomaterials. It generally involves the residues, which are not involved in catalysis, i.e. ε-amino group of lysine residues [105]. The cross-linking agents used for this purpose are glutaraldehyde, epichlorohydrin, adipoyldichloride. However, glutraldehyde has the versatility to react with several functional groups of proteins, such as amine, thiol, phenol and imidazole [106]. Cross-linking of enzyme is rapid, simple to perform and has wide applicability. Sometimes a small proportion of the enzyme molecules act as support, thus there is a possibility of decrease in the enzyme activity. The agents can also interfere with the enzyme activity, especially at higher concentrations.

3. CNT based biosensors

As for the basic characteristics of a useful biosensor, its response should be predictable so that it can be relied on with the magnitude of changes in the biological environment, and the sensor should be sensitive and specific. A new class of biosensors referred to as nanobiosensors, which includes nanoparticles and nanotubes, has been developed, that combines the advantages of nanomaterials. CNTs have small size and excellent electrochemical and electrical properties (approximately 100 times greater ability to conduct electricity than copper wires), CNTs are well suited for transduction of electric signals generated upon recognition of a target, and hence they play an important role in recent development of

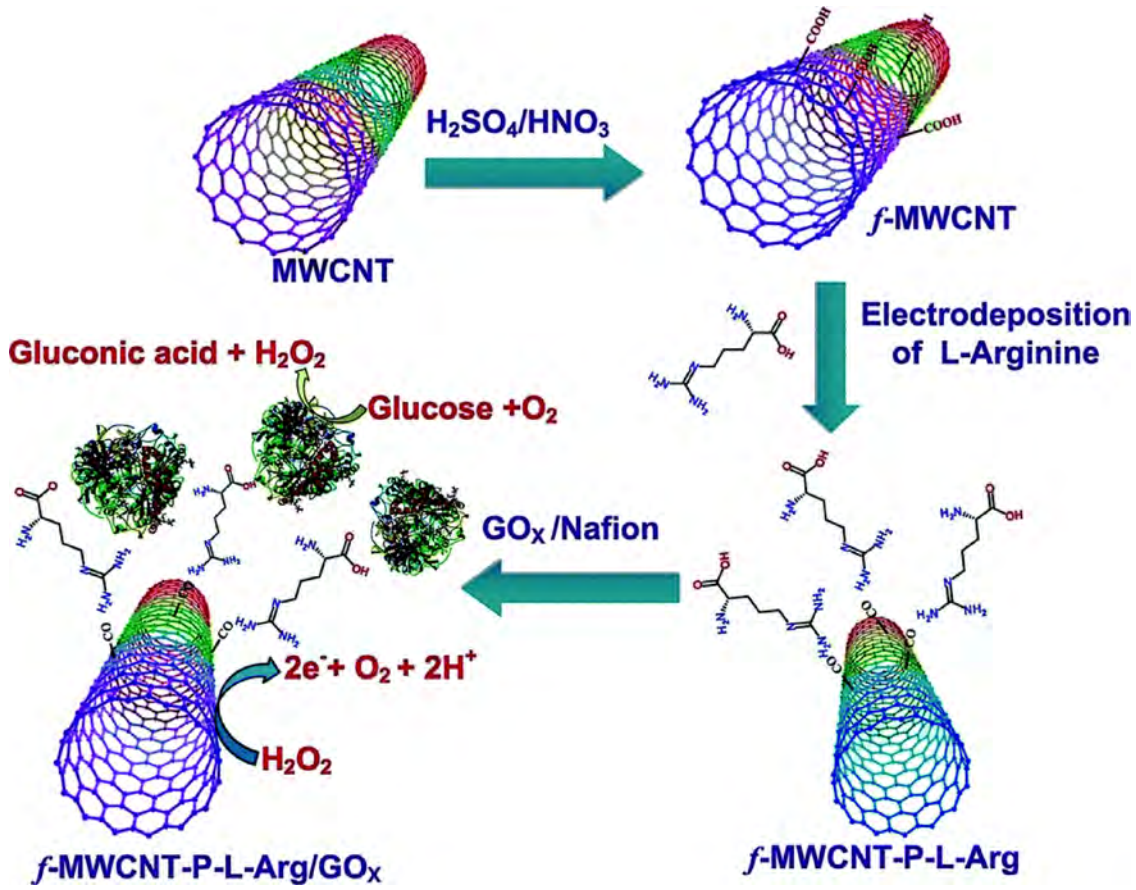

Fig. 8. Construction of glucose biosensor based on the electro polymerization of a poly-L-arginine film (P-L-Arg) onto functionalized multiwalled carbon nanotubes (*f*-MWCNTs)/glassy carbon electrode (GCE) *via* electrostatic attraction with glycoprotein glucose oxidase (GOx). Reproduced from [114] with permission of The Royal Society of Chemistry.

enzyme-based biosensors [22,107–112]. Electrochemical biosensors are popular due to their low-cost, relatively fast response times, ease of use, and small size. CNTs with their small size and excellent electrochemical properties have been found to be either equal or superior to most other electrodes detect ions, metabolites and protein biomarkers [113]. Therefore, they are commonly used in the recent development of enzyme-based biosensors. Different classes of CNT biosensors conjugated to enzymes and DNA, for early-stage detection of biomarkers of various diseases including cancer are briefly described here.

3.1. Oxidases based biosensors

3.1.1. Glucose oxidase

Glucose oxidase (GOx) is a large, dimeric protein having molecular weight of 160 kDa. It contains one tightly-bound flavin adenine dinucleotide (FAD) unit per monomer, seated deep inside the enzyme. These redox active prosthetic groups are not covalently bound and may be released from the protein during denaturation. The active site where glucose binds is just above the FAD binding site, in a deep pocket shown with a star. The oxidation of glucose to gluconolactone is performed by FAD cofactor:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2$$

Amperometric glucose biosensors are most deeply studied because glucose detection can be done very accurately by these sensors for the diagnosis of diabetes mellitus [93,101].

Detection of glucose is one of the most frequently performed analyses in medicine. It has advantages of simplicity and quickness. But there are still some problems such as narrow linear range, low sensitivity and stability. CNTs with their special properties can potentially offer solution to the above problems.

A simple and fast approach for the construction of a novel glucose biosensor based on the electro polymerization of a poly-L-arginine film (P-L-Arg) onto functionalized multiwalled carbon nanotubes (f-MWCNTs)/glassy carbon electrode (GCE) via electrostatic attraction with glycoprotein glucose oxidase (GOx) has been reported (Fig. 8) [114].

In one report CNTs with sulfonic groups were used to construct a highly sensitive glucose biosensor, where in Pt nanoparticles were supported on sulfonated MWCNTs modified glassy carbon electrode. The results show that GOD/Pt/sulfonated-MWCNTs/GC glucose biosensor has detection sensitivity of 0.56 μA/mM [115]. Table 1 represents the comparative data for different glucose biosensors.

The AFM and SEM images of only GOx film show small spherical voids surrounded by small bead like structures (Fig. 9 (a) and (a′)). Large bead like structure do not contain any spherical voids due to filling up of the voids by GAD (Fig. 9 (b) and (b')). Similarly, Fig. 9 (c) and (c′) show closely packed larger bead like structures, which validate higher GOx loading due to larger surface area of GCNT [13].

The basic concept of the glucose biosensor is based on the fact that the immobilized GOx catalyzes the oxidation of β-D-glucose by molecular oxygen producing gluconic acid and hydrogen peroxide [121]. In order to work as a catalyst, GOx requires the redox cofactor flavin adenine dinucleotide (FAD). FAD works as the initial electron acceptor and is reduced to $FADH_2$.

$$\text{Glucose} + \text{GOx-FAD}^+ \rightarrow \text{Glucolactone} + \text{GOx-FADH}_2$$

Table 1
Comparison of different CNT based glucose biosensors.

Electrode	Sensitivity ($\mu AmM^{-1} cm^{-2}$)	Linear range (mM)	K_s (s^{-1})	Electrochemical technique used	Ref.
PPMH/GC-electrode	–	Upto 2	1.32	Amperometry	[116]
GOx-PVA-MWCNT	8.67	0.1–20	1.58	Cyclic Voltametry	[117]
CNT/Gox	2.40	0.04–1	1.08	Cyclic Voltametry	[118]
MWCNT/Au/GOx	2.52	0.1–10	–	Amperometry	[119]
Pt nanoparticles on the LD-ACNTs	3.8	Upto 3	–	Cyclic voltametry and chronoamperometry	[120]
ERGO-MWCNT/GOx/Nf	–	0.01–6.5	3.02	Amperometry	[66]
CNT/Colloidal Au/PDDA/Gox	2.50	0.5–5.2	1.01	Amperometry	[9]
CNT/Polypyrrole/GOx	0.095	0.25–4	–	Amperometry	[10]
MWCNT/ZnO/GOx	4.18	0.2–27.2	1.66	Amperometry	[11]
CNx-MWCNT/Gox	13	0.02–1.02	4.6	Amperometry	[12]
Gelatin MWCNT/GOx	2.47	6.3–20.09	1.08	Amperometry	[13]

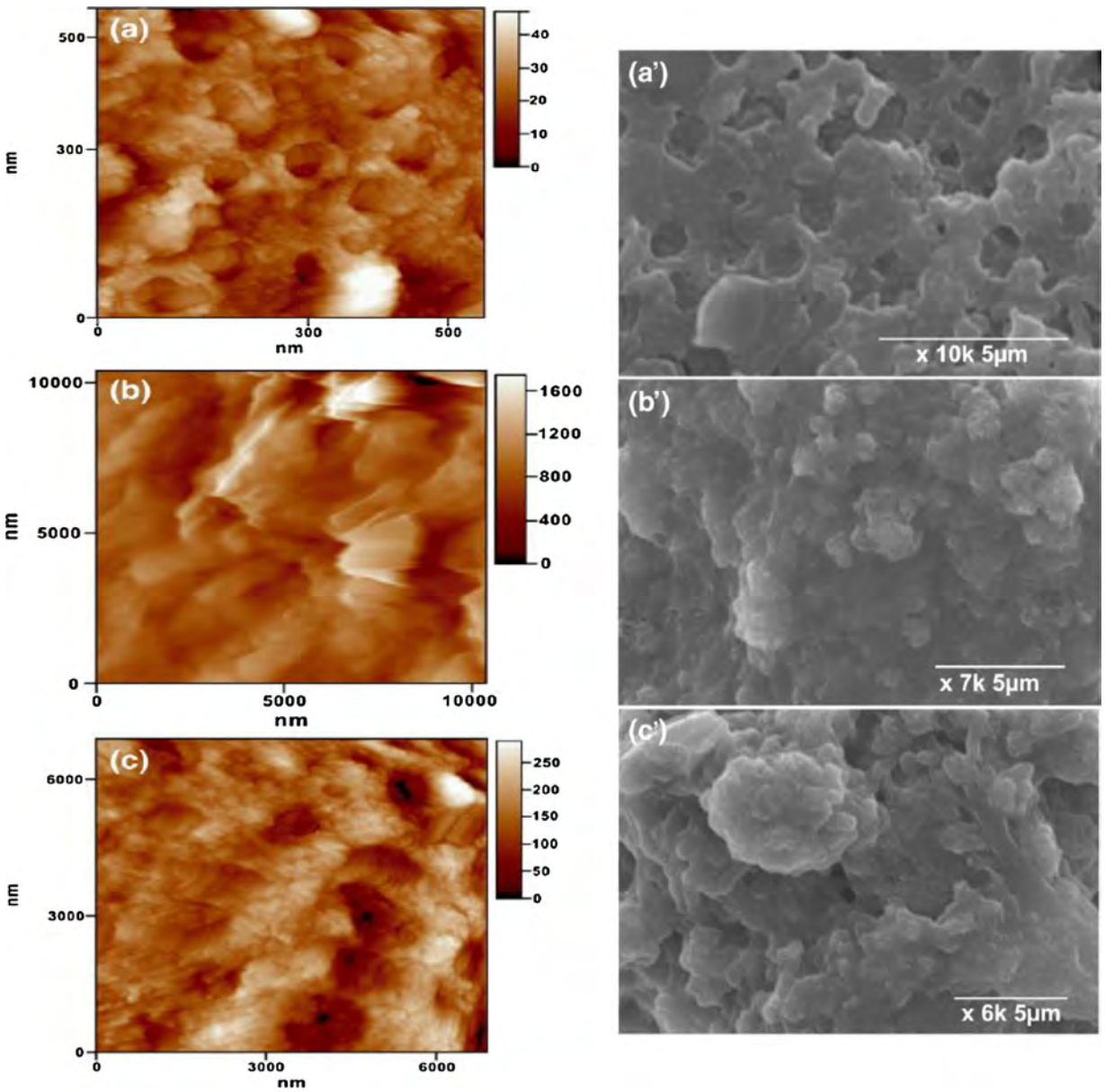

Fig. 9. AFM images of (a) GOx, (b) DCNT/GOx/GAD and (c) PVA-CNT/GOx/GAD films. SEM images of (a′) GOx, (b′) DCNT/GOx/GAD and (c′) PVA-CNT/GOx/GAD films, Reproduced from [13], Copyright (2011), with permission from Elsevier.

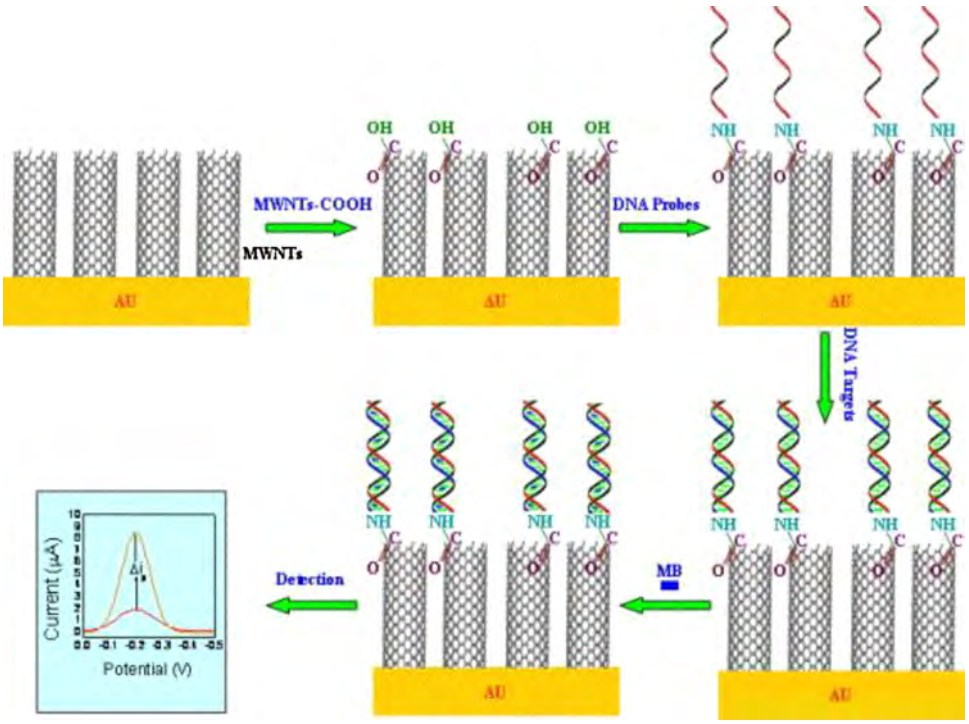

Fig. 10. Scheme for the fabrication of DNA/self-assembled MWNTs modified gold (Au) electrodes and detection of target DNA sequences. Reproduced from [138], Copyright (2004), with permission from Elsevier.

The cofactor is regenerated by reacting with oxygen, and forming hydrogen peroxide.

$$GOx\text{-}FADH_2 + O_2 \rightarrow GOx\text{-}FAD + H_2O_2$$

Hydrogen peroxide is oxidized at a catalytic, classical platinum (Pt) anode. The electrode easily recognizes the number of electron transfers, and this electron flow is proportional to the number of glucose molecules present in blood [122].

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

3.1.1.1. Monitoring of glucose. Three general strategies are used for the electrochemical sensing of glucose: (i) by measuring oxygen consumption, (ii) by measuring the amount of hydrogen peroxide produced by the enzyme reaction or (iii) by using a diffusible or immobilized mediator to transfer the electrons from GOx to the electrode. The quantification of glucose can be achieved by electrochemical detection of the enzymatically liberated H_2O_2. Recently, Wang et al. demonstrated that the overpotential of H_2O_2 can be greatly reduced at CNT-based electrodes [115], indicating the promising applications of CNTs in constructing glucose electrochemical biosensors. The hybrids of CNTs and some noble metal nanoparticles, such as palladium, platinum, gold, copper and iridium, have also been demonstrated to lower the H_2O_2 oxidation/reduction overpotential efficiently. Besides the above materials, the immobilization methods effectively influence the activity and the quality of enzyme electrodes.

3.1.2. Cholesterol oxidase based biosensors

The determination of cholesterol is of great significance in clinical diagnosis of coronary heart disease, arteriosclerosis, myocardial infarction and brain thrombosis. Among the various biosensors of cholesterol, cholesterol oxidase (ChOx)-based electrochemical biosensors are especially attractive because they offer a simple, rapid and inexpensive means of quantifying cholesterol [93,123]. Amperometric cholesterol biosensors are based on the ChOx enzyme, which catalyzes the oxidation of cholesterol to 4-cholesten-3-one and generating H_2O_2 in the presence of oxygen. The reaction is presented below:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} 4 - \text{Cholesten} - 3 - \text{one} + H_2O_2 \quad (2)$$

The quantification of cholesterol can be achieved via electrochemical detection of the enzymatically liberated H_2O_2. Guo el al. proposed a CNT-based amperometric cholesterol biosensor through the LBL (layer-by-layer) deposition of PDDA (poly(diallyl dimethyl ammonium) chloride) and ChOx on an MWNT-modified gold electrode, followed by electrochemical generation of a non-conducting PPD (poly(o-phenylenediamine)) PPD film as the protective coating [93]. This biosensor displayed high sensitivity with a linear range up to 6.0 mM and a detection limit of 0.2 mM. Tan et al. developed an amperometric cholesterol biosensor based on sol-gel CHIT/silica and MWCNT organic-inorganic hybrid composite material [124]. The results showed that the analytical performance of the biosensor can be improved greatly after introduction of MWCNTs. Shi et al. recently fabricated a novel H_2O_2 sensor by intercalating Pt nanoparticle decorated CNTs (CNT-Pt) prepared using a chemical reduction method on the surface of a waxed graphite electrode [125].

3.1.3. Other CNT-oxidase biosensors

Reliable monitoring of lactate is essential for clinical diagnostics, sports medicine, biotechnology and food analysis. Rubianes and Rivas used CNT/mineral-oil paste, containing lactate oxidase, for amperometric monitoring of lactate [126]. Accelerated electron transfer reaction of hydrogen peroxide generated by the CNT-based paste electrode offered a rapid low-potential (0.10 V) detection of the substrate. Similarly, CNT/mineral-oil paste configuration containing polyphenol oxidase was employed for amperometric monitoring of polyphenolic compounds with high sensitivity. In the presence of oxygen, tyrosinase catalyzes the oxidation of phenolic

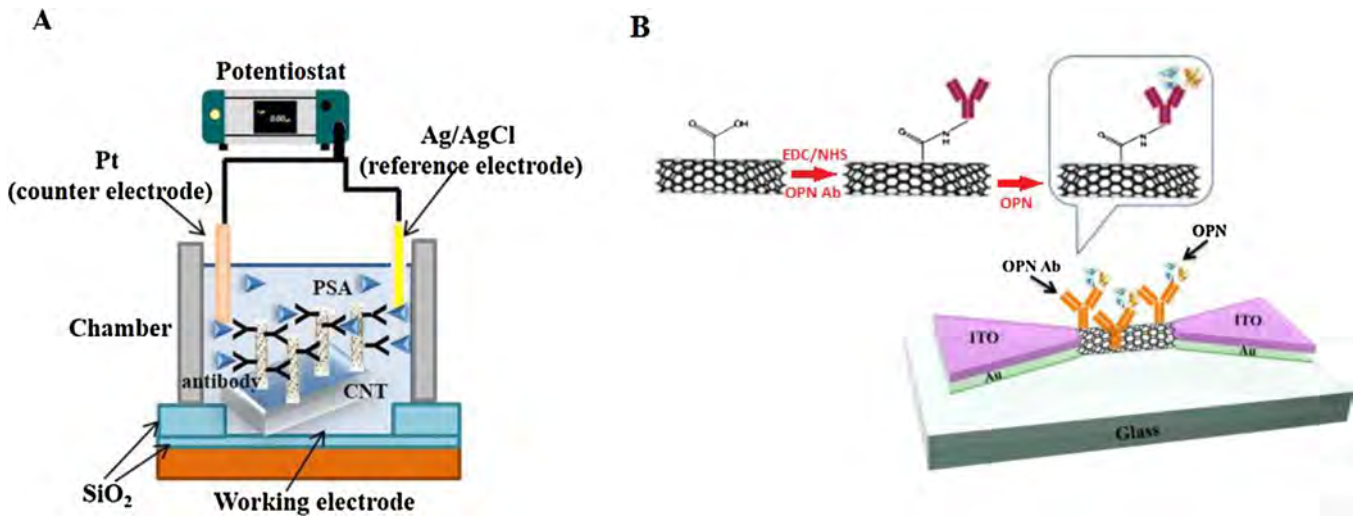

Fig. 11. A- CNT based potentiometric biosensor for PSA sensing where PSA antibodies were coated of CNT-arrayed electrodes Reproduced from [141], Copyright (2007), with permission from Elsevier, B- SWCNT based immunosensors for OPN detection, where OPN antibodies were immobilized on the SWCNT surface. Reproduced from [142], Copyright (2015), with permission from Elsevier.

compounds to quinones, which can be electrochemically detected at the electrode [19].

3.2. Dehydrogenases based biosensors

Similar to the oxidases, some dehydrogenases are also employed to construct various electrochemical biosensors, including alcohol-dehydrogenase (ADH), D-fructose dehydrogenase (FDH) and glucose dehydrogenase (GDH). Tsai et al. reported amperometric ethanol biosensor based on poly(vinyl alcohol)–MWCNT-ADH biocomposite with a sensitivity of 196 $nAmM^{-1}$, linear range up to 1.5 mM, and a response time of about 8 s [14]. The response of a dehydrogenase electrochemical biosenor is based on current generated due to the electrooxidation of NADH that is produced in the enzymatic reaction.

$$\text{Substrate} + NAD^{+} \xrightarrow{\text{Dehydrogenase}} \text{Product} + NADH \quad (3)$$

In this process, NAD^{+} behaves as an enzymatic mediator. The alcohol dehydrogenases-based electrochemical biosensors have application in direct detection of ethanol in alcoholic beverages such as beer, red wine and spirit.

3.3. Other enzymes based biosensors

Electrochemical biosensors for other enzymes such as acetylcholinesterase, alkaline phosphatase, organophosphorus hydrolase, and urease are also available. Xu et al. fabricated voltammetric urease and acetylcholinesterase biosensors by immobilizing the enzymes with a sol-gel hybrid material on the surface of the SWCNT modified electrode [18]. These biosensors are used for the determination of urea and acetylthiocholine. Yu et al. developed highly sensitive organophosphorus pesticides biosensors by immobilization of acetylcholinesterase onto amino functionalized CNTs [127]. Lenihan et al. proposed a new approach to the modification of CNTs with biomolecules for the development of biosensors by immobilizing alkaline phosphatase using an LBL technique in which a protein layer is developed on the surface of CNTs by incubation with streptavidin [128]. Trojanowicz et al. prepared biosensor for paraoxon (pesticide) by the physical adsorption of organophosphorus hydrolase on the surface of modified MWCNT [129]. Joshi et al. fabricated a disposable biosensor by using acetylcholinesterase for the detection of organophosphorus (OP) insecticides [130].

Peroxidases are oxidoreductases, catalyze a variety of reactions in the presence of peroxides such as hydrogen peroxide. Horseradish peroxidase (HRP) is exploited by several researchers to fabricate hydrogen peroxide biosensors. Hydrogen peroxide biosensors are constructed by using MWCNTs as co-immobilization matrix for the immobilization of HRP along with electron transfer mediator MB and by cross-linking of HRP and BSA composite film [131,132]. Lin et al. developed a biosensor with CNT-modified screen-printed electrode for amperometric detection of organophosphorous pesticides [133]. This disposable biosensor is based on the inhibition of acetylcholinesterase and the CNT-promoted detection of the hydrogen peroxide by co-immobilized choline oxidase. One more biosensor employed for the detection of organophosphorous compounds is based on the amperometric biosensing of the substrates of organophosphorous hydrolase (OPH) through the formation of p-nitrophenol product [134,135].

3.4. DNA aptamer based biosensors

DNA aptamer based biosensors are used for rapid, simple and inexpensive testing of genetic and infectious diseases. These biosensors work on the principle of nucleic acid recognition. The electrochemical hybridization biosensors rely on the electrical signal generated as a result of hybridization of single-stranded (ss-) DNA probe immobilized onto the transducer surface with target DNA strand. The use of CNTs enhances the performance of these biosensors. The hybridization of DNA targets could be monitored by CNT-modified electrode providing a label free DNA analysis with $Ru(bpy)^{2+}$mediated guanine oxidation. By this method the hybridization of less than a few attomoles of oligonucleotide targets can also be detected [135,136]. Further, DNA aptamer based biosensors are used for detection of metal ions, small metabolites, organic dyes and other organic molecules, peptides, proteins, cancer cells and infectious microorganisms [137] and the references there in]. DNA aptamers have smaller size, greater stability and cost effectiveness compared to antibodies and other proteins [137]. Besides, the possibility of preparing wide range of aptamers with high reproducibility and high degree of stability make them ideal recognition elements [137].

MWCNT coated gold electrodes are used to detect the presence of target DNA sequences. DNA probes adsorbed on self-assembled MWCNTs are employed for this purpose (illustrated in Fig. 10). DNA hybridization on the electrodes is assessed by following the

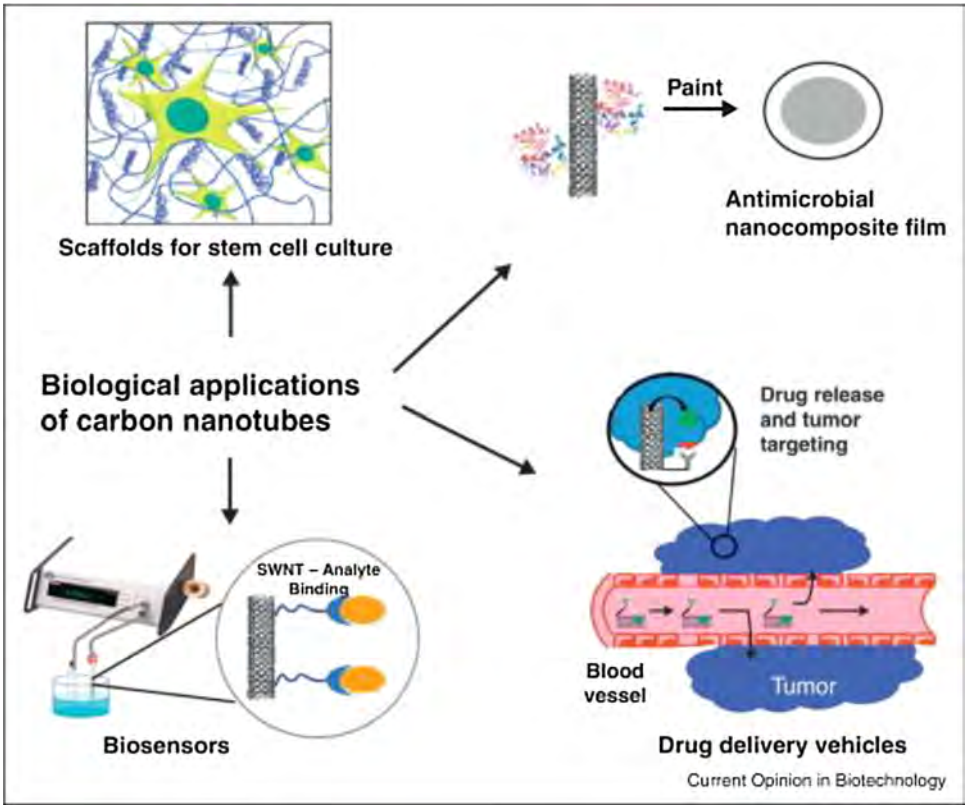

Fig. 12. Key biological applications of carbon nanotubes as biosensors, scaffolds for tissue engineering, vehicles for drug delivery in cancer therapeutics and formation of antimicrobial surfaces, Reproduced from [160], Copyright (2014), with permission from Elsevier.

changes in the voltammetric peak of the indicator methylene blue (MB) [138]. Differential pulse voltammetry was used by Cai et al. for the detection of DNA hybridization on a DNA-functionalized CNT array using daunomycin as a redox label [139]. There is another report on amino-functionalized DNA covalently bound to the carboxylic groups aligned on SWCNTs adsorbed on a gold electrode. Hybridization of the DNA with ferrocene-labelled complementary oligonucleotide is followed here with cyclic voltammetry, which results in a reversible electrochemical response [140].

3.5. CNT based biosensors coated with antibodies for the detection of biomarkers

PSA (Prostate-specific antigen) is detected using CNT-arrayed electrode coated with anti-PSA antibodies (Fig. 11. (A)) [141]. Pt wire counter electrode and Ag/AgCl reference electrode are present along with it. Similarly for the detection of OPN, (Osteopontin) immunosensors made up of glass substrate coated with OPN immobilized SWCNTs are used (Fig. 11(B)) [142]. Zheng and collaborators developed folic acid- functionalized polydopamine-coated CNTs for the electrochemical detection of HeLa and HL60 cancer cells over-expressing the folate receptor [143]. More recently, a nanobiosensor was engineered for the detection of liver cancer cells (HepG2) by using real time electrical impedance sensing. The surface of the indium tin oxide electrode is coated with assembly of CNT multilayers and antibodies to epithelial cell adhesion molecules [144].

Lemer et al. developed hybrids of a genetically engineered antibody and a carbon nanotube (EDC/NHS functionalized) transistor for detection of prostate cancer biomarkers OPN [145].

Kim and co-workers presented a simple and sensitive method for the real-time detection of a prostate cancer marker (PSA-ACT complex) through label-free protein biosensors based on a carbon nanotube field effect transistor (CNT-FET) [146].

Recently, a gold nanoparticle-decorated MWCNT-ionic liquid electrode has been modified into an impedimetric immunosensor. This immunosensor is used to detect human epidermal growth factor receptor 2 (HER2), which is a biomarker for breast cancer [147]. Both SWCNTs and MWCNTs functionalized with DNA strands were developed by Shobha et al. for sensing PSA present in blood samples for early-stage detection of prostate cancer [148]. Au-Ag alloy-coated MWCNTs were used as sensing interface for ultrasensitive detection of volatile biomarkers of MGC-803 gastric cancer cells by Zhang et al. [149] and specific detection of femtomolar-level gastric cancer biomarker miRNA-106a was done by Daneshpour and co-workers [150].

4. Other biomedical applications of carbon nanotubes

The unusual and very interesting physicochemical properties CNTs, such as ordered structure with high aspect ratio, mechanical strength, electrical conductivity, thermal conductivity, metallic or semi-metallic behavior, high surface area and ultra-light weight [151] make CNTs amenable materials for a variety of applications viz. for the growth of cells for tissue regeneration, delivery systems for a variety of diagnostic or therapeutic agents or as vectors for gene transfection (Fig. 12) [152–160]. Some of the applications are presented here [161].

4.1. Monitoring of metabolic compounds

The changes in metabolome signal differentiation and development of cell types. Monitoring of metabolites in embryonic and mesenchymal cell cultures can provide real-time information of cell line status. Conventional methods can successfully monitor a few

process variables, like pH, pO_2, electronic impedance, and temperature in bioreactors. Highly sensitive nano-biosensors developed in the recent times using carbon nanotubes can detect compounds such as lactate and glucose [162,163] at very low concentrations, suggesting possibility for development of detectors for a variety of compounds.

5. Conclusion

The extraordinary electrochemical properties of CNTs have paved the way for their use as platforms for the construction of a wide range of electrochemical biosensors with improved analytical behavior [164]. They combine several exceptional physical, chemical, electrical, and optical characteristics properties which make them one of the best suited materials for the transduction of signals associated with the recognition of analytes, metabolites, or disease biomarkers. Besides, CNTs can serve as scaffolds for immobilization of biomolecules on their surface. The electrocatalytic activity of CNT based electrodes towards hydrogen peroxide and NADH permits effective low-potential amperometric biosensing of numerous important substrates. The enhanced electrochemical reactivity is coupled with resistance to surface fouling to confer high stability to the detector. Electrochemical DNA biosensors can greatly benefit from the use of CNT support platforms and from the enhanced detection of the product of the enzyme label or the target guanine. Such interdisciplinary efforts hold hope for development of new generation of CNT-based biosensors for a wide range of applications. Importantly, CNT based biosensing has potential for in vivo detection with less cytotoxicity, high sensitivity, and long-term stability for reliable point-of-care diagnostics under physiological conditions. Additionally, these applications are not restricted to the medical field but have varied applications in the food, water purification, and agricultural industries among many others. It is thus fair to say that the real biosensing applications of CNTs lie ahead in the future.

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