Multiwalled carbon nanotubes bound beta-galactosidase: It's activity, stability and reusability

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Abstract

Carbon nanotubes (CNTs) based biosensors are recognized to be a next generation building block for ultrasensitive and fast biosensing systems. This article starting with a brief history on CNTs provides an overview on the recent expansion of research in the field of CNT-based biosensors. This is followed by the discussion on structure and properties related to CNTs. Furthermore, the basic and some newly developed synthetic methods of CNTs are summarized. In this chapter, we used polyaniline cobalt multiwalled CNTs to immobilize β-galactosidase, by adopting both noncovalent and

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covalent strategies. Herein, the methodologies of both techniques have been discussed in detail. The η (effectiveness factor) values for nanocomposite bound β-galactosidase by physical adsorption and covalent method were calculated to be 0.93 and 0.97, respectively. The covalently bound β-galactosidase retained 92% activity even after its 10th successive reuse as compared to the adsorbed enzyme which exhibited only 74% of its initial activity. CNT armored enzymes demonstrated remarkably high catalytic stability at both sides of temperature and pH-optima along with easy recovery from the reaction medium which can be utilized in various biotechnological applications. Lastly, the scientific and technological challenges in the field are discussed at the end of this chapter.

Abbreviations

AC	activated carbon
AOx	alcohol oxidase
APS	ammonium persulfate
CNTs	carbon nanotubes
CVD	chemical vapor deposition
DWCNTs	double walled carbon nanotubes
EDTA	ethylene diamine tetra acetic acid
Fc	ferrocene
FTIR	Fourier transform infrared spectroscopy
GA	glutaraldehyde
GCE	glassy carbon electrode
GOx	glucose oxidase
HRP	horse radish peroxidase
MNPs	magnetic nanoparticles
MWCNTs	multiwalled carbon nanotubes
NC	nanocomposite
NM	nanomaterial
NPs	nanoparticles
ONPG	o-nitrophenyl β-D-galactopyranoside
PEI	polyethylenimine
SAED	selection area electron diffraction
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SWCNHs	single walled carbon nanohorns
SWCNTs	single walled carbon nanotubes
TEM	transmission electron microscopy

1. Introduction

The catalytic function of β-galactosidase, βGS (EC.3.2.1.23) is to hydrolyze lactose into its monomers that is glucose and galactose. It has wide

applications in food processing industry, such as to enhance sweetness, solubility, flavor and digestibility of dairy products (Panesar, Kumari, & Panesar, 2010; Singh, Kumar, Mittal, & Mehta, 2016). However, majority of enzymes are fairly unstable and their industrial application is often restricted by a lack of long-term operational stability and the technically challenging recovery process (Sheldon & van Pelt, 2013).

In order to make enzyme utilization in biotechnological processes more favorable, different methods for cost reduction have been put into practice and, immobilization is one of them. Immobilization process enhances stability of the enzyme under both storage and operational conditions. Most importantly, it provides a facile separation of the enzyme from the product hence protein contamination of the product is minimized or avoided altogether (Saifuddin, Raziah, & Junizah, 2013). Immobilization also facilitates enzyme recovery which thereby helps to reduce the cost because the enzyme can be used repeatedly (Khan & Husain, 2018; Sheldon & van Pelt, 2013). Also, it can preclude intramolecular processes, e.g., autolysis, proteolysis and aggregation. The choice of support and the method of immobilization used are the utmost factors taken into consideration prior immobilization.

Recent advances in nanotechnology have provided a wealth of diverse nano-scaffolds that can be used as support for enzyme immobilization. Nano-biocatalysis integrating the biocatalyst and nanoscale materials is drawing great attention as innovative technology. The premise of using nanoscale structures for enzyme immobilization is to reduce diffusion limitations and maximize the functional surface area to increase enzyme loading. Among the various nanomaterials (NMs) used till date, carbon-based NMs have emerged to be one of the most promising ones as they possess honeycomb like structure, thereby providing larger surface area for efficient enzyme loading (Khan, Husain, & Naqvi, 2016).

1.1 Structure and properties of CNTs

Carbon nanotubes (CNTs), also known as buckytubes, were discovered in 1991 when the Japanese electron microscopist Sumio Iijima published his first report on multi-walled carbon nanotubes (MWCNTs), eventually followed by the discovery of single-walled CNTs, SWCNTs (Ijima, 1991). Since then, CNTs have emerged most intensively studied nanostructured materials as evidenced by thousands of papers published on it every year (Balasubramanian & Burghard, 2005). Due to the exceptional properties they possess, they have been employed in a variety of applications that

attracts both academic and industrial interest. CNTs are long hollow tubular structures made up of sp^2 carbon atoms and a nanoscale diameter with one or more walls. The well ordered arrangement of carbon atoms linked via sp^2 bonds provides them the strength and stiffness.

Depending on the number of walls, CNTs can be classified into SWCNTs and MWCNTs. Generally, CNTs are comprised of several concentric rings or cylinders of increasing diameter commonly known as MWCNTs. They can be extended to a diameter of about 100 nm and the distance between the two walls is similar to the distance between the two graphene layers in graphite, 3.5 Å (Balasubramanian & Burghard, 2005). Double walled CNTs (DWCNTs) bridge the gap between SWCNTs and MWCNTs by possessing the properties of both kinds of CNTs. They are made of only two concentric cylinders. More specifically, DWCNTs resemble SWCNTs with respect to their small diameter, length and ability to form bundles, however, their mechanical stability is remarkably high compared to SWCNTs, when covalently functionalized (Yang, Chen, Ren, Zhang, & Yang, 2015). Furthermore, the outer wall of DWCNTs can be functionalized without affecting mechanical and electrochemical properties of the inner tube similar to MWCNTs (Pumera, 2007).

CNTs are well suited for a wide variety of challenging biomedical applications in the field of medicine and engineering; Fig. 1 (Chen & Chatterjee, 2013; Gomez-Gualdrón, Burgos, Yu, & Balbuena, 2011). CNTs-based

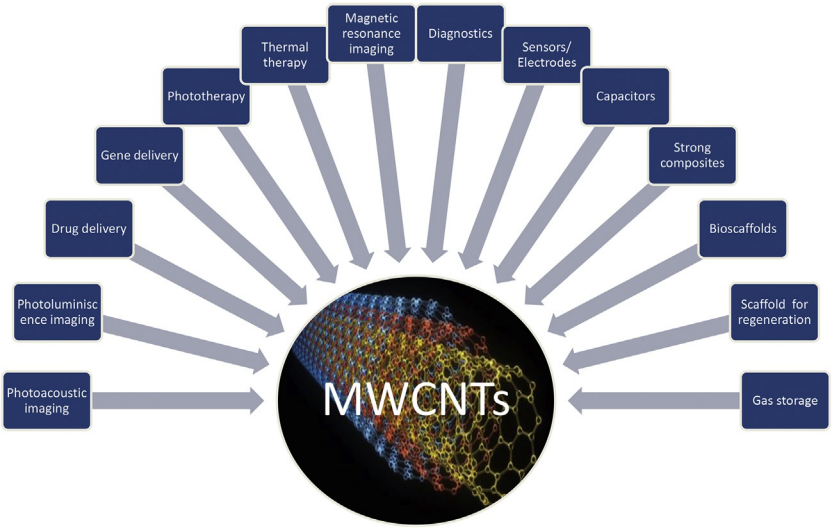

Fig. 1 Applications of CNTs in biomedical area of research.

materials can have a profound impact on electronic and optoelectronic devices, chemical sensors, nanocomposites, optical biosensor and energy storage (Arugula & Simonian, 2014; Boujtita, 2014; Pisoschi, 2013). It is well known that the material properties (e.g., electrical or optical) of CNTs are very sensitive to be affected by exposure to biomolecules and this has led to the investigation, by a number of groups of these materials as sensing elements for biosensors (Hernandez-Ibanez et al., 2016; Jasim, Wajidullah, Shi, Lin, & Yang, 2017). Additionally, their unique structure provides an exceptional electric-current-carrying capacity along their length (1000× higher than copper wires) which leads to attractive CNT-based new electrode material (Abdalla, Al-Marzouki, Al-Ghamdi, & Abdel-Daiem, 2015). The properties possessed by CNTs, such as fast electron transportation high thermal conductivity, excellent mechanical flexibility and good biocompatibility, make them an ideal choice in the fabrication of sensitive electrochemical biosensor. New applications of CNT-based NM in electrochemical sensors and biosensors are now on the increase with the advent of nanotechnology (Andreescu, Njagi, & Ispas, 2008).

1.2 Synthesis and functionalization of CNTs

A number of different routes to synthesize CNTs have been demonstrated over the recent years. The three main synthesis techniques for CNTs production are arc discharge, laser ablation and chemical vapor deposition, CVD (Gougis, Tabet-Aoul, Ma, & Mohamedi, 2014; Park, Vosguerichian, & Bao, 2013). CNTs generally obtained by the arc method or hydrocarbon pyrolysis are multi-walled, having several graphitic sheets or layers. Single-walled carbon nanohorns (SWCNHs) are horn-shaped single-walled tubules with a conical tip. They are generally synthesized by laser ablation of pure graphite without using metal catalyst with high production rate and yield, and typically form radial aggregates (Manawi, Ihsanullah, Samara, Al-Ansari, & Atieh, 2018). Table 1 demonstrates various methodological processes employed in the synthesis of CNTs along with their outcome in detail.

One of the major drawbacks associated with CNTs to be used in biosensor and biomedical applications is their tendency to aggregate in bundles through strong interactions which are difficult to disrupt rendering these materials incapable of achieving their full potential. However, functionalization of CNTs by attaching functional groups on their surface helps to solubilize them and hence facilitate their use in a myriad of applications

Table 1 Detailed description of synthetic methods of CNTs.

Method	Process	Result	Reference
Arc discharge	Two graphite rods in water bath at different voltage	High yield CNTs of good quality	Lakshmi and Khan (2014)
	Two graphite electrodes submerged in different media	Nanocarbon structures of different dimensions	Vasu, Pramoda, Moses, Govindaraj, and Rao (2014)
	Instead of metal catalyst and vacuum devices, strong oxidizing agents HNO_3/H_2O_2 are used	Improves purity of MWCNTs	Singhal, Singh, Singla, and Dharamvir (2013)
	The discharge is maintained in a magnetic field	High quality MWCNTs	Singh, Dharamvir, and Singhal (2013)
	During synthesis physical forces are applied	Relatively straight and defect free MWCNTs	Berkmans, Ramakrishnan, Jain, and Haridoss (2013)
Laser ablation	Binary catalysts combined with Fe, Co and Ni (transition metals)	Different types of carbon nanostructures	Jedrzejewska et al. (2013)
	Irradiation of CO_2 laser in continuous wave mode onto a boron containing graphite target at room temperature	Fine crystalline structure of MWCNTs	Yuge et al. (2012)
CVD	Co used as catalyst (at 700°C) with the use of hydrogen to acetylene gas ratio 25:25 Scm	High yield MWCNTs	Kishore and Pandurangan (2012)
	Using Ni/MgO as catalyst and CH_4 in microfluidized bed photochemical deposition	High purity MWCNTs with less defect	Fu, Cui, Wei, and Ma (2014)
Solvothermal	At low-temperature (180°C)	MWCNTs bundles	Krishnamurthy and Agarwal (2013)
	At temperature 200°C for 10 h	Magnetic MWCNTs with alterable structure	Xiao et al. (2012)

(O'connell et al., 2001; Tasis, Tagmatarchis, Georgakilas, & Prato, 2003). In fact, the functionalization of CNTs has become a pre-requisite in order to enable facile fabrication of nanodevices. Among all the functionalization approaches, physical, chemical or combined modifications have been exploited for their homogeneous dispersion in common solvents to improve their solubility and application.

Physical modification utilize the mechanical means such as ultrasonic, milling, crushing and friction to activate CNTs surface in order to change their surface morphology (Fujigaya & Nakashima, 2008). This method can increase CNTs' internal energy and surface activity and therefore make the tubes react with or attached to other materials, to attain the purpose of the surface modification. At present, the large shear force or ultrasonic processing is often used to disperse CNTs (Hasan, Crawford, & Ivanova, 2013; Spitalsky et al., 2009).

One possibility of CNT functionalization is filling the inner cavity with molecules, known as endohedral functionalization (Huang et al., 2013; Ye, Jo, Jeong, & Lee, 2013). Additionally, metallofullerenes were encapsulated in CNTs as well as noble metals (e.g., Au, Ag, Pt, Pd), yielding metallic nanowires (Huang et al., 2013; Meng et al., 2013). In general, chemical approach can be either covalent or non-covalent in nature. Various ways by which we can covalently functionalize CNTs have been demonstrated in Fig. 2. Non-covalent type of functionalization possesses several

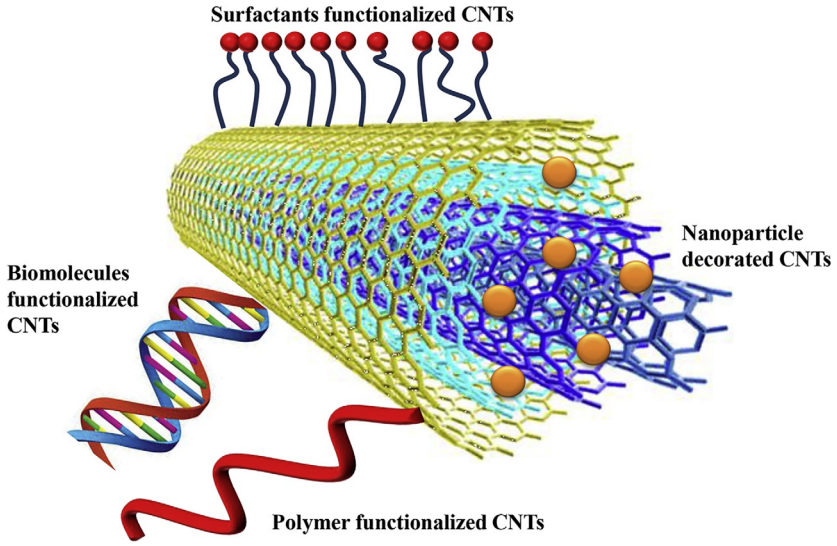

Fig. 2 Different methods of covalent functionalization of CNTs.

advantages over covalent method such as the preservation of structural homogeneity and electrical properties of CNTs. However, these procedures involve ultrasonication, centrifugation and filtration steps.

For ideal covalent or non-covalent functionalization coating on CNTs, some points have to be kept in mind (Tîlmaciu & Morris, 2015). First, the coating used should be non-toxic and biocompatible in nature. Second, it should be stable enough to avoid detachment from the nanotube's surface in biological solutions, and lastly it should bear active functional groups so as to provide interaction of CNTs with other molecules in order to prepare subsequent CNT bioconjugate. To summarize, it has been proven through various studies that surface functionalized CNTs can behave in a biologically different and safer manner, compared to their pristine counterparts (Mananghaya, Santos, & Yu, 2017; Marchesan, Kostarelos, Bianco, & Prato, 2015).

1.3 Application in biosensing

With the outburst of nanotechnology which includes NM with distinguished physicochemical properties, a new class of biosensors referred to as nanobiosensors have been developed. These types of biosensors have unprecedented combination of NM exhibiting properties notably their small size and large surface/volume ratio along with the functionality of "macro"- biosensors in order to facilitate sensitivity and lower detection limits (Holzinger, LeGoff, & Cosnier, 2014). While designing biosensors, the immobilization of enzymes on the transducer surface is an important and decisive step. Many studies have been performed on the immobilization of enzymes due to the known benefits of the enhancement in the property of the enzyme which is convenient in the handling, ease of separation, reduced product cost and conceivable increase in thermal and pH stability (Bucko et al., 2012; Khan, Husain, & Ahmad, 2018). Among various industrially important enzymes, βGS has been extensively utilized as a prototype enzyme due to its adaptability as a biosensor for lactose detection and to provide lactose-free milk to lactose intolerance patients worldwide.

The selection of appropriate immobilization technique affects the biosensor performance like sensitivity, selectivity and stability, as it affects the enzyme orientation, loading, mobility and structure. Nanoparticles-based enzyme immobilization and their potential applications in biosensors have been described in a review paper (Ansari & Husain, 2012). Furthermore, different kinds of techniques applied for the immobilization of βGS and its significances in food industry has been explained in detail in a review article (Panesar et al., 2010).

To obtain the best immobilized enzyme, it is highly recommended that the characteristics of support and enzyme should be known. 3D conformation of an enzyme is liable to alterations because it is balanced by the optimization of counter forces like, hydrophilic interactions, Columbic force, hydrogen bonds, and van der Waals interactions. Essentially, enzymes can pivot inside out while using a hydrophobic support, losing its activity because proteins in general are hydrophobic in their interior and hydrophilic in the exterior (Netto, Toma, & Andrade, 2013). These immobilization techniques have been mainly employed for βGS to advance electrochemical, optical, or gravimetric enzymatic biosensors.

In recent years, CNTs have received great attention as promising carbon-based nanoelectronic devices. Fig. 3A indicates the data based on the articles published related to CNT-biosensors in the past 15 years and according to the list of publications, China occupies a prime position in developing such biosensors based on CNTs (Fig. 3B). The large surface area and good electrical conductivity of CNTs allow them to act as "electron wire" between the redox center of an enzyme or protein and an electrode's surface, which make them an excellent material for the design of electrochemical biosensors (Husain, 2017, 2018a; Mundra, Wu, Sauer, Dordick, & Kane, 2014). Table 2 shows the recent developments in biosensors based on SWCNT/MWCNT, while Table 3 lists different enzyme biosensors based particularly on MWCNTs, their respective analyte, linearity range and limit of detection.

Because of the unprecedented properties possessed by CNTs, as mentioned in previous section, a large number of CNT-conjugates have been designed for detection of DNA biomarkers, cell surface sugars, protein receptors, hormones, antibodies, enzymes and various metabolites (Tîlmaciu & Morris, 2015). Depending on their mechanism of target recognition and transduction, these biosensors are broadly subdivided into electronic transducers, electrochemical CNT-biosensors, immunosensors, and optical CNT-based biosensors. Functionalized CNTs have been used as electrochemical sensors and many review papers concerning CNTs-electrochemical sensors have been highlighted (Dai, 2007; Daniel et al., 2007; Gao, Guo, Liua, & Huang, 2012; Husain, 2018a; Jacobs, Peairs, & Venton, 2010; Yang et al., 2015).

Das and Goswami (2013) immobilized an octameric alcohol oxidase (AOx) (Mr 675 kDa) obtained from *Pichia pastoris* on MWCNT-nafion (MWCNT-Nf) matrix. The complex encapsulated with polyethylenimine (PEI) on gold electrode, showed a redox peak at 0.21 V (vs Ag/AgCl electrode at pH 7.5) for oxidation of alcohol. The electron transfer rate constant

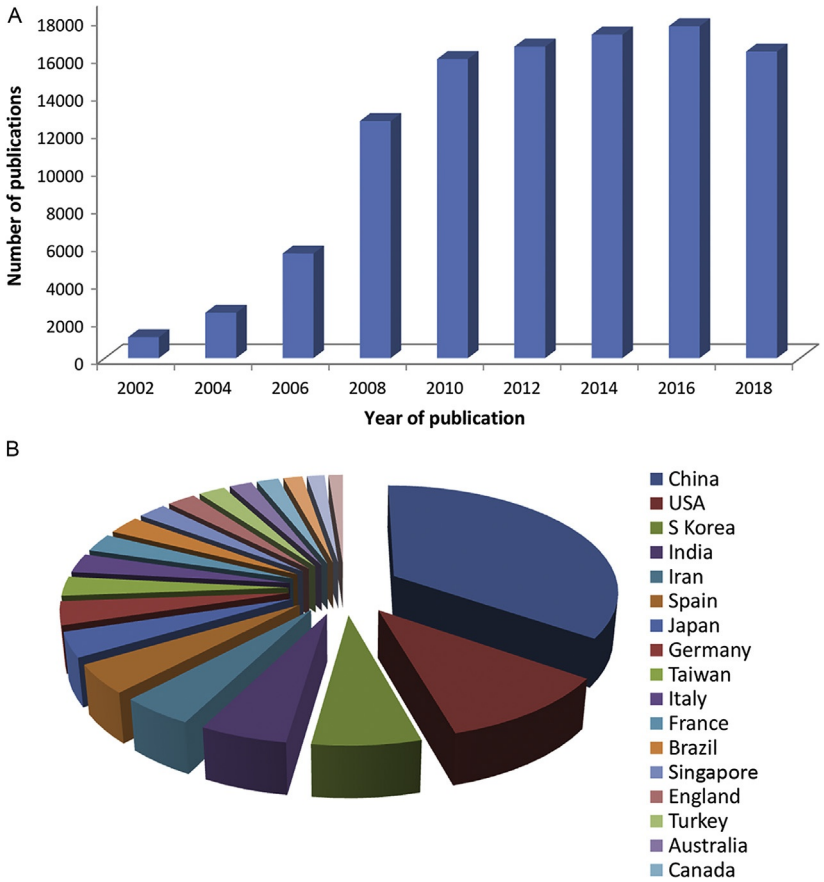

Fig. 3 (A) Graphical representation of number of published papers of CNT-based biosensors in last 15 years. This data was searched using Google scholar and includes regular articles, review articles and book chapters. (B) Top 20 countries of research community working on CNTs-based biosensors.

and surface coverage of the immobilized enzyme were $1.69 \pm 0.15\,s^{-1}$ and $2.43 \times 10^{-12}\,mol\,cm^{-2}$, respectively. Au-MWCNT-Nf-AOx-PEI bioelectrodes for alcohol exhibited a linear response in the range of 8–42 μM and detection limit of 5 μM. Moreover, these bioelectrodes retained ~90% of the original response even after 4 weeks of storage in potassium phosphate buffer, pH 7.5 at 4 °C.

Chinnadayyala et al. (2014) developed a third generation biosensor for improved detection of alcohol in liquid samples. They reported an alcohol oxidase, AOx with a two-fold increase in efficiency (K_{cat}/K_m) by physical entrapment of the activator ferrocene (Fc) in the protein matrix through a

Table 2 Recent developments in biosensors based on SWCNT/MWCNT.

CNT biosensor	Type of biosensor	Analyte	Reference
CNTs	Electrochemical	Ions, metabolites and protein bio-markers	Wang and Dai (2015)
MnO_2-CNT/Pt	Enzymatic	Catechin	Vilian et al. (2015)
MWCNTs	Cholesterol	Cholesterol in blood	Li, Liao, Hu, Ma, and Wu (2005)
Chitosan-CNTs	Electrochemical	Nitric oxide	Santos, Rodrigues, Laranjinha, and Barbosa (2013)
Functionalized-MWCNTs	Electrochemical	Epinephrine	Prasad, Prasad, Tiwari, and Madhuri (2013)
Functionalized-MWCNTs	Electronic	Rat striatum	Kress et al. (2014)
Chitosan-SWCNTs	Electronic	Cellular nitric oxide	Li, Liu, et al. (2005)
SWCNTs	Electronic	Epidermal carcinoma cells	Jin et al. (2010)
Pt/CNTs	Amperometric biosensor	Cysteine	Fei et al. (2005)
CNT arrays	Amperometric biosensor	Biomarker	Yuichi et al. (2009)
MWCNTs-biopolar electrode	Amperometric	Prostate-specific antigen	Feng, Pan, Zhang, Zhu, and Chen (2004)
MWCNTs-P450	Amperometric	Breast cancer	Baj-Rossi, De Micheli, and Carrara (2012)
Tricosane/f-SWCNTs	Electrochemical	Lung cancer	Liu et al. (2011)
D-(+) galactose-SWCNTs	Electrochemical	Cancer marker galactin-3	Park et al. (2011)
	Electrochemical	HeLa and HL60	Zheng, Zhang, Zou, and Jun-Jie (2012)
SWCNTs/MWCNTs	Amperometric	Prostate cancer, PSA detection in blood samples	Shobha and Muniraj (2015)
Au-Ag/MWCNTs	Amperometric	MGC-803 gastric cancer cells	Zhang et al. (2014)

Table 3 Various MWCNTs—enzyme based biosensors, their analytes, linearity range of measurement and detection limit.

Sensor	Analyte	Limit of detection	Linearity range	Sensitivity	Reference
Glucose oxidase/NH_2-MWCNT	Glucose	9 μM	17–650 μM		Khodadadei, Ghourchian, Soltanieh, Hosseinalipour, and Mortazavi (2014)
MWCNT-nafion HRP-Chitosan ferrocene entrapped polyethylenimine	Ethanol	2 μM	5–3000 μM	1 μM	Chinnadayyala, Kakoti, Santhosh, and Goswami (2014)
MWCNT-nafion HRP-Chitosan alcohol oxidase polyethylenimine	Ethanol	5 μM	8–42 μM	0.264 μM	Das and Goswami (2013)
Au NP-MWCNTs	Uric acid	–	0.01–0.8 mM	10 and 20 $mg L^{-1}$	Chauhan and Pundir (2011)
MWCNT-acetyl choline esterase	Carbofuran	–	10^{-10}–10^{-3} $g L^{-1}$	10^{-12} $g L^{-1}$	Zhang, Li, Ma, Xiong, and Qu (2013)
Carboxylated MWCNT sorghum leaf oxalate oxidase	Oxalate	–	8.4–272 μM	0.0113 μA μM^{-1}	Yadav, Devi, Kumari, Yadav, and Pundir (2011)
MWCNT—Lysozyme	Lysozyme	–	2.5 mM $(Fe CN)_6^{3-/4-}$	42.09 μg mL^{-1}	Silva et al. (2014)
MWCNT-nafion cysteamine modified tyrosinase	Dopamine	–	0.05–100 μM	1 μM	Canbay and Akyilmaz (2014)
Glucose oxidase-ZnO NPs-MWCNT	Glucose	–	0.1–6 μM	12.5 mA m^{-1} cm^{-2}	Wang et al. (2009)
Glucose oxidase-silicon dioxide Fe_3O_4@SiO_2/MWCNT	Glucose	–	1 μM–30 mM	800 nM	Baby and Ramaprabhu (2010)
Au NPSs Graphene MWCNT	Glucose	–	4.8 μM	29.72 mA mM^{-1} cm^{-2}	Vanegas et al. (2014)
Ni-MWCNT	Glucose	0.021 μM	0.05–12 mM	70 mA mM^{-1} cm^{-2}	Başkaya et al. (2017)
Palladium Ni-MWCNT	Glucose	0.014 μM	0.01–1.00 mM	90 mA mM^{-1} cm^{-2}	Koskun, Şavk, Şen, and Şen (2018)

simple microwave-based partial unfolding technique. The sensor was fabricated by immobilizing Fe entrapped alcohol oxidase, FcAOx and sol–gel chitosan film coated horseradish peroxidase (HRP) on MWCNT modified GCE through layer-by-layer technique. The amperometric biosensor exhibited a linear response to alcohol in the range of 5.0×10^{-6}–30×10^{-4} mol L^{-1} with a detection limit of 2.3×10^{-6} mol L^{-1}, and a sensitivity of 150 μA mM^{-1} cm^{-2}. The biosensor response was steady for 28 successive measurements completed in a period of 5 h and retained ~90% of the original response even after 4 weeks when stored at 4 °C.

In a study MWCNTs were amine-functionalized by a process of dielectric barrier discharge plasma treatment. The amine-functionalized MWCNTs were subsequently fixed on glassy carbon electrode (GCE) and glucose oxidase (GOX) was immobilized as a model enzyme on the modified GCE. The obtained enzyme electrode was used for glucose biosensing with a linear range of 17–646 μM and sensitivity of 12.3 μA/mM cm^2. Based on the signal to noise ratio of 3, the detection limit was estimated to be 9 μM. The Michaelis–Menten constant for immobilized GOX was found to be 480 μM (Khodadadei et al., 2014).

Functionalized MWCNT supported with highly monodisperse Ni NP modified on GCE (Ni@f-MWCNT/GCE) were synthesized through microwave assisted method and examined for non-enzymatic glucose sensing in ionic liquids by cyclic voltammetry and chronoamperometry. The results demonstrated a good linear span of 0.05–12.0 mM and a detection boundary of 0.021 μM. Furthermore, in the amperometric signal of the electrodes after 200th cycles, no significant change was observed (Başkaya et al., 2017).

In a more recent study, a novel glucose sensor was prepared by in situ reduction technique. A highly sensitive activated carbon (AC) decorated on monodisperse nickel and palladium alloy NC were fixed on a GCE (Ni-Pd@AC/GCE NCs) and GOX was immobilized on the modified carbon electrode. In addition, the Ni-Pd@AC/GCE NC showed a very low detection limit of 0.014 μM, a wide linear range of 0.01 mM^{-1} mA and a very high sensitivity of 90 mA mM^{-1} cm^{-2} (Koskun et al., 2018).

The combination of polymers with carbon NM for the preparation of chemical sensors has opened new avenues of research due to their biocompatibility, excellent sensitivity and selectivity. Among the various conducting polymers, polyaniline (PANI) is one of the most considerable, due to its high electrical conductivity, unsophisticated synthesis, and environment friendly nature (Das, Prusty, Adhikary, & Kalra, 2015). In recent past,

it has gained much recognition in biosensor applications, since it acts as an efficient mediator for electron transfer in the redox reaction. Cobalt (Co), because of its electrical and thermal properties is also extensively employed for the development of nanoparticle (NP) based biosensors. Therefore, we synthesized polyaniline doped cobalt MWCNTs NC (PANI/Co/MWCNT-NC) in order to achieve combinatorial effect for successful immobilization of βGS isolated from *Aspergillus oryzae*.

2. Methodology part

2.1 Synthesis of PANI/Co/MWCNT-NC

General safety: Safety glasses, latex, or nitrile gloves and lab coat.

Chemicals and reagents

1. Multiwalled carbon nanotubes (purity ≥95% wt%, 10 nm diameter)—Central Drug House Private Limited, New Delhi, India
2. Sodium dodecyl sulfate (SDS)—Central Drug House Private Limited, New Delhi, India
3. Ammonium persulfate (APS)—Central Drug House Private Limited, New Delhi, India

Equipment

1. Beaker (50–500 mL)
2. Stirrer/hot plate
3. Analytical balances
4. pH meter
5. Sonicator
6. Magnetic stirrer with bar
7. Hot air oven

Methodology

Method adopted by Giri, Das, and Kalra (2012) after some minor modification was used for the synthesis of PANI/Co/MWCNT-NC.

1. In a beaker, mix 150 mL of 1 M HCl and 50 mL of 0.2 M sodium dodecyl sulfate and subsequently add 60 mg of MWCNT into it.
2. Sonicate the mixture for 30 min at room temperature.
3. In another beaker, take 1 mL of aniline monomer in 50 mL of 1 M HCl and add calculated amount of $CoCl_2$ (3%, w/v) by stirring continuously to achieve a homogenous suspension.
4. Mix both the solutions prepared in the previous steps and sonicate for another 10 min.

5. Add 50 mL of 1 M HCl containing 2.04 g of APS drop by drop in stirring condition to the solution for the complete polymerization of the monomer.
6. Incubate the mixture at 0–5 °C for 24 h.
7. Finally, filter the resulting precipitate and wash with distilled water and ethanol.
8. Dry the precipitate well in a vacuum oven at 60 °C for 48 h for further studies.

2.2 Characterization of PANI/Co/MWCNT-NC

General safety: Safety glasses, latex, or nitrile gloves and lab coat.

Chemicals and reagents

1. Potassium bromide (AR Grade)—Sisco Research Laboratories (SRL), India
2. Sodium acetate (AR Grade) buffer (pH 4.5, 0.1 M)—SRL, India
3. PANI/Co/MWCNT-NC (diluted solutions)

Equipment

1. Carbon coated copper grids-300 mesh
2. JEOL-2100 transmission electron microscope, Japan
3. Sputter coater-JEOL JFC 1600, Japan
4. JSM-6510 LV scanning electron microscope, Japan
5. FTIR-Perkin Elmer instrument, USA (Spectrum-II, wave no. 4000-400).

Methodology

The free and enzyme bound NC preparations were characterized by TEM (JEOL-2100, 200 κV), SEM (JSM-6510 LV, 15 κV) and FT-IR (Perkin Elmer instrument, USA, Spectrum- II, wave no. 4000-400).

1. For TEM studies, prepare the samples by drop coating diluted solutions on carbon coated copper grids at normal atmospheric conditions.
2. Prepare small samples from PANI/Co/MWCNT-NC and air dried immobilized βGS for SEM studies. Mount these samples on carbon tape on a copper stub, followed by 60 nm of gold deposition by means of a sputter coater.
3. For FT-IR, crush the samples and thoroughly mix with KBr to form a transparent pellet.
4. Finally, UV–vis spectrophotometer (Shimadzu, UV-1800) was acquired to measure the enzyme concentration.

The immobilization procedure as to how the enzyme was attached on/to the NC has been described in the subsequent section.

2.3 Immobilization of βGS on PANI/Co/MWCNT-NC

General safety: Safety glasses, latex, or nitrile gloves, lab coat.

Chemicals

1. Sodium acetate (AR Grade) buffer (pH 4.5, 0.1 M)—SRL, India
2. PANI/Co/MWCNT-NC (600 mg)
3. βGS (*Aspergillus oryzae*)—Sigma-Aldrich Chemicals Co. (USA)

Materials and equipment

1. Falcons (15 mL)—Tarson, India
2. Microtubes (1.5 mL)—Eppendorf, India
3. Shaking incubator—Biogen. India
4. Micro-pipettes (20.0–100.0 μL, 0.1–1.0 mL)
5. Ultrasonicator—Life Care
6. Magnet (6000G)
7. UV–vis spectrophotometer (Shimadzu, UV-1800)
8. Centrifuge-REMI Cooling Centrifuge

Methodology

1. First, vary the concentration of PANI/Co/MWCNT-NC from 2 to 18 mg and then independently adsorb free βGS on varying concentrations by continuously shaking the mixture in sodium acetate buffer, pH 4.5 at 25 °C.
2. Second, differ the enzyme concentration from 1 mg mL^{-1} to 5 mg mL^{-1}, keeping the NC concentration (10 mg) fixed.
3. In second set of experiments, where βGS is covalently attached to the support, vary NC concentration from 2 to 10 mg, while keeping the glutaraldehyde (GA) concentration fixed at 2%.
4. Furthermore, vary the concentration of GA from 0.5% to 3.0% (v/v) by maintaining the concentration of NC at 6 mg.
5. Calculate immobilization yield in all cases and take the highest yielding parameters to progress further.
6. For simple adsorption method, suspend and sonicate PANI/Co/MWCNT-NC (250 mg) in 0.1 M sodium acetate buffer, pH 4.5 for 20 min.
7. For covalent method, mix NC (250 mg) with 2%, (v/v) GA in continuous stirring condition for 2 h.
8. Centrifuge the activated support at $3000 \times g$ for 10 min at 4 °C and wash thrice with assay buffer of 0.1 M sodium acetate, pH 4.5 in order to remove traces of GA.
9. Load βGS (24.67 U) individually in both types of colloidal suspensions.

10. Stir the mixture slowly for 5 h (simple adsorption) and 2 h (covalent) and then centrifuge at $3000 \times g$ for 10 min at 4 °C.
11. Remove the unbound enzyme by washing thrice with assay buffer and preserve the precipitate at 4 °C for further studies.
12. Also, store the supernatant and the washes at 4 °C for the calculation of immobilization yield (Husain, Ansari, Alam, & Azam, 2011).
13. Determination of immobilization yield.
    The immobilization efficiency was determined in terms of immobilization yield as follows:

$$\text{Immobilization yield } (\%) = \text{A} - \text{B}/\text{A} \times 100\%$$

where A is the initially added activity of βGS in the immobilization system and B is the residual enzyme in the supernatant and washing solution after the immobilization process.

2.4 Stability studies of immobilized βGS

Chemicals and reagents

1. βGS—Sigma-Aldrich Chemicals Co. (USA)
2. Glycine-HCl (pH 2.0 and 3.0)—SRL, India
3. Sodium acetate buffers, pH (4.0, 4.5 and 5.0)—SRL, India
4. Sodium phosphate buffers, pH (6.0 and 7.0)—SRL, India
5. Tris HCl buffers, pH (8.0 and 9.0)—SRL, India
6. Crushed ice
7. Galactose (1.0–5.0%, w/v)—SRL, India
8. *o*-Nitrophenyl β-D galactopyranoside, ONPG (0.1–1.0 mM)—SRL, India

Equipment

1. Water bath (30–110 °C)—Scientech Instruments, Delhi
2. Ice maker and crusher—Labman
3. Ice box
4. Microtubes (1.5 mL)—Eppendorf, India
5. UV–vis spectrophotometer (Shimadzu, UV-1800)

Methodology

1. *pH-activity profiles* (Ansari & Husain, 2010)
   (a) Prepare different buffers of varying pH including glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0, 4.5 and 5.0), sodium phosphate (pH 6.0 and 7.0), and Tris–HCl (pH 8.0 and 9.0).
   (b) Set the molarity of each buffer as 0.1 M.

(c) Incubate the free and both types of immobilized enzyme preparations (0.04 U) in the buffers of different pH for 30 min and then perform activity assay by monitoring their activity in a spectrophotometer at 405 nm.

(d) Assume the activity at pH 4.5 as control (100%) for the calculation of residual percent activity.

2. *Temperature-activity profiles* (Ansari & Husain, 2010)

(a) Assay the activity of free and immobilized βGS (0.04 U) at different temperatures starting from 20 °C to 80 °C in 0.1 M sodium acetate buffer, pH 4.5 for 15 min.

(b) Consider the enzyme activity at 50 °C as control (100%) for the calculation of residual percent activity for free and both types of immobilized enzyme at other temperatures.

3. *Thermal denaturation* (Ansari & Husain, 2010)

(a) Incubate free and immobilized βGS (0.04 U) at 60 °C in 0.1 M sodium acetate buffer, pH 4.5 for varying times.

(b) Take out aliquots of each preparation (20 μL) in triplicates at gaps of 15 min and chill them quickly in crushed ice for 5 min.

(c) Bring the enzyme to room temperature and measure their activity.

(d) Define the activity of enzyme without incubation at 60 °C as control (100%) for the calculation of residual percent activity at each of the respective reactions.

4. *Effect of galactose* (Ansari & Husain, 2010)

(a) Incubate free and immobilized enzyme preparations (0.04 U) in the presence of various concentrations of galactose (1.0–5.0%, w/v).

(b) Evaluate the activity of enzymes in 0.1 M sodium acetate buffer, pH 4.5 at 37 °C by performing routine enzyme assay.

(c) Consider the activity of enzyme without added galactose as control (100%) for the calculation of remaining percent activity.

5. *Reusability of immobilized enzyme* (Husain et al., 2011)

(a) Take immobilized enzyme preparations (0.04 U) in triplicates for assaying the activity of the enzyme after repeated use.

(b) Perform the reaction in 1.5 mL microtubes for 10 min at 37 °C

(c) Post-incubation, centrifuge the mixture at 3000 × *g* for 10 min at 4 °C.

(d) Decant the supernatant and promptly add 0.1 M sodium carbonate into it, in order to terminate the reaction.

(e) After each assay, wash the immobilized enzyme with 0.1 M sodium acetate buffer, pH 4.5, by centrifugation at 3000 × *g* for 10 min.

(f) Store the obtained pellet in assay buffer at 4 °C and repeat this process for 10 successive days.

(g) Consider the activity determined after the first cycle as control (100%) for the calculation of residual percent activity after repeated use.

6. *Determination of kinetic parameters*

Evaluate the kinetic parameters K_m and V_{max} of free and immobilized βGS from Michaelis–Menten plot by measuring their initial rates by varying the concentrations of the substrate, ONPG (0.1 − 1.0 mM) in 0.1 M sodium acetate buffer, pH 4.5 (Khan et al., 2016).

2.5 Genotoxicity assessment of PANI/Co/MWCNT-NC

Reagents and chemicals

1. pBR322 DNA—Thermo Fischer scientific (USA)
2. Ethylene diamine tetra acetic acid (EDTA)—SRL, India
3. Tris—SRL, India
4. Bromophenol Blue—Merck & Co., USA
5. Glycerol—Merck & Co., USA
6. Ethidium bromide—Sigma-Aldrich Chemicals Co. (USA)

Equipment

1. Beakers (500–1000 mL)
2. Incubator
3. Microtubes (1.5 mL) with stand-Eppendorf, India
4. Electrophoretic gel apparatus
5. UV-transilluminator

Methodology

Plasmid nicking assay (Khan, Husain, & Ansari, 2013).

(a) Prepare a reaction mixture (30 μL) containing 10 mM Tris–HCl buffer, pH 7.5, pBR322 DNA (0.5 μg) with free enzyme (0.04 U), PANI/Co/MWCNT-NC (50 μg), and immobilized enzyme (10 μL). Incubate this for 2 h at 37 °C.

(b) Add 10 μL of solution containing 40 mM EDTA, 0.05% (w/v) bromophenol blue (tracking dye), and 50% (v/v) glycerol after incubation.

(c) Perform electrophoresis in submarine 1.0% (w/v) agarose gel.

(d) View and photograph ethidium bromide stained gel under a UV-transilluminator.

2.6 Assay of βGS

The ONPG hydrolyzing activity of free and bound βGS can be assayed by measuring the release of *o*-nitrophenol from ONPG at 405 nm (Ansari & Husain, 2012).

(a) Perform the reaction by continuous shaking in a total volume of 2.0 mL containing 1.60 mL, 1.65 mL and 1.68 mL of 0.1 M sodium acetate buffer for free, simply adsorbed and covalently immobilized enzymes, respectively, pH 4.5, 0.04 U βGS (Free—100 μL, adsorbed—50 μL, cross-linked—20 μL) and 0.3 mL of 20 mM ONPG at 37 °C for 15 min.

(b) Terminate the reaction by the addition of 2.0 mL of 1.0 M sodium carbonate solution and read product formation spectrophotometrically at 405 nm.

(c) The one unit (1.0 U) of βGS activity is defined as the amount of enzyme that catalyzes the release of 1.0 μmol of *o*-nitrophenol ($\varepsilon_m = 4500\,L^{-1}\,mol^{-1}\,cm^{-1}\,min^{-1}$) under standard assay conditions.

2.7 Estimation of protein

(a) Calculate protein concentration according to the method as described by Lowry, Rosenbrough, Farr, and Randall (1951).

(b) Use Bovine serum albumin as a standard.

2.8 Statistical analysis

(a) Each value represents the mean for three independent experiments.

(b) Perform statistical analysis for comparison between free and both types of immobilized βGS by one way ANOVA test.

(c) Plot the data expressed in various studies using origin 6.1 and express with standard deviation of error (±).

(d) Lastly, consider *P*-values ≤ 0.05 as statistically significant.

3. Results and discussion

3.1 Characterization studies of free and βGS bound PANI/Co/MWCNT-NC

Native and βGS bound PANI/Co/MWCNT-NC was characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and Fourier transform infrared (FT-IR). The TEM images of the free and the enzyme bound NC were illustrated in Fig. 4A–C. The coating of PANI on the CNT was confirmed by the presence of thin border all along

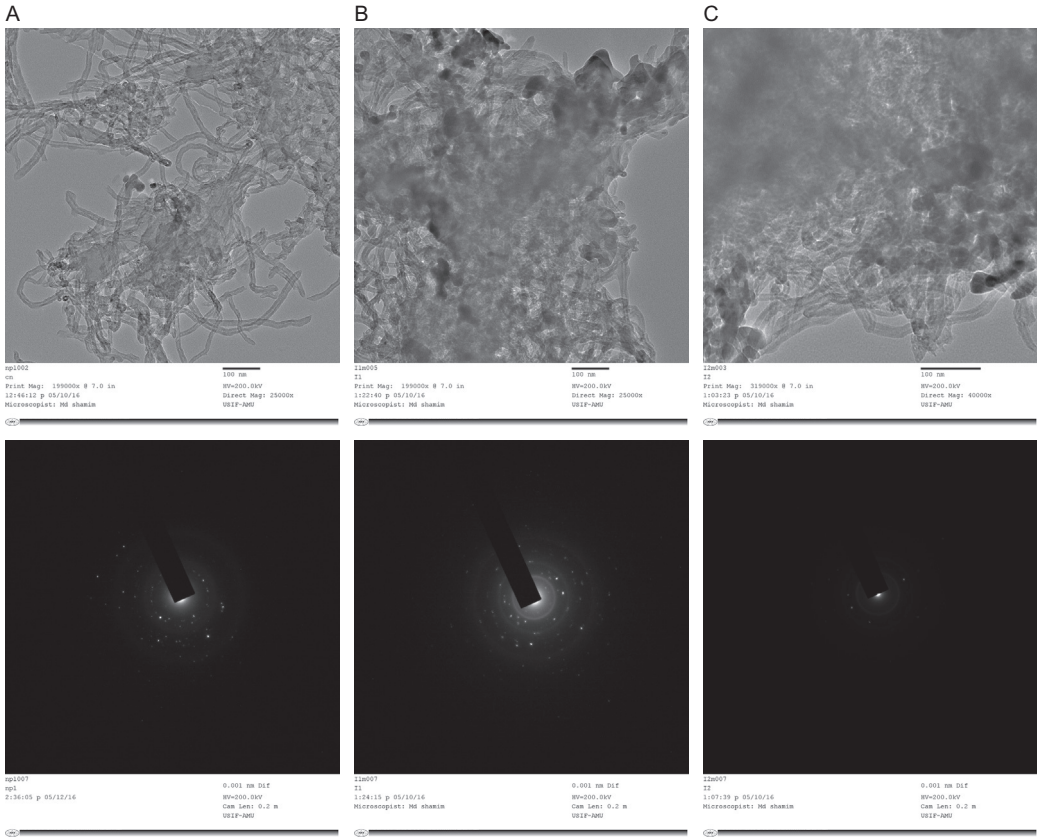

Fig. 4 TEM images and SAED patterns for free and enzyme bound NC. The figure shows (A) free PANI/Co/MWCNT-NC, (B) NC adsorbed βGS, and (C) NC covalently bound βGS. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701*.

the tubular structures (Zhao et al., 2012). The particle size of the NC was found in the range of 18–23 nm. The incorporation of Co can be visualized in TEM images by the presence of small black spots between the PANI coated CNTs. The binding of the enzyme on the surface of NC via adsorption and covalent binding methods has been compared in Fig. 4B and C. The covalent coupling method allowed binding of more enzymes compared to the adsorbed βGS. The selection area electron diffraction (SAED) pattern indicates that the NC is crystalline in nature. However, the immobilization process altered SAED pattern. Post-immobilization, the adsorption procedure made the NC highly crystalline in nature with concentric rings clearly visible in the SAED pattern (Fig. 4B), whereas in the case of GA treated NC, its crystallinity was decreased. Adsorption method is simple and does not involve any use of chemicals, while the covalent binding necessitates the use of GA and covalent bonds are formed in between the enzyme and the matrix. The surface morphology of the bound and free NC was studied by SEM. According to the SEM images, the surface characteristic of the NC was significantly changed upon immobilization of the enzyme by both the methods (Fig. 5). Rigid binding was observed in case of cross-linked enzyme.

The FT-IR spectra were recorded for free PANI/Co/MWCNT-NC, NC adsorbed enzyme and βGS covalently attached to the NC in the range of 500–4000 cm^{-1} (Fig. 6). The characteristic peaks at 1493 and 1570 cm^{-1} for benzenoic and quinonic type rings signify the presence of PANI in the NC, respectively (Zhao et al., 2012). Peak at 1300 cm^{-1} corresponds to

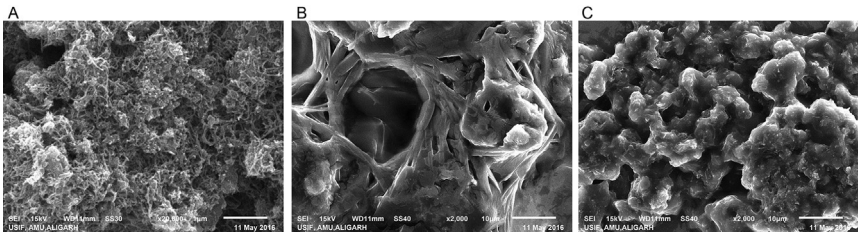

Fig. 5 SEM images for free and enzyme bound NC. The figure illustrates (A) free PANI/Co/MWCNT-NC, (B) NC adsorbed βGS, and (C) NC covalently bound βGS. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701.*

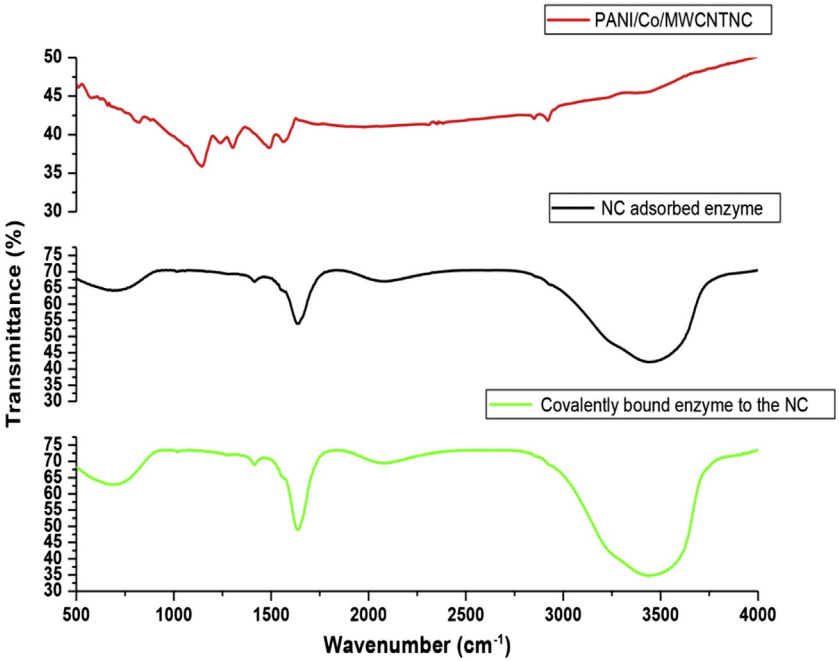

Fig. 6 FT-IR spectra for free and enzyme bound NC. The figure depicts spectra of free PANI/Co/MWCNT-NC, NC adsorbed and NC covalently bound βGS. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701.*

C—N stretching bond. The band which appeared at 1238 cm^{-1} in the spectrum obtained for NC indicated that Co interacts with the nitrogen atoms in the PANI chain (Giri et al., 2012). The characteristic peak for MWCNT was observed at 1148 cm^{-1} which corresponds to C═C. The binding of βGS on PANI/Co/MWCNT-NC was confirmed by the absorption bands obtained for adsorbed and covalently attached βGS. The stretching of carbonyl group of βGS was observed in both types of immobilized enzyme preparations as viewed by broadening of peaks from 3131 to 3653 cm^{-1}. Similar findings have been reported earlier, where broadening of peaks was observed from 3220 to 3447 cm^{-1} (Khan et al., 2016). The peak at 1647 cm^{-1} further signifies the presence of amide linkage. However, the peak intensity decreased in case of covalently immobilized enzyme ensuring

a strong interaction between protein and support. Identical results have been observed when the same enzyme was immobilized on functionalized magnetic nano graphene (Shafi, Khan, Khan, & Husain, 2019).

3.2 Immobilization studies of adsorbed and covalently bound βGS

The adsorption of protein onto CNT involves both hydrophobic and electrostatic interactions. Hydrophobic interactions involve the binding of side chains of hydrophobic amino acids of proteins with sidewall of CNT. In electrostatic interactions, the π-electrons on the surface of CNT interact with π-electrons of the aromatic ring of amino acids (e.g., phenylalanine and tryptophan). Furthermore, the negative charges present on the surface of support interact with positive charges of the enzyme. This type of immobilization is also referred to as immobilization by ionic exchange. Apart from electrostatic and hydrophobic interactions, hydrogen bonds and non-specific adsorption can also play a significant role in the adsorption of enzyme onto CNT surfaces (Husain, 2016, 2018b; Saifuddin et al., 2013).

GA is one of the most widely used cross-linking agents for surface modification of enzyme and the support. It interacts with primary amino groups of protein by intermolecular cross-linking the enzyme molecules immobilized in/on any type of support. GA can be used in two ways to covalently bind the enzyme with the support. In the first method, initially enzyme is adsorbed on the aminated support which is followed by covalent coupling via GA (Ali, Husain, Alam, & Ahmad, 2017). The advantage of this method is to provide rigidity and multi-point covalent attachment while the drawback lies in the loss of enzyme activity and stability, because the surface amino groups of the enzyme are modified. The second method involves the treatment of pre-activated aminated support by GA. The functionalized NC is then allowed to react with the free amino group of the enzyme (Ashraf & Husain, 2011a, 2011b; Barbosa, Torres, Ortiz, & Fernandez-Lafuente, 2012; Siddiqui & Husain, 2019).

In this work, second method has been employed in which GA was used to create reactive aldehyde groups on the support. These free aldehyde groups present on the activated support subsequently reacts with exposed amino groups of the enzyme. In the covalent method, two parameters were analyzed. The maximum immobilization yield of 97% was achieved when 6 mg of dry weight of support was treated with 2% (v/v) GA solution. GA has been used to in two ways to covalently bind the enzyme with NC. In first method, initially βGS was adsorbed on the aminated support which is

Table 4 Immobilization of βGS on PANI/Co/MWCNT-NC.

Method of immobilization	Enzyme activity loaded, X, (U)	Enzyme activity in washes, Y, (U)	Enzyme activity bound on PANI/Co/MWCNT-NC		Immobilization yield (%), B/A × 100
			Theoretical (X − Y) = A	Actual/ immobilized = B	
Adsorption	24.67	0.087	24.58	22.84	93 ± 0.73
Covalent binding	24.67	0.002	24.66	24.00	97 ± 0.54

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, ≤5%.

Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor. *International Journal of Biological Macromolecules, 105*, 693–701.

followed by covalent coupling with GA. At higher concentration of GA, there was a minor loss in immobilization yield. It might be due to higher number of aldehyde groups or protein molecules cross-linking with each other. These conformational changes in the protein molecules might decrease its activity (Ali et al., 2017; Zhu et al., 2012).

Table 4 demonstrates the immobilization yield of βGS obtained via both methods. Schematic representation of immobilization of βGS on functionalized-CNTs by both methods has been shown in Fig. 7. Several earlier workers have also employed GA as a cross-linking agent in the process of enzyme immobilization (Musthapha, Akhtar, Khan, & Husain, 2004; Zhu et al., 2012). The use of GA, as cross linker has prevented leaching of enzyme from the support. There was a marginal increase in the immobilization yield in case of covalently bound enzyme. The improvement in immobilization yield of the enzymes by GA coupling has also been described by earlier workers (Ashraf & Husain, 2010, Gibriel, Amin, Yassien, El Banna, & Khaled, 2014).

3.3 Stability, reusability and kinetic studies of free and PANI/Co/MWCNT-NC bound βGS

3.3.1 Effect of pH and temperature

pH-activity profile for soluble and immobilized βGS has been illustrated in Fig. 8A. The soluble enzyme exhibited a sharp pH-optimum at 4.5, while immobilized enzyme showed a broadening in pH-optima. The covalently attached βGS demonstrated broader pH-optima compared to adsorbed enzyme. At pH 2, the free enzyme retained only 19% of the original activity,

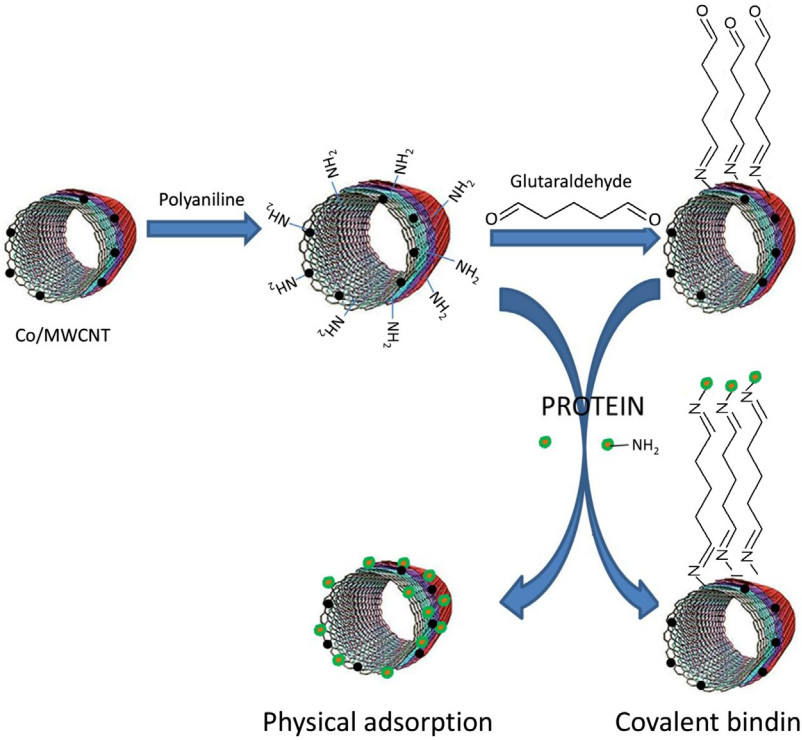

Fig. 7 Over all reaction steps involved in the functionalization of PANI/Co/MWCNT-NC and subsequent immobilization of βGS by physical adsorption and covalent attachment. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701.*

while the adsorbed enzyme could maintain 51% activity and covalently bound enzyme retained about 68% activity under similar experimental conditions. A shift in pH-optima after immobilization of enzymes has also been earlier reported by various researchers (Bayraktar, Serilmez, Karakas, Bicak-Celem, & Onal, 2011; Shafi et al., 2019). Depending on the nature and surface charges on the support as well as the enzyme, the pH in the vicinity of enzyme molecule may differ and hence alter the pH-optima of the immobilized enzyme.

Effect of temperature on the activity of free, NC adsorbed and covalently attached βGS was investigated in the range of 20–80 °C (Fig. 8B). Temperature-optima for soluble and NC adsorbed enzymes was found to be same at 50 °C. However, the covalently bound enzyme exhibited

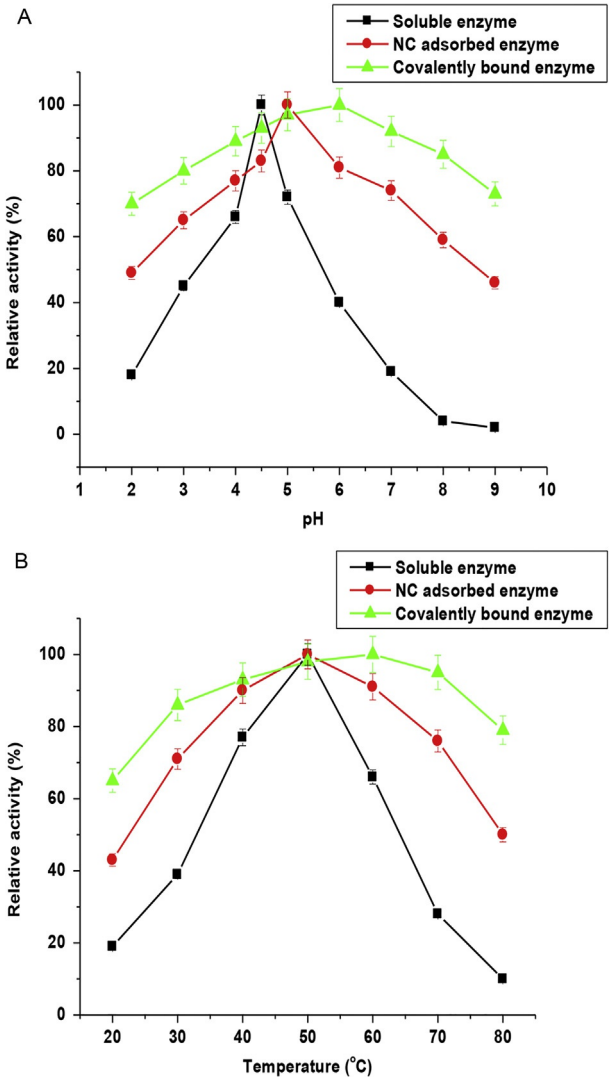

Fig. 8 Stability studies of free and PANI-Co-MWCNT-NC bound βGS. The figure shows (A) pH-activity and (B) temperature-activity profiles. Data represents mean ± standard deviation of three individual experiments performed in duplicates, *P*-value ≤ 0.05. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor*. International Journal of Biological Macromolecules, 105, *693–701*.

maximum activity at 60 °C. The soluble enzyme lost 90% activity when incubated at 80 °C for 15 min, whereas the NC adsorbed enzyme retained 50% activity and the βGS covalently immobilized to NC retained about 80% of the maximum activity under identical incubation conditions. Ansari and Husain (2010) have earlier shown that there was a shift in temperature-optima from 50 °C to 60 °C in case of cross-linked Con-A-cellulose adsorbed βGS. The stability and activity retention exhibited by the immobilized enzyme in a broad range of temperature can be explained by the phenomenon of multi-point attachment which provides rigidity to the enzyme, thereby preventing enzyme conformational changes. The increased enzymatic activity under those conditions, however, is not caused by the generation of a more active enzyme form, but by avoiding distortion of the enzyme structure (Ansari & Husain, 2011). Guidini, Fischer, Santana, Cardoso, and Ribeiro (2010) immobilized *Aspergillus oryzae* βGS on the ionic exchange resin Duolite A568 by ionic-binding and covalent coupling method. They found that the covalently bound enzyme was significantly more stable against the extreme conditions of pH and temperature compared to simply adsorbed enzyme.

Fig. 9A illustrates the thermal denaturation plots for soluble and immobilized enzyme preparations. The soluble enzyme lost almost 95% of the initial activity after 2 h incubation at 60 °C. However, the adsorbed enzyme retained 46% activity, while the covalently immobilized βGS appeared more stable and maintained 78% of its activity at the end of 2 h incubation. The immobilization matrix imparts high rigidity to the enzyme, thus providing enzyme a greater resistance against heat inactivation.

3.3.2 Effect of galactose

For the construction of biosensors, greater importance is given on the activity of the immobilized enzyme in the presence of higher concentration of galactose which is considered to be a competitive inhibitor for βGS catalyzed reaction (Haider & Husain, 2009; Park & Oh, 2010). Fig. 9B explains the effect of increasing concentrations of galactose on the activity of free and immobilized βGS. The βGS covalently bound to NC was found to be most resistant against galactose inhibition and thus it retained 70% of its initial activity in the presence of 5% galactose. However NC adsorbed βGS nearly lost 66% of its activity. The interaction between the matrix and enzyme plays a vital role in the protection of enzyme inhibition. Rigidity of the enzyme due to multi-point covalent attachment may be the possible reason for

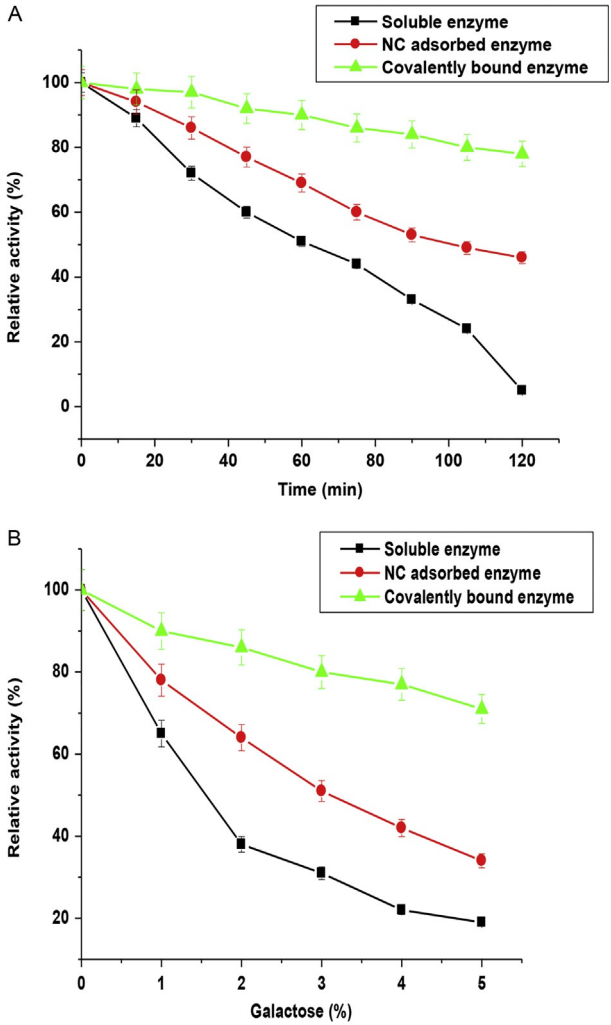

Fig. 9 Stress inactivation studies of free and PANI-Co-MWCNT-NC bound enzyme. The figure depicts (A) effect of temperature and (B) effect of galactose. Data represents mean ± standard deviation of three individual experiments performed in duplicates, *P*-value ≤ 0.05. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701*.

providing resistance against inhibitor in βGS covalently immobilized on NC due to more protection in its native conformation (Khan, Husain, & Bushra, 2017; Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernandez-Lafuente, 2013).

3.3.3 Determination of kinetic parameters

The K_i, K_m and V_{max} values obtained for soluble and immobilized enzyme preparations are listed in Table 5. The K_{iapp} values were found to significantly increase in βGS covalently bound to NC. These values infer that the covalently immobilized enzyme was comparatively more stable as compared to the adsorbed or the soluble enzyme and less affected by galactose inhibition. The K_m values were found to decrease from 0.5 to 0.4 mM for adsorbed enzyme and 0.3 mM for covalently attached enzyme, indicating higher substrate-enzyme affinity (Mateo et al., 2004). A slight decrease in V_{max} values was also observed in case of immobilized enzyme suggesting a decrease in activity retention by the enzyme during binding. Ye, Jiang, and Xu (2007) have immobilized lipase on chitosan-modified poly(acrylonitrile-co-maleic acid) membrane surface and they observed a similar pattern in kinetic parameters by showing a decrease in K_m and a very slight decrease in V_{max} in the bound enzyme compared to the native form.

3.3.4 Reusability of immobilized enzyme

Reusability of enzyme provides cost effectiveness for its use in an economically viable enzyme catalyzed process (Haider & Husain, 2007). The PANI/Co/MWCNT-NC adsorbed enzyme retained about 74% activity while the covalently bound βGS maintained 92% of its initial activity even after 10th successive reuse (Fig. 10A). The covalently immobilized βGS was found to be more rigid than the adsorbed enzyme because of the covalent linkages formed between the NC and enzyme which subsequently reduces the chances of leaching of the enzyme from the support or stabilizing native conformation. The activity of the βGS immobilized on GA treated κ-carrageenan gel beads retained 60% of its initial activity even after 20 reuses (Elnashar et al., 2014).

Table 5 Kinetic parameters of free and PANI/Co/MWCNT-NC bound βGS.

Enzyme	K_i (mM)	K_m (mM)	V_{max} (μmol min^{-1} mL^{-1})
Free βGS	0.5 ± 0.14	0.5 ± 0.12	0.04 ± 0.005
Adsorbed βGS	1.1 ± 0.32	0.4 ± 0.08	0.04 ± 0.004
Cross-linked βGS	11.1 ± 1.3	0.3 ± 0.05	0.03 ± 0.002

Each value represents the mean of three independent experiments performed in duplicates with average standard deviation of ≤5%.

Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor. *International Journal of Biological Macromolecules, 105*, 693–701.

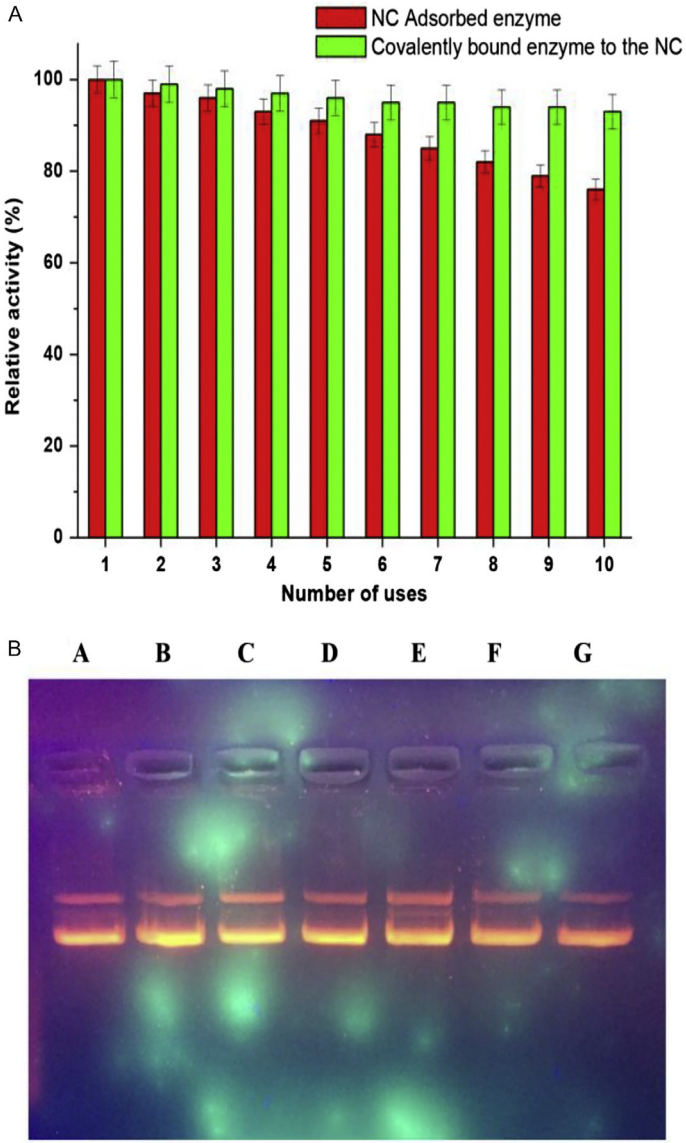

Fig. 10 Reusability and genotoxicity assessment of free and enzyme bound NC. The figure illustrates (A) reusability of PANI-Co-MWCNT-NC adsorbed and covalently attached βGS, *P*-value ≤ 0.05 was considered statistically significant. (B) Plasmid nicking toxicity assay: Lane A denotes pBR322 DNA alone, Lane B & C signify pBR322 DNA with PANI-Co-MWCNT-NC (50 μg), Lane D & E denotes pBR322 DNA incubated with adsorbed enzyme, while Lane F & G contain pBR322 DNA with covalently bound βGS. *Adapted from Khan, M., Husain, Q., & Bushra, R. (2017). Immobilization of β-galactosidase on surface modified cobalt/multiwalled carbon nanotube nanocomposite improves enzyme stability and resistance to inhibitor.* International Journal of Biological Macromolecules, 105, *693–701*.

3.4 Genotoxicity assessment of free and βGS bound PANI/Co/MWCNT-NC

To assess the genotoxicity of the support, plasmid nicking assay was performed. It is one of the most sensitive assays to detect the effect of various toxicants on the integrity of the DNA. The pBR322 plasmid was incubated in the presence of free NC and both types of immobilized enzyme preparations. Fig. 10B shows the agarose gel with similar band pattern in all the cases, which was insignificantly different to that of control. The NC matrix used for the immobilization of βGS did not induce any kind of breakage in pBR322 DNA revealing its non-toxic nature hence it is compatible and safe to be employed in biotechnological applications (Khan, Husain, & Ahmad, 2018; Khan et al., 2017).

4. Conclusion

With the growing demands of our society, in particular healthcare issues associated with aging of the population, next generation medical diagnostics require implementation of rapid, sensitive and cost effective alternatives to the more traditional immunological assays currently used. Similarly, the detection of pathogens and toxic compounds in our environment constitute major threats and sensitive detection procedures are required to address concerns for global security. An ever growing number of biosensing devices and strategies have been developed since the Clark oxygen electrode came to existence in the 1950s. However, NM has clearly provided new and attractive platforms to engineer biosensors with enhanced sensitivity and performance. In particular, the structural, electronic, and optical properties combined by CNTs offer a wealth of opportunities to develop new generation nano-tools for biosensing applications, especially for probing disease markers. The electronic properties of CNTs are extremely well suited for electrochemical, amperometric, simpedometric, or field-effect transduction of signals, but their particular optical properties and their ability to penetrate readily through biological membranes also make them suitable candidates for the development of photoacoustic imaging sensors. However, when proposing a biosensor, it is always recommended to take into consideration that nanoscale materials, although small can be sensed by the body as intruders and consequently attacked. Therefore, issues related to their length, diameter, lifetime, stability, durability, mechanical properties, body adjustment, and toxicity must be evaluated by a scientific and systematic

method. Indeed, when used in their pristine state, directly after synthesis, CNTs contain impurities and can have harmful effects. Nonetheless, when purified and surface-functionalized, their toxicity is drastically decreased and they represent optimal platforms for all kinds of applications. CNTs have comparable dimensions to redox proteins and can be used as effective electrical wiring/connectors with redox enzymes. However, better control of the chemical and physical properties of the CNT-biosensors is still needed. For example, the separation process for different types of CNTs, the miniaturization of the sensors and the in vivo stability need to be addressed to meet feature requirements.

Eventually, a clear understanding of the structure–function relationships will be convenient to support and guide experimental efforts toward the most promising carbon nanostructures with improved biosensing abilities. Thus, fabrication of new CNTs-based optical biosensors and synthesis of new CNT fibers is still a focus of future works in biosensing field. Clearly, the development of CNT-based biosensors is a multi-faceted challenging work that needs a well cooperation between materials scientists, who are developing new materials, and engineers, who are committed to fabricate the new micro/nanodevices such as bionanosensors. Therefore, the greatest and the last challenge is how to build a joint platform that allows the efficient discussion, learning and cooperating between physicists, chemists, and electrical/mechanical engineers.

Although recent works have made many amazing advances in this field, there are still some challenges and further works may mainly include the following five aspects. The first one is cost effectiveness. The high price of CNT materials and CNT-based enzymatic biosensors has become the main problem which seriously restricts their development and widespread applications (Yan, Chan-Park, & Zhang, 2007). Reducing these costs is a complex challenge and will almost certainly require the integration of materials research and nanotechnology. Non-enzymatic sensor based on CNTs is a potential choice which has been attracted by some research groups (Zhu et al., 2012). Novel nanotechnologies such as nanoimprint lithography and soft lithography are also helpful. Both chemical and physical properties of functionalized CNTs strongly depend on the ambient conditions, such as temperature and pH. Thus, the second challenge is how to prolong the thermal stability and lifetime of CNT-based biosensors in application of CNTs. Using NC materials instead of expensive and fragile enzymes to detect biomolecule has shown improved thermal tolerance, long-term stability, and cost effectiveness. Except that, some studies have indicated that the

sensitivity and the detection limits is significantly enhanced using NC materials combined with CNTs and metal (Au, Pt, etc.) for biosensor applications. With the sustainable development of new NM combined with CNTs, characterization of these new materials at the molecular level is essential and a key scientific challenge. In parallel with experimental studies, molecular modeling must be further developed as a tool to predict the performance of new materials for a given biomolecule. Such computational methods will enable a quick evaluation of these novel materials.

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