



Historical Perspective

Recent advances in carbon nanotubes-based biocatalysts and their applications

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ABSTRACT

Enzymes have been incorporated into a wide variety of fields and industries as they catalyze many biochemical and chemical reactions. The immobilization of enzymes on carbon nanotubes (CNTs) for generating nano biocatalysts with high stability and reusability is gaining great attention among researchers. Functionalized CNTs act as excellent support for effective enzyme immobilization. Depending on the application, the enzymes can be tailored using the various surface functionalization techniques on the CNTs to extricate the desirable characteristics. Aiming at the preparation of efficient, stable, and recyclable nanobiocatalysts, this review provides an overview of the methods developed to immobilize the various enzymes. Various applications of carbon nanotube-based biocatalysts in water purification, bioremediation, biosensors, and biofuel cells have been comprehensively reviewed.

1. Introduction

Enzymes are highly effective and efficient biocatalysts having high practical applications in pharmaceuticals, biosensors, and biofuel cells. They accelerate the rate of biochemical reactions and assists in reducing the number of steps required for the completion of the reaction [1]. They exhibit high specificity, are environmentally benign and economic which increases their practical applicability and demand within the chemical industries [2]. Enzymes are active proteins that accelerate and catalyze many biochemical reactions [1]. The enzymes in their natural form are found to be delicate and are highly susceptible to denaturation in case of extreme environmental conditions such as pH, temperature, and organic solvents leading to their underutilization [3–5]. Therefore, as an attempt to ameliorate their stability, the enzymes are commonly immobilized on a solid support which is termed enzyme immobilization and is revealed to be facilitating the improvement in catalytic selectivity, reusability, stability in different reaction conditions, and their heterogeneous form making it easy to separate them from the reaction mixture, thereby generating high purity products [6–8]. These properties fascinate researchers for the development and improvement of enzymes for better performance.

The materials utilized as a support for enzyme immobilization can be

categorized into organic, inorganic, and hybrid/composite [2]. The selection of a relevant immobilization method along with the support material majorly depends on the nature and conditions of the catalytic process along with the kind of enzyme being utilized [9]. However, the choice of the right support material is an important decision that has to be taken due to its direct impact on the properties of the biocatalytic systems [10]. The support suitable for the enzyme immobilization should have certain characteristics and requirements such as providing a sufficiently large internal surface for having a great geometrical confirmation with the enzyme [11,12]. The support should offer protection to the enzyme structure from severe conditions and factors thereby ameliorating the possibility of enhanced catalytic activity [13]. There should be high affinity and interaction between the functional groups of both the enzyme and support. Once the interplay has taken place, the reactive groups involved in the immobilization should be efficient enough to improve the stability of the enzyme–support [14–17].

Inorganic support materials like inorganic oxides, carbon-based materials are generally distinguished by their high thermal and chemical stability along with exceptional mechanical resistance [18]. Additionally, their well-developed porous structures ensure high sorption properties and high surface area for efficient enzyme immobilization

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[19] On the other hand, synthetic polymers are capable of facilitating the covalent binding of enzymes during the absence of cross-linking agents. Their characteristic properties such as high protein affinity along with biocompatibility restraints the negative effects thereby enhancing their utilization [2]. The efficacy of the nanomaterial's can be further improvised by the method of surface functionalization which includes grafting of the specific beneficial functional groups on their surface to develop nanomaterial's having desirable properties and characteristics [20]. By doing so, the dispersibility and interactions of the nanoparticles with enzymes changes, thereby causing a notable alteration in the catalytic activity of the immobilized enzyme [21]. Even though several attempts have been done for immobilizing the enzymes on nanomaterials, consecutive studies based on the influence of nanomaterial properties like composition, morphology, and surface chemistry on the structure and functionality of conjugated enzymes have to be discussed more. Therefore, studies based on investigating the changes in the properties of the enzymes after immobilization is highly pivotal for the interpretation of enzyme-nanomaterial interactions [22].

Among the various immobilization supports discussed in the literature, carbon nanotubes (CNTs) are one of the supports which largely conform to the requirement as a stable enzyme support material [23,24]. Web of science data for the last 5 years by typing the keywords “carbon nanotubes” and “enzyme biocatalysts” as shown in Fig. 1. One can see the importance of the subjects with respect to publications (a), countries citing those articles (b), number of citations from 2017 to 2021 (c).

CNTs are gaining a significant amount of interest to serve as a matrix for immobilization due to their larger specific surface area, better adsorption capacity, mass transfer resistance, and a greater enzyme loading capacity when compared with the traditional counterparts like silica and other polymeric materials [25]. They are composed of carbon (sp^2) in the form of graphite flakes which are rolled to form a cylindrical structure [26]. They possess distinctive physical and chemical characteristics like high mechanical strength, a better surface-to-volume ratio, good dispersibility, inertness, compatibility, high conductivity, and good biocompatibility thereby enhancing their utilization in many fields [27,28].

As compared to other enzyme carriers, CNTs consists of several advantages such as superior electronic properties, rapid electron transfer kinetics, and a large surface to volume ratio. In addition, the designing of sensors modified by CNTs has been revealed to be possessing distinctive properties including heightened sensitivity, low-level

detection of limit, and rapid electrode kinetics when compared to other carbon-based support materials [29]. Similarly, CNTs are also capable of undergoing additional functionalization majorly at their surface via solubilizing chains or focussing on specific target molecules thereby improvising their superiority in practical applications including biofuel cells or the biomedical sector something which is lacked and not seen in the case of other nanostructures [30].

Table 1 summarises the various physical properties of different forms of carbon materials which include graphite, diamond, fullerene, SWCNTs, and MWCNTs. From the table, it can be easily seen that the CNTs (SWCNTs and MWCNTs) are much better in terms of electrical, thermal, and other physical properties which they possess over the other carbonaceous materials. Owing to such advantageous and superior properties, CNTs are revealed to be having higher applicability in various fields including thermal conductors, energy storage, structural materials, catalyst supports, biological applications, along with air and water filtration.

The research in recent years has been trying to interpret and understand the underlying physicochemical mechanisms related to the performance of enzymes immobilized on the nanoparticle supports [33]. The studies indicate that these underlying mechanisms and reactions are complex and that the specific attributes of enzyme-nanoparticle conjugates have been correlated with ameliorated enzyme functionality. These properties include mass transport, enzyme density, morphology, and surface chemistry of the nanoparticle, along with the enzyme orientation [34,35]. An enhancement in the effectiveness of the enzymes, when immobilized onto the nanoparticles due to various reasons has been reported. Also, the various characteristics such as the configuration, orientation, along density of the enzymes are both controllable and achievable by varying the nanomaterial surface chemistry and have also shown increased target-specificity [36,37].

2. Surface modifications and structural variations in CNTs and proteins: an effective strategy for fine-tuning the applicability of nanohybrids

The modifications associated with both the outer CNT surface and tertiary structure of proteins influence the overall nanohybrid properties were extensively reported in the literature. Therefore, gaining a better insight into the interaction between the CNTs and proteins for achieving the controlled synthesis of nanohybrids is highly recommended. The major reason for effective interaction between the CNTs and the proteins

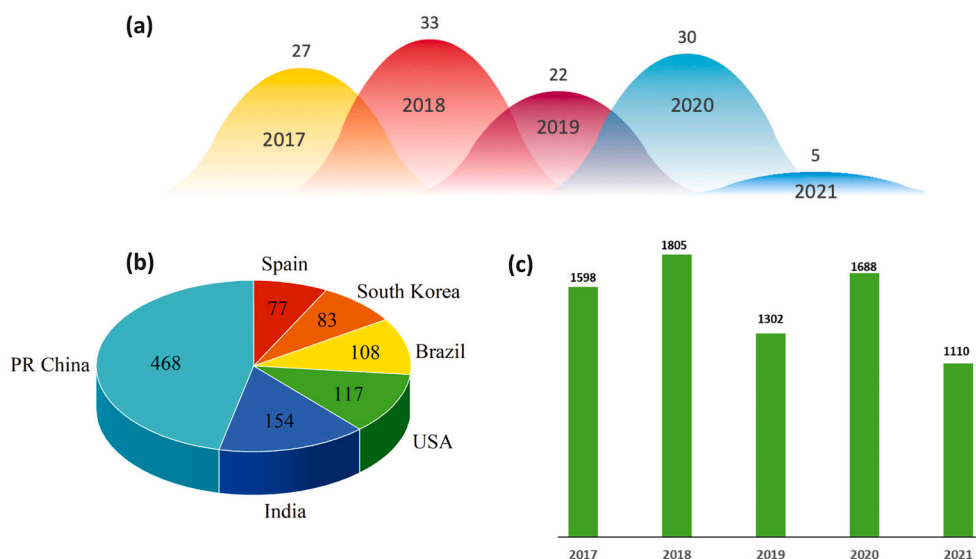


Fig. 1. Web of science data by typing the keywords “carbon nanotubes” and “enzyme biocatalysts”. The figure shows the importance of these areas with respect to publications (a), countries citing those articles (b), and citations data for the last 5 years (c).

Table 1
Physical properties of different forms of carbon materials.

Property	Graphite	Diamond	Fullerene	SWCNT	MWCNT	Reference
Specific gravity (g/cm ³)	1.9–2.3	3.5	1.7	0.8	1.8	[31]
Electrical conductivity (S/cm)	4000 ^p , 3.3 ^c	10 ⁻² –10 ⁻¹⁵	10 ⁻⁵	10 ² –10 ⁶	10 ³ –10 ⁵	[31]
Electron mobility (cm ² / (V s))	2.0 × 10 ⁴	1800	0.5–6	~10 ⁵	10 ⁴ –10 ⁵	[31]
Thermal conductivity (W/(m K))	298 ^p , 2.2 ^c	900–2320	0.4	6000	2000	[31]
Thermal stability in air (°C)	450–650	<600	~600	>600	>600	[31]
Tensile strength (MPa)	4.8–76	2800–2930	11.5	75	150	[32]
Young's modulus (GPa)	4.1–27.6	1050–1210	32–54	900–1700	1800 average, 690–1870	[32]
Resistivity (Ω.m)	7.94–11 × 10 ⁻⁸	1 × 10 ¹⁰ –1 × 10 ²⁰	1 × 10 ⁻⁹	10 ⁻⁶		[32]
Density (Mg/m ³)	1.61–2.49	3.44–3.58	1.65	1.7–1.9	1.7–2.1	[32]

is attributed to the presence of van der Waals forces of interaction among the π - π stacks between organic groups of protein and CNTs surface [38]. Some of the methods that can be employed for the synthesis of CNTs while considering the structural properties are the arc discharge method, CVD, and laser vaporization of graphite. Practicing the above-mentioned methods would result in gaining control over the purity and physical characteristics such as the length and diameter of the synthesized CNTs. For instance, CNTs possessing large diameter offers a higher number of contact sites for protein interaction as compared to those having smaller diameters. Similarly, large-diameter CNTs having a shorter length provide superior rigidity resulting in the reduced chances of bending thereby improving the overall stability [39]. Apart from the influence of physical properties of CNTs, the types of CNTs involved also plays a paramount role in understanding the spatial arrangement between the CNTs and proteins. A study confirmed this by employing different types of CNTs towards stabilizing the α -helical peptide structures on CNT surfaces which resulted in the sensing of various CNTs by means of their physical properties by the structures of the developed peptides [40]. The CNTs having smaller diameters were loaded with a higher number of protein chains thereby resulting in reduced cytotoxicity because of the influence of the surface area and diameter of CNTs [41]. Moreover, the various forces of interaction including electrostatic forces along with π - π stack also have an impact on the interplay due to the presence of organic groups on the CNTs and the protein surface. The adsorption between the proteins and CNTs can also be ameliorated by the enhancement of the surface modification density of the CNTs. The functionalization density on the CNT surface has the ability to induce non-competitive or competitive type of inhibition activities both of which have a profound effect on the biological properties of the developed nanohybrid [42]. The former one gets induced during the utilization of CNTs possessing lowered functionalization density and the CNTs having superior functionalization density induces the latter. The existence of various kinds of fabrication methods in combination with the surface modification techniques and purification processes towards the preparation of CNTs paves ways for modifying and fine-tuning of the structural properties such as length and diameter of CNTs, surface modification density, for attaining better and effective interaction in the nanohybrids.

On the other hand, various kinds of protein structures also have a varied influence on the interplay occurring between the CNTs and proteins. For an effective binding between the CNTs and proteins and for the formation of a nanohybrid, the structure and dimensions of proteins are highly crucial. One of the ways of gaining control over peptide-self-assembly processes for the construction of templates for binding of CNTs is by the utilization of polypeptide chains possessing multiple directionalities [43]. Literature studies conducted have revealed that the proteins or peptides having amphiphilicity is an initial and paramount property as it has a profound influence on binding nature [44]. The presence of Amino acid (AA) residues in proteins and peptides act as the major binding force when it comes to affinity towards CNTs [45]. Also, it is the same AA residues that contribute towards the total free energy of peptides and proteins which leads to the evaluation of the interaction between the CNTs and proteins even more important [46]. One such

method for evaluating is by analyzing the refractive indices of individual AA that are being utilized [47]. Also, the binding of a peptide head-to-tail cyclization structure reduces the confirmation degree of freedom resulting in stabilization and optimization of geometrical space in the case of the α -helical segment [48]. One such instance involved is the tryptophan exhibiting the highest affinity towards CNTs and phenylalanine showing the least affinity towards CNTs among a group of 20 proteinogenic [49]. Even the aromatic content enacts a paramount role in the interactions occurring between the CNTs and peptides. For instance, results obtained from atomic force microscopy and optical absorption spectroscopy revealed atomic residual content assisted towards a dispersion of CNTs in a given solution and substantiated that atomic residual content-rich peptide sequences exhibit higher affinity towards CNTs [50]. The importance of hydrophobic chains at the end of the center associated with amphiphilic peptides towards the absorption of peptides on the surface of CNTs were reported [51]. For gaining a better understanding regarding the different binding behaviors towards CNTs, various proteins such as pepsin, papain, lysozyme (LYS) along bovine serum albumin (BSA) were utilized. Among the four proteins, LYS and BSA had the ability to cause individual dispersion of SWNTs in an aqueous solution which was not observed in the case of pepsin and papain. The heat-induced denaturation performed during the sonication was considered to be affecting the selectivity of proteins during the process of dispersion [52]. The next main factor influencing the protein-CNT interaction is the complementarity in the shape as it can also be used as a tool for measuring the protein-CNT interactive energy [53]. A study, therefore, utilized an amphiphilic peptide helix consisting of different contents of α -helical termed nano-1 for solubilizing CNTs in an aqueous solution. On increasing the peptide content associated with the CNT surface, the helical secondary structure of protein started gaining better stability. Once the absorption on the CNT surface is begun, the helical secondary structure starts losing its stability which is attributed to the minor α -helical content [54]. More research focusing on the influence and changes in the protein spatial structure on interaction with CNT surface needs to be explored. The literature studies conducted, therefore, indicate the ideology of working towards gaining control over protein spatial structure as a central key for analyzing non-covalent interaction.

3. Functionalisation of CNTs as a prerequisite for enzyme immobilization

CNTs in their native form are inert and insoluble in many solvents hence they cannot react or bind directly with the enzymes [55]. The reactivity and solubility of CNTs can be improved by their surface functionalization. This is done by attaching different functional groups to the CNT surface which increases their affinity towards the enzyme [56]. These chemical groups facilitate the interaction of CNTs with enzymes of interest through Van der Waal interactions, covalent or hydrogen bonding interactions, and increases the specificity of the reaction site between CNTs and enzymes [57]. Moreover, surface functionalization of CNTs can increase their dispersibility in various solvents and compatibility for conjugation with the enzymes [58,59].

The proper knowledge of the active sites of the enzyme is highly recommended in order to protect it from denaturation during the process of functionalization and immobilization [60,61]. The active sites of enzymes can be protected using protecting groups such as *n*-tert-butoxycarbonyl during these processes. The facile removal of these protecting groups, once the desired immobilized enzyme has been obtained is considered as an advantage offered by them [62]. This assists in sustaining the enzyme activity after a variety of chemical reactions occur during the process of immobilization.

Various methods of functionalization have been proposed and utilized based on the chemical groups attaching to the CNT surface [63,64]. The methods of functionalization are categorized into three types such as acid functionalization, amine functionalization, and hydroxyl group functionalization as shown in Fig. 2.

3.1. Acid functionalization

The carbon nanotubes (CNTs) are functionalized by attaching the carboxylic acid (CO_2H) group to pristine CNTs. The CO_2H group present on the nanotubes facilitates easy attachment of the enzyme as NH_2 group of the enzyme and CO_2H group forms stable amide bond ($-\text{NH}-\text{C}=\text{O}$) [65]. In this method, CNTs are treated with an acid mixture and it gets oxidized (attaching oxygen-containing group on the surface [66]). For this, the concentrated acid mixture of nitric acid and sulphuric acid in a ratio of 1:3 by volume was utilized [67]. The pristine MWCNT was mixed with the concentrated acid mixture (HNO_3 and H_2SO_4) which was subjected to an ultrasonic bath. This treatment led to the generation of holes on the surface of MWCNT to which functional groups can be attached easily. The MWCNT does not disperse in water well but the attachment of the carboxylic group turns the CNTs hydrophilic by polarising the surface [68]. Also, the defective sites of MWCNT are more prone to oxidation such as ring defects and dangling [69]. The presence of carboxylic acid groups gives a suitable binding site for the enzyme which has to be immobilized. When the enzyme was immobilized on

functionalized MWCNT, there was a formation of an amide bond between the carboxylic acid group of f-MWCNT and the amine group from cellulose. This makes the MWCNT-enzyme link stronger, stable and contributes to reducing enzyme leaching [67]. Another such study involved the immobilization process in which the laccase enzyme was aminated. Among the several supporting materials which were evaluated as the support system of MWNT, cellulose nitrate exhibited the highest enzyme loading of 0.286 U/mg MWNT. Also, the immobilized enzyme exhibited (above 95% of its initial activity was maintained after 10 cycles of reaction) enhanced stability when tested against temperature, pH, and acetone as compared to its soluble counterpart. It was understood that the membranes made up of cellulose nitrate could be utilized in biotechnological and biomedical applications involving immobilized enzymes [70].

The presence of functional groups on CNTs was confirmed with the use of FTIR studies. When the FTIR spectrum of pristine MWNT and functionalized MWNT were compared as shown in Fig. 3, various significant peak areas present at different wavelengths were noted which includes peaks at 1643 cm^{-1} indicates $\text{C}=\text{O}$ stretching mode of carboxylic group, peak at 2914 cm^{-1} ($\text{C}-\text{H}$ stretching), peaks at 3426 and 1061 cm^{-1} ($\text{O}-\text{H}$ and $\text{C}-\text{O}$ functional group stretching) were observed. The presence of these peaks supports the presence of a carboxylic group on MWNTs. Moreover, the presence of $\text{C}=\text{C}$ stretching indicates that MWNT has retained its structure after functionalization [67].

3.2. Amine functionalization

Chemical functionalization of CNTs with an amino group makes them much desirable by introducing reactive groups on pristine nanotubes [71]. Amine functionalization of CNTs has various applications including its application in enzyme immobilization. Techniques for CNT functionalization with the amino group include CNT treatment with acid, grafting or wrapping of nanotubes with a thin layer of a polymer chain, or a combination of these methods. These reactions are

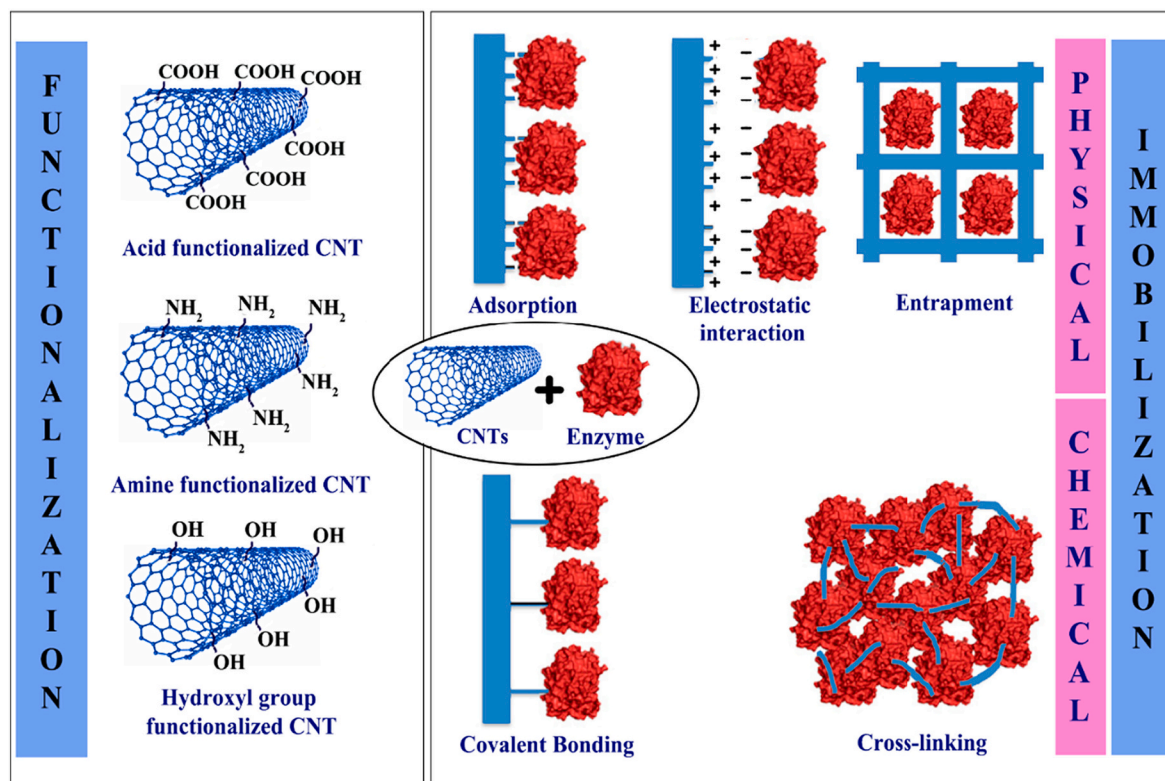


Fig. 2. Schematic representation of the various methods of functionalization and enzyme immobilization.

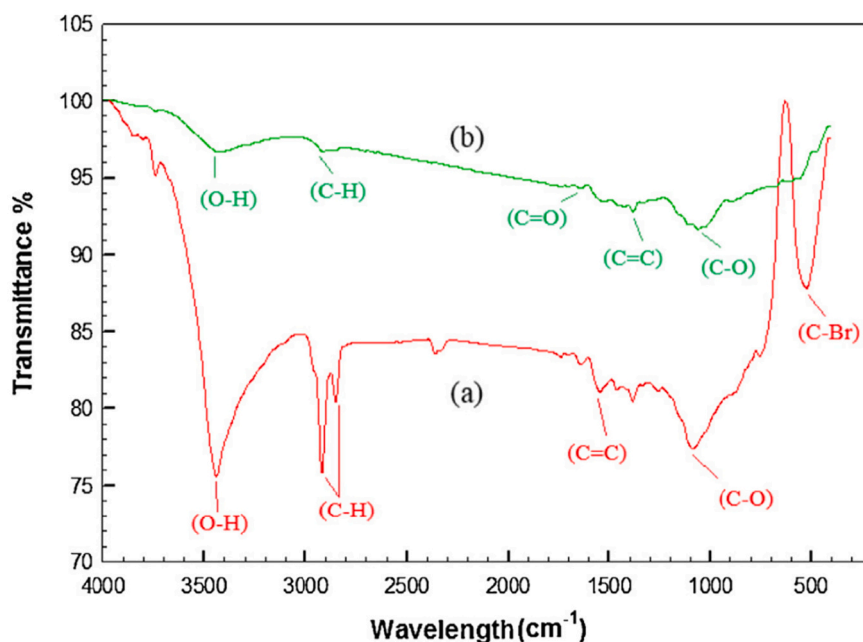


Fig. 3. FTIR spectra of (a) MWNT (b) acid-functionalized MWNT. Adapted with permission from ref. [67]. Copyright 2014 Elsevier.

complicated, require controlled reaction conditions and a long reaction time. Also, the utilization of ethylenediamine (EDA) as cross-linking amino reagent can assist in reducing the complexity of the reaction [72].

The process of amine functionalization begins with the oxidation of pristine MWCNT using the concentrated acid mixture. This makes the inert MWCNT more reactive towards any functional group. For this, the pristine MWNT is treated with a mixture of conc. HNO_3 and H_2SO_4 in the ratio of 1:3 v/v. This mixture (MWNT + acid mixture) and kept for reflux. The resulting oxidized nanotube is dispersed in ethylenediamine (EDA). As a result of this reaction, amino-functionalized MWNT are synthesized in which amino groups from EDA are attached to the nanotube structure [73]. In a similar way amine functionalization of MWCNTs was achieved by a facile process in which the starting material, 0.3 mg MWCNT-COCl was dispersed in *N,N*-dimethylformamide (DMF) using ultrasonic agitation. The resultant was added with 5 mL ethylenediamine under nitrogen and eventually, the mixture was heated at 70 °C with constant stirring for about 72 h. Finally, the product is vacuum filtered, washed with acetone, and dried at 80 °C [74]. Electrochemical functionalization is another method for attaching amino groups to CNTs. It is considered a low destructive method when compared to other chemical methods [75]. Also, it does not include immersing CNTs into bulk solutions and using several chemical reagents. The process requires the utilization of two graphite electrodes as reaction cells and a solution is prepared by dissolving NaCl in water, pristine MWCNT, ethylenediamine together. This solution is then transferred to an electrochemical cell and a voltage of 15 V was applied to the cell for 45 min resulting in the functionalization of amino group on pristine MWCNTs [75].

Amine functionalized MWCNT is more efficient when the enzyme to be immobilized has more electrophilic centers since the NH_2 group present on amine-functionalized MWCNT are nucleophilic. Otherwise, the conditions of solutions used during immobilization reactions should be altered, or else the enzyme should be modified to facilitate immobilization [76]. In certain cases, it was observed that NH_2 groups get protonated and turn into NH_3^+ which can take part in ionic interactions with the enzyme and make the enzyme stable on aminated CNT. Therefore, the amine group plays a paramount role in the covalent binding of enzymes and also helps in binding with cross-linkers like glutaraldehyde for enzyme immobilization [77].

3.3. Hydroxyl group functionalization

The hydroxyl group functionalization involves the attachment of the OH group to MWCNT which makes it a site for electrostatic interactions and reactions [78]. Hydroxyl group functionalization increases CNT's affinity for enzymes by increasing the number of reactive sites. In a study, H_2O_2 was used as an oxidizing agent and was reacted with pristine MWNT which introduced a hydroxyl group on its surface [79]. In the work done by M. Premalatha et al., OH functionalized MWNT was prepared by mixing MWNT with Dowtherm A (a mixture of biphenyl $\text{C}_{12}\text{H}_{10}$ and diphenyl oxide) with the purity of >95%. OH, the functional group is nucleophilic due to the lone pairs on the oxygen atom [80].

The acid functionalization method is effective in making CNTs water-soluble however draws concern for environmental safety. Functionalization of CNTs mainly utilizes concentrated acids, commonly a mixture of H_2SO_4 and HNO_3 which produces hazardous wastes [81]. Therefore, the present research work focuses on the development of an eco-friendly and environmentally benign method for the functionalization of CNTs using a solution of 30% H_2O_2 through autoclaving and sonication. However, hydrogen peroxide generates non-toxic and non-hazardous residue through a simple oxidation process [82]. The process can yield two types of functionalization on the surface, CO_2H groups, and OH groups. Two possibilities arise because, at high-temperature H_2O_2 decomposes to two different radicals, OH radicals and OOH radicals [83]. However, OH radical is most likely to attack CNTs as they are more reactive giving rise to hydroxyl functionalized CNTs. The presence of hydroxyl group on the CNT surface was confirmed by FTIR analysis of pristine CNT and hydroxyl functionalized CNT [82]. The FTIR spectrum showed characteristic OH stretching frequency at 1562 cm^{-1} . The result confirms hydroxyl group functionalization on CNTs [82]. Similarly, sidewall functionalization of SWNT has been achieved through fluorine displacement reactions to give rise to hydroxyl nanotubes [84]. During the process, fluorine was displaced from fluoronanotubes using a series of glycerols and diols in the presence of alkali, and the fluoronanotubes utilized in the study were prepared by fluorination of SWCNT that can act as a precursor for displacement reaction. The study conducted revealed that the fluorine present in fluoronanotubes acts as an efficient electron acceptor, thereby exhibits strong interaction with nucleophilic reagents and facilitates hydroxyl group functionalization [85]. At present time, nanomaterials are being utilized in many research and

industrial fields. Exposure to nanomaterials can lead to adverse human health and discarding industrial waste into the open area has a negative impact on the environment. In several studies, it was also found that hydroxyl functionalization of CNTs reduces negative environmental impact and decreases cytotoxicity when compared to pristine CNTs [86].

All three functionalization techniques assist in improving the susceptibility of CNTs towards enzyme immobilization. However, as per the findings from the literature and our understanding, most of the studies utilize the acid functionalization method due to ease of preparation and efficiency. Also, increased solubility of the acid-functionalized CNTs in various solvents makes acid functionalization more desirable than others [87,88]. Most of the common enzymes like lipase, peroxidase, amylase are compatible with the acid group present on acid-functionalized CNTs. Moreover, the presence of the acid group assists in physical as well as chemical methods of immobilization. When CNTs gets dispersed in the solvent, the CO_2H group gets deprotonated and helps in electrostatic interaction with the positively charged enzymes leading to physical adsorption [15]. The acid groups present can also be made to react chemically with the enzymes leading to the formation of a stable amide bonds with the enzyme leading to chemical immobilization. Additionally, no severe structural damage on the surface of CNTs was observed during acid functionalization [89]. The hydroxyl group, on the other hand, is not considered as an ideal choice for the functionalization process because most of the functional groups present on enzymes are nucleophilic which are non-reactive towards the OH group present on functionalized MWNT [90]. Also, the hydroxyl groups are revealed to be offering low solubility to the CNTs. A few of the issues that need to be addressed in the case of the functionalization process in upcoming research in this field include the location of functionalization,

distribution, and quantifying the amount of functional group.

4. Strategies involved in enzyme immobilization

Enzymes being a biocatalyst holds great importance in the food, pharma, and chemical industries. But its industrial application is limited as it is non-recyclable, denaturises in extreme conditions of pH and temperature, and difficult to handle [91,92]. Studies have shown that enzyme immobilization can be utilized for controlling the enzyme configuration, orientation, along with density, therefore, improving the target specificity and activity of the enzymes [34,35]. Attachment of multiple enzymes along with the nanoparticle morphology plays a crucial role in increasing the localized density of the enzymes [93]. Similarly, by careful and strategic orientation and attachment of the support-binding pocket of the immobilized enzyme with the nanoparticle surface, enzyme orientation can be managed [94]. Also, the presence of the Brownian motion has enhanced the primary interaction between the enzyme and support [95]. Likewise, secondary interactions such as electrostatic attraction have also been revealed to contribute towards increased enzyme-support interaction [96]. These interactions lead to the subsequent increase in the concentration of the substrate near the periphery of the nanoparticle as opposed to the bulk environment which would furthermore increase the activity of the immobilized enzymes on the nanoparticles as compared to those soluble in solution [97]. The process of enzyme immobilization on a suitable support material is the key to overcome enzymatic limitations such as the selection of optimal matrix and suitable immobilization techniques. There is no ubiquitous material for the immobilization of enzymes but according to our study, CNTs have gained more attention in this field. As a result of

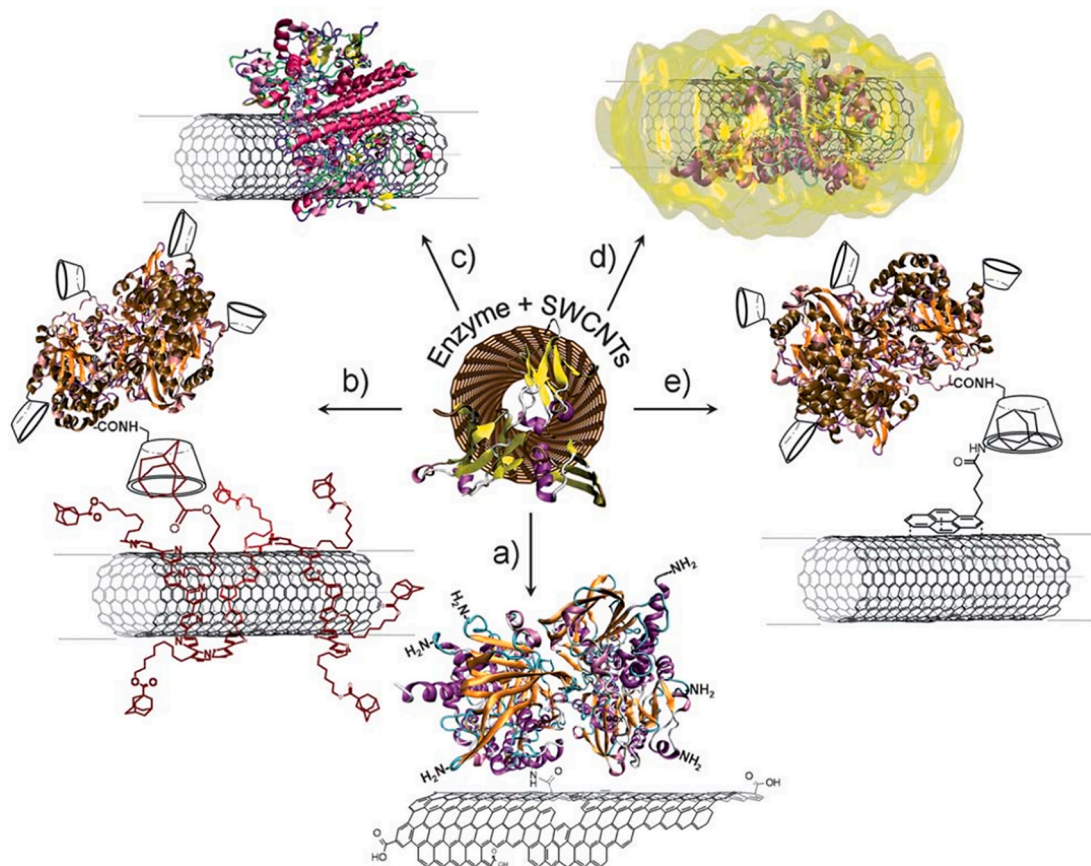


Fig. 4. Immobilization strategies of enzymes on SWCNTs (a) covalent binding via amide coupling with the carboxylic acid groups of oxidized nanotubes; (b) electrochemical coating of nanotubes with affinity partners and subsequent immobilization of affinity counterpart modified enzymes; (c) adsorption of enzymes on SWCNTs via hydrophobic or electrostatic interactions; (d) entrapment of enzymes in a polymer matrix formed around SWCNTs; and (e) immobilization via affinity interactions onto functionalized nanotubes. Adapted with permission from ref. [98]. Copyright 2011 The Royal Society of Chemistry.

this, different techniques have emerged for enzyme immobilization [98] which has been categorized into two types: physical and chemical immobilization as depicted in Fig. 4.

4.1. Physical immobilization

Physical immobilization involves no strong link or bond formed between enzymes and CNTs. The enzymes are bound to CNTs via physical forces of interaction [99–101]. Based on the type of force bonding enzyme and CNTs, physical methods are classified into three types; adsorption, electrostatic interaction, entrapment.

4.1.1. Physical adsorption

The adsorption is considered as a non-covalent process that includes enzymes getting adsorbed on the surface of CNTs through physically attractive forces by preserving the native conformation [102]. For the adsorption process to take place, MWCNT is dipped in the solution of enzymes and the resultant is shaken well and is left undisturbed for some time which allows the adsorption process to take place. Then the solution is washed to remove unadsorbed enzymes from the surface and to reduce crowding [28]. The enzymes develop several physical interactions with the f-MWNT and get stabilized through weak forces like van der Waals, pie-pie stacking, hydrogen bonding, and hydrophobic interactions [103]. The hydrophobicity is revealed to be playing a major role in stabilizing enzymes on the support [104]. Studies involving the immobilization of cellulase enzyme on functionalized MWNT which was stabilized with the help of π - π stacking interaction have been analyzed [105]. Michele and co-workers studied the effect of MWCNTs functionalization and their application as support for the immobilization of *Burkholderia cepacia* lipase (BCL) in the resolution of (RS)-1-phenyl-ethanol utilizing vinyl acetate. This facile and effective process of physical adsorption of BCL onto MWCNTs-A resulted in ameliorated catalytic efficiency and also confirms that the MWCNTs offer support for BCL immobilization and also renders prospective opportunities for the applications in (RS)-1 resolution [79]. Thus, immobilization through physical adsorption is more applicable for industrial purposes because it is easily segregated from the end product after reaction and can be used several times [67].

4.1.2. Electrostatic interaction

Certain enzymes are immobilized in functionalized MWNT with the help of electrostatic interactions. The electrostatic interactions are developed between enzymes and f-MWCNT due to opposite charges present on both the enzyme as well as the f-MWCNT [15]. The choice of support depends on the charge present on the enzyme surface and in case if the enzyme is positively charged, then a functionalized MWCNT possessing a negative charge (COO⁻) is utilized. For example, laccase being positively charged gets immobilized on a deprotonated carboxylic acid group of functionalized MWCNT [106]. The laccase from *Trametes versicolor* was immobilized on chitosan, MWCNT. A strong electrostatic interaction was found to be formed between the amine group of functionalized MWCNT and the enzyme gave rise to an efficient biocathode [107]. The NH₂ group present on functionalized CNTs and enzymes may get protonated and produce a positive charged NH₃⁺ group which helps in electrostatic interaction [108]. Several studies have shown that the enzymes immobilized by this methodology exhibit lower catalytic current stability and activity [109]. Also, the electrostatic attractions can get contrived under different surrounding conditions which leads to its restriction to certain researches.

4.1.3. Entrapment

The entrapment method involves capturing enzymes physically between the 3D structures of CNTs [110]. This leads to the restriction of enzymes to progress comfortably inside the structure which makes this method different from the adsorption technique. There is no forces and bonds present between enzymes and MWCNT but the physical boundary

of CNTs which restricts enzyme to leave the surface [111]. One of the major facts to be considered is that the size of the enzyme should be less than the pore size of the CNTs [112]. The substrate which is to be catalyzed by the enzyme is made to pass through MWCNT in which enzymes are trapped. The product formed after the catalysis of the enzyme moves out of the CNTs and the enzyme is retained by a carbon nanotube [113]. However, the presence of enzyme leakage associated with entrapment leads to its underutilization. The laccase enzyme immobilized on MWNT was utilized for water purification and the resultant was used for the elimination of bisphenol A from water and showed 89.9% better catalytic activity than soluble enzyme. As a result, the tensile strength, storage capacity, and operational capacity were revealed to be enhanced [114]. Various studies conducted describe that the enzymes have also been entrapped inside other materials like silica microparticles, PAN-biochar, cytodextrin, and copper alginate beads [106].

4.1.4. Layer-by-layer approach

The layer-by-layer approach has been one of the recent techniques for the immobilization of enzymes. This facilitates the formation of enzyme multilayers on CNTs. The biocatalyst shows enhanced activity on increasing the number of enzyme layers on the substrate [115]. The alternate assembly of opposite charged enzyme layers provides a better platform for the effective adsorption of enzymes on CNTs [116]. Dhriti Nepal and co-workers developed a substrate incorporating alternate layers of DNA-SWNT (anionic) and lysozyme-SWNT (cationic) adsorbed via strong electrostatic interactions such as van der Waals and π - π interactions [117]. Functionalization of carboxylic acid on CNT generates negatively charged groups on the surface which promote the adsorption of positively charged poly(dimethyldiallylammoniumchloride), followed by the assembling of horseradish peroxidase (negatively charged) [118]. The developed nanocomposite enhanced the ultrasensitive determination of alpha-fetoprotein. Among the various conventional methods, the LBL approach is preferred as it involves consecutive stacking of materials and prevents the denaturation of glucose oxidase (GOx) as LBL follows the physical adsorption approach [119]. Such adsorption techniques for enzyme immobilization are advantageous as they can preserve the protein structures, intrinsic electronic structures, and CNT properties.

4.2. Chemical immobilization

4.2.1. Covalent bonding

In this immobilization technique, the functional groups present on enzymes and the functional group present in enzymes interact to form a covalent bond between enzyme and f-MWCNT which leads to the formation of the stable enzyme-MWCNT complex [120]. The immobilized enzymes possessing amide bonds show good catalytic activity over many cycles. In a study done by Aurelia Zniszczoł, lipase was covalently immobilized on f-MWCNT which showed 78% of initial activity after 50 cycles [121].

The enzyme structure is altered and its activity is reduced but enzymes are not able to leave the nanotubes and enzyme leaching is not observed [122]. In the research done by Abdelmageed M. Oathman et al., laccase was immobilized on MWCNT through covalent bonding in which laccase showed excellent operational stability of 95% after 10 cycles. Stability towards temperature and pH were also seen to be improved (21% for soluble laccase and 49% for immobilized laccase) [70]. In a similar study, MWCNTs were filled with iron oxide and linked to polyamidoamine for developing a supermagnetic matrix for *B. cepacia* lipase immobilization Fig. 5. The maximum activity recovery of immobilized enzyme was observed to be 1716% and specific activity increased to 77,460 U/g-protein which is 17 folds higher than soluble lipase. Also, the enzyme matrix framework was easily separable from the reaction mixture using a magnet as the framework possesses magnetic properties [123,124].

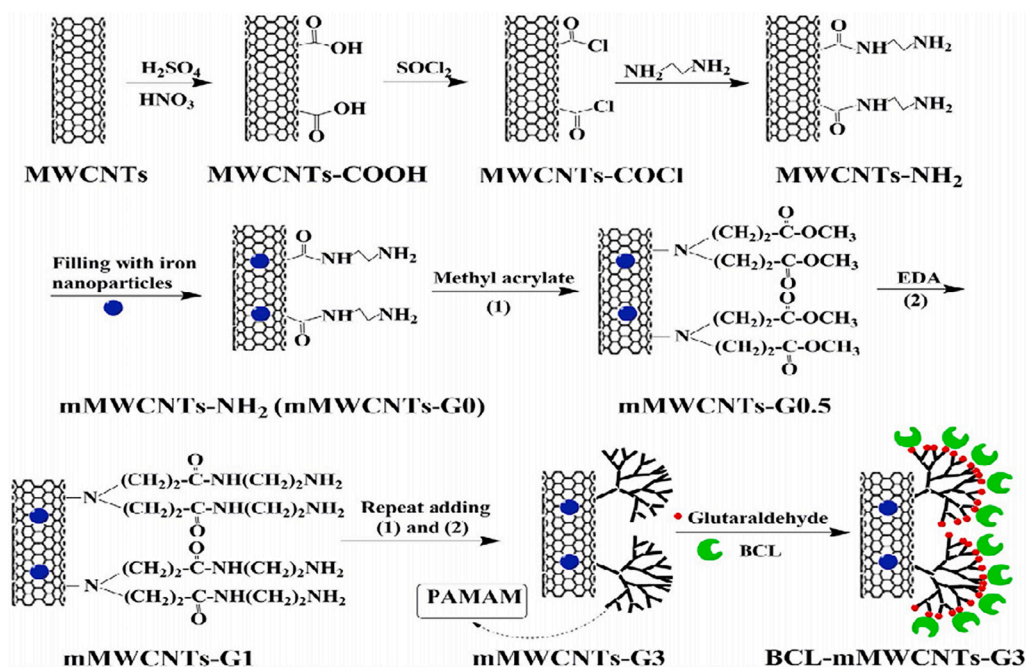


Fig. 5. Schematic illustration of magnetic iron oxide nanoparticles filled CNTs modified with PAMAM dendrimers for BCL immobilization. Adapted with permission from ref. [123]. Copyright 2017 Nature.

4.2.2. Cross-linking

In this method, one linking agent conjugates enzymes together like beads are attached by the thread [125]. The chemical groups on one enzyme react with one end of the linking agent. The free end of the linking group attaches with the second enzyme possessing two enzymes at its end. This linking agent holds the enzymes together and can vary in size. The common group used for linking is glutaraldehyde as it is readily available and inexpensive [126]. There are three different types of cross-

linking methods adapted for enzyme cross-linking; intramolecular, intermolecular, and intersubunit [127]. In the enzyme-MWCNT structure, the CNTs are present in the center and the enzymes linked by cross-linkers are wrapped around the cylindrical structure along the central axis thereby the tertiary structure of enzymes is stabilized [128]. Tyrosinase (TYR) was immobilized on CNT using enzyme adsorption, precipitation, and crosslinking (EAPC) approach to prepare a highly sensitive biosensor Fig. 6. The enzyme was precipitated and crosslinked

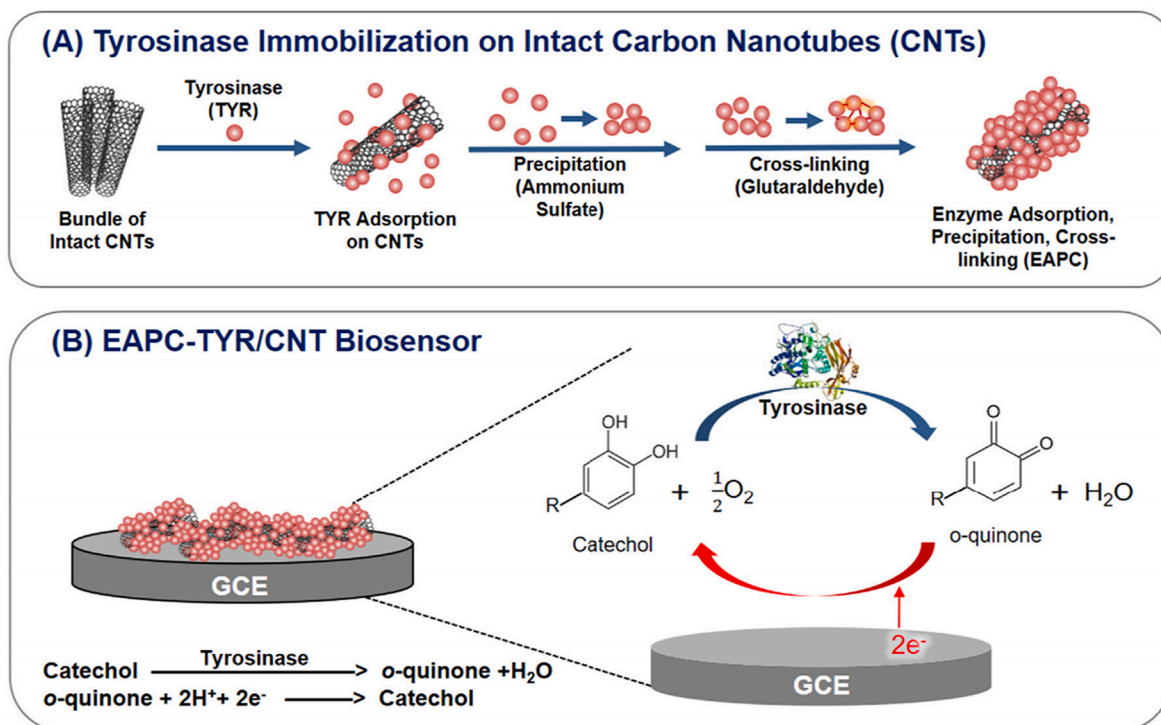


Fig. 6. Schematic illustrations of (A) EAPC protocol, consisting of enzyme adsorption, precipitation, and crosslinking, and (B) electrochemical reactions occurring in the EAPC electrode on the glassy carbon electrode. Adapted with permission from ref. [129]. Copyright 2019 Elsevier.

to prepare the sample for EAPC. The resultant biosensor formed showed high activity due to higher enzyme loading per unit weight of CNTs as a result of effective enzyme crosslinking. The specific enzyme activity of EAPC was observed due to structural enzyme deformation upon enzyme crosslinking and serious mass transfer limitation as there is high enzyme loading [129].

Chemical immobilization is a promising approach towards developing a stable enzyme nanotube conjugate and it provides various advantages over physical methods. No significant enzyme leaching takes place when the enzyme binds through covalent linkage [130]. The binding of enzymes on CNTs takes place at a specific position which often leads to reutilization up to many cycles which in turn makes it economic. The tertiary structure of enzymes is stabilized around CNTs through attractive forces [130]. Despite having several advantages, chemical immobilization faces some major drawbacks. The immobilized molecules should retain their structure and activity as the precursor but during chemical immobilization, the structure of enzymes gets modified. Due to this, steric modification of enzymes is also possible after covalent interaction [131]. Hence the enzyme activity is altered [132,133]. Improper functionalization and orientation of CNTs may affect the immobilization process as the functional group on CNTs are crucial in binding with the enzymes [134]. Additionally, the utilization of strong oxidants and concentrated acids during CNT functionalization is not an environmentally benign alternative [135]. Therefore, the chemical immobilization process is not advisable for industrial applications.

As per our understanding, it is ascertained that the physical methods show several advantages over the chemical methods of immobilization such as higher yield, retainment of the enzyme activity, and structure due to the non-occurrence of a chemical reaction during the physical immobilization process [104,136]. Also, it is observed that there is no

chemical modification of enzyme happening which results in the easy release of the enzyme from the CNTs [137]. Various techniques can be employed for separating enzymes from the CNTs and in addition, the facile nature of this method makes it more desirable at the industrial level for achieving economic immobilization. However, at the same time, there are certain drawbacks of this method that is challenging to be rectified. This includes the adsorbed enzymes undergoing facile leaching and lack of stabilization along with the durability of the enzyme tertiary structure in the case of MWCNT [138]. Moreover, after certain cycles of utilization, the enzymes get strayed away from the CNTs leading to further complications including the risk of the partial unfolding of protein which in turn can disrupt the biological functioning [139,140].

5. Applications of enzyme immobilized CNTs

Enzyme immobilized CNTs have been comprehensively developed and utilized over the last few decades as they have been proven to be an innovative technique for overcoming all limitations of native enzymes and improving their performance. Enzymes immobilized on CNTs are reported for several applications in various fields such as biosensing, biofuel cells, water purification, food industry, and environmental remediation. CNTs find their application in biosensors and biofuel cells due to their excellent electronic communication [141]. The principle allowing the mass transfer of electrons between enzymes and electrodes through CNTs is used to detect analytes in biosensors and form products in biofuel cells [142]. The use of CNTs increases the surface concentration of enzymes thus having great turnover rates of substrates. Application of soluble enzymes for water purification and environment remediation was limited as enzymes lose their activity when they are revealed to the actual environmental conditions and have poor storage

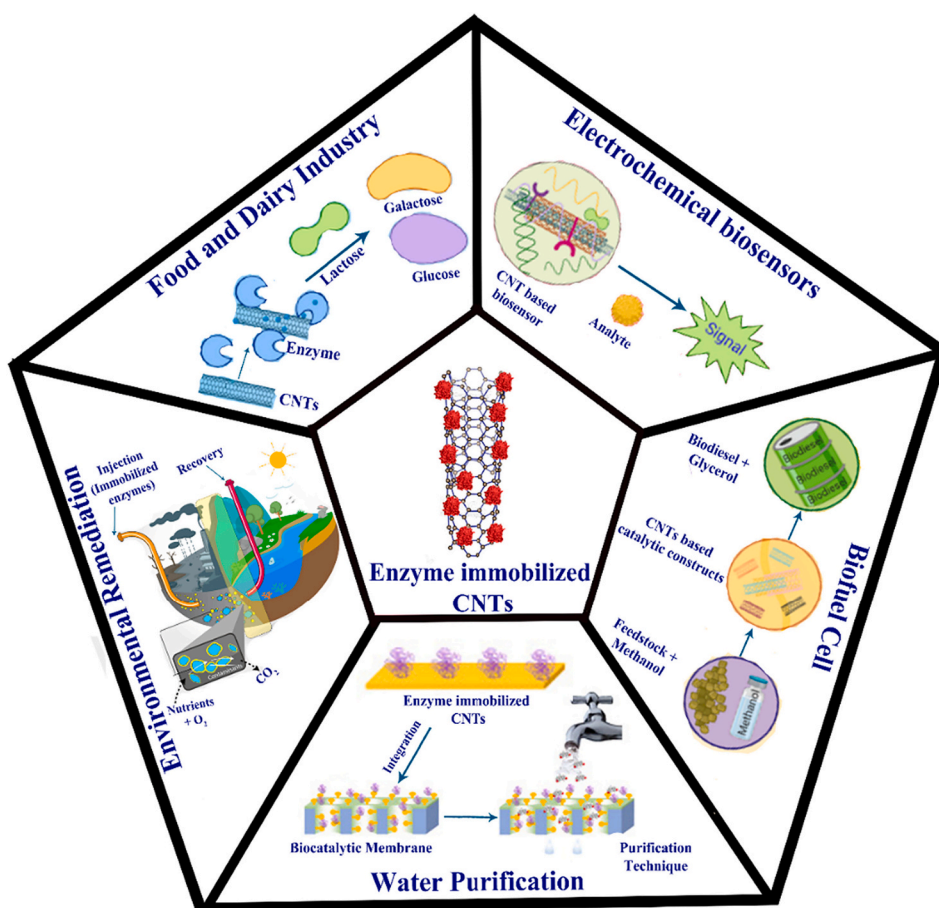


Fig. 7. Applications of enzyme immobilized CNTs.

and operational stabilities. However, the immobilization of enzymes offers various advancements in its applications as shown in Fig. 7.

5.1. Water purification

The CNT-based biocatalysts are used for the degradation of various types of pollutants from water [143]. The CNTs offer various advantages during water purification from their side which includes frictionless water transport system due to their hollow structure which can cause a reduction in the general pressure-driven forces and thereby the total energy obligation. Also, the cytotoxic properties of CNTs can hamper and tackle the micro-contaminants present in highly contaminated water and can also reduce the fouling of the thereby ameliorating the value and utilization of current membrane technologies [144].

Two of the major challenges associated with the utilization of CNTs for their application in water purification include improving their solubility and getting rid of the contaminants such as metal catalysts and non-nanotube carbonaceous agents [145]. Incomplete removal of the contaminants can lead to complications in the observed catalytic behavior and misleading total electrocatalytic activity [146]. In addition, they can even contribute to various other harmful effects including a decrease in the immobilization efficiency, enzyme's conformation changes, along with the alteration of its catalytic activity thereby, making purification a paramount step [147]. The solubility of CNTs in water solution can be enhanced by functionalization using various methodologies mentioned above in the text. Even though several chemicals, physical and even a combination of both the purification methods have been developed and adopted for the removal of impurities and improving the solubility of CNTs, limitations such as degradation of CNTs, and shortening in the length and burning under extreme heat and excessive chemicals have therefore become a tough hurdle which needs to be solved [148]. The purification of CNTs can be done by two methods which are chemical and physical purification. Among the two of them, chemical purification has been revealed to be more effective and having an upper hand which is attributed to their improved selectivity, sensitivity, availability, faster rate kinetics of the oxidation of carbonaceous impurities, along with being economic [145]. The physical methods on the other hand are removing graphitic sheets and carbon nanospheres from the CNTs, however, this is relatively ineffective, and laborious [145].

A facile chemical purification method was utilized for the synthesis of pure MWCNTs with desired properties imperative for designing a suitable platform for 3,4-POD immobilization followed by decorating the MWCNTs with -COOH functionality which led to the enhancement of solubility of the MWCNTs [147]. In addition, the covalent attachment was utilized due to its advantageous properties thereby leading to a strong and durable CNT-enzyme attachment during the process. The resulting biocatalyst was revealed to be decontaminating the 3,4-DHBA and could even assist in the understanding of an enzyme behavior at nanointerfaces [147].

The use of biocatalyst is found to have certain advantages as compared to the conventional methods such as distillation, membrane technologies, coagulation, and filtration which are having limitations like reduced stability, selectivity, and specificity [149,150]. Biocatalysis has high applicability for water purifications whereas the conventional chemical transformations of water pollutants are highly implausible [151,152]. The presence of CNTs improved certain properties of biocatalysts such as high mechanical stability, broad pH and temperature ranges, and functionality in unfavorable environments thus enhancing their utilization in various fields. Also, it has been found out that recovered biocatalyst can be utilized many times, thereby making the method highly economic [153]. Thereby, the biocatalyst can perform few chief functions with utmost selectivity and sensitivity in water purifications such as (i) binding and pre-concentration of the pollutants; (ii) elimination and/or degradation of the pollutants, and (iii) sensing and monitoring of the pollutants [147]. Also, the biocatalyst can be

utilized for the removal or reduction of total water contaminants and for also generating commercially valuable products from intractable organic pollutants present in water [30].

A one-pot methodology for the fabrication of enzymatically active CNT paper for organophosphate (OP) bioremediation by immobilizing organophosphate hydrolase (OPH) onto CNTs was studied by Mechrez et al. [154]. The studies conducted revealed a remarkable reduction in the concentration of MOX concentration which is majorly attributed to in situ hydrolyses of MOX by the immobilized OPH throughout the filtration procedure. It was the distinctive merging of the high enzyme loading capacity within the CNT membranes along with their high mechanical integrity that paved for developing a miniaturized conventional bio-catalytic system [154].

Biocatalytic degradation of phenol from wastewater has been achieved utilizing Fe_3O_4 /nanotubes composites which were synthesized by the hydrothermal method [155]. The composite was employed as a support for immobilizing horseradish peroxidase by covalent binding. The study revealed that the enzyme activity and loading ratio increased with the increase of enzyme immobilization and later the slope became smooth indicating that the combination was achieved and showed good temperature and pH stability along with reusability. The immobilized enzymes exhibited the highest phenol degradation at the phenol concentration of 100 mg/L and therefore could be utilized as a potential tool in the phenol removal from wastewater [155].

The rapidly growing chemical and other industries generate huge volumes of dyes that are discharged into the aquatic bodies [156]. Due to their chemical nature, composition, and quantities, they have an adverse effect on the aquatic system which makes their degradation even more [157]. For this, a biocatalyst based on immobilized laccase on crosslinked polymethacrylate (PMMA)/carbon nanotubes (CNTs) have been utilized as shown in Fig. 8. The fabrication of PMMA-coated CNTs (PMMA-CNTs) was achieved by miniemulsion polymerization, followed by the activation using glutaraldehyde (GA) [158]. The biocatalyst system was revealed to be having high reusability as it could maintain around 50% of the original activity even after being reutilized for 10 batches. Also, the immobilized laccase exhibited higher storage capacity, could work at a broad range of temperatures, and had optimum pH stability as well. The immobilized laccase was revealed to be showcasing remarkable decolorization towards both the methylene blue and orange II, and the decolorization reached upto 96% and 74%, respectively. Also, the added advantage that the decolorization rate was reduced by about 10% after 10 successive cycles improves its application in the removal of azo dyes from wastewater samples [158].

5.2. Environmental remediation

The ever-increasing environmental pollution caused by cyanate, heavy metals produced as a result of various anthropogenic activities such as mining and other industries possesses a major threat to the environment [159–161]. Therefore, the utilization of biohybrids incorporating both enzymes and CNTs as support material is an ideal choice for tackling the present-day environmental crisis. The presence of van der Waals forces and π - π interaction in CNTs makes them an environmentally benign adsorbent for the removal of heavy metal from the water [162]. The iron-oxide-filled multi-walled carbon nanotubes (m-MWCNTs) covalently immobilized with recombinant cyanate hydratase (rTl-Cyn) have been utilized for the simultaneous elimination of both cyanate and heavy metals from the wastewater sample as represented in Fig. 9. For the immobilization process, a cyanate rTl-Cyn concentration of 0.6 mg was chosen as the low concentration of enzyme during the immobilization process would have increased the bonding between enzyme and linker, leading to enhanced activity [163]. The higher activity of the immobilized enzyme at alkaline pH of 8.0 was attributed to the improvement in enzyme stability through covalent immobilization onto the support and therefore improves its utilization in cyanurated waste remediation. Also, the m-MWCNT-rTl-Cyn was able to retain more

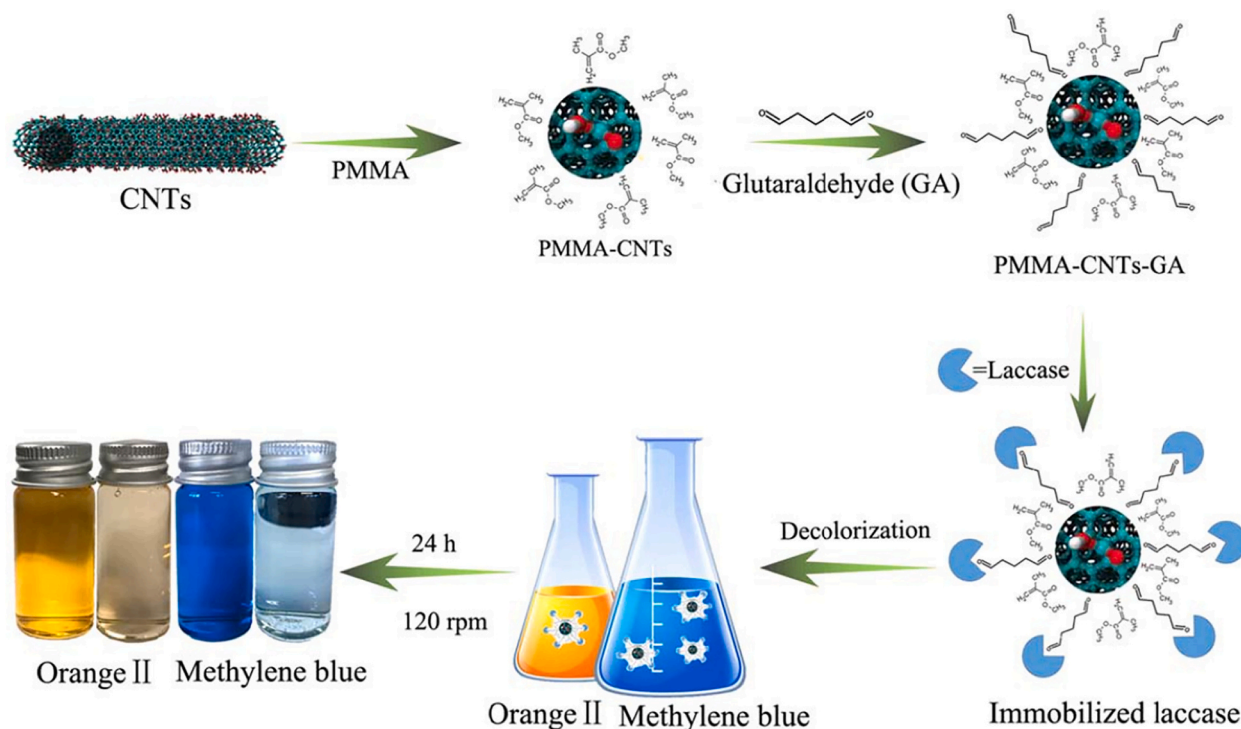


Fig. 8. Schematic representation of experimental protocols for the immobilization laccase and the decolorization of dyes. Adapted with permission from ref. [158]. Copyright 2019 IOP Science.

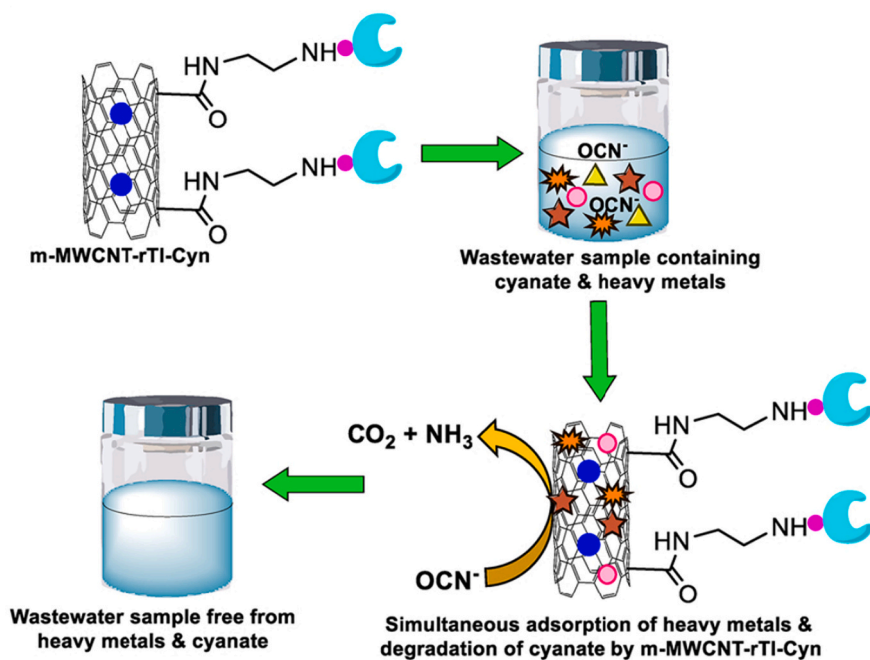


Fig. 9. Schematic illustration of removal of heavy metals and cyanate from waste water sample utilizing immobilized cyanate hydratase on magnetic-MWCNT. Adapted with permission from ref. [163]. Copyright 2019 Elsevier.

than 94% of its initial activity after 10 consecutive cycles and exhibited great reusability and storage stability. Moreover, this novel work depicted that the m-MWCNT-rTI-Cyn was successful in the simultaneous reduction of the concentration of chromium (Cr), iron (Fe), lead (Pb), and copper (Cu) by 39.31, 35.53, 34.48, and 29.63%, respectively along with the concentration of cyanate by $\geq 84\%$ thereby ameliorating its application wastewater treatment [163].

Even though the utilization of CNTs as pollutants adsorbents is emerging, certain limitations such as lack of conversion of the adsorbed pollutants into a soluble form, alteration in the surface properties, and inability in adsorbing high concentration of adsorbate affect its utilization [164]. However, to surpass these shortcomings, recently, researchers are focussing on the ideology of immobilizing enzymes on the CNTs ultimately enhancing their utilization by a synergic effect [165].

Aniline is another compound that is highly utilized in the production of rubbers, dyes, and other industrial effluents [166]. However, aniline being a carcinogen can lead to some serious impacts and thereby needs to be eliminated from the environment [167]. Literature studies reveal that *Delftia* sp. XYJ6, a highly promising bacteria has a robust capacity in biodegrading the aniline and the initial aniline at lower concentrations. However, higher concentrations of aniline are toxic to the *Delftia* sp. XYJ6 and therefore no or reduced biodegradation takes place [168]. During the biodegradation, aniline is initially transformed into catechol catalyzed by aniline dioxygenase as a first product, which is later biodegraded to cis, cis-muconic acid catalyzed by the catechol 1,2-dioxygenase of *Delftia* sp. XYJ6 as a secondary product. Also, the Cis-, cis-muconic acid can be further biodegraded to various smaller compounds [168]. By making use of this information, Yan et al. studied the synergistic effect produced by both the CNTs and the enzymes of *Delftia* sp. XYJ6 in the eradication of aniline [169]. It was revealed that the MWCNTs exhibited higher adsorption of aniline as compared to the single-walled counterparts. Nonetheless, the adsorption of the protein of *Delftia* sp. was found to be higher in the case of the SWCNTs [169]. This would have been due to the presence of two factors - adsorption and enzymatic biodegradation. The CNTs were capable of adsorbing both proteins of *Delftia* sp. XYJ6 and aniline would have resulted in the reaction between them on the CNT surface resulting in the enhanced removal of aniline. In the case of enzymatic biodegradation, even though, higher amount of aniline was absorbed by MWCNTs, the biodegradation enzymes of *Delftia* sp. XYJ6 experienced higher difficulty in entering and maintaining contact with the aniline absorbed on the layered structures of MWCNTs, thereby restricting the reaction area leading to less improvement of aniline biodegradation. Additionally, the presence of covalent and non-covalent interactions such as hydrophobic, π -stacking, etc. could be the reason for multiple adsorptions of molecules on CNTs surface. The synergistic effect produced ensured rapid enzymatic biodegradation of the aniline and could be potentially applied in the aniline removal from the wastewater [169].

Another major challenge to the environment is the presence of water-soluble micro-pollutants which are normally found in trace concentrations and originate from the anthropogenic compounds utilized in pharmaceutical, food, personal care products, and other chemical industries [170]. The fact that they have a long life and exhibit resistance to the conventional biodegradation methods increases their adverse effects on the beings and the entire environment as well. Even though various conventional methods including ozonation, oxidation, and membrane filtration have been utilized for the efficient elimination of these micro-pollutants, limitations such as problems related to disposal, high operation costs, etc. have led to the underutilization of these techniques [171–173]. This led to the development of an enzymatic membrane reactor (EMR) which consists of an enzyme immobilized on a support membrane which allows efficient enzymatic transformation happening concurrently with the selective mass transfer process across the membrane [30]. The properties of EMR such as easy integration combined with continuous operation and flow-through configuration allows the efficient separation of enzymes from the end products thereby enhancing product-involved inactivation of enzymes. The five most harmful and frequently found micropollutants in water bodies - bisphenol-A (BPA), carbamazepine (CBZ), diclofenac (DCF), clofibric acid (CA), and ibuprofen (IBF) were disparate together to regulate the one-pot degradation test to replicate the empirical wastewater treatment conditions. Then CNTs were coated on the PVDF membrane for the enzyme immobilization which ensured effective stability and cross-linking. The study conducted revealed that mass transfer between the biocatalytic CNTs layer was attributed to the flow-through EMR configuration. Also, it was observed that the CNTs biocatalytic membrane was successful in enhanced removal of all five micropollutants and showcased significant satisfactory stability, efficacy, reusability, and ensured minimum fouling [30].

Laccase enzyme is found to be having the capacity to a broad range of

substrates such as phenols, polyamines, dyes, aromatic substrates through a single-electron oxidation mechanism [174]. It is this wide substrate specificity exhibited by the laccase enzyme which enhances its utilization in bioremediation [175]. However, immobilization of the laccase enzyme is highly crucial as it is expendable and can lose its activity when exposed to natural environmental conditions, exhibits imperfect storage and operational capacities usually, and is not economic [176]. The electrospun fibre membranes act as a remarkable support material in case of enzyme immobilization owing to the remarkable characteristics showcased by them like high specific surface area, enhanced porosity, and high connectivity [114]. Owing to the excellent properties exhibited, different studies involving MWCNTs modified EFMs have been conducted and all of them have delivered satisfactory results. By making use of this information, Dai and co-workers utilized MWCNTs-LCEFFMs for the elimination of bisphenol A (BPA) from water samples. The emulsion electro-spinning technique was employed for improving the activity and stability of LCEFFMs and also triblock copolymer PEO-PPO-PEO (F108) as an emulsifier which allowed dispersion of laccase and MWCNTs in electro-spinning solution. The MWCNTs-LCEFFMs was revealed to be having a removal efficiency rate above 90%, along with a degradation efficiency reaching above 80%, and showcased ameliorated adsorption efficiency. Also, the MWCNTs-LCEFFMs showed significant endurance to natural conditions such as pH and temperature [114].

A comparison of different enzyme immobilized carbon nanomaterials towards phenolic compound degradation exhibited exciting results [177]. The different carbon nanomaterials included MWNTs, OMWNTs, GO, and fullerene (C60) being utilized in addition with the enzyme as nanobiohybrids for the degradation of bisphenol A (BPA) and catechol as model phenolic contaminants. The group made use of physical adsorption for the immobilization in place of covalent bonding. It was observed that the immobilized enzymes had a remarkable reduction in the reaction rates when compared to the soluble laccase enzyme which was attributed to the nanomatrix-induced diffusional limitation. Also, the MWNT- exhibited the highest effectiveness factor η when compared to its carbon counterparts. The heterogeneity index values were found to be much lower than 1 in the case of MWNT and OMWNT which is attributed to the presence of interstitial and groove areas in addition to the external surface for adsorption. The difference in the aggregation properties can also influence the adsorption capacities. For example, the presence of van der Waals interactions in the case of CNTs leads to the aggregation of bundles whereas GO sheets have high dispersibility due to the large specific surface area along with the abundant oxygen-containing surface functionalities [177]. In particular, even though the particle-laccase conjugates exhibited a low reaction rate during the kinetic studies, the overall removal efficiency was least or not affected. Also, it was noticed that the comprehensive conversion efficiencies in the case of BPA (excluding adsorption by matrix) were found to be 97.7% and 98.9% in GO- and MWNT-immobilized systems, respectively. The catechol also underwent % enzymatic conversion in the case of the same two systems. Overall, it was revealed that the immobilization of laccase enzyme on the carbon nanomaterials remained active for longer periods, and also the reduced reaction rates did not usher to the declined efficiency under sufficient period [177].

Laccase immobilized CNTs have been utilized for improving the dye removal from the wastewater samples [178]. The laccase-CNTs nanocomposites expressed better pollutant removal efficacy and also exhibited excellent catalytic degradation of the pollutants as compared to the conventional counterpart when tested under various operational conditions such as CNT concentration, laccase ratio, pollutant concentration, temperature, and Reynolds number. The addition of the laccase enzyme was highly influential in dye removal ability as the laccase enzyme assisted in the prevention of agglomeration by broadening the extent between the CNTs. Furthermore, with the addition of laccases, the space between CNTs widened and the agglomeration of CNTs shrank, which stimulates more effective adsorption sites. In the end, the

removal rate exceeded 90%, after the 100 min of adsorption operation. Also, the removal rate change with temperature was higher in the case of CNTs when compared to laccase-CNTs which is attributed to the limited growth space and better temperature stability. The uniform distribution of dye in the CNTs resulted led to the amelioration in mass transfer efficiency of pollutants leading to both CNTs and Laccase-CNTs achieving an enhanced excellent dye removal ability (above 93%). The synergism of adsorption and catalytic biochemical degradation led to Laccase-CNTs having a higher particle diffusion as compared to CNTs [178]. Also, the adsorption thermodynamics studies revealed that the adsorption capacity and rate were enhanced due to the efficient enzyme immobilization. The characterization studies including the SEM scanning, BET-specific surface area, and FTIR spectroscopy revealed that the CNTs possesses tubular porous structure having large specific surface area combined with pore capacity thereby providing more active adsorption sites for trapping of the micro-pollutants and exhibited high dye removal efficiency due to “highly efficient regeneration” effect [178].

Another major type of detrimental issue regarding the environment is related to the burning of sulphur-containing compounds in crude oil which ultimately leads to the production of sulphur dioxide [179]. An example of a sulphur-containing compound causing major environmental pollution is dibenzothiophene (DBT) and its derivative which is difficult to remove and therefore scientists have turned attention to its removal which in general is called desulfurization [180]. One of the convenient routes for the removal of DBT includes the utilization of certain oxidases and peroxidases that can be employed to convert DBT to dibenzothiophene sulfoxide (DBTO) and dibenzothiophene sulfone (DBTO₂) in mild conditions. Later, the generated DBTO and DBTO₂ can be separated by extraction or employing microbial transformation [181]. For this, bioaffinity-based enzyme immobilization is considered one of the best strategies as it ensures oriented immobilization of the enzyme on support and displays lower or reduced negative effects on the enzymes [138]. The enzyme loading studies showed that the reduced changes in the conformation of the Con A on SWNTs-DM, lead to enhanced affinity capture, (2) the generation of SWNT-DM-Con A complexes was capable of reducing the steric hindrance in case of the immobilized enzymes, thereby increasing the capture ability, (3) moreover, the further diffusion of SWNTs by DM, resulted in enhanced surface area for Con A binding and thereby leading to the higher number of enzymes being captured [182]. The effectiveness of an immobilization strategy indicated by the specific activity showed interesting values as the specific activity of Lac using the self-assembly immobilization strategy was found to be five times higher when compared with the Lac acquired utilizing the direct adsorption strategy. These variations could be due to the alterations in the conformations caused by the glycoproteins on SWNTs in the case of the two different immobilization strategies. Also, the thermo stability studies depicted the fact that in the case of the WNT-DM-Con A support, the thermo stability of both the immobilized enzymes showed remarkable enhancement when compared to that of the soluble enzymes. This circumstance was attributed to the specific affinity between the Con A on the support and the glycosyl present on the surface of the n enzymes that have the ability to stabilize the 3-Dimensional structure of the enzymes [182].

5.3. Biosensors

Biosensors have become an indispensable part of present-day healthcare due to the demand for personalized medicine and cheaper diagnostic tools [24]. Biosensors can detect the relevant molecules of our interest and are highly sensitive and selective. They are also immensely utilized in the field of food safety and quality control for detecting the toxic components present in the food compounds [183]. The fact that biosensors can solve various challenges present within the healthcare system enhances their requirement and development with state-of-the-art technology [184,185]. The most probable materials that

can be utilized for the fabrication and development of biosensors having both high sensitivity and selectivity are nanoparticles, CNTs, nanowires, quantum dots, and graphene quantum dots. Among the various types of nanomaterials, CNTs have been implemented into biosensor devices due to their unique optical and electrical properties. Also, they can be further modified chemically to increase their selectivity towards relevant molecules [186]. When enzymes are immobilized on them, the CNTs can detect biomolecules of our interest as enzymes and are highly recognized for their selectivity and sensitivity. A biosensor generally consists of three components: a biological recognition element, a transducer, and a signal processing unit [186]. In case of an enzyme-based biosensor, enzymes serve as recognition element whereas matrix (CNT) act as a transducer. The principle behind biosensing is the detection of the presence of a specific analyte by monitoring certain variations happening during enzymatic reactions such as a change in proton concentration, release or uptake of certain gases, light and heat emission, absorption, or reflectance [186]. Biosensors can be classified into four types based on the changes that need to be monitored during sensing: optical, electrochemical, thermistors, and piezoelectric biosensors [186].

Optical biosensors monitor changes such as absorbance, reflectance, fluorescence, luminescence, and refractive index of the surface during the reaction [186]. One of the most earliest and essential clinical applications of optical biosensors is in the detection of glucose present in both blood and urine. The first biosensor for glucose sensing was commercialized in 1957 after which much researches have been carried out in the field of glucose sensing [187]. Detection of glucose is of great significance in clinical biology for the treatment of diabetes [188]. An instrument that can continuously indicate glucose concentration in blood has been effective in regulating human health and monitoring the function of various organs [189]. Due to the usefulness of glucose detection, numerous researches have been carried out for the development of an appropriate biosensor for glucose sensing. Optical biosensors for detecting glucose are an indirect method involving two reactions that are catalyzed by two enzymes [131]. The test strip for glucose detection has immobilized glucose oxidase and peroxidase enzymes. The glucose oxidase enzyme catalyzes the oxidation of glucose forming gluconic acid and H₂O₂. The H₂O₂ formed in this reaction reacts with orthotolidine in presence of peroxidase to give a deep blue-coloured product whose concentration can be monitored thereby monitoring the concentration of glucose in urine [190]. Fig. 10 shows that glucose oxidase (GOx) was immobilized on ten different glassy carbon electrodes out of which a maximum amount of enzyme immobilization of 2.9 nM/cm² was observed on pristine multiwalled carbon nanotubes (PMWCNT) with high stability in a study [191]. The nanotube enzyme framework showed a standard potential of −444 mV and possess perfect redox peaks with peak separation (ΔE_p) of 22 mV. The PMWCNT/GOx displayed a lower limit of detection (45 μ M) and the Michaelis-Menten constant was found to be 2.24 mM. It was revealed that the sensitivity of the biosensor remained unaffected in the presence of other electroactive groups and that the PMWCNT is proved to be an excellent matrix as it reduces electron tunnelling distance which promotes direct electron transfer between enzyme and electrode. The study conducted showcases the applicability of the pristine MWCNT as an immobilization matrix for various biosensing applications due to its easy handling nature and electrode preparation steps [191].

The electrochemical biosensor measures the change in current signal, potential, and conductance during the chemical reactions in which there is a change in the ionic concentration of the solution. The electrochemical biosensor has been highly utilized for the detection of certain biomolecules such as glucose and hydrogen peroxide [192,193]. Electrochemical biosensor offers both direct and indirect methods of measurements. Indirect measurement is done by measuring the enzymatic product of the biomolecule electrochemically since the biomolecule itself is electrochemically inactive [194]. For example, glucose is not electrochemically active but its products formed after enzymatic

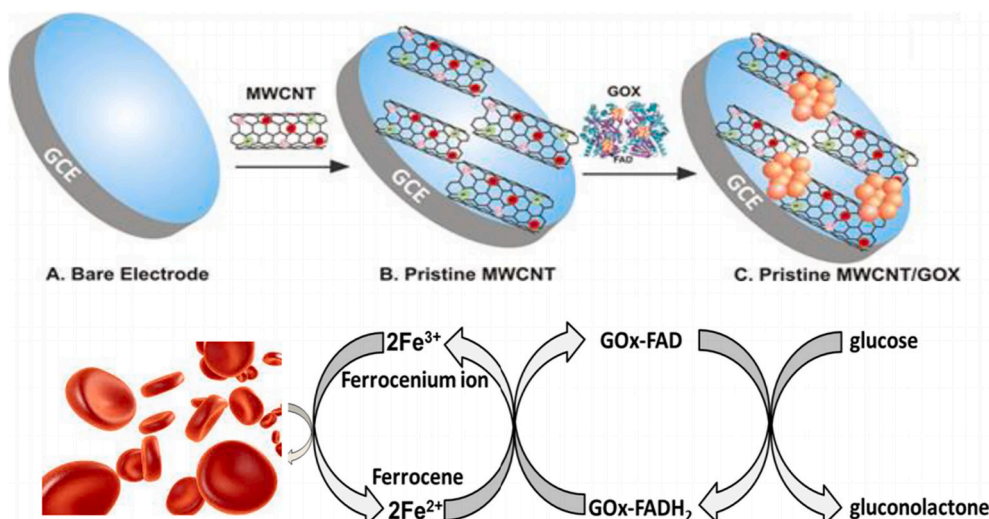


Fig. 10. (A) Bare GCE, (B) PMWCNT Modified GCE, (C) GOx Entrapped PMWCNT/GCE Preparation and Its Bioelectrocatalysis Pathway for Glucose Analysis. Adapted with permission from ref. [191]. Copyright 2019 American Chemical Society.

action (H₂O₂ and NADH) can be conveniently measured [195,196].

In an early study, individual semiconducting SWCNT was used as a glucose biosensor which consisted of glucose oxidase being attached to the SWCNT in a controlled manner. Upon the addition of glucose to the support, a significant increase in conductance was observed. The increase in pH is a result of GOx activity and its conformational change during the conversion of glucose to gluconolactone. It was found that after immobilization, the GOx coated nanotubes are sensitive to glucose and provide real-time monitoring of glucose in the solution [197,198].

Cholesterol biosensors have been developed for the indirect measurement of cholesterol using cholesterol oxidase, to carry our redox reactions. The enzyme was immobilized onto a gold-MWNT electrode which was wrapped with a cross-linked chitosan-room temperature ionic liquid matrix [199]. For analyzing galactose levels in the blood sample, a galactose biosensor was developed offering high reliability with a relative standard deviation below 2% and recovery index between 101.2 and 102.7% [200]. Youngho Wee et al. and I.M. Apetrei et al. obtained the desired biosensor for the detection of phenolic compounds by dispersing CNTs in an aqueous tyrosinase solution [201]. Phenolic compounds are found abundantly in many natural products like fruits and vegetables. These compounds increase the shelf life and quality of fruits and vegetables but they are also known for their toxicity. Quantitative evaluation of phenolic compounds is vital in studying natural product quality, plant-based pharmaceutical formations, and environment monitoring [129]. Shuna Liu et al. developed a biosensor by immobilizing alcohol dehydrogenase on SWNT for ethanol sensing. Ethanol sensors are utilized in various applications like monitoring the chemical reaction, breathe analysis, quality control, and biomedical productions [202]. The biosensor formed had a high electrocatalytic activity towards ethanol oxidation with high stability, reproducibility, and higher biological affinity [203]. The electrocatalytic activity of the SWCNT modified electrode is higher as the electrochemical NADH oxidation is catalyzed by lowering the over potential significantly. This is because SWCNT has a larger surface area for accomplishing enzymatic reactions than the bare electrode [203]. The direct measurements of analytes are accomplished through direct electron transfer of electrons from redox centers (enzymes) to electrode surface through a conducting material [183]. Determination of glucose is also accomplished through direct electron transfer using an electrochemical sensor. The enzyme is connected to the electrode surface through CNTs. CNTs offer a good connection between enzymes and electrodes and an efficient electron transfer linking the electrode and enzyme is observed as shown in Fig. 11 [183]. The electrodes can donate or accept electrons from the enzymes

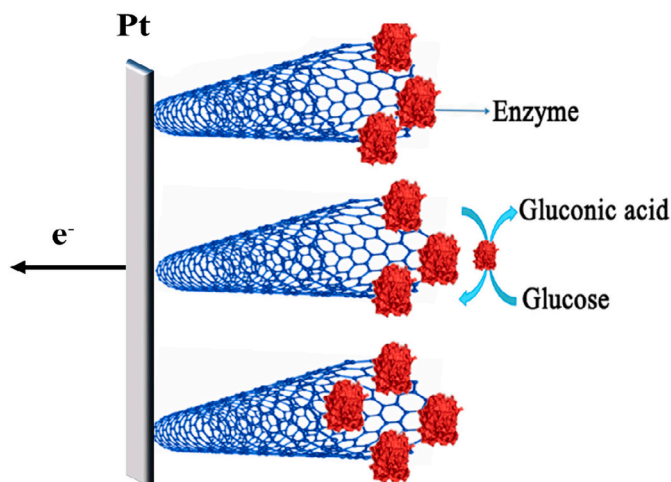


Fig. 11. Schematic representation of glucose biosensor showing the direct transfer of electrons.

where redox reactions take place.

CNTs offer high electronic conductivity and stability in all mediums. If glucose is present in the sample, the biosensor triggers bio-electrocatalytic reactions, and a sharp peak in voltammograms is observed [204]. The cathodic current increases due to the enzymatic reactions accompanying a decrease in anodic current. This method provides direct quantification of glucose in the sample. Electrochemical biosensors give accuracy, precision, speed, simplicity, and economic tool for the detection of biomolecules [204]. In an analogous study, nanocomposites with MWCNT embedded in metal-organic coordination polymers (MOCs) were prepared to serve as a matrix for GOx immobilization. Formation of MWCNT-MOCs one-pot entrapped GOx exhibited enhanced mass transfer over MOCs. The result concludes that MWCNT-MOC modified electrodes present superior enzymatic catalysis performance of greatly enhanced sensitivity (136 $\mu\text{A cm}^{-2} \text{mM}^{-1}$) and a lower detection limit. The reason attributed to the high sensitivity of nanocomposites is the ultra-high entrapment ratio of enzymes (nearly 100%) resulting in good mass transfer efficiency. Thus, MWCNT serves as an efficient matrix for enzyme immobilization and function as an electrochemical biosensor with high performance [205].

Likewise, in Fig. 12 an electrode was developed by immobilizing

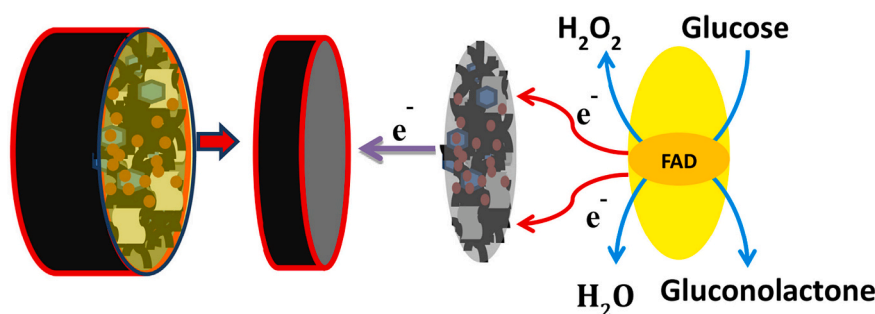


Fig. 12. Mechanism for glucose detection by GOx/AuNP/PANI/rGO/NH₂-MWCNTs biosensor. Adapted with permission from ref. [206]. Copyright 2019 Elsevier.

Glucose oxidase on MWCNT/polyaniline/reduced graphene oxide/gold nanoparticle giving rise to a highly sensitive glucose sensor [206]. The resultant nanomaterial showed good reproducibility (90.2% $n = 7$), low detection limit (64 μM), less K_{mapp} i.e., Michaelis–Menten constant (0.734), and good sensitivity (246 $\mu\text{A cm}^{-2} \text{ mM}^{-1}$). The glucose biosensor was used to detect glucose concentration levels in blood serum via amperometric technique. The GOx catalyzed reaction generates electrons which are easily transferred between analyte and electrode thus leading to the high sensitivity of the sensor. The novel third-generation glucose biosensor was found to be economic, offered high portability and flexibility along with added features such as high sensitivity and excellent reproducibility [206]. Similarly, a glucose biosensor based on chitosan-GOx was immobilized on polypyrrole (PPy)-Nafion (Nf) functionalized MWCNT for developing a high-performance glucose biosensor [207]. The resultant nanohybrid composite imparts a large surface area for GOx immobilization resulting in high enzyme loading, therefore, improving the sensitivity. The biosensor developed showcased a large linear range from 0.01 mM to 4.7 mM with a high correlation coefficient ($R^2 = 0.9992$), had the highest sensitivity of 2860.3 $\mu\text{A mM}^{-1} \text{ cm}^{-2}$, and a low detection limit of 5 μM . These results show that the developed biosensor had higher sensitivity and a wide linear range when compared to the previously reported PPy modified GCE-based glucose biosensors [207]. Moreover, the bio-nanohybrid composite (CHGOx/PPy-Nf-fMWCNTs) delivers a biocompatible environment, which is responsible for increasing the electrocatalytic activity of the immobilized enzyme and assists electron transfer rate (Fig. 13). Also, the cross-linking between the CH and GOx restricts the leaching of GOx and sustains bioactivity. The additional properties such as high anti-interference ability, longevity combined with reusability and storage life along with acceptable glucose detection in the real serum samples [207].

Fig. 14 shows that electrochemical biosensors have gone through many evolutionary changes over years. First-generation biosensors are established on the electronegativity of the receptor substrate or the product. Second-generation sensors depend on artificial redox mediators. Third-generation sensors are based on the direct electron transfer between redox-active enzymes and electrodes [208].

5.4. Biofuel cell

With the increase in utility of energy, there is a demand for building a promising tool for energy production [209]. The classical methods include the combustion of fossil and nuclear power plants but since they are non-renewable, they cannot match the great demand for energy production [210]. Biofuel cells are a sustainable and encouraging alternative to attain high energy production [211]. Biofuel cells directly produce energy from biologically renewable catalysts and fuels. Biofuel cells include both enzymatic and microbial fuel cells based on the nature of the catalyst and electron transfer process [212]. Enzyme-based biofuel cells are based on redox reactions that are catalyzed by enzymes. The redox reaction involves the oxidation of fuel at the anode and the reduction of oxidants at the cathode. Fuels for oxidation can be sugars, alcohols, or hydrogen and common oxidants are O₂ and H₂O₂. As a result of these reactions, chemical energy is directly converted to electrical energy [213]. An enzymatic fuel cell consists of three components: electrodes, enzyme, and conducting material placed between electrode and immobilized enzyme as illustrated in Fig. 15. In electrochemical systems, slow electron transport and poor mass transport of substrate are major rate-limiting steps. Thus, the two major requirements for developing high-performance biofuel cells are; (1) Establishing a long-lasting and efficient electric communication between enzymes and electrodes, (2) Efficient supply of fuel and oxidant to the enzymes [213]. In this

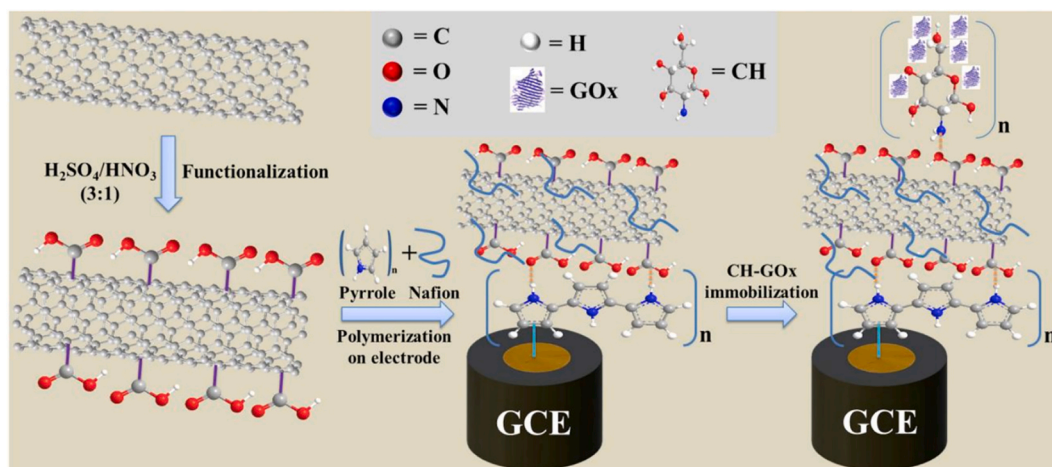


Fig. 13. Schematic of glucose biosensor fabrication strategy showing MWCNTs functionalization with carboxylic groups, electrochemical polymerization on GCE after adding fMWCNTs, pyrrole monomers, and Nf, and CH-GOx immobilization, respectively. Adapted with permission from ref. [207]. Copyright 2016 Elsevier.

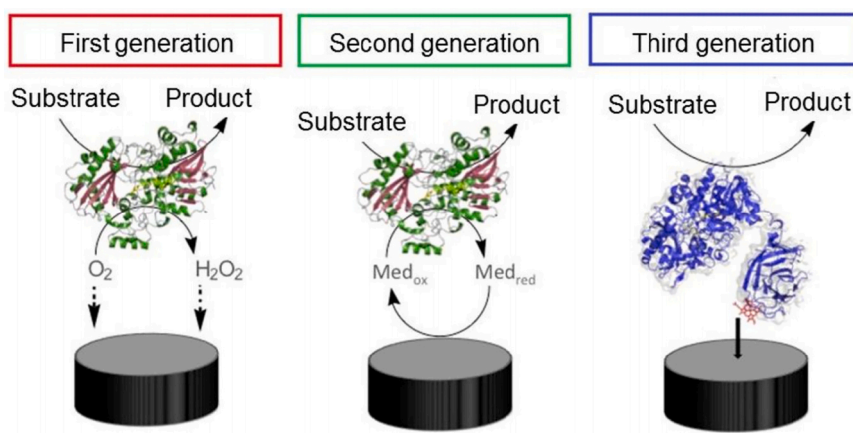


Fig. 14. Schematic illustration of first-, second-, and third-generation amperometric enzymatic biosensors. Adapted with permission from ref. [208] Copyright 2017 MDPI.

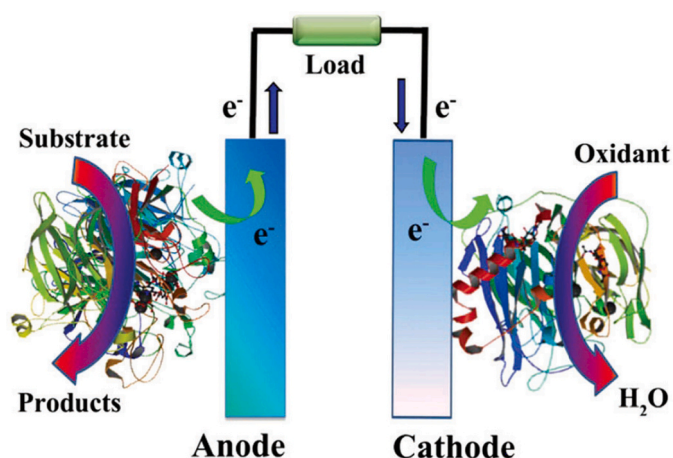


Fig. 15. Schematic representation of a membrane-less EBFC. Adapted with permission from [212]. Copyright 2016 The Royal Society of Chemistry.

context, nanomaterials with high conductivity provide a breakthrough in this field [214,215].

Due to conducting nature of CNTs, placing them in the proximity of active sites of enzymes facilitates mediate electron transfer (MET) and direct electron transfer (DET) between enzymes and electrodes [216]. Apart from having a large surface area for enzyme immobilization and transferring electrons to the electrode, CNTs possess a porous structure

allowing the mass transfer of reactants and products in a biological system. CNTs due to this reason, have been widely utilized for modifying various kinds of electrodes in enzyme-based biofuel cells [217]. Also, the fact that there is an abundance of enzymes that are efficient in oxidizing, various molecules at the anode provides ample opportunity for generating different fuels for the biofuel cell systems. Glucose is one of the majorly utilized fuels in implantable devices, however, there are biofuel cells that also utilize ethanol, and other sugars as fuels [218]. A glucose-based biofuel cell has a glucose dehydrogenase-based anode that oxidizes glucose and a laccase-based cathode that reduces oxygen present in the air as shown in Fig. 16 [213].

Also, the presence of chemically inert, hydrophobic, and dense p-p stacking graphene sidewalls in CNTs makes them an ideal candidate as a support material for the redox mediators. Therefore, they are generally utilized for shuttling the electron transfer processes in biocatalysts [219,220]. The enzyme-based fuel cells were first introduced in 1964 by Yahiro and his team-leading to the number of advances in this field [221]. Yiming yan et al. developed SWNT based glucose/ O_2 biofuel cell using glucose dehydrogenase as anode biocatalyst. The system made use of NAD^+ acting as the cofactor and laccase derived from *Trametes versicolor* as the cathode biocatalyst for O_2 reduction. It was found out that the use of SWNTs was assisting in providing a facile and productive route to enzymatic BFCs with a high voltage. Also, the glucose/ O_2 fuel cell formed offered high voltage and an effective means of generating high-performance energy sources [222]. The direct electron transfer between the laccase and the SWNTs was observed to be an important criterion for the development of the biocathode because of the

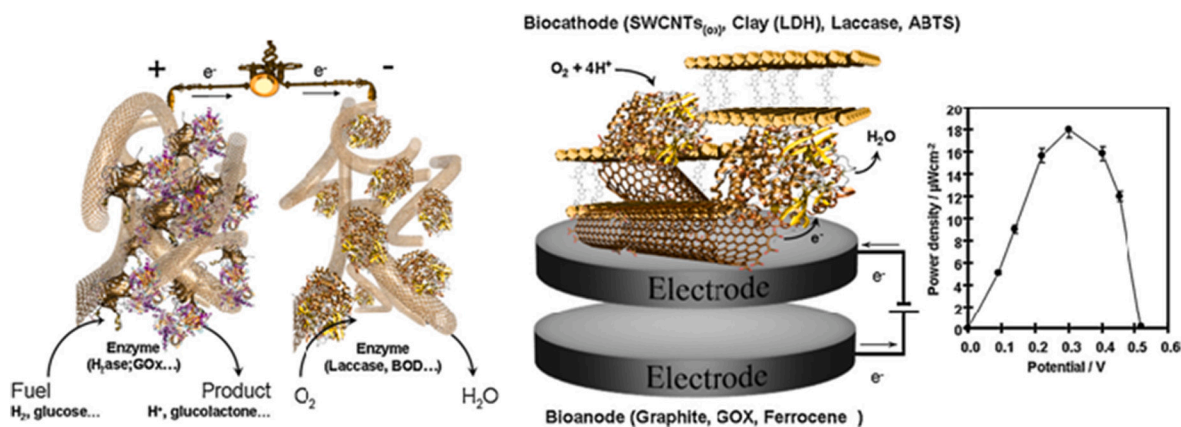


Fig. 16. Schematic representation of SWCNT-LDH-ABTS laccase biocathode connected to a graphite-GOx-mediator bioanode to form a biofuel cell and enzymatic fuel cell based on CNTs. Adapted with permission from ref. [213]. Copyright 2012 Elsevier.

involvement of low overpotential during O_2 reduction. The studies done confirmed that the O_2 reduction at the laccase-SWNT biocathode in the phosphate solution (pH 6.0) was observed at 0.60 V versus Ag/AgCl (0.80 V vs. NHE), a value much closer to the equilibrium value of $E'O_2/H_2O$ (0.87 V vs. NHE), thereby suggesting the requirement a low overpotential for O_2 reduction at the prepared laccase-SWNT biocathode for a productive and facile electron transport [223,224].

Similarly, glucose can be paired with bilirubin oxidase to be used as the cathodic enzyme. This combination gave rise to the highest performing biofuel cell with the power density of $740 \mu W cm^{-2}$ at 0.57 V which is higher than other biofuel cells previously developed with glucose. High power density is a result of mass transfer of product and reactants between electrode and enzyme, the substantial sum of enzymes wired to electrodes, and fast enzyme turnover rate [225]. The cyclic voltammetry studies confirmed that the voltammetry peaks at a rate of $20 mVs^{-1}$ were much narrower and closer to each other in the case of the CNF as compared to the modified CF electrode. Also, the voltage difference of the peaks is $\Delta EP = 36 mV$, compared with $\Delta EP = 55 mV$ for the CF along with the decrease in the overpotential for oxygen reduction resulted in the rapid charge transport through the catalytic film made of CNTs. Also, a fivefold and a tenfold increase in the oxidation and reduction current peaks and the performance respectively in the case of the CNF was observed, thereby proposing that not all redox centers are connected to the electrode surface of CFs. Also, an overall stabilization and much efficient access to a higher fraction of redox polymer centers were observed which was attributed to the ameliorated porosity contributed by CNFs [225].

Another commonly used fuel cell as shown in Fig. 17, H_2/O_2 fuel cell having hydrogenase enzymes at anode and bilirubin oxidases at the cathode for O_2 reduction [226]. Hydrogen is a good candidate for fuel for clear transportation in polluted urban areas since its combustion generates only water and the enzyme needed for its oxidation is easily available [142]. Hydrogenase has an excellent turnover frequency up to $21,000 s^{-1}$ for individual enzymes and is present in many microorganisms for H_2 transformation. H_2/O_2 fuel cells were discovered later than glucose fuel cells and their advancement has remained challenging as

hydrogenase is highly sensitive to oxygen [227]. The CNT functionalization using bio-inspired molecular complexes is a facile technique for the preparation of robust, CO-tolerant, noble-metal-free, and bidirectional electrocatalytic nanomaterials for H_2 evolution and uptake and is highly compatible with the conditions encountered in classical proton-exchange membrane devices [226]. These materials can be synthesized in a single or double step from CNTs and functionalized complexes and later employed for electrode deposition. The cyclic voltammetry studies of an SWCNT-hydrogenase gel showed an $18 mA/cm^2$ hydrogen oxidation current at 0.25 V vs SCE. On comparison of oxidation currents for a SWCNT-hydrogenase gel with control gels, as expected, the H_2 oxidation was obtained from hydrogenase-containing gels only. A strong increase in H_2 oxidation current was obtainable in the presence of SWCNT thereby showing the relevance of CNT aqueous gels being used as an ideal candidate for support in the case of hydrogenases in a fuel cell anode [226].

Alcohol also serves as fuel in enzyme-based biosensors and contributes to green energy production. Ethanol/ O_2 fuel cell was developed by Sidney Aquino Neto et al. having alcohol dehydrogenase at anode and cathode was prepared with bilirubin oxidase. The electrode system made use of MWCNTs-decorated gold metallic nanoparticles to provide a large surface area to the hybrid electrode [228]. Bioelectrodes using MWCNTs showed better performance than the one using other metallic nanoparticles in the earlier studies [229]. The mode of the electron transfer system affects the enzyme operation thus varying the current density of biofuel cells. MWCNT facilitates both MET and DET in the bioanode system giving different power values using the same enzyme. DET based bioanode lacks high current density, having maximum power values of $22 \pm 4 mW cm^{-2}$ and a maximum power density of $226 \pm 21 mW cm^{-2}$ through MET [114]. Despite methanol being a good fuel, ethanol is used extensively for making alcohol-based fuel cells as methanol is hazardous for human health [230].

Enzymatic fuel cells possess remarkable advantages over conventional fuel cells due to their renewability, low cost, biodegradability, specificity towards the substrate, and low overvoltage. There exists a drawback of enzyme-based fuel cells due to the electrical insulation of

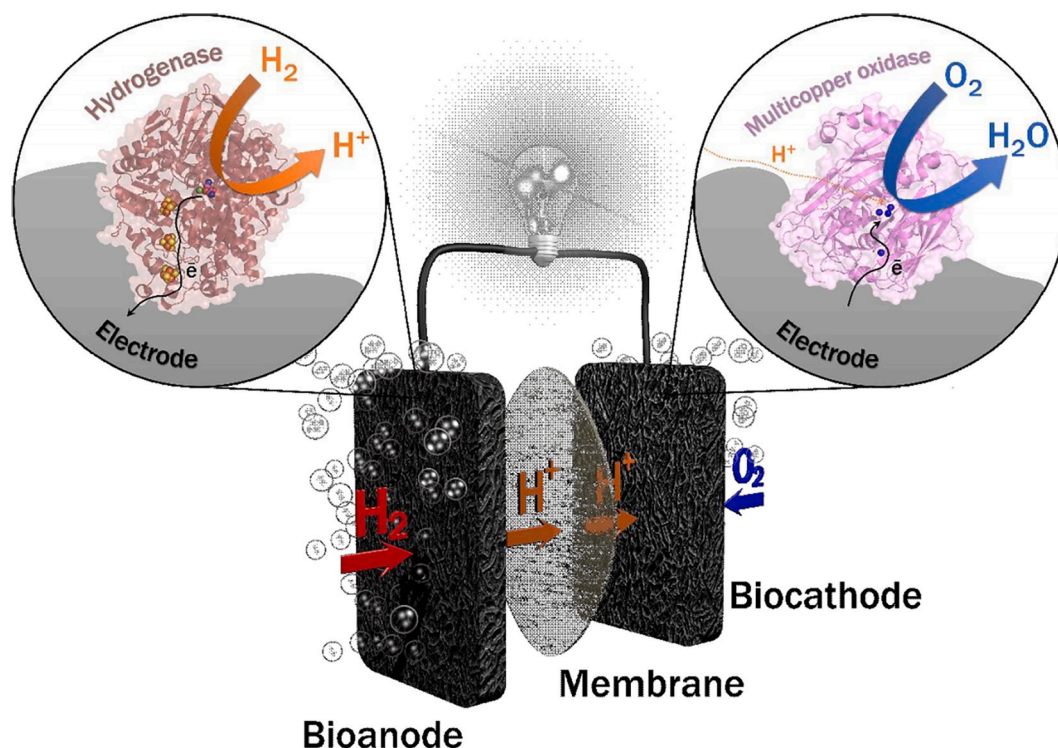


Fig. 17. Schematic representation of H_2/O_2 fuel cells. Adapted with permission from ref. [226]. Copyright 2017 The Royal Society of Chemistry.

enzyme active sites by their neighboring protein. This hinders the application of enzyme immobilized fuel cells. This can be overcome by following appropriate immobilization techniques and understanding catalytic enzymes [212].

5.5. Food and dairy industry

Milk and dairy products are a vital part of the human diet. The lactose present in dairy products plays a crucial role in development and growth during the early stages of life. It helps in the adsorption of basic elements like calcium, magnesium, phosphorus and helps in the growth of acidic microflora inside the intestinal tract [231]. However, the excess consumption of lactose can lead to lactose intolerance as the β -galactosidase present in the gut is incapable of hydrolyzing such a great amount of lactose. β -galactosidase or lactases is commonly known for hydrolyzing lactose into glucose and galactose [232]. The undigested glucose present in the intestine leads to symptoms of lactose intolerance like vomiting, abdominal pain, loose stool, nausea, etc. Lactose intolerant people are advised to reduce lactose intake or consume lactose-free dairy products. However, the deficiency of lactose can lead to health issues like galactosemia, cataract, and osteoporosis [233]. The glucose and galactose formed as a result of hydrolysis increase the sweetness and flavor of dairy products. β -galactosidase is also utilized for the production of galacto-oligosaccharides (GOS) that have great pharmaceutical properties as it helps in maintaining the gut environment by restoring the growth of beneficial bacteria of the intestine and improves the immune system [234]. Therefore β -galactosidase is a crucial enzyme in the dairy and food industry to produce a lactose-free product and for enhancing the taste of food products. Other than milk, cheese also contains a large amount of lactose. During the production of cheese, a massive amount of whey is generated and it is discarded into water bodies which increases its COD and BOD levels. In this aspect, β -galactosidase is used as a recycling agent which converts whey into ethanol and other products. Hence β -galactosidase is also useful in the

cheese industry for the eco-friendly production of cheese [235]. In a study, an eco-friendly hydrothermal process was adopted to synthesize two nanocomposites (NCs) i.e., MWCNT-WS₂ and MWCNT-SnO₂. β -galactosidase was extracted from *Lens culinaris* and was immobilized on the nanocomposites using a glutaraldehyde crosslinker (Fig. 18) with an immobilization efficiency of 91.5% and 88% on WS₂ and SnO₂ respectively [236]. The NCs formed showed a remarkable increase in the rate of hydrolysis of whey lactose. MWCNT-WS₂ hydrolyses 97% whey lactose in 1.5 h and MWCNT-SnO₂ hydrolyses 92% whey lactose in 2 h at optimal conditions. From the above observations, it can be found that MWCNT-WS₂ showed better reusability and catalytic properties compared to MWCNT-SnO₂ due to its hetero functional nature. CNTs in the above system facilitate the diffusion of the substrate to the enzyme and release of product [236]. Similarly, β -galactosidase was immobilized on functionalized 3D MWCNT-MoS₂ with an immobilization efficiency of 93% using a hydrothermal route [126]. The matrix was activated using glutaraldehyde for the cross-linking of the enzyme and the efficiency of lactose hydrolysis showed potential recyclability of 50% after 21 cycles. The nanobiocatalysts offered wider pH and temperature operational range when compared to native form as a result of immobilization. The results showed an excellent increase in storage stability and thermal stability as it could retain 92% of residual activity after eight months [237].

6. Future outlook and trends

The CNTs as an exceptional material are found to be having an enormous ability to be utilized in combination with enzymes towards advancement in future intelligent and smart biosensors having unprecedented applicability in various sectors including biology, biochemistry, catalyst, and biophysics. Enzyme immobilized CNTs can be utilized for checking the anti-microbial activity and anti-adhesive properties thereby making it competitive and effective when compared with the conventional anti-microbial methods. They can also have applicability

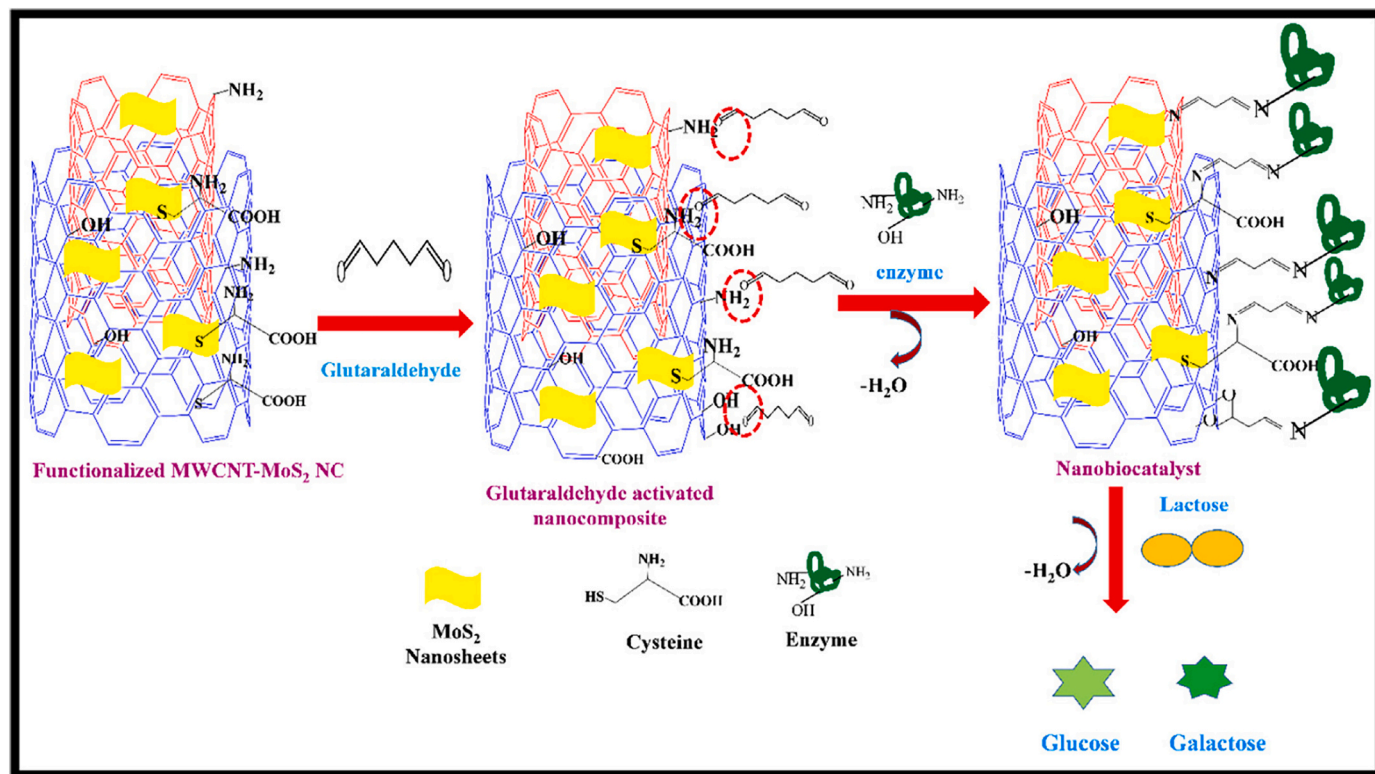


Fig. 18. Scheme of immobilization of *lens culinaris* on functional MWCNT-MoS₂ NCs producing nanobiocatalyst for lactose based dairy industries. Adapted with permission from ref. [237]. Copyright 2019 Elsevier.

towards the development of optoelectronic materials. Also, they can act as a suitable candidate for designing transparent, resilient, stretchable electronic materials. Another possible application of enzyme immobilized CNTs can be towards the development of microbial dihydrogen production and abiotic dihydrogen storage thereby leading to an environmentally benign and green hydrogen economy. Also, for overcoming the limitations associated with the utilization of noble metal catalysts such as platinum, nanohybrids can be employed for making the method much more effective and economic. Enzyme immobilized CNTs can be also used by synthetic organic chemists for the development of novel protocols in the synthesis of industrial and biologically important scaffolds. They can be used in oxidative/reductive coupling, electrophilic/nucleophilic addition, radical-based, and migration/rearrangement reactions. In addition, these strategies can be employed for procuring important precursors for the construction of complex molecules. Furthermore, advanced studies can be conducted towards the development of a highly stable and efficient enzyme immobilized CNT system by deciphering valuable information associated with protein sequences, 3D structures, reaction mechanisms along with the physical and chemical properties of CNTs.

Table 2 summarises the various types of enzymes that have been immobilized on CNTs using various immobilization techniques and their application in chemical and biotechnological fields. From Table 2, it can be seen observed that MWNTs are being considered as the ideal immobilization matrix when compared with the SWNTs. The reason being that MWNTs can be produced with high purity without a catalyst, and their bulk synthesis is easily achievable. Also, it can be noticed that most of the synthesized biocatalysts are being employed in biocatalysis followed by their utilization in biosensors, and biofuel cells. Moreover, both the physical and chemical immobilization techniques are being preferred by the researchers as they have their advantages and limitations.

7. Conclusion

The immobilization of enzymes on solid support increases its utility for commercial purposes by overcoming all the limitations of soluble enzymes. However, the immobilization of enzymes on support materials faces certain challenges such as finding a suitable matrix for

immobilization and appropriate technique. From the wide range of research conducted for discovering the ideal candidate as the matrix material, CNTs have emerged as the favorite materials due to their advantages over other matrices used. CNTs can be functionalized with different chemical groups to elevate their affinity for enzymes as the functional group provides a site for efficient binding. This review discusses and summarises the various techniques used for the immobilization of enzymes on CNTs. These techniques are categorized into physical and chemical techniques. From our understanding, we can conclude that none of the technique can be termed a universal technique as each one of them have their assets and limitations. Depending on the purpose of using an immobilized enzyme, the appropriate technique can be adopted. Physical methods are preferably used by industries due to the simplicity of the process and also due to their high yield of immobilization. However, physically immobilized enzymes undergo enzyme leaching over various cycles of use and therefore may lead to their underutilization. The chemical method gives a stable enzyme matrix but there is a modification in the structure of the enzyme observed and the process of immobilization is complex which limits the usage of this method to a certain extent. This gives scope for more studies to be done to formulate methods of immobilization that reduces enzyme leaching without altering enzyme structures and activity.

The CNTs are found to be having an enormous ability to be utilized as an exceptional material towards the evolution of high-efficiency enzymatic biofuel cells. Their porous, hollow along with the thin diameters assists them by making the contact area much accessible to the active sites of the enzymes, thereby performing a chief part for MET and enables in certain cases DET. The conductive nature of the CNTs enables a facile and steady flow of the electrons that can easily within the 3-D matrix paving way for ameliorated efficiency of the bio-converted energy. The CNTs can be highly beneficial in the biomedical field as they can be utilized in implantable and as pacemakers and sensors. The next hurdle to take control is improving the lifetime in order to be highly competitive with presently used sealed batteries. The CNT-enzyme conjugates or the nanohybrids can be used as an encouraging platform for the advancement of next-generation molecular transporters, tools for intracellular delivery, and drug supports for targeted delivery. Also, the ability of CNTs to assist indirect shuttling of electrons through the enzyme active sites makes the CNT-enzyme-based electrode systems

Table 2
CNTs as a support matrix for enzyme immobilization and their applications.

Enzyme	Immobilization method	Immobilization matrix	Application	Reference
Glucose oxidase	Entrapment	MWCNTs-MOCs	Glucose biosensor	[205]
Alcohol dehydrogenase	Electrostatic interaction	SWNT	Ethanol biosensor	[203]
<i>Cerrena unicolor</i> laccase	Adsorption	CNTs	Biofuel cell, biosensor	[238]
Horse radish peroxidase	Adsorption	MWNT	Biofuel cell, biosensor, H ₂ O ₂ detector	[239,240]
β -Glucosidase	Adsorption	MWNT	Biocatalysis	[241]
β -galactosidase	Covalent bonding	MWNTs	Hydrolysis of lactose	[236]
<i>Candida rugosa</i> lipase	Covalent bonding	MWNT	Biocatalysis	[242,73,121]
Lipase	Electrostatic interaction	MWNT	Biocatalysis	[243]
NADH oxidase	Covalent bonding	MWNT	Biocatalysis	[244]
Cellulase	Physical adsorption	MWNT	Biofuel cell, food industry	[67,245]
lipase	Physical adsorption	MWNT	(RS)-1 resolution.	[79,97,26,246,247]
Peroxidase	Covalent bonding	MWNT	Biocatalyst for oxidation	[248]
Lysozyme	Adsorption	MWNT	Biocatalysis of large substrate	[249]
<i>Myceliophthora thermophila</i> laccase	Covalent bonding	MWNT	Paper, dye, food processing industry	[70]
Laccase	Cross-linking	MWNT	Degradation of molecules	[250]
Laccase	Encapsulation	MWCNTs-LCEfMs	Bisphenol A removal	[114]
Amylo glucosidase	Adsorption	MWNT	Biosensor	[251]
<i>Candida Antarctica</i> Lipase	Adsorption	MWNT	Biocatalysis	[21]
Tannase	Cross-linking	MWNT	Nanobiocatalyst	[28]
Lignin peroxidase	Covalent bonding	MWNT	Dye decolorization	[252]
Recombinant dioxygenase	Covalent bonding	SWNT	Degradation of hydrocarbon intermediates	[253]
Hydrogenase	Covalent bonding	MWCNT-Pes	Biocatalysis	[254]
D-amino acid oxidase	Covalent bonding	MWNT	Synthesis of pharmaceutical compounds	[255]
D-lactate dehydrogenase	Adsorption	SWNT	Lactate biosensor	[256]
Acetylcholine esterase	Covalent bonding	MWNT	Sensing particular pesticides	[257]
Glucose dehydrogenase	Covalent bonding	MWNT-PMG	Biofuel cell	[258]

multifaceted and enhances their applicability as catalysts, biosensors, and biofuel cells. One of the major challenges regarding the utilization of nanohybrids is to understand the reciprocity between the desired protein and the carbon nanostructure. Additional research studies that focus on developing a rational design for the carbon-based nanobiohybrids, considering the toxicity and the cost-effectiveness of this conjugate, are decisive for their application in the sensing and biomedical field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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