Chemistry Central Journal

ChemistryCentral

Research article

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Enhancement of lipase activity in non-aqueous media upon immobilization on multi-walled carbon nanotubes

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Published: 29 November 2007

Chemistry Central Journal 2007, **1**:30 doi:10.1186/1752-153X-1-30

Received: 4 August 2007
Accepted: 29 November 2007

This article is available from: http://journal.chemistrycentral.com/content/1/1/30

Abstract

Background: Immobilization of biologically active proteins on nanosized surfaces is a key process in bionanofabrication. Carbon nanotubes with their high surface areas, as well as useful electronic, thermal and mechanical properties, constitute important building blocks in the fabrication of novel functional materials.

Results: Lipases from *Candida rugosa* (CRL) were found to be adsorbed on the multiwalled carbon nanotubes with very high retention of their biological activity (97%). The immobilized biocatalyst showed 2.2- and 14-fold increases in the initial rates of transesterification activity in nearly anhydrous hexane and water immiscible ionic liquid [Bmim] [PF6] respectively, as compared to the lyophilized powdered enzyme. It is presumed that the interaction with the hydrophobic surface of the nanotubes resulted in conformational changes leading to the 'open lid' structure of CRL. The immobilized enzyme was found to give 64% conversion over 24 h (as opposed to 14% with free enzyme) in the formation of butylbutyrate in nearly anhydrous hexane. Similarly, with ionic liquid [Bmim] [PF6], the immobilized enzyme allowed 71% conversion as compared to 16% with the free enzyme. The immobilized lipase also showed high enantioselectivity as determined by kinetic resolution of (±) 1-phenylethanol in [Bmim] [PF6]. While free CRL gave only 5% conversion after 36 h, the immobilized enzyme resulted in 37% conversion with > 99% enantiomeric excess. TEM studies on the immobilized biocatalyst showed that the enzyme is attached to the multiwalled nanotubes.

Conclusion: Successful immobilization of enzymes on nanosized carriers could pave the way for reduced reactor volumes required for biotransformations, as well as having a use in the construction of miniaturized biosensensor devices.

Background

There has been considerable interest in the use of nanomaterials in biochemical applications [1-4], in particular the immobilization of proteins on nanomaterials [3-8], with nanotubes having lately attracted considerable attention [3,5-8]. Both single-walled carbon nanotubes (SWNTs) and multi-walled carbon nanotubes (MWNTs) have been used as matrices.

Immobilization is carried out using a variety of approaches [9,10], including adsorption [5,7] and covalent immobilization [6,8], techniques on which various

studies have been carried out. A key parameter in any immobilization is the retention of the protein's biological activity. In this respect adsorption is assumed to be superior to other approaches, which generally utilize harsher conditions [9,10]. In most cases reported hitherto [5-8], the retention of enzymic activity upon immobilization on nanotubes, has not been high, with reports describing enzymes retaining 1–20% of their biological activity [7,8]. This is similar to the early work on macroscopic matrices and it is expected that better results would be obtained once better experience/strategies with respect to nanotubes (as matrices) were in place. It is noteworthy, however, that two recent studies report retention of biological activities over the range 55–100% [11,12].

There do not, however, appear to be any reports on the evaluation of enzymes immobilized on carbon nanotubes in non-aqueous media, which, both in the contexts of biocatalysis and bioanalysis, offer great potential [13-17].

This study reports the preparation and characterization of a high activity *Candida rugosa* lipase (CRL) immobilized on multi-walled carbon nanotubes by adsorption. Numerous applications have been found for lipases in non-aqueous media [13-17], with the *Candida rugosa lipase* one of the most frequently used in biotechnology [18]. Yet there is another reason for which a lipase was chosen for this study. Most lipases have a 'lid', which opens up upon interaction with hydrophobic surfaces, enabling them to assume an 'active conformation'[18]. Given that the surface of nanotubes is hydrophobic [3], the choice of a lipase was considered worthwhile in developing a nanobiocatalyst with high activity.

Results and Discussion

Adsorption of lipases on MWNT

It is well known that the retention of biological activity of an enzyme upon immobilization on a surface (immobilization efficiency) is dependent upon the number of enzyme molecules loaded onto a given surface area [9]. Table 1 shows the variation in immobilization efficiency with different quantities of enzyme, to which the same amount of nanotubes was exposed, with the highest immobilization efficiency obtained being 0.53. It has been reported that in the case of some lipases, the presence of an inert protein, such as albumins, gave higher immobilization efficiencies with hydrophobic surfaces like accurel™ [19].

Table 1: Immobilization of *Candida rugosa* lipase on MWNT. Different units of CRL were immobilized on 1 mg of MWNTs. The hydrolytic activity was determined on MWNTs, supernatant and washings. All experiments were carried out in duplicate and the results within each pair differed by < 5%.

Loaded Units	Supernatant + washings	Expressed activity (B)	Theoretical activity (A)	B/A
0.74	0.04	0.30	0.70	0.42
1.48	0.24	0.65	1.24	0.52
2.9	0.29	1.39	2.60	0.53
5.8	1.63	2.04	4.17	0.48

The commercial preparation of CRL that has been used contains 13 μg protein/mg solid. Given the high surface area of nanotubes, 2.9 units (entry 3 Table 1, corresponding to immobilization efficiency of 0.53) constitute a very low protein load region. As pointed out by Bosley and Pielow [19], in such situations the enzyme attempts to maximize its contact with the surface, leading to undesirable conformational changes and hence loss of activity. This is overcome by the addition of other proteins that block the 'high affinity' sites on the support, or simply reduce the surface area available to the enzyme. Table 2 shows that the presence of bovine serum albumin did influence immobilization efficiency in a positive manner. With an optimum amount of BSA, an immobilization efficiency of 0.97 could be obtained. This implies that almost all the enzyme molecules immobilized on the MWNT retained their full hydrolytic activity.

Catalytic performance in non-aqueous media

While many applications have been found for immobilized lipase, such as fat splitting *etc* in the conventional aqueous medium, the last two or three decades have witnessed many interesting applications in nearly-anhydrous organic solvents [13-17]. These applications are based upon esterification/transesterification reactions, which become possible in such media owing to the absence of bulk water. Table 3 gives the initial rates of transesterification in dry hexane. The increased initial rates (in non-aqueous media) with immobilized forms of an enzyme are believed to be due to decreased mass transfer limitations. Enzyme powders in organic media are believed to form physical aggregates that diminish the substrate's access to the enzyme active site [9,13,15-17]. The immo-

Table 2: Immobilization of *Candida rugosa* lipase on MWNTs in presence of BSA. CRL (3 units) were immobilized on 1 mg of MWNTs in presence of varying amount of BSA. The hydrolytic activity was determined on MWNTs, supernatant and washings. All experiments were carried out in duplicate and the results within each pair differed by < 5%.

BSA amount	Supernatant + washings	Expressed activity (B)	Theoretical activity (A)	B/A
0	0.29	1.39	2.60	0.53
0.25	0.61	1.55	2.39	0.64
0.5	1.07	1.52	1.93	0.78
1	1.53	1.35	1.47	0.88
2	1.66	1.31	1.34	0.97
3	2.22	0.54	0.78	0.69

Table 3: Initial transesterification rates exhibited by *Candida rugosa* lipase immobilized MWNTs in different reaction media. All experiments were carried out in duplicate and the results within each pair differed by < 5%.

Lipase preparations	Reaction medium	Initial rates (μmoles min^{-1} h^{-1})	Time increase
pH tuned		0.24	1
Immobilized on MWNTs (In presence of BSA)	Hexane	0.36	1.5
Immobilized on MWNTs		0.54	2.25
pH tuned		0.12	1
Immobilized on MWNTs (In presence of BSA)	[Bmim] [PF_6]	1.08	9
Immobilized on MWNTs		1.62	14

bilization leads to 'spreading' enzymes on the surface. In addition to this, the immobilization itself also leads to stabilization of the native enzyme structure. In the case of CRL, it is likely that a third factor plays an important role. Most of the lipases, including CRL, have a unique structural feature – a lid or flap consisting of an amphiphilic alpha-helix peptide covering the active site. The presence of an interphase (example water-organic interphase) moves the lid and exposes the active site [20]. Hydrophobic supports are also known to cause similar molecular changes [18,21]. Such 'open lid' conformations are known to be more active.

Interactions with the hydrophobic surfaces of nanotubes are expected to result in the 'open lid', as well as more active lipase conformations. Another observation was that in such media, the lipase immobilized in the absence of BSA gave somewhat higher initial rates. This may be explained by the fact that enzymes in nearly anhydrous media are extremely rigid molecules. In fact, it has been reported that some enhancement of flexibility either through urea denaturation [22] or three-phase partitioning [23] results in higher enzymic biological activity. It is not unlikely that the conformational changes leading to limited denaturation as a result of interactions with the carbon nanotube had acquired the necessary conformational flexibility. The interactions of the enzyme with the carbon nanotube in the presence of BSA, on the other hand, had a very close to native, and hence normal, rigid structure.

Lately, ionic liquids have also emerged as an alternative reaction medium for enzyme catalysis [24,25]. Described as 'green solvents' and 'designer solvents', these room temperature ionic liquids release practically no volatile organic compounds into the atmosphere [24-26]. The water immiscible ionic liquids have generally been reported as being better reaction media than water miscible ionic liquids for enzyme catalysis [24,25], with [Bmim] [PF6] being one of the most frequently used water immiscible ionic liquids for enzyme catalysis [24,25,27,28].

The same reaction, that is, formation of butylbutyrate, was also carried out in [Bmim] [PF6]. The lipase immobilized on MWNT (in the absence of BSA) showed a 14-fold increase in the initial rates of transesterification over those shown by pH tuned lyophilized powders (Table 3).

Biotransformations with the lipase immobilized on MWNT

As the lipase immobilized on MWNT showed higher initial transesterification rates in hexane, its utility in the biotransformation was evaluated by the conversion of ethyl butyrate to butyl butyrate over larger reaction periods. Figure 1 shows that CRL immobilized on MWNT gave much better conversion rates. The immobilized enzyme gave 64% conversion after 24 h, whereas the free enzyme gave 14% conversion after the same reaction period. Even in [Bmim] [PF6] the immobilized enzyme was a far better catalyst (Figure 2). The immobilized

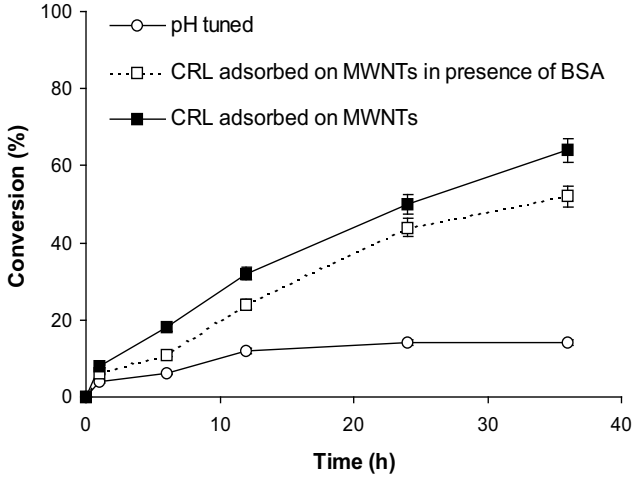


Figure 1
Transesterification of ethyl butyrate with butanol in hexane. Each point represents the outcome of a pair of readings.

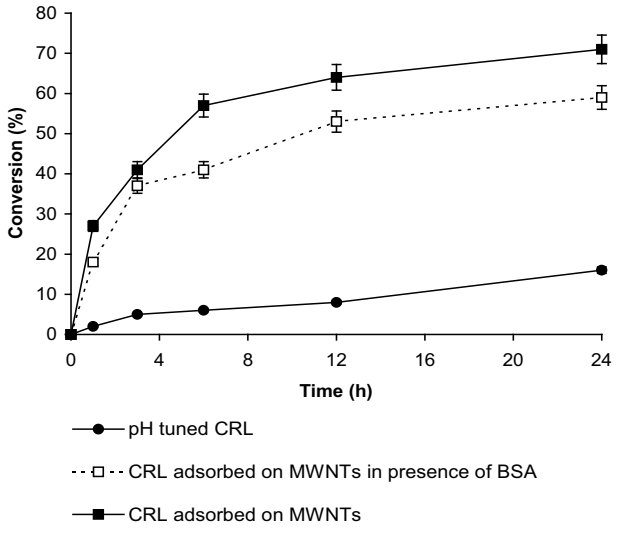


Figure 2
Transesterification of ethyl butyrate with butanol in [Bmim][PF6]. Each point represents the outcome of a pair of readings.

enzyme showed 71% conversion, whilst the free enzyme gave 16% conversion after 24 h. Again, the enzyme immobilized in the absence of BSA performed better. In a control where only MWNTs without enzyme were added, no transesterification activity could be obtained either in hexane or in the ionic liquid.

Enantioselectivity of lipase immobilized on MWNT

The kinetic resolution of racemates to obtain chiral compounds has emerged as a major application of enzymes [15-19,29]. Table 4 shows that free enzyme showed poor transesterification rates during kinetic resolution of (±) 1-phenyl ethanol. However, the enzyme immobilized on MWNT showed significant improvement in percentage conversion with a very high enantiomeric excess of > 99. An enatioselectivity of 360 (with a 36 h reaction time) was calculated according to Chen's equation [30] (note: in the kinetic resolution, 50% would be the maximum). No transesterification could be obtained when a control was run in which only MWNTs were added without lipase.

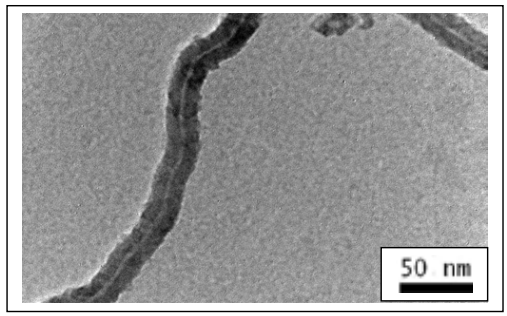
TEM of CRL immobilized on MWNT

TEM images (Figure 3) showed that the enzyme was actually present on the MWNT. In many cases of immobilized enzymes being used in organic media (immobilization on celite is a well-known example), it is believed that an immobilization matrix like celite simply acts as a dispersing agent reducing mass transfer constraints [31]. Thus such systems do not constitute true immobilized enzymes. In the present instance, TEM showed that the system could be described as CRL immobilized on

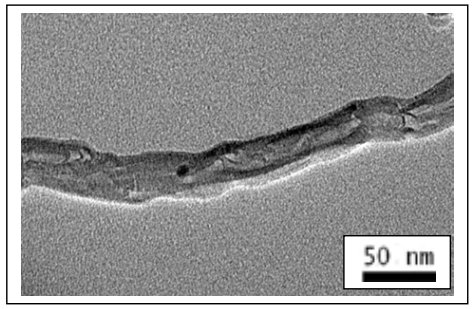
Table 4: Transesterification of (±) 1-phenyl ethanol in ionic liquid with *Candida rugosa* lipase immobilized on MWNTs. All experiments were carried out in duplicate and the results within each pair differed by < 5%.

Entry	Lipase preparation	Time (h)	Conversion (%)	ee (%)	E[a]
1	Free CRL	12	1	-	-
2	Free CRL	24	2	-	-
3	Free CRL	36	5	-	-
4	CRL adsorbed on MWNTs	12	17	>99	250
5	CRL adsorbed on MWNTs	24	34	>99	350
6	CRL adsorbed on MWNTs	36	37	>99	360

[a]Enantioselectivity (E) = ln [1 - c(1 + eep)]/ln [1 - c(1 - eep)], where c = ees/(ees + eep).



(a)



(b)

Figure 3
(a) TEM images of MWNTs; (b) CRL absorbed on MWNTs.

MWNT. Comparing the diameters of the nanotubes with (20 ± 5 nm) and without the enzyme (30 ± 5 nm) (the diameter values represent the average of 10 TEM images in each case), it appears that lipase was physically present on the nanotubes. The enzyme seemed to cover the entire surface of the nanotube, reflecting the evenness of the enzyme 'coating'. Given the propensity of the lipase to form aggregates on surfaces [32], it was thought very unlikely that only a monolayer of lipase molecules had formed on the nanotubes.

Conclusion

There is increasing interest in the miniaturization of technological devices, with studies on the immobilization of enzymes on nanotubes, part of this trend. The present work illustrates that lipase-nanotube systems have some unique features, for instance immobilization on nanotubes actually led to considerable enhancement of enzymic activity in non-aqueous media. In this study, we have reported for the time the performance of an enzyme on carbon nanotubes in non-aqueous media.

Experimental

CRL was a gift from Amano Enzyme Inc., Nagoya, Japan. Multi-walled carbonnanotubes (OD = 10–30 nm, ID = 3–10 nm, length = 1–10 μm, purity 90 + %, Cat. No. 636517) and p-Nitrophenylpalmitate (p-NPP) were obtained from Sigma Chemical Co., St. Louis, USA. The ionic liquid was from Acros Organics, USA. The purity of [Bmim] [PF6] as specified by the vendor was 99.6% (by HPLC) with water content of 0.05% v/v by Karl-Fisher. All solvents were of low water grade (< 0.005% water (vv^{-1})) and obtained from J. T. Baker, USA. These solvents were further dried by gentle shaking with 3 Å molecular sieves (E. Merck, Mumbai, India).

Adsorbtion of Lipase on MWNTs

The MWNTs (1 mg) were dispersed by sonication (Elma transsonic digital ultrasonic unit, model T 490 DH, Germany) at a fixed frequency of 40 kHz and at 110 W power rating) in 0.5 ml of 50 mM phosphate buffer pH 7.0 for 30 min, followed by addition of 0.5 ml of the lipase solution (containing varying amount of the lipase). The mixture was kept at 20°C with constant shaking at 200 rpm for 3 h. After, incubation, mixture was centrifuged at 8,000 *g* for 10 min at 20°C. The supernatant was removed and MWNTs were washed with 1 ml of 50 mM phosphate buffer at pH 7.0 until no hydrolytic activity was detected in the washings.

Transesterification reaction

Ethyl butyrate (60 mM) and *n*-butanol (120 mM) were added to a vial containing 1 ml of hexane/ionic liquid followed by addition of free lipase/lipase adsorbed (10 mg) on MWNTs. The reaction mixture was incubated at 35°C at 200 rpm. The aliquots were withdrawn at different time periods and analyzed by GC. A control was run in which MWNTs were added without lipase.

Protein estimation

The protein concentration was determined according to Bradford's method, using bovine serum albumin as the standard protein [33].

Lipase assay

The hydrolytic activity of lipase was monitored by following the rate of hydrolysis of p-nitrophenylpalmitate spectrophotometrically at 410 nm [34].

GC analysis

The alkylesters were analyzed on Agilent Technologies 6890 N network GC systems, USA, with a flame ionization detector. The capillary specifications were: a column length of 30 m, internal diameter of 0.25 mm. A nitrogen carrier gas at a constant pressure of 4 Kg/cm^2 was used. The column oven temperature was programmed over the range of 150 to 250°C at 10°C min^{-1} with injector and detector temperatures at 240 and 250°C, respectively.

Kinetic resolution of (±)-1-phenylethanol

The alcoholysis between (±)-1-phenylethanol (1 mmol) and vinyl acetate (1 mmol) was carried out in 1 ml ionic liquid followed by addition of CRL/CRL immobilized on MWNTs at 30°C with constant shaking at 250 rpm. The samples were taken out at different time intervals and analyzed by HPLC. A control was run in which MWNTs were added without lipase.

HPLC analysis

For HPLC analysis, 50 μl of the reaction mixture was extracted with 500 μl n-hexane-propan-2-ol (97.5:2.5) mixture. The extract was analyzed by HPLC using Chiracel OD12 RH column (Diacel, Japan). The eluent consisted of 96.5% (vv^{-1}) n-hexane, 3% (vv^{-1}) propan-2-ol and 0.5% (vv^{-1}) ethanol with a flow rate of 1 ml min^{-1} and detection was carried out with a UV detector at 254 nm.

Transmission electron microscopy (TEM)

Transmission electron micrographs were recorded on a FEI Philips Tecnai F20 (200 kV, FEG) instrument. A drop of MWNTs/lipase adsorbed on MWNTs dispersed in distilled water was placed on a copper grid and dried.

Authors' contributions

SS performed almost all the experiments and drafted sections on materials and methods and drew figures. KS carried out immobilization for recording TEM and their analysis. She was also involved with drafting the entire manuscript at the revision stage. MNG participated in the design of experiments, discussions, interpretation of

results and writing of most of the manuscript. All authors approved the final manuscript.

Acknowledgements

This work was supported by funds obtained from Department of Science and Technology, Govt. of India (DST) in the form of a 'Core group grant for applied Biocatalysis'.

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Biotechnology Advances 29 (2011) 889–895

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Enzymes immobilized on carbon nanotubes

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ARTICLE INFO

Article history:
Received 26 January 2011
Received in revised form 13 July 2011
Accepted 13 July 2011
Available online 23 July 2011

Keywords:
Carbon nanotubes
Enzyme immobilization

ABSTRACT

Enzyme immobilizations on carbon nanotubes for fabrication of biosensors and biofuel cells and for preparation of biocatalysts are rapidly emerging as new research areas. Various immobilization methods have been developed, and in particular, specific attachment of enzymes on carbon nanotubes has been an important focus of attention. The method of immobilization has an effect on the preservation of the enzyme structure and retention of the native biological function of the enzyme. In this review, we focus on recent advances in methodology for enzyme immobilization on carbon nanotubes.

Contents

1. Introduction

Practical use of enzymes has been realized in various industrial processes, and is being expanded in new fields, such as fine-chemical synthesis, pharmaceuticals, biosensors, and biofuel cells (Kim et al., 2006). To improve enzyme stability, enzymes have generally been studied with the enzymes immobilized on a solid support (Laurent et al., 2008). Nanomaterials can serve as excellent supporting materials for enzyme immobilization, because they offer the ideal characteristics for balancing the key factors that determine the efficiency of biocatalysts, including surface area, mass transfer resistance, and effective enzyme loading (Jia et al., 2003; Kim et al., 2006; Wang, 2006; Wang et al., 2010). Among the various nanostructure materials, such as nanoparticles, nanofibers, and nanotubes, carbon nanotubes (CNTs) have been the focus of much research. CNTs are produced by various methods, such as arc discharge (Anazawa et al., 2002; Ebbesen and Ajayan, 1992; Wang et al., 2004), laser ablation (Guo et al., 1995), and chemical vapor deposition (Li et al., 1996; Resasco et al., 2002). CNTs consist of graphitic sheets that have been rolled up into a cylindrical shape with lengths in the micrometers, and diameters up to 100 nm (Tasis et al., 2006). CNTs exhibit extraordinary mechanical, electrical, and thermal properties as well as biocompatibility (Shim et al., 2002; Zhang and Henthorn, 2010). Enzyme immobilization is a promising biotechnological application of CNTs (Chen et al., 2003; Gao and Kyratzis, 2008; Hansen et al., 2010; Zhang et al., 2009), especially for fabrication of

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0734-9750/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2011.07.007

biosensors and biofuel cells (Barone et al., 2005; Lee et al., 2010a,b,c; Song et al., 2006; Tsai and Chiu, 2007; Willner et al., 2009). CNTs offer unique advantages including enhanced electronic properties, a large edge plane to basal plane ratio, and rapid electrode kinetics (Jacobs et al., 2010). CNT-based sensors generally have higher sensitivities, lower limits of detection, and faster electron transfer kinetics than traditional carbon electrodes. To fully explore the potential of the enzyme-CNT complex, it is essential to find optimal methods for enzyme immobilization (Taft et al., 2004; Veetil and Ye, 2007; Zhang et al., 2009).

Two main types of CNTs, single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs), have been used to immobilize enzymes. A MWNT is comprised of several layers of graphite surrounding a central tubule, whereas a SWNT only has the central tubule without the graphitic layer. SWNTs are attractive for their higher surface area for enzyme interaction, but MWNTs are desirable for their easier dispersibility and lower cost. Potential biotechnological applications of CNTs have captured the imagination of many researchers (Huang et al., 2002). Noncovalent and covalent conjugations have been reported for the immobilization of various enzymes (Gao and Kyratzis, 2008). Noncovalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT-enzyme complex (Gao and Kyratzis, 2008). Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted. Functionalization of CNTs with organic, polymeric, and biological molecules can provide biocompatible nanotube composites with specific groups on their surface. CNT composites can provide a basis for specific immobilization of an enzyme. No matter what method is used, the main challenge is promoting the stable attachment of enzymes while maintaining their activity and function as closely as possible to their native state (Pedrosa et al., 2010). The performance CNT-enzyme complexes is affected by a combination of the nanotube chemistry and immobilization method. This review will focus on methods for enzyme immobilization onto the surface of carbon nanotubes.

2. Non-covalent enzyme immobilization

For the immobilization of enzymes on CNTs, compared to covalent methods, the noncovalent approach is considered to be a more promising technique, because it preserves the conformational structure of the immobilized enzymes (Matsuura et al., 2006; Nepal and Geckeler, 2006). Enzymes can be adsorbed on a range of surfaces of CNTs; high surface loadings of enzymes or the crowding of the enzyme on the surface may help to prevent inactivation due to surface spreading (Karajanagi et al., 2004). Molecular dynamics simulations have shown that, in the organic solvent heptane, the hydrogen bonds at the active site of the lipase can be better preserved when the lipase is immobilized on the surface of the CNT (Feng et al., 2011). Higher activity was reported by enzymes physically adsorbed onto CNTs (Cang-Rong and Pastorin, 2009).

Adsorption is a commonly used noncovalent approach, which involves the enzyme being physically adsorbed onto the carbon nanotubes (Asuri et al., 2006a; Karajanagi et al., 2004). The adsorption typically involves bathing the CNTs in a solution of the enzyme and shaking the sample to allow time for the physical adsorption onto the surface to occur and then rinsing away enzyme that is not adsorbed. This technique can be carried out by direct physical adsorption onto CNTs or adsorption with the assistance of substances such as polymers, surfactants, and linking molecules. The former is usually a nonspecific adsorption, and the latter can be a specific one.

2.1. Direct physical adsorption

With the direct physical adsorption method, the interacting force between the enzyme and CNT is predominantly a hydrophobic interaction (Gao and Kyratzis, 2008). An enzyme including hydrophobic regions on its exterior can interact with the wall of a CNT through hydrophobic interactions, as illustrated by Fig. 1. The π–π stacking interaction between the sidewalls of CNTs and the aromatic rings also contributes to the adsorption (Matsuura et al., 2006). These hydrophobic and π–π interactions have been widely used to explain the driving force of the direct adsorption of enzyme on CNTs.

Electrostatic interaction has also been demonstrated to play an important role in the adsorption of lysozyme on CNTs (Nepal and Geckeler, 2006). In addition to hydrophobic and π–π interactions, lysozyme interacts with the nanotubes through protonated amino moieties with defect sites of the CNTs at pH values lower than the isoelectric point. At pH levels higher than the isoelectric point, they interact via amine adsorption. The tertiary structures of the lysozyme are preserved well in the lysozyme–CNT complex (Matsuura et al., 2006; Nepal and Geckeler, 2006).

In another example, the hydrogen bonding interactions between the carboxylated CNTs and proteins supports the adhesion of the enzyme complex onto CNTs (Yu et al., 2010); the complex is formed by crosslinking glucose oxidase, 2,5-dihydroxybenzaldehyde, and bovine serum albumin. For direct physical adsorption, the amounts of enzymes adsorbed on CNTs are affected by various factors, such as the nature of the enzyme, the surface chemistry of the CNT, and operation variables. Some enzymes, for example β-glucosidase, chymotrypsin, and soybean peroxidase, can be adsorbed on CNTs in large quantities at 630, 670, and 575 μg protein per mg of CNT, respectively (Gao and Kyratzis, 2008; Gomez et al., 2005; Karajanagi et al., 2004).

As CNTs have a natural affinity for diverse enzymes, the adsorption is spontaneous when enzymes come in contact with CNTs. However, the hydrophobic, nanoscale environment of a CNT can influence enzyme structure and function. Circular dichroism and Fourier transform infrared spectroscopy reveal that enzymes have structural changes upon adsorption, and some enzymes have

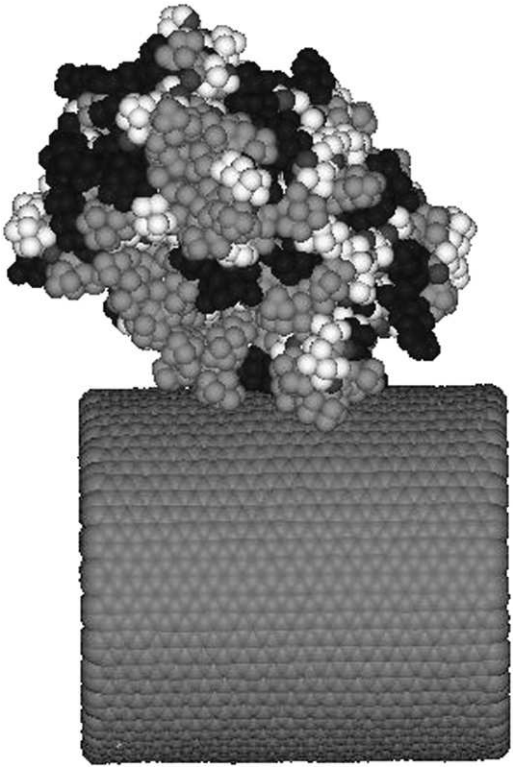

Fig. 1. Schematic illustration of lipase adsorbed on carbon nanotubes by molecular dynamics simulation. The hydrophobic parts are in green, hydrophilic parts in blue.

significant loss of alpha-helix content. For example, horseradish peroxidase endures a loss of about 35% in the alpha-helix content of its secondary structure (Das and Das, 2009), and the losses in the alpha-helix content for soybean peroxidase and R-chymotrypsin are 22% and 43%, respectively (Karajanagi et al., 2004). The nature of the enzyme influences the extent of its denaturation upon adsorption. For soybean peroxidase, the hydrophobic pocket preferentially interacts with the CNT, while for R-chymotrypsin, it must at least partially unfold in order to interact with the CNT surface (Karajanagi et al., 2004). Even though the denaturation of enzymes occurs, CNTs can stabilize enzymes at elevated temperatures and in organic solvents to a greater extent than conventional flat supports (Asuri et al., 2006a). It has been hypothesized that the highly curved surfaces of CNTs are unfavorable for enzyme denaturation in harsh conditions and suppress lateral interactions between adjacent adsorbed enzymes.

In some cases, enzyme adsorption on CNTs can be carried out under sonication due to the formation of carbon nanotube bundles. The lysozyme structure is not affected very much by sonication (Nepal and Geckeler, 2006; Xie et al., 2010). This may be due to the stability of lysozyme at high temperatures. However, for most enzymes, it is not advisable to attempt enzyme adsorption onto CNTs under sonication. Sonication can create large pressure gradients (Brown et al., 2005). Furthermore, structural modifications of protein, such as loss of secondary structure, may be induced by these large pressure gradients (Chalikian and Macgregor, 2009). During sonication, heat from the tip of the apparatus increases the dispersion temperature to approximately 60–70 °C (Matsuura et al., 2006), and the secondary structure and conformation of proteins are cooperatively denatured upon heating (Murayama and Tomida, 2004).

2.2. Enzymes adsorbed onto the CNTs functionalized with polymers

Polymers and biomolecules have been used to functionalize CNTs. The functionalized CNTs (f-CNTs) have good aqueous dispersibility, and the formation of the enzyme–CNT complex is facilitated. f-CNTs can have molecular recognition and binding specificity for enzymes (Chen et al., 2003; Mu et al., 2008), due to the combination of many molecular properties such as hydrophobicity, electrostatic interactions, hydrogen bonding, and steric properties.

Polymers coated on CNTs can provide negatively and positively charged functional groups on the surface of CNT–polymer complexes. In the fabrication of biosensors, enzymes have been adsorbed onto nanotube composites through electrostatic interactions (Lee and Tsai, 2009; Lee et al., 2010c; Wu et al., 2009). For example, the surface of SWNTs can be positively charged by coating poly(sodium 4-styrenesulfonate) combined with ionic liquids (Wu et al., 2009), and glucose oxidase can be immobilized onto the SWNTs as illustrated by Fig. 2. The electrocatalytic activity of electrodes based on the SWNT–enzyme complex is affected by the nature of ionic liquids. Chitosan encapsulated CNTs have a positively charged surface; alcohol dehydrogenase has been immobilized through electrostatic interaction (Lee and Tsai, 2009), and the ethanol biosensor exhibits high sensitivity, a low detection limit, and stable short-term stability. The porous structure formed by the sulfonated polyaniline network grafted onto the surface of CNTs is suitable for the enzyme immobilization (Lee et al., 2010c). This kind of electrode exhibits direct electron transfer for glucose oxidase with a fast heterogeneous electron transfer rate.

2.3. Enzymes adsorbed onto the CNTs functionalized with biomolecules

Enzymes can be specifically bound to CNTs functionalized with biomolecules. The DNA-based biomolecular recognition principle has been applied to carbon nanotubes to construct CNT–DNA electrochemical sensors (Boozer et al., 2004; Moghaddam et al., 2004), by chemically attaching a CNT electrode with ssDNA chains for hybridization with redox-labeled cDNA chains. The binding of DNA onto CNTs appears to be primarily driven by energetically favorable π–π stacking interaction, with the plane of the aromatic nucleotide bases oriented parallel to the surface of the nanotube (Li et al., 2005; Martin et al., 2008; Zheng et al., 2003). For the fabrication of bioelectrodes, single stranded DNA was wrapped on CNTs, and enzymes were immobilized onto DNA-wrapped CNTs (Lee et al., 2010b). The bioelectrode exhibited increased activity and stability of glucose oxidase and laccase, and power production was enhanced. The most important role for the DNA link is enabling the site-addressable binding of redox proteins to carbon nanotubes (Taft et al., 2004; Withey et al., 2008). Enzymes glucose oxidase (GOx) and alcohol dehydrogenase (ADH) labeled with the complementary ssDNA tags can recognize their binding address and adhere to the CNTs functionalized with the complementary strands, as illustrated by Fig. 3. Transducers based on the enzyme–CNT complexes have demonstrated high electron transfer rates. Other molecules, for example streptavidin (Yim et al. (2005) and flavin adenine dinucleotide cofactor (Patolsky et al., 2004), have also been used as the linking molecules between enzymes and CNTs. Biotinylated DNAzyme has been specifically bound onto CNTs, which were attached with streptavidin via stable amide linkages (Yim et al. (2005). The DNAzymes are highly active when conjugated to the water–soluble CNTs. Similarly, glucose oxidase has been adsorbed onto CNTs functionalized with the flavin adenine dinucleotide cofactor (Patolsky et al., 2004). The NAD + or NADP + cofactors functionalized SWNTs are able to form affinity complexes with alcohol dehydrogenase or glucose dehydrogenase (Yan et al., 2007).

2.4. Enzymes adsorbed onto CNTs with assistance of surfactants

Enzymes can be adsorbed onto CNTs with the assistance of surfactants. CNTs coated with the surfactant Triton X-100 can specifically bind to streptavidin as shown in Fig. 4 (Chen et al., 2003). Co-assembling has also been demonstrated to be an efficient method

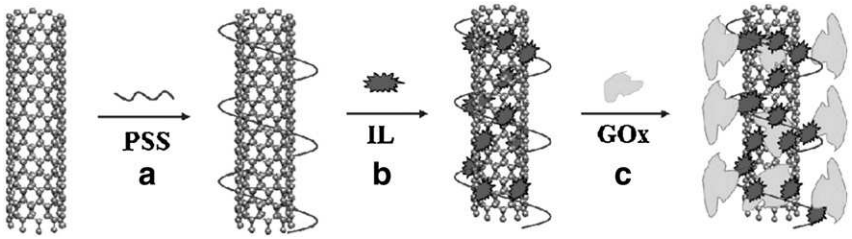

Fig. 2. Schematic illustration of the immobilization of glucose oxidase (GOX) on the surface of SWNTs by using LBL deposition. PSS: poly (sodium 4-styrenesulfonate); IL: ionic liquid. From (Wu et al., 2009) with permission.

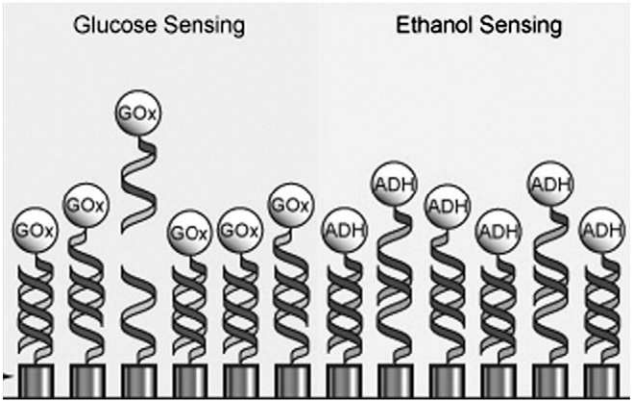

Fig. 3. Conjugation of enzymes tagged with single-stranded DNA to the tips of designated CNTs functionalized with the complementary strands. From (Withey et al., 2008) with permission.

(Yan et al., 2005). Enzymes (horseradish peroxidase and cytochrome *c*) and surfactants (sodium dodecyl sulfate, cetyltrimethylammonium bromide, and Triton X-100) have been co-assembled onto carbon nanotubes. The CNT nanohybrids were demonstrated to facilitate interfacial electron transfer of the proteins with enhanced faradic responses. Coadsorption of Triton X-100 (Shim et al., 2002) or Triton X-405 with poly(ethylene glycol) onto CNTs can be used for selective immobilization of enzymes (Panhius et al., 2003). For the adsorption of enzyme, CNTs are sonicated in the solution of a surfactant such as sodium cholate (McDonald et al., 2007). When the suspension is mixed with [FeFe] hydrogenase, the enzyme binds to the CNTs more strongly than sodium cholate and displaces the sodium cholate to gain access to the CNT surface (McDonald et al., 2007).

2.5. Layer-by-layer technique for immobilization of enzymes

A layer-by-layer (LBL) approach has been adopted for immobilization of enzymes. It permits the coating of various enzymes, producing multilayer enzyme films on CNTs. The biocatalytic activity can be increased by increasing the number of enzyme layers assembled on CNTs (Bi et al., 2009; Wang et al., 2006; Yu et al., 2006). The electrostatic LBL adsorption based on the alternating assembling of oppositely charged layers has been demonstrated to be an effective method for the coating of enzyme on CNTs by the formation of ordered and stable multilayer films (Bi et al., 2009). For

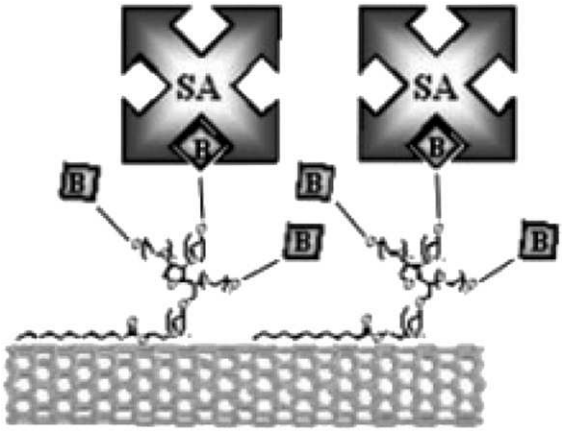

Fig. 4. Scheme for streptavidin (SA) recognition with a nanotube coated with biotinylated Tween. From (Chen et al., 2003) with permission.

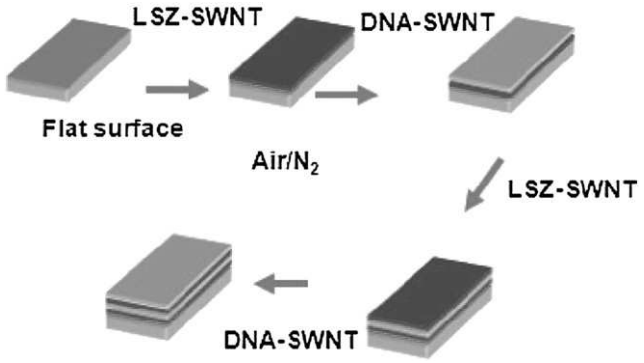

Fig. 5. Schematic diagram of LBL assembly of LSZ-SWNT and DNA-SWNT. LSZ: lysozyme. From (Nepal et al., 2008) with permission.

example, LBL coatings were prepared by the alternating assembly of cationic lysozyme-SWNT and anionic DNA-SWNT (Nepal et al., 2008), as shown in Fig. 5. The strong electrostatic interaction between the DNA and protein plays an important role in the assembly; van der Waals and π–π attractions are also likely to have played a significant role in interlayer adhesion. The nonleaching coatings exhibited robust mechanical properties and long-term protection against bacterial colonization. In a similar work, CNTs were treated in acids to generate negatively charged carboxylic functionalized groups (Bi et al., 2009). Then positively charged poly(dimethyldiallylammoniumchloride) was assembled, followed by the assembling of negatively charged horseradish peroxidase. The enzyme-coated CNTs could achieve ultrasensitive detection of alpha-fetoprotein. Fig. 6 presents a stepwise LBL assembly of alkaline phosphatase multilayers on a carbon nanotube template. The nanotube composite dramatically amplified the electrochemical detection of proteins or DNA (Munge et al (2005).

In addition, matrixes have demonstrated their usefulness in enzyme immobilization. Subtilisin Carlsberg was adsorbed onto SWNTs, and the SWNT–enzyme conjugates were dispersed in poly(methyl methacrylate) (PMMA) (Asuri et al., 2007). The PMMA–CNT–enzyme films were extremely stable, and a high percentage of the initial activity was retained. A conductive matrix was created by integrating biologically derived silica with SWNTs (Ivnitski et al., 2008). The matrix was used for glucose oxidase immobilization. The immobilized enzyme was proven to be stable and catalytic activity for the oxidation of glucose was retained. The enzyme–CNT–silica composite could be successfully integrated into functional bioelectrodes for biosensor and biofuel cell applications.

Advantages of adsorption methods for the immobilization of enzymes are obvious, such as improved preservation of protein structures, intrinsic electronic structures, and CNT properties. However, durability and leaching are always concerns for the application of adsorption methods. Instead, covalent attachment of enzymes can result in high stability and reusability.

3. Covalent linking

3.1. Direct covalent linking enzymes onto CNTs

Covalent immobilization of enzymes on CNTs has been demonstrated by inducing the reaction of the free amine groups on the surface of a protein with carboxylic acid groups that are generated by sidewall oxidation of CNTs and subsequent activation using carbodiimide (Asuri et al., 2006b; Huang et al., 2002; Jiang et al., 2004), as presented in Fig. 7. The method has been widely applied to the covalent immobilization of proteins on carboxylated CNTs (Gao and Kyratzis, 2008). For some enzymes, the enzyme-loadings are higher

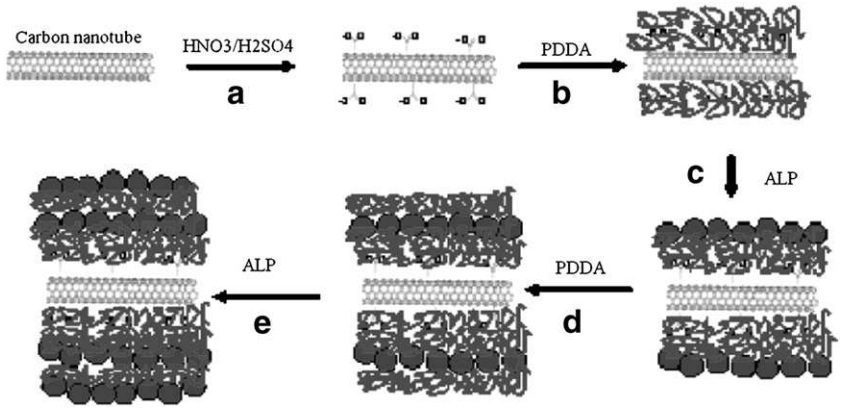

Fig. 6. Schematic of layer-by-layer electrostatic self-assembly of alkaline phosphatase-polyion on carbon nanotube template. PDDA: poly (diallyldimethylammonium chloride) polymer; ALP: alkaline phosphatase. From (Munge et al., 2005) with permission.

than 1000 μg enzyme per mg of CNTs (Ji et al., 2010; Zhang et al., 2009). For the fabrication of biosensors and biofuel cells, enzymes have been covalently attached onto carbon nanotubes (Li et al., 2011; Wang et al., 2003, 2005; Zhao et al., 2009). For fundamental studies, a broad range of enzymes have been covalently attached onto CNTs (Asuri et al., 2006b). The conjugates are stable at high temperatures, providing a unique combination of useful attributes such as low mass transfer resistance, high activity and stability, and reusability. Covalently attached lipase on CNTs has demonstrated advantages over free lipase in catalysis in organic solvent (Ji et al., 2010). The immobilized lipase greatly improves the conversion of the substrate compared to the native lipase. It has been analyzed that the uncovered surface of CNTs may promote the accessibility of substrate to the enzyme and the CNTs can facilitate heat transfer. The influence of nanotube type has been demonstrated by covalently attaching organophosphorus hydrolase on SWNTs and MWNTs. It has been demonstrated that enzymes on SWNTs have much higher activity than those conjugated to MWNTs (Pedrosa et al., 2010).

3.2. Covalent attachment of enzymes onto CNTs with linking molecules

Linking molecules are frequently used for covalent immobilization of enzymes onto CNTs. They bind to CNTs through hydrophobic and π–π interactions (Kim et al., 2009; Pang et al., 2010), and also covalently bind the enzyme through, for example, an amide bond (Alonso-Lomillo et al., 2007; Besteman et al., 2003). Linking molecules present advantages in the immobilization of enzymes. With 1-pyrenebutanoic acid succinimidyl ester, the high reactive succinimidy ester groups were covered on CNTs (Kim et al., 2009), facilitating the horseradish peroxidase immobilization. With aminopyrene, the amino functional groups were introduced uniformly on the CNT surface (Pang et al., 2010), and the immobilized laccase showed higher electrocatalytic activity and better stability than the laccase immobilized on the pristine CNTs. In another example, amino groups were introduced onto CNTs through multistep reactions, and the hydrogenase was immobilized by the formation of amide bonds, resulting in adequate orientation of hydrogenase molecules attached to the CNTs (Alonso-Lomillo et al., 2007). Linking molecules can provide specific sites for CNTs to immobilize enzymes (Dinu et al., 2010; Wang et al., 2010). The activity of perhydrolase S54V was greatly improved when immobilized on CNTs coated with the poly (ethylene glycol) based spacer, as the spacer could prevent non-specific enzyme attachment (Dinu et al., 2010). Wang et al. (2010) cloned the annotated NADH oxidase gene from the *Bacillus cereus* genome and overexpressed the clone with pET30 vector encoding N-terminal 6× His-tag. The His-tagged NADH oxidase was then immobilized onto SWNTs functionalized with $N_\alpha N_\alpha$-bis(carboxy-methyl)-l-lysine hydrate, as illustrated by Fig. 8. The resulting nanoscale biocatalyst demonstrated good loading capacity and stability while retaining 92% maximum activity of the native enzyme. Due to the specific immobilization, no enzyme purification step was necessary before immobilization.

4. Conclusions

Enzyme immobilization on carbon nanotubes allows researchers to take advantage of the fascinating properties of CNTs. The activity of immobilized enzymes is influenced by the methods and procedures of immobilization. Noncovalent approaches have been more studied than covalent methods. Using noncovalent approaches, enzymes can be less denatured upon immobilization and the intrinsic electronic structure and properties of CNTs are preserved. Recently, more attention has been paid to the controlled immobilization of enzymes on CNTs. To that end, specific groups are introduced onto CNTs by functionalization with organic, polymeric, and biological molecules. Through the functional groups, enzymes can be specifically and precisely bound onto CNTs. It is also necessary to study how the linking molecules interact with enzymes and affect the enzyme structure and the arrangement of enzymes on CNTs. These results can significantly contribute to preparing a CNT-enzyme complex with a specific functionality.

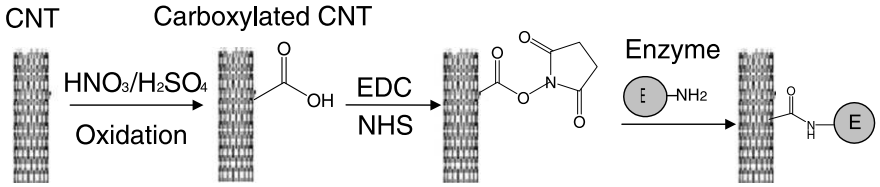

Fig. 7. Schematic representation of conjugation of enzymes to carboxylated CNTs using EDC in the presence of NHS. EDC: N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride; NHS: *N*-hydroxysuccinimide.

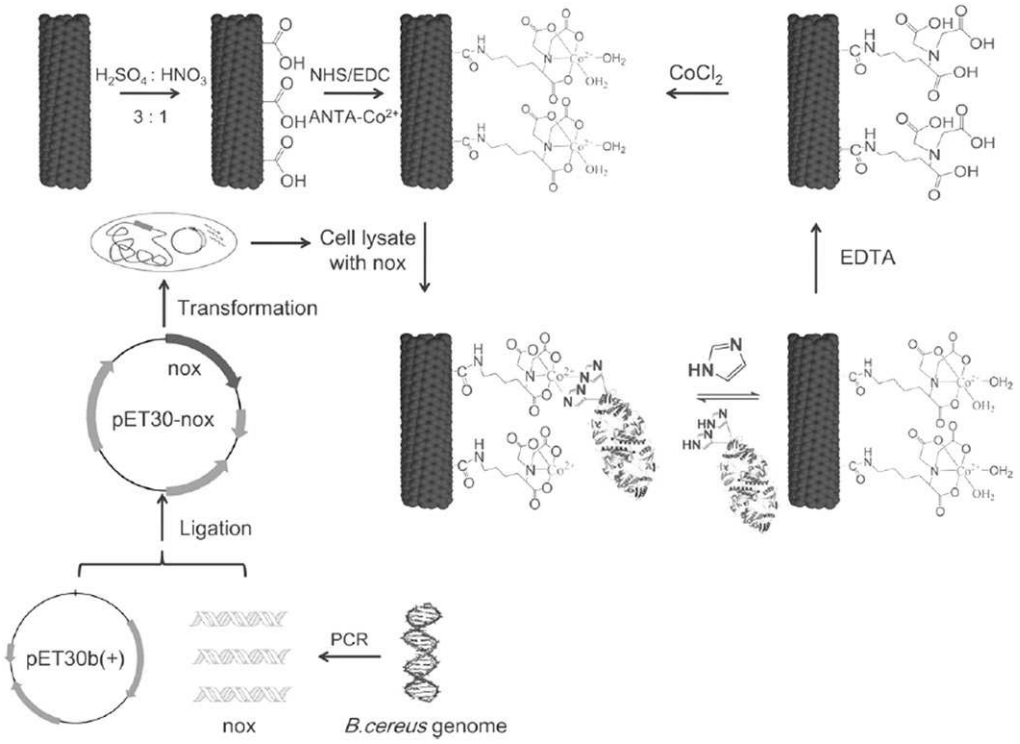

Fig. 8. Scheme of reversible immobilization of NADH oxidase on functionalized SWNTs. From (Wang et al., 2010) with permission.

Acknowledgements

This work was supported by the National Science Foundation of China (21076018), the National Basic Research Program of China (2007CB714302, 2011CB200905), and the Program for New Century Excellent Talents in University.

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