Nanoscale biocatalysis system with novel NADH oxidase

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NANOSCALE BIOCATALYSIS SYSTEM WITH NOVEL NADH OXIDASE

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To My Parents

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List of Abbreviations

ADH	Alcohol dehydrogenase
ADP	Adenosine dephosphate
AFM	Atomic force microscopy
AhpC	Peroxidase gene
AhpF	Hydrogen peroxide-forming NADH oxidase gene
AhpR	Alkyl hydroperoxide reductase system
ANTA	N_{α},N_{α} -bis(carboxymethyl)-L-lysine hydrate
<i>A.xylanus</i>	<i>Amphibacillus xylanus</i>
<i>B.cereus</i>	<i>Bacillus cereus</i>
CNS	Carbon nanosphere
CNS-nox	CNS-ANTA- Co^{2+} -nox conjugate
DLS	Dynamic light scattering
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide
FAD	Flavin adenine dinucleotide
FTIR	Fourier transform infrared
GC	Gas chromatography
GlyDH	Glycerol dehydrogenase
His-tag	Histidine tag
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
K_M	Michaelis-menton constant

<i>L.lactis</i>	<i>Lactococcus lactis</i>
MWCNT	Multi-walled carbon nanotube
MWCNT-nox	MWCNT-ANTA-Co ²⁺ -nox conjugate
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADH/SS	NADH binding redox-active disulfide-containing domain
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NHS	<i>N</i> -hydroxysuccinimide
Nox	NADH oxidase
NTD	N-terminal redox-active disulfide-containing domain
PCR	Polymerase chain reaction
Prxs	Peroxiredoxins
S.A.	Specific activity
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
<i>S.mutans</i>	<i>Streptococcus mutans</i>
<i>S.typhimurium</i>	<i>Salmonella typhimurium</i>
SWCNT	Single-walled carbon nanotube
SWCNT-AhpC	SWCNT-ANTA-Co ²⁺ -peroxidase conjugate
SWCNT-GlyDH	SWCNT-ANTA-Co ²⁺ -glycerol dehydrogenase conjugate
SWCNT-nox	SWCNT-ANTA-Co ²⁺ -nox conjugate
TTN	Total turnover number
UV-Vis-NIR	Ultraviolet-visible-near infrared
V_{max}	Maximum reaction rate
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Abstract

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Chemical transformations catalyzed by immobilized biocatalysts have attracted lots of attention in industry because bioprocessing can be carried out under mild conditions. Immobilized biocatalysts have higher stability during reaction and can be easily recovered from the reaction mixture. In recent years, development in nanotechnology has opened new avenues for enzyme immobilization. The nanoscale biocatalysts have been applied as biosensors, in fuel cells and in biocatalysis. One of the major obstacles that hinder the application of immobilized biocatalysts is the low enzyme activity retention after immobilization. Common immobilization methods, including adsorption, covalent binding and entrapment, often cause enzyme leaching, enzyme 3D structure change and diffusion resistance. As a result, development of an efficient immobilization approach with high activity retention is of great interest to improve catalytic efficiency.

This dissertation describes a robust strategy that can achieve high activity and enhanced stability after enzyme immobilization on nanomaterials. In this study, his-tagged enzymes are immobilized on different nanomaterials based on the specific interaction between histidine-tag and nanomaterials modified with Co^{2+} terminated

nitrilotriacetate group. Nanomaterials were functionalized by acid and then amidated with $N_\omega N_\alpha$ -bis(carboxymethyl)-*L*-lysine. We have successfully immobilized different enzymes, including NADH oxidase, peroxidase, and glycerol dehydrogenase on single-walled carbon nanotubes and applied the same immobilization scheme to others nanomaterials, such as multi-walled carbon nanotubes and carbon nanospheres. Compared with the classical immobilization methods, this approach can well retain native enzyme activity and enhance enzyme stability. Furthermore, the immobilized nanoscale biocatalyst can be regenerated. In another word, the supporting materials are reusable. We applied this immobilization approach not only to single enzymes, but also to multi-enzyme systems, including *in situ* cofactor regeneration systems and consecutive reaction systems. The immobilized cofactor regeneration system has given twice the product yield of that of the free enzyme system. We anticipate that this non-covalent specific immobilization method based on the interaction between histidine-tagged enzymes and nanomaterials can provide an alternative choice for enzyme immobilization.

Chapter 1

Introduction

Chemical transformations catalyzed by enzymes have been broadly utilized to produce important chemicals such as chiral alcohols, amino acids, pharmaceuticals, and biofuels industrially. Native enzymes usually have low stability during catalytic processes. Thus, enzyme immobilization becomes important in industry, which provides a cost-effective solution to develop enzymes with high stability as well as activity retention in both aqueous and organic media.

Classical enzyme supporting materials, including glass beads and porous membranes, have been widely used to immobilize enzymes in the past. However, enzyme immobilization on these materials usually causes low loading capacity and activity retention because of the relatively low surface/volume ratio and strong mass-transfer limitations compared to nanomaterials. Other immobilization methods have also been employed to attach enzymes to supporting materials since the advent of surface chemistry. Nevertheless, non-specific immobilization approaches decrease enzyme catalytic efficiency because of enzyme leaching, enzyme 3D structure loss, and diffusion resistance.

To overcome the limitations of above mentioned immobilization approaches, new strategies have been developed. With the development of nanotechnology in the last few decades, nanotechnology-inspired biocatalyst systems have attracted lots of interest from scientists and engineers because nanomaterials have high surface

area/volume ratio, low mass-transfer limitation, and provide excellent particle mobility in reaction.

Specific immobilization approaches include azide-alkyne cycloaddition, Staudinger ligation, antibody-antigen systems, the biotin-avidin system, and the polyhistidine- $\text{Co}^{2+}/\text{Ni}^{2+}$ system. Among these methods, polyhistidine- $\text{Co}^{2+}/\text{Ni}^{2+}$ system has captured much attention in recent years. Glucose oxidase and horseradish peroxidase were successfully immobilized on different nanomaterials as biosensors. Polyhistidine tagged proteins have been widely used in downstream bioprocessing, the specific and reversible interaction between the His tagged enzyme and $\text{Co}^{2+}/\text{Ni}^{2+}$ ion greatly facilitates protein purification.

In this dissertation, we are going to focus on enzyme immobilization, which is based on the specific interaction between His-tagged enzymes and nanomaterials functionalized by N_{α},N_{α} -bis (carboxymethyl)-L-lysine hydrate(ANTA). The resulting nanomaterial-enzyme conjugates were proven to have good activity retention and excellent stability improvement.

This thesis consists of seven chapters. We first develop and validate the enzyme immobilization method by different nanoscale supporting materials. Then, we apply the same immobilization approach on two multi-enzymatic systems, one with consecutive reactions and the other with *in situ* cofactor regeneration.

Chapter 1 provides the motivation and aims that are stimulated by the obstacles encountered in enzyme immobilization. Chapter 2 focuses on the background of

enzyme immobilization and NADH oxidase, a nicotinamide cofactor regenerating enzyme. An overview of immobilization methods, supporting materials, NADH oxidase structure and proposed mechanism will also be presented. Chapter 3 describes an effective approach to immobilize NADH oxidase on single-walled carbon nanotubes (SWCNTs) with stability improvement as well as excellent activity retention. The complete enzyme immobilization, characterization, and regeneration process have been described in details. Chapter 4 focuses on the application of this immobilization method on other nanomaterials such as multi-walled carbon nanotubes (MWCNT) and carbon nanosphere (CNS). The kinetics and stability of these conjugates will be compared and discussed. Chapter 5 expands the immobilization strategy to a multi-enzymatic system with consecutive reactions, which are catalyzed by a two-enzyme system, alkyl hydroperoxide reductase. The overall activity, stability, and amounts of intermediate have been investigated by using different ratios between two individual enzymes. Chapter 6 exhibits the application of immobilization method to an *in situ* cofactor regeneration system. The amounts of nicotinamide cofactor NAD^+ , NADH/NAD^+ ratio, and the pH of the multi-enzymatic system are investigated in order to find out the optimal conditions to maximize the bioproduction of an important pharmaceutical intermediate. Conclusions and proposals for future works are included in Chapter 7.

Chapter 2

Background

Enzymes are nanoscale biocatalysts, which participate in metabolic pathways and help maintain the stability of intracellular microenvironment in many organisms [1-3]. Reactions catalyzed by enzymes can be carried out under mild conditions [4]. Because of their high activity, specificity, and enantioselectivity [5-9], enzymes have attracted a lot of scientific attention and been widely pursued in various applications, including chemicals synthesis [10], pharmaceutical production [11], biosensors [12] and biofuel cell fabrication [13]. The large-scale process usually requires both active and stable biocatalysts [14]. However, the low operational stability of native enzymes cannot fulfill the requirements [15]. Extensive studies have been launched on improving enzyme stability, such as enzyme modification (genetic and enzymatic level) [16-19], medium engineering [20, 21], and enzyme immobilization [22, 23].

2.1 Enzyme immobilization

Among the enzyme stabilization methods, enzyme immobilization onto a supporting material has been widely investigated [15, 24-26]. Common immobilization methods include adsorption [27], covalent binding [28, 29], entrapment [15], and encapsulation [30]. Compared with free enzyme, immobilized enzyme can have greatly improved stability during operation and be easily recovered. Enzyme immobilization can also

broaden the application of enzyme from an aqueous environment to non-conventional conditions [2, 15, 26, 31]..

Activity retention and stability improvement are two essential aspects to evaluate immobilized enzyme. In order to prepare immobilized enzyme with high quality, three elements—the enzyme, the supporting material, and the immobilization method, need to be considered during the design and execution process. This chapter will focus on the introduction to the different enzyme immobilization methods, supporting materials, and the enzyme of interest in this work, NADH oxidase.

2.2 Immobilization approaches

Generally, immobilization methods have been divided into two categories: nonspecific and specific, according to the interaction specificity between enzyme and supporting material. The nonspecific approaches include adsorption, covalent binding, entrapment, and encapsulation. The specific approaches are classified into covalent and noncovalent categories.

2.2.1 Nonspecific immobilization approaches

Most of the nonspecific immobilization methods have been developed and extensively applied beginning in the second half of the 20th century. Various immobilized enzymes have been prepared through these approaches and applied in industrial processes. Despite the decline in enzyme activity after immobilization, the

nonspecific immobilization method has become an indispensable part in industrial bioconversion. This section will focus on the introduction of the four basic nonspecific immobilization methods.

2.2.1.1 Adsorption-based immobilization

Adsorption-based enzyme immobilization (Figure 2-1) is prepared by physical adsorption of the enzyme onto the supporting material. In the early 20th century, it was first developed by attaching invertase on charcoal to keep its catalytic activity [32]. However, this method did not attract attention from industry until 1960s—amino acid acrylase had the honor to be immobilized on diethylaminoethyl (DEAE)-cellulose via physical adsorption in large-scale application [33].

Adsorption can be generally classified into different categories according to various interactions between enzyme and supporting material: non-specific adsorption (enzyme is absorbed by non-specific interactions such as hydrogen bonds, hydrophilic interactions, and *van der Waals* forces) [34], hydrophobic interaction (interaction between the hydrophobic domain of enzyme and the corresponding material) [35], electrostatic interaction (charge-charge interaction) [36] and biological interaction (interaction between ligands) [37].

Adsorption-based immobilization was continuously studied owing to its intrinsic advantages. Firstly, the immobilization process is simple [26]. The immobilization process can be easily carried out under mild conditions. Second of all, in contrast with covalent methods, no chemical reaction occurs between the supporting material and

the enzyme; hence, an enzyme adsorbed on the supporting material may keep its activity [38, 39].

Several oxidoreductases have been successfully immobilized through physical adsorption. One model enzyme that has been extensively studied is horseradish peroxidase (HRP) because of the luminescent or fluorimetric products that allow it to be detected and quantified. HRP has been reversibly immobilized on novel boronate affinity gel particles and the conjugate shows 90.12% of activity retention [40]. Moreover, the activity retention of HRP has also been studied by immobilizing it on cellulose microfibers coated by titanium oxide. The decrease in activity retention (~50%) is attributed to the interaction of HRP with titanium oxide, which may cause the substrate diffusion to the active site of HRP [41]. Recently, HRP has been adsorbed on single-walled carbon nanotubes (SWCNTs) by sodium cholate suspension-dialysis method and SWCNT-HRP conjugate maintains 98% of the native enzyme activity [39]. Glucose oxidase (GOx) belongs to another important industrial oxidoreductase family and has been extensively investigated. Various supports, such as cellulose and porous glass beads have been used for the immobilization of GOx [42]. In recent years, GOx has been immobilized on the nanomaterials by adsorption and used as biosensor. GOx has been immobilized on SWCNTs by a linking molecule that bound with SWCNTs via *van der Waal* force and used as a single-molecule biosensor with pH sensitivity [27].

However, because of the weak force between the enzyme and the supporting material, enzyme leaching by desorption forces is the most significant limitation of this approach [26]. For example, the change in pH of the reaction environment caused by the products can vary the charge on protein surface, which may weaken the electrostatic interactions. Therefore, several derivative/modified adsorption methods have been developed to solve this limitation by combining adsorption with other methods. For instance, the crosslinking approach has been introduced to post-treat conjugate to prevent enzyme leaching [43]. Recently, another possible method for preventing desorption is to use short peptide. A 12-mer polypeptide library is first generated by bacteria phage and certain peptides with high affinity with nanomaterials are discovered by high throughput screening using phage display [44]. Depending on this technology, fusion enzyme could be made by coupling with the 12-mer polypeptide and immobilized on supporting material. The approach might alleviate enzyme leaching and avoid enzyme 3D structure change because the bond energy between the polypeptide and the nanomaterial is higher than adsorption but lower than covalent coupling. Another feature of this method is that 12-mer polypeptide could be customized according to diverse supporting materials but it may take long time to find the appropriate peptide.

Figure 2-1 Illustration of adsorption-based enzyme immobilization

2.2.1.2 Covalent immobilization

Since the 1970s, covalent enzyme immobilization (Figure 2-2) has become one of the most important methods in enzyme immobilization [45, 46]. Compared with adsorption, a covalent bond provides much stronger interaction between the enzyme and the supporting material [15]. Thus, the main drawback of the adsorption method, enzyme leaching from supporting material, can be minimized by this method.

The covalent bonds are formed by chemical reactions between active amino acid residues on the enzyme surface and functional groups on the supporting material. Among the twenty standard amino acids, only nine of them have active sidechains that can react with functional groups on supporting materials. In order to react with these amino acids, the supporting material need be functionalized first to have the corresponding functional groups. Some essential requirements have been established to achieve efficient immobilization [47]: i) active groups on both the enzyme and the

supporting material should be able to carry out certain reactions, ii) active groups should have less spatial confinement in order to have easy access to each other, iii) both the active amino acid residue and the functional group need to be activated prior to immobilization.

Many dehydrogenases have been covalently immobilized successfully. Alcohol dehydrogenases (ADH), which facilitate the inter-conversion between ketones and alcohols, were immobilized on different supporting materials. ADH was covalently coimmobilized with formate, formaldehyde, and glutamate dehydrogenases on polystyrene nanoparticles to produce methanol from CO₂. The accumulation yield of methanol was 127% with 80% activity retention after 11 reusing recycles [48]. ADH from *Rhodococcus ruber* was also covalently immobilized by linear dialdehyde glutaraldehyde on amino-functionalized glass beads, magnetic carrier, and nanodiamonds. The activity retentions were in between 45% and 62% [49]. Magnetic nanoparticles coated by chitosan with alpha-ketoglutaric acid (Fe₃O₄/KCTS) were used to immobilize ADH from *Saccharomyces cerevisiae* and the immobilized ADH retained 65% of the original activity [50]. However, the main shortcoming of covalent immobilization is the low activity retention [26]. For example, a Schiff base [51], can be nonspecifically formed between the supporting material and four different amino acid residues (lysine, histidine, cysteine, and tyrosine) [52, 53]. These nonspecific interactions may change the enzyme structure and cause the enzyme to lose its native catalytic configuration and activity. In order to solve the problem, some modified

immobilization methods have been developed (Section 2.2.2.1) using specific covalent bonds, and the resulting conjugate can retain much better activity [54, 55].

Figure 2-2 Illustration of enzyme immobilization via covalent bond

2.2.1.3 Entrapment

Enzyme entrapment (Figure 2-3) is an immobilization method that confines the enzyme molecules into a matrix. It was first reported in 1950s that an enzyme can be physically entrapped into a gel matrix with activity retention [56]. However, no special attention was given to this method until Bernfeld and Wan used acrylamide with the cross-linker *N,N*-bis-acrylamide to immobilize antigens and seven enzymes in the lattice by polymerization in 1960s [57].

Figure 2-3 Illustration of enzyme entrapment in mesoporous material.

As for the entrapment procedure, enzyme is first dispersed in medium solution, such as polymeric precursor, and then the physical barrier with confined enzymes is fabricated by either physical or chemical method, including cross-linking, polymerization, and physical gelation [15, 58]. The porous matrix should be permeable to substrate molecules in spite of the entrapment method.

Several porous materials have been used to entrap enzymes, especially mesoporous materials. Mesoporous silica, such as SBA-15 and MCM-41, has been widely used to immobilize enzymes with high loading capacity [59, 60]. *Cytochrome c* was immobilized via two different mesoporous materials, MCM-41/28 and MCM-41/45 [61]. Other materials, such as amino-functionalized mesostructured cellular foams (AF-MCFs) with large mesopores, were also introduced to immobilize glucose oxidase as biosensor [62].

Compared with covalent immobilization, entrapment may keep three-dimensional structure better owing to no direct reaction between the enzyme and the supporting

material [47]. Therefore, this method can be used for enzymes that are deactivated in covalent immobilization. However, the main weakness of this method is that it can cause serious mass transfer resistance and decrease in enzyme catalytic performance. One of the reasons for the raised mass transfer resistance is the uncontrollable pore size distribution [63]. Enzyme leaching is also another problem owing to the large pore size and precursor used [64, 65]. The functionalities on the precursor could be assorted to the inner and active categories according to the interaction with enzymes during immobilization. Besides forming the physical barrier, the precursor with the active functionalities could also react easily with the enzymes compared to the precursor with inner functionalities. This entrapment using the precursor with active functionalities could be regarded as the immobilization with post-modification, which also minimizes the enzyme leaching.

2.2.1.4 Encapsulation

Different from entrapment, enzyme encapsulation (Figure 2-4) is the formation of physical membrane barrier around the enzyme. Encapsulation can be categorized into four classes: solid shell formed around the liquid droplet interface, phase inversion, template leaching, and post-loading encapsulation [47]. The membrane pore size is an important parameter in enzyme encapsulation design. In order to avoid leaching, the membrane pore must be smaller than enzyme. On the other hand, the pore size should be much larger than substrate molecules to mitigate diffusion limitation. Compared to covalent immobilization, encapsulation is usually carried out under mild conditions

and the enzyme in the capsules is not modified by chemical reagents. The 3D structure of the enzyme, therefore, could be retained by this method. Moreover, multiple enzymes can be immobilized simultaneously to construct a multi-enzyme system for sequential reactions.

Figure 2-4 Illustration of enzyme encapsulation.

NADH oxidase coupled with 3 α -hydroxysteroid dehydrogenase was immobilized in collodiol microencapsulation to oxidize androsterone to androstanedione with cofactor regeneration in CSTR reactor [66]. Other enzymes, such as horseradish peroxidase and catalase, have been immobilized by encapsulation before. Horseradish peroxidase has been encapsulated in a nanogel by surface acryloylation and *in situ* aqueous polymerization. Similar Michaelis-Menten constant (K_M) of free and immobilized enzyme indicate that the polymer shell does not affect substrate transport significantly [67]. Controlled polymer multilayer coating was also used to encapsulate catalase, and preserved its activity [68]. However, some factors may affect activity retention of encapsulated enzymes. i) Similar to entrapment, encapsulation can also

cause mass transfer limitations owing to the small pore size of membrane and membrane thickness. The investigation found when encapsulated urease in nylon capsule, the optimum reaction pH and the K_M value of immobilized urease were determined by the thickness of the membrane [69]; ii) The characteristics of the membrane precursor sometimes may lead to the negative effect. For instance, urease encapsulated in the capsule made by poly diallyl dimethyl ammonium chloride and sulfate cellulose can only retain 35~38% of its initial activity due to the interaction between urease and sulfate cellulose [70].

2.2.2 Specific enzyme immobilization approaches

Traditional methods, including adsorption-based enzyme immobilization, covalent binding, entrapment, and encapsulation, belong to non-specific enzyme immobilization approaches. In order to improve enzyme activity retention, specific enzyme immobilization methods have been developed recently, which can be divided into two categories—covalent and non-covalent [53].

2.2.2.1 Specific covalent immobilization

Although most of the covalent immobilization methods belong to non-specific immobilization, the new covalent strategies, appending covalent adducts to the enzymes, have overcome this problem. These covalent adducts on the enzymes can help the formation of covalent bonds between the enzymes and the supporting materials specifically.

Azido, a chemically inert group, has been exploited by using either azide-alkyne cycloaddition (Figure 2-5A) [55, 71] or Staudinger ligation (Figure 2-5B) [54, 72, 73]. Reactions are carried out between azido groups on the enzyme and alkyne/phosphate on the supporting material. For instance, Ronald T. Raines and colleagues appended azido groups to the C-terminus of bovine pancreatic ribonuclease (RNase A) and reported specific interaction between enzyme and its supporting material [74]. Duckworth, et al. (2006) also used a similar method via introducing an azido group to the C-terminus of farnesyltransferase (PFTase) to immobilize the enzyme on agarose beads [75]. However, this “chemoenzymatic” method needs to be carried out in multiple steps and only the C-terminus could be functionalized [53], which limits its application only to the enzymes that have the catalytic site at its C-terminus.

Figure 2-5 Reaction of specific enzyme immobilization by (A) azide-alkyne cycloaddition [76] and (B) Staudinger ligation [72]:

Another powerful strategy uses the specific site-directed protein mutants. A unique amino acid residue with a specific side-chain functional group is introduced to the target enzyme we are interested via site-directed mutagenesis. The mutated enzyme then could be attached to the supporting material with a controlled orientation through the functional group [77]. In these amino acids, cysteine has been widely introduced to the enzyme owing to its thiol group that is covalent coupled with thiopropyl- or organomercurial-functionalized supporting materials (Figure 2-6).

Figure 2-6 Enzyme immobilization by specific covalent reaction between cysteine residue on the enzyme and thiopropyl-functionalized supporting material.

Arg331 on the mutated L-lactate dehydrogenase (LDH, Gln102Arg / Cys97Gly) from *Bacillus stearothermophilus* was replaced by Cys residue and immobilized on thiopropyl-Sepharose gel. The activity of the immobilized mutated LDH reached ~56% of the original 331Cys-LDH activity and the decreased activity was considered as the diffusion limitation in the gel particles [78]. Subtilisin with a single cysteine

mutation (S246C / S145C) was immobilized on thiopropyl Sepharose 6B. The catalytic efficiency (k_{cat}/K_M) decreased with the internal substrate diffusion effect [79]. This approach recently has been taken into the development of antimicrobial peptide (AMP)-functionalized microcapacitive electrode arrays to detect pathogenic bacteria. AMP magainin I displaying the broad-spectrum activity toward Gram-negative bacteria was integrated into the microfluidic flow cell by cysteine thiol group. The biosensor achieved the sensitivity of 1 bacterium/ μl [80]. However, the method cannot be applied to either H_2O_2 -forming or H_2O -forming NADH oxidase, because Cys residues are located at the redox-active catalytic sites that participate into the catalytic process [81-84].

2.2.2.2 Specific non-covalent immobilization

Non-covalent specific enzyme immobilization usually utilizes the biological interactions such as biotin-avidin [85] and polyhistidine- $\text{Co}^{2+}/\text{Ni}^{2+}$ [86]. The benefits of using non-covalent interactions are: i) the interaction between enzyme and supporting material is specific and stable [52] and ii) the immobilization is reversible, in other words, the supporting material can be recycled when the enzyme loses its activity.

2.2.2.2.1 Biotin-avidin system

Biotin is a water-soluble B-complex vitamin, which is vital for cell growth [87], fatty acid synthesis [88], and amino acid catabolism [89]. Biotin has a fused ring structure containing an ureido (tetrahydroimidizalone) ring with a tetrahydrothiophene

ring attached by valeric acid. Avidin, a homotetramer protein with M_w of 69 kDa, is produced from the white of bird's egg [90]. In Figure 2-7, each avidin subunit can bind one molecule of biotin with a tight and specific non-covalent bond ($K_D \approx 10^{-15}$ M) [91].

Figure 2-7 Reaction of biotin and avidin (PDB code: 1RAV) to the biotin-avidin complex (PDB code: 1LDQ). By using ligand explorer from PDB, biotin ($C_{11}H_{18}N_2O_3S$) is indicated in the red box and 2-(Acetylamino)-2-Deoxy-a-D-Glucopyranose ($C_8H_{15}NO_6$) is indicated in the black box.

The biotin-avidin system has several advantages over antibody-antigen systems. First of all, biotin-avidin conjugate can be rapidly and stably formed owing to the low dissociation constant [92]. Secondly, biotin-avidin can be used in fluorescence applications such as fluorescence activated cell sorting (FACS) because of the high fluorochrome/protein ratio [93]. Finally, biotin-avidin can mitigate the background fluorescence noise when using a heavily fluorescein-labeled antibody [94]. Because

of these advantages, several research groups have used this method to prepare protein-material conjugates. Marassa, et al performed the enzyme-linked immunosorbent assay (ELISA) using this method to detect mosquito 28 blood-fed *Culex quinquefasciatus* [95]. Horseradish peroxide-labelled avidin (HRP-avidin) coupled with biotinylated anti-E2 antibody have been used to determine estradiol (E2) [85]. However, this kind of interaction is rarely used in enzyme immobilization owing to the high isoelectric point of avidin (~ 10) [96], which allows it to bind to proteins with negative charge nonspecifically.

2.2.2.2.2 Polyhistidine- $\text{Co}^{2+}/\text{Ni}^{2+}$ system

The chelation between polyhistidine-tag ($6 \times \text{His-tag}$) and bivalent metal (Co^{2+} , Ni^{2+} , Cu^{2+} , Fe^{2+} or Zn^{2+}) is another popular non-covalent specific interaction. The imidazole ring of the histidine sidechain can be coordinated with metal ions to form stable conjugate [97, 98]. The $6 \times \text{His-tag}$ can be added to either N- or C-terminus of the enzyme, implying a broader application range than specific covalent immobilization. Because of its ease of use, high specificity and affinity, this interaction has been widely employed in protein purification, known as immobilized metal affinity chromatography (IMAC), and also becomes a potential choice for enzyme immobilization (Figure 2-8). To use this method for immobilization, the recombinant enzyme needs to fuse a $6 \times \text{His-tag}$ at either N- or C- terminus, which mainly depends on the location of enzyme active site. The supporting material needs

to be functionalized by either nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) in order to capture the bivalent metal ion.

Figure 2-8 Scheme of using His-tag to immobilize enzyme on functionalized SWCNTs.

The interaction between a His-tag and a metal ion has been widely used to prepare protein-nanomaterial conjugates in biosensor fabrication by immobilizing green fluorescent protein (GFP) or its derivatives, such as cyan fluorescent protein (CYP), on functionalized nanomaterials. It was reported that NTA- Ni^{2+} modified Fe/Pt and Co/ Fe_2O_3 core/shell magnetic nanoparticles had high specificity with His-tagged GFP and the conjugates showed outstanding fluorescence [99, 100]. Besides fluorescent proteins, NTA modified nanoparticles can also be used as probes to directly detect certain proteins. Superparamagnetic Fe_3O_4 nanoparticles were first synthesized and coated with NTA to detect streptopain [101]. The NTA- Ni^{2+} modified silica nanoparticles also demonstrated high specific binding with estrogen receptor α ligand binding domain [102].

Until now, most proteins that have been immobilized using a His-tag have been fluorescent proteins, polypeptides, and only a few enzymes such as horseradish peroxidase and T4 DNA ligase (Table 2-1). Although many recombinant His-tagged enzymes are purified by affinity chromatography, only a few have been immobilized on materials through the His-tag and the characteristics of these conjugates have not been thoroughly investigated yet. Since this kind of specific interaction has a high potential to be developed as a robust enzyme immobilization strategy, it will be the focus of this thesis.

Table 2-1 Various types of His-tagged immobilized proteins

Protein	Supporting Material	Ligand	Reference
Horseradish peroxidase	Au nanoparticle	Nitrilotriacetic-Co (II)	[86]
Green fluorescence protein	Au/FePt/Fe ₂ O ₃ nanoparticle	Nitrilotriacetic-Ni (II)	[103]
Green fluorescence protein	SmCo _{5,2} /Fe ₂ O ₃ nanoparticle	Nitrilotriacetic-Ni (II)	[99]
D-Amino acid oxidase	Magnetic bead	Nitrilotriacetic-Ni (II)	[37]
Photosynthetic reaction center	SWCNTs	Nitrilotriacetic-Ni (II)	[52]
MS2 coat protein	SiO ₂ /Fe ₃ O ₄ core/shell nanoparticle	Nitrilotriacetic-Co (II)	[104]
Phosphopeptides	Fe ₃ O ₄ nanoparticle	Nitrilotriacetic-Ni (II)	[101]
Green fluorescence protein	PPL-g-PEG	Nitrilotriacetic-Ni (II)	[105]
Green fluorescence protein	Gold nanoparticle	Nitrilotriacetic-Ni (II)	[106]
Peptide (AB-G5HG2)	CdSe/ZnS core/shell quantum dot	Nitrilotriacetic-Ni (II)	[107]
T4 DNA ligase	γ-Fe ₂ O ₃ nanoparticle	Iminodiacetic-Cu (II)	[108]
Maltose binding protein	Multivalent chelator head	Tri-Nitrilotriacetic - Ni (II)	[109]
Green fluorescence protein	Agarose bead	Nitrilotriacetic-Ni (II)	[97]
Human estrogen receptor alpha	SiO ₂ -TMR-NTA nanoparticle	Nitrilotriacetic-Ni (II)	[102]

2.3 Supporting Materials

Supporting materials are closely correlated with the performance of immobilized enzyme because the performance of immobilized enzymes will be affected by the characteristics of the supporting material, such as surface area/volume ratio and surface morphology. In the following section, supporting materials have been divided into two categories, macroscale and nanoscale.

2.3.1 Macroscale supporting materials

From the outset, scientists and engineers used macroscale materials, such as glass bead and different membranes, as supporting materials to immobilize enzymes. These materials are convenient to obtain and easy to functionalize. Uncountable number of immobilized enzymes have been made, which accelerated the process of biocatalyst application. Transparent porous silicate glass helped to encapsulate copper-zinc superoxide dismutase (CuZnSOD) and the conjugate had similar optical adsorption spectrum as the free enzyme [110]. Calcium alginate-silicate beads were used to immobilize β -glucosidase and the conjugate demonstrated excellent operational stability of [111]. However, because of the relative low surface area/volume ratio and strong mass-transfer resistance [2], enzyme immobilization on macroscale materials causes low loading capacity and poor performance.

2.3.2 Nanoscale supporting materials

Nanotechnology involves the combination of physics, chemistry and even biology in research and development. With the development of physics and chemistry, new nanomaterials have been synthesized, characterized and functionalized, which are applied in fabrication of different kinds of devices in electronics [112, 113] and biomedical diagnosis [114, 115]. Biomolecules, including nucleic acids, peptides, and proteins, may tailor their structures and functions, and even gain novel characteristics when integrated with nanotechnology [116-118]. Novel hybrids, therefore, can be generated by combining nanotechnology and biotechnology with new physicochemical properties and better biocompatibility.

Nanomaterials (carbon nanotubes [119-121], nanoparticles [12, 98, 102], fullerenes [122], nanofibers [123-125], and mesoporous/nanoporous materials [126-128]) were used as solid support, providing platforms for enzymes to improve their functions [26]. Nanomaterials can provide upper limits on enzyme-efficiency-determining factors such as high surface area/volume ratios, enzyme loading capacity, high surface curvature, and low mass transfer resistance [2, 22, 129, 130]. Moreover, nanomaterials help expand the field of enzyme application from bioengineering to electronic engineering.

2.3.2.1 Carbon nanotubes

Carbon nanotubes (CNTs) were first discovered in 1991[131] and are divided into two categories: Multi-walled carbon nanotubes (MWCNTs) rolled from multiple

graphene sheets [131] and single-walled carbon nanotubes (SWCNTs) rolled from single graphene sheet [132]. CNTs have broad industrial applications owing to their extraordinary electronic, thermal and mechanical properties. They have been applied in various sensors [133], memory devices [134], and solar cells [135]. In the biological field, modified biocompatible CNTs were used in cell imaging [136] and drug delivery [137].

CNT has been used as supporting material in enzyme immobilization in recent years. Soybean peroxidase (SBP) and α -Chymotrypsin (α -CT) have been immobilized on SWCNTs by physisorption. The secondary structure of immobilized enzymes was investigated by FTIR and both immobilized enzymes substantially increased the content of α -helix with a decrease in that of β -sheet [138]. Subtilisin Carlsberg (SC) and horseradish peroxidase (HRP) were attached to SWCNTs by covalent binding and the residual activity of either immobilized enzyme was around 50% [24]. MWCNTs have been used as a supporting material to bind with SBP and *Candida antarctica* Lipase B (CALB) covalently and the immobilized enzymes can retain 50~65% of the initial enzyme activity [139].

2.3.2.2 Carbon nanosphere

Carbon nanospheres (CNSs) are prepared by pairing of heptagonal and pentagonal carbon rings and have porous carbon nanostructure [140]. Two categories of CNSs have been defined according to diameter: graphitic carbon onion (2 ~ 20 nm) [141, 142] and less graphitic carbon nanospheres (50 ~ 1000 nm) [143]. The monodisperse

CNSs have been synthesized with higher surface area with appropriate pore size and used as electrochemical capacitor [144]. It was also reported previously that a hybrid prepared by gold nanoparticles and CNSs was conjugated with HRP-labeled antibody and used for amplified electrochemical immunoassay to detect protein and DNA [145]. Moreover, CNSs were synthesized by a one-step pyrolysis method and served as support of PtRu catalyst [146]. However, to the best of our knowledge, CNSs have not been employed as supporting materials for biocatalysis purpose.

One of the focuses of this thesis is enzyme immobilization on CNTs, which has tubular hollow structure with nm range diameter. CNTs offer high surface to volume ratio compared with nanowires. Its nonporous structure can help minimize mass transfer resistance compared to porous nanomaterials. The CNT surface can be broadly functionalized which makes it easy to attach enzymes and also facilitates dispersion in solution. Furthermore, CNTs can be easily recovered by filtration or centrifugation. Compared with MWCNTs, SWCNTs have attracted more interests because of its high surface area/volume ratio and surface curvature. Moreover, SWCNT-enzyme conjugates have the potential to be used as biosensors. Both CNSs and CNTs belong to carbon nanomaterials but have different nanostructures, tubular hollow versus spherical. In addition, CNSs have porous structure, whereas CNTs are nonporous nanomaterials. The kinetic comparison of immobilized enzymes on CNSs and CNTs will be discussed in this work.

2.4 NADH oxidase (nox)

NADH oxidase (nox) is a useful biocatalyst for regenerating nicotinamide cofactors and has been identified in numerous organisms such as *Salmonella typhimurium* (*S. typhimurium*), *Amphibacillus xylanus* (*A. xylanus*), *Lactococcus lactis* (*L. lactis*), and *Streptococcus mutans* (*S. mutans*) [81, 147-150]. There are two categories of nox: hydrogen peroxide-forming NADH oxidase (nox-1) and water-forming NADH oxidase (nox-2). Both belong to the flavoprotein family, with nox having two redox-active disulfide centers and one FAD per unit. The reactions catalyzed by NADH oxidases are as followed [150, 151]:

Nox-1 is also part of the alkyl hydroperoxide reductase system (AhpR) that has been known as the antioxidant system in anaerobic bacteria [81, 148-151]. AhpR belongs to the category of non-heme, non-selenium proteins and catalyzes the reduction of organic hydroperoxide and hydrogen peroxide to corresponding alcohol and water. AhpR is composed of two enzymes: nox-1 (gene name *ahpF*) and peroxidase (gene name *ahpC*). Peroxidase is a representative of a large family of cysteine-base peroxidases and contains two redox-active cysteine disulfide bonds per homodimer [152, 153]. Nox-1 and peroxidase are part of the global regulon of OxyR

transcription factor and the nucleotide sequence of peroxidase is usually located just upstream of *nox-1* in the genome [151].

2.4.1 NADH oxidase 3D structure

The 3D structure of *nox* from *S.typhimurium* was solved previously (PDB 1HYU) (Figure 2-9) [154, 155]. *Nox* is a homodimer and each subunit is composed of three domains: N-terminal redox-active disulfide-containing domain (NTD, residues 1 ~196), FAD binding domain (residues 210 ~ 327 and 450~521) and NADH binding redox-active disulfide-containing domain (NADH/SS, residues 328 ~ 449) [154].

Figure 2-9 3D structure of *nox* homodimer (PDB code: 1HYU). NTD with its redox-active disulfide bond (Cys129-Cys132) and FAD domain are connected by the flexible linker. Another redox-active disulfide bond (Cys345-Cys348) is located at NADH/SS domain. The bound flavin cofactor in the FAD domain is also shown [154].

2.4.2 Catalytic mechanism of alkyl hydroperoxide reductase.

The mechanism of AhpR from *S. typhimurium* and *S. mutans* was proposed by Leslie B Poole's group (Figure 2-10) [81, 147, 148, 150, 154]. Nox shows one redox-active disulfide center in both N- terminal and NADH/SS domain, while AhpC features an inter-subunit disulfide bond at the active site. Two redox-active disulfide centers of nox are significant for electrons shuttling. At the beginning, FAD obtains the electrons from NADH and becomes FADH₂, followed by giving the electrons to the C-terminal active site. Electrons located at C-terminal active site are then transferred to the N-terminal active site. Electrons from the N-terminal disulfide center of nox are transferred to the active site of AhpC when nox and AhpC temporarily interact [155]. The activated AhpC reduces alkyl hydroperoxide or hydrogen peroxide to corresponding alcohol or water [148, 156].

Figure 2-10 Proposed mechanism of alkyl hydroperoxide reductase system from *S. mutans* [150].

2.4.3 NADH oxidase as cofactor regenerating enzyme

Nicotinamide cofactor-dependent oxidoreductases are an important enzyme category and have been widely used in industry to synthesize chiral compounds, such as hydroxyl acids, chiral alcohols, and amino acids [157]. Many oxidoreductive reactions need these nicotinamide cofactors as electron donors or acceptors. There are two categories of nicotinamide cofactors, the oxidized form NAD(P)^+ and the reduced form NAD(P)H . The high cost of nicotinamide cofactors is one of the main obstacles of applying oxidoreductases in industry (NADH : SGD 790/g (Cat. no.: N4505) and NAD^+ : SGD 929/g (Cat. no.: N1636). Prices are obtained from Sigma-Aldrich, Singapore). Therefore, cofactor regeneration is necessary for oxidoreductase application. Compared with chemical and electrochemical methods [158, 159], enzyme-catalyzed cofactor regeneration method provides an efficient and robust strategy and can be carried out under mild conditions [160] Therefore, enzyme-based cofactor regeneration attracts the interest from industry.

Several NAD(P)^+ -regenerating enzymes have been researched in the past: lactate dehydrogenase (LDH) [161, 162], alcohol dehydrogenase (ADH) [162-164] and NADH oxidase [84, 151]. Among them, NADH oxidase is the best candidate because: i) NADH oxidase uses oxygen as the substrate, in other words, no other substrates need to be added, ii) the irreversible reaction catalyzed by NADH oxidase can drive production reaction towards product formation direction, and iii) the product is much easier to separate compared to using LDH or ADH.

2.4.4 Amino acid alignment of NADH oxidases

NADH oxidase has been identified from *L.lactis*, *S.mutans*, and *A. xylanus* in the past [149-151]. Here in this work, I am going to use the annotated nox from *Bacillus cereus* (*B.cereus*). As shown in Figure 2-11, the amino acid level identity of between nox from *B. cereus* and other organisms is as high as 47.4% and the strong similarity level is up to 24.4%. The catalytic center motif CxxC is found near both N- and C-terminus. Another motif of flavin / NAD(P)H binding site GxGxxG is also found in the middle of the nox (*B. cereus*) sequence.

	130	140	150	160	170	180	
<i>A. xylophilus</i>	DFVTYVSLT	CQNCPD	VVQALN	MSVVNPNI	HTMVEGSA	FKDEVDRLN	ILAVPSVYLNGE
<i>B. cereus</i>	HFESYISLS	CHNCPD	VVQALN	VMSVLNPG	ITHMIDGA	AFKEEVESKD	IMAVPTVYLNGE
<i>L. lactis</i>	HFETYVSLT	CHNCPD	VVQALN	TMSILNPNI	SHTMIEGGM	FQEEVEAKG	IMAVPTVFKNGE
<i>S. mutans</i>	HFETYVSLT	CHNCPD	VVQAFN	IMSVVNPNI	SHTMVEGGM	FKDEIEAKG	IMSVPTVYKDGT
	. * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *						
Prim. cons.	HFETYVSLTCHNCPD	VVQALN	4MSV2NPNI	2HTM2EGG2	FK2EVEAKG	IMAVPTVY	2NGE
	190	200	210	220	230	240	
<i>A. xylophilus</i>	FMASGRMT	IEDILGHL	GSGIDASEL	NEKDPD	VLVI	GGGPAG	ASSAIYAARKGIRVGI
<i>B. cereus</i>	SFGSGRMT	LEELAKM	GNGPDASEL	SDKDPY	DVLV	GGGPAG	ASAIYAARKGIRTGIVA
<i>L. lactis</i>	EFHNGRAS	LEELLEK	ITGPESSE	AFDDKDPY	DVLV	GGGPAG	ASAIYAARKGIRTGLIA
<i>S. mutans</i>	EFTSGRAS	IEQLDL	IAGPLKEDAF	DDKGV	FDVLVI	GGGPAGN	SAAIYAARKGVKTGLLA
	: . * * : : * : * : : . . . : * . : * * * : * * * * * : * * * * * : : * : * : *						
Prim. cons.	EF4SGR222EE2L	4KIGG24DAS	224DKDP2	DVLV2GGGPAG	ASAIYAARKG	IRTG2IA	
	310	320	330	340	350	360	
<i>A. xylophilus</i>	LDNGAVLKS	KTIILATGAR	WRDIGVPGE	KEYKNKGVAY	C	THCDAPL	FEKGHVAVVGGGNS
<i>B. cereus</i>	LENGAILKS	KSIVSTGAR	WRNVGVPGE	AEFKNKGVAY	C	PHCDGPLFTG	KDVAVIGGGNS
<i>L. lactis</i>	LENGALLKS	KSVIISTGAR	WRDLGIPGE	QEFKTKGVAY	C	PHCDGPLFAG	KDVAVIGGGNS
<i>S. mutans</i>	LDNGAHLKA	KTAVLALGAK	WRKINVPEGE	KEFFNKGVTY	C	PHCDGPLFTD	KKVAVIGGGNS
	* : * * * * * : * : * : : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *						
Prim. cons.	L2NGA4LKSK2	VIL2TGARWR	DIGVPGEKE	FNKGVAYC	PHCDGPLFTG	KDVAVIGGGNS	

Figure 2-11 *B.cereus* nox amino acid alignment with other known nox. Identity: 47.4%; strong similarity: 24.4%. CxxC: Catalytic Center; GxGxxG: flavin and NAD(P)H binding site.

2.5 Novelty of the thesis

The thesis will focus on single/multi enzyme immobilization on different nanomaterials, using the specific interaction between His-tagged enzymes and modified supporting materials. The novelty of this work is as follows: i) it is a specific enzyme immobilization method that is universally applicable on different

nanomaterials, including SWCNT, MWCNT and CNS. Common enzyme immobilization methods that use non-specific immobilization methods often cause enzyme leaching, enzyme activity decrease, 3D structure change and mass-transfer resistance. The immobilization method proposed in this work has overcome the foresaid limitations. The immobilized enzymes result in very high activity retention and good stability, ii) the immobilization process is reversible. Even if the immobilized enzyme loses its activity after long-term reaction, the reversible immobilization feature enables the reusability of the supporting material without sacrificing activity or loading capacity, and iii) to our knowledge, all of the enzyme immobilization methods published so far require pure enzyme, but with our method, cell lysate can be used directly in enzyme immobilization with good specificity, which avoids expensive enzyme purification steps and decreases the overall cost of bioprocessing.

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Chapter 3

Specific and reversible immobilization of NADH oxidase on functionalized carbon nanotubes

3.1 Introduction

Recent developments in nanomaterials have opened new avenues for industrial biocatalysis [1, 2]. In theory, nanomaterials can serve as ideal supporting materials for enzyme immobilization because they can provide the upper limits on enzyme-efficiency-determining factors such as surface area/volume ratio, mass transfer resistance and enzyme loading capacity [1]. However, common immobilization methods (adsorption [3], covalent binding [4], crosslinking [5] and encapsulation [6]) have limited applicability owing to enzyme leaching [7], 3D structure loss [8], and strong diffusion resistance [9]. Moreover, costly enzyme purification steps are needed before immobilization.

NADH oxidase (nox) was investigated in this study because of its high potential in industrial applications. Oxidoreductases account for 30% of industrial scale biocatalysts [10], and most oxidoreductases need nicotinamide cofactors (NAD(P)^+ and NAD(P)H) to complete their reactions. Owing to the high cost of nicotinamide cofactors, an effective *in situ* cofactor regeneration is essential for industrial synthesis using oxidoreductases [11]. NADH oxidase, which catalyzes oxygen and NADH to either hydrogen peroxide or water, is an excellent cofactor regeneration candidate [12, 13]. Single-walled carbon nanotubes (SWCNTs) were chosen as the enzyme

supporting material because SWCNTs can be broadly functionalized and have good dispersion in solution compared to other nanomaterials such as nanoporous matrices, nanoparticles and nanofibers. Additionally, because of the small diameter of SWCNTs, the mobility of the immobilized particle would not be greatly affected [14]. Furthermore, since SWCNTs are the nonporous materials, mass transfer resistance will be limited during catalysis [9].

In this work, we show an efficient immobilization method based on the specific interaction between His-tagged nox (H_2O_2 -forming NADH oxidase) and single-walled carbon nanotubes functionalized by N_α, N_α -bis(carboxymethyl)-L-lysine hydrate (ANTA) without requiring enzyme purification. This nanoscale biocatalyst has overcome the foresaid limitations, and demonstrates good loading capacity and stability while maintaining 92% maximum activity of the native enzyme. We further demonstrate that the immobilization is reversible and can retain activity for several loading cycles.

3.2 Materials and methods

3.2.1 Materials

Hydrogen peroxide, β -Nicotinamide adenine dinucleotide hydrate (NAD^+), Bradford reagent, cobalt (II) chloride, N_α, N_α -bis(carboxymethyl)-L-lysine hydrate (ANTA), 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide (EDC), nitric acid ,

sulfuric acid, *N*-hydroxysuccinimide (NHS), *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES), ampicillin sodium salt, kanamycin sulfate, potassium phosphate dibasic, potassium phosphate monobasic, potassium bromide, sodium chloride, tryptone, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (Singapore). Dithiothreitol (DTT), β -Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), flavin adenine dinucleotide (FAD), urea, calcium chloride, rubidium chloride, manganese chloride tetrahydrate, yeast extract and agarose were purchased from Merck (Singapore). Low melting agarose was from Nusieve. Gel Star Stain was purchased from Cambrex. Restriction endonucleases and T4 ligase were purchased from Fermentas and New England Biolabs. *Bacillus cereus* (*B. cereus*) ATCC 14579 genome was purchased from ATCC. Super purified HiPCO[®] single-walled carbon nanotubes were from Unidym (USA, D: 0.8~1.2 nm, length: 100~1000 nm, surface area per unit mass: 1315m²/g). Ni²⁺ Sepharose High Performance resin (6% agarose, 0.989g/ml) was bought from Amershan (GE Healthcare, D: ~34 μ m).

3.2.2 Cloning, overexpression and purification

The *nox* gene (*ahpF*, NC_004722) from *B. cereus* ATCC 14579 was isolated from its genome by polymerase chain reaction (PCR) using *Taq* PCR Core Kit (QIAGEN, Research Biolabs, Singapore) and gene specific primers 5'-AGATCTGACCATGGCGATGATACTAGATGCAGATATAAAAAACACA-3' and 5'-CTCGAGGGATCCCTATTAGTTACGGATTAAGTAATCAAATGC-3' (The

concentration of each primer in PCR reaction is 0.5 μ M). Equal volume of each primer solution was combined and subjected to 30 cycles of denaturation (30 s at 94 $^{\circ}$ C), annealing (30 s at 50 $^{\circ}$ C) and extension (90 s at 72 $^{\circ}$ C) reactions. Purified PCR product was then cloned into the pDrive vector (PCR Cloning kit, QIAGEN, Singapore). The recombinant plasmid was digested with restriction endonucleases *Bgl* II and *Xho* I (New England Biolab), and the nox gene fragment was cloned into the pET30b(+) vector. The recombinant plasmid pET30-nox was transformed into *E. coli* BL21(DE3) cells. Cells were cultured in Luria-Bertani (LB) broth with 30 μ g/mL kanamycin at 200 rpm, 37 $^{\circ}$ C. Protein expression was induced with 200 μ M IPTG when OD at 600nm reached 0.6 ~ 0.8. The cell pellets were harvested after 3h of overexpression at 37 $^{\circ}$ C. To obtain the cell lysate, the cell pellet from 200mL culture was suspended in 10mL pH 7.0, 50 mM potassium phosphate buffer (PPB) and sonicated for 12 $\times$ 30 sec at an interval of 30 sec. Nox was purified with immobilized metal affinity chromatography method (IMAC; Gravatrap Ni²⁺ column, GE Healthcare). 20 mM pH 7.4 PPB containing 500 mM NaCl and 500 mM imidazole was used as the elution buffer. PD-10 column (GE healthcare) was used to remove the salts from the solution. Proteins concentrations were measured after 5 min incubation with Bradford Reagent using Biophotometer (Eppendorf, Singapore).

3.2.3 SWCNT modifications

SWCNT-COOH: SWCNTs powder (10 mg) was treated with HNO₃/ H₂SO₄ (1:3) for 3 h at 40 $^{\circ}$ C in a water-bath sonicator. After the treatment, the SWCNTs dispersion

was adjusted to pH 3~5 by NaOH. The SWCNT suspension was filtered with a 0.2 μm nylon membrane and washed with 20 mM pH 7.5 HEPES buffer three times. Finally, SWCNT-COOH was suspended in 20 mM pH 7.5 HEPES buffer.

SWCNT-ANTA- Co^{2+} : ANTA was dissolved in 20 mM pH 7.5 HEPES buffer with excess CoCl_2 to form ANTA- Co^{2+} . Excess Co^{2+} was precipitated by NaOH. The resulting $\text{Co}(\text{OH})_2$ was removed by centrifugation for 10 min at 8,000 rpm. The supernatant containing ANTA- Co^{2+} was collected for further reaction. SWCNT-COOH dispersed in 20 mL HEPES buffer reacted with 100 mM NHS and 20 mM EDC to form SWCNT-NHS ester complex. The ester complex was then mixed with ANTA- Co^{2+} to produce SWCNT-ANTA- Co^{2+} complex. The byproducts and excess NHS and EDC were removed by washing the complex with 20 mM pH 7.5 HEPES buffer. Finally, SWCNT-ANTA- Co^{2+} complex was suspended in 20 mM pH 7.5 HEPES buffer.

3.2.4 SWCNTs characterization

SWCNTs samples at different stages during functionalization, as well as initial SWCNTs samples, were characterized by FTIR spectroscopy and UV-vis-NIR. SWCNTs dispersed in aqueous solutions were measured by a Varian Cary 5000 UV-vis-NIR spectrophotometer. SWCNT dispersions were further filtered with 0.2 μm nylon membranes, and then tubes on the membrane were evaporated to dry in a 100°C oven. The dried SWCNTs samples were mixed with KBr powder, and pressed into thin films for FTIR measurement on a DIGILAB FTS 3100 FTIR instrument. The IR

spectra were averaged over 96 scans from 1000 to 4000 cm^{-1} . The spectra were blank subtracted and baseline corrected using Revolution 4.0 Pro software.

3.2.5 Atomic force microscope (AFM)

AFM was conducted using a MFP 3D microscope (Asylum Research, Santa Barbara, CA) with a cantilever (Arrow NC, Nanoworld) in ac mode. The scan rate was set to 1 Hz at various scan sizes. About 20 μL of sample solutions were dropcasted on a freshly cut mica surface followed by drying and rinsing with deionized water.

3.2.6 Enzyme immobilization

Cell lysate was incubated with both SWCNT-ANTA- Co^{2+} complex and sepharose resin at 4 $^{\circ}\text{C}$ overnight and the resulting conjugates, SWCNT-ANTA- Co^{2+} -nox (SWCNT-nox) and sepharose-nox, were collected by centrifugation. Both conjugates were washed with pH 7.4 HEPES containing 20mM imidazole three times to remove nonspecifically bound proteins. For loading capacity, 20mM, pH7.4, HEPES with 500 mM NaCl and 500 mM imidazole has been used to elute enzyme from SWCNTs. The enzyme concentrations in the elution were measured by Bradford assay.

3.2.7 Conjugates and free enzyme activity assay

The activity of free and immobilized enzyme was measured by the decrease of NADH absorbance at 340 nm (ϵ : 6220 $\text{M}^{-1}\text{cm}^{-1}$), using DU-800 spectrophotometer (Beckman Coulter, Singapore). The standard conditions were 8 μM FAD (free

nox)/24 μ M FAD (SWCNT-nox)/68 μ M (sepharose-nox) incubation for 5 min in air saturated 50 mM pH 7.0 PPB at 30°C. The K_M value of NADH was measured under standard conditions with NADH concentration varying from 0 to 400 μ M.

3.2.8 Total turnover number measurement

A specific amount of SWCNT-nox/free nox was added to the air-saturated 50 mM pH 7.0 PPB buffer with/without 5 mM DTT at 30 °C. NADH was continuously added until the enzyme could not react any longer as described before [12, 13].

3.2.9 Reversible immobilization

The “old” nox enzyme was eluted off SWCNTs with 20 mM pH 7.4 HEPES buffer containing 500 mM imidazole and 500 mM NaCl. After centrifugation, the complex was washed with 20 mM, pH 7.5 HEPES buffer before dispersion. Newly prepared cell lysate was mixed with the dispersed complex overnight for enzyme reloading.

The “old” Co^{2+} was removed from SWCNT-ANTA- Co^{2+} complex with 20 mM pH 7.5 HEPES buffer containing 20 mM EDTA and 500 mM NaCl, and washed with 20 mM pH 7.5 HEPES buffer. The “new” Co^{2+} was attached by incubating SWCNT-ANTA with 50 mM CoCl_2 solution overnight. Newly prepared cell lysate was added to the regenerated complex after sonication.

3.2.10 Reusability Measurement

The reusability of SWCNT-nox and sepharose-nox was measured by their residual activity at standard conditions. After each reaction, MWCNT-nox and CNS-nox were separated by centrifugation and then washed three times by 20 mM, pH 7.5 HEPES.

3.3 Results and discussion

3.3.1 SWCNT-nox

Figure 3-1 outlines the scheme of this work. The annotated nox gene from the *B. cereus* genome was cloned by polymerase chain reaction (PCR), and overexpressed with pET30b(+) vector encoded with N-terminal His-tag. It is well-known that His-tagged proteins can attach to transition metal ions such as Co^{2+} or Ni^{2+} capped with nitrilotriacetate groups. This knowledge has been exploited to bind His-tagged proteins with nanomaterials [15]. Here, we functionalized the SWCNT surface with a Co^{2+} terminated nitrilotriacetate group, which results in specific interaction between the SWCNTs and His-tagged NADH oxidase. First, carboxylic groups were introduced to the SWCNT surface by acid treatment. Then, the carboxylic groups were activated and subsequently amidated by NHS-esters with ANTA- Co^{2+} . The reaction yields of SWCNTs in each step of the functionalization are measured by using UV-Vis spectrophotometer at the wavenumber 11750 cm^{-1} [16]. After acid treatment, SWCNT-COOH yield is around 2.5 mg (~25% of the initial amount),

which is attributed to the cutting operation of these oxidizing acids with the shorten rate about 130 nm hour^{-1} [17]. The final SWCNT-ANTA- Co^{2+} yield are around 2.2 mg ($\sim 22\%$ of the initial amount).

Figure 3-1 Scheme of reversible immobilization of NADH oxidase (*B. cereus*) on functionalized SWCNTs.

Figure 3-2 FTIR and UV-Vis-NIR spectra of the stepwise modification of SWCNTs and nox immobilization. (A) FTIR spectrum of SWCNT modification. (1) Spectrum of SWCNT-ANTA- Co^{2+} complex. Peaks at 3309, 3012, 2960, 1732, 1629, and 1394 cm^{-1} correspond to $-\text{NH}$ stretching, $-\text{CH}_2$ group, $-\text{CH}_2$ group, $-\text{CO}_2^-$ group, $\text{C}=\text{O}$ stretching and $\text{C}-\text{O}$ stretching, respectively. (2) Spectrum of SWCNT-COOH. Peaks at 3433, 1643, 1322, and 1188 cm^{-1} correspond to $-\text{OH}$ stretching, $\text{C}=\text{O}$ stretching, $-\text{CH}$ stretching and $-\text{CH}$ stretching, respectively; (3) Super purified raw HiPCO[®] SWCNTs. (B) UV-Vis-NIR spectrum. (1) Super-purified raw HiPCO[®] SWCNTs in 2% sodium cholate (blue); (2) SWCNT-COOH (green); (3) SWCNT-ANTA- Co^{2+} complex (red); (4) SWCNT-nox (magenta).

The ultraviolet-visible-near-infrared (UV-Vis-NIR) spectrum and Fourier transform infrared spectroscopy (FTIR) (Figure 3-2 A&B) confirm the successful synthesis of SWCNT-ANTA-Co²⁺ complex with the estimated yield around 22%. Cell lysate instead of purified enzyme was used during immobilization, which effectively eliminated the costly enzyme purification step. We found that NADH oxidase in the cell lysate can bind specifically to SWCNT-ANTA-Co²⁺ complex through its N-terminal His-tag, and produce SWCNT-nox (Figure 3-3 A&B). The leaching studies were conducted by measuring enzyme concentration and activity in 20mM, pH 7.5 HEPES and PPB buffers. Both enzyme concentration and activity remain the same within a week, which demonstrates that leaching is negligible with this immobilization method.

Figure 3-3 SDS-PAGE of nox immobilization A: Binding specificity of nox. Lane A: cell lysate; Lane B: imidazole elution sample from control (SWCNT-COOH and nox mixture); Lane C: imidazole elution sample from SWCNT-nox; Lane D: protein molecular weight marker. This picture shows that nox (~60 kDa) from cell lysate can bind specifically to the SWCNT-ANTA-Co²⁺ complex but not to SWCNT-COOH. B: Protein content in cell lysate. Lane A: protein molecular weight marker; Lane B: original cell lysate; Lane C: insoluble part; Lane D: cell lysate after incubation with SWCNT-ANTA-Co²⁺ complex. This picture shows that the amount of overexpressed nox in cell lysate obviously reduced after incubation with SWCNT-ANTA-Co²⁺ complex.

Figure 3-4 AFM micrographs of functionalized SWCNTs and SWCNT-nox. (A) Well dispersed SWCNT-ANTA- Co^{2+} complex before enzyme immobilization. (B) SWCNT-nox (C)&(D) Topographic height profiles along lines shown in (B) crossing a SWCNT and a SWCNT-enzyme conjugate.

We analyzed SWCNT-nox by using atomic force microscope (AFM) to determine its morphology and visualize the interaction between SWCNT-ANTA-Co²⁺ complex and nox. Figure 3-4A shows the presence of individual SWCNT and small SWCNT bundles, which implies that SWCNT-ANTA-Co²⁺ complex can be well dispersed in aqueous solution. The average tube length and diameter after modification are estimated to be 700 ± 100 nm and 1.0 ± 0.3 nm, respectively. Figure 3-4B shows the conjugate after enzyme immobilization and nox is found to be attached to the SWCNT surface. The height of SWCNT-enzyme conjugate is around 6 nm (Figure 3-4D), while the height of SWCNT alone is around 1 nm (Figure 3-4C). Double peaks with similar height are observed on the immobilized enzyme (Figure 3-4D), which may indicate that a homodimer is bound to the SWCNT surface. This result suggests that nox from *B. cereus* could share a similar homodimer structure as nox from *Salmonella typhimurium* (1HYU)—the two proteins have 55% amino acid identity with each other [18].

3.3.2 Loading capacity

The loading capacity is found to be ~0.47 mg enzyme/mg SWCNTs, which is close to the reported immobilization capacity on carbon nanotubes by either covalent binding or adsorption [2, 3]. By comparison, when using commercially available sepharose bead as supporting material, it has much lower loading capacity (0.01 mg enzyme/mg sepharose bead). The high loading capacity of SWCNTs is probably owing to its high surface area per unit mass of SWCNT. If we assume that one His-tag coordinates with one Co²⁺ ion, the ligand density on the modified SWCNTs is estimated to be 3.7×10^{11} groups/cm².

3.3.3 Kinetic comparison between free nox and immobilized nox

A major criterion to evaluate the performance of enzyme immobilization is the retention of native enzyme activity after immobilization. We found that SWCNT-nox could retain 92% of native enzyme maximum activity after immobilization (Figure 3-5 & Table 3-1), whereas sepharose-nox could only maintain 17%. The K_M of NADH, which characterizes enzyme affinity for substrate, is about 53 μM for both free nox and SWCNT-nox, and 113.6 μM for sepharose-nox, suggesting that the diffusion resistance is negligible for SWCNT-nox but strong for sepharose-nox. Our method shows much better activity retention compared with other immobilization methods for nanomaterials: carbon nanotube-enzyme immobilization by physical adsorption usually keeps 40~70% of the native enzyme activity [3, 19]; covalent attachment often maintains about 50% or less owing to the changes in enzyme 3D structures [2]; the encapsulation method was reported to retain only 30% of the glucose oxidase native activity on mesocellular carbon foam [20]. The high activity retention of SWCNT-nox may be attributed to the specific interaction between modified SWCNTs and His-tag and possible preservation of enzyme 3D structure during catalysis [15]. The low activity of sepharose-nox could be due to the porous structure of sepharose that may cause strong diffusion resistance [9].

Figure 3-5 Saturation curves of free nox (-□-), SWCNT-nox (-■-) and sepharose-nox (-Δ-) in air saturated pH 7.0, 50 mM PPB at 30 °C. (A) FAD; (B) NADH

Table 3-1 Kinetics and loading capacity comparison

	V_{max} (U/mg)	$K_{M.NADH}$ (μM)	$K_{M.FAD}$ (μM)	Loading capacity (mg/mg)
Free nox	27.7	53.3	8.1	N/A
SWCNT-nox	25.7	53.4	23.7	0.47
Sepharose-nox	4.9	113.6	68.3	0.01

3.3.4 Stability comparison between SWCNT-nox and free nox

An important advantage of enzyme immobilization is the stability improvement, which expands the range of conditions suitable for enzyme function in industrial applications. We have investigated SWCNT-nox stability on three aspects: storage stability, thermal stability and operational stability. Figure 3-6A shows that the storage stability of SWCNT-nox in 20 mM pH 7.5 HEPES is much better than that of the free nox at 4 °C and slightly better at 20 °C. The half-life of free nox is around 300 h at 4 °C, while that of SWCNT-nox half-life is over 1500 h. Furthermore, SWCNT-nox can preserve 70% of its initial activity even after 1000 h at 4 °C.

SWCNT-nox shows activities over a broader range of temperatures than the native nox (Figure 3-6B). The conjugate retained a specific activity of 10.8 U/mg even when temperature reached 90 °C, whereas free nox was totally denatured at 70 °C. SWCNT-nox also demonstrates higher optimal temperature (50 °C) when compared with free nox (37 °C). Its activation energy E_a was calculated to be 35.45 kJ/mol (based on the Arrhenius plot), higher than that of free nox (26.41 kJ/mol). The thermal stability enhancement after immobilization may be due to the constraints on protein folding--SWCNTs may confine the enzyme into a comfortable size of space and restrict enzyme conformational mobility [1, 21]. High degree of SWCNT surface curvature could also enhance the enzyme stability [22]. Another factor that may improve SWCNT-nox stability is the high enzyme loading capacity of SWCNTs. It was reported previously that when enzyme loading is low, the enzyme tends to alter its 3D structure and maximize contacts with supporting materials, whereas with high enzyme loading, the contacts with the carriers become minimized and the enzyme tends to keep its configuration [23]. Because of the spatial confinement, SWCNT surface curvature and high enzyme loading capacity, nox might minimize its structure change

even under extreme conditions and therefore demonstrates much better stability than free nox at high temperatures.

The operational stability of both free nox and SWCNT-nox was found to be limited by catalytic turnover. Both SWCNT-nox and free nox enzyme show excellent operational stability indicated by total turnover numbers (TTN) above 90,000 (Figure 3-6C). Similar TTN between the two indicate that the operational stability is not affected by the supporting material SWCNTs but rather by the enzyme itself. Previous work on nox (*Salmonella typhimurium*) suggested that over-oxidation of the Cys residue at the enzyme active site could lead to enzyme inactivation [24]. We also found that exogeneous reductive reagent dithiothreitol (DTT) had negligible effect on TTN, which may be due to a second thiol acting as a stabilizing nucleophile at the enzyme active site.

Figure 3-6 Free nox and SWCNT-nx stability. (A) Storage stability of free nox and SWCNT-nx at 4 °C (left) and 20 °C (right). Free nox at 4 °C (-△-), free nox at 20 °C (-□-), SWCNT-nx at 4 °C (-▲-), and SWCNT-nx at 20 °C (-■-). (B) Thermal stability of free nox (-■-) and SWCNT-nx (-□-). Optimum temperature: 37 °C (free nox) and 50 °C (SWCNT-nx). (C) TTN of free nox (red bar) and SWCNT-nx conjugate (blue bar) with/without 5 mM DTT in 50 mM pH 7.0 PPB at 30 °C.

3.3.5 SWCNT-nox regeneration

We further demonstrated that our immobilization method is reversible and can retain high enzyme activity after enzyme reloading. “Old” nox could be completely eluted off from SWCNT-ANTA- Co^{2+} complex by incubating in imidazole solution. SWCNT-ANTA- Co^{2+} complex showed no activity after nox removal, and the enzyme activity was only found in the elution solution. “New” nox enzyme could be loaded easily onto the recovered SWCNT-ANTA- Co^{2+} complex. Figure 3-7A illustrates that this process has been repeated twice. Reusability of the same SWCNT support and that the activity of the freshly loaded enzyme at each cycle is ca. 92%, and the loading capacity of SWCNT-ANTA- Co^{2+} complex also remains above 90%. SDS-PAGE results (Figure 3-8) demonstrate that the interaction between nox and SWCNT-ANTA- Co^{2+} complex was specific during reloading. The amount of the eluted nox was almost the same as the nox immobilized. This indicates that SWCNT-ANTA- Co^{2+} complex can be reused in enzyme immobilization process with significant cost saving.

Moreover, SWCNT-ANTA- Co^{2+} complex can be regenerated when its loading capacity is deteriorating. As illustrated in Figure 3-1, “old” Co^{2+} can be removed by ethylenediaminetetraacetic acid (EDTA), and “new” Co^{2+} can be reloaded by incubating SWCNT-ANTA in CoCl_2 solution. The loading residue of the regenerated SWCNT-ANTA- Co^{2+} complex was found to be 80%, and the enzyme activity residue was 86% (Figure 3-7B). The decrease in loading and activity may be due to the Co^{2+} loss during the regeneration process.

Figure 3-7 SWCNT-nox regeneration. (A) Activity (red) and loading residue (blue) of SWCNT-nox during the reloading cycles. The conjugate showed less than 1% activity and loading residue after imidazole elution. Activity was measured in 50 mM, pH 7.0 PPB with 24 μ M FAD and 200 μ M NADH at 30 °C. (B) Activity (red) and loading residue (blue) of SWCNT-nox during Co²⁺ regeneration. The binding of Co²⁺ on the SWCNT-ANTA complex is repeated once upon removal with EDTA in 20 mM HEPES buffer

Figure 3-8 Nox specificity to SWCNT-ANTA-Co²⁺ complex during enzyme reloading. Lane A: protein weight marker; Lane B, D and F: cell lysate; Lane C: elution sample from the SWCNT-nox during the first loading; Lane E: elution sample from the SWCNT-nox during the second loading; Lane G: elution sample from the SWCNT-nox during the third loading.

3.3.6 Reusability of SWCNT-nox

The reusability measurement is to assess the industrial application potential of SWCNT-nox and sepharose-nox. During ten rounds of recycling, SWCNT-nox always shows better activity retention than sepharose-nox (Figure 3-9). Specifically, even after 10 cycles SWCNT-nox could still retain ca. 70% of its initial activity, higher than that of sepharose-nox (~60%). The activity loss may be due to enzyme deactivation during recycling process [25].

Figure 3-9 Reusability study on residual activity of SWCNT-nox (red) and sepharose-nox (blue)

3.4. Conclusions

Our preliminary investigations indicate that other enzymes, such as the glycerol dehydrogenase (GlyDH) from *E. coli*, can be immobilized on SWCNTs with the same method as well. The resulting SWCNT-GlyDH conjugate can also maintain high native enzyme activity. This process opens up a simple route of enzyme immobilization on SWCNTs without costly enzyme purification. The application of immobilized enzymes could be used in bioreactors including fixed-bed PFR, fluidized bed reactor, tapered fluidized bed reactor, and continuous stirred-tank reactor (CSTR) with membrane module and recovered by filtration/centrifugation. Additionally, the reversible immobilization feature enables the reusability of the supporting materials without sacrificing on enzyme activity or loading capacity, which could significantly lower the cost in possible large scale applications.

3.5 References

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Chapter 4

Activity and stability comparison of immobilized NADH oxidase on multi-walled carbon nanotubes, carbon nanospheres, and single-walled carbon nanotubes

4.1 Introduction

In recent years nanotechnology-inspired immobilized enzyme systems have attracted a lot of attention and been applied in areas of biocatalysis [1], biofuel cell fabrication [2], and biosensor preparation [3]. Theoretically, nanomaterials can provide the upper limits in balancing the key factors that determine the performance of immobilized biocatalysts, including low mass transfer resistance, high surface area/volume ratios, and high enzyme loading capacity [4]. Additionally, enzyme unfolding is limited as nanomaterials confine enzyme molecules into a space of comparable size [5]. Nevertheless, common immobilization methods, which includes physical adsorption [6], covalent binding [7], entrapment [8] and encapsulation [9], often have constraints in enzyme leaching [10], three dimensional structure change [11] and mass transfer resistance [12]. In order to overcome the foresaid limitations, we developed an effective method to immobilize His-tagged enzyme onto functionalized single-walled carbon nanotubes (SWCNTs) previously [13]. The resulting SWCNT-enzyme conjugate demonstrated good enzyme activity retention and much better stability than the native enzyme.

Here in this work, we intend to apply the same immobilization method on other nanomaterials, including both multi-walled carbon nanotubes (MWCNTs) and carbon nanospheres (CNSs), and compare their activity and stability after immobilization.

We used the same enzyme, hydrogen peroxide-forming NADH oxidase (nox) from *B. cereus*, which has been cloned and characterized in Chapter 3, to facilitate the comparison. Many oxidoreductases that catalyze the redox reactions in organism require nicotinamide cofactors, such as L-glutamate dehydrogenase [14], L-amino acid dehydrogenase [15], and carbonyl reductase [16]. Nox, which catalyzes oxygen and NADH to hydrogen peroxide or water plus NAD^+ , is an excellent nicotinamide cofactor regenerating enzyme—nox uses oxygen as its substrate and the irreversible cofactor regeneration reaction can drive the production reaction to completion [17, 18]. We chose MWCNTs as our supporting materials since MWCNTs are structurally similar to SWCNTs, but their diameters can range from a few nanometers to dozens of nanometers. Compared to SWCNTs, MWCNTs are commercially available at relative lower price, which makes it more feasible for industrial applications [19]. CNSs were chosen as the enzyme supporting material because CNS can be easily prepared in a large scale from the hydrothermal treatment of glucose solutions under mild conditions [20, 21]. The as-prepared CNSs with their diameters tunable in the range of 200-800 nm possess abundant surface functional groups such as $-\text{OH}$ and $-\text{COOH}$ for further chemical modification of other molecules [20, 22].

In this study, we modified both MWCNT and CNS with N_α, N_α -bis(carboxymethyl)-L-lysine hydrate (ANTA) by a similar method as reported previously (reference) and immobilized the His-tagged nox based on the specific interaction between the His-tag and the Co^{2+} terminated nitrilotriacetate groups of the supporting nanomaterials. We then compared activity, storage stability, thermal stability, and operational stability of different nanomaterial-enzyme conjugates. It was found that the size difference of the supporting materials would play an important role in the activity and stability of the nanoscale biocatalyst.

4.2 Materials and Methods

4.2.1 Materials

Bradford reagent, cobalt (II) chloride, N_{α},N_{α} -bis(carboxymethyl)-L-lysine hydrate (ANTA), nitric acid, tryptone, 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide (EDC), sulfuric acid, *N*-hydroxysuccinimide (NHS), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), ampicillin sodium salt, kanamycin sulfate, potassium phosphate dibasic, potassium phosphate monobasic, isopropyl β -D-thiogalactopyranoside (IPTG), potassium bromide and sodium chloride were purchased from Sigma-Aldrich (Singapore). Dithiothreitol (DTT), β -Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), flavin adenine dinucleotide (FAD), urea, calcium chloride, rubidium chloride, manganese chloride tetrahydrate, and yeast extract were purchased from Merck (Singapore). FloTubeTM 9000 multi-walled carbon nanotubes were purchased from CNano Technology Ltd (China, D_{MWCNT} : 10~20 nm, surface area: 280 m²/g). CNSs were synthesized according to previously published methods [21, 22]. Briefly, 0.48 mM glucose solution was prepared and then transferred to a sealed Teflon-lined stainless steel autoclave at 180 °C for 8 h. When reaction finished, ethanol and distilled water were used to wash the black-brown precipitates several times and dried at 60 °C for 6 h.

4.2.2 Nox overexpression and purification

The detailed cloning, overexpression and purification process was described in our published article [13]. To be brief, *E. coli* BL21 (DE3) cell containing pET30b(+)-nox recombinant plasmid was cultured in Luria-Bertani (LB) broth with 30 μ g/mL kanamycin at 200 rpm, 37°C. Protein expression was induced by adding 200 μ M

IPTG when OD at 600 nm reached 0.6 ~ 0.8. After cell lysis by sonication, nox was purified and desalted by immobilized metal affinity chromatography (IMAC; Gravatrapp Ni²⁺ column, GE Healthcare, Singapore) and PD-10 desalting column (GE healthcare, Singapore). Nox purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and its concentration was measured after 5 min incubation with Bradford Reagent using Biophotometer

4.2.3 MWCNT and CNS modifications

Carboxylic acid groups were created on MWCNTs following a formerly reported protocol [23]. MWCNTs were treated with acid mixture (HNO₃ / H₂SO₄ = 1:3 v/v) in a water-bath sonicator at 40 °C for 3 h. The MWCNT suspension was filtered through a 0.2 µm nylon membrane and washed with 20 mM, pH 7.5 HEPES buffer. The MWCNT-COOH was suspended in HEPES buffer. Since CNSs already has carboxylic acid groups on its surface, the acid treatment is not necessary.

MWCNT-COOH dispersed in 25 ml HEPES buffer was first activated by 50 mM NHS and 20 mM EDC to form MWCNT-NHS ester complex. ANTA was dissolved in HEPES buffer with excess CoCl₂ to form ANTA-Co²⁺. Extra Co²⁺ was precipitated by NaOH and Co(OH)₂ was removed by centrifugation for 10 min at 5,000 × g. The supernatant containing ANTA-Co²⁺ was added to MWCNT-NHS ester complex to produce MWCNT-ANTA-Co²⁺ complex. The complex was filtered, washed extensively and stored in 20 mM, pH 7.5 HEPES buffer. The same procedure was employed to functionalize CNSs to obtain CNS-ANTA-Co²⁺.

4.2.4 MWCNT and CNS characterization

MWCNT and CNS samples at different stages during modification, as well as initial samples, were characterized by FTIR spectroscopy. MWCNT dispersions were filtered through 0.2 μm nylon membranes and CNSs samples were centrifuged at 12,000 $\times$ g. MWCNTs and CNSs were collected by filtration and centrifugation, respectively, and both were dried in a 100°C oven. The dried samples were mixed with 100 mg KBr powder, and pressed into thin films for FTIR measurement (FTS 3100, DIGILAB, USA). The IR spectra were averaged over 96 scans from 1000 to 4000 cm^{-1} . The spectra were blank subtracted and the baseline was corrected by Revolution 4.0 Pro software.

4.2.5 Enzyme immobilization

MWCNT/CNS-ANTA- Co^{2+} complex were incubated with cell lysate at 4 °C overnight. MWCNT/CNS-ANTA- Co^{2+} -nox conjugates (MWCNT/CNS-nox) were collected by centrifugation and washed with 20 mM, pH 7.4 HEPES containing 20 mM imidazole three times. MWCNT/CNS-nox were then suspended in 20 mM, pH 7.5 HEPES. In order to measure enzyme loading capacity, free nox was eluted from MWCNTs and CNSs with 20 mM, pH7.4 HEPES buffer containing 500 mM NaCl and 500 mM imidazole and the protein concentrations were measured by Bradford assay.

4.2.6 Conjugate activity assay

MWCNT-nox and CNS-nox activity was measured by monitoring the decrease of NADH absorbance at 340 nm (ϵ : 6220 $\text{M}^{-1}\text{cm}^{-1}$) using a DU-800 spectrophotometer (Beckman Coulter, Singapore). The standard conditions were set as follows: 20 μM

FAD (MWCNT-nox)/44 μ M FAD (CNS-nox) in air-saturated 50 mM, pH 7.0 PPB with 200 μ M NADH at 30°C. Both K_M and V_{max} values were determined under standard conditions with NADH concentration varying from 0 to 400 μ M.

4.2.7 Enzyme stability measurement

4.2.7.1 Effect of temperature

The effect of temperature was investigated by changing reaction medium temperature between 20 and 90 °C. The activity of both MWCNT-nox and CNS-nox was measured under standard conditions. The relative activity of both conjugates was calculated by assigning the maximum value of the specific activity as 100%.

4.2.7.2 Storage stability

The storage stability of both MWCNT-nox and CNS-nox was investigated at 4 °C. The activity of MWCNT-nox and CNS-nox was measured at regular time interval under standard conditions with 200 μ M NADH.

4.2.7.3 Reusability

The reusability of MWCNT-nox and CNS-nox was measured by their residual activity at standard conditions. After each reaction, MWCNT-nox and CNS-nox were separated by centrifugation and then washed three times by 20 mM, pH 7.5 HEPES.

4.2.8 Enzyme deactivation assay

The deactivation of free nox, SWCNT-nox, MWCNT-nox and CNS-nox was tested at 50 °C and 90 °C in 50 mM, pH 7.0 PPB, 50 μ M FAD. Residual activity of each

sample was determined with 200 μ M NADH as described above and calculated by the ratio of the residual enzyme activity to its initial activity.

4.2.9 Total turnover number measurement

A specific amount of MWCNT-nox or CNS-nox was added to the air-saturated 50 mM, pH 7.0 PPB buffer with/without 5 mM DTT at 30 $^{\circ}$ C. NADH was continuously added during the measurement until the enzyme could not react any longer [17, 18].

4.3 Results and Discussion

4.3.1 MWCNT-nox and CNS-nox

The scheme of enzyme immobilization on MWCNTs and CNSs is illustrated in Figure 4-1A and Figure 4-1B, respectively. We functionalized both MWCNT and CNS surface with a Co^{2+} terminated nitrilotriacetate group that could facilitate the binding of His-tagged NADH oxidase. Pristine MWCNTs was treated with acid mixture of HNO_3 and H_2SO_4 , and the activated MWCNTs and CNSs were then amidated by *N*-hydroxysuccinimide (NHS)-esters with ANTA- Co^{2+} . The functional chemical groups on both MWCNT-ANTA- Co^{2+} and CNS-ANTA- Co^{2+} complex were confirmed by Fourier transformed infrared spectroscopy (FTIR), respectively. The FTIR spectra of both complex display N-H ($3200\text{-}3300\text{ cm}^{-1}$), C-H ($2850\text{-}3050\text{ cm}^{-1}$), C=O ($1550\text{-}1750\text{ cm}^{-1}$) and C-O ($1200\text{-}1300\text{ cm}^{-1}$) peaks as expected from the chemical structure (Figure 4-2A and Figure 4-2B). MWCNT-nox and CNS-nox were constructed by incubating MWCNT-ANTA- Co^{2+} and CNS-ANTA- Co^{2+} complex with cell lysate containing His-tagged nox, respectively. In order to verify enzyme

binding specificity to the functionalized nanomaterials, the bound enzyme was eluted off MWCNT-nox and CNS-nox by imidazole and the elution was checked with SDS-PAGE, respectively. Both samples show one single band on SDS-PAGE (Figure 4-3A Lane H & Figure 4-3B Lane H), which indicates that enzyme binding is specific. The amount of nox in the original cell lysate was found decreased after incubating with either MWCNT-ANTA- Co^{2+} or CNS-ANTA- Co^{2+} complex.

The loading capacity was found to be 0.196 mg enzyme/mg MWCNTs and 0.052 mg enzyme/mg CNSs, respectively. The enzyme loading capacity of modified MWCNTs is similar to the published loading capacity of MWCNTs using covalent binding method (0.172~0.203 mg enzyme/mg MWCNTs) [23].

Figure 4-1 Scheme of specific immobilization of NADH oxidase (*B. cereus*) on functionalized (A) MWCNTs and (B) CNSs.

Figure 4-2 FTIR spectra of the stepwise modification of MWCNTs and CNSs. (A) FTIR spectrum of MWCNT modification. (1) Spectrum of pristine MWCNTs. (2) Spectrum of MWCNT-COOH. Peaks at 3438, 1697, and 1566 cm^{-1} correspond to hydroxyl groups, carboxyl groups and carbonyl groups. (3) Spectrum of MWCNT-ANTA- Co^{2+} complex with N-H (3211 cm^{-1}), C-H (3029 and 2962 cm^{-1}), C=O (1735 and 1642 cm^{-1}) and C-O (1238 cm^{-1}) respectively. (B) FTIR spectrum of CNS modification. (1) Spectrum of CNS-ANTA- Co^{2+} complex with N-H (3319 cm^{-1}), C-H (2926 and 2847 cm^{-1}), C=O (1626 and 1588 cm^{-1}) and C-O (1256 cm^{-1}) respectively. (2) Spectrum of CNS-COOH. Peaks at 3424, 1706, and 1615 cm^{-1} correspond to hydroxyl groups, carboxyl groups and carbonyl groups.

Figure 4-3 SDS-PAGE of nox overexpression and immobilization on (A) MWCNTs and (B) CNSs. Lane A: standard protein marker; Lane B: overnight cell culture; Lane C: uninduced cell culture; Lane D: cell after induction; Lane E: cell lysate; Lane F: purified nox; Lane G: cell lysate after incubation with MWCNT/CNS-ANTA-Co²⁺ complex; Lane H: imidazole elution sample.

4.3.2 Kinetic comparison

Retention of native enzyme activity is one of the most important features to evaluate the immobilization process. We found that the $V_{\max}$ values of MWCNT-nox and CNS-nox were 23.5 U/mg and 21.5 U/mg, respectively, corresponding to 87% and 78% that of the native enzyme activity (Table 4-1 & Figure 4-4). The controls (nox exposed to MWCNT-COOH and CNS-COOH), on the other hand, did not show any activity at all, implying no non-specific adsorption of nox on these two complexes. As demonstrated in our previous work, a similar enzyme immobilization method was also applied on SWCNT, and the resulting SWCNT-nox was found to retain 92% of native nox maximum activity [13]. These results suggest that this site-specific immobilization method can be successfully applied to different carbon nanomaterials with decent enzyme activity retention. We then compared substrate binding affinity through their K_M values. As shown in Table 1, CNS-nox exhibits much higher K_M value (88.7 μM) than MWCNT-nox (50.6 μM) and SWCNT-nox (53.4 μM), implying strong steric hindrance and diffusion limitation in CNS-nox [24, 25]. On the other hand, the K_M value of both MWCNT-nox and SWCNT-nox is close to that of native nox, implying negligible diffusion limitation in both cases. As the size of the supporting material increases, enzyme specificity (k_{cat}/K_M) and catalytic turnover k_{cat} also decrease. The increase in supporting material size (D_{SWCNT} : 0.8~1.2 nm, D_{MWCNT} : 10~20 nm and D_{CNS} : 741 ± 28 nm) often comes with increase in K_M and decrease in $V_{\max}$ as demonstrated previously [26], which is possibly due to the mobility decrease of immobilized enzyme with increased material size.

Figure 4-4 Lineweaver-Burk plots obtained from free nox (■), MWCNT-nox (▲), and CNS-nox (Δ) in air-saturated 50 mM, pH 7.0 PPB at 30 °C.

Table 4-1 Kinetic comparison between free and immobilized nox

Sample	K_M (μM)	V_{max} (U/mg)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1}\mu\text{M}^{-1}$)
Free nox	53.3	27.7	1648.4	30.9
SWCNT-nox	53.4	25.7	1539.7	28.8
MWCNT-nox	50.6	23.5	1412.3	27.9
CNS-nox	88.7	21.5	1289.4	14.5

4.3.3 Storage and thermal stability

One of the major advantages of enzyme immobilization is its stability improvement. We first examined the storage stability of both MWCNT-nox and CNS-nox at 4 °C over a period of ~1000 h. As shown in Figure 4-5, both immobilized enzymes exhibit better storage stability than native enzyme, which is similar to the case of SWCNT-nox. The estimated half-lives of MWCNT-nox and CNS-nox are around 800 h and 450 h, respectively, which are higher than free nox (~300 h).

The temperature effect on free and immobilized nox is shown in Figure 4-6. Both MWCNT-nox and CNS-nox have the similar bell shaped temperature profiles as SWCNT-nox. Nox immobilization on nanomaterials results in thermal stabilization of nox conjugates, as reflecting in the elevated optimal temperature (T_{opt}) and broader temperature range. The T_{opt} of MWCNT-nox and CNS-nox can be achieved at 50 °C and 60 °C, respectively, which are higher than that of free nox. Free nox can only tolerate temperature up to 70 °C, whereas all of the immobilized enzyme can still survive. Even when temperature goes up to 90 °C, MWCNT-nox and CNS-nox can still have around 45% residual activity. These results indicated that MWCNTs and CNSs are similar to SWCNTs in preserving enzyme activity in a denaturing environment.

To assess the influence of different nanomaterials on thermal stability, we also studied the deactivation of free nox and immobilized enzyme at 50 °C and 90 °C. We found that all of the immobilized enzymes are remarkably more stable than native

enzyme (Figure 4-7A). The estimated half-lives ($\tau_{1/2}$) of both SWCNT-nox and MWCNT-nox at 50 °C are above 90 min, while that of CNS-nox is around 55 min, all higher than that of native enzyme (~25 min). We proceeded to examine the stability of immobilized enzyme at extreme condition of 90 °C, where native nox is completely deactivated (Figure 4-7B). SWCNT-nox displays the best thermal stability among all immobilized enzymes with the half-life of ~60 min; double that of MWCNT-nox (~30 min) and 12-fold higher than that of CNS-nox (~5 min).

We notice that the stability of SWCNT-nox is better than those of MWCNT-nox and CNS-nox. The discrepancy in stability among three conjugates may be attributed to the lateral interactions between adjacent enzyme molecules [27]. The interactions might induce enzyme denaturation since enzyme molecule on the surface of supporting material would interact with the adjacent molecule at high surface coverage [28] and enzyme secondary structure could be altered with the increase in enzyme concentration (Γ_{abs} , mg/m²) [27]. Compared with SWCNT-nox, nox attached on MWCNTs and CNSs shows higher surface coverage and thus a higher Γ_{abs} (Table 4-2), suggesting possible higher lateral interactions occurring between the neighboring nox molecules on MWCNTs and CNSs. Moreover, the lateral interactions could be suppressed by highly curved supporting materials [29]. A smaller diameter of supporting material implies a larger surface curvature between adjacent nox molecules. Among three different supporting materials, SWCNTs has the smallest diameter (0.8~1.2 nm) compared with MWCNTs (10~20 nm) and CNSs (741 ± 28 nm). As a result, the high surface area and surface curvature of SWCNTs may lead to

the lowest lateral interactions between adjacent nox molecules and thus SWCNT-nox has the best stability.

Figure 4-5 Storage stability of free nox, MWCNT-nox, and CNS-nox in 20 mM, pH 7.5 HEPES buffer at 4°C. (■) free nox, (▲) MWCNT-nox, and (Δ) CNS-nox

Figure 4-6 Temperature profiles of (A) MWCNT-nox and (B) CNS-nox. Optimum temperature: 50 °C (MWCNT-nox) and 60 °C (CNS-nox).

Table 4-2 Loading capacity and surface coverage comparison

Samples	SWCNT-nox	MWCNT-nox	CNS-nox
Loading capacity (mg enzyme/mg material)	0.471	0.196	0.052
Surface area (m ² /g)	1315	280	58.9
Γ_{abs} (mg/m ²)	0.356	0.712	0.883
Surface coverage (%)	24.9	49.8	62.8

Figure 4-7 Thermostability of free nox, SWCNT-nox, MWCNT-nox, and CNS-nox in 50 mM, pH 7.0 PPB at (A) 50 °C and (B) 90 °C. (■) free nox, (□) SWCNT-nox, (▲) MWCNT-nox, and (Δ) CNS-nox.

Table 4-3 Nox half-life comparison on the various supporting materials

	Half life			
	4 °C (h)	20 °C (h)	50 °C (min)	90 °C (min)
SWCNT-nox	1500	100	90	60
MWCNT-nox	800	75	90	30
CNS-nox	450	55	55	5

4.3.4 Operational stability

The operational stability of native enzyme and immobilized enzymes was found to be limited by enzyme catalytic turnover. We evaluated the operational stability by Total Turnover Number (TTN). As shown in Figure 4-8, the TTN of free nox, MWCNT-nox and CNS-nox are very similar to each other ($\sim 9 \times 10^4$), suggesting all have excellent operational stability and different supporting materials do not affect enzyme operation stability, i.e. TTN is determined by enzyme itself. These findings are consistent with the findings of SWCNT-nox [13]. Previous work on nox from *S.typhimurium* suggested that the over-oxidation of the cysteine residue at the nox active site may cause enzyme inactivation. Exogenously added reductive reagent DTT shows no improvement on the enzyme TTN in all cases, which may be due to a second thiol acting as a stabilizing nucleophile at the enzyme active site [30]

4.3.5 Reusability

Enzyme immobilization makes it feasible to reuse enzymes since it can be easily recovered from reaction medium. The study reveals that the residual activity of immobilized nox decreases when the recycle number increases (Figure 4-9). After ten cycles, the residual activity of MWCNT-nox is around 65%, close to that of SWCNT-nox (as shown previously), while the activity of CNS-nox drops to 55%. The drop in enzyme activity may be due to the deactivation of nox which are relatively densely packed on the surface of CNSs during batch operation [31].

Figure 4-8 TTN of free nox, MWCNT-nox, and CNS-nox with (red bar)/without 5 mM DTT (blue bar) in 50 mM pH 7.0 PPB at 30 °C.

Figure 4-9 Reusability of MWCNT-nox (■) and CNS-nox (Δ).

Table 4-4 Economics comparison between all three immobilized nox

	SWCNT-nox	MWCNT-nox	CNS-nox
Specific Activity (U/mg)	25.7	23.5	21.5
Loading capacity (mg/mg)	0.47	0.196	0.052
Cost of material (SGD/g)	520.7 ^{b,e}	12.2 ^{c,e}	30.4 ^d
Cost of material per 1000 U immobilized nox (SGD) ^a	43	2.6	27

a. Condition: 50 mM, pH 7.03 PPB at 30 °C

b. Price from Carbon Solution, US

c. Price from CNano Technology, China

d. Price from Sigma-Aldrich, Singapore

e. Exchange rate (USD to SGD and RMB to SGD) based on Currency (Dec 24th, 2010)

4.4 Conclusions

Our investigations indicate that other nanomaterials of lower costs, such as MWCNTs and CNSs, can be used as supporting materials to immobilize enzymes with the same method as well. The resulting MWCNT-nox and CNS-nox conjugate can also maintain high native enzyme activity. The results validate that enzyme immobilization by His-tag can be broadly used not only on SWCNTs, which opens up a gate to apply this method to large-scale preparation without costly enzyme purification. It is also shown that the material properties, such as surface area and surface curvature, affect the performance of immobilized enzyme. The similar immobilization method may also be applied to other nanomaterials with surface functional groups.

We also compare the economics of nox immobilization process using SWCNTs, MWCNTs, and CNSs. The unit price of carbon nanopowder (D: ~500 nm, Cat. no.: 699624, Sigma-Aldrich) stands for CNSs, the most similar products we could find from Internet. As shown in Table 4-4, when we need 1000 U of immobilized nox, MWCNTs is the most economic supporting material to immobilize nox, which is attributed to the balance among material cost, activity retention, and loading capacity. Moreover, the reusability of MWCNT-nox is better than that of CNS-nox described in section 4.3.5. However, the real cost of CNSs should be much cheaper than the price we obtained from Sigma-Aldrich as CNSs could easily be prepared in the large scale with a simple substrate under the mild conditions. Although the cost of using

SWCNTs is the much more expensive than that of MWCNTs and CNS, SWCNT-nox exhibits the best performance in our investigations. Therefore, we will use SWCNTs as the supporting materials in the following investigations.

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Chapter 5

Characterization of immobilized multi-enzyme alkyl hydroperoxide reductase system on functionalized single-walled carbon nanotubes

5.1 Introduction

Many studies have employed enzymes in various chemical transformations because of their high activity, specificity, and selectivity [1, 2]. Biotransformation often involve either a mono-enzymatic system that conducts one specific bioconversion at a time or a multi-enzymatic system that carries out consecutive enzymatic reactions to yield diverse products such as alcohols, amines, and enantiomerically pure compounds [3, 4]. An efficient and clean multi-enzymatic system can be used in either multi-step complex synthetic routes via metabolic networks *in vivo* or isolated biocatalysts *in vitro*, such as the enzymatic production of methanol from CO₂ [5], the conversion of L-asparagine to glutamate [6], and conversion of different phenylpropionicacids to stilbene and flavonoid compounds [7].

The use of cell-free multi-enzymatic systems has several advantages compared to whole-cell systems [8, 9]: i) the reduced complexity and ease of reaction control, including optimization of biocatalyst and substrate concentrations, change of reaction mediums, and variation of enzyme ratios; ii) it is not necessary to consider the substrate toxicity to the system; and iii) the higher purity of final products because of the absence of side-reactions and metabolite contaminations. However, the relatively

low stability of isolated biocatalysts often hampers the application to large-scale biocatalytic process [10].

Enzyme immobilization on nanoscale supporting materials allows the stability of biocatalysts to be remarkably increased with activity retention as well as easy recovery from the reaction medium [11]. In recent years, multi-enzymatic systems immobilized on nanomaterials have attracted much interest [12-15]. Nonetheless, the difficulty lies in the loss of enzyme activity with nonspecific immobilization approaches, such as adsorption, entrapment and covalent binding, which often cause enzyme leaching [16], enzyme 3D structure change [17], and mass-transfer resistance [18]. Our lab has previously demonstrated an efficient enzyme immobilization method based on the specific interaction between His-tagged enzyme and N_{α},N_{α} -bis(carboxymethyl)-L-lysine hydrate (ANTA) functionalized SWCNTs, MWCNTs and CNSs. The performance of these immobilized enzymes overcomes the foresaid limitations and shows outstanding activity retention [19]. From the observations in Chapter 4, SWCNT-nox shows the best activity retention and stability improvement among all nox-nanostructures we have tested, probably owing to the small diameter of SWCNTs and low enzyme surface coverage. Therefore, SWCNTs have been chosen as the supporting material in the following investigations.

In this study, we will apply the same method on alkyl hydroperoxide reductase (AhpR), which is composed of two enzymes— H_2O_2 -forming NADH oxidase (nox, encoded by *ahpF*) and peroxidase (encoded by *ahpC*) [20]. AhpR can protect strains

from the toxic effects caused by organic hydroperoxides. It has been found in many anaerobic bacteria such as *Salmonella typhimurium* (*S. typhimurium*) [21], *Streptococcus mutans* (*S. mutans*) [22], *Amphibacillus xylanus* (*A. xylanus*) [23], and *Lactococcus lactis* (*L. lactis*) [20]. The reactions catalyzed by AhpR are as follows and the overall reaction of AhpR is identical to the water-forming NADH oxidase when oxygen is the substrate [24, 25].

Previous work on AhpR proved that AhpC has no activity when nox is absent [26]. A proposed mechanism of AhpR (*S. typhimurium*) demonstrates that AhpC requires electron transport from the N-terminal disulfide bond of nox to be active [23]. Here, we proposed to apply the non-covalent specific immobilization method used in the mono-enzyme system to the consecutive biocatalytic reaction system of nox/AhpC with supporting materials SWCNTs. Compared with other bienzymatic systems, AhpC is not an independent enzyme—it obtains electrons from nox and reduces the intermediate generated by nox. The collision between the two immobilized enzymes is expected to enable desired consecutive biotransformation reactions. The overall AhpR activity and stability, nox/AhpC ratios, and the H₂O₂ amount during reaction were investigated.

5.2 Materials and methods

5.2.1 Materials

Bradford reagent, ampicillin, kanamycin, potassium phosphate, sodium chloride, tryptone, cobalt chloride, nitric acid, sulfuric acid, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), N_{α},N_{α} -bis(carboxymethyl)-L-lysine hydrate (ANTA), 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide (EDC), N -hydroxysuccinimide (NHS), N -(2-hydroxyethyl) piperazine- N' -(2-ethanesulfonic acid) (HEPES), and isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. Dithiothreitol (DTT), β -Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), and flavin adenine dinucleotide (FAD) were from Merck. Low melting agarose was obtained from Nusieve (Research Biolabs, Singapore). *Taq* DNA polymerase and T4 DNA ligase were from New England Biolabs (Research Biolabs, Singapore). Restriction endonucleases *Bgl*II and *Xho*I were purchased from Fermentas (BST Scientific, Singapore). Super purified HiPco[®] Single-walled carbon nanotubes were purchased from Unidym (USA, D : 0.8–1.2 nm, length: 100–1000 nm, surface area per unit mass: 1315 m²/g).

5.2.2 *ahpC* cloning, overexpression and purification

The *ahpC* gene from *B. cereus* ATCC 14579 was amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase with primers 5'-AGATCTGACCATGGCGATGTTATTAATCGGCACAGAAGTAA-3' and 5'-CTCGAGGGATCCCTATTAGATTTTGCCTACAAGGTCAAG-3'. The PCR

products were purified by Gel Extraction Kit (QIAGEN) and then cloned into pDrive vector with blue/white screening features (PCR Cloning kit, QIAGEN, Research Biolabs, Singapore). Recombinant plasmid pDrive-*ahpC* was obtained by miniprep (QIASpin Miniprep kit, QIAGEN), and checked with DNA sequencing. The plasmid was then digested by restriction endonucleases *Bgl*II and *Xho*I, cloned to pET-30b (+) (Novagen) to construct pET30b-*ahpC*, and transferred into *E.coli* BL21(DE3) competent cells (Stratagene, ITS). AhpC was overexpressed at 37°C with 200 µM IPTG when absorbance reached 0.6~0.8 at 600 nm. After 3-h overexpression, cell pellet was collected by centrifugation and lysed in 50 mM, pH 7.0 HEPES buffer by sonication at 30 s × 8 with 30 s intervals. AhpC was purified by immobilized metal affinity chromatography using Gravatrap Ni²⁺ column (IMAC, GE Healthcare) and desalted by PD-10 column (GE Healthcare) according to manufacturer's instructions. The purity of AhpC was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration of AhpC was measured after 5 min incubation with Bradford Reagent using Biophotometer (Eppendorf). Nox (hydrogen peroxide-forming) from *B. cereus* was also involved in the experiment and the cloning, expression, and purification strategies have been previously described in section 3.2.2.

5.2.3 SWCNTs modification

SWCNTs were first treated with HNO₃/H₂SO₄ mixture (HNO₃/H₂SO₄ = 1 : 3), activated by NHS/EDC in 20 mM, pH 7.5 HEPES buffer, and reacted with ANTA-

Co²⁺, according to a previously reported protocol [19]. SWCNTs modification was detected by Fourier transform infrared spectroscopy (FTIR). Functionalized SWCNT samples were filtered with 0.2 µm nylon membrane and wash by distilled water for three times. Samples were dried in a 100 °C oven and pressed into thin films with KBr powder. FTIR spectra were obtained by using a FTS 3100 (DIGILAB, USA) instrument. The IR spectra were averaged over 96 scans from 1000 to 4000 cm⁻¹.

5.2.4 Enzyme immobilization

The cell lysis was carried out in 20 mM, pH 7.5 HEPES buffer by sonication (30 s × 8) and centrifuged at 30, 000 × g for 30 min. The supernatant was incubated with SWCNT-ANTA-Co²⁺ complex at 4 °C overnight. SWCNT-ANTA-Co²⁺-AhpC conjugate (SWCNT-AhpC) was purified and washed with 20 mM, pH 7.5 HEPES buffer containing 20 mM imidazole for three times. SWCNT-AhpC was suspended in 20 mM, pH 7.5 HEPES buffer. Nox was also immobilized to SWCNT-ANTA-Co²⁺ complex using the same immobilization procedure and the detailed information referred to section 3.2.6. In this study, nox and AhpC are separately immobilized on SWCNT-ANTA-Co²⁺ complex in the following experiments.

5.2.5 Circular dichroism

The secondary structure of free AhpC and SWCNT-AhpC was detected by circular dichroism spectroscopy (CD). CD measurement was carried out by using a ChiraScan circular dichroism spectrometer (Applied Photophysics, United Kingdom) with constant N₂ flushing. The far-UV CD spectra (200~260 nm) were obtained in 10 mM,

pH 7.0 potassium phosphate buffer containing ~0.34 mg/ml enzyme in a 1-mm path length quartz cuvette at room temperature. Each spectrum is obtained from the average reading from three consecutive scans. The contents of α -helix and β -sheet were calculated based on the mean residue ellipticity at 222 and 218 nm. The spectrum of SWCNTs was also acquired under the same conditions.

5.2.6 AhpR activity with different nox/AhpC ratio

The activity of free and immobilized AhpR system was measured by tracking NADH absorbance decrease at 340 nm (ϵ : 6220 M⁻¹cm⁻¹) with a DU 800 spectrophotometer (Beckman Coulter, USA). Standard conditions include air-saturated 50 mM, pH 7.0 potassium phosphate buffer (PPB) containing 200 μ M NADH and 8 μ M FAD (free nox) / 23.95 μ M (SWCNT-nox) at 30 °C. Four combinations of AhpR system (nox/AhpC, SWCNT-nox/AhpC, nox/SWCNT-AhpC, and SWCNT-nox/SWCNT-AhpC) with different nox/AhpC molar ratios (100 : 1, 10 : 1, 1 : 1, 1 : 30, and 1 : 50) was tested under standard conditions. In this study, the activity of free nox is assigned to 100%. “Relative activity” is defined as the activity of AhpR system over the activity of equal amount of free nox.

5.2.7 Enzyme stability measurement

5.2.7.1 Storage stability

Storage stability of free AhpC and SWCNT-AhpC was examined at 4 °C and 20 °C over 1000 h. The activity of both free and immobilized AhpC was measured under standard conditions with the molar ratio of nox/AhpC at 1:1.

5.2.7.2 Total turnover number

Four combinations of AhpR system with different nox/AhpC molar ratios (100 : 1, 10 : 1, 1 : 1, and 1 : 30) were added into the air-saturated 50 mM, pH 7.0 PPB buffer with/without 5 mM DTT at 30 °C. NADH was added until AhpR could no longer catalyze the reaction [27].

5.2.8 Amplex red assay

The oxidation of 9-acetylresorufin (Amplex Red, Molecular Probe, Invitrogen, Singapore) catalyzed by horseradish peroxidase (HRP) was used to detect H₂O₂ quantitatively. The reaction is carried out according to a strict 1:1 stoichiometry between H₂O₂ and Amplex Red, and generates the fluorescent product resorufin (ϵ : 54,000 M⁻¹cm⁻¹, excitation: 530 nm, and emission: 590 nm). Samples from free nox, SWCNT-nox, and four combinations with different nox/AhpC molar ratios (1: 1, 1: 10, 1: 50, and 1: 100) were pipetted into a 96-well microplate after reacting with 200 μ M NADH under standard conditions, respectively. Working solution (50 μ l) containing Amplex Red reagent and HRP was subsequently added to the 96-well

microplate and the reaction lasted for 30 min in dark at room temperature. The fluorescence of resorufin was detected by a microplate reader (Tecan Infinite[®] 200 Pro, SciMed, Singapore).

5.3 Results and discussion

5.3.1 Cloning, overexpression and purification of AhpC

The *ahpC* gene has been successfully amplified by PCR with genomic DNA from *B.ceresus* as template. Nucleotide sequence of *ahpC* was confirmed by DNA sequencing, showing one silent mutation (GAA to GAG) at position Glu164 when compared to the annotated gene sequence from NCBI. Overexpressed AhpC reveals prominent band on SDS-PAGE (Figure 5-1A Lane C), and there are no other unspecific proteins after purification (Figure 5-1A Lane F). The open reading frame (ORF) of AhpC encodes the enzyme with a molecular weight of ~25 kDa.

5.3.2 SWCNTs modification and enzyme immobilization

SWCNTs were treated with acid mixture in the presence of NHS/EDC, and functionalized by nitrilotriacetate group terminated with Co²⁺. The resulting complex enables the specific attachment of His-tagged AhpC. FTIR spectra were used to confirm the functionalized SWCNTs. As shown in Figure 5-2, SWCNT-COOH shows typical -OH (3421 cm⁻¹) and C=O (1620 cm⁻¹) peaks, and the spectrum of SWCNT-ANTA-Co²⁺ complex includes peaks of N-H (3348 cm⁻¹), C-H (3026 cm⁻¹), C=O (1610 cm⁻¹), and C-O (1348 cm⁻¹) as expected.

The immobilization specificity of AhpC was confirmed by SDS-PAGE. We found that AhpC in the cell lysate could bind to SWCNT-ANTA-Co²⁺ complex specifically (Figure 5-1A Lane I), not to the control SWCNT-COOH (Figure 5-1A Lane H). After immobilization, the amount of AhpC in cell lysate decreases dramatically (Figure 5-1B Lane C) compared with the original cell lysate (Figure 5-1A Lane B). These results indicate that His-tagged AhpC can be specifically immobilized on SWCNT-ANTA-Co²⁺ complex.

In order to determine the loading capacity of AhpC, we investigated three different AhpC to SWCNTs ratios (5:1, 10:1, and 15:1, w/w). The immobilization capacity was found to be ~0.78 mg enzyme/mg SWCNTs at all ratios examined.

5.3.3 Circular dichroism

To assess the secondary structure of AhpC after immobilization, we used CD spectroscopy. Figure 5-3 displays the CD spectra of free AhpC, SWCNT-AhpC, and modified SWCNTs in the far-UV region. We verified that modified SWCNT did not contribute to the CD spectra of SWCNT-AhpC. The mean residue ellipticity at 222 nm and 218 nm is used to quantify α -helix and β -sheet content. The α -helix or β -sheet content of SWCNT-AhpC is similar as native AhpC (Table 5-1), reflecting that the attachment of AhpC to modified SWCNTs does not change the protein secondary structure. The retention of AhpC conformation may be attributed to the specific interaction between His-tagged enzyme and SWCNT-ANTA-Co²⁺ complex.

Figure 5-1 SDS-PAGE of AhpC overexpression, purification and immobilization. (A) AhpC from overexpression to immobilization. Lane A: protein weight marker; Lane B: uninduced cell culture; Lane C: cell culture induced by IPTG; Lane D: cell lysate; Lane E: insoluble part; Lane F: purified AhpC; Lane G: cell lysate; Lane H: imidazole elution sample from control (SWCNT-COOH and AhpC mixture); Lane I: imidazole elution sample from SWCNT-AhpC. (B) Protein content in cell lysate. Lane A: protein weight marker; Lane B: original cell lysate; Lane C: cell lysate after incubation with SWCNT-ANTA- Co^{2+} complex. This figure shows that the amount of overexpressed AhpC in cell lysate obviously reduced after incubation with SWCNT-ANTA- Co^{2+} complex.

Figure 5-2 FTIR spectra of SWCNT stepwise modifications. (1) Spectrum of the SWCNT-ANTA-Co²⁺ complex. Peaks at 3348, 3026, 1610, and 1348 cm⁻¹ correspond to -NH, -CH, C=O, and C-O, respectively. (2) Spectrum of SWCNT-COOH. Peaks at 3433 and 1643 cm⁻¹ correspond to -OH and C=O. (3) Super purified SWCNTs.

Figure 5-3 Circular dichroism spectra of free AhpC (■), SWCNT-AhpC (▲), and modified SWCNTs (□).

Table 5-1 Secondary structure of free AhpC and SWCNT-AhpC

Sample	α -helix (%)	β -sheet (%)
Free AhpC	30.6 ± 0.40	42.7 ± 0.75
SWCNT-AhpC	28.9 ± 0.91	41.6 ± 0.28

5.3.4 Activity with different nox/AhpC ratio

Activity retention is one of most important features to evaluate the immobilized enzyme. The activity retention of four combinations (nox/AhpC, nox/SWCNT-AhpC, SWCNT-nox/AhpC, and SWCNT-nox/SWCNT-AhpC) has been investigated with different nox/AhpC ratios (Figure 5-4). When the molar ratio of nox/AhpC is at 100:1 or 10:1, i.e., when nox is in large excess of AhpC, the relative activity of all four combinations is close to 100%, which suggests that nox dominates the activity of AhpR system as the amount of AhpC is too little to make any differences. The close relative activity of all combinations also implies that the activity of SWCNT-nox is similar to free nox, which is consistent with our previous findings [19].

The relative activity of all combinations increases from ~150% to ~200% when nox/AhpC ratio varies from 1:1 to 1:50. More AhpC in the AhpR system lead to increase in overall activity, which suggests that more electrons have been transferred from nox to AhpC and more hydrogen peroxide has been scavenged [21, 26]. Free AhpR system demonstrates the best activity among four combinations. After enzyme immobilization, SWCNT-nox/SWCNT-AhpC system can still keep ~ 87% of free AhpR activity. The satisfactory activity retention may be owing to the specific interaction between modified SWCNTs and His-tagged enzymes, which might result in enzyme structure preservation during immobilization [28]. In addition, the similar mobility through Brownian motion between nanoparticle-immobilized enzymes and free enzymes could also help retain high activity [15, 29].

Figure 5-4 Relative activity of both free and immobilized AhpR system with the different nox/AhpC ratios. nox/AhpC (red); nox/SWCNT-AhpC (blue); SWCNT-nox/AhpC (yellow) and SWCNT-nox/SWCNT-AhpC (green).

5.3.5 AhpR stability

Stability improvement is another important parameter to evaluate the immobilized enzyme. As shown in Figure 5-5, the storage stability of SWNCT-AhpC has been improved significantly compared with free AhpC at 4 and 20 °C. SWCNT-AhpC stored at 4 °C can still maintain >90% activity after 1000 h, whereas free AhpC can only keep ca. 60% activity. The estimated half-life of SWCNT-AhpC at 20 °C is around 1200 h, which is twice the half-life of free AhpC (~550 h). Factors such as high surface curvature [30, 31], high loading capacity [32], and nanospatial confinement [11] may cause the elevated enzyme stability.

The operational stability of free and immobilized AhpR system was investigated by measuring their total turnover numbers (TTN). All combinations systems, free nox/AhpC (Figure 5-6A), SWCNT-nox/free AhpC (Figure 5-6C), free nox/SWCNT-AhpC (Figure 5B), and SWCNT-nox/SWCNT-AhpC (Figure 5-6D) exhibit excellent operational stability with similar TTN at $\sim 9 \times 10^4$, indicating that immobilization has no influence on enzyme operational stability. Neither AhpC nor SWCNT-AhpC enhances the TTN of either nox or SWCNT-nox, pointing out that the deactivation of AhpR is not caused by hydrogen peroxide produced through nox but by hydrogen peroxide turnover or release [27]. Despite adding exogenous DTT into the reaction system, the TTN of AhpR is not affected in all combinations, which is possibly owing to the second thiol that behaves as a stabilizing nucleophile at the active site of nox

[27]. Therefore, the operational stability of AhpR is determined by the overoxidation of cysteine residue at nox active site, not by SWCNT, AhpC, or exogenous DTT [33].

Figure 5-5 Storage stability of free AhpC and SWCNT-AhpC. Free AhpC at 4 °C (▲), free AhpC at 20 °C (△), SWCNT-AhpC at 4 °C (■), and SWCNT-AhpC at 20 °C (□).

Figure 5-6 Total turnover number of both free and immobilized AhpR system with different nox/AhpC ratios. (A) nox/AhpC. (B) nox/SWCNT-AhpC. (C) SWCNT-nox/AhpC. (D) SWCNT-nox/SWCNT-AhpC. With 5 mM DTT (blue) and without 5 mM DTT (red).

5.3.6 Amplex red assay

Hydrogen peroxide generation was determined by Amplex Red assay through detecting resorufin fluorescence at 590 nm. We found that both free and SWCNT-nox produced almost the same amount of hydrogen peroxide, similar to the published results [23, 34]. The hydrogen peroxide production of four combinations with different nox/AhpC ratios was also measured. As indicated in Figure 5-7, supporting material SWCNTs do not facilitate or block the electron transfer between nox and AhpC. When nox/AhpC ratio is 1:1, ~50% less hydrogen peroxide is produced in comparison with free or immobilized nox alone. When nox/AhpC ratio is 1:10, only 35% hydrogen peroxide is left after reaction. The incomplete turnover of hydrogen peroxide at those nox/AhpC ratios may be attributed to the unproductive collisions between nox and AhpC in reaction mixture [27]. When the ratio reaches above 1:50, hydrogen peroxide can hardly be detected. Therefore, either free or immobilized AhpC has to be present in vast excess to be an effective scavenger.

Figure 5-7 Hydrogen peroxide generation of both free and immobilized AhpR system with different nox/AhpC ratios. Free nox (pink); SWCNT-nox (violet); nox/AhpC (red); nox/SWCNT-AhpC (blue); SWCNT-nox/AhpC (yellow); and SWCNT-nox/SWCNT-AhpC (green).

5.4 Conclusions

In summary, we have demonstrated that the non-covalent specific immobilization approach via the specific interaction between His-tagged enzyme and modified nanomaterials can also be applied on a consecutive biocatalytic reaction system, such as AhpR. The immobilized AhpC showed improved storage stability after immobilization. Different nox/AhpC ratios can affect the overall free/immobilized AhpR activity but not the operational stability. With the increase of AhpC amount in AhpR, the intermediate hydrogen peroxide decreases and both the immobilized AhpR and free AhpR generate similar amount of hydrogen peroxide.

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Chapter 6

Glycerol dehydrogenase immobilization on single walled carbon nanotubes for 4-Hydroxy-2-Butanol production via cofactor regeneration

6.1 Introduction

Nicotinamide cofactor-dependent oxidoreductases are widely used in bioindustry to synthesize chemicals such as chiral compounds, hydroxyl acids or amino acids [1-3]. However, the high cost of these nicotinamide cofactors NAD(P)H/NAD(P)^+ is one of the major obstacles for the industrial application of oxidoreductase [4]. To solve this problem, an *in situ* cofactor regeneration system is often adopted. Compared with chemical and electrochemical methods [5-6], enzyme-catalyzed cofactor regeneration method provides efficient and robust solution and can be carried out under mild conditions [7].

Several enzymes have been studied previously to regenerate NAD^+ from NADH, including alcohol dehydrogenase with acetaldehyde [8], L-lactate dehydrogenase (LDH) [9] and NADH oxidase (nox) [10-11]. Nox is a better candidate compared to other cofactor-regenerating enzymes for the following reasons: i) nox uses oxygen as its substrate and no more additional substrate is needed ii) the synthesis reaction can shift its equilibrium towards the product formation direction because of the irreversible reaction catalyzed by nox. It was reported previously that nox was

coupled with alcohol dehydrogenase to produce (*S*)-1-phenylethanol, an important pharmaceutical/agrochemical intermediate [12].

In recent years, enzyme immobilization on nanomaterials has caught a lot of attention in areas such as biosensor [13-14], fuel cell [15-16], and biocatalysis [17-18]. Compared with the research on single enzyme immobilization, multienzyme systems are less investigated. Nevertheless, many important reactions are catalyzed by multienzyme complexes, such as cofactor regeneration system. For multienzyme immobilization, in addition to the problems usually encountered with single enzyme immobilization, such as enzyme leaching [19], strong diffusion resistance [20] and loss of enzyme 3D structure [21], the ratios of individual enzymes, the optimal reaction conditions of multienzymes and sometimes, the cofactor amounts need to be adjusted to achieve an optimal overall product yield.

We have demonstrated an efficient enzyme immobilization method previously based on the non-covalent specific interaction between His-tagged enzyme and functionalized single-walled carbon nanotubes (SWCNTs) [22]. Compared with other enzyme immobilization methods, such as covalent binding and physical entrapment, the advantages of this method are: i) immobilized enzyme tends to have high activity retention and good stability [23]; ii) immobilized enzyme in a favorable orientation during catalysis [24]; iii) the immobilization is specific and reversible [25].

In this study, we intended to test the same immobilization procedure on a multienzyme system, using H₂O₂-forming NADH oxidase, the nicotinamide cofactor

regenerating enzyme, and glycerol dehydrogenase (GlyDH) to produce 4-hydroxy-2-butanone (4H2B) (Figure 6-1). 4H2B is an important pharmaceutical intermediate for the synthesis of the anticancer drug doxorubicin, steroid, and pesticide [26-28]. GlyDH can oxidize secondary alcohols to their ketone forms with cofactor NAD(P)⁺, such as the oxidation of 1,3-butanediol (1,3-BDO) to 4H2B [29]. Our group has successfully improved the native GlyDH (*E.coli*, encoded by *gldA*) activity by 26-fold through protein engineering approaches [30], and the GlyDH variant was used in this study

In this chapter, we applied the non-covalent specific immobilization process to immobilize His tagged-GlyDH on functionalized SWCNTs first. The resulting conjugate showed excellent activity retention and enhanced stability. We then employed both immobilized GlyDH and nox (hydrogen-peroxide forming, referring to Chapter 3) in a cofactor regeneration system to produce 4H2B from 1,3-BDO, with free multienzyme system as control. The amounts of nicotinamide cofactor NAD⁺, the NADH/NAD⁺ ratio, and the pH of the multienzyme system were investigated in order to find out the optimal conditions to maximize 4H2B yield. Our results showed that the immobilized cofactor regeneration system could largely increase the 4H2B bioconversion.

Figure 6-1 Bioconversion of 4H2B from 1,3-BDO with *in situ* cofactor regeneration

6.2 Materials and methods

6.2.1 Materials

Bradford reagent, cobalt(II) chloride, *N*_α*N*_α-bis(carboxymethyl)-L-lysine hydrate (ANTA), 1-ethyl-3-[3'-(dimethylamino)propyl]carbodiimide (EDC), nitric acid, sulfuric acid, β-Nicotinamide adenine dinucleotide (NAD⁺), 1,3-butanediol (1,3-BDO), 4-hydroxy-2-butanone (4H2B), methylene dichloride, *N*-hydroxysuccinimide (NHS), *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), dichloromethane, isopropyl β-D-1-thiogalactopyranoside (IPTG), and catalase were bought from Sigma-Aldrich. β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), and flavin adenine dinucleotide (FAD) were purchased from Merck. Super purified HiPco[®] Single-walled carbon nanotubes (SWCNTs) were purchased from Unidym (USA). *E. coli* BL21 (DE3) containing mutated *gldA* gene was from our previous work with one more point mutation L173A.

6.2.2 GlyDH overexpression and purification

E. coli BL21 (DE3) containing pET21b-*gldA* plasmid was grown in *Luria-Bertani* (LB) broth with 100 µg/ml ampicillin at 37 °C. Gene expression was induced by 0.2 mM IPTG when OD at 600 nm reached 0.6~0.8. GlyDH was purified with immobilized metal affinity chromatography method (IMAC; GE healthcare, Singapore) and desalted by PD-10 desalting column (GE Healthcare, Singapore). GlyDH concentration was measured by 5-min incubation with Coomassie protein assay reagent and its purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

6.2.3 SWCNTs modification and GlyDH immobilization

The modification of SWCNTs followed the procedure described in our former article [22]. To be brief, SWCNT-COOH was first prepared by treating pristine SWCNTs with HNO₃ and H₂SO₄ mixture, and then activated by NHS/EDC. ANTA-Co²⁺ was attached to the activated SWCNT surface by carbodiimide crosslinking. SWCNT-ANTA-Co²⁺ complex was purified by filtration and dissolved in 20 mM, pH 7.5 HEPES buffer.

Cell lysate containing His-tagged GlyDH was incubated with SWCNT-ANTA-Co²⁺ complex overnight at 4 °C. SWCNT-ANTA-Co²⁺-GlyDH conjugate (SWCNT-GlyDH) was separated by centrifugation, washed with 20 mM, pH 7.5 HEPES containing 20 mM imidazole three times, and suspended in 20 mM, pH 7.5 HEPES buffer. GlyDH loading capacity was determined by measuring GlyDH concentration

after it was eluted from SWCNTs with 20 mM, pH7.5 HEPES buffer with 500 mM NaCl and 500 mM imidazole.

6.2.4 Free and immobilized GlyDH activity measurement

GlyDH activity on 1,3-BDO was measured by detecting the increase of NADH absorbance at 340 nm (ϵ_{NADH} : 6220 M⁻¹cm⁻¹) by a DU 800 UV-Vis Spectrophotometer (Beckman Coulter, USA). Standard conditions were set as 30 °C, 0.56 mM (free GlyDH)/0.6 mM (SWCNT-GlyDH) NAD⁺ in 50 mM, pH 9.5 K₂CO₃/KHCO₃ buffer. The K_M and V_{max} values of both free and immobilized GlyDH were determined under standard conditions with 1,3-BDO concentration varying from 0 to 500 mM.

6.2.5 Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded by a ChiraScan circular dichroism spectrometer (Applied Photophysics, United Kingdom) with constant N₂ flushing. The measurements were conducted in 10 mM, pH 7.5 potassium phosphate buffer containing 0.4 mg/ml GlyDH in a 1-mm path length quartz cuvette at 25 °C. The spectrum of each sample was averaged over five scans (200~260 nm). The mean residue ellipticity at 222 and 218 nm was used to quantify the content of α -helix and β -sheet in GlyDH.

6.2.6 Effect of cofactor concentration

Cofactor effect on 4H2B conversion was tested in 50 mM, pH 9.5 $\text{K}_2\text{CO}_3/\text{KHCO}_3$ buffer containing 100 μM FAD and 100 mM 1,3-BDO at 30 $^{\circ}\text{C}$, with NAD^+ concentration varying from 0.5~10 mM. When determining the effect of NADH, NAD^+ concentration was fixed at 5 mM while the NADH/ NAD^+ ratio changed between 5:1 and 1:5. After 12-h reaction, each sample was extracted by methylene dichloride, filtered with 0.2 μm membrane, and quantified by gas chromatography (GC).

6.2.7 GC analysis

The 1,3-BDO and 4H2B contents were detected by an Agilent 6890N gas chromatography (USA) and analyzed by Agilent Chemstation. GC was equipped with a HP-5 phenyl methyl siloxane capillary (30.0 m $\times$ 320 μm $\times$ 0.25 μm nominal, Agilent, USA) and the temperature was set isothermally at 110 $^{\circ}\text{C}$. Helium gas (He) was used as the mobile phase with constant pressure at 14.98 psi. 2- μl samples were injected each time with the injector temperature at 250 $^{\circ}\text{C}$. Absorbance peaks were obtained with a Flame Ionization Detector (FID) operating at 250 $^{\circ}\text{C}$.

6.2.8 Bioconversion of 1,3-BDO to 4H2B

4H2B bioconversion in both free and immobilized cofactor regeneration systems was investigated. Reaction conditions were set as 5 mM NAD^+ , 100 μM FAD and 100

mM 1,3-BDO at 300 rpm, 30 °C. The optimal reaction conditions, including GlyDH/nox ratio and pH value, were determined as follows.

To find out the optimum GlyDH/nox ratio, reactions were carried out in pH 9.5, 50 mM K₂CO₃/KHCO₃ buffer for 24 h with different GlyDH/nox ratio (5:1, 3:1, 1:1, 1:5, and 1:10). All samples were extracted by methylene dichloride, filtered with 0.2 µm membrane, and 1,3-BDO and 4H2B were quantified by GC.

To determine the optimum pH, reactions were conducted in different pHs for 12 h, with pH 7.0, 7.5, and 8.0, 50 mM potassium phosphate buffer (PPB) and pH 9.0, 9.5, and 10.0, 50 mM K₂CO₃/KHCO₃ buffer, respectively, while the GlyDH/nox ratio was fixed at 1:5 for free-enzyme system and 1:1 for immobilized-enzyme system.

6.2.9 Reusability

The reusability study of cofactor regeneration system was tested under the same reaction conditions as described above with SWCNT-GlyDH as control. The bioconversion reaction was allowed to continue for 30 min. Catalysts were recovered by centrifugation and washed with 20 mM, pH 7.5 HEPES buffer thrice.

6.3 Results and discussion

6.3.1 GlyDH immobilization and kinetics studies

Pristine SWCNTs were treated with HNO_3 and H_2SO_4 mixture, activated by NHS/EDC, and amidated with ANTA- Co^{2+} as described previously [22]. SWCNT-GlyDH was prepared by directly incubating SWCNT-ANTA- Co^{2+} complex with cell lysate containing His-tagged GlyDH. To check GlyDH binding specificity, we eluted the enzyme off the conjugate with imidazole and checked the elution with SDS-PAGE. There was only one band of the same molecular weight as GlyDH (~ 39 kDa) on SDS-PAGE and the GlyDH amount in cell lysate also decreased after incubating with SWCNT-ANTA- Co^{2+} complex (Figure 6-2B, Lane C). The observation indicated that SWCNT-ANTA- Co^{2+} complex could bind to the His-tagged GlyDH specifically. The enzyme loading capacity was found to be 0.734 mg GlyDH/mg SWCNTs.

Table 6-1 Kinetic characteristics of free and immobilized GlyDH

Enzyme	$K_{\text{M, 1,3-BDO}}$ (mM)	$K_{\text{M, NAD}^+}$ (μM)	V_{max} (U/mg)	k_{cat} (min^{-1})	$k_{\text{cat}}/K_{\text{M, 1,3-BDO}}$ ($\text{min}^{-1}\text{mM}^{-1}$)	$k_{\text{cat}}/K_{\text{M, NAD}^+}$ ($\text{min}^{-1}\mu\text{M}^{-1}$)
Free GlyDH	63.3	568.3	159.4	6588.2	104.1	11.6
SWCNT-GlyDH	84.1	608.7	163.6	6761.1	80.4	11.1

One key parameter to evaluate immobilization process is the enzyme activity retention. As shown in Figure 6-2A & Table 6-1, the V_{max} of SWCNT-GlyDH (163.6 U/mg) is almost the same as free GlyDH (159.4 U/mg), which is probably because the immobilization process does not alter the structure of GlyDH. To prove this, circular dichroism (CD) spectroscopy was carried out to assess the secondary structures of both free and immobilized GlyDH. As displayed in Figure 6-2B, the immobilized GlyDH has similar α -helix content at around 38% as free GlyDH (Table 6-2), implying that the functionalized SWCNTs do not affect enzyme secondary structure during immobilization. This may explain for the ca. 100% enzyme activity retention measured. As for NAD^+ and 1, 3-BDO binding affinity, SWCNT-GlyDH demonstrates a slightly higher K_M value than free GlyDH, possibly owing to mass transfer resistances and steric hindrances [31]. The specificity of SWCNT-GlyDH (k_{cat}/K_M) also decreases due to the increase in K_M .

Table 6-2 Secondary structure of free GlyDH and SWCNT-GlyDH

Sample	α -helix (%)	β -sheet (%)
Free GlyDH	38.1 ± 1.1	32.2 ± 0.9
SWCNT-GlyDH	37.3 ± 1.2	32.9 ± 0.4

Figure 6-2 SDS-PAGE of GlyDH purification and immobilization. (A) SDS-PAGE of GlyDH from overexpression to purification. Lane A: protein weight marker; Lane B: overnight cell culture; Lane C: uninduced cell culture; Lane D: 0.2mM IPTG induced cell culture; Lane E: cell lysate; Lane F: purified GlyDH (M_w : ~39 kDa). (B) SDS-PAGE of binding specificity of GlyDH with SWCNTs. Lane A: cell lysate; Lane B: imidazole elution sample from control (SWCNT-COOH and GlyDH mixture); Lane C: cell lysate after incubation with SWCNT-ANTA-Co²⁺ complex; Lane D: imidazole elution sample from SWCNT-GlyDH; Lane E: protein weight marker.

Figure 6-3 Characteristic comparison of both free GlyDH and SWCNT-GlyDH. (A) Saturation curve of 1,3-BDO for free GlyDH (-■-) and SWCNT-GlyDH (-□-) on 1,3-BDO in 50 mM, pH 9.5 K₂CO₃/KHCO₃ buffer at 30 °C. (B) CD spectra of free GlyDH (■), SWCNT-GlyDH (Δ), and SWCNTs (Control, O)

6.3.2 Thermal and storage stability

One common phenomenon after enzyme immobilization is an increase in enzyme stability. We first investigate the thermostability of both free and immobilized GlyDH by incubating them at 60 °C (Figure 6-4A). After 30 min incubation, SWCNT-GlyDH loses around 10% activity, while the activity of native GlyDH sharply decreases and only ~70% is retained. The estimated half-life ($\tau_{1/2}$) of free GlyDH is approximately 240 min, which is shorter than that of SWCNT-GlyDH (~400 min). Moreover, we have measured the storage stability of both free GlyDH and SWCNT-GlyDH at 4 °C and 20 °C (Figure 6-4B). SWCNT-GlyDH also exhibits better storage stability than free enzyme in both cases and the estimated half-lives of SWCNT-GlyDH are ~4500 h (4 °C) and ~1400 h (20 °C), longer than those of free GlyDH (~2300 h at 4 °C and ~1000h at 20 °C). The stability enhancement is probably due to the spatial confinement of the enzyme [32], SWCNT surface curvature [33], and high enzyme loading capacity [34].

6.3.3 pH Profile of free and immobilized GlyDH

The effects of pH on both free and immobilized GlyDH activity are shown in Figure 6-5. Both free GlyDH and SWCNT-GlyDH have similar bell shape pH profiles and share the same optimum pH of 9.5, which suggests that the catalytic microenvironment of GlyDH is not changed significantly by supporting material SWCNTs [35]. Furthermore, it is also observed that SWCNT-GlyDH has less sensitivity to pH than free GlyDH. The pH studies of SWCNT-GlyDH here will help

to determine the optimum pH value of the coupled bioconversion reactions later in this work.

Figure 6-4 Stability of free and immobilized GlyDH in 50 mM, pH 9.5 $\text{K}_2\text{CO}_3/\text{KHCO}_3$ buffer. (A) Thermostability of free GlyDH (-■-) and SWCNT-GlyDH (-□-). Both free and immobilized GlyDH were incubated at 60 °C in 50 mM, pH 9.5 $\text{K}_2\text{CO}_3/\text{KHCO}_3$ buffer. (B) Storage stability of free GlyDH and SWCNT-GlyDH. (-■-) Free GlyDH at 4 °C, (-□-) free GlyDH at 20 °C, (-▲-) SWCNT-GlyDH at 4 °C and (-Δ-) SWCNT-GlyDH at 20 °C.

Figure 6-5 Effect of reaction medium pH on the activity at 30 °C. (-■-) free GlyDH and (-□-) SWCNT-GlyDH.

6.3.4 Effect of cofactor concentration on 4H2B bioconversion

The bioconversion from 1,3-BDO to 4H2B was carried out with a *in situ* cofactor regeneration system, using SWCNT-GlyDH from this work to oxidize substrate 1,3-BDO to 4H2B, and SWCNT-nox from our previous work to regenerate NAD^+ from NADH [22]. Free enzyme system (GlyDH and nox) served as a control. The concentration of substrate 1,3-BDO and product 4H2B were detected by GC (Figure 6-6).

We have studied the influence of nicotinamide cofactor concentration on both free and immobilized enzyme systems. The 4H2B yield of both systems rapidly rises until $[\text{NAD}^+]$ reaches 5 mM (Figure 6-7). When $[\text{NAD}^+]$ is above 5 mM, the yield of 4H2B gradually increases and achieves saturation. A possible explanation for this phenomenon is that the K_{M,NAD^+} value of GlyDH or SWCNT-GlyDH is around 0.57~0.61 mM, and the active site of GlyDH/SWCNT-GlyDH is already saturated with NAD^+ when its concentration is above 5 mM, which is approximately 10-fold the K_{M,NAD^+} value of GlyDH or SWCNT-GlyDH. Therefore, in our further studies, the bioconversion reaction condition was set at 5 mM NAD^+ .

Figure 6-6 Gas chromatography spectra of (A) 1,3-BDO standard; (B) 4H2B standard; and (C) reaction mixture by using HP-5 phenyl methyl siloxane capillary at 14.98 psi and 110 °C. The retention time of 4H2B and 1,3-BDO is 1.3 min and 1.4 min, respectively.

Figure 6-7 Effect of NAD⁺ concentration on 4H2B bioconversion. (-■-) free GlyDH and nox; (-□-) SWCNT-GlyDH and SWCNT-nox.

6.3.5 Effect of GlyDH/nox ratio on 4H2B bioconversion

The balance between the two enzymes, GlyDH and nox, is essential to construct an efficient cofactor regeneration system, hence we also investigated the influence of various GlyDH/nox ratios on the yield of 4H2B. Both the free GlyDH and SWCNT-GlyDH have approximately the same specific activity at pH 9.5, while SWCNT-nox (22.65 U/mg) demonstrates much better activity than free nox (5.06 U/mg). Moreover, since both nanoparticle-enzyme conjugates have better stability than free enzymes at 30 °C, the immobilized enzyme system always exhibits higher yield than free enzyme system at all GlyDH/nox ratios examined (Figure 6-8A). The best GlyDH/nox ratio (w/w) was found to be 1:5 for the free-enzyme system with a yield of about 22%, and 1:1 for the immobilized-enzyme system with 4H2B production around 37%.

We have also tested the synthesis of 4H2B by co-immobilizing GlyDH and nox on SWCNT-ANTA-Co²⁺ complex with different initial GlyDH/nox ratios (3:1, 1:1, and 1:5). The yield of 4H2B turned out to be less than 33%, which is lower than separate enzyme immobilization on SWCNTs. The reason for the drop in yield may be the ratio of GlyDH to nox on the nanotubes is not at optimum after co-immobilization. Since it is very difficult to determine enzyme ratio with co-immobilization method, we did not use this approach for further studies.

6.3.6 Effect of pH on 4H2B bioconversion

Another important factor that may affect 4H2B production is the pH of the bioconversion reaction. Since the optimum pH value of free GlyDH (9.5) is different from free nox (7.0), to find the optimum pH of the cofactor regeneration system, we tested both immobilized and free enzyme systems under different pH conditions. The yield of 4H2B from immobilized-enzyme system was found to be always higher than free-enzyme system (Figure 6-9 & 6-10A). Both systems have a similar optimum pH (9.5), the same as GlyDH, indicating that GlyDH is the dominant enzyme in the cofactor regeneration system. The best yield of 4H2B from the immobilized enzyme system is 37%, higher than that of the free enzyme system (18%).

The possible explanation is that the specific activity of SWCNT-nox is 22.65 U/mg at pH 9.5, close to its optimum value (23.65 U/mg, Table 6-3), while that of free nox activity is only 5.06 U/mg at pH 9.5. The elevated activity could help SWCNT-nox regenerate NAD^+ faster and drive the production reaction equilibrium towards 4H2B generation [10]. On the other hand, when using free or immobilized GlyDH alone, the conversion of 4H2B is just around 5% (Figure 6-10B).

The conversion of 4H2B is faster during the first three hours and the formation of 4H2B is proportional to the consumption of 1,3-BDO at all pH tested. During the first 3-h reaction period, the production of 4H2B in the immobilized enzyme system is higher (13% ~ 18%) than free-enzyme system (~10%), which might be owing to the higher activity from SWCNT-nox. During the later 9-h reaction, 4H2B production

rate becomes slower, which may be due to the shift of reaction equilibrium and product inhibition.

Table 6-3 Activity of free and immobilized nox at different pHs

Enzyme	pH					
	7.0	7.5	8.0	9.0	9.5	10.0
Free nox	23.41	16.89	13.65	2.76	5.06	5.25
SWCNT-nox	23.65	21.21	19.63	14.20	22.65	20.15

Activity was measured in 50 mM PPB (pH 7.0~8.0)/HEPES (pH 9.0~10.0) buffer with 200 μ M NADH at 30°C.

Figure 6-8 Yield at various GlyDH/nox ratios in 50 mM, pH 9.5 $K_2CO_3/KHCO_3$ buffer at 30 °C after 24-h reaction. Standard deviations were calculated from triplicate experiments.

Figure 6-9 Comparison of 4H2B bioconversion between free and immobilized cofactor regeneration systems in different pH reaction mediums

(A) pH 7.0, (B) pH 7.5, (C) pH 8.0, (D) pH 9.0, (E) pH 9.5, and (F) pH 10.0) at 30 °C. (-■-) 4H2B found in reaction mixture; (-□-) 1,3-BDO left in the reaction mixture.

Figure 6-10 4H2B bioconversion at different pH conditions. (A) cofactor regeneration system with free (blue bar) and immobilized (red bar) enzymes. (B) free GlyDH (blue bar) and SWCNT-GlyDH (red bar).

6.3.7 Reusability

Free enzymes are usually very difficult to recover from reaction mixture [37]. On the other hand, one of the advantages of using immobilized system is that enzyme recovery becomes feasible. We have checked the reusability of the cofactor regeneration system with SWCNT-GlyDH and SWCNT-nox, using SWCNT-GlyDH as control. The production of 4H2B is 6.5 mM after the first round of reaction, but the conversion drops to 1.2 mM after 11 reusing cycles (Figure 6-11). Conversely, the production of 4H2B from SWCNT-GlyDH is quite stable—it could produce 1.4 mM 4H2B after the first round reaction and still able to generate 1.0 mM 4H2B even after 11 reusing cycles. We found the yield of 4H2B of the cofactor regeneration system is similar to that of SWCNT-GlyDH after 11 cycles, indicating that SWCNT-GlyDH is still stable while the deactivation of SWCNT-nox becomes dominant in the cofactor regeneration system. The TTN of cofactor regeneration system is around 2.3×10^4 base on NAD^+ regeneration in 50 mM, pH 9.5 $\text{K}_2\text{CO}_3/\text{KHCO}_3$ buffer at 30 °C, the value of which is similar with that of SWCNT-nox (2.5×10^4) at the same condition. Nox TTN limitation is probably due to the overoxidation of the Cys residues at its active site [38].

Figure 6-11 4H2B yield with recycling of immobilized enzymes. Cofactor regeneration system using SWCNT-GlyDH and SWCNT-nox (Red bar) and SWCNT-GlyDH (blue bar).

6.4 Conclusions

A cofactor regeneration system, including both GlyDH and nox, were immobilized on the surface of functionalized SWCNTs for 4H2B synthesis. The 4H2B yield increased with cofactor NAD^+ concentration when $[\text{NAD}^+]$ was below 5mM. When $[\text{NAD}^+]$ was above 5 mM, different NADH/ NAD^+ ratios did not affect the production of 4H2B. We found that the optimum pH for the immobilized multi-enzyme system is pH 9.5, which is also the optimal pH of GlyDH/SWCNT-GlyDH. The best GlyDH/nox ratio for the immobilized enzyme system was 1:1, while that of the free enzyme system was 1:5. The overall 4H2B yield was increased from 18% to 37% after enzyme immobilization. Thus, we believe that this non-covalent specific immobilization approach via the specific interaction between His-tagged enzyme and modified nanomaterials can also be applied on multi-enzyme system with good overall production rate.

6.5 References

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Chapter 7

Summary and Future Research

Intensive research efforts have been invested in enzyme immobilization from the second half of the 20th century. Although enzyme immobilization has demonstrated many benefits in enzyme stability and specificity improvement, enzyme activity retention remains to be a major challenge. This could be due to i) The lack of detailed three-dimensional structural information for immobilization design; ii) immobilization technologies do not make a balance between stability improvement and activity retention of the enzyme.

This thesis attempts to make progress on the following aspects: (1) efficient enzyme immobilization method with good loading capacity and activity retention (2) the relationship between the performance of immobilized enzyme and the properties of different supporting materials (3) immobilization of multi-enzymatic system on nanomaterials. Three factors may influence the immobilization efficiency: (1) enzyme, (2) immobilization method, and (3) supporting material. In this study, three different nanomaterials (SWCNTs, MWCNTs and CNSs) were chosen as supporting materials.

In this study, we took advantage of the chelation between His-tag and the bivalent metal ion Co^{2+} and immobilized enzymes on modified nanomaterials. NADH oxidase (nox) is an attractive nicotinamide cofactor regenerating enzyme, and thus, it was chosen as model enzyme to develop the immobilization method in this study. The nox gene was cloned from *B. cereus*, overexpressed in BL21(DE3), and purified by IMAC. The activity of native nox is 27.72 U/mg. To obtain SWCNT-nox, SWCNTs were functionalized with carboxylic groups first, and subsequently amidated with

ANTA-Co²⁺ using NHS/EDC. His-tagged nox can be directly and specifically immobilized on SWCNT-ANTA-Co²⁺ complex from cell lysate without enzyme purification. The resulting SWCNT-nox can maintain >92% activity compared with the native nox and the loading capacity is 0.47 mg enzyme/mg SWCNTs. The stability of SWCNT-nox has been greatly improved with the increased optimum temperature and storage half-life. SWCNT-nox can still retain ~70% of the enzyme initial activity after ten reusing cycles. When nox completely lose its activity, “old” enzymes can be removed from SWCNTs and the conjugate can be regenerated with fresh enzymes. The new conjugate can maintain ca. 92% loading capacity and activity compared to the original.

In order to broaden the application of our immobilization method, other nanomaterials such as MWCNTs and CNSs were also investigated as supporting materials. MWCNT-nox and CNS-nox have been prepared by the same immobilization method and both MWCNT-nox and CNS-nox can retain around 87% and 78% activity, respectively, compared to that of free nox. All three nanomaterials belong to carbon materials with different morphologies and diameters and the relationship between enzyme performance and material properties were investigated. It was found that the material diameter determines the activity retention of the immobilized nox, while enzyme surface coverage and material surface curvature determine the lateral interactions between adjacent enzyme molecules on the supporting material, which may affect the enzyme stability after immobilization.

To investigate the multi-enzymatic immobilization system, AhpR was chosen as model enzyme system for consecutive transformation study. AhpR is composed of two enzymes, nox and AhpC. AhpC was cloned, overexpressed and immobilized on

functionalized SWCNTs. SWCNT-AhpC demonstrates good activity retention compared with free AhpC in the presence of nox. Activities of four alkyl hydroperoxide reductase system combinations have been examined. With all combinations, intermediate hydrogen peroxide still exists in the reaction mixture until the ratio of nox/AhpC increases to 1:50, indicating that SWCNTs do not affect the intermediate formation and the serial reaction of AhpR.

GlyDH, a nicotinamide cofactor-dependent oxidoreductase, can oxidize secondary alcohols to their corresponding ketones. Its activity towards substrate 1, 3-BDO has been improved through directed evolution method by 26-fold in our group before. Here, we employed GlyDH in a multi-enzyme cofactor regeneration system, together with nox, to produce an important pharmaceutical intermediate 4H2B. SWCNT-GlyDH can maintain~100% of GlyDH activity on 1, 3-BDO and improved its thermal and storage stability. The amount of NAD^+ is no longer the limiting factor to 4H2B production when $[\text{NAD}^+]$ reaches 5 mM. The immobilized enzyme system SWCNT-GlyDH and SWCNT-nox has the similar optimum pH value (9.5) as the free nox/GlyDH system. The yield of 4H2B doubled after immobilization. The optimum GlyDH/nox ratio shifts from 1:5 (free) to 1:1 (immobilized).

This work has successfully developed an efficient specific immobilization method with excellent activity retention and stability improvement. By working with different enzymes and materials, this method has demonstrated its broad application potential. The detailed and clear insight of enzyme performance related to immobilization has not been well established yet. Therefore, the following studies are proposed for future studies:

(1) Research on the relationship between enzyme performance and immobilization process still lags behind. Due to the lack of guidance from theoretical information, the decision on enzyme immobilization strategy rarely depends on the rational design but mostly on trial-and-error process. More efforts, therefore, should be invested to help understand the immobilization process for the application of enzymes in industry. For instance, investigations could be carried out on understanding covalent immobilization by using Förster resonance energy transfer (FRET). A fluorescence quencher could be functionalized onto the supporting material with attachment of different donor chromophores to the amino acids that could form covalent bond with the supporting material. After the immobilization procedure, fluorescence from chromophores could be detected by fluorospectrometer. The specific amino acid forming covalent bond with the supporting material would be determined by the quenched fluorescence, because the emitted energy from the donor is transferred and quenched by the quencher when the distance between them is less than 10Å. By identifying these amino acids, we may be able to engineer the current enzyme to minimize the non-specific covalent bond formation.

(2) The applications of immobilized enzymes should also be investigated in a broader field, such as medical diagnosis, biological fuel cell and biosensor, because of the increased demand of using enzyme biosensor in clinical and environmental applications. With immobilized enzymes, biosensors can improve the detection resolution and biofuel cells could achieve the high energy conversion efficiency. Although many tests have been conducted in research laboratories, the industrialization is still plagued by the efficiency and life-time. The possible solutions come from three aspects: first, the enzyme stability, which could be increased by protein engineering and enzyme immobilization; second, the cost of materials and enzymes, which could be cut down by enlarging the production scale and reducing

purification steps; third, the device fabrication techniques, which could be improved by the development of mechanical engineering and semiconductor manufacture.

Appendix

List of publications

1. Liang Wang, Li Wei, Yuan Chen, and Rongrong Jiang, Specific and reversible immobilization of NADH oxidase on functionalized carbon nanotubes. *Journal of Biotechnology* (2010), 150, 57-63.
2. Liang Wang, Rong Xu, Yuan Chen, and Rongrong Jiang, Activity and stability comparison of immobilized NADH oxidase on multi-walled carbon nanotubes, carbon nanospheres, and single-walled carbon nanotubes, *Journal of Molecular Catalysis B-Enzymatic* (2011), 69(3-4); 120-126.
3. Liang Wang and Rongrong Jiang, Reversible His-tagged enzyme immobilization on functionalized carbon nanotubes as nanoscale biocatalyst (book chapter of “Nanoscale Biocatalysis—Methods in Molecular Biology”, Humana Press) (In press)
4. Liang Wang, Hongfang Zhang, Chi-Bun Ching, Yuan Chen, and Rongrong Jiang, Nanoparticle-supported bioproduction of 4-hydroxy-2-butanone via *in situ* cofactor regeneration (Submitted)
5. Liang Wang, Yuan Chen, and Rongrong Jiang, Nanoparticle-supported consecutive reactions catalyzed by alkyl hydroperoxide reductase (Submitted)