Preliminary study on immobilization of plant esterase on functionalized multi-walled carbon nanotubes (MWCNTs) for biosensor application

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Abstract. The functionalization of multi-walled carbon nanotubes (MWCNTs) is extremely important to increase their solubility. This study reported the efficient functionalization of MWCNTs via acid treatment introducing carboxyl group at the side wall of MWCNTs by mixing the concentrated nitric and sulfuric acid. Plant esterase enzyme extracted from wheat flour called alpha naphthyl acetate esterase (ANAE) was immobilized onto functionalized MWCNTs via covalent attachment of amine group in ANAE and carboxyl group of functionalized MWCNTs. In order to characterize the functionalization of MWCNTs and immobilization of enzyme, Fourier transform infrared (FTIR) spectroscopy have been used to confirm the existence of these functional groups. The FTIR spectrums revealed that the several peaks formed at 1992.39, 2324.14, 2880.85 and 3029.51 cm\(^{-1}\) which can be assigned to the C=O symmetric stretching, O−H stretch from strongly hydrogen-bonded −COOH, H−C stretch modes of H−C=O in the carboxyl group, and O-H stretch from carboxyl groups (O=C−OH and C−OH) respectively. The immobilization of ANAE on functionalized MWCNTs confirmed by observation of the peak at 3646.81 cm\(^{-1}\) and 3850.91 cm\(^{-1}\) introduced the strong amide linkages between carboxylic acid groups and amine group on the MWCNTs. This proposed framework can be applied for fabrication of screen printed electrode as biosensor for detection of pesticide.

1. Introduction

Widespread use of the pesticides resulted in accumulation of its residue in soils and water and which may enter food chain, and increase the exposure to humans [1]. Pesticide can be divided into many classes in which the most commonly used pesticide is organophosphorus (OP) compound such as methyl parathion and dichlorovos [2]. The OP pesticides can cause cholinergic toxicity in the human body. This is due to their inhibitory effect of OP pesticides on acetyl cholinesterase (AChE) enzyme activity, which is a key enzyme for nerve transmission resulting to the neurological problems [3]. Therefore, rapid determination and reliable quantification for OP compound have become increasingly important. Enzyme based electrochemical biosensors as the most promising alternative to detect pesticides have emerged during the past about ten years [4]. The detection activity can be measured by employing different transduction techniques such as amperometry, potentiometry, spectrometry and thermometry for detection of different substrates or products of the enzymatic reaction [5] . Previous studies have developed many enzyme inhibition biosensors by using AChE. However, this enzyme need to extract from animal tissue such as the electric eel and human erythrocytes which is not convenient to use [6].

Thus, an esterase called alpha naphthyl acetate esterase (ANAE), extracted from plants used in this study since has a similar sensitivity as AChE [7]. ANAE will be immobilized with functionalized MWCNTs for further investigation on inhibition esterase to detect OP pesticide.

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Carbon nanotubes (CNTs) have been frequently used as support for sensors because of the unique properties of this material, such as the high surface area ratio, excellent biocompatibility, low density. Therefore, it is considered a potential candidate as support for enzyme binding by chemical functionalization [8]. However, the CNTs form stable aggregates due to emerging Van der Waals type interactions and these aggregates are quite difficult to disperse with enzyme [9]. Thus, chemical functionalization of nanomaterials is a well-established technique for grafting desirable functional groups such as carboxyl and amine groups onto their surface to obtain nanomaterials with desired properties.

In this study, the multi-walled carbon nanotubes (MWCNTs) were used rather than single wall carbon nanotubes (SWCNTs) as it possesses higher tensile strength properties due to their structural arrangement. The MWCNTs undergo functionalization by acid treatment. This treatment involves the uses of concentrated acid mixture of nitric acid and sulfuric acid at a ratio of 1:3 (v/v) in order to incorporate carboxyl group at the termini end and/or side wall of the MWCNTs followed by facilitate the immobilization of enzyme onto MWCNTs [8]. These functional groups could react with enzyme which consist of amine group to form peptide bond.

Enzyme immobilization on nanostructured materials presents some advantages over the bulk solid materials, namely the high surface area which can lead to higher enzyme loading, efficient nanoscale dispersion and feasible surface functionalization. Efficient immobilization of the enzyme onto the carbon nanotube while maintaining its native catalytic activity is the key consideration to develop ultrasensitive enzyme based biosensors [10]. Immobilized enzyme is defined as the enzyme either bound or adsorbed onto the surface of an insoluble support. Besides, an immobilized enzyme has a limited movement in space either entirely or to a small limited region. The existing immobilization methods include direct physical adsorption, physical entrapment in polymers or sol–gel, cross-linking, covalent attachment and self-assembling [10]. In this research, enzyme was immobilized onto MWCNTs through the covalent attachment generated between carboxylic groups on the functionalized MWCNTs and amine groups from enzyme. Functionalization of MWCNTs as well as the immobilization of enzyme on functionalized MWCNTs was confirmed by comparing the wavelength of spectrums of pristine MWCNTs, functionalized MWCNTs, and immobilized MWCNTs-COOH (MWCNTs-COOH-ANAE) and ANAE samples obtained from Fourier transform infrared (FTIR) spectroscopy.

2. Materials and method

2.1 Materials

Commercial wheat flour was purchased from a local market in Malaysia) for extraction of ANAE. Multiwall carbon nanotubes (MWCNTs) (95% pure, 1.5 µm in length and with a 15–30 nm outer diameter) purchased from Sigma Aldrich. Disodium hydrogen phosphate, sodium dihydrogen phosphate anhydrous and all materials were obtained from the Department of Biotechnology Engineering (IIUM).

2.2 Methods

2.2.1 Functionalization of MWCNTs-COOH

The functionalization was obtained by mixing 1g of MWCNTs with the 60 mL of concentrated sulfuric acid and 20 mL concentrated nitric acid with a 1:3 (v/v) proportion in round bottom flask. The heating mantle was set to 60°C for the mixture to undergo reflux system within 1 hour. In order to stop the reaction, this mixture was diluted with 600 mL of distilled water [11]. After cooling at room temperature, the mixture was then filtered using 0.45 µm of PTFE membrane with a vacuum filtration system repeatedly using 6 L of deionized water due to low pH of concentrated acid until it reached neutral pH 7. The collected mixture was then dried at 105°C within 2 hour under vacuum [11]. The pristine MWCNTs treated in mixed acid were denoted as MWCNTs-COOH.
2.2.2 Immobilization of ANAE
The immobilization part of enzyme on the functionalized MWCNTs was achieved by covalent attachment method. 4 mg of functionalized MWCNT weighted and mixed with 2 mL of 0.05 M phosphate buffer at pH 7 to prepared 2 mg/mL mixture. Next, 4 mg/mL of ANAE solution was prepared by dispersing 8 mg of ANAE into 2 mL 0.05M phosphate buffer at pH 7 to dilute the ANAE. The prepared solution consists of functionalized MWCNTs, ANAE, and phosphate buffer were mixed together in centrifuge tube followed by incubation at a temperature of 30°C, 200 rpm speed for 2.5 h. After incubation, the mixture was then centrifuged at 3800 rpm for 12 min. In order to wash and remove the unbound protein the mixture repeatedly dispersed into fresh phosphate buffer solution and centrifuged at least 4–5 times to wash. After left to air dry for 24 h, the collected conjugated denoted as MWCNTs-COOH-ANAE can be used for further analysis [12].

2.2.3 Characterization of functionalized MWCNTs and immobilized ANAE
Fourier-transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas [13]. FTIR was used to characterize (1) the functional groups on functionalized MWCNTs which were carboxyl group and (2) the immobilization of ANAE on functionalized MWCNTs. The samples that undergo for FTIR were labelled as pristine MWCNTs, MWCNTs-COOH, ANAE, MWCNTs-COOH-ANAE at the INHART laboratory, IIUM, Gombak. Appearance of new functional groups were deliberated with wavelength 400 to 4000 cm⁻¹.

3. Results and discussion
3.1 FTIR analysis for functionalization
FTIR was used to study the functional groups attached to the sidewalls of the MWCNTs by measuring how much light a sample can absorb at each wavelength [12]. Figure 1 showed (a) the pristine MWCNTs and (b) functionalized MWCNTs on FTIR spectrum with the wavelength between 400 to 4000 cm⁻¹.

![Figure 1: FTIR spectra: (a) pristine MWCNTs and (b) functionalized MWCNTs](image_url)
Based on Figure 1 (a), the hexagonal structure of pristine MWCNTs was confirmed by formation of a peak at 1416.57 cm\(^{-1}\), indicates the stretching of the carbon double bonding (C=C) on the side wall defect in MWCNTs. Next, in Figure 1 (b) the peak shifted at 1807.16 and 1992.39 which can be assigned to the C=O symmetric stretching. The peak at 3029.51 corresponding to the O-H stretch from carboxyl groups (O=C−OH and C−OH), while the peak at 2086.30 and 2324.14 can be associated with the O−H stretch from strongly hydrogen-bonded −COOH. The H−C stretch modes of H−C=O in the carboxyl group resulted from an absorption peak at 2880 [13]. Thus, these results suggested that carboxylic acid groups have been successfully introduced onto the surfaces of MWCNTs via acid treatment.

3.2 FTIR analysis for Immobilization
To confirm ANAE immobilization on the functionalized MWCNTs, the FTIR spectra was taken in FTIR spectrometer. Figure 2 demonstrates FTIR spectra for ANAE Figure 2 (a) and functionalized MWCNTs-ANAE in Figure 2 (b) with the wavelength between 400 to 4000.

![Figure 2: FTIR spectra: (a) functionalized MWCNTs–ANAE and (b) ANAE](image)

In order to compare both pristine ANAE and immobilized MWCNTs-COOH several significant peak areas at different wavelengths were observed. Based on Figure 2 (a), the peak 1242.23 could be accredited to C-N stretching. Then, the peaks 1667.38 cm\(^{-1}\) characterized the in-plane amide N-H deformation mode. Followed by, the prominent peak at 1769.97 can be assigned to the N-H scissoring mode. In addition, peaks at 2822.23 and 2881.85 can be attributed to asymmetric and symmetric C-H stretching. Next, the peaks shifted to 3200–3900 can be attributed to N-H stretching. In comparison with functionalized MWCNTs in Figure 2 (b), it can be observed that the
peaks 3646.81 and 3850.91 introduced the strong amide linkages between carboxylic acid groups and amine group on the functionalized MWCNTs [14].

4. Conclusion
From this study, it can be concluded that all of objectives were achieved for both functionalization of MWCNTs with carboxyl group and immobilization of ANAE on the functionalized MWCNTs. This confirmation characterized using FTIR since it is very informative for studying the functional groups attached to the sidewalls of the MWCNTs. By comparing the result of FTIR for pristine MWCNTs and MWCNTs-COOH, it showed the presence of carboxyl group and amine group on immobilized MWCNTs-COOH. This MWCNTs-COOH-ANAE will be used for fabrication of screen printed electrode as biosensor for detection of pesticide.

References
[1] Essumang D K, Togoh G K, and Chokky 2009 Bull. Chem. Soc. Ethiop. 23(1) 19–27.
[2] Wang J, Xia Q, Zhang A, Hu X and Lin C 2012 J. Zhejiang Univ. Sci. B 13(4) 267–273.
[3] Alavanja M C R 2009 Rev. Environ. Health 24(4) 303–309.
[4] Bucur B, Munteanu F D, Marty J L and Vasilescu A 2018 Biosensors 8(2) 1–28.
[5] Hayat A and Marty J L 2014 Sensors (Switzerland) 14(6) 10432–10453.
[6] Tanimoto de Albuquerque Y D and Ferreira L F 2007 Anal. Chim. Acta 596(2) 210–221.
[7] Jun Hou C, He K, Yang L, Huo D, Yang M, Huang S, Zhang L and Shen C 2012 World J. Microbiol. Biotechnol. 28(2) 541–548.
[8] Favero G, Fusco G, Mazzei F, Tasca F and Antiochia F 2015 Nanomaterials 5(4) 1995–2006.
[9] Putzbach W and Ronkainen N J 2013 Sensors (Switzerland) 13(4) 4811–4840.
[10] Vatanpour V, Esmaeili M, Hossein M, and D. Abadi 2014 “filtration membranes embedded by amine-functionalized multi-walled carbon nanotubes,” vol. 466, pp. 70–81, 2014.
[11] Zhao Z, Yang Z, Hu Y, Li J, and Fan X 2013 Appl. Surf. Sci. 276(476–481).
[12] Anirudhan T S, Jalajamony S and Sreekumari S S 2012 Appl. Clay Sci. 65–66 67–71.
[13] Peng H, Alemany L B, Margrave J L and Khabashesku V N 2003 J. Am. Chem. Soc. 125(49) 15174–15182.
[14] Hasegawa S, Horike S, Matsuda R, Furukawa R, Mochizuki K, Kinoshita Y and Kitagawa S 2007 J. Am. Chem. Soc. 129(9) 2607–2614.

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