

Thesis Title	Enzyme Amplified Labeling on Carbon Nanomaterials: Application to Immunoassay
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Abstract

Amplified labeling platforms consisting of layers of enzyme on carbon nanomaterials have been developed. The first label used tyrosinase immobilized on carbon nanotubes as an electrochemical label for immunoassay. The second label used a multienzyme system consisting of alkaline phosphatase and diaphorase immobilized on graphene nanoparticles for immunoassay.

The first amplified labeling platform was developed to enhance the ability of an electrochemical immunoassay. The platform consisted of multi-wall carbon nanotubes (MWNTs) supporting layers of the enzyme tyrosinase (TYR), which provide an amplification cycle due to substrate recycling at the electrode. The immobilization of multi-layered TYR on the surface of MWNTs by using stepwise layer-by-layer deposition was demonstrated. The optimal assay conditions were obtained after experimenting with the pH and applied potential. Two TYR layers on the MWNTs provide the maximum response towards catechol. The results indicated that the conjugation of MWNTs and TYR exhibited a high sensitivity toward the catechol substrate (320.8 mA.M^{-1}), with a short response time (2 s). Kinetics studies of the TYR on MWNTs toward catechol substrate were performed. The result found that $k_{\text{cat}} / K_{\text{M}}$ was one order of magnitude less than the value for free tyrosinase. MWNTs-TYR conjugation was applied to labeling for an electrochemical immunoassay detecting *Salmonella* Typhimurium. Via rabbit anti-salmonella polyclonal antibodies. The results indicate that the system provide a detection limit for *Salmonella* Typhimurium approximately 340 CFU/mL in pure culture and 10^3 CFU/mL in milk samples (spike). The system also provide a colorimetric detection route by monitoring the absorbance of the enzyme product at 410 nm, and provide a detection for *Salmonella* Typhimurium of approximately 10^3 CFU/mL.

The second nano-structured platform consisted of graphene nanoparticles (GO) and bi-enzyme layers of alkaline phosphatase (ALP) and diaphorase (DI) to enhance the detection ability for *Salmonella* Typhimurium detection. The immobilization of multi-layer enzyme on the surface of GO was based on a stepwise layer-by-layer electrostatic deposition. The surface of GO allowed more enzyme molecules to adsorb compared with MWNTs, based on optical measurements. The kinetics of GO/PAH/DI modified SPEs were investigated. k_{cat}/K_M of the enzyme could not be determined due to the unproportional response of the system towards PAP concentrations. The result of electrochemical study of the second nano-structured platform indicate that the response was not largely amplified and could not be applied to immunoassay.

Keywords: Alkaline phosphatase / Carbon nanotubes/ Diaphorase / Electrochemical Immunosensor / Enzyme recycling/ Graphene nanoparticles / Layer-by-layer / Tyrosinase

หัวข้อวิทยานิพนธ์	การใช้เอนไซม์ติดฉลากบนอนุภาคคาร์บอนระดับนาโนเพื่อเพิ่ม สัญญาณการตรวจวัด ในการประยุกต์ใช้กับเทคนิคทางอิมมูโน วิทยา
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บทคัดย่อ

งานวิจัยที่ศึกษาครั้งนี้มุ่งเน้นการพัฒนาตัวติดฉลากขยายสัญญาณการตรวจวัด ที่ประกอบด้วยชั้นของ
เอนไซม์บนอนุภาคคาร์บอนระดับนาโนเมตร ตัวติดฉลากชิ้นแรกใช้เอนไซม์ไทโรซิเนสตรึงบน
อนุภาคคาร์บอนนาโนทิวส์เพื่อใช้ในการตรวจวัดทางอิมมูโนวิทยาโดยอาศัยเทคนิคทางเคมีไฟฟ้า ตัว
ติดฉลากชิ้นที่สองใช้เอนไซม์อัลคาไลน์ฟอสฟาเทสและโคอะเฟอเรสตรึงบนอนุภาคกราฟีนระดับนา
โนเมตรเพื่อใช้ในการตรวจวัดทางอิมมูโนวิทยา

ตัวติดฉลากเพิ่มสัญญาณตัวแรก ได้รับการพัฒนา เพื่อจุดประสงค์ในการเพิ่มขีดความสามารถในการ
ตรวจวัดทางอิมมูโนวิทยาโดยอาศัยเทคนิคทางเคมีไฟฟ้า ซึ่งประกอบด้วยคาร์บอนนาโนทิวส์ชนิด
หลายชั้น (multi-wall carbon nanotubes) และชั้นเอนไซม์ไทโรซิเนส ซึ่งสามารถขยายสัญญาณได้โดย
คุณสมบัติของการนำสารตั้งต้นกลับมาใช้ใหม่ที่ผิวหน้าอิเล็กโทรด การตรึงชั้นของเอนไซม์ไทโรซิเนส
บนพื้นผิวของคาร์บอนนาโนทิวส์โดยการใช้เทคนิคชั้นต่อชั้น (layer-by-layer) และได้ศึกษาหาค่า
สภาวะที่เหมาะสมของเอนไซม์ต่อปัจจัยความเป็นกรดเบสและศักย์ไฟฟ้าที่ใช้ในการทดลอง จาก
การศึกษาพบว่าไทโรซิเนสจำนวนสองชั้นบนพื้นผิวคาร์บอนนาโนทิวส์ให้สัญญาณสูงสุดต่อสารตั้ง
ต้นคาทาคอล และผลการทดลองบ่งชี้ว่าการตรึงเอนไซม์ไทโรซิเนสบนคาร์บอนนาโนทิวส์นั้นมีความ
ไวสูงต่อสารตั้งต้น ($320.8 \text{ mA}\cdot\text{M}^{-1}$) ภายในระยะเวลาสั้น (2 วินาที) จากการศึกษาจลนศาสตร์ของ
เอนไซม์ไทโรซิเนสบนคาร์บอนนาโนทิวส์ที่มีต่อสารตั้งต้นคาทาคอลพบว่า ค่า k_{cat} / K_M ของเอนไซม์
มีค่าเลขยกกำลังน้อยกว่าเอนไซม์ไทโรซิเนสอิสระอยู่หนึ่งลำดับ เอนไซม์ไทโรซิเนสบนคาร์บอนนา
โนทิวส์ได้ถูกนำไปใช้เป็นฉลากในการตรวจวัดทางอิมมูโนวิทยาโดยอาศัยเทคนิคทางไฟฟ้าเคมีเพื่อ

ตรวจวัดเชื้อซัลโมเนลลา โดยอาศัยแอนติบอดีต่อเชื้อซัลโมเนลลา ผลการทดลองบ่งชี้ว่าระบบสามารถให้ค่าจำกัดของการตรวจวัด (LOD) เชื้อซัลโมเนลลาที่ 340 CFU/mL ของเชื้อซัลโมเนลลาที่เพาะเลี้ยงในอาหารเลี้ยงเชื้อและ 10^3 CFU/mL ในตัวอย่างนมที่เดิมเชื้อ นอกจากนี้ระบบติดฉลากยังสามารถให้ผลการตรวจวัดทางแสงโดยการวัดค่าการดูดกลืนแสงของผลิตภัณฑ์ที่เกิดจากเอนไซม์ที่มีความยาวคลื่น 410 นาโนเมตร จากผลการทดลองพบว่าการตรวจวัดโดยวิธีทางแสงต่อเชื้อซัลโมเนลลาให้ค่าจำกัดการตรวจวัดที่ 10^3 CFU/mL

ตัวติดฉลากที่เป็นโครงสร้างระดับนาโนเมตรตัวที่สองประกอบด้วย อนุภาคกราฟีนระดับนาโนและ เอนไซม์สองชนิดคืออัลคาไลน์ฟอสฟาเทส (ALP) และไดอะเฟอเรส (DI) เพื่อใช้เป็นตัวเพิ่มความความสามารถในการตรวจวัดเชื้อซัลโมเนลลา การตรึงเอนไซม์สองชนิดบนอนุภาคกราฟีนอาศัยเทคนิคชั้นต่อชั้น (layer-by-layer) เพื่อให้เกิดการจับกันแบบไฟฟ้าสถิต จากการใช้การตรวจวัดทางแสงพบว่าพื้นผิวของอนุภาคกราฟีนนั้นมีผลทำให้เอนไซม์เข้าจับตัวบนพื้นผิวของกราฟีน มากกว่าพื้นผิวของคาร์บอนนาโนทิวป์ส์ ผลจากการศึกษาจลนศาสตร์ของเอนไซม์ไดอะเฟอเรสบนกราฟีนที่ตรึงลงบนอิเล็กโทรดพบว่ามีความไม่สัมพันธ์กันของสัญญาณที่ตอบสนองต่อความเข้มข้นของสารตั้งต้น (PAP) ผลการทดลองพบว่าตัวติดฉลากตัวที่สองที่ประกอบด้วยอนุภาคกราฟีนระดับนาโนร่วมกับเอนไซม์สองชนิดไม่สามารถขยายสัญญาณเพิ่มได้ และไม่สามารถนำไปใช้เป็นตัวติดฉลากในการตรวจวัดทางอิมมูโนวิทยาโดยอาศัยเทคนิคทางเคมีไฟฟ้า

คำสำคัญ : กราฟีนนาโนพาร์ทิเคิล / คาร์บอนนาโนทิวป์ส์ / ไดอะเฟอเรส / เลเซอร์บายเลเยอร์ / ไพโรซิเนส / อัลคาไลน์ ฟอสฟาเทส / เอนไซม์รีไซเคิล / อิเล็กโทรเคมีคัล อิมมูโนเซนเซอร์

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NOMINATION SYMBOLS

C_E	enzyme concentration	mol L^{-1}
C_c	catechol concentration	mol L^{-1}
D	diffusion coefficient	$\text{cm}^2 \text{s}^{-1}$
E	potential	V
E_{pc}	cathodic peak potential	V
E_{pa}	anodic peak potential	V
i_{pc}	cathodic peak current	A
I	electric current	A
L	thickness	m
t	time	S
V_{ss}	reaction rate	V s^{-1}
V	voltage	V
k_{cat}	turnover number	s^{-1}
K_M	the Michaelis constant	mol L^{-1}

LIST OF ACRONYMS

ALP	alkaline phosphatase
Ab	antibody
Ag	antigen
Ag/AgCl	silver/silver chloride reference electrode
BSA	bovine serum albumin
Ca	catechol
CNTs	carbon nanotubes
CV	cyclic voltammetry
CVD	chemical vapor deposition
DPV	differential pulse voltammetry
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EIA	enzyme immunoassay
GO	graphene oxide
DI	diaphorase
IgG	Immunoglobulin G
LOD	limit of detection
LBL	layer-by-layer
MWNTs	multi-walled carbon nanotubes
MWCNTs-COOH	multi-walled carbon nanotubes carboxylic acid functionalized
NADH	nicotinamide adenine dinucleotide
PBS	phosphate buffer solution
PAH	poly allylamine hydrochloride
PAPP	para-amiophenylphosphate
PQI	para-Iminoquinone
SWNTs	single-walled carbon nanotubes
SPE	screen-printed carbon electrodes
SEM	scanning electron microscopy
SDS	sodium dodecyl sulfate
Tyr	tyrosinase
TMB	3,3',5,5'-tetramethylbenzidine
UV-vis	ultraviolet-visible
WE	working electrode