THE OPTIMIZATION OF GRAPHENE SENSING LAYER AGAINST *Escherichia coli*

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I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged

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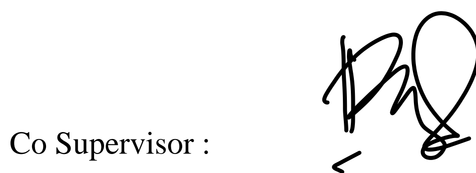

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This thesis is dedicated to my beloved family. My father, mother, husband, and siblings for their endless support, love, and prayers.

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ABSTRACT

In the last decade, biosensor have been developed to detect *E. coli.* The system is complex with a receptor detecting specific target and producing a signal transducer to be a readout data. Previous *E. coli* sensors lacked selectivity, that potentially could detect other bacteria. In order to rectify this problem, this study aims to investigate the immobilization of anti–O and K *E. coli* antibodies on a SiO_2/graphene film through the usage of a Pyrene butyric acid N–hydroxysuccinimide (PBANHS) linker. The investigation used some instruments like Raman spectroscopy, Atomic Force Microscopy (AFM), Field Effect Scanning Electron Microscopy (FE–SEM), and Current–Voltage (I–V) meter. In this research, the antibody was successfully immobilized on SiO_2/graphene evidenced by the presence of pyrene (C–C) peak at <1000 cm^{-1} and (S–H) hybridization peak at 2506.25 cm^{-1} as PBANHS/anti–O and K *E. coli* antibody fingerprint in Raman spectra. Graphene height surface distribution increased 7.893 nm after PBANHS assembly and 0.364 nm after antibody immobilization. On the other hand, graphene maximum height decreased 0.46 nm after PBANHS assembly and 0.33 nm after antibody immobilization. Based on the electrical resistance, the sensing layer was able to detect *E. coli* against *Staphylococcus aureus* (*S. aureus*) with resistance difference 3.97 Ω and Limit of Detection (LOD) 16 CFU/mL. FE–SEM image shows the dispersion and attachment of *E coli* on the surface of the sensing layer, compared to the clustering of *S. aureus*. This new investigation lead to a new potential of specific immobilized anti–O and K *E. coli* antibodies on SiO_2/graphene film as a selective sensing layer on *E. coli* sensor system.

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ABSTRAK

Dalam dekad yang lalu, biosensor telah dikembangkan untuk mengesan *E. coli*. Sistem ini kompleks dengan reseptor yang mengesan sasaran tertentu dan menghasilkan transduser isyarat untuk menjadi bacaan. Sensor *E. coli* sebelumnya tidak mempunyai selektiviti, yang berpotensi dapat mengesan bakteria lain. Untuk membetulkan masalah ini, kajian ini bertujuan untuk menyiasat imobilisasi antibodi anti–O dan K *E. coli* pada lapisan graphene melalui pautan Pyrene asid butirik N–hidroksisuccinimida (PBANHS). Penyelidikan ini menggunakan beberapa instrumen seperti: Raman spektroskopi, Atomic Force Mikroskopi (AFM), Field Effect Scanning Elektron Mikroskopi (FE–SEM), dan Elektrik–Voltan (I–V) meter. Dalam penyelidikan ini, antibodi berjaya diimobilisasi pada SiO_2/graphene yang dibuktikan dengan adanya puncak pirena (C–C) pada <1000 cm^{-1} dan (S–H) puncak hibridisasi pada 2506.25 cm^{-1} sebagai cap jari PBANHS/antibody anti–O dan K *E. coli* dalam spektrum Raman. Taburan permukaan ketinggian graphene meningkat 7.893 nm selepas pemasangan PBANHS dan 0.364 nm selepas imobilisasi antibodi. Sebaliknya, ketinggian maksimum graphene menurun 0.46 nm selepas pemasangan PBANHS dan 0.33 nm selepas imobilisasi antibodi. Berdasarkan nilai kerintangan elektrik, lapisan penginderaan menunjukan kepilihan lebih tinggi untuk mengesan *E. coli* berbanding *Staphylococcus aureus* (*S. aureus*) dengan perbedaan nilai kerintangan elektrik 3.97 Ω an Had Pengesanan (LOD) 16 CFU/mL. Lampiran imej FE–SEM *E. coli* juga menunjukkan penyebaran di atas permukaan daripada *S. aureus* yang dilekatkan pada satu tapak yang dipeluwap. Penemuan penting ini membawa kepada potensi baru antibodi anti–O dan K *E. coli* tertentu pada graphene sebagai lapisan penderia selektif pada sistem pengesan *E. coli*.

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LIST OF SYMBOLS AND ABBREVIATIONS

|Z|	– Absolute impedance
μL	– Microlitre
μm	– Micrometre
z″	– The imaginary impedance component
λ	– Lambda
π–π	– Pi–pi
Φ	– Phi
Ω	– Ohm
1D	– One dimension
2D	– Two dimension
3D	– Three dimension
A	– Adenine
AFM	– Atomic force microscopy
AgCl	– Silver chloride
Au	– Gold
AUT	– 11–amino–1–undecanethiol hydrochloride
BSA	– Bovine serum albumin
CCA	– Chromocult coliform agar
CFU	– Colony forming unit
CFU/mL	– Colony forming unit per millilitre
CLSI	– Clinical and laboratory standard institute
cm	– Centimetre
cm^2/V	– Centimetre square per volt
Cu	– Copper
CuNPs	– Copper nanoparticles
CVD	– Chemical vapour deposition
Cys–gold	– Cysteine gold

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DAEC – Diffusely adherent *E. coli*
dH_2O – Distilled water
DI water – Deionized water
DMF – Dimethylformamide
DNA – Deoxyribonucleic Acid
DTSP – 3,3′–Dithiodipropionic acid di (N–hydroxysuccinimide ester)
DTT – Dithiothreitol
E. coli – *Escherichia coli*
EAEC – Enteroaggregative *E. coli*
EDC –1–ethyl–3–(3–dimethylaminopropyl carbodiimide hydrochloride
EHEC – Enterohaemorrhagic *E. coli*
EIEC – Enteroinvasive *E. coli*
EIS – Electrochemical impedance spectroscopy
ELISA – Enzyme linked immunosorbent assay
EPEC – Enteropathogenic *E. coli*
EQR – Environmental quality report
ETEC – Enterotoxigenic *E. coli*
FE–SEM – Field emission scanning electron microscopy
FET – Field effect transistor
G – Guanine
Glc*NA*c – N–acetylglucosamine
GO – Graphene oxide
GUD – D–Glucuronidase
H – Flagellar code in *E. coli* serotyping, hours, hydrogen
h – Hour
HPLC – High performance liquid chromatography
HRP – Horseradish peroxidase
HUS – Haemolytic uremic syndrome
Hz – Hertz
I – Current
I_D/I_G – Ratio of D and G peak
I_{ds} – Bias current
Ir – Iridium
I–V – Current and voltage

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K – Spring constant, capsular code in *E. coli* serotyping
LGI – Lectin glycoprotein interaction
LOD – Limit of detection
LPS – Lipopolysaccharide
MCH – 6–Mercapto–1–hexanol
mg – Milligram
MiNT SRC – Microelectronic and nanotechnology shamsuddin research centre
mL – Millilitre
mM – Millimolar
mRNA – *Messenger* ribonucleic acid
MUA – Mercapto undecanoic acid
MUG – 4–methyl–umbelliferyl β–D–glucuronide
Mur*NA*c – N–acetylmuramic acid
NH_2 – Amide
NHMS – National health and morbidity survey
NHS – N–hydroxysuccinimide
Ni – Nickel
nm – Nanometre
No – Number
O – Antigen code in *E. coli* serotyping
OMPs – Outer membrane proteins
PBA – Pyrene butyric acid
PBANHS – Pyrene butyric acid N–hydroxysuccinimide
PBS – Phosphate buffer saline
PCR – Polymerase chain reaction
Pd – Palladium
pH – log $[H^+]$ (power of hydrogen)
PMMA – Poly (methyl methacrylate)
Pp – Pages for APA style
PSPD – Position sensitive photo detector
Pt – Platinum
R – Resistance
Ra – Surface roughness

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Rfb – Gene code for the majority of the enzymes involved in O antigen biosynthesis
RMS – Root mean square
RNA – Ribonucleic acid
Rpm – Revolutions per Minute
rRNA – *Ribosomal* ribonucleic acid
Rz – Maximum height
S – Sulphur
S. aureus – *Staphylococcus aureus*
S. thypirium – *Salmonella thypirium*
SAM – Self–assembled monolayer
SEM – Scanning electron microscope
Si – Silicon
SiO_2 – Silicone dioxide
SPIMs – Screen printed interdigitated microelectrode
SPR – Surface plasmon resonance
ssDNA – *Single–stranded DNA*
STEC – Shiga toxin producing *E. coli*
Stx2 – Shiga toxin
T – Timine
TiO_2 – Titanium dioxide
TMB – 3,3',5,5'–tetramethylbenzidine
TPa – Tonnes per annum
U – Uracil
USA – United states of America
UTHM – Universiti tun hussein onn Malaysia
UV – Ultraviolet
V – Voltage
Vol. – Volume
v/v – Volume/volume 100 mL
W/mK – Watts per meter–Kelvin
WGA – Wheat germ agglutinin
WHO – World Health Organization
w/v – Weight/volume 100 mL

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CHAPTER 1

INTRODUCTION

1.1 Background of study

In the last decade, biosensor solved the problem of individual samples' detection. Biosensor is an analytical device that contains immobilized biological compartments which can detect a target analyte by using specific and targeted reactions, thus allowing for rapid analysis of a sample. A biosensor consists of biocatalyst/bioreceptor, and a transducer which requires high selectivity [17]–[19]. A wide array of biomolecules have been used as bioreceptors for the purpose of bacteria detection, including antibodies, nucleic acids, phages, and aptamers [20]–[23]. In particular, antibodies have been the preferred element due to their ability to bond to specific antigens in the immune system, easy extraction by engineering methods, and working naturally after being functionalized. Nucleic acids, aptamers, and their derivatives are less used because some species have low copy number of Deoxyribonucleic Acid (DNA), while phages are difficult to isolate, small, and potentially dangerous in the environment [24], [25].

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E. coli has been spreading a big disease as World Health Organization (WHO) stated in May 2017 that 525.000 children under 5 years old died. It happens every year due to disease related to *E. coli* contamination. At the same time, there are nearly 1.7 billion cases of childhood diarrheal disease across the globe each year. Generally, pathogenic *E. coli* are found in food, animals, humans, and all sort environment as the human faecal waste spreading among them. The cell wall of *E. coli* plays an important role in mobile activity for survival in various environments, like adhering to another cell or surface. As an enteric bacteria, lipopolysaccharides on the cell wall have O–polysaccharides towards the exterior of the lipopolysaccharides, and K capsular

polysaccharides as the capsular identity that occupy about 75% of bacterial surface area [26]. O–polysaccharides, coded by specific genes such as *rfb*, are O–antigens that can bind to anti O–polysaccharides [27]. Anti–O and K *E. coli* antibodies molecules can be easily engineered from animals, making production easy. Moreover, anti–O and K *E. coli* antibodies can be immobilized on a surface, thus allowing them to be used as a bioreceptor in biosensors. The use of cross–linkers help to connect the antibody onto the surface during the process of immobilization in the functionalization of the antibody [28].

Graphene provides carboxyl groups with many superior characteristics. With a special two–dimensional (2D) structure and *sp2* carbon bonds, graphene is a nanomaterial that has strong carrier mobility and electrical conductivity at room temperature (up to ~10,000 $cm^2/V\ s$), a large surface area (SSA of 2600 m^2g^{-1}), and good biocompatibility [29]–[31]. It has been used to develop electrical components in many devices, including biosensors. Some linkers have been developed in the biosensor formation. Pyrene butyric acid N–Hydroxysuccinimide (PBANHS) is one of the developed linkers as an activator reagent for the carboxylic acid, which consists of hydrophobic headgroup π–π stacking system. Succinimidyl ester groups are strongly bound to the nucleophilic substitution by amine groups on the antibody. However, the usage of PBANHS decreased sensing layer preparation both time reaction and cost without using 1–ethyl–3–(3–dimethylaminopropyl carbodiimide hydrochloride) (EDC) coupled to N–hydroxysuccinimide (NHS) reaction to activate carboxylic acid group [32].

The sensing layer is the main part of the biosensor leading to the advance probed which distinguishes the target bacteria from non–target bacteria [20]. Different assemblies and formations of the sensing layer have been explored. These formations produced different outputs due to the optimization of the sensing layer. Yang *et al* (2016) developed an *E. coli* sensor with gold electrode–based electrochemical impedance. Electrochemical transducers produce an electrical charge between the electrode and electrolyte of oxidation–reduction reaction (redox) [33]. When the bacteria attach to the surface, electron transfer will be blocked and impedance is increased [34]. However, electrochemical transducers need additional reagents called electrolytes as the sample medium causes redox reactions that increase the complexity of the biosensor test.

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Kim *et al* (2002) explored the functionalization of antibodies for *E. coli* detection. *E. coli* antibodies were immobilized through an optical system based on surface plasmon resonance, a reflection of light from a thin metal film that shifts the reflectance index when any binding occurs on the sensor [35]. The optical approach led to high fabrication costs and complex reactions because it required labels to detect *E. coli*. Furthermore, *E. coli* antibodies were also functionalized as bioreceptors on a graphene film through PBANHS linkers which provide amine linkage for antibody immobilization by π–π interaction with the graphene film [36]. Most of the graphene sensors were modified in–field effect transistor (FET) by measuring the bias current (I_{ds}) by the signal process, and would produce a signal response when *E. coli* solution was added onto the sensor's recording chamber. Graphene acted as a conducting channel across two metal electrodes, namely source and drain, which the current was conveyed. Nevertheless, FET was still more costly than the general common transistor [37]–[39].

Wibowo *et al* (2018) fabricated an *E. coli* sensor based on the graphene resistivity by relying on the negative charge of *E. coli*. The resistivity of the graphene decreases when the number of bacteria increases, causing an increase in current flow due to the negative charge of *E. coli* which induced current via holes in the graphene. When the number of bacteria increased from 4 to 273 CFUs on the sensing layer, the resistance of the the graphene gradually decreased from 4.371 to 3.903 Ω. Unfortunately, because this simple approach did not use specific biomolecules to optimize selectivity, it detected other negatively charged particles such as markers, dust, or other bacteria, thus making it incompatible for usage as an *E. coli* sensor [40].

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1.2 Problems statement

In recent years, *E. coli* biosensors have been developed. However, these sensors are not selective enough, detecting other analytes that causing unreliable results. It is understood that the sensor element is a core component of biosensors. Hence, it is crucial that these elements have the appropriate selectivity, sensitivity, and stability. Briefly, graphene has a wide surface, high carrier mobility, and is a biocompatible material. It is a promising candidate for the sensing layer scaffold. Additionally, anti–O and K *E. coli* antibodies are easily synthesized. As the selective agents, these

antibodies could bind to O–lipopolysaccharides and K–capsular polysaccharides on the cell wall of *E. coli* that occupy as the most part on bacterial surface. Pyrene butyric acid N–hydroxysuccinimide (PBANHS) is known as the most used linker on graphene and antibody immobilization. However, the method of immobilization, compaction, and formation of antibodies onto the graphene film is still in a problem and need to be optimized.

1.3 Objectives of study

1. To immobilize anti–O and K *E. coli* antibodies on graphene film using PBANHS linkers
2. To characterize the assembled PBANHS linkers and immobilized anti–O and K *E. coli* antibodies on graphene film using AFM, FE–SEM and Raman Spectroscopy
3. To test the electrical resistance of graphene sensing layer for *E. coli* detection

1.4 Scopes of study

1. The anti–O & K *E. coli* antibodies immobilization was conducted via PBANHS linker
2. Commercial 0.5 × 0.5 cm 2D SiO_2/graphene was used as the sensing layer
3. The characterization of graphene sensing layer development was carried out using AFM, FE–SEM and Raman Spectroscopy
4. The graphene sensing layer was tested via I–V measurement on its electrical resistance
5. FE–SEM analysis was conducted to investigate the selectivity of the graphene sensing layer against *E. coli* and *S. aureus*

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CHAPTER 2

LITERATURE REVIEW

2.1 *Escherichia coli*

2.1.1 Structure of *Escherichia coli*

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E. coli is commonly seen under microscope with hemispherical caps in a cylindrical tube. The cell covered with the envelope contains of three layers as special characteristic of Gram–negative bacteria. Cytoplasmic membrane, peptidoglycan or murein layer, and the outer membrane with lipopolysaccharide lie down at the outer cell [41]. Outer membrane as the cell wall is 20 nm thick barrier and highly impermeable with asymmetric bilayer. Biological membrane generally is structured in a lipid bilayer with partial asymmetry. Differentially, outer membrane created unique impermeable layer with composed lipopolysaccharide (LPS) molecule in outer leaflet contains of phosphorylated sugar chains in various length in formation of hydrophobic lipid A. A tight membrane enables overcoming environmental molecules with cation divalent cross–link anionic LPS system while hydrated saccharide chains avoid the ingress an active molecule to surface, and the inner hydrophobic refuse hydrophilic substances. Outer membrane proteins (OMPs) is responsible in nutrients that the cell needs through selective absorption [42].

Peptidoglycan (murein) is also the main structural that contributes in preserving the integration of cell by withstanding the turgor formed between cytoplasmic membrane and the outer membrane. Moreover, peptidoglycan also contributes to the cell shape maintenance and provides as a substrate for anchoring other components of other cell envelope like proteins and teichoic acids [43]. The core peptidoglycan consists of linear glycan strands cross–linked from short peptides. The

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