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SENSITIVE *Leptospira* DNA DETECTION USING TAPERED OPTICAL FIBER SENSOR COATED WITH CARBON QUANTUM DOTS

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By

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This thesis presents a study of *Leptospira* DNA detection via tapered single mode optical fiber (SMF). The tapered region of the optical fiber was functionalised by sensing layer so that any reactions with the sample of interest would change the sensing layer properties. The specific capture probe DNA was designed to hybridise with complementary target *Leptospira* DNA. The specificity was detected using non-complementary target DNA while the cross-reactivity was tested with genomic *Leptospira* DNA. The urge to develop this sensor is purposedly to fulfill the demand in medical technology that is against leptospirosis. Leptospirosis is a disease infected from *Leptospira* bacteria to human. The issue arises when leptospirosis has vague clinical signs and likely to be under-diagnosed which may lead to human fatal. Moreover, the current diagnostic available is inadequate to get rapid, accurate, and easy detection. Therefore, alternative detection of *Leptospira* bacteria that addresses these issues is highly important in medical diagnostic for leptospirosis.

In this PhD project, a novel and sensitive tapered optical biosensor was developed for detection of *Leptospira* DNA. The operation of tapered optical fiber technology platform was based on the change in optical properties upon hybridization of the desired single strand DNA (ssDNA) to capture probe DNA. It involved a modification of SMF by tapering process to enhance evanescent field interaction with the surrounding. Apart from testing the bare tapered fiber, the modified optical fiber was also coated with carbon quantum dot (CQDs) to improve the DNA sensing capability. Following that, the tapered region of the optical fiber was functionalised by incubating process using simple and commercialized chemicals to link with the probe DNA. Two types of 16s ribosomal RNA gene (rrs) probe DNA (non-terminated and amine-terminated)

were used in this study. Detection was achieved with the occurrence of hybridisation between the probe DNA and its complementary DNA, in this case, target ssDNA. The surface morphologies of functionalised bare tapered optical fiber were studied using scanning electron microscopy (SEM) while Field Emission Scanning Electron Microscope (FESEM) was used for as-prepared and annealed CQDs coated. Other characterisations conducted were atomic force microscopy (AFM), energy dispersive spectroscopy (EDS) and Raman spectroscopy. Similar characterisations were also performed on *Leptospira* DNA hybridisation.

The transmission spectrum of the DNA-based optical fiber sensor was measured in the 1500 – 1600 nm wavelength range. It was discovered that hybridisation shifts of the wavelength for all testing were linearly proportional with the increase of the complementary DNA concentrations from 0.1 nM to 1.0 nM. The sensitivities of the functionalised bare tapered optical fiber detection using non-terminated and amine-terminated probe towards DNA were measured to be 1.2876 nm/nM and 1.7301 nm/nM, respectively. Meanwhile the signal response enhancement was established using tapered optical fiber coated with CQDs. The sensitivities obtained for as-prepared and annealed CQDs coated on tapered optical fiber for non-terminated probe DNA were 1.8295 nm/nM and 2.3211 nm/nM, respectively while for amine-terminated probe were 2.104 nm/nM and 2.7621 nm/nM, respectively. The findings indicate the sensor high specificity when minimal shift was detected for non-complementary DNA using all functionalised bare or CQDs coated on tapered optical fiber. This novel sensor was also able to distinguish between genomic DNA of *Leptospira* serovars against Clostridium difficile as the control sample. In conclusion, the work presented in this thesis lays the development of a sensitive and effective biosensor for *Leptospira* bacterial detection based on their specific DNA sequence analysis. This groundwork of tapered optical biosensor is highly potential for real time zoonotic disease diagnosis with in situ measurement capability at very low (femtomolar) target concentrations.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGESANAN SENSITIF *Leptospira* DNA MENGGUNAKAN PENGESAN GENTIAN OPTIK TIRUS DISALUTI TITIK-TITIK KUANTUM KARBON

Oleh

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Tesis ini membentangkan satu kajian pengesanan *Leptospira* DNA melalui gentian optik tirus mod tunggal (SMF). Bahagian tirus gentian optik tersebut difungsikan oleh lapisan pengesanan supaya sebarang reaksi dengan sampel akan mengubah sifat lapisan pengesanan. Tangkapan prob DNA spesifik direka untuk menghibridkan DNA sasaran pelengkap *Leptospira*. Kespesifikan itu dikesan menggunakan DNA sasaran bukan pelengkap sementara reaktiviti silang diuji dengan DNA genom *Leptospira*. Gesaan membangunkan pengesan ini adalah untuk memenuhi permintaan dalam teknologi perubatan yang menentang leptospirosis. Leptospirosis adalah penyakit yang dijangkiti daripada bakteria *Leptospira* kepada manusia. Isu ini timbul apabila leptospirosis mempunyai tanda-tanda klinikal yang samar dan kemungkinan untuk kekurangan diagnosis yang mana barangkali mengakibatkan kematian manusia. Lebih-lebih lagi, diagnostik semasa yang ada tidak mencukupi untuk mendapatkan pengesanan pantas, tepat dan mudah. Oleh itu, pengesanan alternatif bakteria Leptospira yang menangani isu-isu ini adalah tahap yang sangat penting dalam diagnosis perubatan untuk leptospirosis.

Dalam projek PhD ini, pengesan-bio optik tirus yang asli dan sensitif telah dibangunkan untuk mengesan DNA *Leptospira*. Operasi teknologi gentian optik tirus adalah berdasarkan perubahan sifat optik ke atas hibridisasi utaian tunggal DNA (ssDNA) yang diingini terhadap prob DNA tangkapan. Ia melibatkan pengubahsuaian SMF dengan proses penirusan untuk meningkatkan interaksi medan evanescent dengan persekitaran. Selain daripada menguji gentian tirus terdedah, gentian optik yang diubah suai juga dilapisi dengan titik-titik karbon kuantum (CQDs) untuk memperbaiki keupayaan pengesanan DNA. Selepas itu, bahagian gentian optik difungsikan

dengan proses inkubasi menggunakan bahan kimia mudah dan komersil untuk dihubungkan dengan prob DNA. Dua jenis gen prob DNA 16s ribosomal RNA (rrs) (tiada-akhiran dan amino-akhiran prob DNA) digunakan dalam kajian ini. Pengesanan dicapai dengan berlakunya hibridisasi antara prob DNA dan DNA pelengkap, dalam kes ini, ssDNA sasaran. Morfologi permukaan gentian optik tirus terdedah telah dikaji menggunakan mikroskopi pengimbasan elektron (SEM) manakala mikroskopi pengimbasan pelepasan elektron (FESEM) digunakan untuk CQDs tersedia dan sepuh lindap yang disalut. Pencirian lain dikaji menggunakan mikoskop daya atomic (AFM), mikroskop penyebaran tenaga (EDS), dan spektroskopi Raman. Pencirian yang sama juga telah dilakukan ke atas hibridisasi DNA *Leptospira.*

Spektrum penghantaran pengesan gentian optik berasaskan DNA diukur dalam julat panjang gelombang 1500 – 1600 nm. Telah dijumpai bahawa hibridisasi anjakan panjang gelombang untuk semua ujian adalah berkadar linear dengan peningkatan kepekatan DNA pelengkap dari 0.1 nM hingga 1.0 nM. Sensitiviti pengesanan gentian optik terdedah dengan menggunakan prob DNA yang tiada-akhiran dan amino-akhiran terhadap DNA telah diukur masing-masing menjadi 1.2876 nm/nM dan 1.7301 nm/nM. Sementara itu, peningkatan tindak balas isyarat telah dikukuhkan menggunakan gentian optik tirus disaluti CQDs. Sensitiviti yang diperolehi untuk CQDs tersedia dan terserap lindap yang disalut pada gentian optik tirus untuk prob DNA yang tiada-akhiran adalah masing-masing 1.8295 nm/nM dan 2.3211 nm/nM, sementara untuk prob DNA amino-akhiran adalah masing-masing 2.104 nm/nM dan 2.7621 nm/nM. Penemuan ini menunjukkan spesifikasi pengesan yang tinggi apabila hanya anjakan minimum dikesan untuk DNA bukan pelengkap menggunakan semua gentian optik terdedah yang difungsikan atau disalutkan dengan CQDs. Pengesan juga dapat membezakan antara DNA genom serovar *Leptospira* terhadap Clostridium difficile sebagai sampel kawalan. Kesimpulannya, kerja dibentangkan dalam tesis ini membincangkan perkembangan pengesan-bio sensitif dan berkesan untuk pengesanan bakteria *Leptospira* berdasarkan analisis urutan DNA spesifik mereka. Asas pengesan-bio gentian optik tirus ini sangat berpotensi untuk diagnosis penyakit zoonotik dalam masa nyata dengan keupayaan pengukuran in situ pada kepekatan sasaran yang sangat rendah (femtomolar).

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LIST OF ABBREVIATIONS

A	Adenine
AFM	Atomic Force Microscopy ()
APTES	3-aminopropyltriethoxysilane ().
C	Cytosine
C	Carbon
C. difficile	Clostridium difficile
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CF_4	Tetrafluoromethane
CHF_3	Fluoroform
CMOS	complementary metal-oxide-semiconductor
CNT	Carbon nanotube
COOH	Carboxylates
CQDs	Carbon Quantum Dots
DENV-3	Dengue Virus 3 Genotype 1
DH_2O	Distilled water
dLPG	Dual peak long period grating
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
DTFBG	Double tilted fiber Bragg grating
EDS	Energy dispersive spectroscopy
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FBG	Fiber Bragg gratings

FESEM	Field Emission Scanning Electron Microscopy
FET	Field-effect transistor
FRET	Förster resonance energy transfer
G	Guanine
GA	Glutaraldehyde
GNP	Gold nanoparticles
GO	Graphene oxide
gyrB	gyrase B subunit
H	Hydrogen
H_2SO_4	Sulfuric acid
HF	Hydrofluoric acid
HNO_3	Nitric Acid
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K_d	Equilibrium dissociation constant
L. interrogans	Leptospira interrogans
L. borgpeteresenii	Leptospira borgpeteresenii
L. kirschneri	Leptospira kirschneri
L. noguchii	Leptospira noguchii
LOD	Limit of detection
LPS	Lipopolysaccharide
MAT	Micro agglutination testing
MB	Molecular beacons
mFBG	Microfiber Bragg grating
MMF	Multimode optical fiber
N	Nitrogen

NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH_2	Amine group
NH_4OH	Ammonia solution
non-cDNA	Non-complementary DNA
O	Oxygen
OH	Hydroxyl group
OMPs	Outer-membrane proteins
OSA	Optical spectrum analyser
PCR	Polymerase chain reaction
PO_4^-	Phosphate backbone
RI	Refractive index
RIE	Reactive-ion etching
rRNA	Ribosomal ribonucleic acid
rrs	16S rRNA gene
SecY	Protein translocase subunit
SEM	Scanning Electron Microscopy
SF_6	Sulfur hexafluoride
Si	Silicon
SiO_2	Silicon dioxide
Si-O-Si	Siloxane
SMF	Single mode optical fiber
SPR	Surface plasmon resonance
ssDNA	Single strands DNA
T	Thymine
TIR	Total internal reflection

UPM	Universiti Putra Malaysia
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
WC	Watson Crick

CHAPTER 1

INTRODUCTION

1.1 Overview

Malaysia is considered as a highly endemic of leptospirosis country due to the suitable weather and climatic conditions that favor the bacteria growth and dissemination [1]. Leptospirosis possesses a threat to human health. The fatality rate caused by leptospirosis within 2004-2009 varied from 1.8% to 7.6% [2]. Therefore, the health authorities have started to look into the matter due to the increasing number of outbreaks, incidences and deaths.

Leptospirosis is a zoonotic infection caused by *Leptospira*; bacteria found in environment. Infection occurs when humans are exposed directly or indirectly to the contaminated environment and carrier host reservoirs [3]. Humans as incidental hosts could suffer from acute infection but sometimes it would be fatal [1]. Infected patient will have *Leptospira* in the blood, attacking essentially all tissues and organs. Eventually, *Leptospira* is excreted in urine for a period of both during and after the infection[4], [5]. The determinations for early diagnosis of leptospirosis are focused towards the direct detection of *Leptospira* or their DNA or antigens in blood, body fluids, urine and tissues [6].

Deoxyribonucleic acid (DNA) is an intracellular property [7] that is commonly used to detect disease infection [8], [9]. This is due to the extracellular properties of bacteria or its antigen tend to lose their specific binding activity due to the fact that the conformation of the most protein might probably be denatured after being removed from their natural environment [10], [11]. Moreover, DNA-based detection can be performed in either live or dead cells and does not require microorganism isolation[12], [13]. The detection can be achieved using specific segment of *Leptospira* DNA from serum, urine, aqueous humor, and a number of post mortem organs [14].

The information from these biological samples is detected using biosensor. Biosensors are simple analytical devices that are able to attach biological molecules with variety of physical transducers. The biological responses or bio-recognition activities on the transducer are converted into a readable digital electronic signal thus providing quantitative or semi-quantitative analytical information [15]. However, most of the biosensors are common in electrical domain and very few in optical domains. The inherent advantages of optical as compared to electrical counterparts are explosion proof, immunity to the radiofrequency disturbance, electromagnetic interference, and also their environmental ruggedness and resistant [16], [17]. One of the most effective optical sensors is using optical fiber as the transducing platform. Its abilities to operate in situ, used for remote sensing, potential of distributed sensing,

reduced setup dimension (small size, light weight, and flexible), minimal invasion for in vivo measurement while providing high sensitivity and specificity create strong interest toward biosensor based on optical fiber [17]–[19].

Optical fiber technology has greatly evolved towards practical applications since 1960s when laser was first invented [20]. Sensing investigation using optical fiber technology was explored in early 1970s using low-loss modern optical fiber [20]. Since then, major effort has been devoted in exploiting optical fibers prospective for sensing in a variety of application areas especially for communication purposes. Many researches have been conducted on selection of suitable design for optical fiber sensors [21]. Innumerable sensors using optical fibers are being used to detect physical and chemical parameters such as strain, temperature, vibration and pH from outside perturbations of the fiber itself. The transmitted light provides information on phase, intensity, wavelength and polarization based on amended local environment parameters. In turn, the transmitted light is transformed the measurands to measurable and quantifiable optical signals. Generally, the sensing capability is accomplished by permitting the transmitted light in an optical fibre to interact with the measurands of the surrounding medium.

The first application of optical biosensor was defined by Lubbers and Opitz in 1975[22]. They initially developed optical fiber-based chemical sensor to detect carbon dioxide (CO_2) and oxygen (O_2) in biological fluids [23]. Since then, rapid development of more sophisticated, selective, sensitive optical biosensors were cultivated. At the same time, new types of optical fiber, optical instrument, advanced optical approaches with various bio-recognition molecules, and new immobilization techniques were also introduced [23]. Integration of technologies from optical fiber technologies and advances in molecular biology practices have led to the speedy improvement of advanced optical biosensor technologies.

New immobilisation technique in optical biosensor is by integrating nanomaterial such as Carbon quantum dots (CQDs). CQDs is focused as it has attracted great interests as a new class nanomaterial, by virtue of their unique characteristic in physicochemical, optical and electronic. For being new nanocarbon member due to their benign, abundant and inexpensive in nature [24], it demonstrated remarkable application in numerous fields in biosensing, bioimaging, optoelectronic devices, pollutant detection, and catalysis [25]. Generally, CQDs is carbon nanoparticles with a size in the nanoscale range (10 nm or less). Due to that, it is likely to have higher surface to volume ratio as compared to the amorphous films. The interaction between the CQDs-nanostructured layers on the sensor platform with the analyte, the sensor is enhanced significantly to produce maximum output signal. As a result, higher sensitivity is normally expected for this nanotechnology approach. Thus, deployments of unique tapered optical fiber as a transducer, together with

CQDs nanomaterial give an upper hand towards novelty and great performance of the sensor.

1.2 Problem statement

Leptospirosis becomes public health concern since the frequent outbreaks that lead to mortality. The main concern on those occurrences is that leptospirosis diagnosis could be difficult to be distinguished from other infectious diseases. The initial symptoms of leptospirosis typically resemble influenza, dengue, or malaria disease [14]. For the reason that the signs are not specific for leptospirosis, cases are often wrongly diagnosed leading to delayed treatment. Without early treatment, the patient may enter into more severe stages, which could lead to kidney failure, meningitis, liver failure, respiratory distress and potentially death [13], [26].

Various molecular diagnostic methods are available to detect leptospirosis. However, the diagnoses are still inadequate for early and specific detection. Routine blood tests are usually nonspecific. Hyponatremia, mild to moderately increased transaminases, mildly raised white blood cell count, and thrombocytopenia are all common [27]. The bacterial culture and microagglutination test (MAT) are frequently used as specific diagnostic test. However, they still cannot be implemented as an early diagnostic test. The *Leptospira* colonies are slow-growing bacteria and for MAT, the practice of using anti-*Leptospira* antibodies can only be detected on day eight after onset of symptoms[28], [29]. MAT is also limited by its complexity to perform and subjectivity as it is serovar-dependent that requires the maintenance of living *Leptospira* [30].

DNA-based diagnostic is an approach to acquire sensitive early detection of infected disease [31]. One of the DNA-based diagnostic tests is polymerase chain reaction (PCR) based test which has shown considerable potentials for rapid and accurate diagnosis. Nevertheless, PCR-based test is not yet widely available and expensive. The operation also requires expertise personnel and the procedure protocol is not standardized that causes difficulties to perform the test [30], [32]. Optical fiber biosensor is an approach to get easy detection procedure. In PCR procedure, the detection needs to replicate and amplify single strands DNA with specific temperature condition. However, this proposed biosensor can be performed in single step detection and direct hybridisation measurement of single strands DNA at room temperature [33].

In term of DNA-based biosensor, others common transducing platform for the biosensor system is electrical-based such as conductometric, potentiometric, and amperometri [34]. Electrical-based biosensor system is low in cost and highly sensitive. Yet, this conventional detection system is known to be prone to electromagnetic interference as well as danger during conducting test.

Samples will have a direct interaction with electrical connection and this condition is dangerous to person who handles the procedure as well as will interfere the samples nature [35]. There are other biosensors reported to detect analytes using fluoroimmunoassay which typically rely on the use of fluorescent markers [36]. However, additional reagents are needed to measure the fluorescence signal outputs by the labelled DNA strands, based on the number of DNA hybridization engagements. [36]. The process is complicated, high in cost, and not likely to have a real-time detection[37].

Due to many of the drawbacks associated with existing diagnostic tests, an alternative and more reliable scientifically-based test for leptospirosis is being pursued. Several applications of tapered optical fiber sensor in the biotechnology and molecular biology realm seem to be promising to identify sensitive and specific detection [14], [38], [39]. In addition, CQDs were proposed to be integrated on the sensing surface to provide large surface area and enhanced optical sensing performance. Larger surface area increased the DNA adsorption on the sensing surface. CQDs served as a semiconductor to create electrons and holes leading to enhanced light absorption [40]. It absorbs more light propagates close to the tapered area coated with CQDs. Thus, with CQDs integration, higher detection sensitivity of the DNA hybridisation could be achieved.

1.3 Research objectives

The aim of this research is to develop a sensitive and specific detection system using tapered optical fiber, which can detect *Leptospira* DNA. The set goals are established by performing the following specific objectives:

1. To deposit, characterise and study the micro-characteristics and element of functionalised layer as well as *Leptospira* DNA hybridisation.
2. To investigate the optical sensing performances of the functionalised layer on developed biosensor and *Leptospira* DNA hybridisation.
3. To analyse CQDs micro-characteristics and their influence on DNA sensing performance.
4. To analyse the optical sensing performances of the CQDs based biosensor towards *Leptospira* DNA hybridisation.

1.4 Research scope

The detection approach is based on the DNA sequences specificity during hybridisation on the bare tapered single mode optical fiber (SMF), CQDs, and annealed CQDs coated on tapered optical fiber. The chosen parameter for tapered region followed the previous work that emphasized on the optimisation of ligand-target protein interaction [41]. Since this work focused on the proof of concept to biomolecules, similar dimension was used assuming it give

highest sensing performance when detecting protein biomolecules. The dimension of tapered region used was 5 mm in length for up and down taper transition. The tapered waist diameter was 12 μm with 15 mm in length. This dimension was fixed the entire experiment. All three types of SMF transducers are characterised on their morphologies and optical properties. The detection system is tapered optical tested on DNA hybridisation activities. The synthetic oligonucleotides comprise the non-terminated and aminated probe DNA with their several target (complementary) DNA concentrations are employed to analyse the sensor sensitivities. The specificity is achieved by detecting non-complementary DNA hybridization response. The potential of the developed detection system is further explored by using real *Leptospira* DNA samples.

1.5 Thesis organisation

This thesis is divided into seven chapters as follows:

Chapter 1 describes the overview, objectives, problem statement and scope of the study. It briefly mentions on optical fiber sensor, optical biosensor, leptospirosis disease and the delinquent arises in leptospirosis detection.

Chapter 2 clarifies in details the epidemiology of the leptospirosis, the *Leptospira* bacteria and the current diagnostic on leptospirosis. The basic fundamental optical fiber sensor is explained on their several types. The principal behind the surface functionalisation and DNA biosensing on tapered optical fiber are also enlightened. At last, this chapter presents a few critical reviews on the reported works based on DNA detection using tapered optical fiber sensor.

Chapter 3 discloses the methodology of the detection system. The sensor fabrication process is particularly elaborated on how the optical fiber is being tapered and in-situ deposition of CQDs onto tapered optical fiber. The procedures to functionalise bare, CQDs, and annealed CQDs tapered optical fiber are also included. As a final step in this system, the immobilization of probe DNA and hybridization target DNA onto functionalised surface are remarked.

Chapter 4 reveals the optical properties of the optimised tapered optical fiber and the sensing performance of the *Leptospira* DNA detection on bare tapered optical fiber. The surface morphologies, physical and optical characterisation of the functionalised layer on the surface tapered optical fiber are declared. The sensor performances of specificity, cross-reactivity, and limit of detection (LOD) are discussed in this chapter. The sensor performances achieved are compared for both non-terminated and aminated probe DNA used.

Chapter 5 reveals the surface morphologies and optical characterisation CQDs when coated on tapered optical fiber. The comparison of sensing performances of specificity, cross-reactivity, and limit of detection (LOD) using non-terminated and aminated probe DNA used are also discussed and compared with chapter 4 findings.

Chapter 6 summaries and concludes the research findings. The highlight of the research novelty and outlines recommendations for future work are stated as well.

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