UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF FLUORESCENCE BASED DNA BIOSENSOR UTILIZING QUANTUM DOT FOR DNA OF GANODERMA BONINENSE

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DEVELOPMENT OF FLUORESCENCE BASED DNA BIOSENSOR UTILIZING QUANTUM DOT FOR DNA OF *GANODERMA BONINENSE*

By

NOREMYLIA BT MOHD BAKHORI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

DEVELOPMENT OF FLUORESCENCE BASED DNA BIOSENSOR UTILIZING QUANTUM DOT FOR EARLY DETECTION OF *GANODERMA BONINENSE*

By

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January 2013

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In this work, fluorescence based DNA biosensor has been developed for detection of *ganoderma boninense* utilizing quantum dot as sensing material. The DNA sensor was fabricated by modification of hydrophobic surface of CdSe/ZnS quantum dot (CdSe/ZnS QD) nanoparticle with mercaptopropionic acid (MPA) to form water-soluble CdSe/ZnS QD. The modified CdSe/ZnS QD was successfully attached to the ssDNA to form CdSe/ZnS QD-ssDNA conjugate.

Attachment of CdSe/ZnS QD with ssDNA and hybridization procedures are divided into procedure 1 and 2. Hybridization of complementary target DNA with CdSe/ZnS QD-ssDNA conjugate and reporter probe labeled with Cy5 is monitored using fluorescence resonance energy transferred (FRET) as detection mode. The FRET of CdSe/ZnS QD-dsDNA is distinguishable from CdSe/ZnS

QD-ssDNA conjugate based on no FRET was observed without target DNA system. Several parameters were studied to determine the optimum conditions of hybridization efficiency including hybridization time and hybridization temperature. The highest FRET intensity was observed at 10 minutes hybridization with hybridization temperature of 45 °C for procedure 1 and 25 °C for procedure 2.

For sensitivity study, decreased of FRET intensity was observed when the concentration of target DNA increased. Limit of detection (LOD) for procedure 1 is 2.4×10^{-13} M and procedure 2 is 1.12×10^{-12} M. Selectivity study for the developed system confirmed that non-complementary DNA shows no FRET signal but only emission of QD. The hybridization did not occur due to QD was not in position with reporter probe for FRET process to occur. This proved that the detection system specific towards target DNA. Reproducibility shows acceptable relative standard deviation (R.S.D) of 1.67 % (n=5) and 0.86 % (n=5) for procedure 1 and 2 respectively. In TEM characterization, the particle size of CdSe/ZnS QD and CdSe/ZnS QD-ssDNA conjugate is within the range of 2 to 10 nm. Agglomeration was observed for CdSe/ZnS QD-ssDNA conjugate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PEMBANGUNAN FLUORESCENCE BERASASKAN BIO-PENGESAN DNA MENGGUNAKAN KUANTUM DOT UNTUK PENGESANAN AWAL *GANODERMA BONINENSE*

Oleh

NOREMYLIA BT MOHD BAKHORI

Januari 2013

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Dalam kerja ini, pendarfluor biosensor berasaskan DNA telah dibangunkan untuk mengesan DNA *Ganoderma boninense* menggunakan kuantum dot sebagai alat pengesan. Pengesan DNA telah direka dengan mengubahsuai CdSe/ZnS kuantum dot (CdSe/ZnS QD) nanopartikel dengan asid merkaptopropionik (MPA) untuk membentuk CdSe/ZnS QD larut air. CdSe/ZnS QD telah berjaya digabungkan dengan ssDNA untuk membentuk CdSe/ZnS QD-ssDNA konjugat.

Langkah pembentukan CdSe/ZnS QD dengan ssDNA dan pengacukan DNA dibahagikan kepada langkah 1 dan 2. Pengacukan DNA sasaran pelengkap dengan CdSe/ZnS QD-ssDNA konjugat dan DNA pembawa yang dilabel, Cy5 telah dipantau dengan menggunakan tenaga resonans pendarfluor (FRET) sebagai mod pengesanan.

Penghasilan FRET yang didapati daripada CdSe/ZnS QD-dsDNA dapat dibezakan dengan CdSe/ZnS QD-ssDNA apabila tiada FRET yang dihasilkan apabila tiada DNA sasaran lengkap dalam sistem ini. Beberapa parameter telah dikaji untuk menentukan keadaan yang optimum bagi kecekapan pengacukan termasuk masa dan suhu pengacukan serta perberbezaan kepekatan DNA sasaran. FRET tertinggi didapati pada masa 10 minit pengacukan dan suhu 45 °C untuk langkah 1 and 25 °C untuk langkah 2.

Bagi kepekaan pengesan, intensiti FRET menurun apabila kepekatan sasaran DNA meningkat. Had pengesanan (LOD) bagi langkah 1 adalah 2.4×10^{-13} M dan langkah 2 adalah 1.12×10^{-12} M. Kajian selektiviti untuk sistem yang dibina telah terbukti bahawa bukan pelengkap DNA sasaran menunjukkan tiada FRET tetapi hanya pancaran isyarat QD. Pengacukan tidak berlaku kerana QD tidak berada dalam kedudukan dengan DNA pembawa untuk proses FRET terjadi. Ini terbukti yang sistem pengesanan ini khusus kepada DNA sasaran. Kebolehulangan menunjukkan sisihan piawai relatif (SPR) adalah 1.67 % (n=5) dan 0.86 % (n=5) untuk langkah 1 dan 2 masing-masing. Dalam pencirian TEM, saiz zarah CdSe//ZnS QD dan CdSe//ZnS QD-ssDNA konjugat menunjukkan julat 2 hingga 10 nm. Penggumpalan berlaku untuk CdSe//ZnS QD-ssDNA.

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I certify that an Examination Committee has met on …… to conduct the final examination of Noremylia Mohd Bakhori on her Master of Science thesis entitled 'Development of Fluorescence Based DNA Biosensor Utilizing Quantum Dot for Early Detection of Ganoderma boninense' in accordance with Universti Pertanian Malaysia (Higher degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the…….

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been dully acknowledged. I also declare that it has not previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

NOREMYLIA BINTI MOHD BAKHORI

Date: 7 January 2013

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LIST OF ABBREVIATIONS

Ad	Adenine
BSR	Basal Stem Rot
CdSe	Cadmium Selenide
CdS	Cadmium Sulphide
CdTe	Cadmium
Cyt	Cytosine
Cy3	Cyanide dye
Cy5	Reporter probe
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
FRET	Fluorescent resonance energy transfer
Gu	Guanine
h	Planck's constant
HDA	Hexadecylamine
InAs	Indium Arsenide
InP	Indium Phosphide
iRBCs	*Plasmodium falciparum*-infected erythrocytes
M	Molar
MBP	Maltose binding protein
MPA	Mercaptopropionic acid
nm	Nanometer

NHS	*N*-hydroxysulfosuccinimide
PbS	Lead sulphide
PCQD	PEGylated-cationic QD
PL	Photoluminescence
PMAO-PEG	Poly(maleic anhydride-alt-1-octadecene)-poly(ethylene glycol)
QD	Quantum dot
S_0	Ground state
S_I	Excited state
s	second
ssDNA	Single-stranded DNA
TBP	Tributylphosphine
TEM	Transmission electron microscopy
TOP	Trioctylphosphine
TOPO	Trioctylphosphine oxide
Thy	Thymine
UV	Ultra violet
ν	Frequency of light
v	Volume
W	Watt
ZnS	Zinc sulphide
ZnSe	Zinc selenide

CHAPTER 1

INTRODUCTION

One of the major problems in oil palm industry is disease known as basal stem rot (BSR). It is caused by a white rot fungus called *Ganoderma boninense* (Paterson, 2007). The presence of *Ganoderma boninense* is predominated as the major cause of the disease that promotes BSR disease in oil palm plantations in Malaysia due to the pathogenic and most aggressive factors (Pancal and Bridge, 2005). Conventional methods to control the spreading of this pathogen have been done such as biological control and cultural practices which only involve in reduce the spreading and costly matter but these approaches still cannot control BSR among oil palm since it involves the genetic factor from the pathogens.

BSR occurs on the oil palm that grows in oil soil types including alluvial soil of the coast, inland, peat and laterite. The attack will cause the destruction of the plant up to 80 % if there is expansion of productive period of the plant. *Ganoderma boninense* attack at the rate of 31 to 67 % and reduce the production of fruit bunches from 26 to 45 percent and causes the loss of millions of dollars. Furthermore, infected plants are expected to die within one to three years after the appearance of the BSR's symptoms. When infected trees fell down, *Ganoderma boninense* in root and stem tissues will still survive until these tissues disintegrate.

BSR attacks starting from the palm root and continue to the stem's bole causing rotten of the rot that prevents supply of water and nutrients from the soil to the whole plant. In this case, the plants situated close to the affected plant also will be attacked by root-to-root contact and thus the spreading of the BSR will occur among plantation. The infections of BSR to oil palm can be considered as damage to oil palm since it causes the appearances of fruiting bodies at the base of the stem, unopened spears, existence of yellowing crown and appearances of deep cracks at the base of the stem (Breton *et al*., 2006). The visible symptoms of BSR only can be observed at very late stage infection whereas more than 50 % of the plant tissues already rotted. In this stage, no amount of chemicals can save the plant and no more chances for the planters to cure the plant or the plant will end to death. Only early detection can help to manage the disease in order to prevent BSR from continue attacking the oil palm. Early management of the disease can help the plant to survive and produce high production of palm oil for the economical benefit to our country.

1.1 Biosensor

Biosensor has a big impact in research and the needs for it are rapidly increasing recently. Generally, biosensor is a sensor that composed of a bioreceptor, a transducer and a signal processor. Bioreceptor is also known as a biological element or biomolecule that captures the analytes in solution. It can be antibodies, nucleic acids or receptors. The transducer transduces bio-recognition processes into measurable signals via a physico-chemical transducer such as

electronic and optical techniques as two major transducers. It also plays an important part in biosensor because it responds to any changes accompanying the reaction. The transducer converts the binding event to the measurable signals which undergo amplification before passed through a microelectronic device that comes out with a readable output. The schematic diagram of biosensor is illustrated in Figure 1.1.

Based on International Union of Pure and Applied Chemistry (IUPAC), a biosensor is defined as an analytical device which converts a biological response into an electrical signal. Biosensors can be classified either based on elements (biological recognition elements) or detection mode (transducers). Biosensor is classified based on element such as enzyme, antibody and DNA are named as enzyme-based biosensor, immunological biosensor and DNA based biosensor respectively. Biosensor is also categorized based on type of detection mode and classified as electronic biosensor, optical biosensor and piezoelectric biosensor.

There are many advantages of biosensors. One of the advantages is sensitive and selective. It is sensitive because biomolecules often possess high affinity toward their target, for example antibodies capture antigens with a dissociation constant at the nanomolar scale. Biological recognition also is very selective. An example is that enzyme and substrate just like lock and key. Such high selectivity often leads to selective biosensor. Biosensor is also portable, inexpensive and can be developed into integrated sensor devices for a very wide application (Song *et al.*, 2006).

There are various types of potential applications of biosensors. The main requirements for a biosensor approach to be valuable in terms of research and commercial applications are the availability of a suitable biological recognition element, identification of a target molecule and the potential for disposable portable detection system. Some examples of them are detection of pathogens (Leonard *et al*., 2003), environmental applications for the detection of pesticides (Skladal and Maschini, 1992) and river contaminants (LaGier *et al*., 2007), determination of drug residues in food (Caselunghe and Lindeberg, 2000) and drug discovery (Durick and Negulescu, 2001).

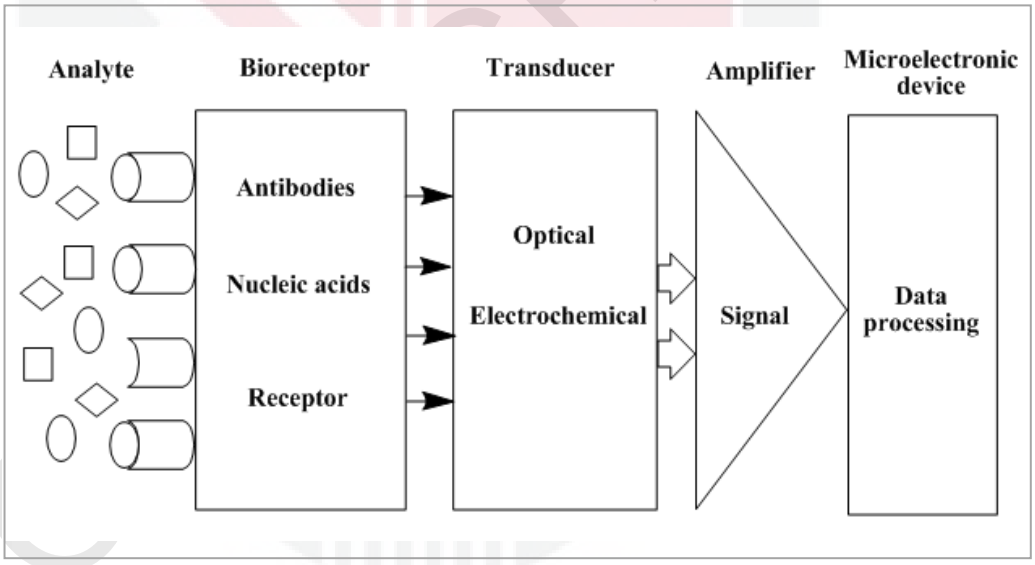

Figure 1.1 Schematic diagram of a biosensor

1.2 DNA biosensor

DNA biosensor involves in the detection of DNA sequences in many areas including clinical, environmental and food analysis. The advantage of the

analysis of gene sequences plays fundamental role in the rapid detection of genetic mutations and infectious diseases. This type of sensor become the major interest since it promises for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to the traditional hybridization.

Conventional method for identification of specific DNA sequences in biological samples are based on the isolation of double-stranded DNA (dsDNA) and further polymerase chain (PCR) to amplify the target sequence of DNA. The PCR products can be subjected to electrophoresis or may be adsorbed onto a suitable membrane and exposed to a solution containing DNA probe before being chemically or enzymatically labeled with radioactive material, chemilumnophore or ligands such as biotin as nucleic acid itself cannot be able to provide any signal (Suman and Kumar, 2008).

The basic operation of DNA biosensor is based on hybridization of DNA. Hybridization is the process of establishing sequence-specific interactions between two or more complementary strands of nucleic acids into a single hybrid (Cugia, 2010). This process involves the complementary coupling between the specific single-stranded DNA (ssDNA) (probe) sequences and another specific ssDNA (target) within analyte, immobilized onto the solid support (transducer). DNA will bind to their complement under normal conditions. The hybridization between the both strands of DNA is possible because the nucleotide bases will form hydrogen bonds only with specific complementary bases as will be discussed later in section 1.4. The stability of the hybridization depends on the

nucleotide sequences of both strands. A perfect match in the sequences of nucleotides produces very stable dsDNA whereas one or more base mismatches impart increasing instability that can lead to weak hybridization of strands (Junhui and Ruifu, 2007). The high affinity of the specific binding reaction of the hybrid offers widely uses as a diagnostic reaction for detecting single point mutations related to genetic disease and for high sensitivity detection of the presence of a particular microorganism in a sample.

DNA biosensor generally composed of three types; optical, electrochemical and piezoelectric DNA biosensor. Optical DNA biosensor is the most frequently used in detection of analytes. It only required the simplest detection unit such spectrophotometer for fluorescence detection. The nucleic acids do not have intrinsic properties that are functional in direct detections so many of the nucleic acid-based assays especially optical setups require a label for detection. The label is chose based on stability, sensitivity and its convenience. Besides that, electrochemical DNA biosensor is useful for sequence-specific biosensing of DNA. After modification with complementary DNA, the electrodes are able to detect electrochemically the target DNA under certain conditions (Chen *et al.*, 1998). It involves the detection of DNA hybridization by monitoring a current at a fixed potential. The immobilization of DNA probes either ssDNA or dsDNA onto the transducer surface plays an important role in the overall performance of the DNA biosensor (Junhui and Ruifu, 2007). Another technique in DNA biosensor is piezoelectric DNA biosensor that is based on quartz crystal that oscillate at a defined frequency when an oscillating voltage is applied allowing

high sensitivity. The change of the frequency which results from adsorption of target DNA that hybridized with the DNA probes immobilized on the crystal is measured. This method has recently emerged as most attractive due to their simplicity, low-cost, sensitivity and real time label-free detection.

Hybridization event from the developed studies were applied in DNA based biosensor for detection of specific DNA pathogens in many studies for examples, an electrochemical DNA biosensor used for detection foodborne pathogens such as *Aeromonas hydrophila* (Tichoniuk *et al*., 2010) and *Escherichia coli* (dos Santos *et al.,* 2012) while Krejcova *et al*. (2012) reported electrochemical biosensor for influenza viruses detection. Besides, optical biosensor involving quantum dot (QD) has been developed also for detection of *Escherichia coli* (Hahn *et al*., 2005) and detection of *Chlamydia trachomatis* pathogen has been reported utilizing gold nanorods (Parab *et al.,* 2010). For *Ganoderma boninense* detection, there are a few existing techniques were developed such as Multiplex Polymerase Chain Reaction (MPCR) (Chong *et al*., 2011), Ganoderma Selective Medium (GSM) (Ariffin and Idris, 1991), combination of PCR and enzyme-linked immunosorbent assay (ELISA) techniques (Utomo and Niepold, 2000) and Fuzzy Inference System (Su'ud *et al*., 2007). All these conventional techniques are time consuming for isolation and analysis of samples. Thus, development of DNA biosensor can overcome the existing techniques because the developed sensor can be use in-site or portable for quick and sensitive detection involving DNA.

1.3 Fluorescence Spectroscopy

Fluorescence spectroscopy is considered as primarily research tool in biochemistry which analysis fluorescence properties of a sample. Fluorescence used extensively in biotechnology, medical diagnostics, DNA sequencing, forensics, and genetic analysis. Fluorescence detection is highly sensitive and we no longer need the expensive or to go through difficulties in handling radioactive tracers for most biochemical measurements.

A typical spectrofluorophotometer contains light source, excitation and emission monochromator, sample cell, detector, amplifier and detector or as shown in Figure 3.1. Sample in solution usually excited by UV light. The common used light source is deuterium lamp. Monochromator is an optical device that can transmit the selected wavelength of light.

The light from an excitation source passes through a monochromator before strikes the sample. The light is absorbed by the sample causes the sample to fluorescence producing fluorescent light at wavelength characteristics of that particular element involved that emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator through the slit before reaches a detector that is placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light. Measuring at a 90° angle will detect only the light scattered by the sample and give better signal-to-noise ratio. The detector measures the absence or presence of the spectrum

extracted for each element and the intensity of the spectrum to perform the qualitative and quantitative analysis of the elements. Then, the signal is amplified before recorded as emission spectrum for readable output display.

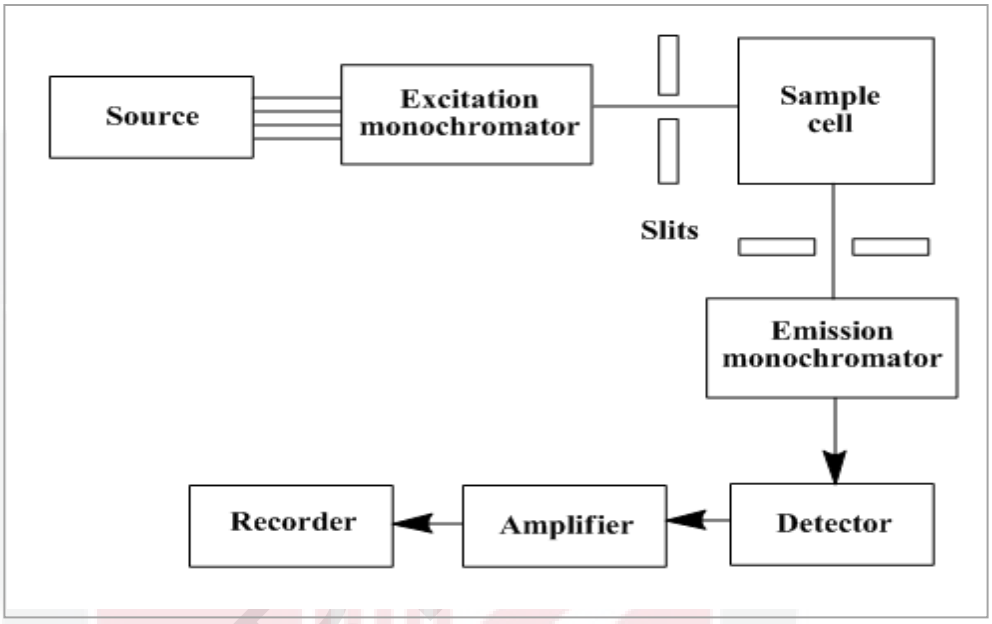

Figure 1.2 Illustrative diagram of fluorescence spectroscopy

1.4 Statement of Problems

The aggressiveness of BSR disease of oil palm has brought the researchers to construct techniques for detection of *Ganoderma boninense*'s DNA. The conventional detection system such as PCR, MPCR, and ELISA are required samples to be isolate and analysis in the lab before the sequences are confirmed. There is no new method has been developed recently for detection of this pathogen. Since BSR disease also can be contributed from other *Ganoderma* species, so we need a new, fast and sensitive method for detection only from

Ganoderma boninense. In order to overcome this problem, DNA biosensor plays important role for the detection of specific infectious agent especially for early diagnosis including optical and electrochemical biosensors for detection of DNA hybridization. In this case, optical biosensor offers great advantages for the highly sensitivity, simple, selectivity and low-cost approach. This method apply fluorescence signal for the modified sensor that has been immobilized with ssDNA which known as a key for further preparation of optical biosensor to detect specific DNA via DNA hybridization process.

In this study, quantum dot (QD) is used as a sensor and involves in monitoring the hybridization event between ssDNA (probe) and its complementary target DNA. Furthermore, the optimization of hybridization condition and the analytical performance were also studied. The hybridization of the system with non-complementary DNA is performed to study the selectivity of the detection system. The detection of the events occurred in the system were based on fluorescence resonance energy transfer (FRET) for detection mode. The hybridization of the complementary target DNA with ssDNA and reporter probe will bring the acceptor, Cy5 and donor QD into close distance, causing the emission of the Cy5 or called as FRET illumination from the donor QD once excited at certain wavelength.

1.5 Objectives

The general objective of the study is to developed DNA biosensor for detection of DNA of *Ganoderma boninense*.

1. To study the interaction of modified quantum dot with ssDNA, dsDNA and non-complementary DNA.
2. To study the effect of different types of hybridization procedures.
3. To investigate the optimum condition and analytical performance of DNA based detection method. Parameters investigated include hybridization time and temperature, different concentration of target DNA and selectivity study.

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