DEVELOPMENT OF NANOPARTICLE SENSORS FOR THE DETECTION AND QUANTIFICATION OF SWINE DNA IN MIXED BIOLOGICAL AND COMMERCIAL SAMPLES FOR HALAL AUTHENTICATION

BY

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SYMBOLS AND ABBREVIATIONS

ANS	Anserine (ß-alanyl-L-1 methylhistidine)
APTES	3-aminopropyltriethoxysilane
BAL	Balenine (ß-alanyl-L-3 methylhistidine)
BLAST	Basic local alignment search tool
BOLD	Barcode of life data systems
bp	Base pair
BSE	Bovine spongiform encephalopathy
CAR	Carnosine (ß-alanyl -L- histidine)
CBIOL	Consortium for the barcode of life
CNT	Carbon nanotube
CE	Capillary electrophoresis
CO1	Cytochrome oxidase 1
Cq	Quantification cycle
Cy3	Cyanine 3
Cy5	Cyanine 5
Cytb	Cytochrome b
DGGE	Denatured gradient-gel electrophoresis
DL	Detection limit
DNA	Deoxy-ribonucleic acid
ds-DNA	Double-stranded DNA
DPN	Dip-pen
EC	European Commission
EG_3-OH	Triethylene glycol
EG_3-COOH	11-mercaptoundecyl tryethylene glycol acetic acid
ELISA	Enzyme-linked immunosorbant assay
EtBr	Ethidium bromode
Eu18SrRNAF	Eukaryotic 18S rRNA specific forward primer
Eu18SrRNAF	Eukaryotic 18S rRNA specific reverse primer
	Eukaryotic 18S rRNA specific TaqMan probe

EU	European Uninon
FAM	6-carboxyfluoresceine
FF	Frank furter
FRET	Fluorescence resonance energy transfer
fg	Femtogram
Fmol L^{-1}	Femtomole per liter
g	Gram
GNP	Gold nanoparticle
h	hour
IABkFQ	3-Iowa black FQ
I_D	Drain current
IDT	Intregated DNA Technologies
IEF	Isoelectric focusing
ISFET	Ion-sensitive field-effect transistor
Kb	Kilo-base pair
LCD	Liquid crystal display
LED	Light-emitting diode
LOD	Limit of detection
L	Liter
Mab	Monoclonal antibodies
Mb	Mega base pair
MB	Meatball
MBM	Meat and bone meal
mg	Miligram
µg	Microgram
µg mL^{-1}	Microgram per mililiter
min	minute
mL	Mililiter
µL	Microliter
M	Molar or mole per liter
mM	Milimolar or milimole per liter

μM	Micromolar or micromole per liter
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIM	Metal-insulator-metal multilayer
MNG	Metal nanogap
MNGD	Metal nanogap devices
MLC	Myosin light chain
MOSFET	Metal-oxide-semiconductor field-effect transistor
MRI	Magnetic resonance imaging
nMOSFET	n-type MOSFET
pMOSFET	p-type MOSFET
Mt-DNA	Mitochondrial DNA
NCBI	National center for biotechnology information
NeCMOS	Nanogap-embedded complementary metal-oxide-semiconductor
ng	Nanogram
ng mL^{-1}	Nanogram per mililiter
nm	Nanometer
nM	NanoMolar
NNI	Natinal nanotechnology initiatives
OC	Osteocalcin
P_{ab}	Polyclonal antibody
PBS	Phosphate buffer saline
PCG	Protein coding gene
PCR	Polymerase chain reaction
pg	Picogram
pM	Picomolar or pecomole per liter
Poly A	Poly adenosine
PSA	Prostate-specific antigen
qPCR	Quantitative PCR
QD	Quantum dot
RMSEC	Root mean square error of calibration

RMSEV	Root mean square error of validation
R^2	Correlation coefficient
RT-PCR	Real-time PCR
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RFLP	Restriction fragment length polymorphism
s	Second
SAM	Self-assembled monolayer
SB	Streaky bacon
SERS	Surface enhanced Raman spectral
SOI	Silicon-on-insulator
SNP	Single-nucleotide polymorphism
SPR	Surface plasmon resonance
ss-DNA	Single-stranded DNA
SSP	Species-specific PCR
STR	Short tandem repeat
SWCNT	Single-walled carbon nanotube
SwcytbF	Swine cytochrome b specific forward primer
SwcytbR	Swine cytochrome b specific reverse primer
SwcytbTqM	Swine cytochrome b specific TaqMan probe
TGGE	Temperature gradient-gel electrophoresis
TMR	Tetramethyl rhodamine
TOPO	Trioctylphosphine
Tox	Thick gate-oxide
Trox	Thin-layer of gate oxide
VNTR	Variable tandem repeat
V_T	Threshold voltage

PEMBANGUNAN PENGESAN PARTIKEL-NANO UNTUK MENGESAN DAN MENGKUANTIFIKASI DNA BABI DI DALAM CAMPURAN BIOLOGI DAN SAMPEL KOMERSIAL UNTUK AUTENTIKASI HALAL

ABSTRAK

Pengesahan komponen terisytihar di dalam daging dan produk-daging sangat penting untuk memastikan ketelusan dalam pelabelan makanan dan untuk melindungi pengguna dari segi kepercayaan, pegangan agama, kesihatan dan usaha gigih. Pulangan tahunan untuk pasaran makanan halal sedunia telah mencapai sehingga 661 billion dollar Amerika pada 2011 dan akan berkembang dalam tahun-tahun yang akan datang. Untuk menghadapi pasaran yang berdaya saing tinggi dan untuk mengaut keuntungan yang berlebihan, penipuan label jenama Halal kerap berlaku. Oleh kerana daging babi dan hasil lebihannya mudah didapati pada harga yang lebih murah, kes penukaran daging halal yang lebih tinggi harganya dengan daging babi yang lebih rendah nilainya dalam produk daging komersial kerap berlaku. Walau bagaimanapun, campuran daging babi dan bahan-bahan lebihannya yang diperolehi dalam makanan Halal dan Kosher adalah satu perkara yang serius kerana sensitiviti agama yang mana babi tidak dibenarkan untuk dimakan oleh umat Islam, Yahudi dan Hindu berdasarkan peraturan agama masing-masing. Oleh itu, peralatan analisis yang sensitif, berkeupayaan tinggi, dan mudah digunakan sangat diperlukan untuk mengesan dan mengira tahap pencemaran daging babi dalam produk makanan Halal dan Kosher.

Kaedah konvensional analisis DNA yang berasaskan kepada tindak balas rantai polymerase (PCR) telah mencapai keupayaan tertinggi dan mempunyai had tertentu dalam mengenalpasti penanda DNA yang pendek dan terbukti boleh dikesan walaupun dalam keadaan pemprosesan makanan yang kritikal. Tambahan pula, kaedah berasaskan PCR bukan sahaja mahal, tetapi juga memerlukan pengesahan produk untuk menghapuskan keraguan dalam situasi tertentu. Pengesanan DNA menggunakan nanopartikel emas (GNPs) adalah sangat baik kerana ia tidak memerlukan apa-apa instrumen yang kompleks dan mahal dan membolehkan pengesanan yang pantas dan boleh dipercayai menggunakan jujukan DNA yang sangat pendek (15-30 pb).

Dalam laporan ini, kami telah membangunkan dua jenis pengesan berasaskan pengesan-GNP. Pengesan pertama ialah pengesan kalorimetri yang membolehkan pengesanan visual daripada amplifikasi dan bukan amplifikasi PCR menggunakan penanda DNA babi (27 dan 25 nukleotida) dalam tempoh 10 minit tanpa apa-apa alat bantuan pada campuran produk daging terproses dan yang belum diproses. Ia juga membolehkan pengesahan keputusan visual yang ditentukan oleh ciri-ciri spektra penyerapan GNPs terkumpul dan tidak terkumpul yang berbeza-beza. Had pengesanan (DL) kaedah ini adalah diantara 4-6 ng μL^{-1} DNA genomik babi. Pengesan kedua adalah probnanobio hibrid yang secara kovalen bersepadu dengan label fluorofor 27-nukleotida pecahan-AluI daripada gen mitokondria cytochrome b (mt-cytb) babi. Ia membenarkan pengesanan dan kuantifikasi banyak salinan mt-DNA babi secara serentak dalam tempoh masa 60 minit dengan bantuan spektroskopi pendarfluor yang sangat efektif dari segi kosnya. Had pengesanan kaedah ini untuk daging babi tercemar yang teruk diautoklaf bersama campuran daging lembu adalah (0.42 ng μL^{-1}) di mana saiz template PCR yang lebih panjang telah terpecah kepada serpihan kecil yang menyebabkan ianya gagal dikesan menggunakan kaedah PCR.

Penanda DNA lebih pendek (15-30 pb) yang sesuai untuk aplikasi biosensor ini telah dihasilkan oleh pencernaan genom mitokondria babi secara dalam-silico dengan menggunakan enzim sekatan AluI. Sebelum aplikasi biosensor, spesifikasi penanda mt-DNA babi diuji dengan membangunkan PCR-sekatan serpihan polymorphism panjang (RFLP) yang singkat (109 pb) seperti prob TaqMan yang digunakan untuk analisis kuantitatif real-time PCR (qPCR) yang mengandungi laman AluI yang sesuai dengan amplikon. Kedua-dua PCR-RFLP dan ujian qPCR didapati sangat berkesan untuk mengesan sehingga serendah 0.0001 ng DNA genomic babi yang tulen dan 0.001 ng daripada pencemaran babi dalam produk daging komersil yang sedia untuk dimakan.

DEVELOPMENT OF NANOPARTICLE SENSORS FOR THE DETECTION AND QUANTIFICATION OF SWINE DNA IN MIXED BIOLOGICAL AND COMMERCIAL SAMPLES FOR HALAL AUTHENTICATION

ABSTRACT

Verification of declared components in meats and meat products is essential to ensure transparency in food labeling and to safeguard consumers' trusts, religious faiths, health, and hard earned fortunes. The annual turnover of the global Halal food market has reached USD 661billion in 2011 and will be proliferating in the coming years. To coup up in highly competitive market and to make an excessive profit, fraudulent labeling of Halal brand is frequently occurring. As pork and pork-derivatives are easily available at cheaper prices, replacement of higher priced Halal meats in commercial meat products with lower valued pork has become quite prevalent. However, the mixing of pork and pork-derived materials in the Halal and Kosher foods is a serious matter as they are not allowed to be consumed by the followers of Islam and Judaism by respective religious laws. Thus, sensitive, dependable, and easy performable analytical tools have long been desired to detect and quantify the minute level of adulterated pork in Halal and Kosher foods.

Conventional methods based on polymerase chain reaction (PCR)-based DNA analysis has reached a ceiling stage and has limitations in detecting shorter-length DNA markers which are proven to be survived in the harsh conditions of food processing. Further, PCR-based methods are not only expensive but also needs product authentication to eliminate ambiguity in certain instances. DNA detection using gold nanoparticles (GNPs) is very promising because it does not need any complex and expensive instrumentations and allows rapid and reliable detection of very short-length (15-30 bp) DNA sequences.

In this report, we have developed two types of GNP-based sensors. The first one is the colorimetric sensor that allowed visual detection of PCR amplified and non-amplified swine DNA markers (27 and 25 nucleotides) within 10 min without any instrumental aids in a mixed background of processed and unprocessed meat products. It also permitted the verification of the visually determined results by the distinct features of the absorption spectra of the aggregated and non-aggregated GNPs. The detection limit (DL) of this method varied between 4-6 ng μL^{-1} of porcine genomic DNA. The second one was the hybrid nanobioprobe that covalently integrated a fluorophore labeled 27-nucleotide *Alu*I-fragment of swine mitochondrial cytochrome b (mt-cytb) gene. It allowed simultaneous detection and quantification of porcine muticopy mt-DNA targets within 60 min with the help of a cost-effective fluorescent spectroscopy. The DL of this method was 0.42 ng μL^{-1} of contaminated pork in severely autoclaved pork-beef binary mixture in which longer-size PCR template were broken down to smaller fragments leading to detection failure by the PCR- methodology.

The shorter-lengths DNA markers (15-30 bp) suitable for biosensor application were developed by in-silico digestion of swine mt-genome with *Alu*I restriction enzymes. Prior to biosensor applications, the swine specificity of the developed mt-DNA markers was tested by developing a very short (109 bp) PCR-restriction fragment length polymorphism (RFLP) assay as well as a TaqMan probe quantitative real-time PCR (qPCR) assay containing appropriate *Alu*I-sites within the amplicon. Both the PCR-RFLP and qPCR assays were effective to detect as low as 0.0001 ng of pure swine-genomic DNA and 0.001 ng of pork contamination in ready-to-eat commercial meat products.

CHAPTER 1

RESEARCH BACKGROUND, OBJECTIVES AND SCOPES

RESEARCH BACKGROUND, OBJECTIVES AND SCOPES

1.1 Introduction

"Halal" is a Quranic term meaning allowed or permitted. Halal food means Shariah compliant hygienic foods and drinks that are prepared following the Shariah law of Islam (Standard, 2009). The global Halal food industry stood at USD 641 billion in 2010 and is estimated to cross USD 661 billion by 2011. If pharmaceuticals and cosmetics are included, the total turnover of global Halal market will exceed to USD 2.3 trillion by 2011 (Badawi, 2011). The current trends show the industry will be proliferating in the coming years to keep space with the ever increasing demands for Halal products.

"Halal Logo" on finished consumer goods is regarded as a symbol of Shariah compliant products and is trusted by 1.8 billion Muslim consumers of the globe (Badawi, 2011). To survive in highly competitive markets and gain additional profit, fraudulent labeling of Halal brand is frequently occurring (Aida,Che Man,Wong,Raha &Son, 2005; Che Man,Aida,Raha &Son, 2007; Murugaiah et al., 2009; Rohman,Sismindari,Erwanto &Che Man, 2011). As minced meat is being increasingly used as additives in most of the modern foods, (Tanabe et al., 2007) verification of labeling on finished foods should ensure food safety (i.e., unexpected occurrence of food allergies), gain consumer trusts, and promotes fair trades in local and international markets. According to EC legislation (178/2002) on food safety (Commission, 2002) all stake holders in a food supply chain must be able to clearly identify all raw materials used in the preparation of food products. In fact, most countries in today's world have regulatory bodies to monitor the labeling practices of consumer goods (Tanabe *et al.*, 2007).

Pork is a potential adulterant in Halal and Kosher food products because of its easier availability and cheaper prices than those of Halal (allowable) meats (Regenstein,Chaudry &Regenstein, 2003; Tanabe *et al.*, 2007). Several molecular techniques based on lipids (Rohman *et al.*, 2011; SzabÓ,FÉBel,SugÁR &RomvÁRi, 2007), proteins (Ayaz,Ayaz &Erol, 2006; Chen,Hsieh &Bridgman, 2004), and DNA (Aida *et al.*, 2005; Che Man,Mustafa,Khairil Mokhtar,Nordin &Sazili, 2010; Rojas et al., 2010; Yusop,Mustafa,Che Man,Omar &Mokhtar, 2011) have been developed for the identification of meat species. Methods based on lipids are not reliable as both the type and content of fat can be significantly manipulated through the cooking process. Susceptibility of protein markers to heat and chemicals has diminished the dependability of the protein-based methods (Asensio,González,García &Martín, 2008). On the other hand, DNA-based molecular techniques have been emerged as a smart analytical tool due to codon degeneracy, higher stability and universal presence of DNA in majority of cells, enabling identical information to be obtained from the same animal, regardless of the tissue of origin (Ballin,Vogensen &Karlsson, 2009). Among the DNA-based methods, polymerase chain reaction (PCR) assays have received huge attention because of its easy operation and extraordinary power to amplify specific target sequences from few copies to easily detectable quantities even in a very complex pool of genomic DNAs (Li &Rothberg, 2004; Rodríguez,García,González,Hernández &Martín, 2005; Sakai et al., 2011). The sensitivity of the PCR assays can be further improved by targeting muticopy gene, such as mitochondrial (mt) gene with shorter-amplicon lengths (Murugaiah *et al.*, 2009; Tanabe *et al.*, 2007).

PCR-assays of shorter amplicon-lengths are preferable for the analysis of compromised samples as they are less affected by degradation, amplify more efficiently, and better resolute in capillary electrophoretic (CE) separation (Aboud,Gassmann

&McCord, 2010; Smith,Vigilant &Morin, 2002). It has been implicated that an amplicon-length of more than 150 bp (>150 bp) is unreliable for the analysis of species in processed food (Rojas *et al.*, 2010). However, a shorter amplicon in PCR assays can be hardly achieved without the compromise of specificity (Hird et al., 2006). A compromised PCR assay is problematic since it cannot be confirmed whether authentic target is detected or not.

Most of the DNA-detection techniques such as DNA microarray involve the labeling of target DNA with a fluorescent, radioactive or oxidizing agents (Teletchea, 2009). However, a labeled-based assay not only involves the additional cost of labeling but also brings error in the final results due to non-specific photobleaching of the fluorescent dye or other labeling agents (Niu, 2004). Recently, label-free methods based on surface plasmon resonance (SPR), electrochemical methods and mass based methods such as quartz crystal microbalance (QCM) and silicon cantilever have been documented (Niu, 2004). A label-free method based on SPR is particularly promising because of its higher sensitivity and operational simplicity. Typically, an SPR method measures the shift in SPR absorption angel at the interface of a metallic support and liquid medium upon target binding. Since the refractive index of the single-stranded DNA (ssDNA) and double stranded DNA (dsDNA) are significantly different, there is a large shift in SPR absorption angle when the target DNA hybridizes with the bound probe (Niu, 2004). Usually, a tag of higher refractive index is introduced into the probe to enhance the SPR sensitivity. A metallic particle such as gold nanoparticles (GNPs) are specially promising for this application because of its large dielectric constant, high density and biocompatibility (Niu, 2004). Actually, colloidal gold particles, covalently or non-covalently linked to oligonucleotide have been applied for the detection of specific sequences and single-nucleotide mismatches for decades (Dubertret,Calame

&Libchaber, 2001; Elghanian,Storhoff,Mucic,Letsinger &Mirkin, 1997; Li &Rothberg, 2004; Maxwell,Taylor &Nie, 2002; Mirkin,Letsinger,Mucic &Storhoff, 1996; Storhoff et al., 2000). However, such studies were limited to the laboratory level model experiments with synthetic oligo-targets. No studies so far practically explored the sequence and mismatch detecting power of the oligo-nanoparticle conjugates in heterogeneous biological samples and processed food products.

1.2 Problem Statements

Species authentication methods based on morphology, lipid and protein markers are not reliable. DNA-based methods based on PCR are reliable but cannot detect shorter DNA targets. Methods based on fluorescent and radio-labeling can detect short oligo targets but incur additional cost and errors in the final results due non-specific photobleaching of the labeled dyes.

1.3 Study Objectives

1.3.1 General Objectives

To develop, characterize, and test nanobiosensor probes for the detection and quantification of shorter swine DNA markers of mitochondrial origins in mixed biological and commercial samples for Halal authentication.

1.3.2 Specific Objectives

i. To develop shorter-length porcine DNA markers suitable for biosensor applications, targeting muticopy genes such as mitochondrial genes. The multicopy genes should ensure available targets even in degraded samples,