ELECTROCHEMICAL BIOSENSOR BASED ON SILICON NANOWIRES/PLATINUM NANOPARTICLES ASSOCIATED WITH LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR DETECTION OF PORCINE DNA IN FOOD

By

NORZILA BINTI KUSNIN

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

ELECTROCHEMICAL BIOSENSOR BASED ON SILICON NANOWIRES/PLATINUM NANOPARTICLES ASSOCIATED WITH LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR DETECTION OF PORCINE DNA IN FOOD

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February 2024

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In the past, issues with food adulteration, such as lard adulteration and the use of pig intestine casing sausage, have been the major issues in Malaysia since they are related to the halal status of the food products. The porcine derivatives include pork fat (lard), porcine gelatin, porcine blood plasma and mechanically recovered meat usually used by the food manufacturers in most countries because of their cheaper price and ready availability. Hence in order to ensure the food and dairy products that are free from the porcine derivatives, there is a requirement of developing sensitive and rapid methods which can trace even the minute composition. In this work, an electrochemical DNA biosensor has been developed based on the fabrication of silicon nanowires/platinum nanoparticles (SiNWs/PtNPs) onto a screen-printed carbon electrode (SPCE) for the detection of *Sus scrofa* mitochondrian DNA (mtDNA) in food utilizing ferrocenylnaphthalene diimide (FND) as indicator. In this study, the morphology and elemental analysis of SiNWs/PtNPs-modified SPCE was analyzed by field emission

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scanning electron microscopy (FESEM) combined with energy dispersive X-ray (EDX), respectively. The cyclic voltammetry (CV) was used to study an electrical contact between the PtNPs and the screen-printed working electrode through SiNWs, while electrochemical impedance spectroscopy (EIS) was used to measure the charge transfer resistance of the modified electrode. Based on the results, it clearly showed that the SiNWs/PtNPs was successfully coated onto the electrode and the effective surface area for SiNWs/PtNPs-modified SPCE was increased 16.8 times as compared with the bare SPCE. The differential pulse voltammetry (DPV) used for the detection of porcine DNA with FND as an intercalator was confirmed for its specific binding to the double-stranded DNA (dsDNA) sequences. The increase of FND peak current was obtained after hybridization detection by fabricated electrode. The optimal performance of SiNWs/PtNPs-modified SPCE for electrochemical detection of porcine DNA was optimize with several parameters; DNA probe concentration (5 μM), immobilization time (24 hours), pH buffer (7.5), different types of salts in the hybridization buffer (NaCl), salt concentration (1.0 M), hybridization temperature (40 °C) and incubation time (20 min), respectively. The developed biosensor showed selective response towards complementary target DNA and able to distinguish non-complementary and mismatched DNA oligonucleotides. The SiNWs/PtNPs-modified SPCE that was fortified with DNA hybridization demonstrated a good linearity in the range of 0.0219 ng/μL to 219 ng/μL (R^2 = 0.96) with the detection limit of 175.2 ng/μL. The developed DNA sensor also showed excellent current reproducibility with a relative standard deviation (RSD) of 2.52% when testing with a series of five modified electrodes under the same preparation batch. The sensor demonstrated good storage stability with slight attenuation from the initial current (week 1) after week 5 of storage. In order to assess the capability of the developed DNA biosensor to reliably

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detect real samples, the application of a suitable amplification method was considered. Loop-mediated isothermal amplification (LAMP) was chosen due to the rapid amplification time and isothermal conditions, which simplify the equipment requirements. The preparation of a specific and amplified target gene using LAMP was investigated. A set of specific primers (F3, B3, FIP, BIP, and LF) were successfully designed to initiate strand displacement and DNA synthesis under isothermal conditions. The amplification parameters, including temperature and incubation time, were optimized, with positive results detected at a stable temperature of 63°C for 60 minutes. The results were visualized through the turbidity caused by the white precipitate of magnesium pyrophosphate, a byproduct of the amplification process. Cross-reactivity studies with various types of meat and processed foods demonstrated good reliability, specifically for detecting porcine species. In conclusion, the DNA biosensor was successfully developed and can be used to detect specific target of porcine DNA in food for halal purposes. This biosensor demonstrates high specificity, making it a valuable tool for ensuring the authenticity of halal-certified products. Its application in the food industry can significantly enhance quality control measures, thus providing consumers with greater confidence in the halal status of their food.

Keywords: DNA biosensor; halal detection; loop-mediated isothermal amplification (LAMP); silicon nanowires

SDG: GOAL 9: Industry, innovation and infrastructure

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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ELEKTROKIMIA SENSOR DNA BERDASARKAN SILIKON NANOWAYAR/PLATINUM NANOPARTIKEL BERSERTA DENGAN AMPLIFIKASI ISOTERMAL BERPANDU GELUNG UNTUK PENGESANAN DNA BABI DALAM MAKANAN

Oleh

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Pada masa lalu, isu mengenai pemalsuan makanan seperti pemalsuan dengan lemak babi dan penggunaan usus babi sebagai sarung sosej, telah menjadi isu utama di Malaysia kerana ia berkaitan dengan status halal produk makanan. Derivatif babi termasuk lemak babi, gelatin babi, plasma darah babi dan daging yang diperoleh secara mekanikal biasanya digunakan oleh pengeluar makanan di kebanyakan negara kerana harganya yang lebih murah dan ketersediaannya yang tinggi. Oleh itu, untuk memastikan produk makanan dan tenusu bebas daripada derivatif babi, terdapat keperluan untuk membangunkan kaedah yang sensitif dan pantas yang dapat mengesan walaupun komposisi yang sangat sedikit. Dalam kajian ini, biosensor DNA elektrokimia telah dibangunkan berdasarkan fabrikasi silikon nanowayar/platinum nanopartikel (SiNWs/PtNPs) ke atas elektrod karbon bercetak skrin (SPCE) untuk mengesan DNA mitokondria *Sus scrofa* (mtDNA) dalam makanan menggunakan ferrosenilnaphthalena diimida (FND) sebagai penunjuk. Dalam kajian ini, morfologi

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dan analisis unsur SPCE yang diubah suai dengan SiNWs/PtNPs dianalisis menggunakan mikroskopi elektron imbasan pancaran medan (FESEM) digabungkan dengan spektroskopi sinar-X penyebaran tenaga (EDX). Voltammetri kitaran (CV) digunakan untuk mengkaji hubungan elektrik antara PtNPs dan elektrod karbon bercetak skrin melalui SiNWs, manakala spektroskopi impendans elektrokimia (EIS) digunakan untuk mengukur rintangan pemindahan cas elektrod yang diubahsuai. Berdasarkan hasil kajian, ia jelas menunjukkan bahawa SiNWs/PtNPs telah berjaya disalutkan pada elektrod dan luas permukaan berkesan untuk SPCE yang diubahsuai dengan SiNWs/PtNPS meningkat sebanyak 16.8 kali berbanding dengan SPCE yang tidak diubah suai. Voltammetry nadi pembezaan (DPV) yang digunakan untuk pengesanan DNA babi dengan FND sebagai penunjuk telah disahkan untuk pengikatan khusus pada urutan jujukan DNA berantai dua (dsDNA). Peningkatan arus puncak FND diperoleh selepas pengesanan hibridisasi oleh elektrod yang difabrikasi. Prestasi optimum SPCE yang telah diubahsuai dengan SiNWs/PtNPs untuk pengesanan elektrokimia DNA babi telah dioptimumkan dengan beberapa parameter; kepekatan probe DNA (5 μM), masa imobilisasi (24 jam), penimbal pH (7.5), pelbagai jenis garam dalam penimbal hibridisasi (NaCl), kepekatan garam (1.0 M), suhu hibridisasi (40 °C) dan masa inkubasi (20 min). Biosensor yang dibangunkan menunjukkan tindak balas selektif terhadap DNA sasaran pelengkap dan mampu membezakan oligonukleotida DNA bukan pelengkap dan tidak sepadan. SPCE yang diubahsuai dengan SiNWs/PtNPs yang diperkukuh dengan hibridisasi DNA menunjukkan kelinearan yang baik dalam julat 0.0219 ng/μL hingga 219 ng/μL (R^2 = 0.96) dengan had pengesanan 175.2 ng/μL. Biosensor DNA yang dibangunkan juga menunjukkan kebolehulangan arus yang sangat baik dengan sisihan piawai (RSD) sebanyak 2.52% apabila diuji dengan lima elektrod yang diubahsuai dalam kumpulan penyediaan yang

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sama. Sensor ini menunjukkan kestabilan simpanan yang baik dengan sedikit penurunan daripada arus awal (minggu 1) selepas minggu ke-5 penyimpanan. Untuk menilai keupayaan biosensor DNA yang dibangunkan untuk mengesan sampel sebenar dengan tepat, aplikasi kaedah amplifikasi yang sesuai telah dipertimbangkan. Amplifikasi isotermal berpandu gelung (LAMP) dipilih kerana masa amplifikasi yang pantas dan keadaan isoterma, yang menyederhanakan keperluan peralatan. Penyediaan gen sasaran yang spesifik dan amplifikasi menggunakan LAMP telah dikaji. Satu set primer khusus (F3, B3, FIP, BIP dan LF) berjaya direka untuk memulakan penggantian utas dan sintesis DNA di bawah keadaan isoterma. Parameter amplifikasi termasuk suhu dan masa inkubasi, telah dioptimumkan, dengan hasil positif dikesan pada suhu stabil 63 °C selama 60 minit. Hasilnya dapat dilihat melalui kekeruhan yang disebabkan oleh mendakan putih magnesium pirofosfat, hasil sampingan daripada proses amplifikasi. Kajian kereaktifan silang dengan pelbagai jenis daging dan makanan yang diproses menunjukkan kesesuaian yang baik, khususnya untuk pengesanan spesis babi. Kesimpulannya, biosensor DNA telah berjaya dibangunkan dan boleh digunakan untuk mengesan sasaran khusus DNA babi dalam makanan bagi tujuan halal. Biosensor ini menunjukkan kepekaan yang tinggi, menjadikannya alat yang berharga untuk memastikan keaslian produk yang disahkan halal. Penggunaannya dalam industri makanan dapat meningkatkan langkah-langkah kawalan kualiti, sekali gus memberikan keyakinan yang lebih tinggi kepada pengguna mengenai status halal makanan mereka.

Kata kunci: Biosensor DNA; pengesan halal; amplifikasi isotermal berpandu gelung; silikon nanowayar

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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
A	Adenine
APTES	Aminopropyl-triethoxysilane
A_{280}	Absorbance value at 280 nm
A_{260}	Absorbance value at 260 nm
B3	Backward outer primer
BIP	Backward inner primer
bp	Base pairs
BSA	Bovine serum albumin
B*st*	Bacillus stearothermophilus
C	Cytosine
CV	Cyclic voltammetry
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
DTDPA	3,3-dithiodipropionic acid
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
F3	Forward outer primer
FESEM	Field-emission scanning electron microscopy
FIP	Forward inner primer

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FLP	Forward loop primer
FND	Ferrocenylnaphthalene diimide
FTIR	Fourier transform infrared spectroscopy
G	Guanine
HPLC	High performance liquid chromatography
HRTEM	High resolution transmission electron microscopy
IDT	Integrated DNA Technologies
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
M	Molar
MB	Methylene blue
mg	Milligram
mL	Millilitre
mM	Milimolar
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nM	Nanomolar
nm	Nanometer
PCR	Polymerase chain reaction
pg	Picogram
pH	Numerical/logarithmic scale to specify acidity or alkalinity
POC	Point of care
ppm	Part per million
PtNPs	Platinum nanoparticles

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qPCR	Quantitative polymerase chain reaction
RT-PCR	Real time polymerase chain reaction
SAM	Self-assembly monolayer
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SiNWs	Silicon nanowires
SPCE	Screen printed carbon electrode
ssDNA	Single stranded DNA
T	Thymine
TBE	Tris-borate-EDTA
TE	Tris-EDTA
T_m	Melting temperature
UV-Vis	Ultraviolet-visible spectroscopy
V	Voltage
V/s	Volt per second
µA	Microampere
µL	Microlitre
µM	Micromolar
Ω	Ohm

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CHAPTER 1

INTRODUCTION

1.1 Background of study

In recent years, legislation has increasingly required companies to disclose all product ingredients, with some developing nations also adopting these mandates. Despite stringent regulations in countries like Canada, Malaysia, Indonesia, and Saudi Arabia, detecting food adulteration remains challenging (Nakyinsige et al., 2012; Rejeb et al., 2021). Accurate labeling is a crucial component for informing consumers about the products ingredients and also promotes fair trade. Changes in lifestyles have led to more processed foods (Vandendriessche, 2008; Zia et al., 2020), resulting in common adulteration and fraud in the food industry for financial gain. In the past, there have been issues arise with halal certification and food adulteration, such as using pig intestine casings and adulterated lard (Man et al., 2007; Lianou et al., 2021). This issue arise because pork byproducts are often used due to their low cost and availability (Aida et al., 2005; Fadzillah et al., 2020). Islam and Judaism prohibit pork, necessitating sensitive techniques to identify even trace amounts of porcine byproducts to ensure halal certification (Windarsih et al., 2022; Febriandika & Hakim, 2023).

Early porcine detection methods, including sodium SDS-PAGE, ELISA, and HPLC, primarily targeted soluble proteins (Alikord et al., 2018). SDS-PAGE separates proteins by size, but interpretation is challenging, especially with heavily processed foods (Galimberti et al., 2013; Zia et al., 2020). HPLC, though accurate, requires

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specialized technicians and costly equipment (El Sheikha et al., 2017), and protein-based tests suffer from cross-reactivity issues (Dooley et al., 2004; Zia et al., 2020). Molecular biology introduced a PCR-based approach for porcine detection, leveraging mtDNA abundance for species identification (Farag, 2020; Montiel-Sosa et al., 2000). While effective, these methods demand advanced equipment and trained personnel (Dhama et al., 2014; Raja Nhari et al., 2023), highlighting the need for alternative, cost-effective detection methods.

DNA electrochemical biosensors, known for their precision, speed, and cost-effectiveness, are attracting considerable attention (Lawal, 2023). These sensors employ a transducer (electrode) and a highly specific biorecognition interface, seamlessly integrating into portable technology (Shanbhag et al., 2023). Crucially, selecting the right DNA matrix is vital for enhancing sensor sensitivity, achieving low detection limits, and maximizing surface area (Leonardo et al., 2021). Nanotechnology advancements have led to the development of silicon nanowires (SiNWs), offering substantial surface-to-volume ratios and sizes ranging from 1 to 100 nm, facilitating efficient electron transport and rapid detection reactions (Abd Rahman et al., 2022; Gao et al., 2011; Choi et al., 2013; Raman et al., 2023). SiNWs enhance interaction with target analytes, improving signal generation and detection sensitivity (Rashid et al., 2023). Platinum nanoparticles (PtNPs) are extensively studied for chemical and biological detection due to their excellent electrical conductivity, biocompatibility, and large surface area (Kishi & Umeda, 2009; Mazzotta et al., 2021). PtNPs offer significant potential for electrode modification, enhancing sensitivity, selectivity, and detection consistency across various biosensor applications (Lian et al., 2012; Tran,

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2023; Dumore & Mukhopadhyay, 2022; Dursun & Gelmez, 2010; Makhlouf et al., 2022; Kalambate et al., 2015).

An electroactive hybridization indicator, as an alternative to hybridization-based sensors (Song et al., 2019), improved electrochemical responses by increasing indicator concentration at the electrode surface during hybridization. For optimal sensitivity and selectivity, it must differentiate between ssDNA and dsDNA and produce a clear, low-potential voltammetric response. Recent research by Derakhshan et al. (2023) demonstrated esculetin's use as an electroactive indicator for detecting Hepatitis B Virus (HBV) DNA at concentrations as low as 3.80 nM. Electroactive indicators, such as ferrocenylnaphthalene diimide (FND), have been developed for higher sensitivity and selectivity. FND, a threading intercalator, shows lower affinity for single-stranded probes and higher affinity for DNA duplexes (Takenaka, 2021). FND stabilizes DNA double helices, protects DNA from degradation, and improves structural integrity. Its redox active ferrocene moieties enable electrochemical sensing and investigation of DNA damage, making it valuable in biosensing and DNA-related redox processes (Yang et al., 2015). Gaiji et al. (2017) synthesized FND for detecting PNA-DNA duplex hybridization with a low detection limit in the femtomolar range.

Loop-mediated isothermal amplification (LAMP) complements PCR-based detection, offering speed, ease of use, robustness, and suitability for in-field testing (Panno et al., 2020; Notomi et al., 2000). Using B*st* DNA polymerase, LAMP enables strand displacement DNA synthesis in an isothermal environment, known for its specificity and sensitivity (Neshani et al., 2023; Thompson & Lei, 2020). LAMP's simplicity makes it affordable and practical, requiring only a heating block or water bath (Liu et

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al., 2012). It amplifies up to 10^9 DNA copies within an hour using six primers targeting specific sequences (Fukuta et al., 2013). Evaluation involves electrophoresis on 2% agarose gels, showing ladder-like patterns or bands indicating a positive response (Zhang et al., 2014).

1.2 Problem statement

Current porcine detection methods have several drawbacks, including time requirements, high costs, the need for skilled personnel, and complex equipment. Electrochemical DNA biosensors have been proposed to overcome these limitations for porcine DNA detection in food. These biosensors offer benefits like continuous monitoring, high sensitivity, rapid response, portability, ease of use, and minimal sample preparation. They are known for their specificity in amplifying DNA targets and quantifying nucleic acid. However, detecting low DNA concentrations in food samples remains challenging. To enhance sensitivity and selectivity, chemically modified electrodes are often used instead of bare electrodes. While promising, existing DNA biosensors for porcine detection face issues like false negatives or positives due to variations in DNA extraction, primer design, and interfering substances. Researchers are exploring nanomaterials as surface modifiers to improve these sensors (Salleh & Hassan, 2023). The use of silicon nanowires (SiNWs) combine with platinum nanoparticles (PtNPs) is not well documented. Key challenges include ensuring the proper DNA immobilization layer on the electrode, minimizing nonspecific interactions, and converting DNA hybridization events into measurable signals. Optimal conditions for DNA layer formation are essential for the sensor's performance.

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Previous studies, such as those by Barakat et al. (2014) and Erwanto et al. (2014), have used PCR for pork detection in food products like meatballs and sausages by targeting the cytochrome b gene. However, PCR is costly, requires skilled professionals, and is time-consuming. Loop-mediated isothermal amplification (LAMP) has been adopted as a quicker, more sensitive, and specific alternative, offering a simple and affordable setup with visual detection. Creating suitable primers is crucial for LAMP's performance. This study used LAMP-amplified products for electrochemical detection, enhancing the current response of targeted DNA due to the abundance of porcine mitochondrial DNA, thus reducing false negatives from low DNA concentrations. We developed a new electrochemical DNA biosensor for detecting porcine DNA in food. This biosensor uses isothermal amplification (LAMP) with differential pulse voltammetry (DPV) detection, offering a quick, accurate, and selective method for halal food analysis.

1.3 Novelty of research

This study is the first to use silicon nanowires (SiNWs) with platinum nanoparticles (PtNPs) as a DNA probe matrix in an electrochemical biosensor. It also pioneers the combination of loop-mediated isothermal amplification (LAMP) and a hybrid ferrocenylnaphthalene diimide indicator for detecting porcine DNA in food. This method replaces traditional agarose gel electrophoresis with SiNWs/PtNPs-based electrochemical sensor, enhancing sensitivity and specificity by monitoring redox current changes. Additionally, the LAMP primer sequences used are newly reported in this research.

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1.4 Research objectives

This study aims to develop a novel, practical, and affordable DNA electrochemical biosensor for detecting porcine DNA in food. The details and specific objectives are listed below:

I. To prepare and characterize SiNWs/PtNPs-modified SPCE for the electrochemical detection of porcine DNA.
II. To immobilize DNA probes on the surface of SiNWs/PtNPs-modified SPCE under optimal concentration for the electrochemical detection of porcine DNA
III. To design and optimize LAMP primers to enhance DNA amplification
IV. To assess the analytical performance of the optimized DNA electrochemical sensor using raw pork, raw beef, raw lamb, raw chicken, pork sausage, and canned pork

1.5 Scope and limitations

This study focused on detecting porcine mitochondrial DNA (mtDNA) in raw meat and specific processed pork products, excluding highly processed foods such as gummies. A limited sample size was utilized to showcase the performance of the DNA biosensor. Future research should encompass a wider variety of food matrices and a more extensive sampling approach to comprehensively validate the biosensor's applicability.

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1.6 Thesis outline

Chapter 1 provides a brief introduction to the significance of halal food for consumers, emphasizing the growing concern over adulteration issues associated with food labeled as halal. Additionally, it addresses the limitations of existing methods used to detect porcine DNA in food samples. These limitations underscore the relevance and primary objective of this research, which aims to develop a simple and sensitive DNA biosensor for the detection of porcine DNA in food. The proposed approach involves utilizing SiNWs/PtNPs as electrode surface modifiers and Loop-Mediated Isothermal Amplification (LAMP) as the DNA amplification technique.

Chapter 2 provides an in-depth exploration of halal issues and previously available methods for detecting porcine DNA. With the research's primary goal of developing a DNA biosensor for porcine DNA detection in mind, the chapter includes reviews on DNA, biosensor, nanomaterials, and hybridization indicators commonly used in biosensor development. Furthermore, it discusses commercially available kits for porcine DNA detection and reviews the steps and applications of loop-mediated isothermal amplification.

Chapter 3 outlines the experimental design and procedures undertaken throughout the research. These include synthesizing platinum nanoparticles, modifying electrodes with SiNWs/PtNPs, characterizing and optimizing the modified electrodes, optimizing DNA probe immobilization and hybridization, assessing their stability, selectivity, and sensitivity. Furthermore, it covers primer design for loop-mediated isothermal amplification, optimization of LAMP conditions, and DNA extraction from food

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samples. Method validation and application to real samples are also addressed within this comprehensive framework.

Chapter 4 discusses the development and application of the DNA biosensor, detailing the factors contributing to the results. It begins with the synthesis of platinum nanoparticles (PtNPs), characterized by HR-TEM and UV-vis for optimal properties. The modification of screen-printed carbon electrodes (SPCE) with silicon nanowires (SiNWs) and PtNPs is then explained, with CV and EIS characterization showing enhanced electrochemical properties. The chapter also covers the optimization of DNA probe immobilization and hybridization conditions, as well as the design and optimization of LAMP primers for specific porcine DNA detection. Effective DNA extraction from food samples and validation using LAMP amplicons are discussed. The biosensor's application to real food samples demonstrates its capability for accurate porcine DNA detection, highlighting the synergistic effects of SiNWs and PtNPs in enhancing performance.

Chapter 5 provides a comprehensive research summary, conclusions, and recommendations for future work.

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