

Design of Immunobiosensors for Detection of Tumor-Associated Anti-P53 Autoantibodies: Method Development

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Dedication

This Thesis is dedicated to the memory of

My father-Mr Adeniyi Tunde John

&

Friend-Ogunlade Michael Temitope

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Abstract

Detection and profiling of circulating tumor-associated autoantibodies (TAABs) are useful for screening and early-stage diagnosis of asymptomatic lung cancer. Immunobiosensor technologies aimed to accomplish the highly sensitive, rapid and low-cost detection of TAABs can improve the early-stage detection of lung cancer. Immunobiosensors for the detection of anti-P53-tumour associated autoantibodies have been developed in this work. The design of sensing interfaces with immobilized P53 protein (P53ag) as a sensing element layer on a solid interface was investigated. Several methods of detecting anti-P53-antibodies (anti-P53ab) were investigated. These methods are label-free detection using electrochemical impedance spectroscopy (EIS) and two label techniques. The label-free electrochemical techniques utilize gold electrode pre-modified with a conducting layer of electrochemically grafted phenylethylamine for covalent immobilization of P53ag. The limit of anti-P53ab detection with the label-free EIS was 103.0 pg.ml^{-1} . The labeled technique developed utilizes fluorescent, and peroxidase-like nanomaterial labeled antibody as a detection probe. For the fluorescence detection, fluorescent silica nanoparticles were synthesized by overloading FITC into the silica matrix and conjugated to detection antibody (anti-IgG). The detection of the anti-P53ab was based on the dissolution of the silica nanoparticles to release the loaded dye as a signal amplification strategy. The fluorescence detection was carried out on a microplate, and magnetic bead modified P53-antigen platforms and limit of detection (LoD) were 42.0 fg.ml^{-1} and 3.3 fg.ml^{-1} for anti-P53ab; respectively. $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ hybrid nanoparticles were synthesized, and their peroxidase-like activity and colorimetric detection were evaluated. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ exhibited comparable activity to HRP. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ was conjugated to protein-G-anti-IgG for the detection of anti-P53ab on a microplate and cellulose paper platforms. The LoD was 20.0 fg.ml^{-1} and 63.0 fg.ml^{-1} for the microplate and cellulose paper platform; respectively. The potential application of the designed immunobiosensor was evaluated in simulated serum samples. The developed sensors showed higher detection sensitivity, stability and had a lower detection limit for anti-P53ab when compared with the ELISA based detection. The results have provided alternative and effective quantification approaches to ELISA and a promising future for multiplexed detection of tumor-associated autoantibodies. The developed methodologies in this thesis could be applied for the detection of other autoantibodies in other cancer types and auto-immune diseases.

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LIST OF ABBREVIATIONS

Abs	Antibodies
AC	Adenocarcinoma
AEBD	Aminoethylbenzene diazonium salt
Anti-P53	Anti-P53-autoantibodies
Anti-IgG	Mouse-anti-IgG (Fc-specific)
Anti-P53ab	Anti-P53-autoantibody
Avg.	Average
Ag AgCl	Silver chloride reference electrode
APA	4-(2-aminoethyl)phenylamine
Au	Gold electrode
CT	Computed tomography scan
CV	Cyclic voltammetry
DLS	Dynamic light scattering
EDS	Energy-dispersive x-ray spectroscopy
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunoassay
FTIR	Fourier transform infrared spectroscopy
FLISA	Fluorescent-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FITC@SiNP	Silica-coated FITC doped nanoparticles
FITC@SiNP-APTES-PEI	Polyethyleneimine grafted FITC doped silica nanoparticles
FITC@SiNP-APTES-PEI-anti-IgG	Anti-IgG conjugated Polyethylene imine grafted FITC doped silica nanoparticles

Fe₃O₄ NPs	Magnetite nanoparticles
Fe₃O₄@SiNP	Silica-coated magnetite nanoparticles
Fe₃O₄@SiNP@APTES-Au@Pd	Gold-palladium core shell nanoparticles supported on Silica coated magnetite nanoparticles
HRP	Horseradish peroxidase
SA	Succinic anhydride
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
ProG	Protein G
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
NSCLC	Non-small cell lung cancer
NLST	National Lung Screening Trial
MAGFLISA	Magnetic nanoparticles linked fluorescent immunoassay.
LMICs	Low and middle income countries
TAA_s	Tumor-associated antigens
TSA_s	Tumor-specific antigens
WHO	World Health Organization

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Motivation

Lung cancer is the leading cause of cancer-related deaths worldwide despite broad advances in surgical and therapeutic developments. The poor survival prognosis of lung cancer is primarily attributable to late diagnosis due to the asymptomatic nature of the disease. Early-stage detection of lung cancer when a tumor is still localized is vital for increasing the survival rate. Therefore, the development of diagnostic tools for screening of high-risk individuals and early-stage detection of lung cancer in the asymptomatic phase is essential in reducing the mortality rate. The motivation behind this study is to develop a highly sensitive and specific, user-friendly, rapid, and affordable immunobiosensors that would be suitable for screening of lung cancer in mobile laboratories, homes and at the doctor's office.

Immunobiosensors that can detect cancer-specific biomarkers in body fluids play a crucial role in cancer diagnosis, prognosis and monitoring of treatment. The essential factors that determine the successful application of immunobiosensors in a screening test and early-stage diagnosis of cancer include the selectivity, sensitivity, and specificity towards the targeted biomarker. Biomarkers that could distinguish between a healthy individual and early-stage lung cancer patients is highly desirable for use in lung cancer screening. Serological tumor-associated autoantibodies as a biomarker of choice have been chosen in this work due to their advantages over other lung cancer biomarkers. Tumor-associated autoantibodies are present at higher concentrations in the serum of an individual with lung cancer and are assayable years before any other tumor biomarkers. With the successful development of pregnancy and HIV/AIDS screening test kits, the majority of immunobiosensors for the generalized screening test applications have focused on colorimetric and fluorescent-based signal transduction methods. These immunobiosensors are cheap, user-friendly and are simple with ease of interpreting results. In recent times, the integration of smartphones and imaging software with optical transduction have allowed for quantification of targeted biomarker and ease of processing of

results. This characteristic will provide a more significant impact in achieving low-cost screening test kits for lung cancer without expensive clinic visits in advanced and low resources settings than other transduction methods. This work investigated the design of colorimetric and fluorescence immunobiosensors for the detection of tumor-associated autoantibodies, and the model analyte was the anti-P53-autoantibody (anti-P53ab).

Nanomaterials are interesting tools with unique physical and chemical properties due to the quantum-size effect when compared to bulk materials. The size, shape, composition, chemical and physical properties of nanomaterials can be tailored to produce biomarkers with unique properties. Nanomaterials are employed for signal amplification in the development of ultrasensitive immunobiosensors. This immunobiosensors has opened the opportunity to achieve the detection of ultra-trace concentrations of disease biomarker in clinical samples. This would be beneficial in detecting lung cancer biomarkers in an asymptomatic individual, thus shifting the late-stage diagnosis of lung cancer to the early-stage diagnosis. Hence, this work explored the unique advantages of nanomaterials (magnetite, gold, silica and palladium nanoparticles) and their composite in the design of an ultrasensitive immunobiosensors for the detection of tumor-associated autoantibodies.

Aim and research objectives

This study aims to develop a highly sensitive low-cost and miniaturizable immunobiosensor for screening and early-stage diagnosis of lung cancer. The present work specifically targeted the detection of tumor-associated autoantibodies with high sensitivity, low detection limits, and increased dynamic range. The aims will be achieved through the following objectives:

- a. To synthesize nanomaterials with enhanced optical and catalytic properties for applications as ultrasensitive signal transduction labels.

- b. To investigate the efficiency of different bioconjugation chemistry for the design of nanomaterial-immunobioprobes.
- c. To characterize the synthesized nanomaterial and nanomaterials-immunobioprobes using various analytical techniques.
- d. To study the effect of bioconjugation on the optical and catalytic properties of the nanomaterials
- e. To fabricate an immunobiosensing platform by immobilization of P53-tumor-antigen onto the solid-support interfaces.
- f. Use of optical transduction techniques (colorimetric and fluorescence) for the development of highly sensitive detection of anti-P53 tumor-associated autoantibodies.
- g. Evaluate the potential application of the fabricated sensing surfaces and nanomaterial-bioconjugates for the detection of anti-P53 antibodies in serum.

Thesis Outline

The structure of this thesis comprises of a chapter on introduction and literature review followed by five research-based chapters. The final chapter presents the conclusions and recommendations for future work. A brief synopsis of the thesis is as outlined below:

Chapter 1: Introduction

This chapter will describe lung cancer, its current diagnostic status and the significance of P53-autoantibody as a diagnostic lung cancer biomarker. The section also provides a theoretical background about the various aspects of immunobiosensor fabrication. Finally, a brief review of metallic nanoparticle (gold, palladium) and fluorescent nanoparticles (fluorescent silica nanoparticles) synthesis and properties for biosensor applications are presented. The review demonstrates that versatile techniques for early-stage lung cancer diagnosis and screening are lacking. Hence the present study is desirable.

Chapter 2: Experimental

This chapter outlines the experimental process, materials, and methods used for the synthesis of the nanomaterials, antibody bioconjugation to nanoparticles and antigen immobilization on solid support surfaces. The chapter will also present an overview of the instrumentation and characterization techniques such as X-ray photoelectron spectroscopy (XPS), Dynamic light scattering (DLS) and other spectroscopic methods used in the course of the development of the immunobiosensors.

Chapter 3: Label-free impedimetric sensing of anti-P53ab

This chapter reports on the use of electrochemical impedance spectroscopy (EIS) to monitor the immobilization of tumor antigen (P53ag) onto the gold electrode. The characterization of the fabricated sensing interface was also reported. The interaction between the immobilized P53-antigen and the anti-P53-antibodies was studied in a label-free experimental format suitable for the diagnostic assay of tumor-associated autoantibodies.

Chapter 4: Fluorescent detection of anti-P53ab

This chapter reports on the synthesis and characterization of fluorescent silica nanoparticles and their conjugation to the anti-IgG antibody. The fluorescent silica nanoconjugates were used as a novel immunobioprobes for detection of tumor-associated autoantibodies. The analytical performance of the immunobioprobes towards the detection of anti-P53 abs was investigated.

Chapter 5: Peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au@Pd

This chapter reports on the synthesis and characterization of silica-coated magnetic nanospheres decorated with bimetallic gold@palladium (Fe₃O₄@SiNP-APTES-Au@Pd). The peroxidase-like activity of the bimetallic nanoparticles towards the oxidation of the colorimetric substrate was investigated. The mechanism of the peroxidase-like activity and the steady-state kinetic parameters were evaluated and compared with that of horseradish peroxidase (HRP). The effect of experimental, environmental factors and storage stability on the peroxidase-like activity of the Fe₃O₄@SiNP-APTES-Au@Pd were evaluated.

Chapter 6: Colorimetric Detection of anti-P53ab

This chapter reports on the bioconjugation of anti-IgG to Fe₃O₄@SiNP-APTES-Au@Pd. The nanobioconjugate was characterized and the effect of the bioconjugation on the peroxidase-like activity was evaluated. The Fe₃O₄@SiNP-APTES-Au@Pd-ProG-anti-IgG was used as a label for the colorimetric detection of anti-P53ab. The anti-P53ab immunobiosensor was developed in a microtitre ELISA plate and cellulose paper platform. The analytical performance of the immunobiosensors was evaluated and compared with other methods.

Chapter 7. Conclusion and recommendation.

References

Chapter 1:

Introduction

1. INTRODUCTION

Understanding the physiological processes associated with lung carcinogenesis is essential for the development of immunology-based biosensors. Immunobiosensors are an essential tool for screening and early-stage diagnosis of cancer. This chapter describes lung cancer, current diagnostic status and the significance of autoantibodies as a lung cancer biomarker. The chapter also introduces the immunobiosensor and the nanomaterials used in this thesis for signal transduction and amplification.

1.1. Lung Cancer Epidemiology

Cancer is a generic term for a complex group of diseases that are characterized by the uncontrolled growth of an abnormal cell in the body [1]. Risk factors that contribute to the development of cancer include exposure to carcinogens, immunosuppression, and infectious diseases. All these factors can cause a genetic mutation that results in cell proliferation and cancer [2,3]. Globally, cancer is the leading cause of disability and death. The mortality rate due to cancer has taken over from cardiovascular diseases and exceeded the number of deaths from HIV/AIDS, malaria and tuberculosis combined [4].

According to the International Agency for Research on Cancer (IARC), in 2018 there were 18.1 million new cases of cancer and about 9.6 million cancer-related death worldwide [5,6]. Cancer incidents and mortality rate have shown significant differences depending on the cancer type, gender and geographical locations [7]. **Figure 1.1** shows the current estimated incidence and mortality rate of cancer worldwide. The most common cancers are prostate, breast, liver, colorectal and lung cancer [8]. The death rates due to prostate, breast and colorectal cancer are low in comparison to other cancer types. The deadliest cancers are liver cancer, stomach cancer, esophagus cancer, and lung cancer. The incidence and mortality rate of lung cancer correlates

proportionally [9]. More than 87 % of people with lung cancer die within one year of being diagnosed, making the disease the most lethal form of cancer worldwide.

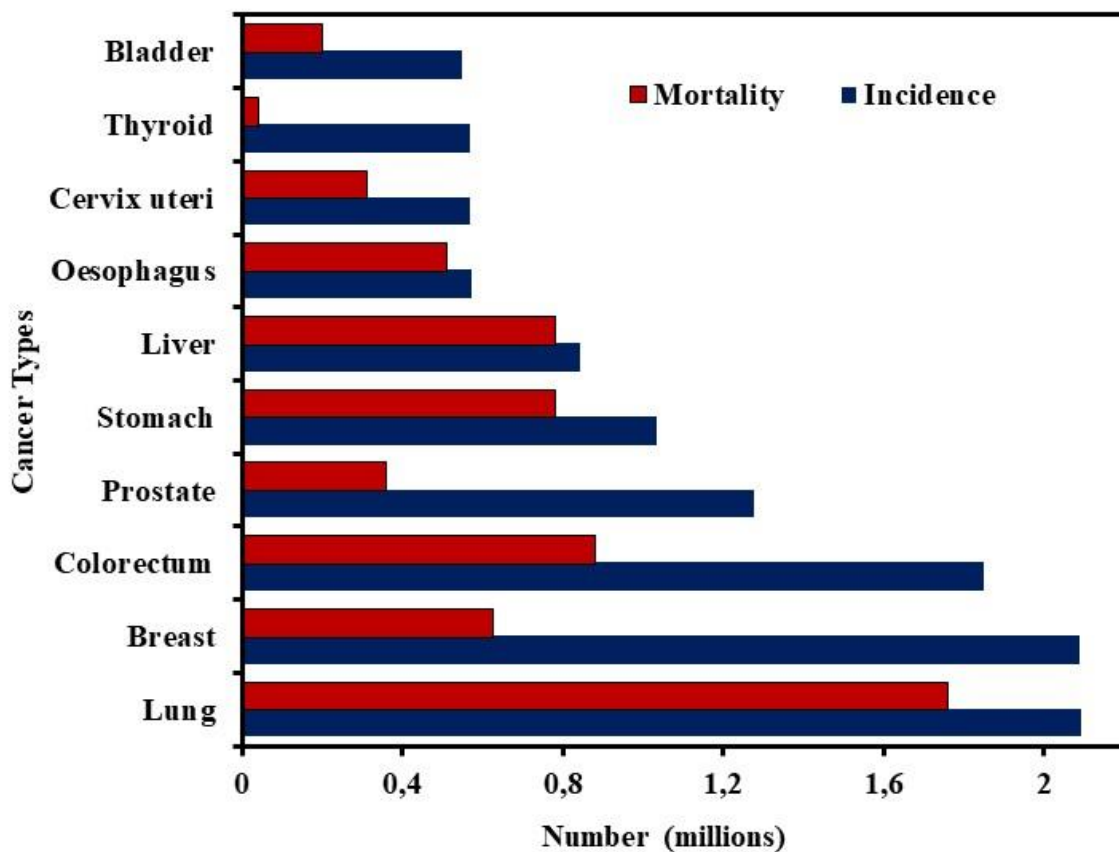


Figure 1.1: Estimated number of cancer incident cases and deaths worldwide. Source: IARC: GLOBOCAN, 2018 [10].

Figure 1.2 shows the pictorial illustration of the human lungs and the presence of the cancerous cell mass within the lungs. The lung is the respiratory organ located in the chest cavity and responsible for the supply of oxygen to the body cells and tissues and the removal of carbon dioxide from the body cells. Lung cancer is a wild growth of abnormal cells that start from the cells in the tissues lining the interior passages of the lungs [11]. The lung is connected to two lymphatic vessels and the pulmonary circulatory system. Therefore, it is easier for the cancer

cells that are shed from the primary tumor sites to enter the blood and metastasize to other organs in the body. The frequent metastatic lung cancer sites are the brain, liver, adrenal glands, lymph node, and bone, leading to poor survival and death [12,13].

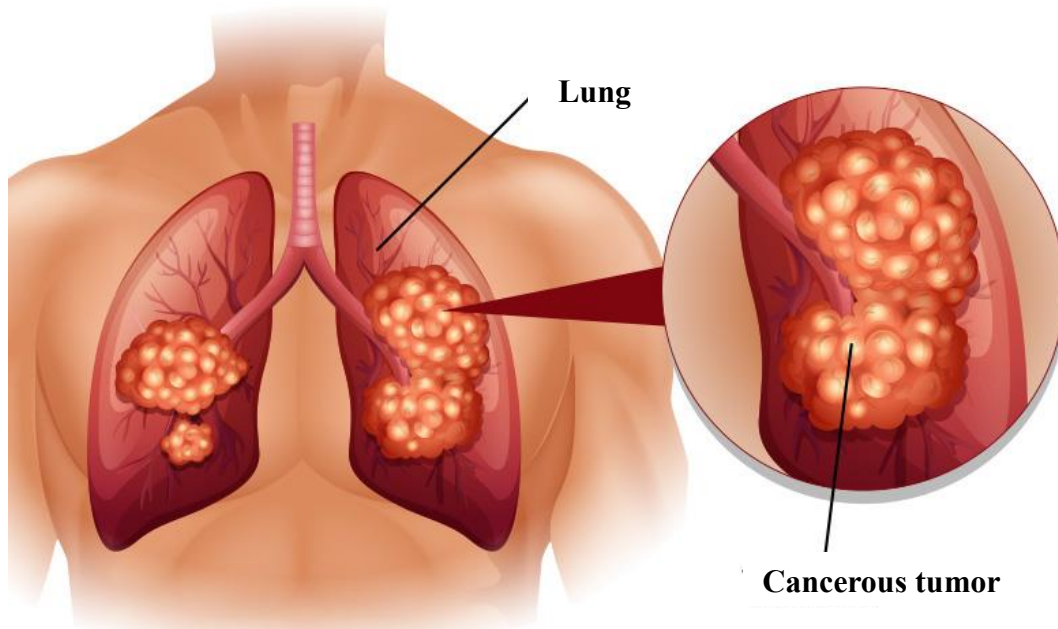


Figure 1.2: Pictorial representation of the lung showing a cancerous mass in the lung.
Source: Storyblocks images: stock-image.

For many decades, lung cancer has remained the leading cause of cancer-related deaths worldwide. It accounts for the deaths of over 1.6 million people every year [1,14]. According to the GLOBOCAN report, in 2018, there were 2.1 million new cases and 1.8 million lung cancer-related deaths worldwide [10,15]. For several decades, the geographical pattern of lung cancer mortality closely follows those of the incident. This is due to high fatality and the relative lack of variability in the survival pattern in different parts of the world [16]. Recent reports have shown that lung cancer incidents and mortality rates are increasing in less developed and low-income economies [17–19]. **Figure 1.3** shows the comparison of lung cancer incident and mortality rates in (a) low income and high-income economies and (b) common cancer incidents and deaths in South Africa. According to the World Health

Organisation (WHO), about 1.2 million new cancer cases and 1.0 million lung cancer deaths were reported in the low and middle-income countries (LMICs) in 2018.

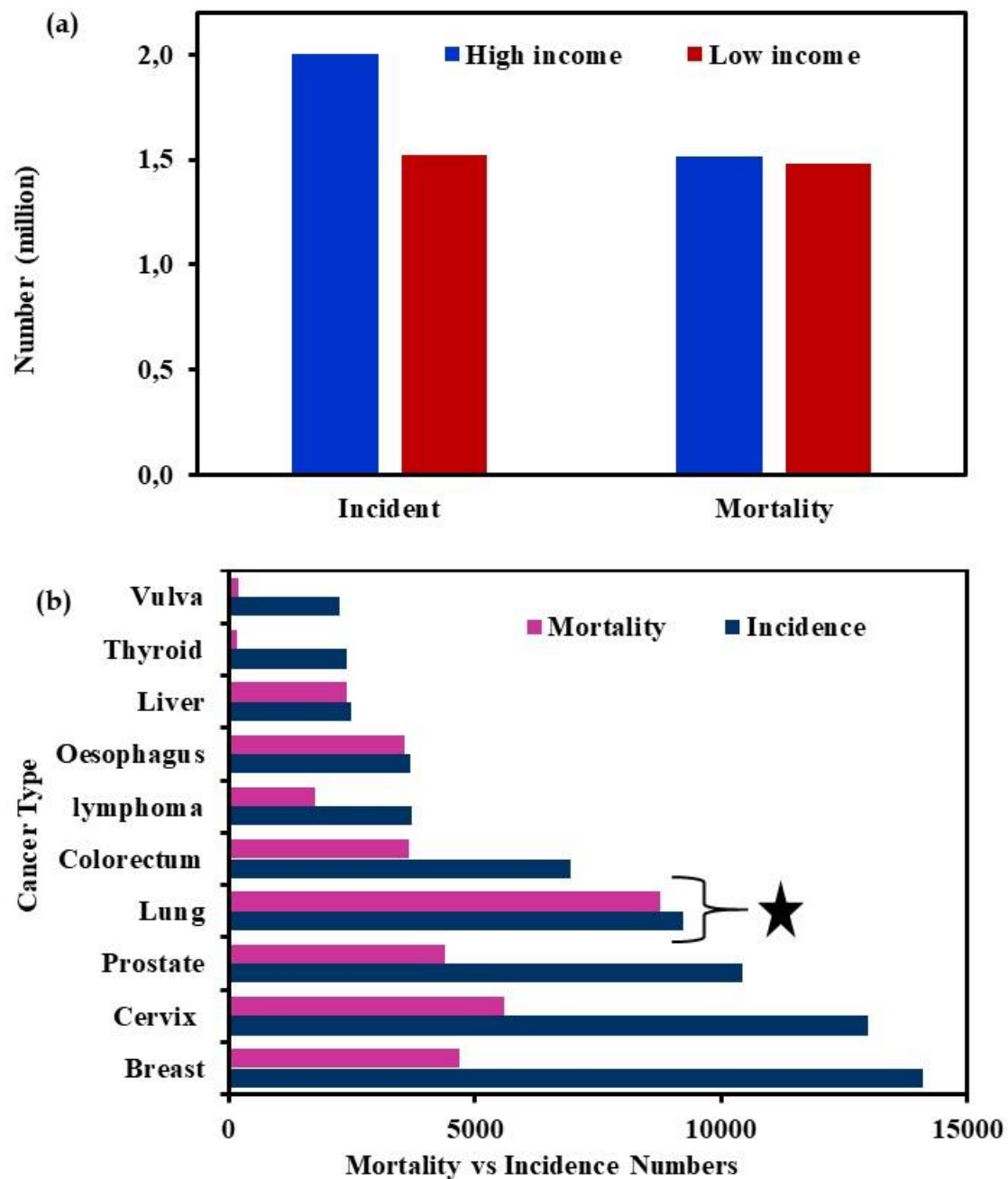


Figure 1.3: Comparison of lung cancer incidence and mortality rates in (a) low-income and high-income economies; (b) the most common cancer incident and death in South Africa [5,20].

LMICs include countries in Africa, Asia, and Latin America. These countries account for about 58% of global lung cancer mortality every year, **Figure 1.3 (a)**. The incidence rates are intermediate in Africa. Notably, the highest rates are about 32 and 29 per 100,000 people in Morocco and South Africa; respectively [21]. The majority of lung cancer patients in the LMICs die within the first year of their diagnosis. The mortality rates were 30 and 28 per 100,000 people in Morocco and South Africa, respectively. Lung cancer is the fourth commonly diagnosed cancer and a leading cause of death in South Africa, **Figure 1.3 (b)**. It accounts for 40 % of total cancer-related mortality in the country. The global cancer observatory report predicted that the incidents and mortality rates of lung cancer in South Africa might increase by 65 % by the year 2030 [8,20]. In most of the LMICs, resources for lung cancer diagnosis are often limited or non-existent. People often lack timely treatment due to the high cost of accessing health facilities. Currently, in these countries, a lung cancer diagnosis is based on self-reporting after the expression of symptoms, usually at the late-stage when treatment can no longer be effective and successful.

1.2. Lung cancer etiology and classification

Multiple genetic, cellular and local tissue transformation to preneoplastic cells are involved in the protracted process that results in lung carcinogenesis [2,11]. The major etiological factor for lung cancer is tobacco smoke. Smoking, exposure to secondhand smoke, radon gas and asbestos are the primary cause of lung cancer [22]. Globally, cigarette smoke is responsible for about 85% to 90 % of all lung cancer cases [23,24]. Cigarette smoke is full of carcinogens such as polyaromatic hydrocarbons and nitrosamine ketone which play a significant role in DNA damage. This causes the cells of the lungs to grow uncontrollably and create a cancerous tumor [11,22]. Studies have shown that lung cancer is a significant challenge in HIV-infected individuals due to the immunosuppression [25], and co-infection with oncogenic viruses such

as human herpesvirus 8 (HPV) and Epstein-Barr virus [26]. This co-infection has lead to an increase in the prevalence of lung cancer in HIV-infected non-smokers. The survival and incidence rates of lung cancer show variations depending on the type of lung cancer, racial or ethnicity differences, and stage at diagnosis [27]. **Figure 1.4** shows the WHO histological classification of lung cancer and associated five years survival rate depending on the stage of detection and histological subtype. Lung cancer is a highly heterogeneous disease. Histologically, lung cancer is classified into two types; non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [28]. SCLC accounts for about 15% of all lung cancer cases, it grows rapidly and often it has metastasized at the time of diagnosis. This makes SCLC the most aggressive form of lung cancer [29]. Less than 5% of NSCLC patient live up to 5-years after diagnosis, and the median life expectancy after treatment is 12 months [30].

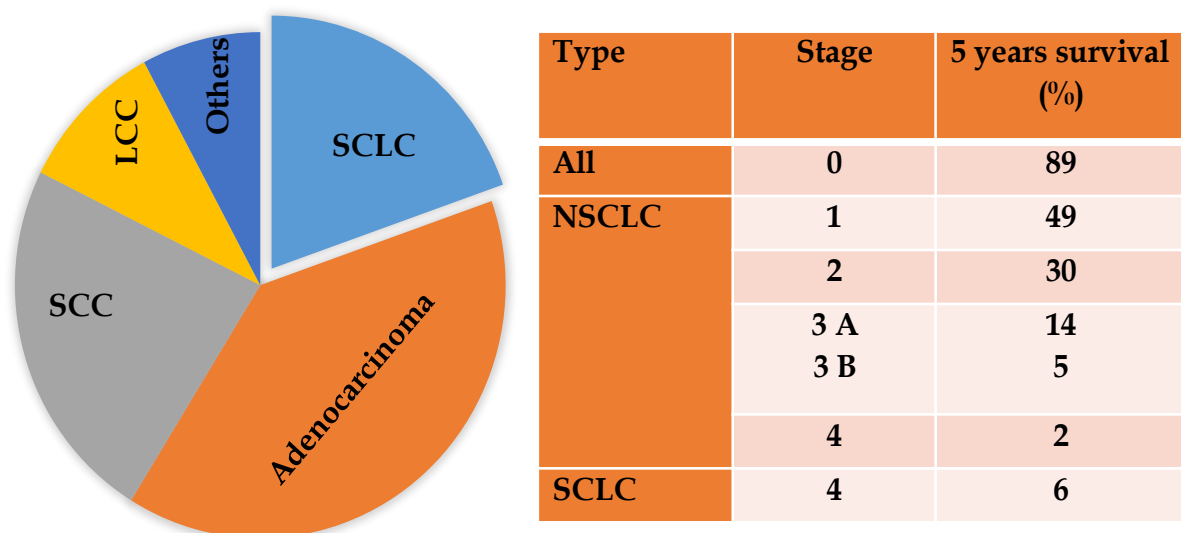


Figure 1.4: WHO histological classification of lung cancer and associated five years survival rate depending on the stage of detection and histological subtype; NSCLC are squamous cell carcinoma (SCC), large cell carcinoma (LCC) and Adenocarcinoma (AC) [28].

NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell anaplastic carcinoma, which accounts for about 85% of all lung cancers. NSCLC affects the cell of the main bronchi and blocks the lungs which results in reduced airflow and death. Sadly NSCLC is always detected at the very late stages, and the 5-years survival rate is 2 % with a median survival time of 8 months after treatment [30]. The majority of lung cancer (75%) are diagnosed at the late stage (stage 4) due to lack of symptoms at the early stage [31]. When lung cancer is diagnosed at an early stage (stage 0), the survival rate for both NSCLC and SCLC is high (89 %). The survival rate decreases drastically after stage 0, with 49 % survival rate at stage 1, and reduces to 2% and 6% at stage 4 for NSCLC and SCLC; respectively. Currently, the patient hardly lives up to five years after diagnosis due to the aggressive nature of lung cancer [30,32]. This necessitates the need for the early-stage diagnosis of lung cancer.

Early-stage diagnosis of lung cancer can be achieved through a screening test. In recent times, temporal analysis has shown a significant decline in lung cancer-associated deaths in some developed countries. The reduction in mortality rate is due to the implementation of lung cancer screening programmes and the diagnosis of asymptomatic lung cancer. For example, through the National Lung Screening Trial (NLST) in the United States (US), lung cancer-associated mortality has declined by 20 % [33,34]. Similarly, randomized screening such as the NELSON trial in the Netherland, Belgium and the Denmark lung cancer screening using low-dose computed tomography (LCDT) imaging have been executed. These screening programmes aimed to achieve early-stage detection of asymptomatic lung cancer [35,36]. Also, in 2017, the European Union recommended guidelines for the LCDT annual lung cancer screening test for reducing lung cancer mortality [33,37]. The success of lung cancer screening emphasizes the need for screening tests to achieve a reduction in lung cancer mortality. However, lung cancer screening tests have not been implemented in the LMICs. This is due to the lack of lung cancer

screening facilities and the high-cost limitations of the currently available screening tests [38,39].

1.3. Lung cancer screening and diagnostic techniques

Screening test which can identify patients with early-stage lung cancer would allow the physician to diagnose lung cancer when treatment is most effective. Early-stage detection reduces the associated mortality rate. Imaging techniques (such as chest X-ray, low-dose computerized tomography (LDCT) scan, magnetic resonance imaging (MRI), and invasive procedures (such as bronchoscopy, endobronchial ultrasound, and fine-needle aspiration biopsy) are currently employed for lung cancer screening and diagnosis. However, these techniques have various limitations listed in **Table 1.1**. Generally, the imaging techniques cannot distinguish between benign and cancerous nodules. The current diagnostic tools are expensive and unsuitable for the screening and point-of-care applications. The limitations of the present techniques have created a demand for the development of technologies that are highly sensitive, radiation-free and non-invasive or minimally invasive for lung cancer screening and early diagnosis. Biosensor-based biomarker quantification provides diagnostic information in a simple and non-invasive approach at a low cost. Thus, a robust sensitive and easy-to-use immunobiosensors for the detection and quantification of circulating lung cancer biomarkers will have significant applications in early-stage detection, monitoring of the efficacy of treatment and relapse after surgery. The immunobiosensor for lung cancer screening should ideally:

- (i) Target the detection and quantification of an appropriate lung cancer biomarker,
- (ii) Objectively predicts the presence of lung cancer at the early stage of carcinogenesis,
- (iii) Support short assay time with minimal sample processing,
- (iv) Inexpensive, easy readout and naked-eye interpretation of results, and

(v) Allow for the multiplexed biomarker detection in a single sample.

Table 1.1: Current techniques for lung cancer diagnosis.

Method	Limitations	Refs
Chest X-ray	• Tumor mass must be about 1-2 cm. • False-positive and negative results. • Expensive. • Require an expert to run and interpret results. • It cannot differentiate between a cancerous and benign tumor.	[40]
Computed Tomography scan (CT-scan)	• Exposure to X-ray. • False-negative results. • Expensive • Located in centralized laboratories.	[40,41]
Magnetic resonance imaging (MRI)	• Use of magnetic field. • False-negative results. • Sophisticated and expensive instrumentation. • Not suitable for all patients.	[42–44]
Positron Emission Tomography (PET) scan	• Exposure to radioactive tracers. • False-negative result. • It cannot detect a tumor of less than 5 mm. • Expensive and sophisticated instrumentation.	[45–47]
Low dose Computed spiral Tomography scan (LCDT-scan)	• False-positive results. • Exposure to ionizing radiation. • Expensive instrumentation. • It is located in centralized laboratories. • Not suitable for all patients. • Requires expert to interpret results.	[48,49]
Sputum cytology	• It results in a high rate of false-negative and positive results. • It causes the degradation of biomarkers due to enzymes in sputum.	[50,51]
Bronchoscopy	• Highly Invasive • Damage the vocal cord. • Intolerable to the patients.	[52]
Fine needle biopsy	• Highly invasive. • Expensive. • Cannot detect metastatic cancer cell.	[53,54]

1.4. Biomarkers for lung cancer diagnosis

A biomarker is a measurable physiological or pathological substance, which is related to the disease. Increasing or decreasing concentrations of a biomarker can be indicative of healthy or diseased associated processes [55]. Tumor-associated biomarkers are biological substances that are produced by cancer cells or made by the human body in response to cancer cells. An ideal lung cancer biomarker should;

- (i) Have 100% accuracy in differentiating between healthy and lung cancer patients.
- (ii) Be clinically sensitive and specific to lung cancer.
- (iii) Precede the neoplastic process so that it can be used for screening early lung cancer.
- (iv) Present in all patients with lung cancer irrespective of genetic differences.
- (v) Be easily assayable at low cost in a minimally invasive or non-invasive manner.

The transformation from a normal to malignant lung epithelial cells arises from several genetic and epigenetic changes [56]. These changes lead to the secretion of oncofoetal antigens, isoenzymes, mucin, tissue-specific proteins, circulatory DNA, micro-RNA, glycoproteins, and proteins of processes such as apoptosis, angiogenesis or proliferation [57]. These tumor biomarkers are found in the blood, sputum, tissue, plasma or serum of individuals with asymptomatic lung cancer at a higher concentration than a healthy individual. In the sputum, cells from cancer sites are the primary targeted protein sources. Some tumor biomarkers exist at higher levels in the lung tissue than body fluids. Tissue-based biomarkers require biopsy which is invasive and uncomfortable [58]. Blood and serum-based circulating biomarkers have the most significant diagnostic value. Blood samples are obtained in a minimally invasive manner. Various genetic and protein-based biomarkers have been reported in the literature for lung cancer diagnosis and prognosis [59–65]. **Table 1.2** shows the list, source, description, and method of detection of some of the candidate gene-based biomarkers reported in the literature for the diagnosis of lung cancer.

Table 1.2: Summary of gene-based diagnostic lung cancer biomarkers.

Biomarker	Source	Description	Method	Refs
Epidermal growth factor receptor (EGFR)	Tissue	- EGFR is a tyrosine kinase receptor gene; it is located on chromosome 7 at position 12. - EGFR mutation occurs within exons 18-21 in lung cancer related to smoking.	PCR, IHC	[66,67]
Anaplastic lymphoma kinase (ALK)	Tissue	- ALK is a tyrosine member receptor of the insulin receptor family. - The gene is located on chromosome 2. - The mutation results in rearrangement which promotes malignant growth and proliferation in NSCLC.	RT-PCR, FISH,	[68]
Kirsten rat sarcoma oncogene (KRAS)	Tissue	KRAS oncogene is located on the long arm of the chromosome.	FISH, RT-PCR,	[69]
ROS proto-oncogene-1 receptor tyrosine kinase (ROS 1)	Tissue	- ROS 1 is a tyrosine kinase member of the insulin family and is located on the long arm of chromosome 6 at position 22. - Ros1 plays a role in epithelial cell differentiation during the development of NSCLC.	FISH, IHC	[70,71]
Human Epidermal growth factor receptor 2 (HER 2)	Tissue	- HER2 is a proto-oncogene located on chromosome 17 at position 12. - HER2 mutations occur in exon 4 of the tyrosine kinase domain and are expressed in adenocarcinoma	FISH	[72]
Tumor suppressor gene (P53 and P16)	Tissue	- P53 and P16 are tumor suppressor genes. - P53 is located on chromosome 17 and regulates cell division and DNA repairs. - Mutation of the P53 gene is known in all histological subtype of lung cancer - P16 is encoded in the CDKN2A gene on chromosome 9. - P16 methylation and loss of heterozygosity are associated with lung cancer patients and smokers.	IHC, PCR	[73]
Circulating micro-RNAs, DNA and RNA	Plasma, serum	Cell-free DNA, RNA, and microRNA originates from tumor cells and circulates in the body fluids.	Cold-PCR, Sanger sequencing	[74,75]

IHC: immunohistochemistry; RT- reverse transcript; PCR: polymerase chain reaction; FISH: fluorescence immunohistochemistry; NSCLC: Non-small cell lung cancer; RT-PCR: a real-time polymerase chain reaction.

1.4.1. Protein-based lung cancer diagnostic biomarkers

Changes in the level of proteins expressed that results from carcinogenesis are more suitable biomarkers for the early-stage diagnosis of lung cancer due to their high selectivity [76]. The immunobiosensor developed in this thesis targeted the detection of the immunological protein-based lung cancer biomarker, specifically anti-P53-autoantibody (anti-P53ab). The protein-based lung tumor biomarkers are discussed in detail due to their advantages over the gene based-biomarkers. The protein biomarkers include aberrantly expressed proteins, inflammatory proteins, oncogene products, tumor antigens and immunoglobins [77].

1.4.1.1. Tumor-Associated Antigen

The immune system protects the body from harmful substances by recognizing antigens and producing antibodies [78]. There are two divisions of antigens; these are heteroantigens (foreign antigen) and autoantigens (self-antigen). Heteroantigens includes substances that are generated by pathogens such as microorganisms. Autoantigens are normal body proteins that are recognized by the immune system as a foreign substance [79,80]. Tumor antigens are autoantigens. Tumor cells are derived from normal host cells whose proteins are recognized as self-molecules to which the immune system is usually tolerant. Like other cancer cells, lung cancer cells express antigens that are secreted into the blood or attached to the major histocompatibility complex (MHC class I or II molecules) on the tumor cell surface [81,82].

Tumor antigens are divided into two classes, that is, tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). TSAs are encoded by a primary frame of gene products that are differentially secreted by cancer cells and not by normal body cells. They are found on the cancer cells and may be encoded by the mutated genes, pseudogenes and products of genetic translocation events. TSAs are related to the tumor suppressor genes and oncogenes such as the P53 antigen, P16 antigen, Annexin A2 and interleukin-6 [83,84]. Tumor-associated

antigens (TAAs) result from aberrant expression of non-mutated proteins that are secreted in response to the development of cancer cells [85]. They are divided into cancer-testis antigens (CTAs), differentiation antigens, oncofoetal antigens, oncoviral antigens and overexpressed antigens [86]. CTAs are a group of proteins encoded by genes that are typically expressed in the male germlines, trophoblasts, and ovaries but not in somatic cells. Gene regulation is disrupted due to cancer and is expressed in proportion to tumor growth [87,88]. Over 250 gene encoding CTAs have been discovered and are broadly divided into CTA-X located on chromosome X and non-CTA X located on autosomes. The occurrence of CTAs in lung cancer has been well documented [87–90]. Studies have shown the expression of new yoke oesophageal-1 testis antigen 1 (NY-ESO-1), melanoma-associated antigens-1 (MAGE A1, MAGEAB2, MAGEB2), sperm-associated antigen 9 (SPAG9), and the cancer-associated gene (CAGE 1) in various histological types of lungs cancer [58,90,91].

The oncofoetal antigens are found on embryonic and fetal tissue but are overexpressed due to the development of lung cancer. These are produced during fetal development in the gastrointestinal tract, and the production stops before birth. They are not naturally detected in healthy adults, except in smokers [92]. Oncofoetal antigens including carcinoembryonic antigen (CEA) and tissue polypeptide antigen (TPA) are suitable biomarkers that are often expressed in Adenocarcinoma. **Table 1.3** shows a list of blood-based diagnostic lung tumor-specific antigens. Although the TAAs and the TSAs are expressed at the early stage of carcinogenesis, most tumor antigens have a very short half-life and exist at low concentrations in the blood [93]. The high dynamics of other protein in the body fluids and the abundance of albumins relative to small antigen concentrations have limited the detection of tumor antigens in blood and serum [94].

Table 1.3: Summary of diagnostic lung cancer antigen biomarkers and the diagnostic cut-off values.

Serum / Plasma biomarkers	Description	Cut-off	Refs
New York oesophageal antigen	- NY-ESO-1 is a 22 kDa cytoplasmic protein encoded on chromosome Xq28. - It is the most immunogenic cancer protein, and the elevated level has been associated with SCC.	-	[7,88]
P53-gene product	P53 protein accumulation in cancerous tissues occurs due to the mutation of P53-gene. The half-life is 6-20 min and is not detected in the blood and serum	0.3 ng.ml ⁻¹	[73]
Carcinoembryonic antigen (CEA)	- CEA is an oncofoetal protein with a molecular weight of 200 kDa when glycosylated. - It is overexpressed in all lung cancer types	3.0 ng.ml ⁻¹	[100,101]
Squamous cell antigen (SCA)	- SCA is a 45 kDa cytoplasmic glycoprotein of the serine protease inhibitor family. - SCA is elevated explicitly in squamous cell carcinoma, adenocarcinoma, and other inflammatory lung diseases.	1.5 ng.ml ⁻¹	[101–103]
C-reactive protein (CRP)	- CRP is a nonspecific but sensitive biomarker of inflammation synthesized in the liver by hepatocytes. - Increased CRP level is found in NSCLC.	8.0 ng.ml ⁻¹	[101,104]
Interleukin-6 (IL-6)	IL-6 induces the synthesis of CRP.	NR	[104]
Cytokeratin-19 fragment (CYFRA 21-1)	- CYFRA 21-1 is a fragment of cytokeratin-19 with a molecular weight of 40 kDa. - It is part of the cytoskeleton in epithelial cell and is overexpressed in NSCLC, SCLC, and AC	3.3 ng.ml ⁻¹	[103,105]
Neuron-specific enolase (NSE)	- NSE is a 78 kDa gamma-homodimer and represents the dominant enolase-isoenzymes found in neuronal and neuroendocrine tissues. - A high level of NSE greater than 100 ng mL⁻¹ suggested the presence of advanced SCLC.	13.0 ng.ml ⁻¹	[105,106]
Pro-gastrin-releasing peptides (ProGRP)	- ProGRP is a gastrin-releasing peptide residue 31-98. - A high level of ProGRP is found in the blood of patients with SCLC.	35.0 pg.ml ⁻¹	[106–108]

NSCLC: Non-small cell lung cancer; SCLC-small cell lung cancer; SCC-squamous cell carcinoma, AC: Adenocarcinoma

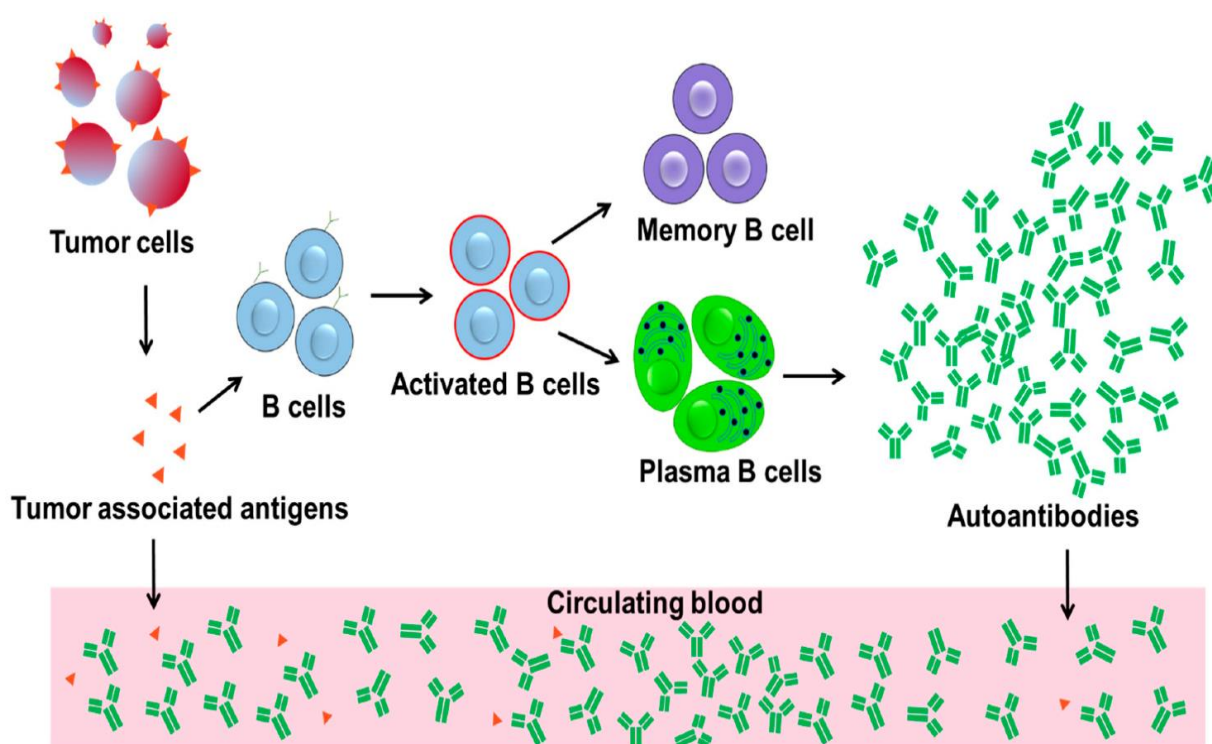
Thus, immunodiagnostic tools targeting the detection of lung cancer tumor antigen for diagnostic and screening tests do not pass clinical trials. However, the antigen-based biosensor has shown the excellent prognostic application in monitoring of response to treatment of advanced-stage lung cancer [95]. The poor diagnostic value of tumor antigens necessitates the need for a target of biomarkers that could overcome the limitations of antigens in the development of an immunodiagnostic sensor for early-stage diagnosis of lung cancer. Circulating tumor antibodies are a solution to antigens and are discussed in detail below.

1.4.1.2. Autoantibodies as a lung cancer biomarker

The body's immune system produces circulating autoantibodies in response to exposure to cancer proteins. These are emerging as promising biomarkers for the early detection of lung cancer. The presence of cancer cells can activate the immune system to respond to a tumor-specific antigen and protect the host against the development of a primary tumor through immunosurveillance [96–98]. However, it has been identified that the immune system also promotes the growth of a primary tumor by a process called Immunoediting. Immunoediting consists of three phases namely; elimination, equilibration, and escape. During the elimination stage, the immune system recognizes tumor cells as foreign substances and eliminates them. Tumor cells that survive the elimination stage enter the equilibrium phase and are mutated to variant tumor cells that are saved for years with increasing resistance to immune attack [99]. The saved tumor cell variants continue to grow uncontrollably and eventually will be detected by the immune system in the escape phase. The tumor cells secrete mutated tumor-associated antigens that distinguish them from normal body cells [96].

Scheme 1.1 shows the schematic illustration of the tumor-associated autoantibody production by the immune system. The tumor antigens are post-translationally modified, overexpressed

or aberrantly degraded in such a way that simulates an autoreactive immune response through the activated B-cell or T-cell receptors [109]. This results in the secretion of a tumor-associated autonomous antibody. An individual B-cells produces 5000-20000 antibodies per minute and undergo mitosis every three days maintaining the enhanced production of many copies of the autoantibodies [110-112]. Thus, amplifying the cancer antigen “signal” and becoming detectable during early-stage lung cancer [113,114]. The concentration of antigens is too low to be detected in the same timeframe. The specificity of autoantibody and amplification of the “tumor signal” can fulfill the high specificity and sensitivity requirement of a biomarker for screening and early-stage diagnosis of lung cancer.



Scheme 1.1: Schematic illustration of tumor antigen and associated autoantibody production and amplification by the immune system [110].

A tumor-associated autoantibody is of human immunoglobins G (IgG) subclass. The IgG is a large molecule with a 150 kDa and composed of four polypeptide chains with identical two 50 kDa heavy chains and two identical 25 kDa light chains. A pictorial representation of an IgG antibody is shown in **Figure 1.5**.

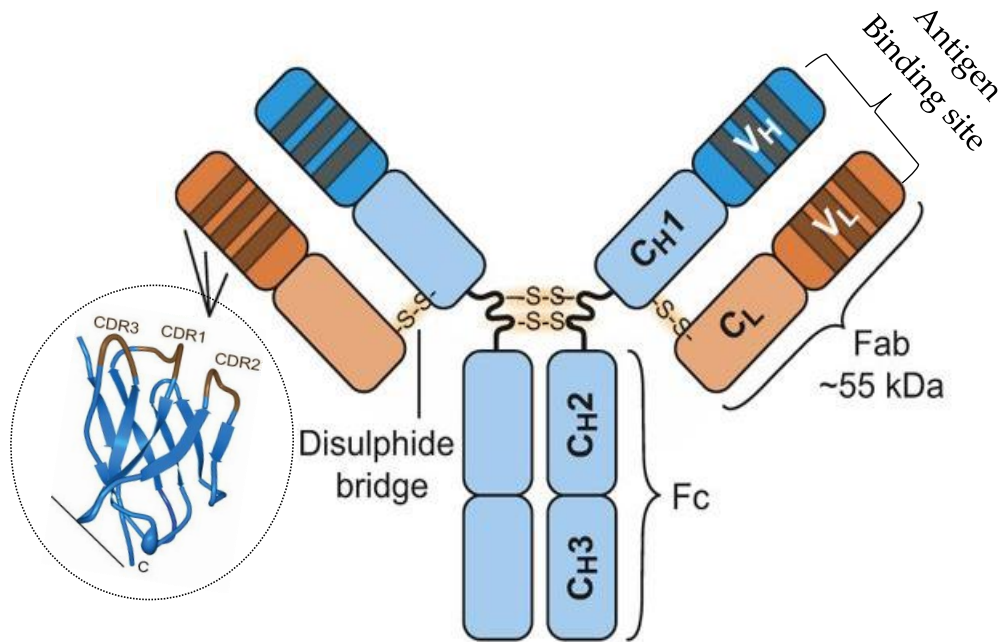


Figure 1.5: Structure of immunoglobulin G (IgG) showing the hypervariable complementary determining regions (CDRs) [115,116].

The heavy chain has three different constant regions (C_H1, C_H2, C_H3) and a variable region (V_H). Each of the light chains has a variable (V_L) and constant (C_L) regions. The fast diversity in IgG initiated during an immune response and B cell development results from the hypervariable complementary-determining regions (CDR1, CDR2, CDR3). The CDRs are generated by somatic rearrangement of the different V, D and L germline genes each forming a specific combination which are the antigen-binding sites in the variable regions. The CDRs determine the specificity of the antibody to the antigen. The diversity of the CDR3 is further enhanced by the insertion or deletions of nucleotides between genes. The CDR3 is the region

that interacts with the antigen, and the diversity of the CDR3 makes IgG more antigen-specific. This antigen-specific immunoglobulin sequence may be shared among different lung cancer patients and could serve as a biomarker for lung cancer screening and early diagnosis. In comparison to detecting tumor antigens, autoantibodies to tumor antigen present numerous advantages as summarised in **Table 1.4**. The autoantibody response to tumor antigen may be expressed for about ten years before the manifestation of cancer [93,117]. Thus, autoantibodies are a more viable target biomarkers in the development of diagnostic tools for screening of individuals at risk of developing lung cancer and early-stage diagnosis [64,93].

Table 1.4: Comparison of a tumor-associated autoantibody and antigen biomarker.

Tumor antigen	Autoantibody to tumor antigen
Overexpressed at an early stage	Overexpressed at an early stage
Secreted by cancer cell	Immune response to the cancer cell and antigen
Low concentration in a body fluid	High level in a body fluid
Short half-life	Long half-life
Unstable outside the body	Stable and persist in serum
Heterogeneous	Heterogeneous

Table 1.5 presents the list of validated blood-based autoantibody biomarkers currently in clinical use for lung cancer diagnosis. Studies have identified autoantibodies in plasma and serum of preclinical stage of lung cancer. Humoral immune response to tumor protein such as the tumor suppressor protein (P53), cancer-associated antigen (CAGE), GBU 4-5, human epidermal growth receptor (HER 2), melanoma antigen-A4 (MAGE A4), New yoke oesophageal-1 testis antigen (NY-ESO-1) and MUC1-antigen have been reported in lung cancer

patients [93,105]. The frequently identified autoantibodies in all subtype of lung cancer are the anti-P53, anti-CAGE-1, anti-SOX2, anti-GBU 4-5, anti-progestin releasing peptide (PGP 9.5), anti-NY-ESO-1 and anti-annexin1 autoantibodies. These autoantibodies have significant diagnostic value for discriminating between a healthy individual and early-stage lung cancer patients [118]. Due to the heterogeneous nature of lung cancer, a single biomarker may have poor diagnostic value. Several studies have shown the improved diagnostic power of autoantibody biomarker panel for the diagnosis of lung cancer. A group of autoantibodies to seven cancer-testis antigens including P53, GAGE 1, PGP 9.5, CAGE, MAGE, GBU 4-5 shows high significant diagnostic power in identifying asymptomatic lung cancer with a specificity of 95 % [88,119]. The healthy individual was identified among NSCLC and SCLC with a sensitivity of 76% to 96%. A specificity of 92% was achieved by detecting a panel of four lung tumor biomarkers (CEA, CYFRA21-1, SCC, and ProGRP) in a cohort of 153 Chinese [120]. The combination of carcinoembryonic antigen, retinol-binding proteins, transferrin, 1-antitrypsin, and squamous cell carcinoma antigen was found to differentiate lung cancer patient from a healthy control [121]. The combination showed a sensitivity of 89.3% and specificity of 84.7%. A panel of six autoantibodies to lung cancer antigen; cancer antigen-125 (CA 125), cancer antigen-19-9 (CA 19-9), CEA, NSE, CYFRA 21-1 and ferritin have achieved a sensitivity of 80.4% for identification of adenocarcinoma. A panel of three autoantibodies; anti-CEA, anti-NSE, and anti-CA-125 achieved higher sensitivity for discriminating clinical lung cancer patients from high-risk individuals [122]. Also, the combination of CYFRA 21-1, CEA and P53 autoantibody panel could achieve 79.3% sensitivity for the diagnosis of lung cancer [123]. Early detection of asymptomatic lung cancer based on the presence of 6-autoantibodies; CAGE, NY-ESO-1, P53, SOX, MAGE, and GBU4-5 in the serum of heavy smokers shows a 91% specificity in identifying asymptomatic lung cancer among healthy individual [124].

Table 1.5: Validated lung cancer autoantibody diagnostic biomarker in clinical use

Autoantibody	Protein function	Abnormality eliciting response	Detection Method
Anti-P53	Tumor suppressor	Mutation	SEREX, ELISA, Western blot
Anti-NY-ESO-1	CTA	Ectopically expressed	SEREX, Phage display, SPA
Anti-MAGE A4	CTA	Ectopically expressed	Phage display, Western blot,
Anti-LAGE	CTA	Ectopically expressed	ELISA
Anti-c-myc	A transcription factor, oncogenic	Mutation	ELISA, Western blot, SPA, Protein array
Anti-CYFRA-21-1	Scaffold protein	Overexpressed	ELISA, Protein array.
Anti-SOX 2	A transcription factor, stem cell	Overexpressed	Western blot, SPA, Protein array
Anti-GBU 4-5	Encode a DEAD box domain	Antigen expression	Phage peptide, Western blot, ELISA.

SEREX: serological analysis of antigen by recombinant cDNA expression cloning; SPA: serological proteome analysis; ELISA: enzyme-linked immunoassay; MAGE A4: Melanoma antigen-A4; NY-ESO-1: Newyoke esophageal-1 testis antigen, CTA-cancer testis antigen.

1.5. Diagnostic value of serum anti-P53ab

Mutations of tumor suppressor gene P53 are the most commonly observed genetic abnormality in human cancer [125]. The mutation of the P53 gene is present in about 50 % of NSCLC and 80 % of SCLC cases [126]. The protein product of the P53 gene is a nuclear phosphoprotein expressed in normal cells. The P53 gene mutation leads to the inactivation of the biological properties and accumulation of non-functional P53-protein in cells [127]. P53-protein is not present in a healthy individual, but accumulation begins at the onset of cancer. The accumulated P53-protein hence acts as antigen [128]. The overexpression and accumulation of mutated P53-protein stimulate an immune response and subsequent production of serological anti-P53 autoantibodies (anti-P53ab). Anti-P53ab is not present in sera of a healthy individual

and patients with a benign pulmonary tumor. However, anti-P53ab concentration increases significantly at the early stage of carcinogenesis. Hence, it can be used as a biomarker for the diagnosis of incipient cancer. Earlier in 1995, Lubin et al. [129] reported that serological anti-P53ab has a high predictive and diagnostic capability with a good correlation between the anti-P53ab concentrations and the onset of carcinogenesis. Various studies have shown that anti-P53ab can discriminate between a potential lung cancer patient and healthy individuals with a specificity higher than 95 % and sensitivity of 20 % [65,123,130,131]. The high specificity indicates a relatively high level of overall diagnostic accuracy for early lung cancer screening. Anti-P53ab have been detected in sera of most heavy smokers and workers exposed to asbestos before the development of lung cancer [132]. Since the P53-protein alteration is the earliest genetic mutation in lung carcinogenesis, anti-P53ab has high pre-neoplastic sensitivity and selectivity [133,134]. It is not expressed in a healthy individual who is not at risk of developing cancer. Due to immune response, the anti-P53ab is expressed at a higher concentration at the earliest stage of carcinogenesis [110]. Thus, the anti-P53ab fulfilled the conditions of an ideal biomarker for lung cancer screening and early-stage diagnosis listed in **section 1.4**. This unique characteristic makes anti-P53ab the most promising TAAbs biomarker for early lung cancer diagnosis. Proteomics and immunoassay techniques have been used to detect anti-P53ab in serum. These includes serological analysis of tumor antigen by recombinant expression cloning (SEREX) [135], phage display [136], serological proteome analysis (SERPA) [117,135], multiple affinitive protein mapping [80,117], hyphenated 2D LC/MS/MS-protein microarray [133,137,138], western blot and enzyme-linked immunosorbent assay (ELISA) [133,139–142]. These techniques have drawbacks in their application for point-of-care as they require expensive instruments and reagents, are time-consuming and are not suitable for applications in resource-limited settings. There is a great demand for systems that can offer simplicity and ease of use while achieving high selectivity, sensitivity, and specificity.

1.6. Biosensor and immunobiosensor

A biosensor is an analytical device incorporating a biological or biological derived sensing element (bio-) onto a signal-transducing element (-sensor). Upon interacting with the analyte, the sensing and the transducing elements generate a signal which is proportional to the concentrations of a specific analyte [143]. **Figure 1.6** shows the schematic representation of the components of a biosensor. The biorecognition elements can be an enzyme, nucleic acids or biologically derived elements such as aptamers, engineered proteins, combinatorial ligands, and imprinted polymers. The signal transducer elements can be optical, electrical, magnetic or micromechanical transducer [144,145]. Biosensors are classified either based on the nature of the bioreceptor or the transduction method used. Depending on the transducer technology employed, biosensors can be electrochemical, optical, magnetic, thermal, mechanical and piezoelectric. Likewise, by the biorecognition elements used. A biosensor can be classified as enzyme biosensor, DNA biosensor, Aptasensors and immunobiosensors [146].

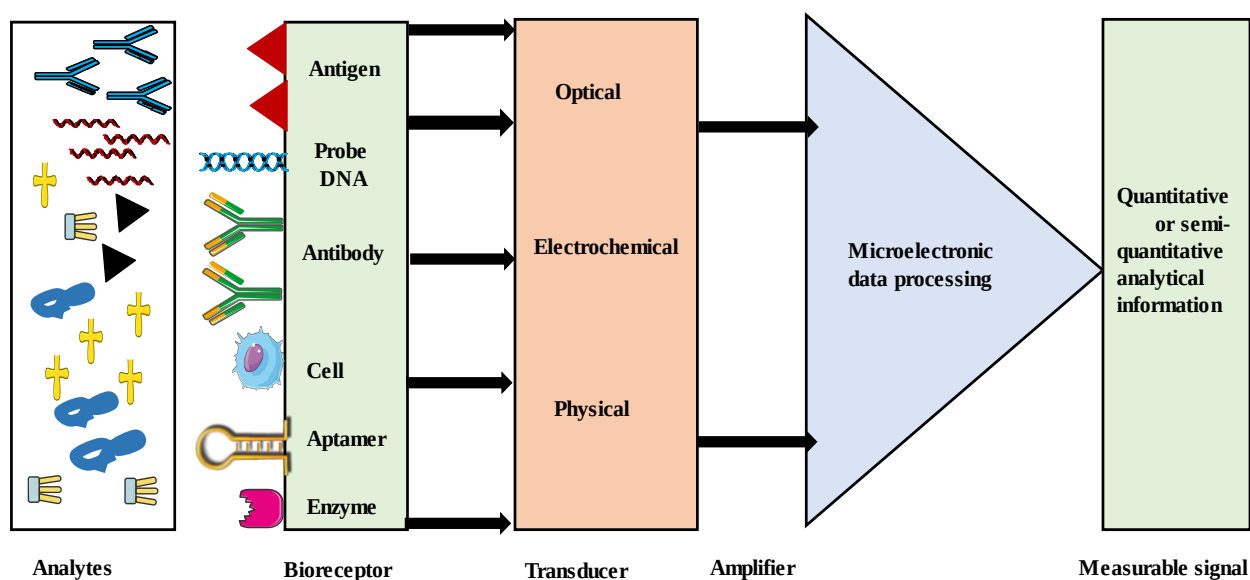
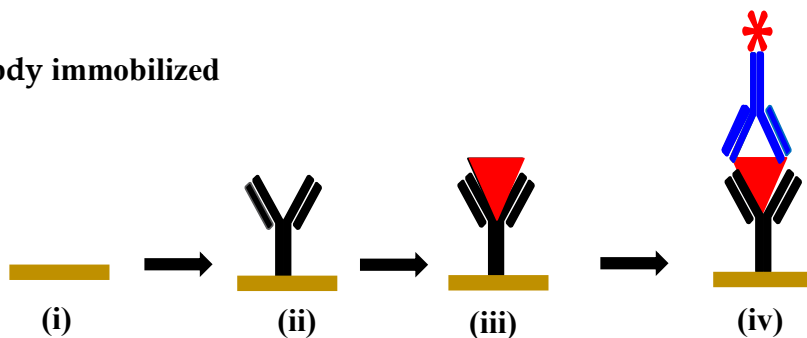


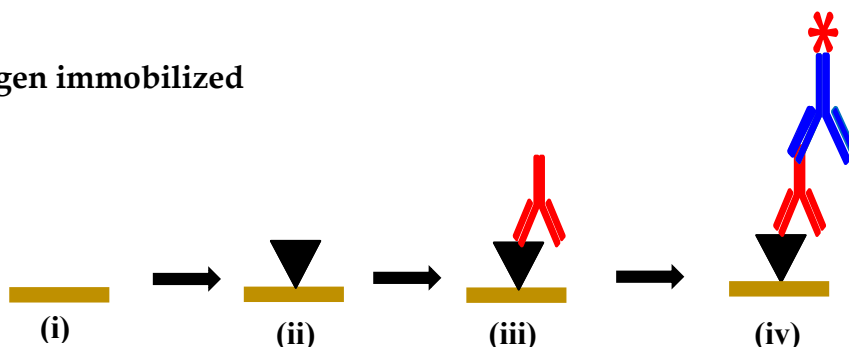
Figure 1.6: Schematic diagram illustrating the principle of biosensor operation and principal components [147].

Immunobiosensors are affinity ligand-based biosensing devices that couple immunochemical reactions to an appropriate transducer. The sensing element is formed using an immobilized antigen or antibody, and the binding effect of the analyte is transferred into a measurable signal by the sensor. Immunobiosensors utilize the specific interactions of an antibody and antigen to form an immunocomplex. Therefore, results in a simple analytical device that can be used to deliver sophisticated analytical results in decentralized locations, in the field, home or the doctor's office. These systems offer advantages such as simple design, low cost, and rapid immunoassay [148]. Immunoassay is a term used to describe the procedure for tests based on immunoreactions [146]. It is classified as either homogenous or heterogeneous immunoassay. In a homogenous immunoassay, the immunoreaction takes place in solution. Heterogeneous immunoassay involves the immobilization of the biorecognition elements on a solid support such as microtiter plate and electrodes. The immunoreaction takes place at the solid support interface. The enzyme-linked immunosorbent assay (ELISA) is the gold standard technique employed for the immunobiosensing of disease biomarkers [149]. The most commonly used configurations are the sandwich and immunometric (antigen-down) assay [150]. **Scheme 1.2** shows the schematic representation of immunobiosensors formats (a) sandwich assay and (b) immunometric assays. The scheme shows (i) immunobiosensor interface, (ii) immobilization of biorecognition element, (iii) label-free detection, and (iv) labeled detection of an analyte of interest. In the sandwich assay, the analyte to be detected (antigen) is bound between two primary antibodies that are the immobilized capture antibody and the detection antibody as shown in **Scheme 1.2 (a)**. The immunometric immunoassay involves binding the antigen to the transducer surface to detect antibodies found in the test samples. These assay formats can either be in a labeled or label-free direct or indirect detection strategy.

(a) Antibody immobilized



(b) Antigen immobilized



Legends:

■ Solid phase

▼ Antigen

Y Antibody

Y* Probe-labelled antibody

Scheme 1.2: Schematic Illustrations of immunobiosensors formats showing (a) sandwich assay, and (b) immunometric assays.

In the label-free direct detection strategies in **Scheme 1.2 (iii)**, the physical changes induced by the formation of the antibody-antigen immunocomplex at the transducer interface is monitored and correlated with the analyte (antigen or antibody) concentrations. The indirect-labeled detection in **Scheme 1.2 (iv)** uses signal-generating labels such as Raman tag, organic dye, enzyme and radioactive label as a signal probe for the detection of the formation of an antigen-antibody immunocomplex. The response of the label is indirectly correlated with the concentration of the analytes. Furthermore, immunobiosensors are not only characterized by their specificity and selectivity towards the analyte but also by essential parameters such as a

dynamic and linear range of detection, the limit of detection and sensitivity. The dynamic range is the range of the concentrations at which a linear signal response to varying concentrations of the analyte is observed. The sensitivity is the amount of signal generated over a concentration range. It is evaluated from the slope of the plot of the sensor response against analyte concentrations in the linear range. By exploiting the linear calibration curve, the limit of quantification (LoQ) and the limit of detection (LoD) are calculated using **Equations (1.1)** and **(1.2)**. The LoD is the lowest quantity of the analyte concentration that can be reliably distinguished from the blank while the LoQ is the lowest concentration of analyte that can be determined with acceptable repeatability and trueness [151].

$$\text{LoD} = \frac{3\sigma}{\text{Sensitivity}} \quad (1.1)$$

$$\text{LoQ} = \frac{10\sigma}{\text{Sensitivity}} \quad (1.2)$$

1.6.1 Techniques for immobilization of biorecognition elements

The analytical performance of the immunobiosensor strongly depends on the immobilization and bioconjugation method adopted for the immobilization of biorecognition elements. The immobilization method affects the stability and activity of the biorecognition elements. The sensitivity of the detection probe also depends on the method adopted for conjugating the detection probe to the antibody. Broadly, immobilization and bioconjugation techniques are divided into physical adsorption, covalent binding, and affinitive binding. These methods present various advantages and disadvantages which are summarised in **Table 1.6**. The work reported in this thesis exploited a few of these methods that are (i) affinitive interaction via protein G, (ii) covalent immobilization via glutaraldehyde crosslinking, and (iii) reductive amination. These techniques are discussed in detail.

Table 1.6: Immobilization and bioconjugation strategies for Immunobiosensor.

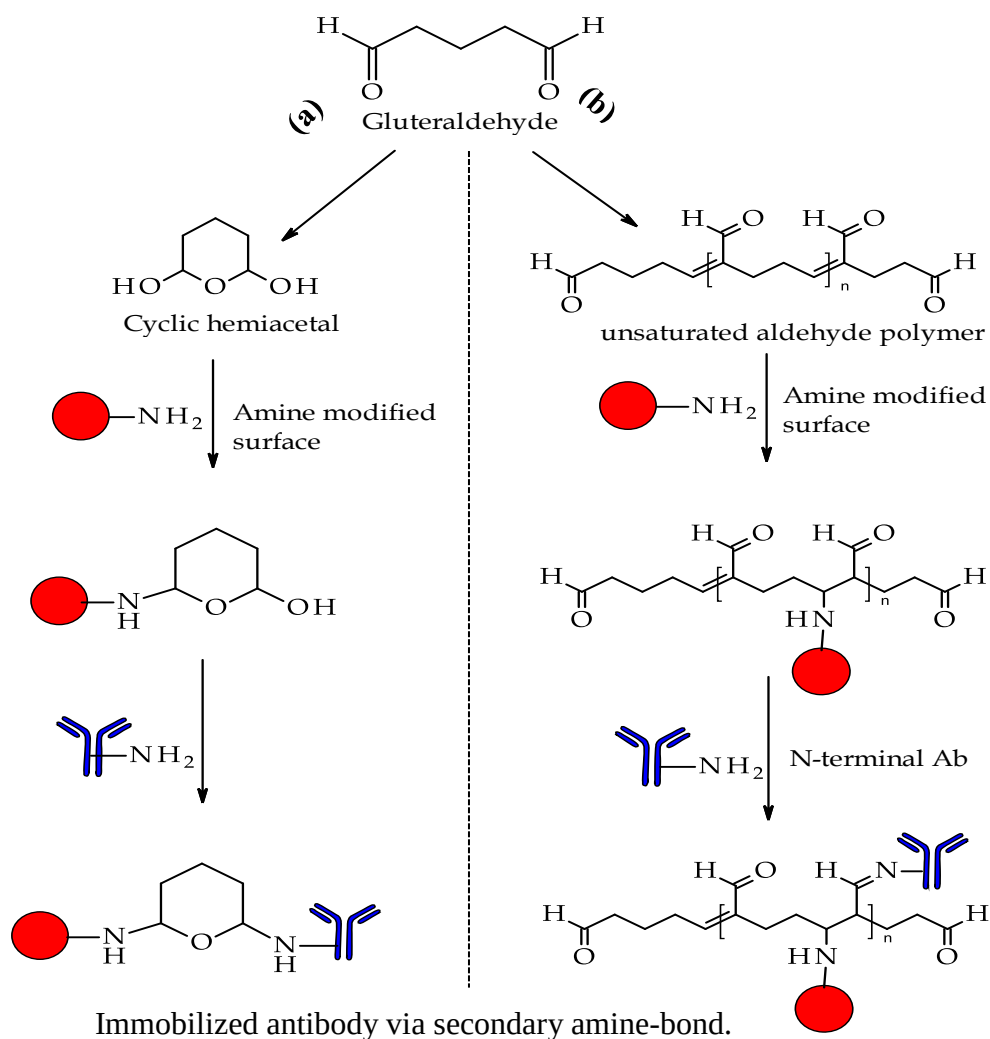
Method	Type of interaction	Advantages	Disadvantages
Physical	• Electrostatic attraction. • Van der Waals. • π- π interactions. • Chemisorption.	• Simple. • Inexpensive. • Rapid.	• Random orientation. • Poor stability. • Reproducibility. • Sensitive to changes in buffer conditions.
Affinity	Affinitive interactions: • Avidin-streptavidin. • Binding of his-tag to the divalent metal ion. • His-tag to His-tag protein. • Protein A or G to IgG.	• Oriented immobilization. • Highly reproducible.	• requires the use of linkers. • Expensive. • Loss of bioactivity. • pH dependence.
Covalent	• Chemical bond formation between the protein and the surface via a crosslinker.	• Reproducible. • Stable conjugate. • Spatial surface patterning. • Orientation can be controlled.	• Expensive. • The protein may be denatured. • Require coupling reagents.

1.6.1.1. Glutaraldehyde crosslinking

Glutaraldehyde is a bis-aldehyde homobifunctional crosslinker that has found extensive use in crosslinking of protein to the amine-functionalized solid support, nano, and microparticles, enzyme or between protein molecule [152]. Glutaraldehyde can react with several functional groups of proteins, and these include amine, thiol, phenol and imidazole groups [153,154]. The relative reactivity of glutaraldehyde with the functional groups follows this trend of amine: ϵ -amine > α -amine > guanidinyll > secondary amine > hydroxyl groups [155]. The unprotected amine groups are the most reactive nucleophile towards aldehyde, and the protein crosslinking involves lysine residue. The lysine residue is exposed in an aqueous medium due to the polarity

of the amine groups. Also, lysine residues are not involved in the bio-recognition sites, which allows moderate cross-linking to preserve protein conformation and biological activities.

Scheme 1.3 shows the activation of amine-modified surfaces with glutaraldehyde and immobilization of antibodies to the modified surface. Commercial glutaraldehyde aqueous solution exists in a polymeric form [156,157]. At acidic or neutral pH, dilute concentrations ($\leq 10\%$) of glutaraldehyde undergoes intramolecular cyclization and exist predominately in the monomeric cyclic hemiacetal form [158,159] as shown in **Scheme 1.3 (a)**. The hemiacetal reacts with an amine by nucleophilic attack at the anomeric carbon bearing the hydroxyl groups resulting in a substitution reaction that forms a stable secondary amine linkage [158,160]. However, under alkaline reaction conditions ($\text{pH} \geq 8.0$), glutaraldehyde exists as an α,β -unsaturated poly-glutaraldehyde polymer [160] as shown in **Scheme 1.3 (b)**. The reactivity of the poly-glutaraldehyde proceeds by Michael-type addition reaction of the amine to the double bonds of the poly-glutaraldehyde polymer. This results in a stable secondary amine linkage [155]. Thus, the covalent linkage between glutaraldehyde and the amine group shows exceptional stability [158]. The monomeric glutaraldehyde unit of the polymeric structure could also react with amine groups resulting in the formation of a Schiff-base product [159]. Schiff-base is further reduced (by sodium cyanoborohydride) to form a stable secondary amine.

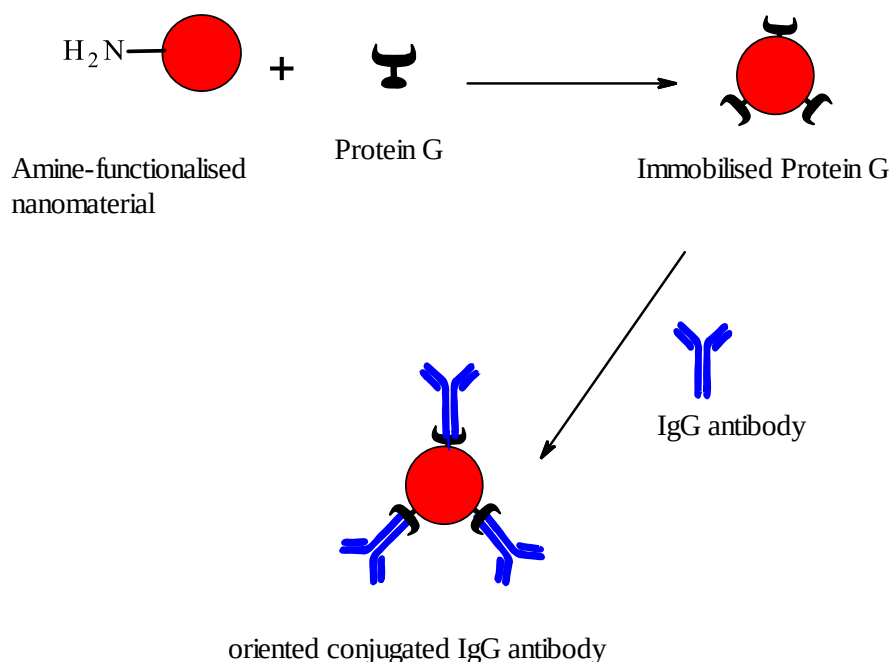


Scheme 1.3: The activation of amine-modified surfaces with glutaraldehyde and immobilization of protein [161].

1.6.1.2. Oriented Affinitive bioconjugation

The immobilization and bioconjugation of an unmodified antibody can be mediated by an intermediate protein covalently coupled to the surface. Such protein mediators are protein G, protein A, polyhistidine (His₆-tag), biotin and streptavidin. Streptavidin-biotin interaction is one of the strongest known non-covalent interactions and requires prior biotinylation of the antibody. However, the antibody is mostly randomly oriented using this approach [162]. Oriented affinitive conjugation of antibody could be achieved with the use of either His-tag

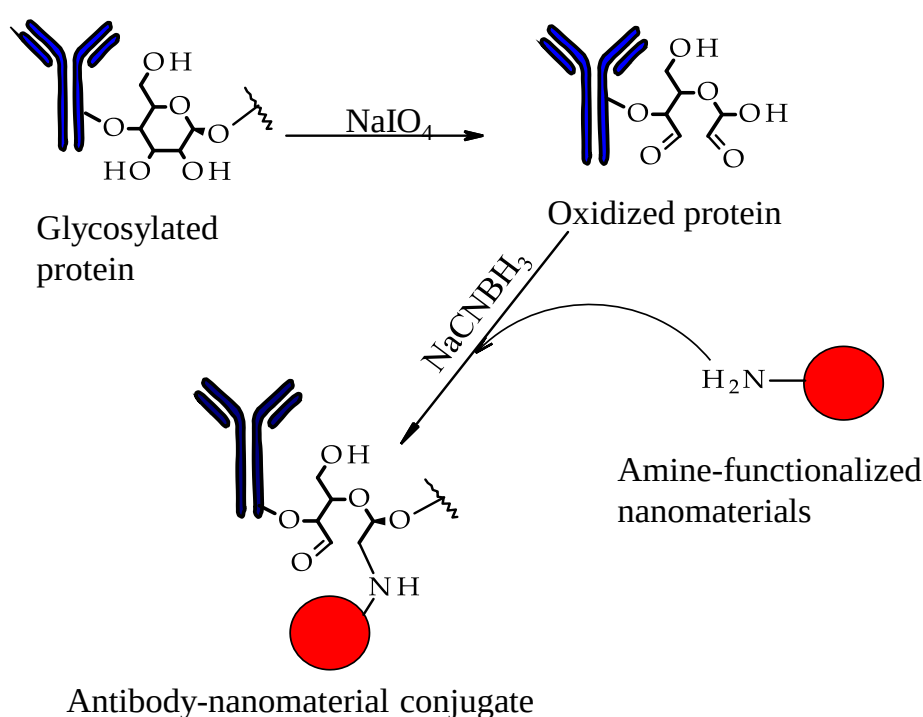
proteins, or Protein A or Protein G (**Scheme 1.4**). The fusion of a polyhistidine to the C- or N-terminal of the recombinant antibody allows for the formation of a stable chelate between the His-tag and divalent metal (Ni, Co, Cu, and Zn)-nitriloacetic acid-modified surface [163,164]. This approach allows for the formation of a tail on orientation without hindrance to the antibody binding sites [165]. Another well documented and simple method for oriented bioconjugation is the Protein G-mediated affinitive binding [104,166–170]. Protein G is an antibody binding protein expressed in streptococcal bacteria. It has two binding domains that are specific to the Fc-portion of immunoglobulins G (IgG). The surface is pre-modified with Protein G before interaction with IgG resulting in a tail-on-orientation of the IgG. Thus the antigen-binding sites of the antibody will be exposed. This approach offers advantages such as a high degree of sensitivity and cost-effectiveness over other affinitive bindings. An improvement in biosensor sensitivity based on oriented immobilization via this approach has previously been reported [166,171,172].



Scheme 1.4: Oriented antibody conjugation via Protein G affinitive interaction [161].

1.6.1.3. Reductive amination coupling to a glycosylated antibody

Scheme 1.5 shows a typical illustration of the reductive amination coupling of the antibody to a solid surface. Some IgG antibodies have an N-glycosylation site at Asn297 in the CH₂ domain of the Fc-region. A mild reducing agent such as sodium periodate can be used to oxidize the diol of the carbohydrate moiety into aldehyde groups. The aldehyde groups can react with an amine or hydrazide group to form a Schiff base or hydrazine which is subsequently reduced with sodium cyanoborohydride (NaCNBH₃) to a stable secondary amine linkage. The Fab region of the conjugated antibody is exposed to antigen binding and to improved immunobinding efficacy.



Scheme 1.5: Covalent oriented immobilization of antibody on nanoparticle via a glycosylated moiety [161].

1.6.2 Signal transducer techniques

The transducer (detector) converts the biorecognition events to measurable and quantifiable signals. The transducer can be classified as optical, magnetic, piezoelectric and electrochemical. The signal transducer techniques adopted in this thesis are described in brief.

1.6.2.1 Electrochemical Transducers

Electrochemical transducers are a promising tool for point-of-care biomarker detection and quantification due to the potential for miniaturization and low-cost [173,174]. In the design of an electrochemical immunobiosensor, an electrically conducting surface (electrode) connected to an electrical circuit is used as a signal-transducing element. The antigen-antibody immunocomplex formation is monitored using an electrical signal which could be an electric current generated (amperometry), a voltage difference (potentiometry), or change in resistance (impedimetry). The work reported in this thesis involves the use of impedimetric and amperometric transduction techniques, and these are discussed in detail.

1.6.2.2. Electrochemical Impedance Spectroscopy (EIS) immunobiosensor

The application of EIS as a transduction technique enables the label-free detection and quantification of antibody-antigen immunocomplex formation. EIS is a powerful informative and non-destructive techniques due to the small voltage excitation used during detection [175]. It has attracted much interest as a tool for studying the immunocomplex reaction (biorecognition event) at the electrode/electrolyte interface by measuring the impedance as a function of applied voltage [176]. Various EIS-based affinity immunobiosensor have been widely investigated for the detection of disease biomarkers by immobilizing antibodies or antigens on the surface of gold or carbon electrodes. EIS has been widely reported for the detection and quantification of tumor antigen biomarkers. This include the detection of murine double minute 2 (MDM2) [177], epidermal growth receptor factor (EGFR) [166,178], alpha-

fetoprotein [179,180], carcinoembryonic antigen (CEA) [181–183], carbohydrate antigen (CA-125) [184], prostate-specific antigen (PSA) [185,186], P53-tumor antigen [187]. The EIS immunobiosensor showed good analytical performance for the detection of the listed tumor biomarkers.

Generally, EIS measurement can either involve a faradaic or non-faradaic process. Non-Faradaic EIS experiments are performed in the absence of a redox probe. Thus only capacitive changes (double layer capacitance) are measured [188]. Faradaic EIS employs an electroactive redox probe in a solution whose heterogeneous electron transfer rate constant is dependent on the antigen-antibody binding [189]. The EIS measurement involves the application of a small AC voltage which is superimposed on the formal potential of the redox couple. A small AC voltage is applied to maintain a thermodynamic equilibrium at the electrode/electrolyte interface to assist in the separation of mass transfer and electron transfer processes. **Figure 1.7** shows the applied AC voltage results in the resulting AC that is measured.

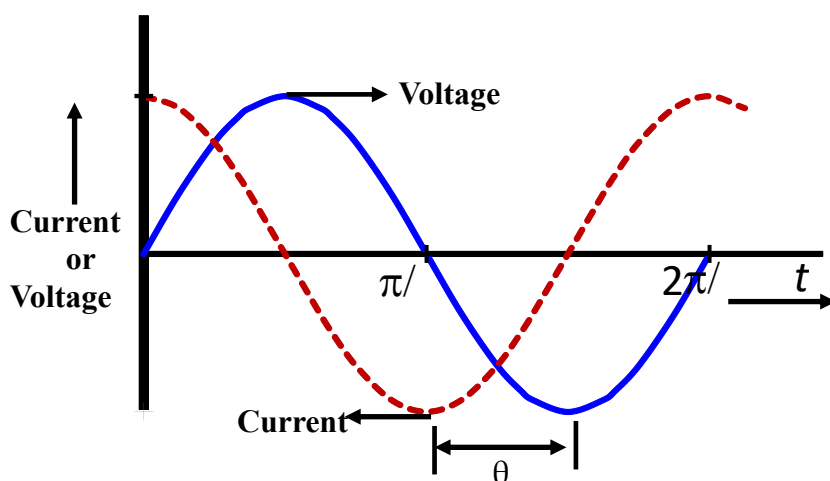


Figure 1.7: Representative diagram of Applied AC voltage and resulting AC response.

The applied AC voltage signal can be expressed as a function of time (V_t):

$$V_t = V_o \sin(\omega t) = V_o \sin(2\pi f t) \quad (1.3)$$

The AC signal obtained with time is the current density, I_t :

$$I_t = I_o \sin(\omega t + \varphi) = I_o \sin(2\pi f t + \varphi) \quad (1.4)$$

where V_o is the amplitude of the voltage signal, ω is the angular frequency ($\omega = 2\pi f$), f is the frequency (Hz), t is the time. I_o is the current amplitude and φ is the phase angle which depends on the impedance of the system.

The impedance of the system (Z) is determined according to Ohms law

$$Z = \frac{V_t}{I_t} = \left(\frac{V_o \sin(2\pi f t)}{I_o \sin(2\pi f t + \varphi)} \right) \quad (1.5)$$

$$Z = Z_0 \frac{\sin(2\pi f t)}{\sin(2\pi f t + \varphi)} \quad (1.6)$$

The Euler's expression (**Equation 1.7**) is employed to represent a complex impedance

$$e^{j\varphi} = \cos\varphi + j\sin\varphi \quad (1.7)$$

where j is an imaginary number and is equals to $(\sqrt{-1})$. **Equations 1.3 and 1.4** are expressed as a function of time

$$V_t = V_o e^{(j\omega t)} \quad (1.8)$$

$$I_t = I_o e^{(j\omega t + j\varphi)} \quad (1.9)$$

Thus, impedance is expressed as a complex number as:

$$Z = \frac{V_t}{I_t} = Z_0 e^{(j\omega t)} = Z_0 (\cos\varphi + j\sin\varphi) = Z' + jZ'' \quad (1.10)$$

The Z' and Z'' represent the real, and the imaginary components of the impedance (Z) and j is a complex number. The EIS data are represented by a Nyquist or Bode plot. The Nyquist plot is a plot of the real part of the impedance (Z') on the X-axis and the imaginary part of the impedance (Z'') on the Y-axis, **Figure 1.8 (a)**. The real impedance is the resistance to the flow of current by the circuit while the electrical energy storage capability of the circuit is reflected by the imaginary (Z'') part of the impedance. The Nyquist plot gives information about the

charge transfer resistance (R_{CT}), solution resistance (R_S), Warburg impedance (Z_W), and the double-layer capacitance (C_{dl}) or constant phase element (CPE). The Bode plot is a plot of the phase angle (θ) against the logarithm of the impedance magnitude ($\log Z$) and the logarithm of the frequency ($\log f$) as shown in **Figure 1.8 (b)**. The Bode plot gives additional frequency information which is not represented in the Nyquist plot.

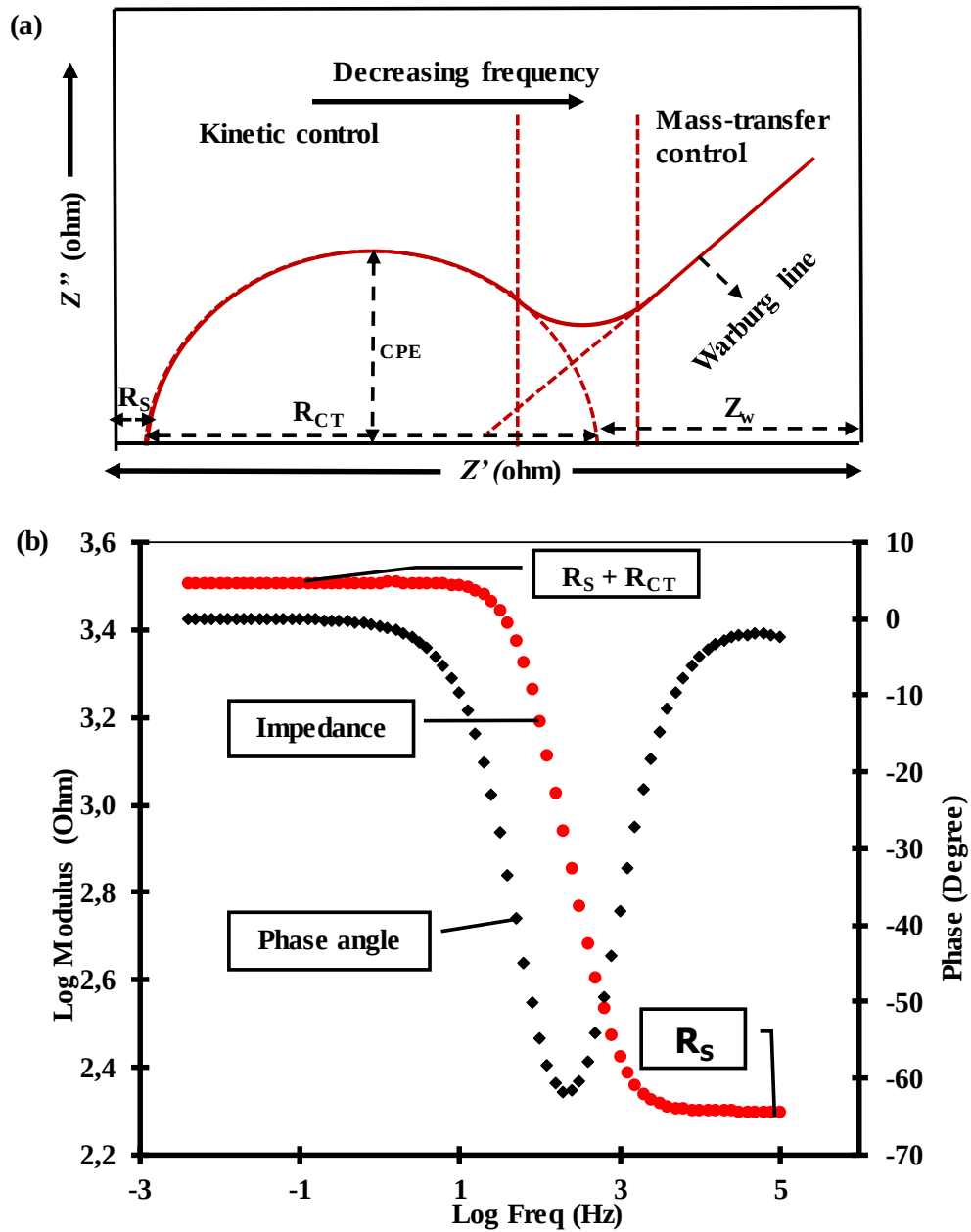


Figure 1.8: Impedance data representation (a) Nyquist plot and (b) bold plot [190].

The impedance of the electrochemical interface is usually analyzed using an electrical equivalent circuit. The most widely used equivalent electrical circuit that often satisfactorily describes impedimetry immunobiosensing systems is the Randles-Sevick equivalent circuit [191]. **Figure 1.9** shows the elements of a Randles-Sevick equivalent circuit. The Randle-Sevick equivalent circuit consists of an active electrolyte resistance (solution resistance, R_s) in series with the parallel combination of the double-layer capacitance (C_{dl}) or constant phase element (CPE) and the charge transfer resistance (R_{CT}).

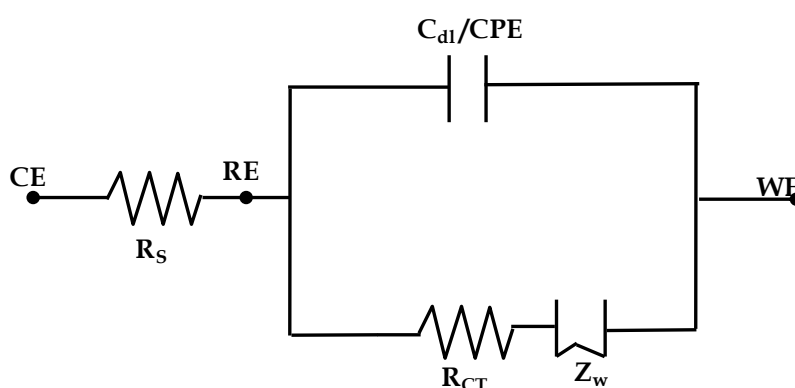


Figure 1.9: Randles-Sevick equivalent circuit for the electrochemical impedance system.

The solution resistance (R_s) depends on the solution along the distance between the working electrode and the reference electrode. The Warburg Impedance (Z_w) gives information about the diffusion of the redox probe through the surface layer. It represents the pseudo-impedance due to the mass transfer, which is the diffusion of ions from the bulk solution to the electrode surface. At lower frequencies, Z_w predominates with a phase angle of 45° in a diffusion control faradaic system. The double-layer capacitance (C_{dl}) or constant phase element (CPE) gives information about the capacitance of the thin film at the electrode surface. For the real practical behavior, the capacitive properties of the double layer are modeled as the constant phase element (CPE).

The impedance of the CPE is given by **Equation (1.11)**:

$$Z_{CPE} = Y_o (j\omega)^{-\alpha} \quad (1.11)$$

The C_{dl} estimate could be evaluated from the CPE using **Equation 1.12**

$$C_{dl} = \frac{(Y_o R_{CT})^{-\alpha}}{R_{CT}} \quad (1.12)$$

where ω is the angular frequency, Y_o is the magnitude of the admittance, while α is an exponent with values between 0 and 1. Y_o and α are not dependent on frequency.

1.6.2.3. Amperometry and Voltammetry immunobiosensor

Amperometry and various forms of voltammetry (differential pulse, linear sweep, square wave, and stripping) are widely used electrochemical methods for the detection of protein biomarkers [192]. In voltammetry, a variable potential is applied, while in amperometry the applied potential remains constant. These methods are designed to measure the current generated by a redox process when an analyte binds to the recognition element. This principle has been widely used in the development of the glucose biosensor, which utilizes a biocatalytic redox enzyme-substrate reaction to generate an electrochemical signal [193,194]. Since immunobiosensor employs affinitive antigen-antibody interactions. They cannot intrinsically act as redox partners in the electrochemical reaction. The analyte is detected indirectly by using an electroactive labeled secondary-antibodies as shown in various reports [195–201]. Most reported immunobiosensor were based on the catalysis of the enzyme-labeled secondary antibodies. The enzymes used are horseradish peroxidase (HRP) or alkaline phosphatase (ALP) that catalyze suitable substrates to form an electroactive product [174,202]. Recent attention has been focused on signal amplification using multi-enzyme and nanomaterial labels. Researchers have reviewed the various aspects of voltammetry, and amperometry immunobiosensor signal amplification based on multi-enzyme and nanomaterials labeled probe [173,203–206]

1.6.2.4. Optical Immunobiosensor

Optical biosensors are light based-sensors that utilize changes in the light absorption, total internal reflection, fluorescence, luminescence and surface plasmon phenomenon to detect and quantify a targeted analyte. A direct optical immunobiosensor measure changes in the optical properties after the formation of an immunocomplex on the sensor surface. Photodetectors collected the optical output and converted into electrical signals that correlate with the concentration of the target analyte. **Table 1.7** shows the summary of the optical phenomena that are employed in optical immunobiosensors. The transducer technique for recognizing the immunocomplex event is the intensity-based optical transduction. This technique relies on either colorimetric, luminescence or fluorescence-based reporter molecule for the detection of a target analyte.

Table 1.7: Optical process employed in optical immunobiosensor

Process	Transduction Technique
Absorption	Intensity measurement
Reflectance	Intensity measurement
Fluorescence	Emission intensity measurement, fluorescence lifetime measurement
Refraction index changes	Total internal reflection, surface plasmon resonance
Optical path	Interferometry
Spectroscopy	Surface-enhanced Raman scattering

1.6.2.5. Colorimetric Immunobiosensor

Colorimetric detection is a well-established transduction technique commonly employed in lateral flow test strips and the enzyme-linked immunosorbent assays [149,207]. The advantages of an easy-to-use, low cost, portable and user-friendly detection strategies makes these the most acceptable and suitable diagnostic tools for point-of-care (POC) diagnosis [208,209]. The quantification of the analyte is based on color development and measurement of color intensity using a low-cost portable spectrophotometer [210,211]. Traditional colorimetric detection is accomplished by monitoring the generation of colored enzymatic products. Enzyme-catalyzed reactions, in which antibody conjugated to an enzyme are used as a signal generation label in an indirect immunoassay format. Although many different types of enzymes have been used in colorimetric immunosensor, phosphatase (ALP) and horseradish peroxidase (HRP) are the two most widely used enzyme [174,212]. The enzyme labels catalyze the conversion of a chromogenic substrate into a colored product which is measured as an indirect signal to quantify the analyte. Various colorimetric substrates such TMB, ortho-phenylenediamine (OPD), 3,3-diaminobenzidine (DAB) and 2,2-azino-bis(3-ethylbenzothiazoline-sulphonic acid (ABTS) are commonly used substrate for HRP enzyme [213]. Nitro-blue tetrazolium chloride (NBT) and p-nitrophenyl phosphate disodium salt (PNPP) are the main substrates used for the alkaline phosphatase enzymes [149,202]. However, natural enzymes are expensive, lack stability in harsh environmental conditions and are difficult to obtain in the highest purity [214]. Also, signal amplification by multiple enzymes labeling is not achievable due to steric hindrance when conjugated to the antibody. Thus the limit of detection is often not sensitive to monitor antibody-antigen immunocomplex formation. This is also a drawback for the detection of low concentrations of cancer biomarkers especially in the early stages of lung cancerogenesis [215]. This limitation calls for the design and development of highly sensitive colorimetric immunoprobes characterized by high sensitivity, stability, fast response and

adaptability for the development of cancer screening test kits. The emergence of the new field of nanozymology has enabled the development of nanomaterials with enzyme-like activity (nanozyme) [216–218]. In contrast to bioenzymes, nanozyme possesses tuneable and efficient catalytic activity, higher stability against stringent conditions, low cost, and have a high surface area for antibody conjugation [214,217]. With these advantages, the work reported in chapter five of this thesis focuses on the synthesis of nanomaterials with enzyme-like activity for the detection of tumor-associated autoantibodies is of international research interest.

1.6.2.6. Fluorescent immunobiosensor

Fluorescence sensing is a widely used optical transduction technique due to its advantages namely: high sensitivity, selectivity, stability, and ease of multiplexing [219]. Typical fluorescence immunobiosensor utilizes a sandwich immunoassay format. The detection of the target biomarker is performed by the formation of a capture antibody-antigen-antibody/fluorophore sandwich structure. In this configuration, the biorecognition element immobilized on the solid support captures the targeted analyte (antigen or antibody) and a secondary antibody conjugated to a suitable label form an immunocomplex with the captured targeted analyte. The fluorescence light emitted from the immunoprobe molecule is detected by the photodetector and converted into electrical signals. The intensity of emitted radiation is proportional to the amount of the targeted analyte. **Figure 1.10** shows the process involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescent by fluorophore-labeled antibodies. A photon of energy (h_{ex}) supplied by the light-emitting diode was absorbed by the ground state fluorophores (S_0) and creating an excited electronic singlet state (S_1') for a finite period typically between 1-10 ns. The fluorophore undergoes conformational changes and the energy of (S_1') is dissipated, yielding a relaxed, excited state (S_1) from which a photon of energy (h_{em}) is emitted on returning to the ground state (S_0). The emitted radiation has lower energy (longer wavelength) than the excitation

energy due to energy dissipation during the excited state. The difference in energy (Stokes shift) allows the emission photon to be detected against a low background, isolated from the excitation photon with a monochromator. The Stokes shift is fundamental to the high sensitivity of fluorescence immunobiosensor [220].

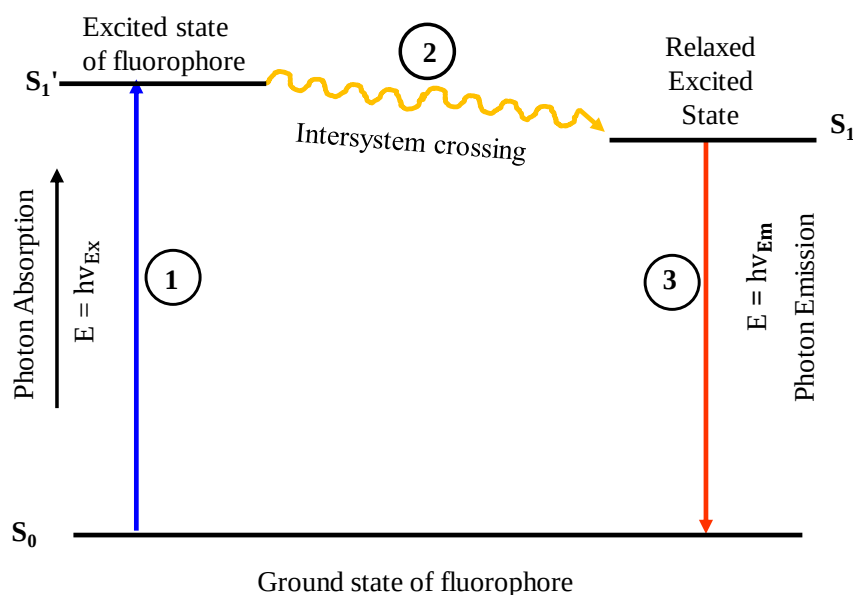


Figure 1.10: Illustration of the process involved in fluorescence emission of the fluorophore

The fluorescence intensity measurement could be demonstrated in two distinct assay formats either using a single fluorescent probe or by the use of two Förster energy transfer (FRET) paired fluorophores [221]. Fluorescent organic dye molecules conjugated to detection antibodies are mostly used fluorescence immunoprobes [222,223]. However, organic fluorophores have intrinsic limitations such as poor photostability, and their fluorescence is quenched at a high antibody-dye ratio [224]. These limitations have made organic fluorophore bioprobes not suitable for the detection of the clinically relevant level of cancer biomarkers at the early stage [225]. Attaining ultrasensitive fluorescent-based detection of a biomarker requires the use of functional fluorescence immunoprobes with enhanced fluorescent intensity, photostability and allows for higher affinity binding with the targeted. Nanoparticle-based

signal amplification strategies have been employed in recent times. Various fluorescent nanomaterial including NaYF₄: Yb, Er nano-phosphors [209,226,227], cadmium telluride based semiconductor quantum dot [219,228–230] have been employed for the enhanced detection of cancer biomarkers.

1.7. Nanomaterials for immunobiosensing application

Nanomaterials enhanced the performance of immunobiosensor in terms of sensitivity, wider linear range and lowered detection limits. Nanomaterials are biocompatible, have a high surface area, chemically stable, and unique physicochemical properties [204,231]. The high surface area and biocompatibility of nanomaterials allow for the immobilization of the biorecognition element (antigen or antibody) in a more significant amount [232]. Researchers have developed various techniques for enhanced biosensor performance by modifying the immunosensing interfaces with functional nanomaterials. Considerable attention has been paid to the use of magnetic nanoparticles (MNP), silica and polymer nanoparticles, metallic nanoparticles, carbon-based nanomaterials, and quantum dots. Different review articles have reported various fields of applications of nanomaterials in enhancing biosensor performance [204,233–235]. The work reported in this thesis focus on the use of nanomaterials as (i) an immunoprobes (carrier of signal generation element), (ii) separators and collectors of the immunocomplex, (iii) artificial enzyme mimetics and (iv) multiple signal generation labels has been investigated.

1.7.1. Nanomaterials as a carrier of the signal element

Nanomaterials are used as carriers for loading numerous signal transduction elements such as enzymes [195,236], dyes [237–239], and metallic nanoparticles [240–242]. Silica nanoparticles have been used as an excellent carrier for dual loading of signal transduction elements and immobilization of detection antibody due to its three-dimensional network

structure. They are widely used due to their biocompatibility which prevents loss of protein activity, stability, the ease of surface modification using organosilane reagents [243]. Fan et al., [244] proposed a thionine loaded silica-based controlled release system with acid cleavage linkage for quantitative analysis of prostate-specific antigen, a low detection limit of 310 fg.ml^{-1} PSA and a wide dynamic range within 0.001 to 50 ng.ml^{-1} was achieved. Other studies have also achieved sensitive detection of protein biomarkers using silica nanoparticles as a carrier for signal transduction probe [245–248]. In optical detection systems, the most common strategy to amplify the signal intensity is to assemble fluorophore molecules within a nanoparticle or polymer matrix [248,249]. The biological recognition event is signaled by multiple dye molecules loaded within the silica nanoparticle and therefore gives an enhanced fluorescence signal that is brighter than a single fluorophore-antibody conjugate.

1.7.2. Nanomaterials as enzyme-mimetic

Nanomaterials have been investigated as enzyme mimetic (nanozymes). As nanocatalyst, nanozyme have many active sites while natural enzyme has only one active site. Thus, they are an efficient catalyst to use in immunobiosensors as signal amplifiers. Nanomaterials such as metallic nanoparticles (Au, Ag, Pd, Pt), transition metal oxides (Fe_3O_4 , CuO, CeO) nanoparticles and carbon-based nanomaterials (graphene, carbon nanotube) and their composites have shown excellent bioenzyme-like activities, such as peroxidase and catalase mimetic [205,250–252]. The intrinsic enzyme mimetics activity of monometallic nanozyme is too weak to be applied for colorimetric immunobiosensor [216]. Bimetallic nanoparticles are considered more beneficial over monometallic counterparts due to their compositional dependent tuneable and unique catalytic activity [251,253,254]. Au/Pd, Au/Cu, Au/Ag, Pd/Fe bimetallic core-shell and alloyed nanoparticles [255,256] due to the synergetic effect from the intermetallic combination have shown superior catalytic performance unachievable with their monometallic counterparts [257,258]. The application of bimetallic gold@palladium supported

on magnetite core-silica nanosphere as peroxidase enzyme mimetics and its use for signal enhancement have been investigated.

1.7.3. Nanomaterials as separators and collectors

Crystals of transition metals (Fe, Co, Ni) and their oxides have magnetic properties [259]. Magnetic nanoparticles (MNPs) have a high surface area, biocompatibility and can be easily separated with the aid of a magnet. Magnetic separation is an efficient way to enrich biomolecules and separate immunocomplexes. MNPs are used for the immobilization of biorecognition elements for easy and rapid separation of bioconjugate without loss of activity. The magnetic nanoparticle can be easily dispersed in the liquid phase, and the immobilized biomolecules can react with the free target in the liquid phase with high efficiency. The immunocomplex formed on the magnetic nanoparticles is easily separated with a magnet. Therefore, the homogenous reaction is combined with heterogeneous separation to achieve sensitive detection of biomolecules. This approach also reduces assay time. Previous studies have developed highly sensitive magnetic-based immunosensors [181,260–262]. The application of this approach in enhancing fluorescence-based detection has been investigated.

1.8. Identified knowledge gap and proposed study

The introduction and literature review chapter has identified various knowledge gaps in the fabrication of sensors for early-stage diagnosis of lung cancer. These limitations include:

- (i) The current methods for the diagnosis of lung cancer are imaging and invasive techniques, which are not suitable for screening of asymptomatic lung cancer,
- (ii) The current techniques for lung cancer diagnosis pose a higher risk to patients due to exposure to ionizing radiation /needle aspiration that could damage the lungs,

- (iii) Most of the current researches on lung cancer biosensor design targeted the detection of a tumor-associated antigen (TAAs). These are expressed at low concentration in asymptomatic lung cancer,
- (iv) TAAs have a short half-life and not stable in body fluids,
- (v) TAAs detection usually fails clinical trials, and
- (vi) They are not suitable for screening and early-stage diagnosis of lung cancer.

These limitations, therefore, necessitate the continued research into biosensor for lung cancer screening. The immunobiosensors studied in this work targets the detection of anti-P53ab. This was because anti-P53abs are present at higher concentrations, more stable and have a longer half-life than antigens in serum. Anti-P53ab is not present in a healthy individual, but the level increases at the early stage of carcinogenesis. Therefore, they have higher diagnostic values in identifying early-stage lung cancer with low false positive and negative results. To the best of the candidate's knowledge, only four biosensors for anti-P53ab detection have been reported in the literature, and their analytical performance is summarised in **Table 1.8**.

Table 1.8: Summary of a reported biosensor for anti-P53-autoantibody detection.

Detection Techniques	Biorecognition element	Linear range (ng/ml)	Low detection limit	Refs
EIS	Synthesized Pentadentate peptide	-	10 fg.ml ⁻¹	[95]
Colorimetric	Human P53 protein	-	0.4 ng.ml ⁻¹	[134]
QCM-D	Human P53 protein	-	20 µg.ml ⁻¹ g	[263]
Square-wave voltammetry	Human P53 protein	0.001 -10	80 fg.ml ⁻¹	[264]
ELISA	Human P53 protein	1.56-100	0.39 ng.ml ⁻¹	[264]

EIS- (electrochemical impedance spectroscopy) and QCM-D (quartz microbalance crystal with dissipation).

From **Table 1.8** it is evident that the use of biosensor technology for the detection of anti-P53ab has not been exhausted. The limitation of the reported systems are listed below:

- The method of immobilizing biorecognition element (P53ag) [95,264] are based on physical adsorption, and such sensing interfaces are not stable.
- They are not reproducible, that is, adsorption of biorecognition element on gold [264].
- They have limited shelf life, that is, utilizes enzymes which denature easily [134].
- The colorimetric sensor developed employed HRP enzyme which suffers from numerous limitations in **Section 1.4**.
- Optical transduction is most suitable for screening and point of care diagnosis in low resources settings than the square wave and QCM-D [263].

Therefore, studies that seek to address these limitations are of interest. The following research was therefore undertaken:

- The design of a stable and reproducible sensing interface by covalent immobilization of the P53ab.
- Synthesize nanomaterials with stable fluorescent and peroxidase mimetics activity as a substitute for HRP as signal transduction labels.
- Investigate the sensing of anti-P53ab in a labeled and label-free manner.
- Investigate the various techniques namely; electrochemical impedance spectroscopy (EIS), fluorescence and colorimetric detection of anti-P53ab.
- Enhancing the detection limits and sensitivity of anti-P53ab by incorporating novel fluorescent and peroxidase-like mimetic nanomaterials for signal enhancement.
- Design a paper-based colorimetric immunobiosensor for anti-P53ab detection based on peroxidase-like mimetic activity.
- Design a fluorescence based immunobiosensor based on magnetic separation and fluorescent silica nanoparticles.

CHAPTER 2:

Experimental

2. EXPERIMENTAL

This Chapter provides details about the reagents, materials, protocols and analytical techniques employed in the fabrication of the immunobiosensors, synthesis of the nanomaterial and antibody conjugation. The detailed methodology and suppliers of the various instruments and equipment used for characterization throughout this thesis were also discussed in detail.

2.1. Reagents and Materials

2.1.1. General Reagents and Materials

Fluorescein-5-isothiocyanate (FITC, isomer 1), tetraethylorthosilicate (TEOS, 99.9 %), 3-aminopropyltriethoxysilane (APTES, 98%), 4-(1,1,3,3-tetramethyl butyl)-phenyl polyethylene glycol-(Triton X-100), polyethyleneimine (PEI), 2-N-(morpholino)ethane-sulfonic acid (MES), N-hydroxysuccinimide (NHS), 4-(2-aminoethyl)phenylamine (APA), 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide hydrochloride (EDC), succinic anhydride (SA), tetrafluoroboric acid (HBF_4), sodium nitrite (NaNO_2), tetrabutylammonium tetrafluoroborate (TBABF_4), Acetonitrile (ACN), phosphoric acid (H_3PO_4), sodium periodate (NaIO_4), polyoxyethylene sorbitan monolaurate (Tween 20), potassium ferricyanide $\text{K}_3\text{Fe}(\text{CN})_6$, palladium chloride (PdCl_2), hydrogen tetrachloroaurate trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99%), potassium ferrocyanide $\text{K}_4\text{Fe}(\text{CN})_6$, iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), iron (II) chloride, sodium citrate tribasic ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), sodium nitrate (NaNO_3), ascorbic acid (AA), 3,3',5,5'-tetramethylbenzidine (TMB), ortho-phenylenediamine (OPD), sodium borohydride (NaBH_4), sodium azide (NaN_3), glutaraldehyde (GA), Ascorbic acid (AA), isopropyl alcohol (IPA), terephthalic acid (TA), 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Sigma-Aldrich. Ammonium hydroxide (NH_4OH , 25 wt %), sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), n-hexane, absolute ethanol, 1-octadecene, hydrogen peroxide (H_2O_2 , 30 %), sodium chloride (NaCl), oleic acid, and sodium oleate was purchased from Minema chemicals (South Africa). H_2PdCl_4 stock solution (10 mM) was prepared by

dissolving PdCl_2 (90 mg) in HCl (20 mM, 50 ml) in a boiling water bath. The 4-2-aminoethylbenzenediazonium tetrafluoroborate (ABED) was synthesized according to a previously reported method [265]. Polycrystalline Au electrodes (0.020 cm^2) and glassy carbon electrode (0.070 cm^2) were purchased from bioanalytical systems (BASi, USA). MaxiSorp black 96 microwell plate, Dynabeads M-270 carboxylic acid, Whatman cellulose No-1 chromatography paper, and Pierce BCA Protein assay kit was purchased from ThermoScientific. Amicon Ultra centrifugal filter units and Pur-A-Lyzer kits were purchased from Merck.

2.1.2 Biologicals

Recombinant Human cellular tumor antigen P53-antigen (CSB-EP24077HU), Mouse anti-human tumor protein P53-monoclonal antibody (CSB-MA0240711A0) and Mouse anti-human tumor protein P53-polyclonal antibody (CSB-PA0788RB) were purchased from Flarebio Biotech (USA). Mouse anti-IgG antibody (Fc-specific, anti-IgG) (M4280), recombinant Protein G (59303), Newborn Calf serum (NCS) and Bovine serum albumin (BSA) were purchased from Sigma Aldrich. The required concentrations of the biomolecules were prepared as per the experimental requirement in phosphate-buffered saline (10 mM PBS, pH 7.4) containing 135 mM NaCl.

2.1.3. Buffer and solvents

The buffers used throughout the thesis were prepared with ultrapure millipore water. Dilution buffer was phosphate buffer saline (PBS, 10 mM, pH 7.4) containing NaH_2PO_4 (0.339 g, 0.0025 mol), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.021 g, 0.0075 mol), sodium chloride (7.89 g, 0.135 mol), potassium chloride (0.20 g, 0.0027 mol). The activation buffer was MES buffer (10 mM, pH 5.5). The coupling buffers were acetate buffer (10 mM, pH 5.0) or MES buffer (10 mM, pH 5.5). The antigen coating buffer was bicarbonate/carbonate buffer (100 mM, pH 9.6) containing

NaHCO₃ (5.76 g, 0.0686 mol) and Na₂CO₃ (3.332 g, 0.0314 mol). The washing buffer (PBST pH 7.4) consists of PBS with the addition of Tween 20 (0.05 % v/v). High blocking buffer (HBB, pH 7.4) consisting of trisaminomethane (1.214 g, 0.01 mol), NaCl (0.029 g, 0.005 mol), Tween -20 (0.5 % v/v), BSA (2.0 % w/v). The substrate buffer-consist of sodium acetate-acetic acid (200 mM). H₂SO₄ (2.0 M) was used as the stopping solution. The dissolution buffer consists of NaOH (0.004g, 0.0001 mol) titrated with HCl to pH 9.0. The pH was adjusted to the required value with NaOH or phosphoric acid depending on the experimental requirement. Cyclohexane (99.5 %), n-hexane, dimethylformamide (DMF), hydrochloric acid (HCl), sulphuric acid (H₂SO₄), hexanol, acetone, chloroform, and absolute ethanol were purchased from B&M Scientific (South Africa). Ultrapure water with a resistivity of 18.2 MΩ.cm obtained from a Milli-Q water purification system (Millipore Corp, Bedford, USA) and used throughout the experiment. All other solvents were of analytical grades and employed directly without purification.

2.2. Equipment and methods

Electrochemical measurements were conducted on an Autolab Potentiostat/galvanostat PGSTAT302N equipped with NOVA 1.10 software and FRA32M for impedance measurement and analysis. The electrochemical data were collected using a conventional three-electrode setup with either a bare or modified gold (Au, r = 0.8 mm), glassy carbon electrode (GCE, r = 1.6 mm) as working electrodes. A platinum wire was used as a counter electrode, and the silver-silver chloride (Ag|AgCl, 3 M KCl) or Ag|AgCl (pseudo-reference) was used as the reference electrode. The potential of the pseudo-reference in aqueous conditions was less than the Ag|AgCl (3 MKCl) by 0.015 ± 0.003 V. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) experiments were recorded in a 1:1 mixture of 1.0 mM K₃Fe(CN)₆ and K₄Fe(CN)₆ redox couple in 0.1 M KCl (pH 7.4). CVs were recorded between -0.2 to 0.6 vs Ag|AgCl (3 M KCl). EIS was recorded in the frequency range between 10 kHz to

100 MHz (50 frequency) at a formal potential of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with an amplitude 5 mV rms sinusoidal modulation. The impedance data were represented in a complex impedance plot, and the fitting program of Autolab was used to analyze the impedance data with an appropriate equivalent circuit. All experiments were conducted at room temperature (25 °C) in a solution first purged with nitrogen gas. A blanket of nitrogen was kept over the solution to prevent diffusion of oxygen into the solution. Before performing the experiments, the bare working electrodes (Au or GCE) were polished with alumina paste on a Buhler felt pad, followed by a series of chemical and electrochemical cleaning to remove contaminants.

The X-ray powder diffraction patterns were recorded on a Bruker D8 Discover equipped with a Lynx Eye detector, using Cu-K α radiation (1.5405 Å, nickel filter). The data were collected in the range from $2\theta = 10^\circ$ to 100° , scanning at 1° min^{-1} with a filter time-constant of 2.5 seconds per step and a slit width of 6.0 mm. Samples were placed on a silicon wafer slide. The X-ray diffraction data were treated using the freely available Eva (evaluation curve fitting) software. Baseline correction was performed on each diffraction pattern by subtracting a spline-fitted to the curved background.

X-ray photoelectron spectroscopy (XPS) spectra were recorded using AXIS Ultra DLD (supplied by Kratos Analytical) using Al (monochromatic) anode, equipped with a charge neutralizer. For the XPS survey scans, the following parameters were used: the emission was 10 mA, the anode (HT) was 15 kV. The operating pressure was below 5×10^{-9} Torr. A hybrid lens was used and the resolution to acquire scans was at 160 eV pass energy in slot mode. The center used for the scans was at 596.0 eV (width of 1205 eV) with steps at 1.0 eV and dwelled time at 100 ms. The high-resolution scans were acquired using 40.0 eV pass energy in slot mode, the center was at 402.0 eV and width at 12.0 eV for N1s at 0.05 eV and dwelled time at 500 ms. XPS data analysis was recorded on with Kratos version 2 program. The core-level

binding energies (BEs) were aligned with the adventitious carbon C1s binding energy of 284.5 eV.

Electron paramagnetic resonance (EPR) spectra were carried out using a Bruker EMX Plus EPR spectrometer, model number: EMP-9.5/12B/P. EPR settings were 0.632 mW for the microwave power, frequency 9.714 GHz, resolution 2048 points, center field 3460 G and 100 G sweep width.

The hydrodynamic size and Zeta potential were measured using Malvern Zetasizer Nano-ZS90 series S equipped with a 633 nm He/Ne laser. The samples were diluted to 1 $\mu\text{g}.\text{ml}^{-1}$ in a suitable buffer. The refractive index and absorption of silica nanoparticles were fixed at 1.25 and 0.01. The temperature was set at 25 °C, and six measurements of 30 runs were carried out in a disposable cuvette. Zeta potential measurements were carried out in a disposable folded capillary cell (DTS 1060).

Transmission electron microscopy (TEM) micrographs were obtained using a Zeiss Libra 120 TEM operating at 80 kV. The materials to be investigated were dissolved in a suitable solvent, dropped onto a formvar carbon-coated copper grid and allowed to dry at room temperature before measurement.

Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (SEM-EDX) images and elemental mapping were carried out using an INCA PENTA FET coupled to VAGA TESCAN using a 20-kV accelerating voltage.

FT-IR spectra were recorded on a Bruker Alpha-model FT-IR Spectrometer (4000 - 650 cm^{-1}) or Perkin Elmer Spectrum 100 FT-IR Spectrometer (4000 - 400 cm^{-1}) equipped with the diamond-ATR sampler. All measurements were recorded at room temperature. 100 scans were taken for each interferogram at 4 cm^{-1} resolution. The background spectrum in the air was

recorded and automatically subtracted from the sample spectrum by the appropriate software (Brucker OPUS 6.5 software) and (Perkin Elmer Spectrum One software).

Thermal gravimetric analysis (TGA) analysis was performed using a Perkin Elmer TGA 4000 analyzer. The investigation was carried out under a nitrogen flow rate of $120 \text{ cm}^3 \cdot \text{min}^{-1}$. The weighed samples (2.0 mg) were heated from 50 to 800 °C at a rate of $10 \text{ }^\circ\text{C min}^{-1}$. The weight loss was calculated based on the percentage differences of the residue to the actual weight of the nanoparticle.

Ground state electronic absorption spectra were obtained on a Shimadzu UV-2550 spectrophotometer in the range of 300-800 nm. Protein absorption at 280 nm was quantified on Thermo Scientific Nanodrop 2000. Quantification of absorption reading in the 96-well plates was acquired on a SpectraMax M5 multimode microplate reader.

Fluorescence excitation and emission spectra were recorded on a Varian Eclipse spectrofluorometer. Quantification of fluorescence emission in the 96-well microtiter plate was carried out on SpectraMax M5e multi-mode microplate reader. The fluorescence excitation was at 450 nm. The digital image of the paper sensor surfaces was captured using a CanoScan 9000F high-resolution flatbed scanner. The captured images were analyzed with image J-software.

2.3. Synthesis of nanoparticles

2.3.1. Synthesis of FITC@SiNP-APTES-PEI (Scheme 4.1)

FITC@SiNP-APTES-PEI nanoparticles containing initial amount of 0.5 %, 1.0 %, 2.0 %, 4.5 %, 6.5 %, and 9.0 % FITC (w/w %) were prepared following a previous reported method with modification [225,266]. The first step was to conjugate FITC to APTES using the following protocol. Absolute ethanol was dried over 3A molecular sieve beads for 48 hours. FITC (5.256 mg, $13.5 \text{ }\mu\text{M}$), APTES (60.0 μl , $257 \text{ }\mu\text{M}$) and dried ethanol (2.0 ml) were added into a dry

glass vial under a nitrogen atmosphere. The mixture was stirred for 20 hours in the dark under a nitrogen atmosphere. The completeness of the reaction was monitored by the disappearance of the isothiocyanate ($-N=C=S$) groups in the FTIR spectra. Ethanol was removed under reduced pressure to obtain the fluorescent organosilane precursor FITC-APTES. In the second step a water-in-oil microemulsion was prepared by mixing n-hexanol (5.4 ml), cyclohexane (22.5 ml), Triton X-100 (5.3 ml), and water (1.5 ml). The solvent mixture was stirred for 10 minutes to form a microemulsion. FITC-APTES conjugate (x mg) and TEOS (300.0 μ l, 280.0 mg) were added to the microemulsion and stirred for 30 minutes. NH_4OH (20.0 μ l) was added to the reaction mixture, and the reaction was stirred for 24 hours in an aluminum foil-wrapped glass flask. TEOS (150.0 μ l) was added to the reaction mixture and stirred for another 24 hours to create a core-shell structure. The microemulsion was broken by adding acetone (30.0 ml). The nanoparticles were then isolated and washed by repeated centrifugation and sonication (3000 rpm, 10 min, five times) with a mixture of water and PBS pH 7.4 (2:1). The synthesized silica nanoparticles were referred to as FITC@SiNP core-shell nanoparticles. The amount of FITC-APTES (x mg) added to the microemulsion was; x = 1.365 mg, 2.761 mg, 5.562 mg, 8.292 mg, 17.819 mg, and 24.677 mg to achieve 0.5 %, 1.0 %, 2.0 %, 3 %, 6.5 % and 9.0 % (w/w) FITC@SiNP; respectively. The calculation of w/w % was based on the amount of FITC-APTES to TEOS added to the microemulsion. The third step involves the covalent conjugation of polyethyleneimine (PEI) to the FITC@SiNP. The FITC@SiNP (20.0 mg) was suspended in dry ethanoic APTES solution (10.0 ml, 2.0 % v/v) and stirred at room temperature for 24 hours. The resultant FITC@SiNP-APTES was purified by repeated washing and centrifugation with ethanol and PBS. The FITC@SiNP-APTES was dispersed in PBS (10.0 ml, pH 7.4). The FITC@SiNP-APTES (1 ml, 2.0 mg.ml⁻¹) was reacted in PBS solution (49.0 ml, pH 7.4) containing glutaraldehyde (5.0 % v/v). The mixture was stirred for 1 hour at room temperature. A high molar ratio of glutaraldehyde (GA) was used to ensure only one aldehyde of GA reacts

with the amine groups on the nanoparticles. The resulting glutaraldehyde modified FITC@SiNP-APTES was washed with PBS five times to remove unreacted glutaraldehyde. Polyethyleneimine (PEI) solution (5.0 % w/v) in PBS (pH 7.4) was added to the glutaraldehyde modified FITC@SiNP-APTES and stirred for 12 hours at 4 °C. The FITC@SiNP-APTES-PEI nanoparticles were collected by centrifuge and purified by repeated washing and sonication in PBS.

2.3.2. Synthesis of Fe₃O₄@SiNP-Au@Pd

2.3.2.1. Synthesis of Fe₃O₄ nanoparticle

Monodispersed magnetite nanoparticles were prepared by the thermal decomposition of the iron-oleate precursor method previously reported in [267] with modifications. Briefly, FeCl₃.2H₂O (5.40 g, 20.0 mmol) was dissolved in water (40.0 ml), sodium oleate (20.0 g, 62.0 mmol), ethanol (50.0 ml), and n-hexane (80.0 ml). The mixture was heated at 70°C for 4 hours. The resulting solution was separated using a separating funnel, and the red organic layer was washed with deionized water (40.0 ml) three times and dried at 80 °C for 12 hours to yield Fe(oleate)₃. The Fe(oleate)₃ was obtained as a reddish-brown sticky solid. The Fe(oleate)₃ (12 g, 13.3 mmol) was dissolved in oleic acid (8.0 ml) and 1-octadecene (70.0 ml) and degassed with N₂ for 30 min at room temperature. The temperature was raised stepwise at 5 °C.min⁻¹ up to 320 °C. Then, the reaction was maintained at 320 °C for 1 hour under a slow stream of nitrogen gas. The solution was cooled to room temperature, and ethanol (250.0 ml) was added to precipitate the Fe₃O₄ nanoparticle. The precipitate was collected by magnetic separation, re-dispersed in hexane and precipitated with ethanol. This process was repeated several times to clean the nanoparticles. The oleic acid capped-Fe₃O₄ was dispersed in cyclohexane at 2.0 mg.ml⁻¹.

2.3.2.2. Formation of silica shell on Fe₃O₄ NPs (Fe₃O₄@SiNP-APTES)

The Fe₃O₄@SiNP core-shell nanoparticles were prepared using the water-in-oil microemulsion method. Triton X-100 (8.9 ml), oleic acid capped-Fe₃O₄ (2.0 mg.ml⁻¹, 3.8 ml), cyclohexane (31.3 ml), H₂O (2.0 ml) and hexanol (3.5 ml) were stirred at 600 rpm for 45 minutes to obtain a transparent golden-yellow solution. NH₄OH (400.0 µl, 25 %) and TEOS (50.0 µl, 0.23 mmol) were added to the mixture and stirred at 400 rpm for 6 hours. Additional TEOS (20.0 µl, 0.09 mmol) was added after every two hours for six consecutive times to grow a silica shell. After the last TEOS addition, the reaction mixture was stirred for 12 hours at room temperature to yield Fe₃O₄@SiNPs. The Fe₃O₄@SiNPs were isolated by adjusting the pH to 2 with cycles of cooling in liquid nitrogen during pH adjustment. Acetone (20.0 ml) was added to break the microemulsion. The Fe₃O₄@SiNPs was washed with a mixture of water and ethanol (1:3 v/v) and collected by magnetic separation. The Fe₃O₄@SiNP were dried at 50 °C in the oven overnight. The Fe₃O₄@SiNPs (40.0 mg) was dissolved in a 50.0 ml mixture of toluene/ethanol (9:1). APTES (500.0 µl) was added and stirred at room temperature for 12 hours to yield Fe₃O₄@SiNP-APTES. The Fe₃O₄@SiNP-APTES were purified by repeated washing with ethanol and then water five times. The Fe₃O₄@SiNP-APTES was isolated by magnetic separation during the washing. The purified Fe₃O₄@SiNP-APTES were dispersed in ethanol (2.0 mg.ml⁻¹).

2.3.2.3. Synthesis of Au seed solution

Small size Gold nanoparticles (Au NPs) were synthesized by the borohydride reduction method. In a typical reaction, HAuCl₄ (4.5 mg, 0.14 mM) and trisodium citrate (1.5 mg, 0.07 mM) was dissolved in water (100.0 ml) and cooled in an ice bath for 30 minutes under a slow stream of nitrogen gas. NaBH₄ (4.5 mg, 0.12 mM) was added quickly to the HAuCl₄ solution and stirred at room temperature for 30 minutes. The solution turned from yellow to ruby-red due to the formation of gold nanoparticles. If the reaction yield of 100 % was achieved, the

number of Au NPs seed was calculated according to **Equation 2.1** [268]. The number of Au NPs particles was estimated to be 4.976×10^{16} particles per milliliter which were converted to $0.83\mu\text{M}$.

$$N_{\text{seed}} = \frac{[\text{Au}] \times M \times 6.02 \times 10^{21}}{\rho \pi D^3} \quad (2.1)$$

where ρ is the density of Au (19.3 g.cm^{-3}), D is the TEM diameter ($3.8 \pm 2.1 \text{ nm}$), $[\text{Au}]$ is the initial concentration of the HAuCl_4 and M is the atomic weight of Au (197.0 g.mol^{-1}).

2.3.2.4. Synthesis of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$

The Au NP seed ($0.83\mu\text{M}$, 45.0 ml) and $\text{Fe}_3\text{O}_4@\text{SiNPs-APTES}$ (4.0 ml , 10.0 mg.ml^{-1}) were stirred for 30 min to form the reddish-brown $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ was separated with a magnet. The collected $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ was washed three times with deionized water to remove undeposited Au NPs. The purified $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ was redispersed in water (2.0 mg.ml^{-1}). Palladium was deposited on the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ by NaBH_4 reduction. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ (19 ml , 2.0 mg.ml^{-1}) was added into a flask under vigorous stirring at room temperature. H_2PdCl_4 (1.0 ml , 10.0 mM) was added and stirred for 45 minutes. Afterward, an ice-cold NaBH_4 solution (0.1 M , 1.0 ml) was added in drops and allowed to react for an extra 30 minutes. The color of the reaction mixture turned from reddish-brown to black indicating the growth of the palladium shell on the Au core to yield $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The resulting nanoparticles were collected by magnetic separation, washed five times with water and dried at 80°C in a vacuum oven. Different volumes (0.2 ml , 0.4 ml , 0.6 ml , or 1.0 ml) of H_2PdCl_4 (10.0 mM) was added to different batches to obtain $\text{Fe}_3\text{O}_4@\text{SiNP-Au@Pd}$ with different palladium concentrations. These were referred to as $\text{Fe}_3\text{O}_4@\text{SiNP-Au}_1@\text{Pd}_1$, $\text{Fe}_3\text{O}_4@\text{SiNP-Au}_1@\text{Pd}_2$, $\text{Fe}_3\text{O}_4@\text{SiNP-Au}_1@\text{Pd}_3$, and $\text{Fe}_3\text{O}_4@\text{SiNP-Au}_1@\text{Pd}_5$ for 0.2 ml , 0.4 ml , 0.6 ml , or 1.0 ml ; respectively.

2.4. Conjugation of anti-IgG antibody onto amine-functionalized nanoparticles

Four different approaches; electrostatic adsorption, glutaraldehyde crosslinking, reductive amination and Protein G oriented immobilization of anti-IgG antibody to $\text{Fe}_3\text{O}_4@\text{SiNP}$ - $\text{Au}_1@\text{Pd}_3$ were evaluated.

2.4.1. Electrostatic adsorption, $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ -anti-IgG_(ads).

The electrostatic adsorption of anti-IgG to the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ was carried out as follows: The nanoparticles (2.0 mg.ml^{-1}) dispersed in PBS buffer (pH 7.2), and the pH was adjusted to 9.5 with K_2CO_3 before the addition of anti-IgG ($100 \text{ }\mu\text{l}$, 1.0 mg.ml^{-1}) and incubated at $4 \text{ }^\circ\text{C}$ with gentle shaking overnight. The $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ -anti-IgG conjugate was purified using magnetic separation. To block non-specific binding sites, the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ -anti-IgG was resuspended in the HBB blocking buffer pH 7.4 containing BSA (2 % w/v) and kept at $37 \text{ }^\circ\text{C}$ for 4 hours. The purification was with magnetic separation and washing with water several times. The final conjugate was suspended in PBS buffer (0.1 %), NaN_3 (0.01 mM) as a preservative and kept at $4 \text{ }^\circ\text{C}$. The conjugate was represented as $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ -anti-IgG_(ads).

2.4.2. Conjugation by periodate method. $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ -anti-IgG_(PI) or FITC@SiNP-APTES-PEI-anti-IgG_(PI).

This method was used for the conjugation of anti-IgG to FITC@SiNP-APTES-PEI and $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES- $\text{Au}@\text{Pd}$ nanoparticles. The carbohydrate moiety on the Fc-portion of the anti-mouse-IgG antibody (anti-IgG) was oxidized to aldehyde following the well-known periodate oxidation protocol [269,270]. Briefly, anti-IgG ($100.0 \text{ }\mu\text{l}$, 1.0 mg.ml^{-1}) was dissolved in sodium acetate buffer (pH 5.5, $400.0 \text{ }\mu\text{l}$) in a dark oxidation tube, NaIO_4 solution (20.0 mM, $500.0 \text{ }\mu\text{l}$) was added and incubated for 30 min in the dark at room temperature. The carbohydrate moiety of the antibody was converted to aldehyde groups as shown in **Scheme**

1.5. The oxidation was stopped by adding glycerol (0.1:1 v/v in PBS buffer, 1.0 ml). The periodate treated anti-IgG antibody solution was transferred into centrifugal filters (Ultra-15 Amicon, 10 kDa MWCO) and was centrifuged to remove excess periodate and glycerol. The aldehyde-anti-IgG antibody was exchanged into PBS (pH 7.4) buffer and finally concentrated to 100.0 μ l using a centrifugal filter (Amicon, 3 kDa MWCO). The aldehyde-anti-IgG (100.0 μ l, 1.0 mg.ml⁻¹) was added to a dispersion of the FITC@SiNP-APTES-PEI or Fe₃O₄@SiNP-APTES-Au@Pd (2.0 mg.ml⁻¹) and stirred for 12 hours at 4 °C. Then sodium cyanoborohydride (40.0 mM, 50.0 μ l) was added and kept at 4 °C overnight to reduce the Schiff base formed. The antibody-conjugated nanoparticles were washed three times and suspended in the HBB blocking buffer pH 7.4 and stirred at 37 °C for 4 hours to block the non-specific binding sites. The nanoparticle-IgG conjugate was purified and suspended in PBS buffer pH 7.4 containing BSA (0.1 %), NaN₃ (0.01 mM) as a preservative and kept at 4 °C. The anti-IgG conjugated nanoparticles were referred to as FITC@SiNP-APTES-PEI-anti-IgG or Fe₃O₄@SiNP-APTES-Au@Pd-anti-IgG_(PI).

2.4.3. Glutaraldehyde cross-linking of anti-IgG antibody

The Fe₃O₄@SiNP-APTES-Au@Pd (2.0 mg.ml⁻¹) was treated with glutaraldehyde, GA (2.5 %) at room temperature for 1 hour. After reacting with GA, the Fe₃O₄@SiNP-APTES-Au@Pd was washed with PBS buffer (pH 7.4) three times to remove excess GA. Then anti-IgG or protein G (100.0 μ l, 1.0 mg.ml⁻¹) was added to the purified GA modified Fe₃O₄@SiNP-APTES-Au@Pd solution and stirred at 4 °C for 12 hrs. The Fe₃O₄@SiNP-APTES-Au@Pd-ProG was purified by magnetic separation to remove excess biomolecules. The non-specific binding site was incubated in the HBB blocking buffer for 4 hours. The anti-IgG (50.0 μ l, 1.0 mg.ml⁻¹) was added to the Fe₃O₄@SiNP-APTES-Au@Pd-ProG and incubated at 4 °C for 4 hours to yield Fe₃O₄@SiNP-APTES-Au@Pd-ProG-anti-IgG conjugate. Two conjugates were obtained following this method that is Fe₃O₄@SiNP-APTES-Au@Pd-ProG-anti-IgG and

$\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd-anti-IgG}_{(\text{GA})}$. The bioconjugates were suspended in PBS buffer pH 7.4 containing BSA (0.1 %), NaN_3 (0.01 mM) as a preservative and kept at 4 °C.

2.4.4. Quantification of anti-IgG conjugation

A supernatant assay was used to quantify the amount of anti-IgG antibody conjugated to the nanoparticles. After coupling the anti-IgG antibody to the nanoparticles, the unbound (excess) anti-IgG was quantified using the Bichinonic acid (BCA) colorimetry protein assay. The BCA solution was prepared in sodium bicarbonate buffer (pH 9.6) containing sodium hydroxide (0.14g, 0.1 mol) and sodium tartrate. Copper (II) sulfate solution was prepared and mixed with 50 parts of the BCA solution. The stock anti-IgG solution prepared in the conjugation buffer and the conjugation buffer eluents (40.0 μl) was added into the well of a microplate. The BCA/copper sulfate solution (160.0 μl) was added to each well and incubated for 60 mins at 37 °C for color development. The absorption was measured. Each sample was analyzed in triplicate, and the average absorption intensity was obtained. The average intensity of the supernatant solution from the conjugation reaction was subtracted from the intensity of the total amount of anti-IgG used for the reaction to acquire indirect information on the amount of anti-IgG conjugated. The concentrations of the anti-IgG were extrapolated from the BCA calibration plot of known concentrations of anti-IgG. The amount of the anti-IgG per milligram of the nanoparticles was calculated using **Equation (2.2)**. The percentage conjugation efficiency (% E) was calculated using **Equation (2.3)**.

$$\frac{[\text{anti-IgG}](\mu\text{g})}{\text{mg of nanoparticles}} = \frac{[\text{anti-IgG}]_0 - [\text{anti-IgG}]_f}{\text{weight of nanoparticle}} \quad (2.2)$$

$$\% \text{ E} = \frac{[\text{anti-IgG}]_0 - [\text{anti-IgG}]_f}{[\text{anti-IgG}]_0} \times 100 \quad (2.3)$$

2.5. Fabrication of anti-P53ab immunobiosensors

2.5.1. Immobilization of P53-antigen (P53ag) onto the gold (Au) electrode (Scheme 3.1)

The pre-modification of the gold electrode with phenylethylamine group (PEA) was through electrochemical grafting of 2-aminoethyl benzene diazonium (AEBD) salts. The synthesis of AEBD was achieved following procedures previously reported in [265,271]. The NMR and FTIR spectra of the synthesized AEBD salt are shown in the supplementary data as **Figure S2.1 and Figure S2.2**; respectively. The FTIR spectrum of AEBD showed the presence of stretching vibrations at 3270 cm^{-1} (N-H), 3112 cm^{-1} (C-H) and 2286 cm^{-1} ($\text{N}\equiv\text{N}$). The presence of the $\text{N}\equiv\text{N}$ stretching vibrations indicates the successful conversion of the arylamino ($-\text{NH}_2$) groups to diazonium ($-\text{N}_2^+$) groups. The ^1H and ^{13}C NMR showed the expected number of protons and carbons indicating the purity and the successful synthesis of the AEBD.

The polycrystalline gold (Au) surfaces were cleaned before modification. This was accomplished by polishing sequentially with $1.0\text{ }\mu\text{m}$, $0.3\text{ }\mu\text{m}$ and $0.05\text{ }\mu\text{m}$ alumina slurry on micro cloth pads, rinsed with water and sonicated for 3 minutes after each stage of polishing. The polished electrodes were immersed in KOH (0.5 M)- H_2O_2 solution (1:1 v/v) for 5 min and subsequently washed with a copious amount of Milli-Q water. Finally, the electrodes were electrochemically cleaned by cycling between -0.2 and 1.5 V vs. (Ag|AgCl, 3 M KCl) at 100 mV.s^{-1} in 0.5 M sulphuric acid until reproducible scans of a cleaned gold electrode were obtained. The cleaned Au electrodes were further washed with water, rinsed with acetonitrile and dried under a continuous flow of nitrogen gas. The cleaned Au electrodes were immersed into an N_2 saturated acetonitrile solution containing 1.0 mM AEBD salt and 0.10 M TBABF₄ as supporting electrolyte. The cyclic voltammograms were recorded between 0.40 to -0.40 V at a scan rate of 100 mV.s^{-1} . This results in the generation of phenylethylamine (PEA) radical. The PEA radical was grafted onto the gold electrode to form a phenylethylamine layer represented as Au-PEA. The Au-PEA was rinsed with deionized water and acetonitrile to

remove physically adsorbed AEED residue. The Au-PEA electrodes were immersed in a succinic anhydride (5 %) solution in DMF for 4 hours to form a carboxylic acid layer represented as Au-PEA-SA. The Au-PEA-SA electrodes were washed with DMF and MES buffer (pH 5.5). The Au-PEA-SA electrode was activated with a mixture of EDC (0.40 M) and NHS (0.10 M) in MES buffer (100 mM, pH 5.5) for 1 hour. The activated electrodes were rinsed with acetate buffer (100 mM, pH 5.5) to remove unreacted EDC/NHS. Subsequently, the activated Au-PEA-SA-NHS electrodes were incubated with the P53ag solution (20.0 μ l, 50.0 μ g.ml⁻¹) in acetate buffer (10 mM, pH 6.5) for 12 hours at 4 °C. The Au-PEA-SA-P53ag was rinsed with PBST (pH 7.4) to remove any unbound P53ag. The Au-PEA-SA-P53ag immunoelectrode was incubated in BSA solution (20.0 μ l, 2.0 %) for 1 hour to block the unreacted succinimide group and the non-specific binding sites. The fabricated immunoelectrode is represented as Au-PEA-SA-P53ag/BSA and was washed with PBS and stored at 4 °C before use.

2.5.2. Immobilization of P53ag onto carboxyl magnetic beads (MBs), Scheme 4.3

The P53ag was covalently immobilized on carboxylic acid-functionalized magnetic beads (MBs) using the EDC/NHS activation and coupling procedure. MBs (200.0 μ l, 2.5 mg) was placed in an Eppendorf tube and washed five times with MES buffer (100 mM, pH 5.2) and magnetically collected. Subsequently, the MBs were suspended in MES buffer (100 mM, pH 5.2) containing EDC (0.40 M) and NHS (0.10 M), mixed on a rotating incubator (750 rpm) at room temperature for 45 minutes. The NHS activated magnetic beads are represented as MB-NHS. The MB-NHS bead was washed twice with MES buffer (pH 5.5, 500 μ l), collected by magnetic separation and resuspended in MES buffer (pH 5.5, 200.0 μ l). The P53ag (50.0 μ g.ml⁻¹) was added to the MB-NHS solution and incubated at 4 °C on a rotatory mixer (500 rpm) for 6 hours. The P53ag modified magnetic bead was referred to as MBs-P53ag. The MBs-P53ag were suspended in the HBB blocking buffer for 4 hours to block the unreacted activated groups

and non-specific binding sites. Following washing of the MBs-P53ag/BSA with PBST three times and then PBS two times. The MB-P53ag/BSA conjugates were separated with a magnet and suspended in PBS buffer (pH 7.4) containing BSA (0.10 %) and 0.095% sodium azide as preservative and kept at 4°C.

2.5.3. Immobilization of P53ag on 96 well microtiter plate, Scheme 4.2 and 6.1

The P53ag was coated onto polystyrene microwell plates. A black Nuc Maxisorp 96 microwell plate was used for the fluorescent detection in **Scheme 4.2**. A clear UltraCruz high binding 96 well plates were used for the colorimetric detection in **Scheme 6.1**. The two plates were modified following the same procedures. The 96 well polystyrene microtiter plates (PMTP) were coated with P53ag ($10.0 \mu\text{g}.\text{ml}^{-1}$, 100.0 μl) diluted in carbonate-bicarbonate buffer pH 9.2 by incubating at 4 °C for 12 hours. After washing the plate with the washing buffer (PBST), the non-specific binding sites on the plate were blocked with 200.0 μl of the HBB blocking buffer at room temperature for 1 hour. Afterward, the P53ag/BSA modified (PMTP-P53ag/BSA) wells were washed with PBST three times and with PBS two times. The PMTP-P53ag/BSA plates were used immediately for analysis.

2.5.4. Immobilization of P53ag on cellulose paper disc. (Scheme 6.2).

Whatman No 1 cellulose paper disk sensor, was produced using a standard paper puncher and treated with sodium periodate (0.5 M, 20.0 μl per disk) for 30 min to create reactive aldehyde groups on the paper. The paper disk was washed with distilled water and PBS buffer to remove unreacted periodate. The paper disk was placed on a piece of filter paper to absorb excess water and dried at room temperature. The periodate-modified paper was stuck onto a transparent plastic strip with the aid of double-sided tape to afford a paper disk sensor chip. Then, the P53ag (2.0 μl , $10.0 \mu\text{g}.\text{ml}^{-1}$) in PBS (pH 7.5) was spotted on the dialdehyde cellulose

paper and incubated at room temperature for 30 minutes. Then the excess P53ag was washed off with PBST buffer. Sodium cyanoborohydride, NaCNBH_3 (1.5 mg.ml^{-1} , $20.0 \text{ }\mu\text{l}$) was added to form a stable covalently immobilized P53ag. The HBB blocking buffer ($50.0 \text{ }\mu\text{l}$) containing skimmed milk (casein) was added and incubated for 10 minutes to block the non-specific binding sites. The P53ag modified paper was washed three times with PBST buffer ($100.0 \text{ }\mu\text{l}$), and twice with PBS ($100.0 \text{ }\mu\text{l}$) and dried at room temperature for 10 minutes. The P53ag /casein modified paper was stored at $4 \text{ }^\circ\text{C}$ before use.

2.6. Evaluation of the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-APTES-Au@Pd}$

The peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ was determined at $25 \text{ }^\circ\text{C}$. TMB (12.5 mM , $100.0 \text{ }\mu\text{l}$) and H_2O_2 (1.0 M , $200.0 \text{ }\mu\text{l}$) were added into acetic acid-sodium acetate (HAc-NaAc) buffer (0.2 M , 2.65 ml). $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ ($100.0 \text{ }\mu\text{g.ml}^{-1}$, $40.0 \text{ }\mu\text{l}$) was added to the mixture and incubated for 10 min before the absorption spectra were recorded. The pH-dependent activities were measured in reaction buffers (pH 1-10) at $26 \text{ }^\circ\text{C}$. Temperature-dependent activities were measured between $15\text{-}80 \text{ }^\circ\text{C}$. The kinetic experiments were conducted at fixed TMB concentration and varying the concentration of H_2O_2 and vice versa for TMB. The absorption spectra of the TMB oxidation products were recorded.

2.7. Procedure for immunometric assay of anti-P53ab

The P53ag/BSA functionalized surfaces (Au electrode, 96-well plate, cellulose paper, and magnetic nanoparticles) were washed three times with PBST and twice with PBS. Then, $50.0 \text{ }\mu\text{l}$ each of the serum ($10.0 \text{ }\%$) or PBS containing anti-P53ab (in a buffer or diluted serum) of different concentrations was added to the corresponding interfaces and incubated at $37 \text{ }^\circ\text{C}$ for 1 hour. Each concentration was analyzed in triplicate. The P53ag/BSA-anti-P53ab modified surfaces were washed three times with PBST and twice with PBS to remove uncaptured anti-P53ab. For the colorimetric or fluorescence-based detection system, the synthesized immuno-

bioprobe; FITC@SiNP-APTES-PEI-anti-IgG (fluorescence) or Fe_3O_4 @SiNP-APTES-Au@Pd-ProG-anti-IgG (colorimetric) (100.0 μl) was added to the P53ag/BSA-anti-P53 immunocomplex surface and incubated at 37 °C for 45 minutes. The unreacted immunobioprobe were washed three times with PBST buffer. For the colorimetric detection, the color was developed by the addition of the colorimetric substrate (100.0 μl) containing TMB (1.0 mM) and 100 mM H_2O_2 in acetate buffer, pH 5.0) and incubated at 37 °C for 5 minutes. The stop solution H_2SO_4 (100.0 μl , 2.0 M) was added. The absorption was measured at 650 nm and 450 nm after the addition of H_2SO_4 . For the fluorescence detection, the dissolution buffer (10.0 mM NaOH) was added and incubated at 40 °C for 30 minutes. The fluorescence emission was acquired at 520 nm with an excitation of 485 nm.

RESULTS AND DISCUSSION

List of Publications

Section of this thesis that has been submitted and is in preparation for publication in peer review journals

1. **Omotayo K. Adeniyi.**, Philani N. Mashazi (2019). Stable thin films of human P53 antigen on the gold surface for the detection of tumor-associated anti-P53-autoantibodies. *Electrochimica Acta* (in Press). <https://doi.org/10.1016/j.electacta.2019.135272>.
2. **Omotayo K. Adeniyi.**, Mashazi, P.N. Highly sensitive fluorescence sensing of circulating tumor-associated autoantibodies using magnetic bead and fluorescent silica nanoparticles. *Optical Materials* (in preparation).
3. **Omotayo K. Adeniyi.**, Sicwetsha Simbogle, Mashazi, P.N. Nanomagnets-silica nanoparticles decorated with Au@Pd for enhanced peroxidase-like activity and colorimetric glucose sensing (Submitted for review). *ACS Applied Materials and Interfaces*.
4. **Omotayo K. Adeniyi.**, Sicwetasha Simbogle., Mashazi, P.N. (2019) Fe₃O₄@SiO₂-Au@Pd nanohybrid as enzyme-mimetics label for the ultrasensitive colorimetric detection of serological anti-P53-autoantibodies. *ACS Applied Materials and Interfaces* (to be submitted)
5. **Omotayo K. Adeniyi.**, Adesina A, Mashazi, P.N. Paper-based enzyme-free colorimetric detection of serological tumor-associated autoantibodies towards early lung cancer screening. *Sensor and Actuators B* (to be submitted)
6. **Omotayo K. Adeniyi.**, Adesina A., Mashazi, P.N. Immunobiosensor based approaches for lung cancer diagnosis: a review of biomarkers, transduction techniques, and signal amplification strategies. *Chemical Reviews* (in preparation).

Sections of this thesis have been presented at the following scientific conferences and symposiums.

1. **Omotayo K. Adeniyi**, Mashazi, P.N. Recent advances in immunobiosensor development for the early-stage detection of lung cancer biomarkers. (The 8th Interdisciplinary Postgraduate Conference (IPGC), Grahamstown, South Africa, 26th to 29th, September 2017; National symposium; Oral presentation).
2. **Omotayo K. Adeniyi**, Mashazi, P.N. Design and evaluation of electrochemical immunobiosensor for detection of tumor-associated autoantibodies (28th Anniversary of World Congress on Biosensors, Biosensor 2018, Miami, Florida, USA, 12th to 15th June 2018; International Conference; Poster Presentation)

Chapter 3:

Label-free impedimetric sensing anti-P53 autoantibodies

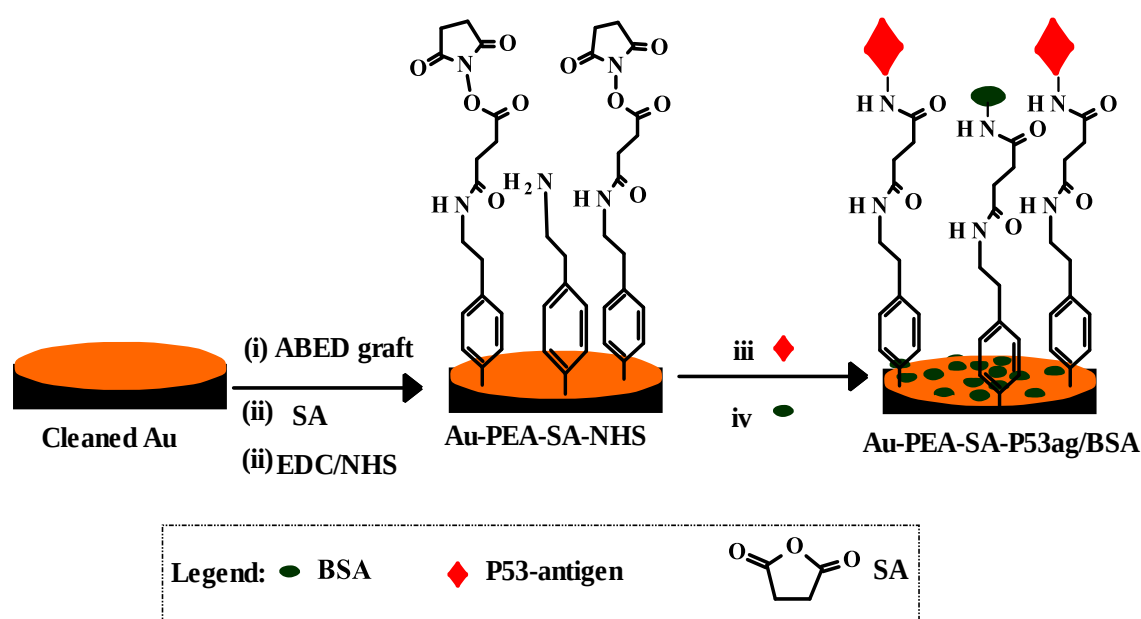
3. Label-free Impedimetric Sensing of anti-P53ab

The design of label-free immunobiosensor for anti-P53ab detection was investigated. The covalent attachment of the P53ag as a biorecognition element onto the gold electrode pre-grafted with phenylethylamine (PEA) monolayer was investigated. Spectroscopic and electrochemical methods were used for the characterization of the modified electrodes. Electrochemical impedance spectroscopy (EIS) as a label-free technique was used to study the interaction between the surfaces attached to P53ag and anti-P53ab in solution.

3.1. Covalent immobilization of P53-antigen onto the gold electrode

The chemistry of surface premodification for the immobilization of sensing elements is an essential factor. It influences the performance of biosensing interfaces as it affects the sensitivity, stability, and selectivity of immunobiosensors [272]. Finding the most suitable platform to give the stable organization of biorecognition elements leads to optimized binding efficiency, selectivity and reproducible signal output. The conventional method for the immobilization of sensing element (P53ag) onto the gold surface takes advantages of the physical adsorption and self-assembly of a tumor-associated antigen on alkyl thiolate monolayer (SAM) through the formation of S-Au and N-Au bond. However, physically adsorbed P53ag are not stable and are often washed off during the immunosensing process. Also, the bond energy of S-Au is $154.4 \text{ kJ.mol}^{-1}$ [273], and the S-Au bond is labile under conditions of high temperature [274], ultra-violet radiation [275], and applied electrical potential. In contrast, the covalent immobilization of P53ag on the gold electrode pre-functionalised with electrochemically grafted phenylethylamine monolayer results in the formation of a more environmentally and electrical stable C-Au bond which has a higher bond energy of 317 kJ.mol^{-1} [276].

Scheme 3.1 shows the step-by-step modification of the gold electrodes surface via electrochemical grafting method and immobilization of P53ag. The first step is the grafting of 4-(2-aminoethyl) benzene radical onto the gold electrode to form Au-PEA followed by carboxylation with succinic anhydride to form an Au-PEA-SA modified electrode. The fabrication of the Au-PEA-SA electrode involves the reaction of the ethyl-amine groups of the Au-PEA with succinic anhydride through a nucleophilic ring-opening reaction.



Scheme 3.1: Schematic representation of the different steps involved in the covalent immobilization of P53ag onto the gold electrode.

The activating effect of the carbonyl groups enables the succinic anhydride to react with the amine and forms a stable bond with the opening of the cyclic ring to afford a carboxylic acid functionalized surface (Au-PEA-SA). The chemical modification of amino-terminated organic film with succinic anhydride for immobilization of protein has previously been demonstrated on a silicon substrate [277,278]. The carboxylic acid on the Au-PEA-SA was activated with EDC/NHS to provide an N-hydroxysuccinimide (NHS) ester reactive surface, Au-PEA-SA-NHS. The NHS ester spontaneously reacts with the amine groups of P53ag to form Au-PEA-

SA-P53ag. The nonspecific binding sites on the gold electrode surface and the unreacted succinimide ester groups were blocked using BSA, and the fabricated immunosensor is referred to as Au-PEA-SA-P53ag/BSA. The details of the method are described in **section 2.6**.

3.2. Characterization of bare and modified gold electrodes

3.2.1 Electrochemical grafting of AEBD onto the gold electrode

Figure 3.1 shows the consecutive cyclic voltammograms for the electrochemical grafting of the AEBD onto the Au electrode. The first scan showed an irreversible reduction peak at 0.17 V vs. Ag|AgCl. The reduction peak corresponds to the electrochemical reduction of the AEBD through a one-electron transfer process resulting in the loss of nitrogen as N_2 and subsequent formation of phenylethylamine (PEA) radical. The peak due to the faradic process observed on the first scan disappeared on subsequent scans. This behavior could be attributed to the chemisorption of PEA radical onto the Au electrode surface. The chemisorbed PEA layer prevented the formation of the PEA radical and their chemisorption during the subsequent scans.

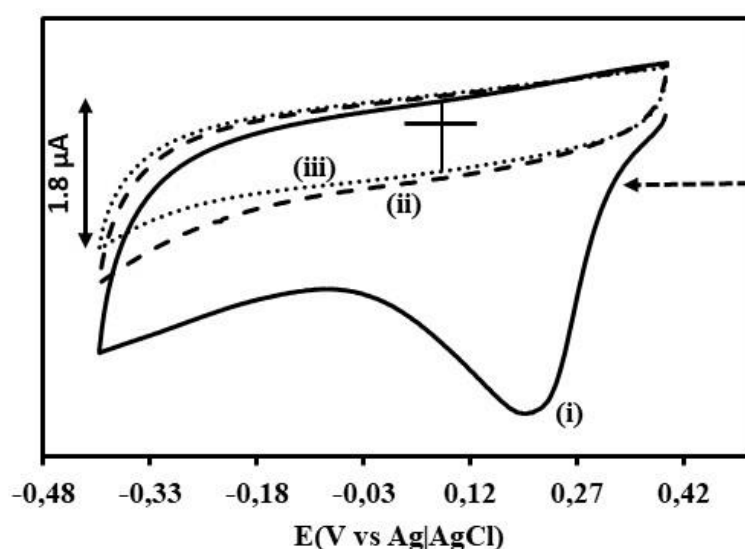


Figure 3.1: Cyclic voltammogram corresponding to the electrochemical grafting of 1.0 mM AEBD salts in ACN containing 0.10 M TBABF₄ at a scan rate of 0.10 V.s⁻¹.

Previous reports have shown that several factors influence the film compactness and surface concentration of the phenyl groups attached to the electrode surface via electrochemical grafting [279,280]. These factors include (i) the concentration of the aryldiazonium salt, (ii) the nature of the modification media, (iii) potential window, and (iv) the number of cyclic voltammetry (CV) cycles. The controlled formation of monolayer based on the number of cyclic voltammetry cycles has been reported [265]. Monolayer coverage has been achieved with a small number of CV cycles of less than five [281]. The number of cycles for the electrochemical grafting was optimized, and three CV cycles were used in this work to achieve efficient antigen immobilization.

The surface coverage of the PEA layer on the gold electrode can be estimated from the amount of the electrochemically reduced AEBD by integrating the charge (Q) under the voltammetric reduction peak. The theoretical AEBD surface coverage on the Au electrode was calculated according to **Equation 3.1** and was found to be $2.11 \times 10^{-9} \text{ mol.cm}^{-2}$.

$$\Gamma = \frac{Q}{nFA} \quad (3.1)$$

where Γ is the surface coverage (mol.cm^{-2}), Q is the charge obtained by integrating the area under the cathodic peak using origin software and was found to be $4.08 \times 10^{-6} \text{ C}$, n is the number of electrons transfer. The calculated surface coverage was of the same order of magnitude ($2.11 \times 10^{-9} \text{ mol.cm}^{-2}$) as that of a closely packed monolayer of phenyl groups on gold ($1.33 \times 10^{-9} \text{ mol.cm}^{-2}$) previously reported by Pinson et al., [282]. However, the theoretical surface coverage overestimates the actual surface coverage on the gold electrode because not all the electrochemically generated PEA radicals are grafted onto the Au electrode.

3.2.2. Cyclic voltammetry (CV) characterization of immunoelectrode

Electrochemical CV experiments were investigated to confirm the electrochemical grafting of the PEA layer and the immobilization of P53ag onto the pre-functionalized gold electrode surfaces. These include the blocking of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple, blocking of gold oxidation and reduction peaks, and inhibition of copper deposition on the gold electrode. The bare Au, Au-PEA, and the P53ag modified immunoelectrode were studied in 2 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl, 2.0 mM CuSO_4 in 0.5 M H_2SO_4 and 0.01 M KOH.

3.2.2.1. Copper deposition

Figure 3.2 (a), shows the cyclic voltammograms of the (i) bare Au, (ii) Au-PEA-SA and (iii) Au-PEA-SA-P53ag/BSA immunoelectrode in 2 mM CuSO_4 (pH 0.5). The electrochemical underpotential deposition (UPD) and over potential desorption (OPD) of copper (Cu) on a polycrystalline Au surface is a well-known process [283,284]. The method was adopted for the characterization of the fabrication of Au-PEA-SA-P53ag/BSA immunoelectrode. The observed CV features in **Figure 3.2 (a) (i)** were similar to those reported in the literature for the bare Au electrode [285]. The bare gold electrode showed two distinct cathodic peaks (**1 and 2**) and two anodic peaks (**3 and 4**), **Figure 3.2 (a)**. The presence of the cathodic peak originates from the UPD of Cu^{2+} on the surface of the bare Au electrode. The cathodic peak at 0.21 V was due to the formation of a regular single Cu^+ monolayer attributed to the $\text{Cu}^{2+}/\text{Cu}^+$ redox process. The peak at 0.090 V corresponds to the deposition of Cu^0 on the bare Au electrode [285]. The anodic peak at 0.060 V and 0.280 V corresponds to stripping of the Cu^0 and oxidation to Cu^+ which was followed by further oxidation of Cu^+ to form Cu^{2+} ; respectively. **Figure 3.2 (a)** shows that the characteristics Cu deposition and stripping peaks were not observed at the modified Au-PEA-SA electrode surface in **Figure 3.2 (a) (ii)** and after the immobilization of P53ag in **Figure 3.2 (a) (iii)**. Also, the magnitude of the current was lowered at the modified electrodes in comparison to bare Au. The PEA-SA and P53ag/BSA

passivate the electrode surface, thus preventing the deposition of Cu onto the gold electrode. This confirms the successful modification of the Au electrode with the PEA for Au-PEA and P53ag/BSA for Au-PEA-SA-P53ag/BSA.

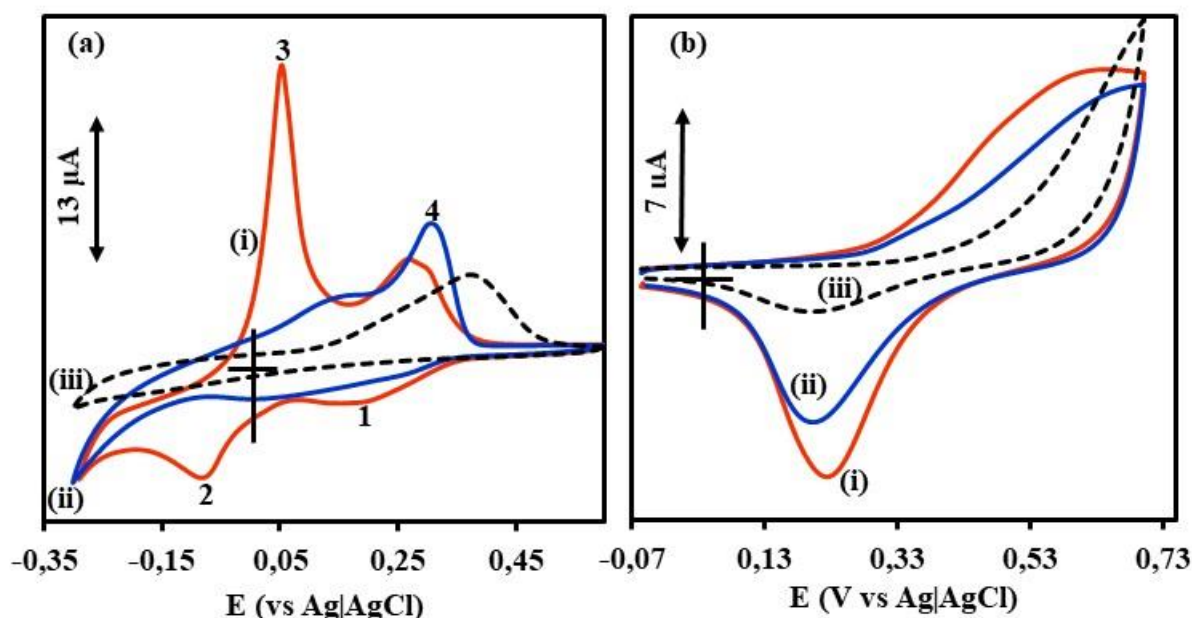


Figure 3.2: Cyclic voltammograms of (i) bare Au (ii) Au-PEA-SA (iii) Au-PEA-SA-P53ag/BSA modified immunoelectrode in (a) 2.0 mM CuSO₄ in 0.5 M H₂SO₄, scan rate 100 mV.s⁻¹ (b) 0.01 M KOH, scan rate 50 mV.s⁻¹.

3.2.2.2. Gold oxidation studies

The blocking of gold oxidation and reduction was also studied in an alkali solution. **Figure 3.2 (b)**, shows the cyclic voltammograms of the (i) bare Au electrode, (ii) Au-PEA-SA and (iii) Au-PEA-SA-P53ag immunoelectrode when cycled in 0.01 M potassium hydroxide (KOH) between -0.07 to 0.70 V vs Ag|AgCl. This characteristic of the gold reaction was used to confirm the various steps in the fabrication of the immunoelectrode. The bare gold electrode **Figure 3.2 (b) (i)**, exhibits well defined anodic and cathodic peaks associated with the gold oxidation and reduction process. The Au oxidation redox process was also observed on the modified Au-PEA-SA electrode surface in **Figure 3.2 (b) (ii)** but there was a decrease in

current density. The observation suggest that the Au-PEA-SA modified electrode is permeable to hydroxyl ions or hydroxyl ions react at pinholes resulting in the decrease in current density. **Figure 3.2 (b) (iii)** shows that the oxidation and reduction peaks were drastically reduced at the Au-PEA-SA-P53ag/BSA modified surface. The biomolecules created an insulating barrier between the underlying surface and solution. This confirms the successful immobilization of P53ag and blocking of non-specific binding sites with BSA forming Au-PEA-SA-P53ag/BSA immunoelectrode.

3.2.2.3. Ferri /Ferrocyanide redox couple studies

The electron transfer properties at the Au-PEA, Au-PEA-SA-NHS, Au-PEA-SA-P53ag, and Au-PEA-SA-P53ag/BSA surfaces were studies and compare using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple. The CVs of the bare Au and Au-PEA-SA were shown in the supplementary information, **Figure S3.1**. The CV of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple exhibited reversible behavior at the bare Au electrodes with a peak to peak separation (ΔE) of 78.0 ± 4 mV. The anodic to a cathodic peak current ratio (I_{pa}/I_{pc}) was approximately 1, **Figure S3.1 (a) (i)**. **Figure 3.3** shows the superimposed CVs of (i) Au-PEA (ii) Au-PEA-SA-NHS, (iii) Au-PEA-SA-P53ag, and (iii) Au-PEA-SA-P53ag/BSA in a mixture of (1:1) 2 mM $\text{K}_3\text{Fe}(\text{CN})_6$: $\text{K}_4\text{Fe}(\text{CN})_6$ solution containing 0.10 M KCl. After the electrochemical grafting of PEA monolayer onto the Au electrodes to form AU-PEA, the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple reversibility remained the same and an increase in the voltammetry peak current was observed. The electrografted PEA layer possessed conducting behavior towards the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple. The observed conducting behavior of the Au-PEA modified electrodes were attributed to the electrostatic attraction between positively charged ($-\text{NH}_3^+$) of the PEA layer at pH 7.4 and the negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions [265].

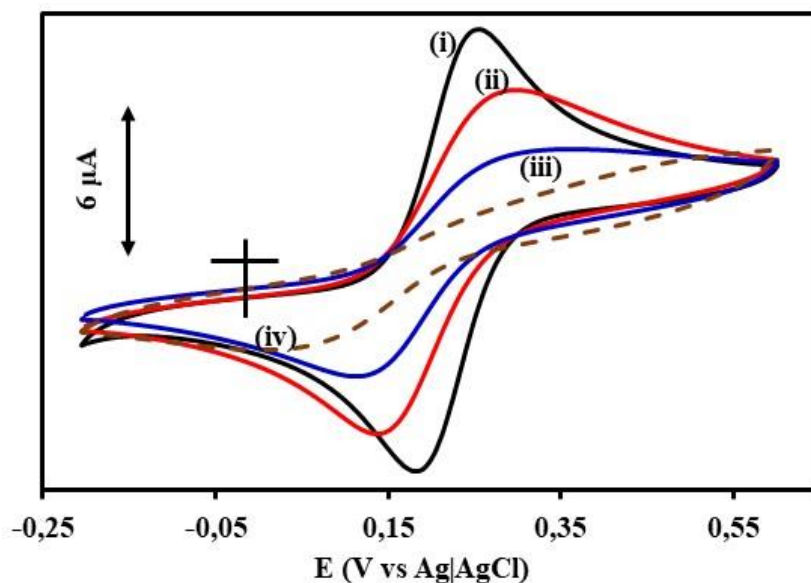


Figure 3.3: Cyclic voltammograms of (i) Au-PEA, (ii) Au-PEA-SA-NHS, (iii) Au-PEA-P53ag, and (iv) Au-PEA-SA-P53ag/BSA in 2.0 mM (1:1) $K_3/K_4Fe(CN)_6^{3-/4-}$.

After the carboxylation of the Au-PEA electrode with succinic anhydride, the CV of Au-PEA-SA in **Figure S3.1 (a) (ii)** shows that the $[Fe(CN)_6]^{3-/4-}$ redox process was inhibited. This was due to the formation of negatively charged carboxyl (COO^-) groups at pH 7.4. The negatively charged (COO^-) at the Au-PEA-SA electrode repels the diffusion of the $[Fe(CN)_6]^{3-/4-}$ ions and inhibits the redox process leading to an increase in ΔE from 78.0 ± 4.0 mV for Au-PEA to 490.0 ± 12.0 mV for Au-PEA-SA. This inhibition of the redox process is consistent with the previous reports on the electrochemical behavior of (COO^-) terminated films on the electrode surfaces [286,287]. After the activation of the Au-PEA-SA electrode with EDC/NHS, the $[Fe(CN)_6]^{3-/4-}$ redox process was restored at the Au-PEA-SA-NHS electrode surface. The ΔE decreases from 490.0 ± 12.0 mV for Au-PEA-SA to 144.0 ± 5.0 mV for Au-PEA-SA-NHS. The restoration of the redox process was attributed to the neutralization of the negatively charged (COO^-) film on the Au-PEA-SA-NHS electrode. The Au-PEA-SA-P53ag/BSA exhibited inhibition of $[Fe(CN)_6]^{3-/4-}$ redox process, **Figure 3.3 (a) (iv)**. The disappearance of the oxidation peak and shift of reduction peak to less negative potential leads to an increase in

ΔE . This was attributed to the insulating and bulky nature of the P53ag and BSA. The increase in the ΔE was accompanied by the reduction in peak current confirming the successful immobilization of P53ag and the blocking of the non-specific binding sites by BSA.

The interfacial electron transfer kinetics of the Au-PEA and Au-PEA-SA-P53ag/BSA immunoelectrodes was investigated by varying the scan rate from 10 to 200 mV.s^{-1} in the $\text{Fe}(\text{CN})_6^{3-/4-}$ redox probe. The cyclic voltammograms of (a) Au-PEA and (b) Au-PEA-SA-P53ag/BSA at different scan rate are shown in supplementary information, **Figure S3.2 (a)** and **(b)**. **Figure S3.2 (c)** for Au-PEA and **Figure S3.2 (d)** for Au-PEA-SA-P53ag/BSA shows the plot of cathodic (I_{Pc}) and anodic (I_{Pa}) peak current against the square root of scan rate (v). Both the I_{Pc} and I_{Pa} peak current increases linearly with the increasing square root of the scan rate confirming diffusion-controlled process [288]. The linear curve fittings follow the regression equations with the slope, intercept and correlation coefficient given in **Equations (3.2) – (3.5)**.

$$I_{\text{Pa(Au-PEA)}} = 0.55 v + 0.7, R^2 = 0.997 \quad (3.2)$$

$$I_{\text{Pc (Au-PEA)}} = - 0.52 v - 0.8, R^2 = 0.996 \quad (3.3)$$

$$I_{\text{Pa (Au-PEA-SA-P53ag/BSA)}} = 0.44 v + 0.2, R^2 = 0.994 \quad (3.4)$$

$$I_{\text{Pc (Au-PEA-SA-P53ag/BSA)}} = - 0.33 v - 0.1, R^2 = 0.992 \quad (3.5)$$

The diffusion coefficient (D) of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox species from the bulk electrolyte solution of the Au, Au-PEA, Au-PEA-SA-P53ag and Au-PEA-SA-P53ag/BSA immunoelectrode surface was calculated from the Randles-Sevick equation (**Equation 3.6**)

$$I_{\text{p}} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \quad (3.6)$$

where C is the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ concentration (2 mM), n is the number of electron transferred ($n = 1$), I_{p} (anodic and cathodic peak current). v is the scan rate (0.1 V.s^{-1}), D is the diffusion coefficient ($\text{cm}^2.\text{s}^{-1}$), A is the Au electrode geometric surface area (0.021 cm^2). The diffusion

coefficients of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ for Au, Au-PEA, Au-PEA-SA-P53ag and Au-PEA-SA-P53ag/BSA immunoelectrode has been obtained to be $8.41 \times 10^{-5} \text{ cm}^2.\text{s}^{-1}$, $8.85 \times 10^{-5} \text{ cm}^2.\text{s}^{-1}$, and $5.01 \times 10^{-5} \text{ cm}^2.\text{s}^{-1}$ and $4.52 \times 10^{-5} \text{ cm}^2.\text{s}^{-1}$; respectively. The higher diffusion coefficient value for Au-PEA indicates faster diffusion of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the Au-PEA electrode. In comparison to bare Au, the faster diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the Au-PEA electrode was due to the electrostatic attractive force by the positively charged surface (NH_3^+) and negative redox $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at pH 7.4. The diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ decreased for the Au-PEA-SA-P53ag and Au-PEA-SA-P53ag/BSA immunoelectrode. The insulating and bulky nature of the immobilised biomolecules blocked the diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions. This resulted in a decrease in the diffusion coefficient from $8.85 \times 10^{-5} \text{ cm}^2.\text{s}^{-1}$ for Au-PEA to 5.01×10^{-5} for Au-PEA-SA-P53ag and 4.52×10^{-5} for Au-PEA-SA-P53ag/BSA.

The surface concentration of the ionic species (C^*) for the Au-PEA ($4.21 \times 10^{-9} \text{ mol.cm}^2$) and Au-PEA-SA-P53ag/BSA ($1.63 \times 10^{-9} \text{ mol.cm}^2$) in the presence of PBS (pH 7.4) with the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ have been estimated from the CV plot using the Brown-Anson model as given in **Equation 3.7 [289]**.

$$I_p = \frac{n^2 F^2 C^* A v}{4RT} \quad (3.7)$$

The decrease in the surface concentration of ionic species from $4.21 \times 10^{-9} \text{ mol.cm}^2$ for Au-PEA to $1.63 \times 10^{-9} \text{ mol.cm}^2$ for Au-PEA-SA-P53ag/BSA confirmed successful immobilization of the insulating P53ag/BSA biomolecule layer on the electrode surface. Thus the decrease in currents at the Au-PEA-SA-P53ag/BSA is due to the immobilization of the biomolecules [290].

3.2.3. Electrochemical impedance spectroscopy (EIS) Characterization

Figure 3.4 shows the Nyquist plots for the (i) Au-PEA, (ii) Au-PEA-SA-NHS (iii) Au-PEA-SA-P53ag and (iv) Au-PEA-SA-P53ag/BSA. The experimental data was represented with the marker, and the fitted data was represented with a solid line. The Nyquist data representation

in **Figure 3.4** exhibited an increase in the diameter of the semicircle (R_{CT}) as the electrode modification occurs. The bare Au and Au-PEA exhibited almost the same Nyquist plot due to the permeability to the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions towards the underlying Au surface. The PEA forms a conducting layer on the Au surface. The Nyquist plot of the Au-PEA-SA modified surface is shown in Figure S3.1 (b). The Au-PEA-SA presents a negative (COO^-) surface charge at pH 7.4. This resulted in the electrostatic repulsion of the negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and an increase in the R_{CT} as shown in the Nyquist plot of the Au-PEA-SA in **Figure S3.1 (b) (ii)**.

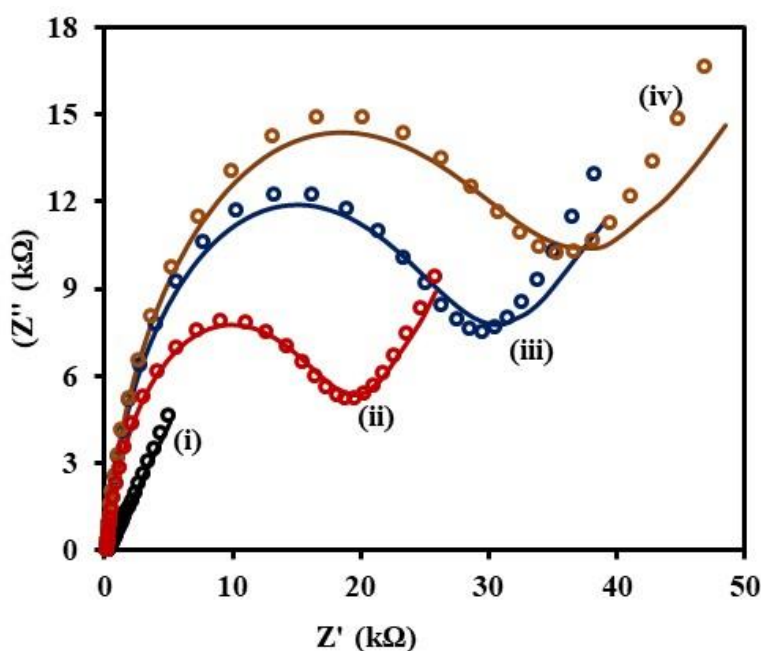


Figure 3.4: Nyquist plots of (i) Au-PEA, (ii) Au-PEA-NHS, (iii) Au-PEA-P53ag, and (iv) Au-PEA-SA-P53ag/BSA immunoelectrode in 2.0 mM (1:1) $\text{K}_3/\text{K}_4\text{Fe}(\text{CN})_6]^{3-/4-}$.

The negatively charged Au-PEA-SA electrode surface was neutralized after the activation of the carboxyl groups with EDC/NHS to form Au-PEA-SA-NHS. The Nyquist plot of Au-PEA-SA-NHS in **Figure 3.4 (ii)** shows that the electron transfer process was restored and a decrease in the diameter of the Nyquist plot (R_{CT}) in comparison to Au-PEA-SA was observed. Further modification with P53ag/BSA to form Au-PEA-SA-P53ag/BSA resulted in an insulating film that blocked the surface from interacting with the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe, **Figure 3.4 (iii)**

and (iv). The Nyquist plot was analyzed quantitatively by fitting with Randles-Sevick equivalent circuit. The solid lines in **Figure 3.4** show the fitted Nyquist data. The results were acceptable if the Chi-square values (χ^2) was less than 5%. The fitted equivalent circuit gave quantitative information of the following elements as circuits shown and discussed in **Chapter 1, Section 1.7.11**. **Table 3.1** shows the summary of the charge transfer resistance (R_{CT}), constant phase element (CPE), heterogeneous electron transfer constant (K_{ct}) and the time constant (τ) data obtained for the Au, Au-PEA, Au-PEA-SA-P53ag, and Au-PEA-SA-P53ag/BSA immunoelectrode. More interest was drawn towards R_{CT} and CPE circuit elements. The R_{CT} decreased from $0.65 \pm 0.06 \text{ k}\Omega$ to $0.39 \pm 0.10 \text{ k}\Omega$ due to the conducting PEA layer. At the Au-PEA-SA, the R_{CT} increased from $0.39 \pm 0.10 \text{ k}\Omega$ for Au-PEA to $54.3 \pm 2.31 \text{ k}\Omega$. The increase in R_{CT} was due to the slow electron transfer kinetics at the Au-PES-SA surface. At the Au-PEA-SA-NHS, the R_{CT} decreased from 54.3 ± 2.31 for Au-PEA-SA to $13.2 \pm 4.20 \text{ k}\Omega$. The decrease in R_{CT} was a result of the neutralization of COOH by NHS functional groups. The activated Au-PEA-SA-NHS spontaneously reacts with P53ag covalently leading to the formation of a stable amide bond. After the P53ag immobilization, the R_{CT} increased from $13.2 \pm 4.20 \text{ k}\Omega$ for Au-PEA-SA-NHS to $30.2 \pm 5.70 \text{ k}\Omega$ for Au-PEA-SA-P53ag. Also, the R_{CT} value increased to $38.2 \pm 7.2 \text{ k}\Omega$ after the blocking of the non-specific binding sites with BSA for Au-PEA-SA-P53ag/BSA. The increase in R_{CT} could be attributed to the insulating nature of the biomolecules. The observed changes in R_{CT} further confirmed the modification of the gold electrode surface. The value of the constant phase element (CPE) of the bare Au electrode increased from $6.23 \mu\text{F}\cdot\text{cm}^{-2}$ to $7.98 \mu\text{F}\cdot\text{cm}^{-2}$ for the Au-PEA modified electrode. The CPE of the Au-PEA-SA-P53ag and Au-PEA-SA-P53ag/BSA immunoelectrodes were found to be $1.36 \mu\text{F}\cdot\text{cm}^{-2}$, and $1.20 \mu\text{F}\cdot\text{cm}^{-2}$; respectively. The CPE values decreased after the immobilization of the P53ag and BSA onto the Au electrode due to the increased double-layer distance between the Au-PEA-SA-P63ag/BSA and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe [291].

Table 3.1: Evaluated EIS parameters of different modified electrode surfaces

Electrode	Rs (kΩ.cm ⁻²)	Rct (kΩ.cm ⁻²)	CPE (μF.cm ⁻²)	k _{CT} (cm.s ⁻¹)	(τ) μs
Au	0.12	0.65 ± 0.06	6.23	1.96 x 10 ⁻⁵	4.05
Au-PEA	0.13	0.39 ± 0.10	7.98	3.72 x 10 ⁻⁵	3.11
Au-PEA-SA-P53ag	0.13	30.2 ± 5.70	1.36	4.22 x 10 ⁻⁷	0.362
Au-PEA-SA-P53ag/ BSA	0.12	38.2 ± 7.20	1.20	3.34 x 10 ⁻⁷	0.520

The heterogeneous electron transfer constant was calculated from **Equation (3.8)** [292]. The estimated heterogeneous electron transfer rate constant (k_{ct}) for the bare Au, Au-PEA, Au-PEA-SA-P53ag and Au-PEA-SA-P53ag/BSA modified electrodes are presented in **Table 3.1**.

$$k_{ct} = \frac{RT}{n^2 F^2 A R_{CT} C} \quad (3.8)$$

The k_{ct} value of the bare Au electrode (1.96 x 10⁻⁵ cm.s⁻¹) increased to 3.27 x 10⁻⁵ cm.s⁻¹ after the formation of Au-PEA. The higher magnitude of k_{ct} for Au-PEA showed that faster electron transfer occurred at Au-PEA. After the immobilization of P53ag and BSA, the value of k_{ct} decreased to 4.22 x 10⁻⁷ cm.s⁻¹ and 3.34 x 10⁻⁷ cm.s⁻¹; respectively indicating a hindered electron transfer process. The slow electron transfer kinetics to the electrode surface is due to the insulating nature of the P53ag and BSA proteins. The inhibition of electron transfer generates low capacitance on the gold electrode surface by the P53ag and BSA. The time constant (τ) for each modified electrode was calculated using **Equation 3.9**, and the estimated τ values were presented in **Table 3.1**.

$$\tau = R_{CT}.C_{dl} = \frac{1}{2\pi f_{max}} \quad (3.9)$$

The τ for the diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the bare Au electrode was found to take 4.05 μs . After the electrochemical grafting of PEA onto the Au electrode, there was a significant decrease in the time constant to 3.11 μs compared to the bare Au electrode. The decrease in τ indicated faster diffusion of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to Au at the Au-PEA/electrolyte surface. The immobilization of P53ag and BSA proteins led to an increased time constant value to 3.62 μs and 5.20 μs ; respectively. This was due to the bulky and insulating properties of the P53ag and BSA. The results confirm the successful fabrication of the Au-PEA-SA-P53ag/BSA immunoelectrode.

3.3. Spectroscopic characterization

3.3.1. X-ray photoelectron spectroscopy (XPS) characterization

XPS analysis was performed to characterize the various steps in gold electrode surface modification as shown in **Scheme 3.1**. **Figure 3.5** shows the survey spectra of (i) bare Au, (ii) Au-PEA, and (iii) Au-PEA-SA-P53ag/BSA immunoelectrode surfaces. Several peaks were observed from the survey spectra. For bare Au in **Figure 3.5 (i)**, the peaks were at binding energies of 82 eV, 284 eV, 365 eV, 400 eV, and 531 eV corresponding to the elements on the gold surface. The peaks were for (Au 4f), carbon (C1s), silver (Ag 3f), nitrogen (N1s), and oxygen (O1s); respectively. The wide-scan spectrum of the cleaned bare Au surface reveals the presence of peaks due to gold as Au 4f and Au 4d. Other peaks due to silver (Ag 3d), carbons (C 1s) and oxygen (O 1s) exist at negligible concentrations. The occurrence of the oxygen and carbon peak on the bare Au was attributed to the exposure of the Au surface to air before measurement and cannot be avoided. This has been previously observed [293].

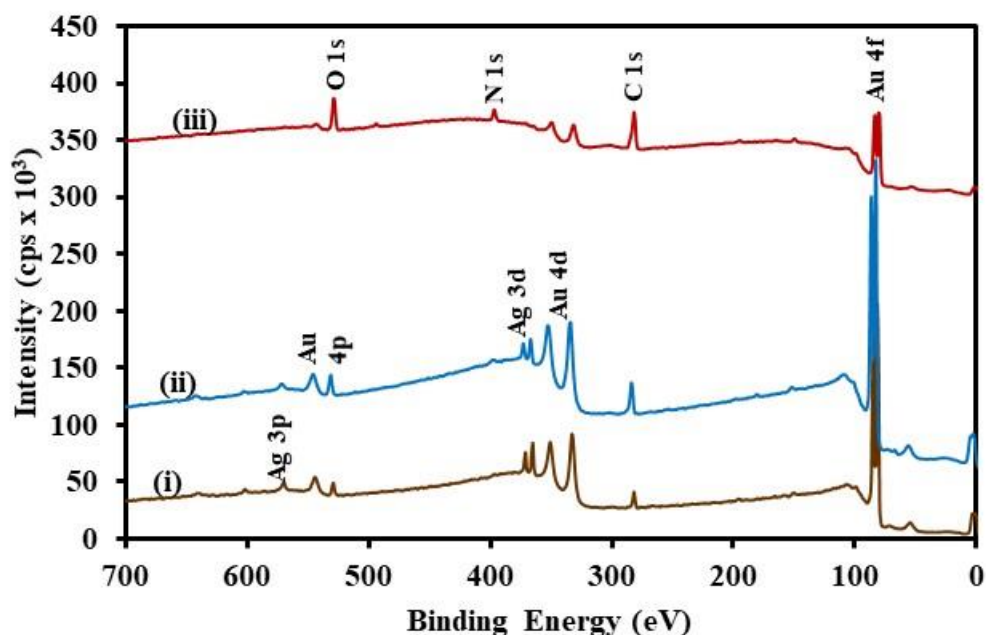


Figure 3.5: XPS survey spectra of (i) bare Au, (ii) Au-PEA, and (iii), Au-PEA-SA-P53ag/BSA.

After the formation of the Au-PEA, the XPS survey scan in **Figure 3.5 (ii)** showed the presence of the elements observed at the gold surface. There was a new N1s peak at 400 eV, and the carbon peak shows an increase in intensity. The nitrogen peak indicates that the PEA layer was grafted onto the bare Au surface. The Au peaks persisted after the PEA grafting, suggesting that a thin film of the PEA layer that allowed the penetration of soft X-ray to the underlying Au surface was formed. The oxygen and carbon increased in intensity. The Au-PEA-SA-P53ag/BSA in **Figure 3.5 (iii)** shows an increase in the intensity of nitrogen, carbon, and oxygen, suggesting that the P53ag is immobilized onto the surface. The peak intensity due to the underlying Au surface (Au 4f, Au 4d) decreases significantly after the P53ag/BSA immobilization. The fact that the Au signal of the Au-PEA-SA-P53ag/BSA is much weaker than the Au-PEA suggests that the immobilized P53ag attenuates the penetration of the X-ray to the underlying gold surface. The attenuation of the Au 4f signal due to PEA and PEA-SA-P53ag/BSA layer was used to provide an estimate of the layer thickness evolution using a

previously reported method [294]. The relative attenuation (I/I_0) of the Au 4f signal was expressed as shown in **Equation 3.10**.

$$\frac{I}{I_0} = e^{(-\frac{d}{\lambda \sin \theta})} \quad (3.10)$$

where I/I_0 is the ratio of Au 4f peak intensities of the modified and bare surfaces, d is the layer thickness, θ is the take-off angle (90°) and the mean free path of the substrate-specific organic layer ($\lambda = 4.12 \text{ mg.m}^2$). The λ is calculated from the Seah and Dench formula (the detailed calculation is provided in the Appendix). The thickness of the PEA layer was found to be approximately 1 nm. The thickness increases to 5.14 nm after the covalent immobilization of the P53ag/BSA. The increase in the surface thickness confirmed the successful modification of the Au surface with PEA and P53ag/BSA layers. The survey spectra were analyzed further to obtain quantitative information. **Table 3.2** shows the elemental composition of the bare and modified surfaces. The Au 4f was 37.1 % at bare Au. This value decreases to 30.5 % upon modifying Au with the PEA layer. This value decreased further to 6.9 % at the Au-PEA-SA-P53ag/BSA surface. The observed decrease in Au 4f atomic percentage (at %) followed a similar trend as that of Ag 3d which disappeared at the Au-PEA-SA-P53ag/BSA surface confirming the surface modification with P53/BSA. The relative atom percentage of C1s and N1s shows an increasing trend from 45.2 % < 54.5 % < 6.2 % for carbon (C 1s) and 0 % < 3.1 % < 9.9 % for nitrogen (N1s) at the bare Au < Au-PEA < Au-PEA-SA-P53ag/BSA surface, respectively. The increase in both the C1s and N1s is attributed to the immobilization of PEA and P53ag/BSA which contains carbon and nitrogen element. The O 1s atomic percentage showed a trend not similar to other elements. The O1s at the bare Au was 12.2 % and decreased to 2.7 % at the Au-PEA surface but further increased to 18.1 % at the Au-PEA-SA-P53ag/BSA surface. Another quantitative assessment of the surface was the atomic ratio of elements. The ratio of C1s: Au 4f and C1s: N1s were investigated. The ratio for C1s to Au 4f was 1.2 % for the bare Au and increased to 1.8 % for Au-PEA and 9.4 % for Au-PEA-SA-P53ag/BSA

surface. The increase confirms the modification of the gold electrode with PEA (Au-PEA) and SA-P53ag/BSA (Au-PEA-SA-P53ag/BSA) as shown in **Scheme 3.1**. The ratio of C1s to N1s also showed an increase of 0.05 % for Au-PEA and 0.16 % for Au-PEA-SA-P53ag/BSA.

Table 3.2: Atomic (%) composition determined by XPS for the modified Au electrode at subsequent stages of modification

Electrodes	Atomic composition (%)					Atomic ratio (%)	
	Au 4f	C 1s	N 1s	O 1s	Ag 3d	C1s/Au4f	C1s/N1s
Bare Au	37.1	45.2	-	12.2	5.7	1.2	-
Au-PEA	30.5	54.4	3.1	2.7	4.6	1.8	0.05
Au-PEA-SA-P53ag	6.9	65.1	9.9	18.1	-	9.4	0.16

3.3.1.2. High-resolution XPS spectra

The high-resolution XPS spectra for the nitrogen (N 1s) and carbon (C 1s) were evaluated. **Figure 3.6** shows the high-resolution C 1s spectra (left) and N1s spectra (right) of (a) Au-PEA (b) Au-PEA-SA and (c) Au-PEA-SA-P53ag/BSA. The data were normalized to the photon flux and scan number before background subtraction. The raw data were shown with a scattered plot and the fitted data with a straight line. After the modification of the Au with PEA, the deconvoluted C1s spectrum of the Au-PEA in **Figure 3.6 (a)** was fitted into three components at 284.9 eV (71.6 %), 286.1 eV (22.7 %) and 288.8 eV (5.7 %). The first component at 284.9 eV was assigned to the alkyl chain (C-C), the phenyl aromatic sp² carbon (C=C) and hydrocarbon C-H of the PEA layer.

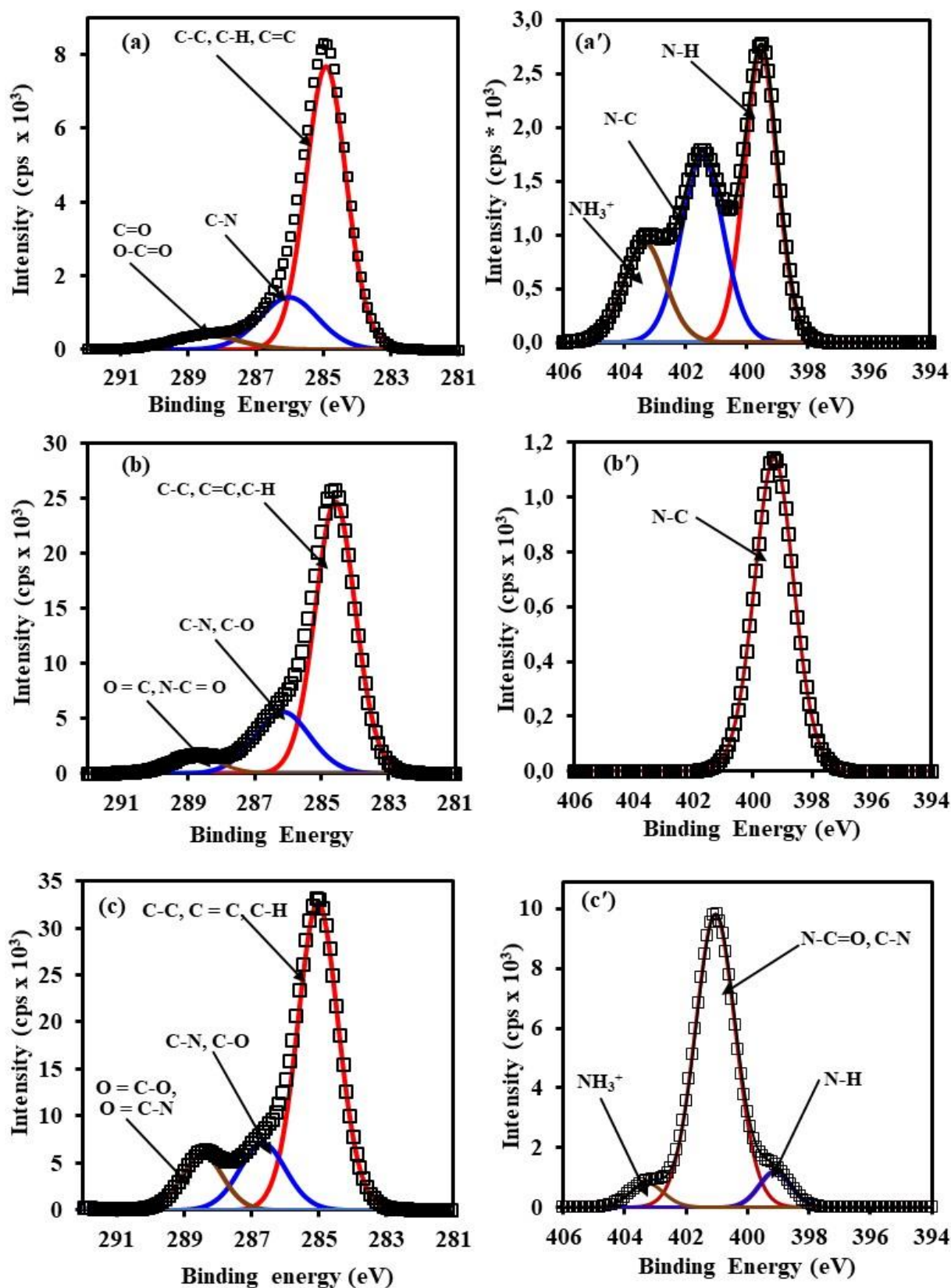


Figure 3.6: High-resolution C1s spectra (left) and N1s spectra (right) of (a) Au-PEA, (b) Au-PEA-SA, and (c) Au-PEA-SA-P53ag/BSA.

The occurrence of the Au-C bonds could not be established by XPS due to the low differences in the electronegativity between carbon ($\chi_{\text{C}} = 2.55$) and gold ($\chi_{\text{Au}} = 2.54$) atoms [294,295]. The component at 286.1 eV was assigned to the carbon bonded to the single bond of amine (C-N) groups. This confirms the successful grafting of the PEA onto the Au electrode and the presence of terminal amine groups at the Au-PEA surface. The high-resolution C1s spectrum of Au-PEA-SA in **Figure 3.6 (b)** was fitted into three components. **Figure 3.6 (b)** showed an increase in the intensity of the components at 284.9 eV (C-C, C-H) and 286.3 eV (C-N, C-O) when compared with the Au-PEA surface. The higher binding energy peak at 288.9 eV was attributed to the amide (N-C = O) and carboxylate (O-C = O) groups after amide coupling and acid functionalization. The presence of the amide bond and carboxylate groups results from the covalent interaction of the SA with the Au-PEA-amine groups and subsequent opening of the cyclic ring respectively. **Figure 3.6 (c)** shows changes in the surface carbon composition after the covalent immobilization of P53ag/BSA. The C1s spectrum of the Au-PEA-SA-P53ag/BSA surface were fitted into three components with binding energies at 285.5 eV (55.8 %), 286.8 eV (27.0 %), and 288.5 (17.3 %). The relative percentage of the C-C, C-H, C=C groups at the Au-PEA-SA (74.1 %) decreased to 17.3 % for Au-PEA-SA-P53ag/BSA. The relative percentages of C-N and HN-CO increased from 19.7 % and 6.5 % for Au-PEA-SA to 27.0 % and 55.8 % for Au-PEA-SA-P53ag/BSA; respectively. The increase in the C-N and HN-CO could be due to the abundant of proline, lysine. Leucine and serine α -amino and α -carboxylic groups present in the P53ag/BSA molecules.

For the N1s region (**Figure 3.6 (a') – (c')**), the deconvolution of the N 1s spectrum of the Au-PEA was fitted into three components at 399.1 eV, 401.1 eV, and 403.1 eV and attributed to the nitrogen bonded to primary amine ($-\text{NH}_2$), nitrogen bonded to carbon (C-N) and protonated amine groups (NH_3^+); respectively [296]. The Au-PEA-SA N 1s spectrum was fitted into one component at 399.9 eV attributed to C-N groups of the amide bond in the Au-PEA-SA. The

Au-PEA-SA-P53ag/BSA surfaces were fitted into three components at 399.1 eV, 401.1 eV, and 403.1 eV. The peak at 401.1 eV and 403.1 eV corresponds to the amide N-C and protonated amine groups respectively [271]. The nitrogen environment in the Au-PEA-SA-P53ag immunoelectrode surface was significantly different from the underlying Au-PEA-SA layer. The main component of the N1s peak of the Au-PEA-SA-P53ag/BSA immunoelectrode has the binding energy at 401.10 eV (85.39%) corresponding to the peptide bonds present on the P53ag/BSA. The N-H component (399.1 eV) could be attributed to the primary amine of the terminal lysine residue of P53ag/BSA. The small component at 403.1 eV could be assigned to protonated amine (NH_3^+) groups from the P53ag/BSA lysine side chain. The quantitative analysis and the corresponding peak positions and assignments are summarized in **Table 3.3**.

Table 3.3: Summary of components position, component percentage and the assignments at different at modified surfaces during immunoelectrode fabrication.

Electrode	Atom	Component Positions (eV)	Assignments	Component (%)
Au-PEA	N 1s	399.1	<u>N</u> -H	44.2
		401.1	<u>N</u> -C	36.4
		403.1	- <u>NH</u> ₃ ⁺	19.4
	C 1s	284.9	<u>C</u> -H / <u>C</u> -C / <u>C</u> =C	71.6
		286.1	<u>C</u> -N	22.7
		288.8	<u>C</u> =O	5.7
Au-PEA-SA	N 1s	399.1	N-C	100
	C 1s	284.9	<u>C</u> -H / <u>C</u> -C / <u>C</u> =C	74.1
		286.2	<u>C</u> -N / C-O	19.7
		288.7	N- <u>C</u> =O / O- C=O	6.5
Au-PEA-SA-P53ag/BSA	N 1s	399.1	<u>N</u> -H	8.5
		401.1	<u>N</u> -C = O	85.4
		403.1	<u>NH</u> ₃ ⁺	6.1
	C 1s	285.50	<u>C</u> -H / <u>C</u> -C / <u>C</u> =C	17.3
		286.30	<u>C</u> -N / C-O	27.0
		288.50	N- <u>C</u> =O / O-C=O	55.8

3.4. Optimization of the experimental variable

The different variables affecting the fabrication and analytical performance of the Au-PEA-SA-P53ag/BSA immunoelectrode were optimized. These were: (i) the number of voltammetric scans for grafting of PEA onto the Au electrode, (ii) the concentration of AEBD, (iii) amount of immobilized P53ag, and (iv) the concentration of BSA in the blocking buffer. The total change in the charge transfer resistance (ΔR_{CT}) after the reaction with anti-P53ab (500.0 ng.mL⁻¹) was the criterion used to assess the sensitivity ($\Delta R_{CT}/R_{CT0}$) of the Au-PEA-SA-P53ag/BSA immunoelectrode for each tested variable. **Figure 3.7** shows the optimization of Au-PEA-SA-P53ag/BSA immunoelectrode variables (a) the effect of electrografting voltammetric scans, (b) the effect of concentrations of immobilized P53ag, and (c) the effect of blocking buffer (BSA) concentration. In **Figure 3.7 (a)**, other experimental conditions were kept constant while the number of electrografting scans used for PEA grafting was varied from 1 to 5 scans. The baseline R_{CT} (blue bars) increases with an increasing number of scans. This showed that the amount of PEA molecule grafted to the Au surface increases. Hence, the amount of P53ag immobilized increases. The sensitivity of the different Au-PEA-P53ag/BSA electrodes fabricated using different scans for PEA grafting was investigated. The sensitivity is represented with the line plot (black line) in **Figure 3.7 (a)**. The sensitivity increase as the number of scans increased up to 3 scans. After which a significant decrease was recorded. The decrease in the sensitivity could be due to either the (i) formation of PEA multilayer at scans greater than 3, (ii) P53-antigen crowding resulting from the higher amount of immobilized P53ag and (iii) steric hindrance which prevents interaction of P53ag with anti-P53ab. Therefore, the AEBD solution of 1.0 mM, with 3 scans between 0.4 to -0.4 was chosen as the optimum AEBD electrografting condition.

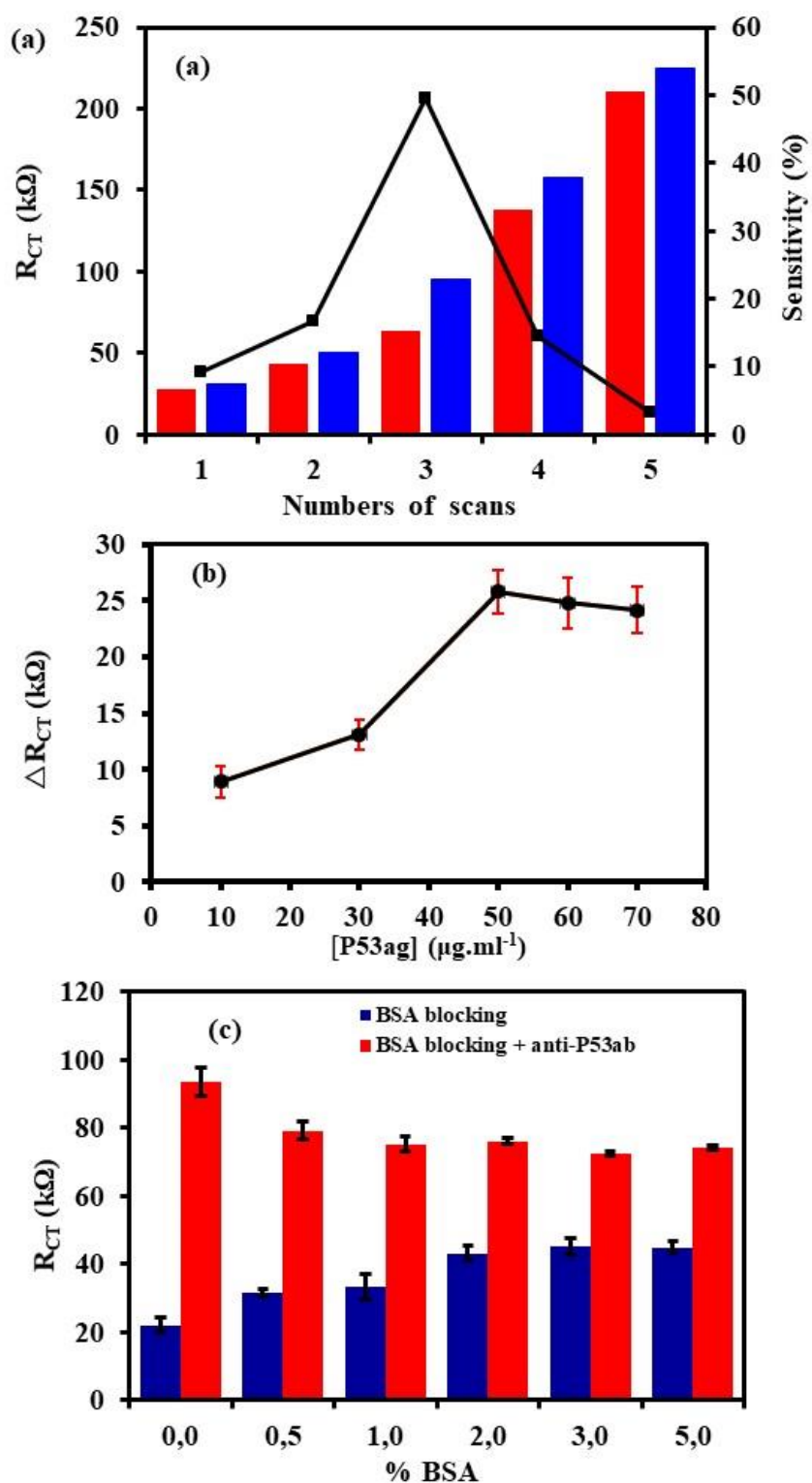


Figure 3.7: Optimization of Au-PEA-P53ag/BSA immunoelectrode variables (a) effect of electrografting voltammetric scans, (b) the effect of the amount of immobilized P53ag, and (c) effect of blocking (BSA) buffer concentrations on the analytical sensitivity.

The effect of the amount of immobilized P53ag on the immunoelectrode response was investigated. In **Figure 3.7 (b)** the change in R_{CT} (ΔR_{CT}) value increases with increasing concentrations of the immobilized P53ag to $50.0 \mu\text{g}.\text{ml}^{-1}$ and decreases afterward. The decrease in the immunoelectrode response at higher P53ag concentrations ($> 50.0 \mu\text{g}.\text{ml}^{-1}$) could be attributed to the saturation of P53ag which results in crowding and steric hindrance. This prevents the effective capture of the anti-P53ab. Therefore, $50.0 \mu\text{g}.\text{ml}^{-1}$ of P53ag was selected as the optimal concentration for the fabrication of Au-PEA-SA-P53ag/BSA immunoelectrode.

The blocking step was optimized to minimize non-specific binding on the immunobiosensor. Different percentage concentrations of BSA (0.0 %, 0.50 %, 1.0 %, 2.0 %, 3.0 %, and 5.0 %) in PBST buffer (pH 7.4, 0.01 % Tween-20) were tested as blocking buffer using a pre-established incubation time of 1 hour. The results are shown in **Figure 3.7 (c)**. The blue bars represent the unblocked immunobiosensor surface and 0 % BSA showed specific and non-specific binding of anti-P53ab onto the immunobiosensor. The baseline signal (R_{CT0}) increases with an increase in the percentage concentration of BSA from 0.0 % to 2.0 % and level off. BSA binds to the non-specific binding sites on the immunoelectrode and suppresses the non-specific binding of anti-P53ab. The optimum specific to non-specific sensor response was found for the 2.0 % BSA concentration. A phosphate-Tween 20 buffer solution (PBST, pH 7.4) containing 2.0 % BSA was selected as the optimum blocking buffer reagent for the Au-PEA-SA-P53ag/BSA immunoelectrode fabrication.

3.5. Impedimetric Detection of anti-P53ab

Anti-P53-antibody (anti-P53ab) has shown high specificity and affinitive binding to the immunodominant epitopes localized in the termini of the human cellular tumor protein P53 antigen [297]. The analytical sensitivity of the Au-PEA-SA-P53ag/BSA immunoelectrode was evaluated by testing against the known concentration of serially diluted anti-P53ab in PBS buffer (pH 7.4). A solution of 1.0 % BSA in PBS buffer (pH 7.4) was taken as a negative

control. The Au-PEA-SA-P53ag/BSA was incubated with the anti-P53ab solution and negative control for 1 hour. **Figure 3.8** shows (a) Nyquist and (b) corresponding Bode plots of the impedance spectra of the Au-PEA-SA-P53ag/BSA immunoelectrode after incubation with different concentrations of the anti-P53ab. The fittings values are presented in **Table 3.5**. The diameter of the semicircle in the Nyquist plot (R_{CT}) of the immunoelectrode increased upon incubation with the anti-P53ab due to the P53ag/P53ab immunocomplex formation. The P53ag/P53ab immunocomplex acts as a kinetic barrier to interfacial electron transfer at the immunoelectrode surface, leading to an increase in R_{CT} . A small noticeable decrease in the value of capacitance (Y_o) was observed with increasing concentrations of anti-P53ab (**Table 3.4**). The reduction in Y_o indicates a reduction in the capacitive behavior of the immunoelectrode due to the increasing coverage with anti-P53ab on subsequent concentration increase.

The corresponding Bode plot upon the immunoreactions at the Au-PEA-SA-P53ag/BSA surface is shown in **Figure 3.8 (b)**. In the high-frequency region ($f > 100$ Hz), the total impedance, $\log |Z|$, is independent of frequency and remains almost unchanged with a phase angle (ϕ) of nearly 0° . This indicates that the solution resistance (R_s) remains unchanged. In the intermediate frequency range (10 – 100 Hz), the $\log |Z|$ is almost a straight-line curve with a phase shift of $71^\circ < \phi < 90^\circ$ indicating pseudo-capacitive behavior of the immunoelectrode. The maximum changes in the impedance modulus lines upon immunoreaction with increasing concentrations of anti-P53ab were obtained in the low-frequency region (< 1 Hz). The phase angles (R_{CT} dominant character) moved towards a lower frequency on immunoreaction with increasing anti-P53ab concentrations. The change in R_{CT} is more prominent than the other circuit elements and confirms the choice of R_{CT} as an element for the sensing signal.

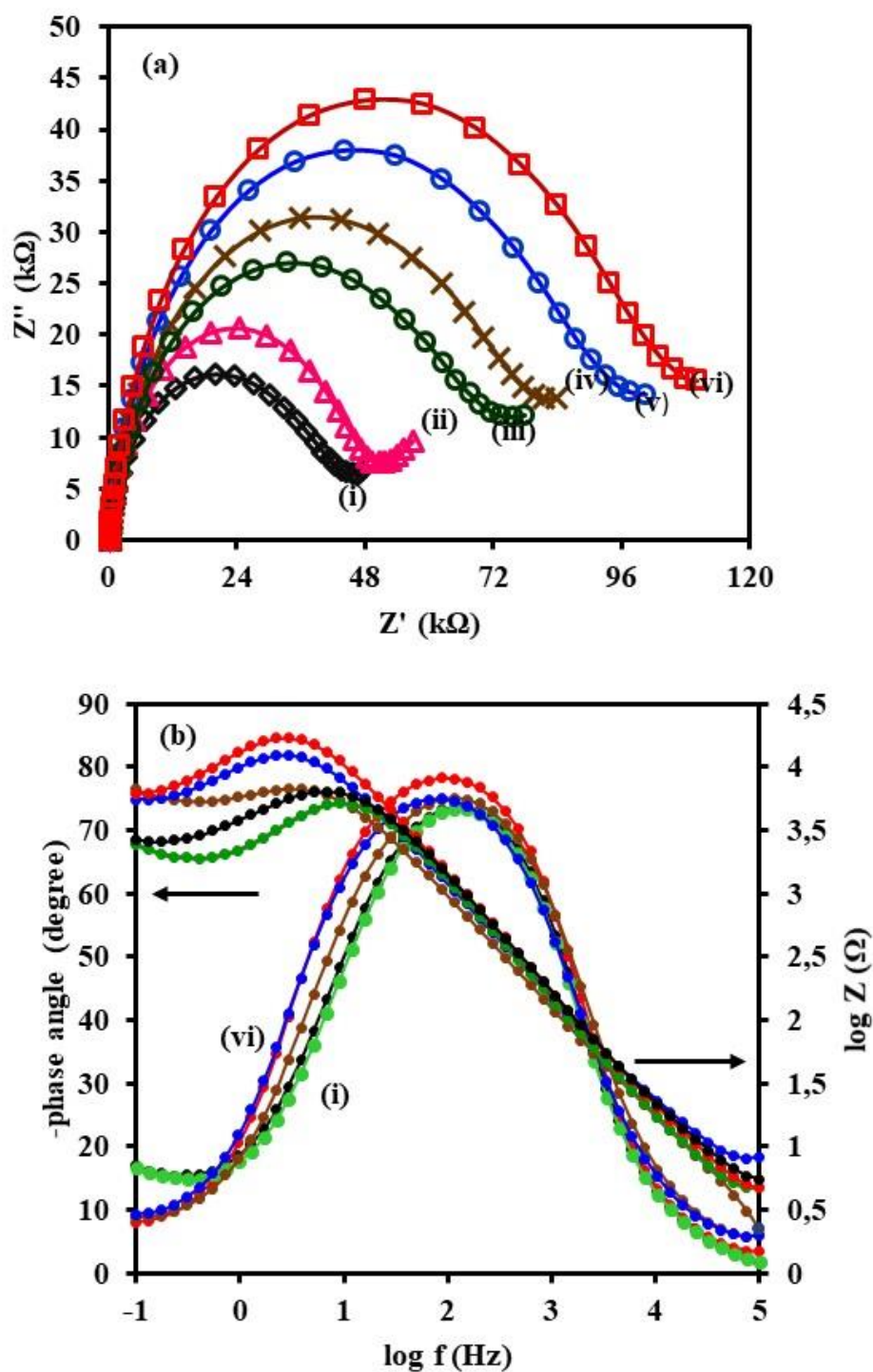


Figure 3.8: (a) Nyquist plot of Au-PEA-P53ag/BSA immunoelectrode after incubation with (i) negative control, (ii) 10 ng.ml^{-1} , (iii) 50 ng.ml^{-1} , (iv) 100 ng.ml^{-1} , (v) 200 ng.ml^{-1} , and (vi) 500 ng.ml^{-1} anti-P53ab.

Table 3.4: EIS characteristics parameters of the immunoelectrode on immunoreaction with different concentrations of anti-P53ab

Anti-P53ab (ng.ml ⁻¹)	R _{CT} (kΩ. cm ²)	Y _o (μF cm ⁻²)	n	Z _w (x 10 ⁻⁵) (Ω cm ²)	χ ² (x 10 ⁻²)	RSD (%) in ΔR _{CT} (n = 3)
Blank	32.3	0.984	0.902	5.39	2.2	-
10.0	41.2	0.887	0.909	5.37	1.9	2.43
50.0	56.8	0.871	0.875	5.88	3.6	5.53
100.0	64.2	0.814	0.897	6.21	1.5	6.50
200.0	74.1	0.819	0.894	5.72	1.1	5.70
500.0	85.5	0.724	0.929	6.82	2.2	5.75
1000.0	95.7	0.665	0.931	6.47	4.5	8.13

Figure 3.9 shows (a) the dose-response curve of R_{CT} vs. [anti-P53ab], and (b) the linear calibration plot of the normalized ΔR_{CT}/R_{CT} against the logarithm of the anti-P53ab concentrations. In **Figure 3.9 (a)**, the curve shows a near sigmoidal relationship between ΔR_{CT} and anti-P53ab concentrations. The ΔR_{CT} increases with increasing concentration of anti-P53ab (1.0 ng.ml⁻¹ to 1.0 μg.ml⁻¹). At higher anti-P53ab concentrations (>100.0 ng.ml⁻¹), a non-linear response was observed. This non-linear response was attributed to the saturation of the immunoelectrode surface due to the binding of all available P53ag to anti-P53ab. The measurement of relative changes in R_{CT} provides more significant information than the absolute ΔR_{CT} values for quantitative sensing applications [298]. The ΔR_{CT} was normalized with the R_{CT} value of the negative control sample (without anti-P53ab) to obtain the analytical calibration curve. **Equation 3.11** shows the equation for the normalization of the sensor signal.

$$\frac{\Delta R_{CT}}{R_{CT}(\text{blank})} = \frac{R_{CT}(\text{anti-P53ab}) - R_{CT}(\text{blank})}{R_{CT}(\text{blank})} \quad (3.11)$$

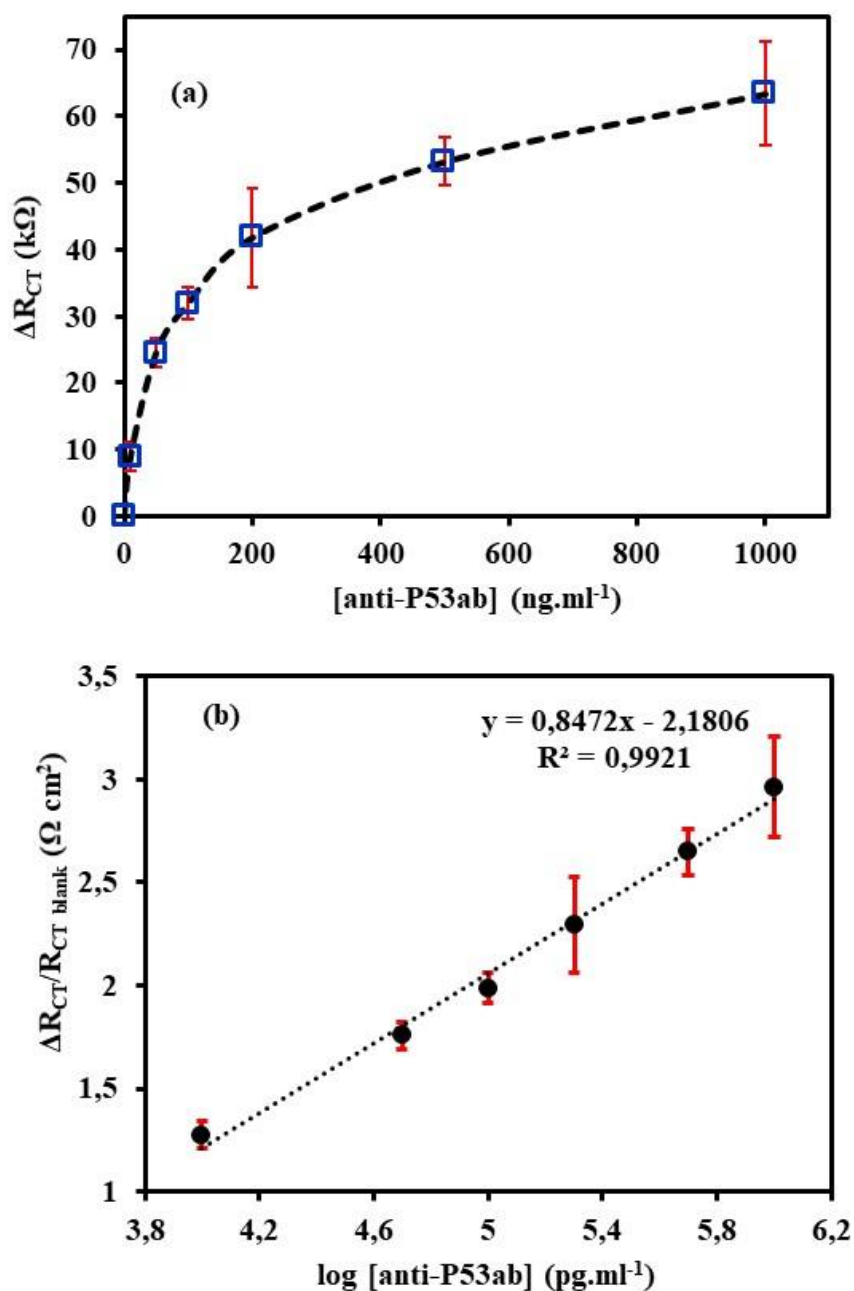


Figure 3.9: Concentrations dependent response of Au-PEA-SA-P53ag/BSA (a) dose-response curve of ΔR_{CT} vs. [anti-P53ab], and (b) calibration plot of the normalized sensor signal against logarithm of [anti-P53ab]. The error bar represents the standard deviation from three replicate experiments.

The calibration plot showed an excellent linear relationship between the normalized sensor response ($\Delta R_{CT} / R_{CT(\text{blank})}$) and the logarithm values of the anti-P53ab concentrations in the

broad range of 1.0 ng.ml⁻¹ to 1.0 µg.ml⁻¹. The linear regression equation from the calibration curve obeys **Equation 3.12**.

$$\left(\frac{\Delta R_{CT}}{R_{CT(blank)}}\right) = 0.847 \log [\text{anti-P53ab}] - 2.181, R^2 = 0.992 \quad (3.12)$$

The sensitivity of the fabricated Au-PEA-SA-P53ag/BSA immunoelectrode has been estimated from the slope of the calibration curve and found to be 0.847 Ω. cm⁻².ng⁻¹ of anti-P53ab. The LoD and LoQ of the immunoelectrode were found to be 0.103 ng.ml⁻¹ and 0.344 ng.ml⁻¹; respectively. The analytical performance of the Au-PEA-SA-P53ag/BSA immunoelectrode was compared with other anti-P53ab immunobiosensor reported in the literature. The analytic parameters were summarised in **Table 3.5**. The LoD is lower when compared to previously reported 20.0 ng.ml⁻¹ [263] for the quartz microbalance biosensor, 1.2 ng.ml⁻¹ for amperometry immunomagnetic biosensor [299] and comparable to 0.390 pg.ml⁻¹ for conventional ELISA [141]. However, the LoD was higher when compared to 88.0 fg.ml⁻¹ in [95] and 10.0 fg.ml⁻¹ [166]. This perhaps was due to the signal enhancement of the introduced gold nanoparticle in [95] and higher affinity of the synthesized pentadentate peptide to the anti-P53ab in [166] used in the two assays; respectively.

Table 3.5: Comparative analytical performance of Au-PEA-SA-P53ag/BSA immunoelectrode with existing systems for anti-P53ab detection.

Biorecognition element	Immobilization techniques	Transducer	Linear range (ng.ml ⁻¹)	LoD	Refs
Pentadentate peptide	Covalent immobilization via SAM	EIS	-	10.0 fg.ml ⁻¹	[95]
P53-antigen	Adsorption onto AuNP	SQWV	0.001 -10	88.0 fg.ml ⁻¹	[166]
P53-antigen	Magnetic micro-carrier	Amperometry	-	1.20 ng.ml ⁻¹	[299]
P53-antigen	Covalent immobilization via SAM	QCM-D	-	2.0 ng.ml ⁻¹	[263]
P53-antigen	Adsorption unto micro well	ELISA	1.56-100	0.390 ng.ml ⁻¹	[141]
P53-antigen	Covalent via electrografting of PEA	EIS	1.0 - 1000	0.103 ng.ml ⁻¹	Present work

3.6. Reusability, reproducibility, and stability of immunoelectrode

The regeneration of the immunoelectrode is an important factor to be considered to investigate the reusability of the immunobiosensor for series of analyses. The 10 % DMSO/formamide (1:1), 0.01 % Tween-20, PBS (0.10 M, pH 7.4) solution was used to break the antibody-antigen immunocomplex. **Figure 3.10** shows the R_{CT} response for the regeneration of the immunoelectrode. The red bars show the R_{CT} of the regenerated baseline and the black bars show the R_{CT} response of the regenerated electrode after the exposure to anti-P53ab. The captured anti-P53ab dissociated from the Au-PEA-SA-P53ag/BSA immunoelectrode to regenerate free epitopes for anti-P53ab binding. The conditions that disrupt these interactions depend on the mode of interaction between the immobilized P53ag and the captured anti-P53abs. The regenerated surface yielded a reproducible baseline, **Figure 3.10 (a red bars)**. About 92 % of the captured anti-P53ab was removed. DMSO/formamide mixture substituted

the vicinity of the P53ag/anti-P53ab immunocomplex, causing the protein folding and removal of captured anti-P53ab [300].

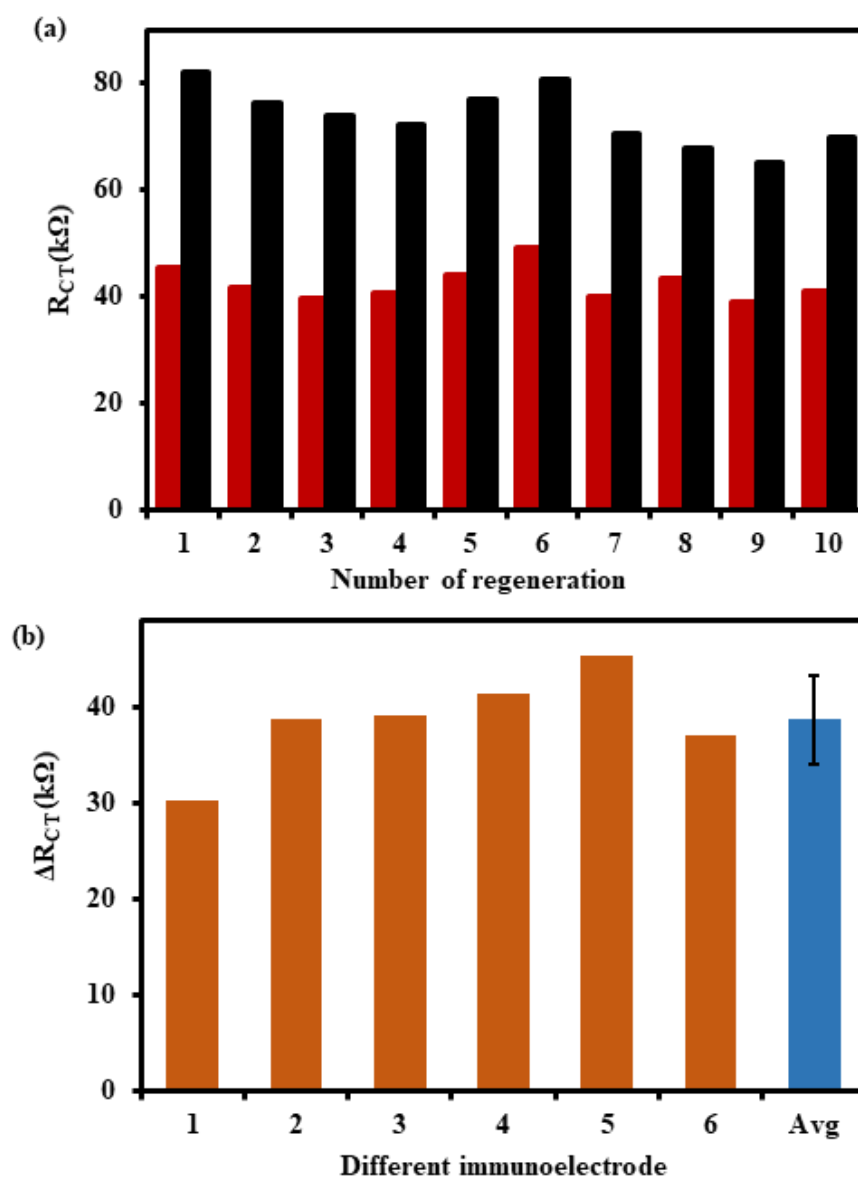


Figure 3.10: (a) Regeneration of Au-PEA-SA-P53ag/BSA with 10% DMSO: formamide (1:1), regenerated baseline (red) and response to anti-P53ab (black), and (b) sensor to sensor reproducibility studies of 6 different immunoelectrodes response to anti-P53ab. Avg = Average.

The regenerated Au-PEA-SA-P53ag/BSA immunoelectrode was incubated in PBS buffer (pH 7.4) at 4°C for 12 hours to allow the reconfiguration of the immobilized P53ag and used to

detect anti-P53ab again. The RSD was 3.2 % for the first seven repeated usages and 9.8 % for the 10th cycle indicating good reusability. The good regeneration ability demonstrated by the immunoelectrode is attributed to the excellent stability of the P53ag on the electrode surface through covalent immobilization. The storage of the immunoelectrode after regeneration showed good reusability and storage stability at 4 °C for 20 days. To evaluate the sensor to sensor reproducibility a series of six Au-PEA-SA-P53ag/BSA immunoelectrodes were prepared and use for detection of anti-P53ab in **Figure 3.10 (b)**. The % RSD of the measurements for the six electrodes was 7.21 %, suggesting the precision and reproducibility was very good.

3.7. Chapter conclusions

This chapter showed the successful fabrication of electrochemical immunobiosensor Au-PEA-SA-P53ag/BSA via the covalent immobilization of human P53-antigen (P53ag) onto a gold surface. The Au-PES-SA-P53ag/BSA showed specific interactions with anti-P53 autoantibodies (ant-P53ab) in solution. The characterization of the Au-PES-SA-P53ag/BSA using electrochemical (CV and EIS) and spectroscopic methods (XPS) confirm the successful fabrication of the immunoelectrode. The recognition of anti-P53ab was confirmed using a label-free electrochemical impedance spectroscopy method. The limit of detection (132.0 pg.ml⁻¹) was lower than the commercial ELISA. The Au-PEA-SA-P53ag/BSA immunoelectrode showed a wide linear range, excellent stability, acceptable reproducibility and higher sensitivity than conventional ELISA and other reported anti-P53 autoantibody immunobiosensors. The excellent stability was attributed to the covalent attachment of P53ag and blocking of non-specific binding sites with BSA. Inter-electrode reproducibility was very good with 7.2 % RSD for n = 6. The intra-electrode reproducibility investigated by the regenerated surface in DMSO/formamide was found to be excellent with 3.2 % RSD for n = 7 and 9.8 % for n = 10.

CHAPTER 4:

Fluorescent detection of anti-P53ab

4. Fluorescent Detection of anti-P53ab

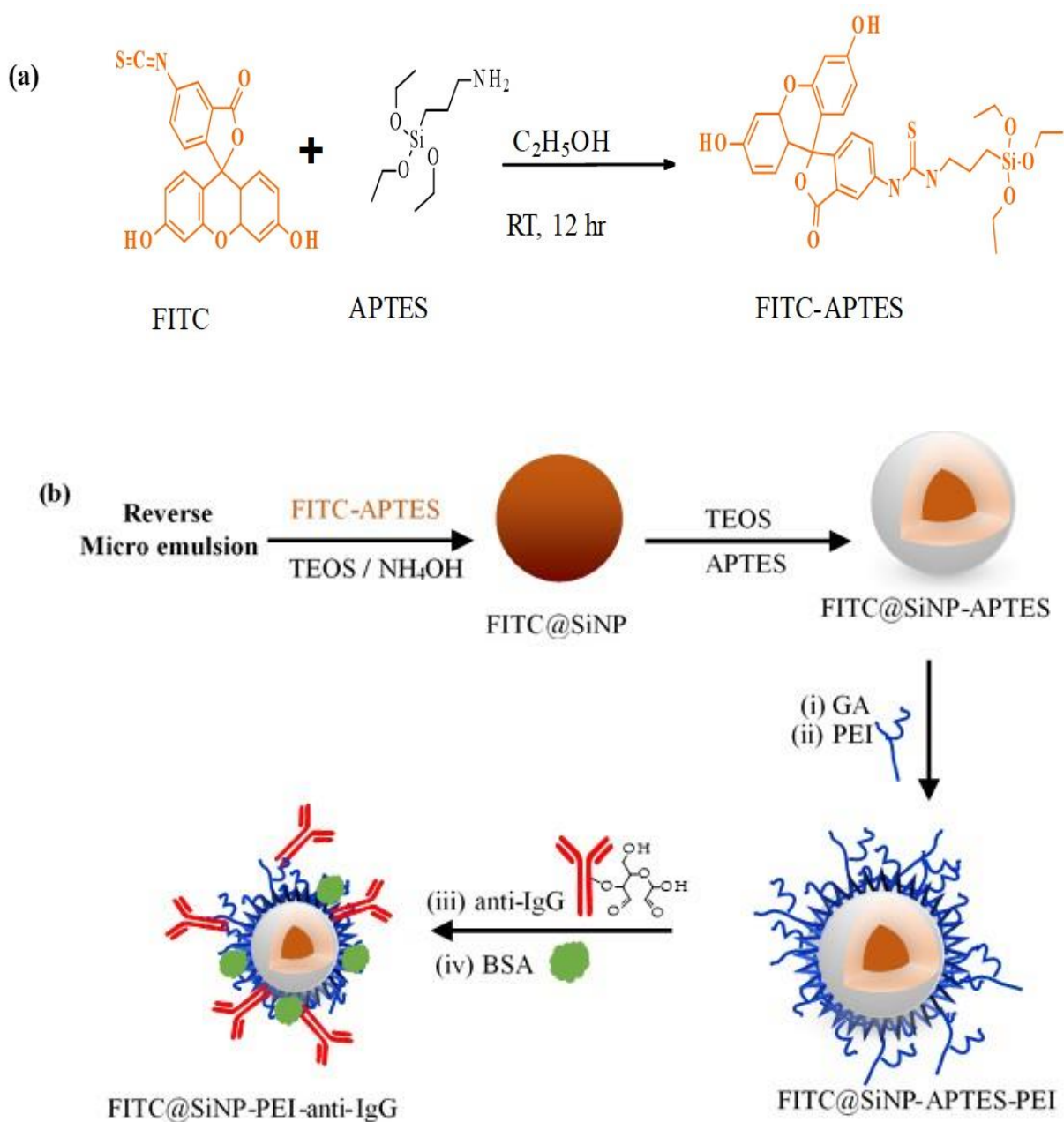
This chapter adopted the covalent immobilization method (EDC-NHS mediated coupling) for the immobilization of the P53ag onto the carboxyl-functionalized superparamagnetic bead. The fabrication of a new fluorescent-silica nanoparticle linked-magnetic bead immunobiosensor will be investigated. Attaining ultrasensitive fluorescent-based detection of anti-P53ab requires the use of functional detection probes with enhanced fluorescent intensity, photostability, and higher affinitive binding with the targeted biomarker (anti-P53ab). Fluorescence immunoassay format adopting signal enhancement is based on the triggered pay-load release of the signaling element. These signaling elements are loaded within nanocarriers such as liposomes [301,302], polymeric carrier [303] and nanoscale materials [241,304]. The nanocarriers have shown more significant advantages in realizing ultrasensitive detection of disease biomarkers. Fluorescence signal enhancement based on FITC-doped silica nanoparticle (FITC@SiNP) dissolution dye-payload release is herein reported. The P53ag/anti-P53ab recognition events could be signaled by many fluorophores encapsulated within a single silica nanoparticle. This will give an enhanced fluorescence signal that is about 30000-fold better than a single organic dye [305]. The signal enhancement could aid the ultrasensitive detection of anti-P53ab in biofluids and reduces the risk of false-negative results. However, high signal amplification is not the only issue. Traditionally, antibodies are covalently conjugated to the nanoparticle through cross-linking techniques targeting the amine residue on the antibodies. The conjugation of antibodies to the nanoparticles via the amine residue often results in the modification of functional important amine groups. Thus, masking of the antigen-binding sites. This results in many fluorescent nanoparticles without functional antibodies on their surfaces to recognize the antigen. The nanoparticles without functional antibody drastically reduce the effect of the enhanced photophysical properties and sensitivity. In this chapter, the synthesis of fluorescent FITC doped core-shell silica nanoparticle (FITC@SiNP) is reported. The surface engineering

for oriented conjugation of anti-IgG-antibody to form FITC@SiNP-APTES-PEI-anti-IgG for application in immunofluorescent detection of anti-P53ab is reported. Ultrasensitive detection of tumor-associated autoantibodies was achieved based on the synergetic signal amplification effect of FITC@SiNP, magnetic bead concentration and silica nanoparticle dissolution fluorescent TURN-ON approach.

4.1. Synthesis of FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe

The development of various hybrid systems incorporating organic-dyes within the silica nanoparticles matrix has been reported [238,306,307]. The enhanced optical characteristic of dye-doped silica nanoparticles in comparison to the incorporated dye is well known. The FITC@SiNP core-shell nanoparticles were synthesized via a process based on alkaline hydrolysis of organosilane precursors within a water-in-oil microemulsion system. The water-in-oil reverse emulsion method is an efficient and robust system for the synthesis of monodispersed spherical nanoparticles [308,309]. **Scheme 4.1** shows the schematic illustration for the synthesis of FITC@SiNP-APTES-PEI-anti-IgG immunobioprobe. In **Scheme 4.1 (a)** FITC was covalently conjugated to APTES via formation of thiourea bond to afford a fluorescent organosilane reagent (FITC-APTES). The FITC-APTES was incorporated into the silica sphere by hydrolysis and co-polymerisation with TEOS within the water-in-oil microemulsion nanoreactor. Under the alkaline condition, the tetra and tri-ethoxy groups of TEOS and FITC-APTES concurrently hydrolyze to silanol groups. The hydrolysis is followed by nucleation and concomitant polymerization to silica nanoparticles with covalently bonded FITC molecules, **Scheme 4.1 (b)**. The obtained nanoparticles were referred to as FITC@SiNP. The FITC@SiNP were coated with a silica shell (FITC@SiNP) to enhance its optical properties and to provide surface silanol groups (Si-OH) for further modification. The surface silanol groups react with APTES to form an amine-modified FITC@SiNP-APTES. APTES forms a self-assembly thin monolayer on the fluorescent silica nanoparticles through a covalent Si-O

bond between APTES and the silanol groups of the silica nanoparticle [310]. Branched-chain polyethyleneimine (PEI) was grafted onto the FITC@SiNP-APTES through glutaraldehyde linkage. The obtained nanoparticle was referred to as FITC@SiNP-APTES-PEI.



Scheme 4.1: The synthesis of the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe.

Branched-chain cross-linkers are more efficient for protein conjugation and also increase the nanoparticles-antibody conjugate stability than the linear chain crosslinkers [311]. The multivalent branched amine-linker arm groups of the PEI provides multiple sites for the efficient covalent conjugation of the anti-IgG antibody. The conformational flexibility of the PEI crosslinker also improves the hydrophilicity of the nanoparticle and reduces non-specific binding of the protein [312]. Fc-directed conjugation of the detection antibody (anti-IgG) to the FITC@SiNP-PEI nanosphere was achieved through reductive amination. The coupling between the primary amine groups of FITC@SiNP-PEI and reactive aldehyde groups on the anti-IgG results in the formation of an imine. The imine undergoes an in-situ nucleophile addition reaction with the hydride moiety of sodium cyanoborohydride to form a stable antibody nanoparticle conjugate (FITC@SiNP-PEI-anti-IgG nanobioprobe). It is necessary to optimize the amount of FITC within the silica nanoparticle without compromising the optical and structural reliability. Different sets of FITC@SiNP with a FITC loading of 0.5% to 9.0 % (w/w) were synthesized. Different amounts of the FITC-APTES derivatives were introduced into the microemulsion while keeping the concentration of the other reagents the same to achieve different % w/w FITC loading.

4.2. Characterization of FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe

4.2.1. Fourier Transform Infrared (FT-IR) characterization

Figure 4.1 (a) shows the FT-IR spectra of (i) FITC, (ii) APTES, and (iii) FITC-APTES. The FITC shows the isothiocyanate ($\text{N}=\text{C}=\text{S}$) and aromatic OH groups stretching vibrations at 2100 cm^{-1} and 3160 cm^{-1} respectively. The FTIR spectrum of APTES shows the characteristic peaks at 1100 cm^{-1} (Si-O-C), 1280 cm^{-1} (C-N), 765 cm^{-1} and 1600 cm^{-1} (N-H wag). The FTIR spectrum of FITC-APTES in **Figure 4.1 (b) (iii)** shows the complete disappearance of the ($\text{N}=\text{C}=\text{S}$) and a more distinct N-H vibration peak. The isothiocyanate group of FITC ($\text{N}=\text{C}=\text{S}$) reacts with the amine groups of APTES to form a thiourea bond. The disappearance of the

N=C=S peak indicated the complete reaction confirming the formation of the FITC-APTES conjugates.

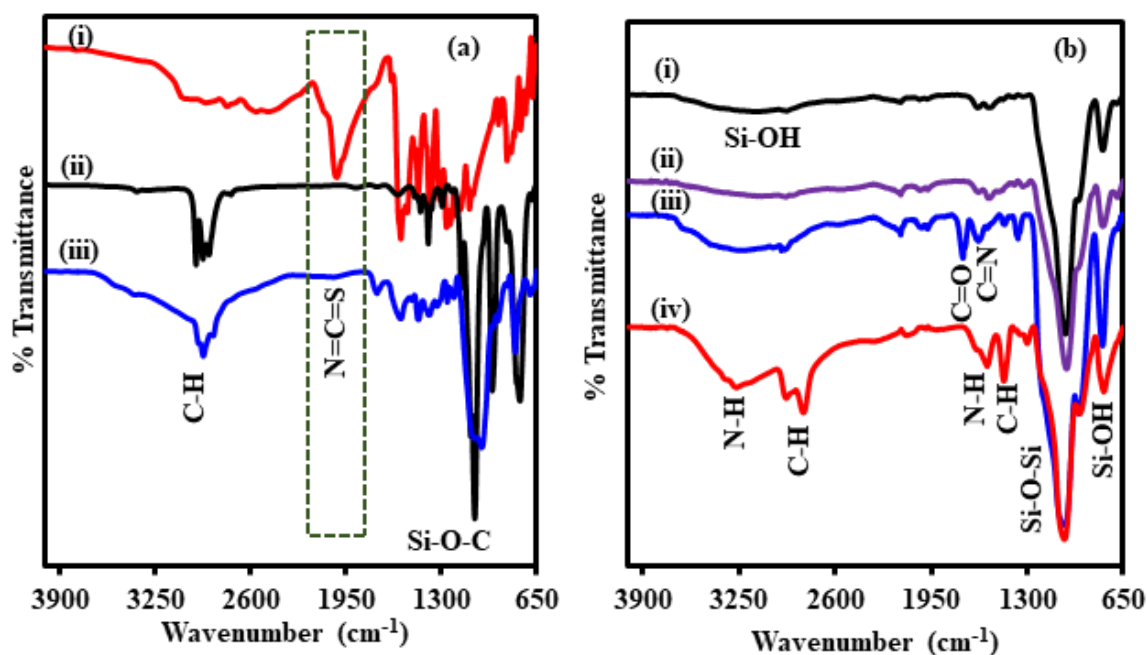


Figure 4.1: (a) FTIR spectra of (i) FITC, (ii) APTES, (iii) FITC-APTES, and (b) FTIR spectra of (i) FITC@SiNP, (ii) FITC@SiNP-APTES, (iii) FITC@SiNP-APTES-GA, and (iv) FITC@SiNP-APTES-PEI functionalized nanoparticles.

Figure 4.1 (b) shows the FT-IR spectra of (i) FITC@SiNP, (ii) FITC@SiNP-APTES, (iii) FITC@SiNP-APTES-GA, and (iv) FITC@SiNP-APTES-PEI modified nanoparticles. All the nanoparticles show characteristic vibrational bands at 1049 cm^{-1} (Si-O-Si) and 796 cm^{-1} (Si-O). The bands at 943 cm^{-1} indicate the presence of surface silanol (Si-OH) groups on the nanoparticles. The observed weak bands in the spectrum of the FITC@SiNP at 3011 cm^{-1} , 2887 cm^{-1} , 1540 cm^{-1} , and 1416 cm^{-1} were attributed to the C-H stretching and bending vibration of aromatic and aliphatic groups from the FITC dye. The intensity of the C-H bands increases after each surface modification with APTES due to an increase in the surface density of the methylene carbons (C-H). After the reaction of FITC@SiNP-APTES nanoparticles with

glutaraldehyde (GA), two new bands at 1640 cm^{-1} and 1728 cm^{-1} appears attributed to C=N and C=O stretching vibrations respectively. The C=N vibrations result from the Schiff bond between the FITC@SiNP-APTES and GA, while the C=O results from the terminal aldehyde groups on the FITC@SiNP-APTES-GA nanoparticles. After the reaction between PEI and FITC@SiNP@SiNP-APTES-GA, the C=O peak disappears indicating the successful grafting of PEI to the nanoparticles through the formation of a stable secondary amine bond. Also, the intensity of the methylene C-H bending vibrations (1433 cm^{-1} and 1305 cm^{-1}) increases due to the contributions of stretching vibrations from the C-H groups of the PEI. The presence of the two weak N-H stretching vibrations at 3236 cm^{-1} and 3346 cm^{-1} on the FITC@SiNP-PEI nanoparticles confirms the existence of terminal primary amine groups.

4.2.2. Thermogravimetric Analysis

Thermal gravimetric analysis (TGA) was performed to assess the extent of surface modification. TGA was carried out in nitrogen with a heating rate of $20\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$. **Figure 4.2** shows the TGA thermogram of (i) FITC@SiNP, (ii) FITC@SiNP-APTES, and (iii) FITC@SiNP-PEI; respectively. The weight loss for the FITC@SiNP was 13.37 % and was considered as the reference for the calculation of the surface functional groups. The difference between the weight loss of the FITC@SiNP-APTES and the FITC@SiNP was 2.15 % which was attributed to the loss of the APTES. A Significant weight loss of 13.56 % was recorded for the FITC@SiNP-PEI nanoparticles. The high percentage of weight loss was attributed to the presence of PEI groups. The TGA result confirms the successful surface modification with APTES and PEI.

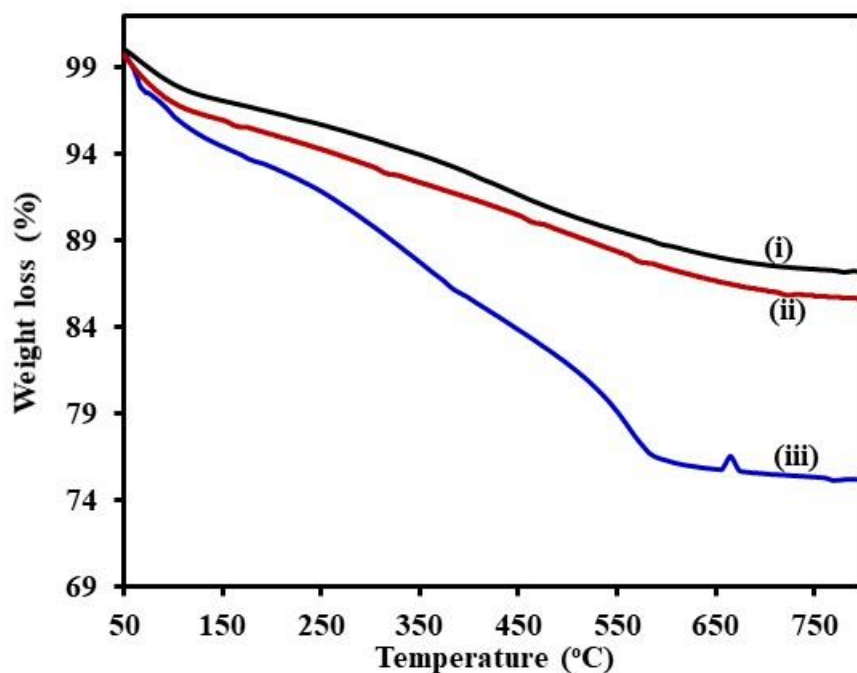


Figure 4.2: TGA thermogram of (i) FITC@SiNP, (ii) FITC@SiNP-APTES, and (iii) FITC@SiNP-APTES-PEI.

4.2.3. Microscopic characterization

The nanostructure, morphology, size, and aggregation state of the synthesized and modified nanoparticles were investigated using a high-resolution scanning electron microscope (HRSEM) and transmission electron microscope (HRTEM). **Figure 4.3** shows the HRSEM micrographs and the corresponding energy dispersive X-ray (EDS) spectra of the (i) FITC@SiNP and (ii) FITC@SiNP-APTES-PEI. The HRSEM images show that the nanoparticles were spherical with uniform and smooth surfaces. The results confirm the high efficiency of the microemulsion methods for the synthesis of spherical and monodispersed nanoparticles. The elemental composition of the nanoparticles was obtained from the EDS analysis of the SEM images. The EDS spectra in **Figure 4.3**, shows that the FITC@SiNP and FITC@SiNP-APTES-PEI were primarily composed of silicon and oxygen with an atomic ratio of approximately 1:2. There were no observable differences in the SEM and EDS of FITC@SiNP and FITC-APTES. The EDS spectra could not confirm the presence of nitrogen

and carbon from FITC@SiNP and FITC@SiNP-APTES-PEI. This could be attributed to a low abundance of nitrogen and carbon relative to the silicon and oxygen atoms.

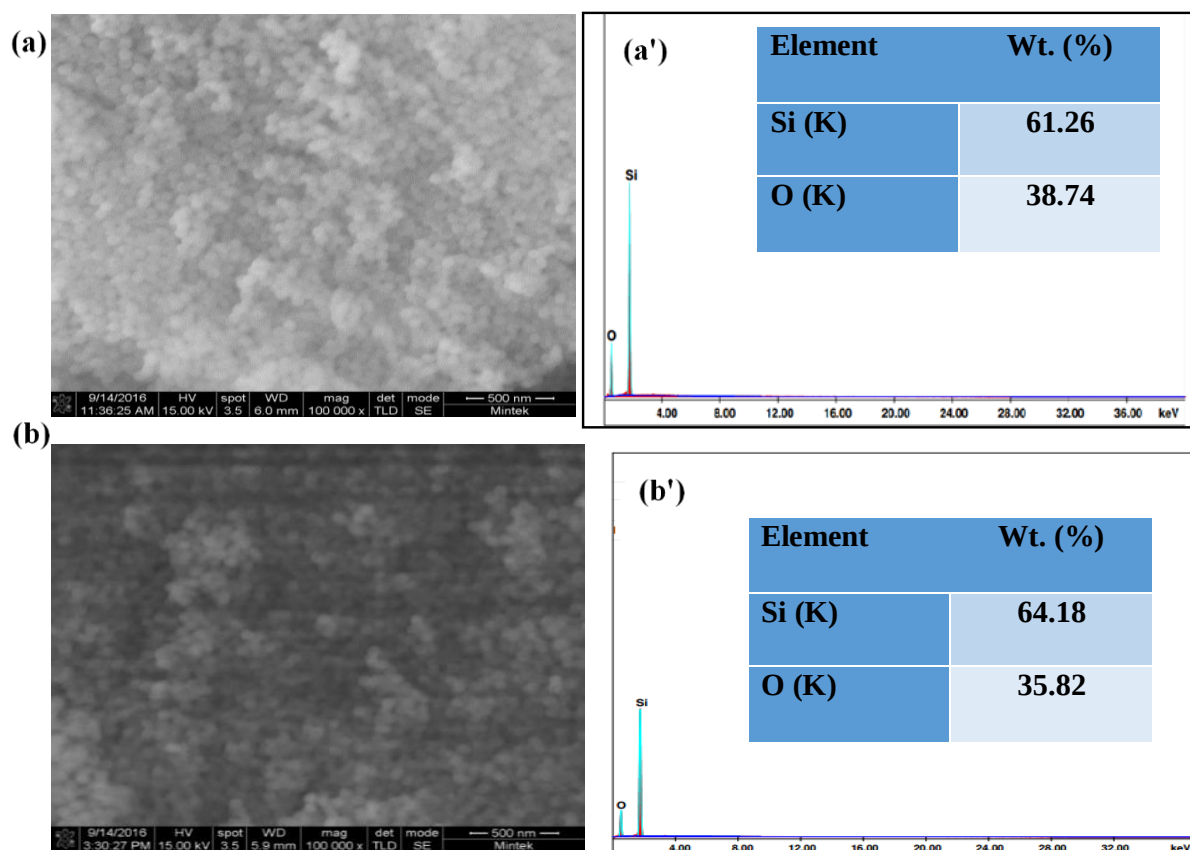


Figure 4.3: HRSEM images of the (a) FITC@SiNP, and (b) FITC@SiNP-APTES-PEI nanoparticles with their corresponding EDS spectra and elemental % weight composition (a') and (b'); respectively.

The size and morphology of the fluorescent silica nanoparticles were measured from the TEM images. **Figure 4.4** shows the TEM images of the FITC-dye loaded FITC@SiNP and their corresponding size distribution histograms. The % (w/w) FITC-dye loading was (a) 0.50 %, (b) 1.0 %, (c) 2.0 %, (d) 3.0 % and (e) 9.0 %. From the TEM images, the FITC@SiNP was spherical, non-aggregating and uniform.

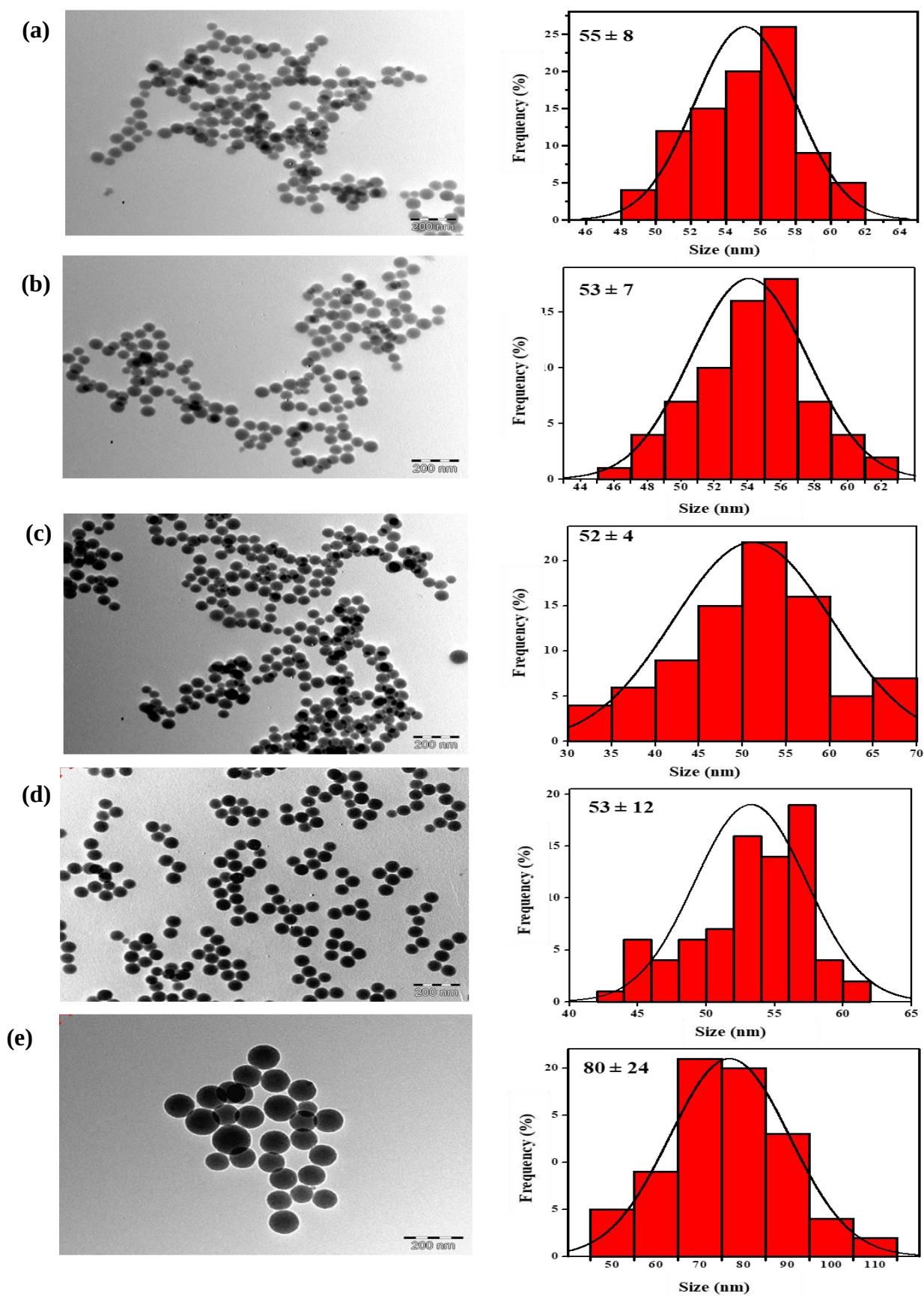


Figure 4.4: TEM micrographs of FITC@SiNP with (a) 0.5 %, (b) 1.0 %, (c) 2.0 %, (d) 3.0 %, and (e) 9.0 % (w/w) FITC-dye loading and their corresponding size distribution histogram.

The average diameter of the FITC@SiNP were 55 ± 8 nm, 53 ± 7 nm, 52 ± 4 nm, 53 ± 8 nm, 58 ± 6 nm, 60 ± 2 nm, and 80 ± 24 nm for w/w % loading of 0.5 %, 1.0 %, 2.0 %, 3.0 %, 4.5 %, 6.5 %, and 9.0 % (w/w); respectively. The TEM image and size distribution histogram of the 4.5 and 6.5 % FITC@SiNP are shown in **Figure S4.1**. The size distribution histograms show that the average diameter of the nanoparticles decreases with increasing FITC-dye loading from 0.5 % to 3.0 %. The decrease in size with the increased amount of FITC loading could be attributed to an increase in the supersaturation concentration of the FITC-APTES. This has been reported before [313]. The classical nucleation theory predicts that supersaturation concentration drives the nucleation rate. An increase in the amount of the FITC-APTES molecules increases the number of nucleating sites leading to the formation of the smaller sized nanoparticle, hence the decrease in the average diameter [314]. However, after 3.0 w/w % loading, the average diameter increases with an increasing rate of dye loading up to 9.0 w/w % (80 ± 24 nm). This could be due to a reduction in the rate of hydrolysis resulting from a decrease in the relative amount of water molecules to the FITC-APTES molecules within the microemulsion system. Hence the nucleation rate is reduced and size increases. The TEM images of the 9.0 % w/w FITC@SiNP shows that the nanoparticles were polydisperse with a size range from 50 nm to 114 nm. At higher dye loading (9.0 w/w %), the FITC-APTES could destabilize the microemulsion system which results in polydisperse nanoparticles. High-resolution TEM (HRTEM) was used to investigate the formation of a core-shell nanostructure and the effect of post surface modifications on the size and morphology of the nanoparticles. **Figure 4.5** shows the HRTEM images of (a) 6.5 % w/w FITC@SiNP and (b) 6.5 % w/w FITC@SiNP-APTES-PEI. The magnified HRTEM image (a') did not show the core-shell nanostructure architect of the FITC@SiNP nanoparticles. This was attributed to the phase homogeneity between the core FITC@SiNP core and the silica coat. The average size of the 6.5 % FITC@SiNP (60 ± 2 nm) increased by 8 nm after the APTES modification

(FITC@SiNP-APTES). The size distribution and HRTEM micrographs of the FITC@SiNP-APTES and the FITC@SiNP-APTES-PEI were similar. Due to the lower contrast of carbon atom compared to silicon, the presence of the PEI branched linker arm could not be visualized under HRTEM. The HRTEM image shows that the FITC@SiNP-APTES-PEI were monodispersed and neither aggregation nor nanoparticles crosslinking was observed.

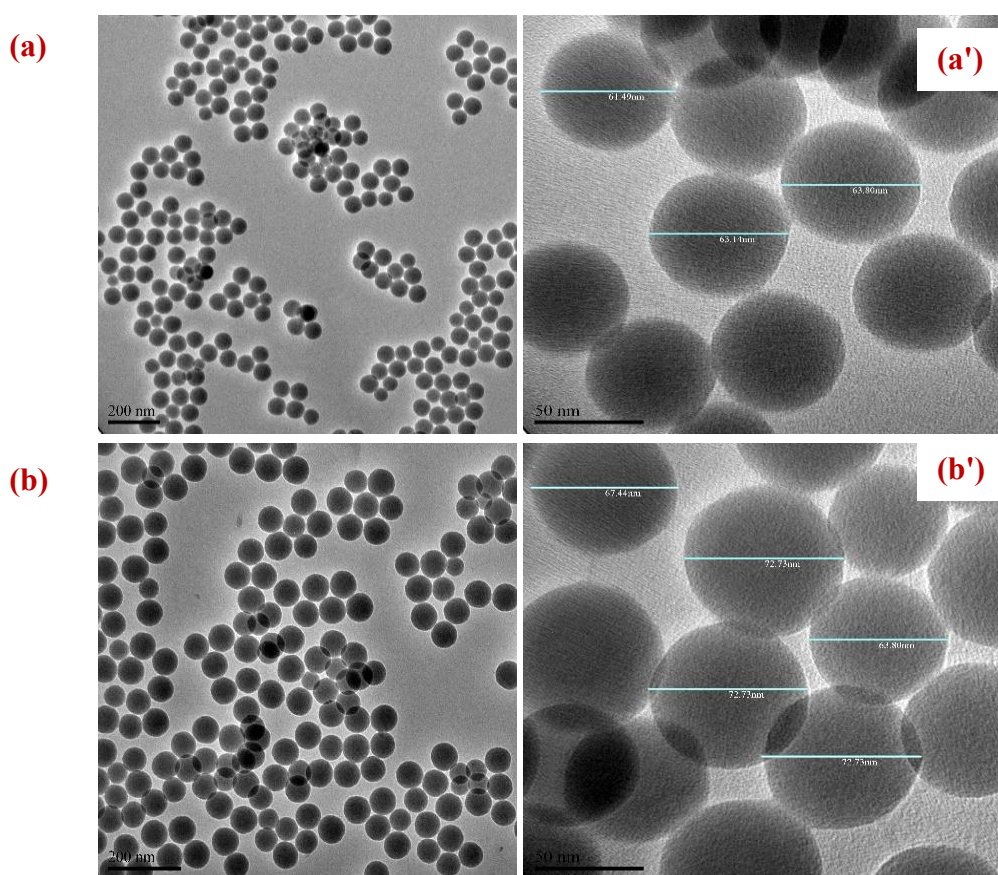


Figure 4.5: HRTEM of (a) 6.5 % FITC@SiNP and (b) 6.5% FITC@SiNP-APTES-PEI. (a') and (b') are the corresponding images at higher magnification

4.2.4. Photon correlation spectroscopic (PCS) characterization

The hydrodynamic diameter (DLS size) and the zeta potential of FITC@SiNP and FITC@SiNP-APTES and FITC@SiNP-APTES-PEI-anti-IgG were investigated. The hydrodynamic diameter, zeta potential and the polydispersity index (PDI) of the FITC@SiNP with different % w/w FITC-dye loading were summarised in **Table 4.1**. The hydrodynamic diameter is a measure of the diameter of the nanoparticles and the hydration sphere that has the same translational diffusion coefficient as the particle [315,316]. The hydrodynamic diameter of all the nanoparticles was greater than the corresponding TEM diameters. The higher hydrodynamic diameter of FITC@SiNP in comparison to the TEM size showed colloidal instability and agglomeration in PBS buffer (pH 7.4). The hydrodynamic diameter increases from 131 ± 2.5 nm for 0.5 % (w/w) to 270 ± 5.4 nm for 3.0 % (w/w) FITC@SiNP. The hydrodynamic diameter decreased afterward.

Table 4.1. Zeta potential, polydispersity index and DLS size of different FITC@SiNP at pH 7.4 compared with TEM size.

(%) w/w	Size (nm)		PDI	zeta potential (mV)
	TEM	DLS		
0.5	55 ± 8	131 ± 2.5	0.282	-29 ± 0.31
1.0	53 ± 7	142 ± 8.0	0.352	-27 ± 0.62
2.0	52 ± 4	179 ± 3.7	0.381	-22 ± 0.37
3.0	53 ± 12	270 ± 5.4	0.322	-16 ± 0.54
4.5	58 ± 6	212 ± 7.1	0.413	-19 ± 0.67
6.5	60 ± 2	189 ± 3.2	0.351	-20 ± 0.94
9.0	80 ± 24	303 ± 6.9	0.727	-17 ± 0.79

The polydispersity index (PDI) also increases with increasing FITC-dye loading. This corroborates the results from TEM that at high w/w loading (9.0 %), FITC@SiNP becomes more polydispersed. The changes in the surface zeta potential decreases with increasing % w/w FITC@SiNP from -29.0 ± 0.31 for 0.5 % w/w to -16.0 ± 0.54 for 3.0 % w/w. The decrease in the zeta potential was attributed to a decrease in the surface silanol groups as the amount of FITC molecules in FITC@SiNP increases. The observed decreasing trend in the zeta potential value corroborates the increasing hydrodynamic diameters as the % w/w increases from 0.5 % and 3.0 % w/w and shows that the FITC@SiNP become more agglomerating at pH 7.4 due to the lowered surface charge. The increase in the zeta potential for the 4.5 % (-19.0 ± 0.67) and 6.5 % (-20.0 ± 0.94) in comparison to the 3.0 % w/w was attributed to significant differences in the TEM size.

The modification steps of the 6.5 % w/w FITC@SiNP was further investigated with zeta potential measurement at different pH values. The other FITC loading follows the same trend. **Figure 4.6** shows the zeta potential of (i) 6.5% FITC@SiNP, (ii) 6.5% FITC@SiNP-APTES, and (iii) 6.5% FITC@SiNP-APTES-PEI as a function of pH. The isoelectric point of the 6.5% FITC@SiNP was at pH 4.8, and the zeta potential was -26.0 mV at pH 7.0, confirming the presence of surface silanol groups (Si-OH). After coating with APTES to form 6.5% FITC@SiNP-APTES, the isoelectric point shifted to pH 5.8. The zeta potential at pH 7.0 was -10.0 mV. A 16.0 mV shift in zeta potential was observed in comparison to the 6.5% FITC@SiNP. After the grafting with PEI, the isoelectric point of 6.5% FITC@SiNP-APTES-PEI was pH 11.0. The zeta potential at pH 7.0 shifted to + 7.9 mV. The increase in the isoelectric point and the zeta potential could be attributed to the presence of the primary amine groups of the PEI molecules. The amine groups are protonated at $\text{pH} < 11.0$ (NH_3^+), neutral (NH_2) at pH 11.0 and deprotonated at $\text{pH} > 11.0$.

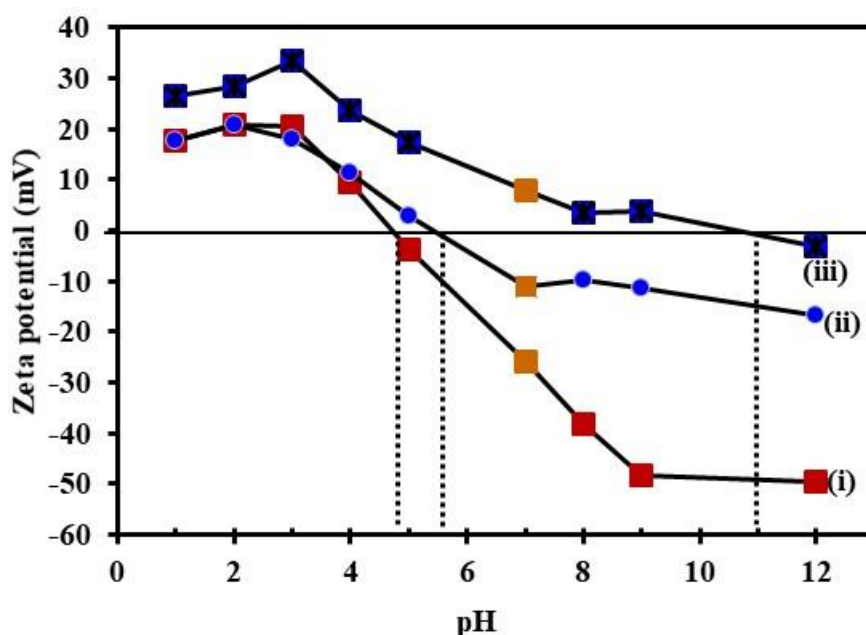


Figure 4.6: Zeta potential of 6.5% (i) FITC@SiNP, (ii) FITC@SiNP-APTES and (iii) FITC@SiNP-APTES-PEI nanoparticles in different pH solution.

The changes in the zeta potential at different pH values confirm the surface modification and surface charges with OH and NH₂ functional groups. Furthermore, the zeta potential values show that the FITC@SiNP and FITC@SiNP-APTES-PEI were colloidal stable at pH > 7.0 and pH < 5.0; respectively for the bioconjugation to form FITC@SiNP-APTES-PEI-anti-IgG.

Figure 4.7 shows (a) hydrodynamic diameter and (b) zeta potential of the nanoparticles in the coupling buffer (pH 8.0). The zeta potential of the FITC@SiNP-APTES and FITC@SiNP-APTES-PEI was found to be -3.5 mV and + 8.6 mV; respectively. The surface charge is not positive enough at this pH to stabilize the nanoparticles in the coupling buffer. Hence, the hydrodynamic diameter of FITC@SiNP significantly increases from 180 nm to 293 nm and 483 nm after the surface modification with APTES and PEI respectively. The zeta potential of FITC@SiNP-APTES-PEI (+8.6 mV) decreases to -14.6 mV after the conjugation of anti-IgG, confirming the immobilization of anti-IgG. The anti-IgG is a protein containing amino acid groups hence the negative charge at pH 8.0 due to the deprotonation of the carboxylic groups (COO⁻). The zeta potential decreases to -20.0 mV after blocking of non-specific binding sites

with BSA. The hydrodynamic diameter of the FITC@SiNP-APTES-PEI (483 nm) decreases to 271 nm after the anti-IgG conjugation due to an increase in the colloidal stability resulting from the negatively charged anti-IgG biomolecules. The hydrodynamic diameter increases to 594 nm after the BSA blocking. The drastic increase in the hydrodynamic diameter could be attributed to the bulky anti-IgG and BSA biomolecule layer on the nanoparticles. The 6.5 % FITC@SiNP-APTES-PEI-anti-IgG/BSA was used to demonstrate the effect of the bioconjugation in pH 8.0. The same results were observed for FITC@SiNP-APTES-PEI-anti-IgG/BSA with other % w/w loadings of FITC.

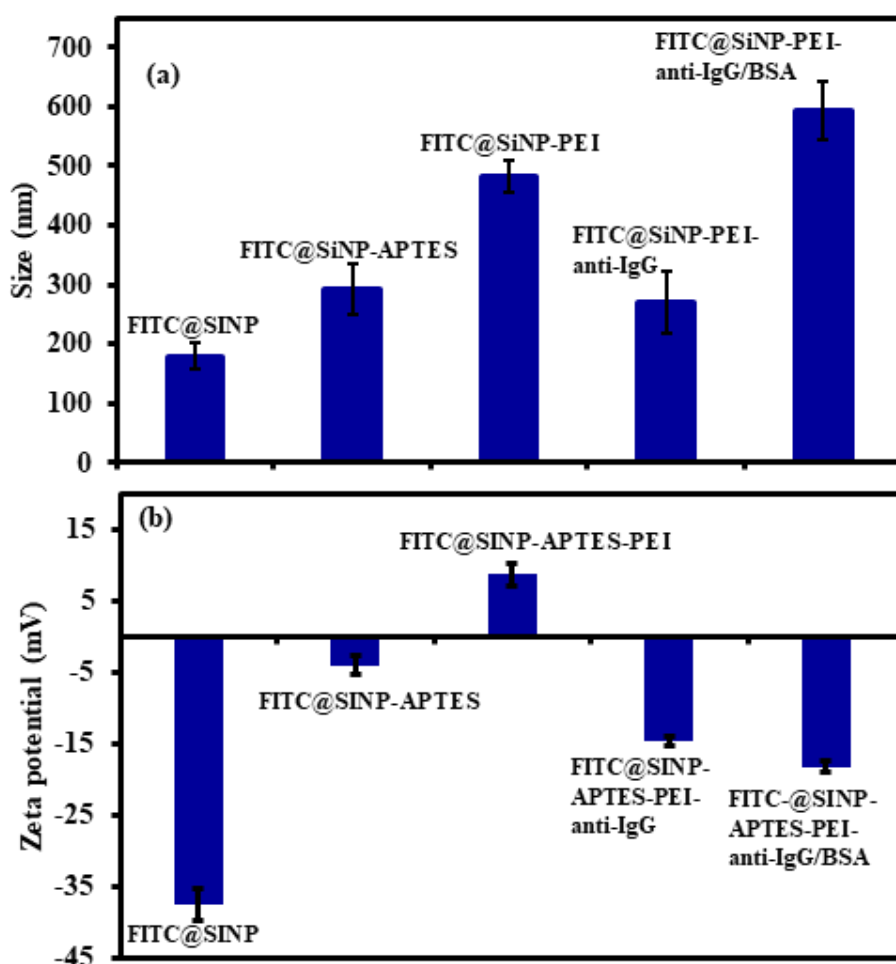


Figure 4.7: (a) Hydrodynamic diameter and (b) zeta potential of the 6.5 % w/w nanoparticles at different stages of the functionalization recorded at pH 8.0.

4.3. Photophysical properties of FITC@SiNP-APTES-PEI

4.3.1. Ground State Absorption

The absorption spectra of free FITC and the synthesized FITC-APTES in PBS are shown in Supplementary information, **Figure S4.2**. The UV-vis absorption spectrum of FITC was characterized by a narrow peak with maximal absorption at 495 nm. The absorption spectrum of the FITC-APTES was typical of the FITC-dye. There were no significant changes in the absorption spectra characteristics of FITC in **Figure S4.2 (i)** and FITC-APTES in **Figure S4.2 (ii)**. The absorption wavelength of the aqueous dispersion of FITC@SiNP-APTES-PEI nanoparticles in **Figure S4.3** was red-shifted by 12 nm in comparison to the aqueous solution of FITC-APTES. The observed shift in the absorption wavelength could be attributed to the solvatochromic effect because the silica nanoparticle matrix is more polar than the water. The effect of % FITC loading on the ground state absorption of FITC@SiNP was evaluated.

Figure 4.8 show absorption spectra of FITC@SiNP with different % w/w FITC loading. The loading was 0.5 %, 1.0 %, 2.0%, 4.5 %, 6.5% and 9.0%. The spectrum of 0.5 % and 1.0 % overlaps on each other and showed a broad absorption.

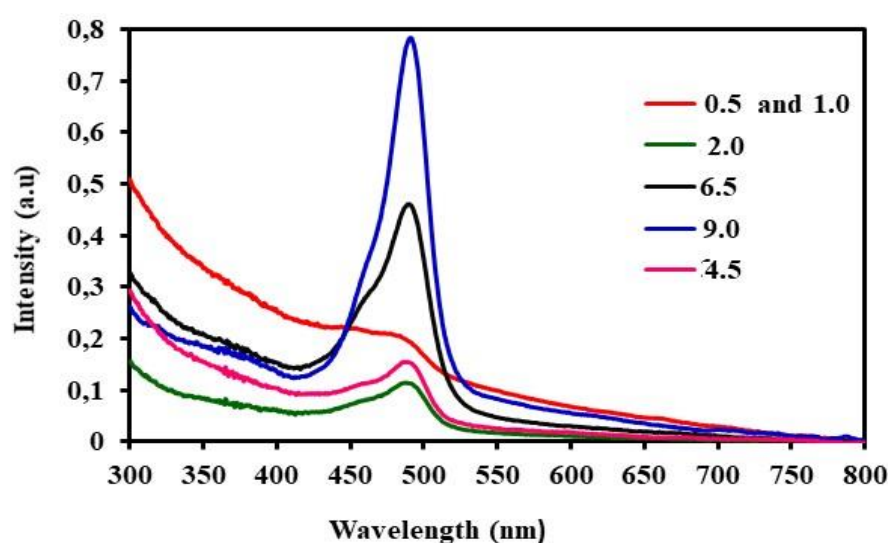


Figure 4.8: UV-vis absorption spectra of FITC@SiNP with different % w/w FITC loading.

In **Figure 4.8** no significant changes in the maxima wavelength value with changes in the % dye loading were observed. It was evident that the absorption intensity increased as the FITC-APTES concentrations increased. This increase in the absorption indicates that more FITC molecules were incorporated within FITC@SiNP. The number of FITC dye molecules per FITC@SiNP was investigated by dissolving an equal amount of the FITC@SiNP in carbonate buffer pH 10.0 at 37 °C. The dissolution occurs due to the hydrolysis of the silica bonds and the release of the loaded FITC molecules. The absorption reading of the dissolved FITC@SiNP was recorded. The absorption intensity was used to calculate the number of FITC molecules per nanoparticles using a molar extinction coefficient of FITC and the TEM size. From the TEM size (diameter, d), the average volume of a single FITC@SiNP was obtained using **Equation (4.1)**.

$$V_{\text{FITC@SiNP}} = \frac{4}{3} \pi \left(\frac{d}{2}\right)^3 \quad (4.1)$$

The total number of FITC@SiNP in the suspension was calculated based on the dry weight and the density of the amorphous silica matrix (2.22 g.cm⁻³) [317]. The total number of FITC molecule (FITC_{tot}) from the dissolved nanoparticles was determined from the absorption intensity after the dissolution using **Equation (4.2)** [318].

$$\text{FITC}_{\text{tot}} = \frac{6.022 \times 10^{23} \times A}{\epsilon l} \quad (4.2)$$

where l is the path length (1 nm), and A is the absorption intensity, ϵ is the molar absorptivity of FITC (73,000 cm⁻¹M⁻¹). The number of FITC molecules per FITC@SiNP is equal to FITC per unit volume divided by the number of FITC@SiNP per unit volume as expressed in **Equation (4.3)**.

$$n_{\text{FITC}} = \frac{\text{FITC}_{\text{tot}}}{n_{\text{FITC@SiNP}}} \quad (4.3)$$

where n_{FITC} is the number of FITC dye molecule per FITC@SiNP, FITC_{tot} is a total number of FITC molecules and $n_{\text{FITC@SiNP}}$ is the number of FITC@SiNP in the suspension. The total number of FITC@SiNP in the suspension, the average volume of the as-synthesized FITC@SiNP and the number of FITC-molecules per FITC@SiNP at different % FITC loading are summarised in **Table 4.2**. The average volume of the FITC@SiNP increases with increasing TEM size. The number of FITC-dye molecules per FITC@SiNP increases with increasing % w/w. The number of FITC-dye molecules per FITC@SiNP increases with increasing % w/w FITC@SiNP.

Table 4.2: Determination of FITC loading using TEM and UV-absorption values

% w/w	TEM size (nm)	Average volume (cm ³)	Nanoparticles in suspension (particles)	FITC molecule in suspension (moles)	FITC per nanoparticle (moles)
0.5	55 ± 8	8.27 x 10 ⁻¹⁷	3.68 x 10 ¹⁵	8.16 x 10 ¹⁷	222 ± 6
1.0	53 ± 7	7.83 x 10 ⁻¹⁷	3.90 x 10 ¹⁵	1.23 x 10 ¹⁸	316 ± 3
2.0	52 ± 4	7.83 x 10 ⁻¹⁷	3.90 x 10 ¹⁵	1.78 x 10 ¹⁸	454 ± 12
4.5	53 ± 12	8.14 x 10 ⁻¹⁷	3.74 x 10 ¹⁵	3.73 x 10 ¹⁸	994 ± 21
6.5	60 ± 2	1.18 x 10 ⁻¹⁶	2.58 x 10 ¹⁵	7.41 x 10 ¹⁸	2290 ± 19
9.0	80 ± 24	2.88 x 10 ⁻¹⁶	1.05 x 10 ¹⁵	9.99 x 10 ¹⁸	9380 ± 81

4.3.2. FITC@SiNP fluorescent emission and quantum efficiency

The fluorescence excitation and emission spectra of the FITC and FITC-APTES conjugate in PBS buffer were shown in **Figure S4.4**. The FITC and FITC-APTES emit green fluorescent radiation with maximum emission wavelength at 518 nm. The emission bands are characteristic of the dominance of anionic species of FITC in solution [223,225]. **Figure 4.9** shows the emission spectra of an aqueous dispersion of different % w/w FITC@SiNP-APTES-PEI nanoparticles in PBS buffer (pH 7.4). The maximum emission wavelength was slightly red-shifted with increasing % w/w loading. The emission wavelength increased from 518 nm for the 1.0 % w/w FITC@SiNP-APTES-PEI to 522 nm for 9.0 % w/w FITC@SiNP-APTES-PEI. The emission spectra of the 6.5 % w/w FITC@SiNP-APTES-PEI nanoparticles (522 nm) was red-shifted by 4 nm in comparison to the FITC-APTES (518 nm). A similar red shift in emission wavelength has been reported for most organic-dye-doped silica nanoparticles [190, 204]. The observed bathochromic shift in the emission spectra of the FITC@SiNP-APTES-PEI was due to the higher polarity of the silica matrix. The emission spectra confirmed the successful synthesis of the FITC@SiNP-APTES-PEI. The effect of the % w/w FITC loading on the fluorescence emission intensity was also investigated. **Figure 4.9** shows the effect of increasing % w/w FITC-dye loading on the fluorescence emission intensity of the FITC@SiNP-APTES-PEI. The fluorescent intensity increases with increasing % w/w FITC loading from 0.5 % (222 ± 6.0 FITC molecules) to 1.0 % (316 ± 3.0 FITC molecules). At higher loading > 1.0 % per FITC@SiNP-APTES-PEI, a decrease in the fluorescence intensity was observed. This fluorescence quenching was attributed to dye-to-dye interaction which results in HOMO foster resonance energy transfer (HOMO-FRET) between interacting FITC dye molecules [318]. The intermolecular spaces between the dye molecules are reduced at higher FITC (> 1.0 %) loading. Therefore, it prompts energy transfer between the FITC molecules. This results in non-radiative energy loss. The 1.0 % with 316 FITC molecules have the ideal

intermolecular space between the dye molecules allowing fluorescence to occur. Thus, 1.0 % w/w loading is optimum to obtain maximum fluorescence intensity of the FITC@SiNP.

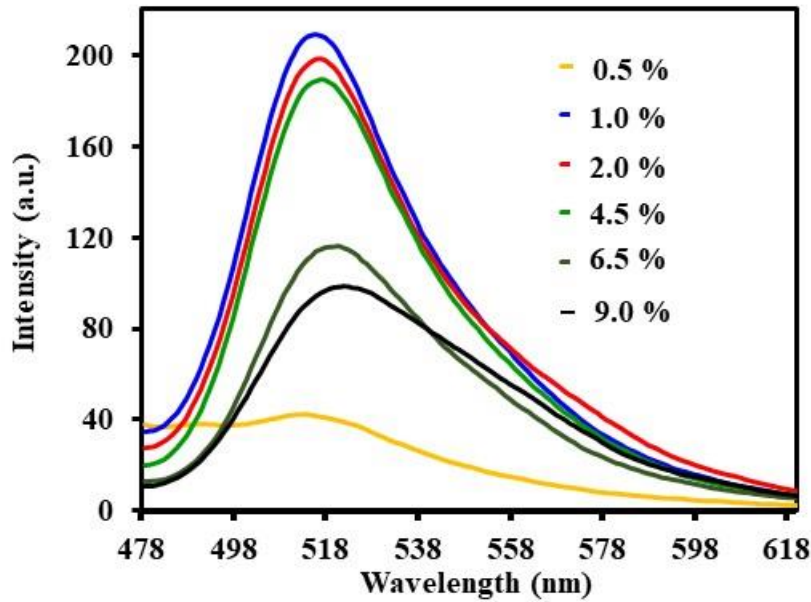


Figure 4.9: Fluorescent emission spectra of FITC@SiNP with different % w/w FITC-loading.

The quantum yield measured the efficiency of the fluorescence process. The fluorescence quantum yield (ϕ_F) express the proportion of the excited FITC@SiNP that deactivates by emitting a fluorescent photon. The fluorescence quantum efficiency of the FITC@SiNP was determined using **Equation (4.4)**.

$$\% \phi_{\text{FITC@SiNP}} = 100 \left(\phi_{\text{Fstd}} \times \frac{F_{\text{FITC@SiNP}} \times A_{\text{std}}}{F_{\text{std}} \times A_{\text{FITC@SiNP}}} \times \frac{\eta_{\text{FITC@SiNP}}^2}{\eta_{\text{std}}^2} \right) \quad (4.4)$$

Where $F_{\text{FITC@SiNP}}$ and F_{std} is the sum of the integrated fluorescence intensities of the FITC@SiNP and the fluorescein standard ($\phi_{\text{Fstd}} = 0.98$) [306] and A_{std} and $A_{\text{FITC@SiNP}}$ was the absorption values of the standard and the FITC@SiNP; respectively. η_{std}^2 and $\eta_{\text{FITC@SiNP}}^2$ are the refractive index of the medium.

The fluorescence quantum efficiency of FITC dye relative to fluorescein standard in the buffer solution (pH 10.0) was found to be 60 %. The fluorescent efficiency of the FITC@SiNP was evaluated before the dissolution of the FITC@SiNP and after dissolution. **Figure 4.10** shows the comparison of the fluorescence efficiency of different % FITC@SiNP (a) before dissolution and (b) after dissolution in the carbonate-bicarbonate buffer (pH 10.0). The quantum efficiency increases with increasing % FITC loading up to 1.0 % (316 ± 3.0 molecules) per FITC@SiNP.

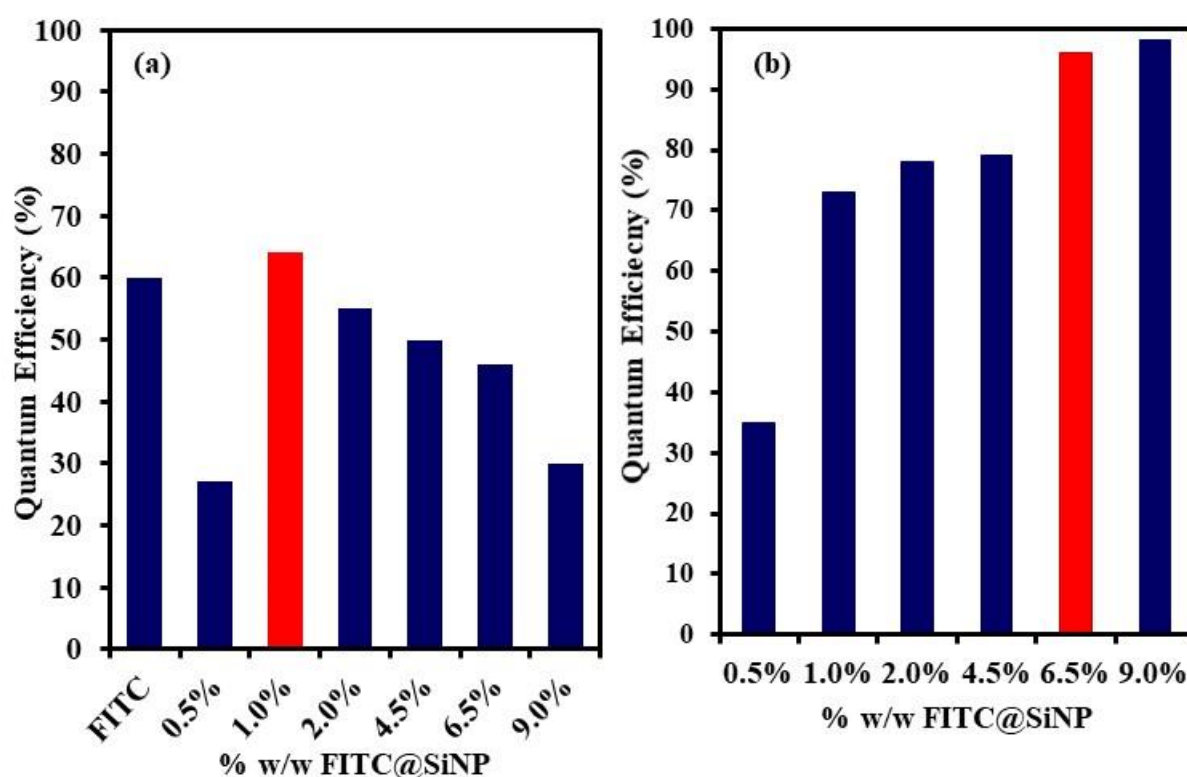


Figure 4.10: Comparison of fluorescent quantum efficiency of different % w/w FITC@SiNP nanoparticles (a) before dissolution (b) after dissolution in sodium carbonate-bicarbonate buffer (pH 10.0).

The fluorescence efficiency of the 1.0 % FITC@SiNP (65%) was about 5 % units higher than the free FITC (60 %) in the PBS buffer. A decrease in the quantum efficiency was observed at higher FITC loading > 1.0 %. The quantum efficiency of the highly loaded FITC@SiNP with 2.0 %, 4.5 %, 6.5 %, and 9.0 % were 53 %, 49 %, 45 %, and 29 %; respectively. These values

were lower than that of the free FITC in PBS (60 %). The lower quantum efficiency of the highly doped FITC@SiNP was attributed to the HOMO-FRET resulting from intermolecular quenching. The quantum efficiency results corroborate the trend observed for fluorescent emission intensity. The fluorescence quantum efficiency after the dissolution of the FITC@SiNP as a function of % w/w FITC loading was also investigated, **Figure 4.10 (b)**. After the dissolution of the FITC@SiNP, the fluorescence efficiency of the dissolved 0.5 %, 1.0 %, 2.0 %, 4.5%, 6.5 % and 9.0 % w/w FITC@SiNP were 35 %, 73 %, 78 %, 79 %, 95 % and 97 %; respectively. These values were higher when compared to their respective undissolved FITC@SiNP. The dissolution of the FITC@SiNP led to the release of the FITC dye molecules from the silica matrix into the aqueous solution. The dissolution of the silica prevents dye-to-dye interaction and HOMO-FRET, hence, the FITC molecules emit fluorescent radiation without interference from their neighboring dye molecules. The increase in the fluorescence efficiency with increasing % w/w FITC@SiNP was due to the increase in the number of liberated FITC molecules. The quantum efficiency of the highly doped FITC@SiNP with 2870 FITC molecules (6.5 % w/w) and 9380 FITC molecules (9.0 % w/w) were 95 % and 97 %; respectively. The fluorescence efficiency is almost close to unity. This confirms that the dissolution of the nanoparticles allows the dye molecules to be free from the effect of the HOMO-FRET observed in the undissolved nanoparticles. The result demonstrates that fluorescent silica nanoparticle dissolution provides an efficient method for enhancement of fluorescent emission of FITC@SiNP.

4.3.3. Effect of various conditions on the dissolution of FITC@SiNP-APTES-PEI

The dissolution of silica nanoparticles is caused by the hydrolysis of the silica (-Si-O-Si) bond and is well reported in the literature [320,321]. The dissolution of silica is accelerated at high temperatures, pH, and in the presence of cations such as sodium and potassium ions. The modification of the silica surface with PEI could affect the dissolution rate of the FITC@SiNP-

APTES-PEI, the optimum conditions for the dissolution of the nanoparticles were evaluated to select the best dissolution buffer conditions that can efficiently release the FITC dyes without modifying its fluorescence properties. The dissolution experiments were carried out for a total period of six hours in six different tubes. After an hour interval of dissolution, residual FITC@SiNP-APTES-PEI in the tube were collected by centrifugation, and the initial colorless buffer solutions turn yellow indicating the release of the FITC dyes into the solution. **Figure 4.11** shows (a) the pictorial representation and (b) time-dependent fluorescence intensity for the dissolution of the FITC@SiNP-APTES-PEI in different buffer systems. The highest rate of dissolution was in NaOH (1.0 mM, pH 10.0). The FITC@SiNP-APTES-PEI was dissolved entirely in the NaOH solution, **Figure 4.11 (a) (iii)**. Residual FITC@SiNP-APTES-PEI were not obtained after one hour of dissolution and centrifugation. This was attributed to the complete hydrolysis of the FITC@SiNP-APTES-PEI Si-O-Si bonding network in NaOH. The carbonate buffer also showed a significant FITC@SiNP-APTES-PEI dissolution. The effect of the dissolution buffer on the fluorescence intensity of the liberated FITC dye was also evaluated. FITC exist in cationic, neutral and anionic forms in solution, making its fluorescence emission and absorption properties strongly pH-dependent. The highest fluorescent intensity of the dissolved FITC@SiNP-APTES-PEI nanoparticles was recorded in the 1.0 mM NaOH (pH 10.0) and the 100 mM carbonate-bicarbonate (pH 10.0) dissolution buffer. Under acidic conditions (1.0 mM HCl) the release of FITC was observed, but the fluorescent intensity was smallest due to the poor fluorescence emission of FITC in acidic solution. Thus, 1.0 mM NaOH (pH 10.0) is considered the most suitable dissolution buffer due to its highest rate of dissolution and the highest fluorescence intensity.

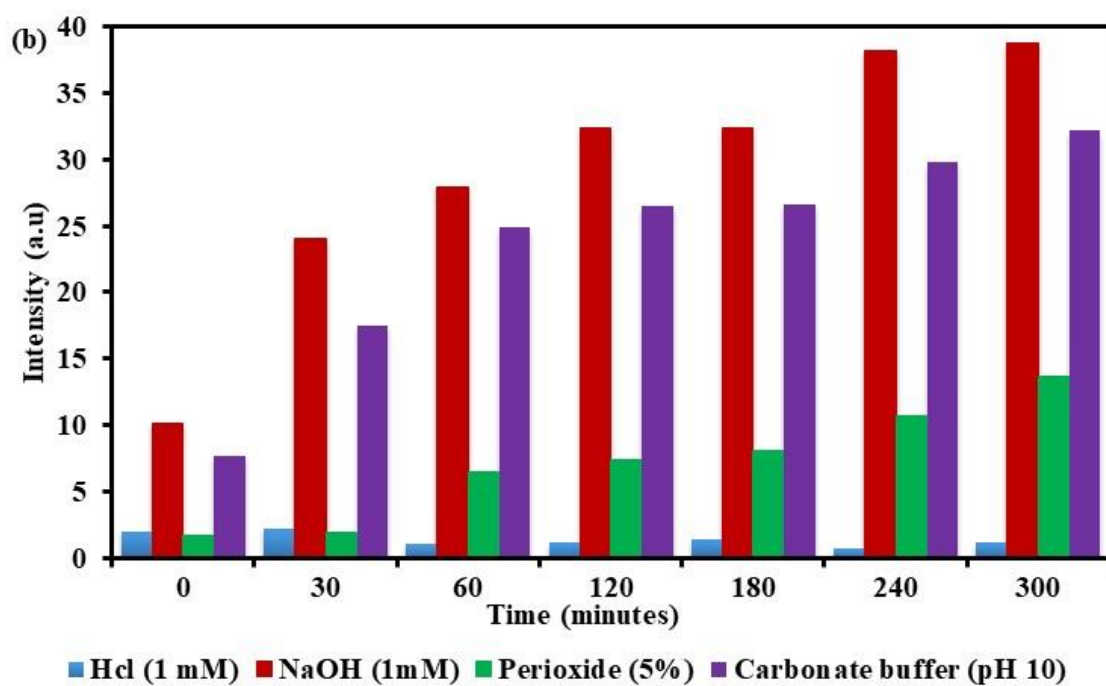
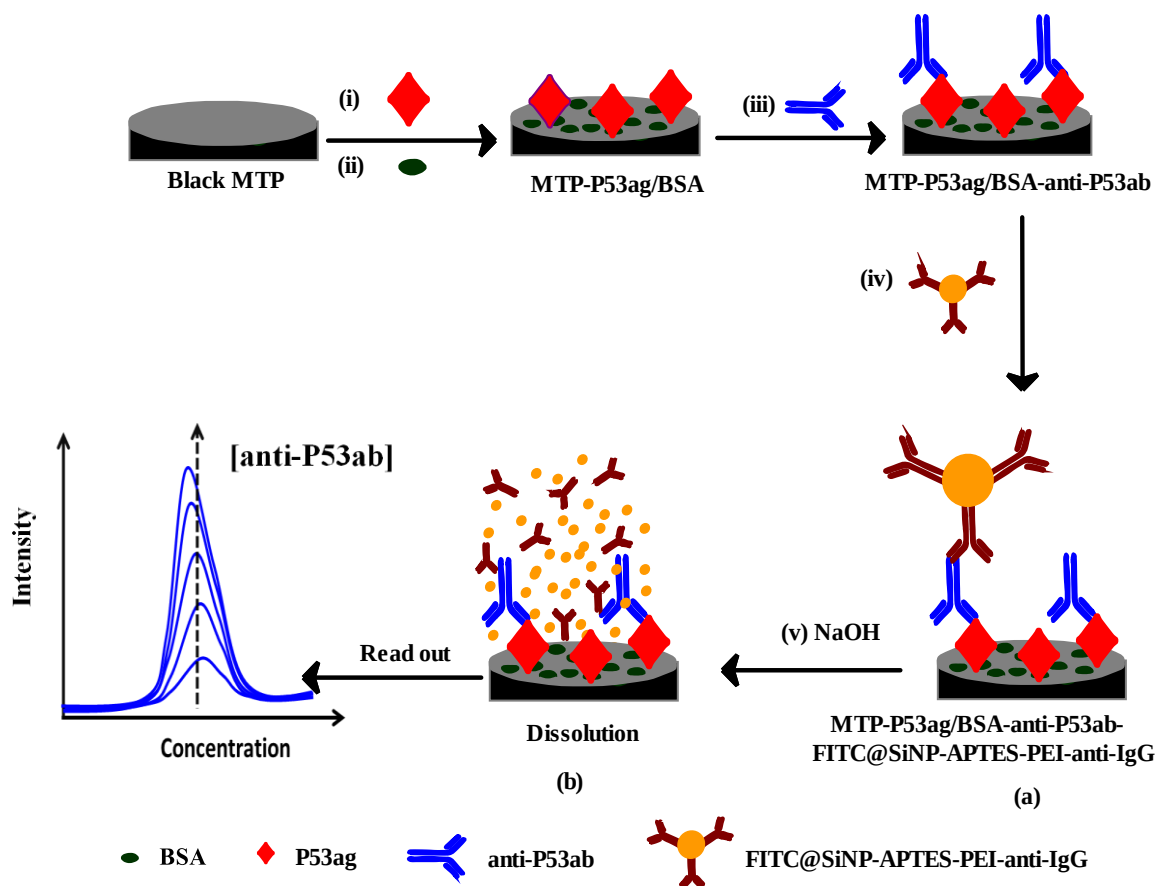


Figure 4.11: (a) Pictorial presentation and (b) time-dependent fluorescent intensity of FITC@SiNP-APTES-PEI in different dissolution buffer systems.

4.4. Fluorescent silica nanoparticle detection of anti-P53ab

The performance of the highly doped 6.5 % FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe was investigated for the detection of anti-P53ab. The choice of the 6.5 % FITC@SiNP-APTES-PEI-anti-IgG was based on the high fluorescence efficiency after dissolution. The immunobiosensor setup was demonstrated in a black microwell plate (MTP) using similar procedures for the commercial enzyme-linked immunoassay (ELISA). The 6.5 % FITC@SiNP-APTES-PEI-anti-IgG was used as the fluorescence detection probe instead of the HRP-anti-IgG/TMB/H₂O₂. **Scheme 4.2** shows a schematic representation of the modification of the black microwell plate with P53ag and the detection of anti-P53ab.



Scheme 4.2: Schematic representation of fluorescent immunobiosensor for anti-P53ab detection based on FITC@SiNP-APTES-PEI.

The P53ag was immobilized onto the microwells of a black 'MaxiSorp type immunoplate'. The blocking of non-specific adsorption sites was achieved using BSA. For biosensing, different concentrations of anti-P53ab in the test serum samples were added onto the MTP-P53ag/BSA modified microwells. The capture of the anti-P53ab from the test serum samples leads to the formation of the MTP-P53ag/BSA-anti-P53ab immunocomplex. The detection step involves anti-P53ab/anti-IgG interaction. The captured anti-P53ab interacts with anti-IgG on the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe. The anti-IgG would specifically bind to the Fc-region of the captured anti-P53ab. After each immunoreaction, the plate was washed with PBST (pH 7.5) to remove unreacted or physically adsorbed species

The dissolution buffer (1.0 mM NaOH, pH 10.0) was added to the immunoreaction plate to dissolve the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe. The dissolution liberated the FITC-dye molecules from the silica matrix. When illuminated by excitation energy, the liberated FITC molecules emit green fluorescence. The emission intensity would be proportional to the amount of the liberated FITC from the captured FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe. The intensity is indirectly proportional to the amount of anti-P53ab in the spiked serum samples. The effect of the 6.5 % FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe dissolution on the analytical performance of the fluorescent detection system was evaluated.

Figure 4.12 shows the comparison of the fluorescent emission spectra (a) before and (b) after an hour of adding the dissolution buffer. The fluorescence emission intensity is higher in wells that contained the anti-P53ab. The negative control and the blank wells were without anti-P53ab. The low fluorescent intensity in the negative control and the blank sample wells suggested that the signal in the positive wells were due to the specific interaction of the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe with the captured anti-P53ab. The fluorescent emission intensities of both the control and positive wells were significantly

enhanced after one hour of exposure to the dissolution buffer. There was an increase in the fluorescent intensity as the anti-P53ab concentrations increased, confirming the dependence of the signal on the concentrations of the anti-P53ab analyte.

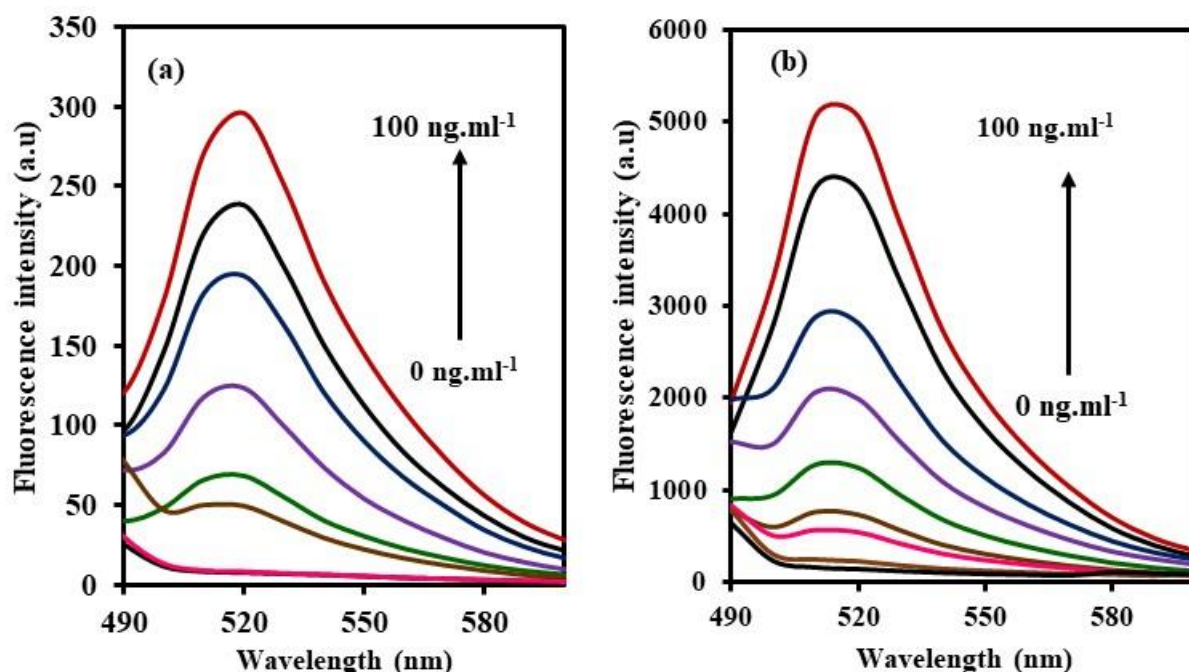


Figure 4.12: Fluorescence emission spectra of the response to different concentrations of anti-P53ab within the range of 0.0 to 100.0 ng/ml; (a) before, and (b) 60 minutes after the addition of NaOH dissolution buffer.

Figure 4.13 shows the (a) concentration dependence response curve and (b) the linear correlation curves for the detection of anti-P53ab for (i) before and (ii) 60 minutes after the addition of NaOH dissolution buffer. The fluorescent signal intensity increases with increasing concentration of the anti-P53ab from 0.1 ng.ml⁻¹ to 100.0 ng.ml⁻¹. The increase in the fluorescent intensity indicates an increase in the formation of the P53ag/BSA-anti-P53ab-FITC@SiNP-APTES-PEI-anti-IgG immunocomplex. It was evident from the dose-response

curves that the fluorescent intensity was significantly enhanced after the dissolution of the captured 6.5 % w/w FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe.

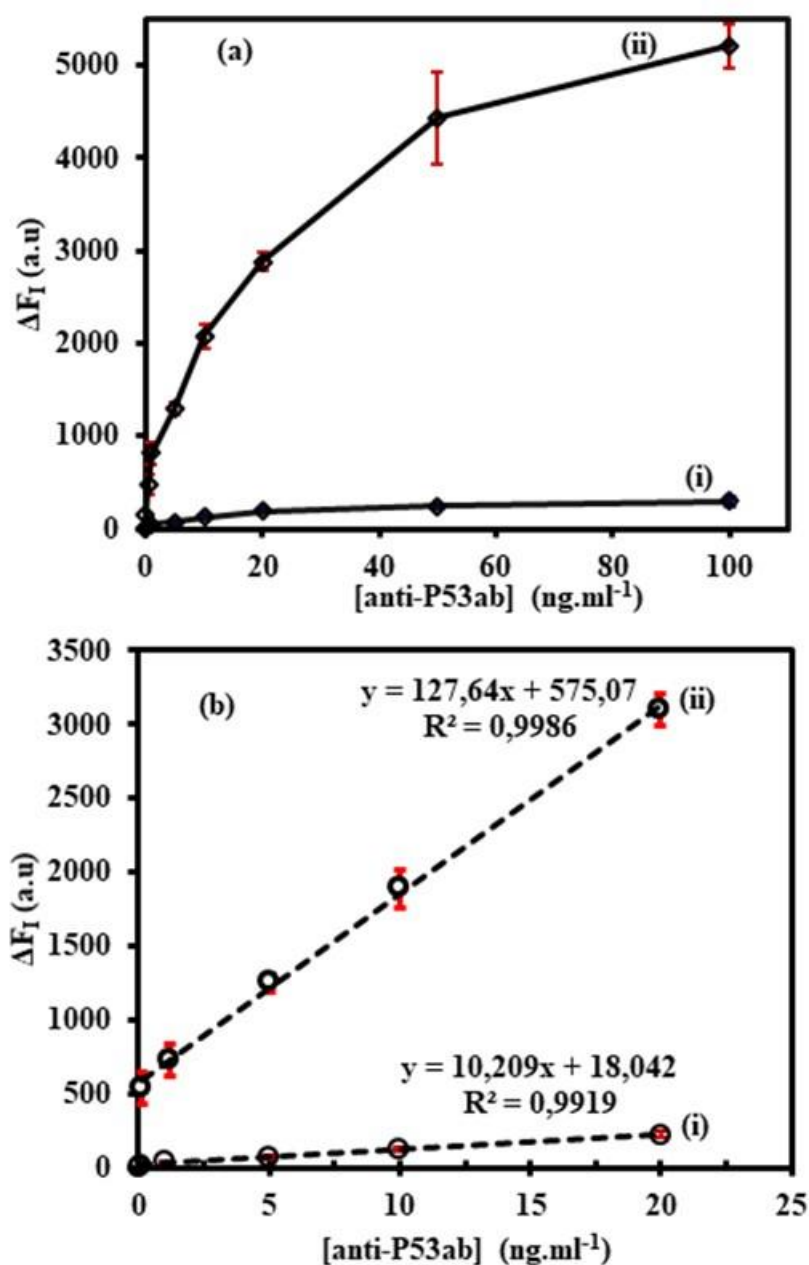


Figure 4.13: (a) dose-response curve, (b) linear calibration curve of fluorescent emission intensity to different concentrations of anti-P53ab (i) before ($t = 0$), and (ii) after ($t = 60$ mins) addition of NaOH dissolution buffer.

The lower fluorescent signal intensity before the addition of the NaOH dissolution buffer was due to fluorescence ‘TURN-OFF’ at high dye loading due to the HOMO-FRET mechanism. The enhancement of the fluorescent signal intensity was due to the ‘TURN on’ of the fluorescent emission of the FITC molecules released from the dissolved 6.5 % w/w FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe into solution. A particle of the 6.5 % w/w FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe releases about 2870 FITC dye molecules after dissolution, thus an enhanced fluorescence emission intensity. Also, the fluorescence intensity of FITC increases with increasing pH and has maximum emission in alkaline solution [322]. The sodium hydroxide dissolution buffer leads to a maxima FITC fluorescence emission. The 6.5 % FITC@SiNP-APTES-PEI-anti-IgG dissolution-based detection yielded a fluorescent intensity enhancement factor of about 23-fold at lower anti-P53ab concentrations (0.1 and 1.0 ng.ml⁻¹). The signal enhancement factor was 17 % at high antibody concentrations (100.0 ng.ml⁻¹) when compared with the signal intensity before dissolution.

There was a good linear relationship between the relative fluorescence intensity ($\Delta F_I = F_I - F_o$) and the anti-P53ab concentration in the range 100.0 pg.ml⁻¹ to 20.0 ng.ml⁻¹, **Figure 4.13 (b)**. F_I and F_o are the fluorescence intensity of the positive and negative control samples. The linear regression equation obtained before and after the addition of dissolution buffer was given by **Equations 4.5 and 4.6**; respectively.

$$\Delta F_I = 10.21 [\text{anti-P53ab}] + 18.04, \quad R^2 = 0.998 \quad (4.5)$$

$$\Delta F_I = 127.64 [\text{anti-P53ab}] + 578.8, \quad R^2 = 0.991 \quad (4.6)$$

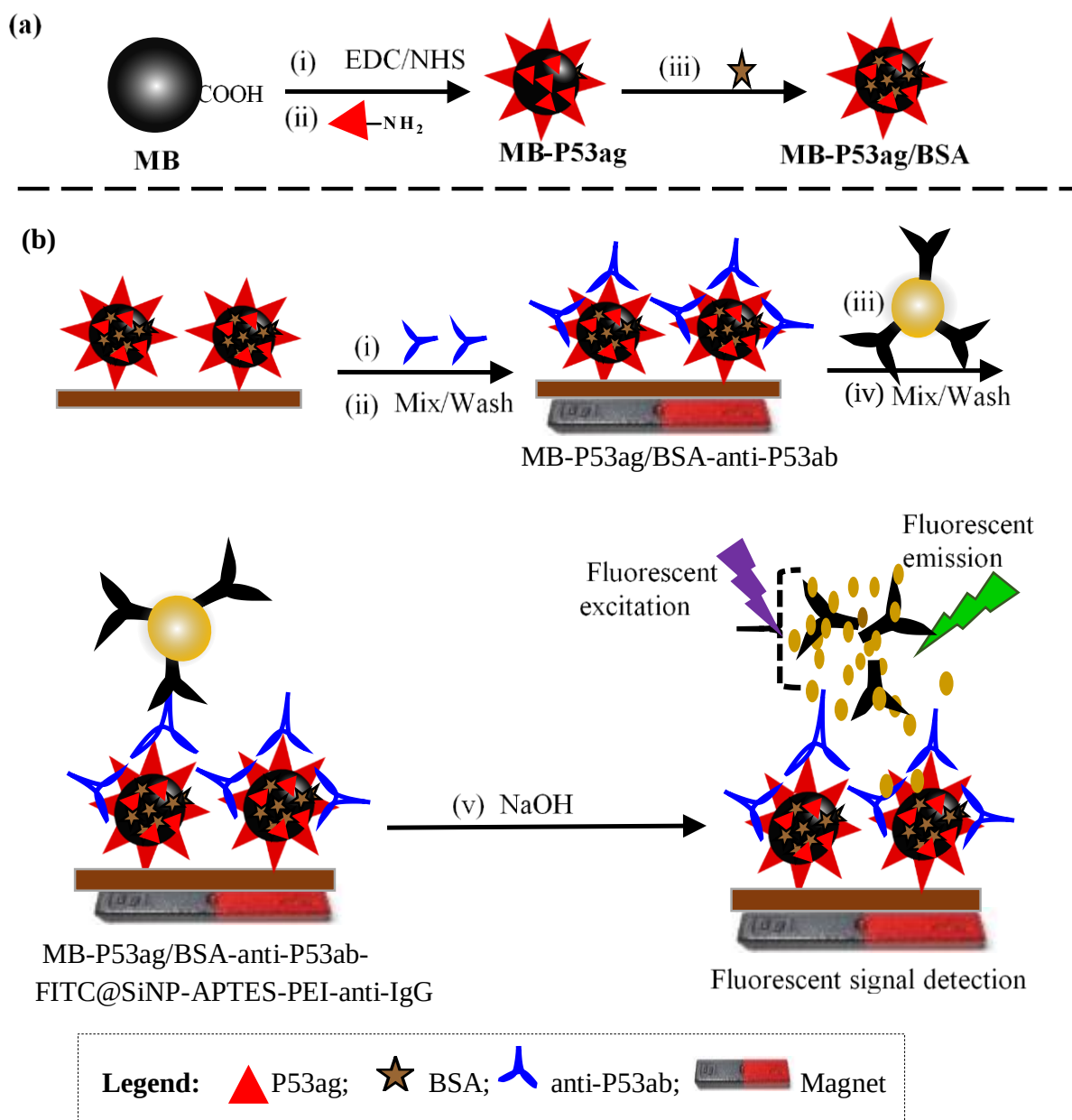
The sensitivity of detection was estimated from the slope of the regression equations and was found to be 10.21 (a.u).ml.ng⁻¹ and 127.64 (a.u).ml.ng⁻¹; respectively. The sensitivity was 12.5-fold higher after the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe dissolution. The LoD was calculated to be 460.0 pg.ml⁻¹ and 42.0 pg.ml⁻¹ for before and after the addition of

dissolution buffer respectively. The LoQ was 1.57 ng.ml⁻¹ and 0.42 ng.ml⁻¹ for before and after the addition of dissolution buffer; respectively. The limit of detection was about 10-fold lower after the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe dissolution. The LoD and LoQ were calculated according to the 3 σ /s criteria described in **section 1.6**. The σ of the negative control samples was 1.568 and 5.361; respectively.

4.5. Magnetic bead-based Fluorescent silica nanoparticles for detection of anti-P53ab

The combination of magnetic beads and fluorescence probes have advantages for rapid detection and high integration for the development of point-of-care lab-on-a-chip immunobiosensor. The development of a lab-on-a-chip immunobiosensor for anti-P53ab detection was conducted as proof of concept. The analytical performance of the FITC@SiNP-APTES-PEI-anti-IgG as the detection nanobioprobe and magnetic bead coated with P53ag as the sensing element was evaluated off-chip. The schematic illustration of the off-chip immunobiosensor fabrication steps is shown in **Scheme 4.3**. The human P53-antigen protein (P53ag) was immobilized on the magnetic bead (MB-P53ag) and incubated with BSA to block the non-specific binding sites. The P53ag/BSA modified magnetic bead (MB-P53ag/BSA) was used to isolate the anti-P53ab from the spiked serum samples leading to the formation of MB-P53ag/BSA-**anti-P53ab** immunocomplex. An external magnet was used to separate the MB-P53ag/BSA-**anti-P53ab** immunocomplex and transferred into a PBST washing buffer solution with mixing to remove non-specifically bound anti-P53ab. The MB-P53ag/BSA-**anti-P53ab** immunocomplex solution and the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe were incubated at room temperature for 30 min. The FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe binds explicitly with the Fc-region of the captured anti-P53ab as previously described. The resulting MB-P53ag/BSA-**anti-P53ab**-FITC@SiNP-APTES-PEI-anti-IgG immunocomplex was collected and washed with an external magnet to separate the unbound FITC@SiNP-APTES-PEI-anti-IgG. The isolated immunocomplex was transferred into the

dissolution buffer and incubated at 40 °C for 30 min to release the loaded FITC-dye molecules into solution. The fluorescent emission intensity was quantified using a fluorescence plate reader. The fluorescent intensity is related to the concentration of the anti-P53ab in the serum samples.



Scheme 4.3: Schematic illustration of (a) covalent immobilization of P53ag on the magnetic bead, and (b) detection of anti-P53ab with the MB-P53ag and FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe.

4.5.1. Optimization of immunoassay conditions

The amount of the MB-P53ag/BSA, the concentration of the FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe, the incubation time, and serum dilution ratio was optimized. **Figure 4.14** (a) shows the effect of increasing MB-P53ag/BSA concentration (1.0 to 250.0 $\mu\text{g}.\text{ml}^{-1}$) on the fluorescent intensity and (b) effect of incubation time on the immunocomplex formation between anti-P53ab with MB-P53ag/BSA. The concentration of anti-P53ab (50.0 $\text{ng}.\text{ml}^{-1}$) and the 50 μl of FITC@SiNP-APTES-PEI-anti-IgG (200.0 $\mu\text{g}.\text{ml}^{-1}$) were kept the same. The fluorescence intensity increases up to 100.0 $\mu\text{g}.\text{ml}^{-1}$ of MB-P53ag/BSA. A further increase in the amount of MB-P53ag/BSA above 100.0 $\mu\text{g}.\text{ml}^{-1}$ does not affect the fluorescence intensity. Therefore, after the addition of 100.0 $\mu\text{g}.\text{ml}^{-1}$, a maximum number of anti-P53ab have been captured, and further increase does not have a significant effect. Thus, 100.0 $\mu\text{g}/\text{ml}$ of the MB-P53ag/BSA was chosen as the optimum concentration.

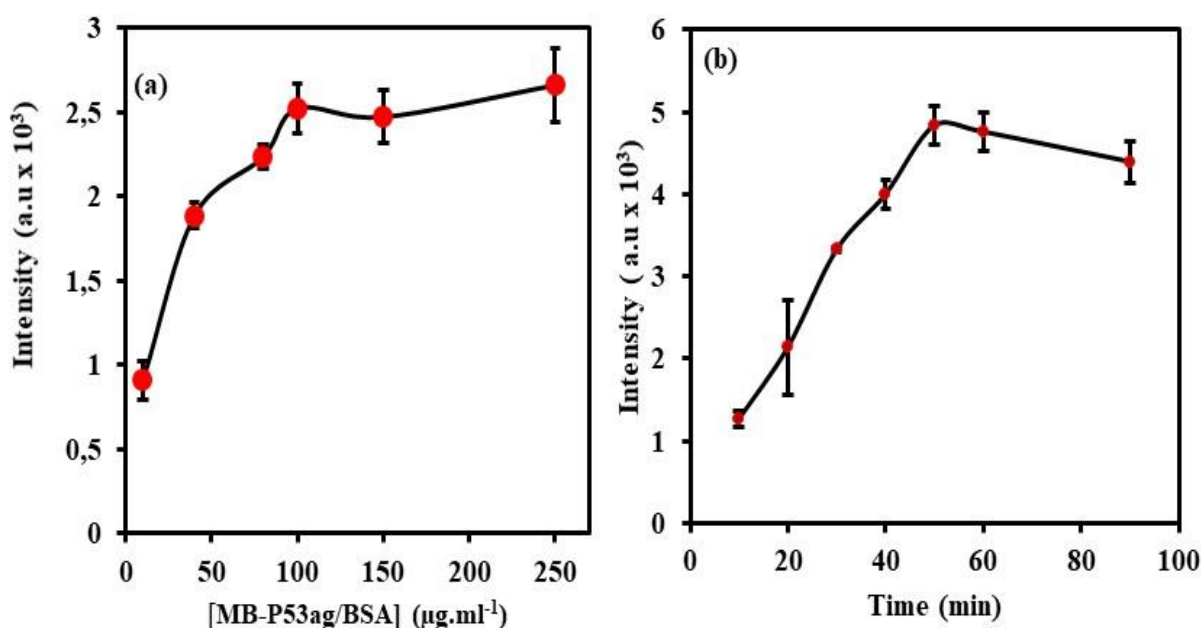


Figure 4.14: Fluorescence intensity of the detection system (a) at different concentrations of MB-P53ag/BSA, and (b) different incubation time upon the addition of 50.0 $\text{ng}.\text{ml}^{-1}$ anti-P53ab.

The time for the incubation (under slow mixing) of the MB-P53ag/BSA ($100.0 \mu\text{g}.\text{ml}^{-1}$) with the anti-P53ab ($50.0 \text{ ng}.\text{ml}^{-1}$) and $50.0 \mu\text{l}$ of FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe ($200.0 \mu\text{g}.\text{ml}^{-1}$) was optimized. **Figure 4.14 (b)** shows that the fluorescent intensity increases linearly with increasing incubation time up to 50 minutes. The dissolution buffer condition and dissolution time were optimized in the previous section, $1.0 \text{ mmol}.\text{L}^{-1}$ NaOH (pH 10.0) and incubation for 60 minutes at 40°C were selected as the optimum condition.

4.5.2. Detection of anti-P53ab using MB-P53ag/BSA and FITC@SiNP-APTES-PEI-anti-IgG

The analytical performance of the MB-P53ag/BSA as anti-P53ab capture and FITC@SiNP-APTES-PEI-anti-IgG was investigated using the optimized conditions. The different concentrations of anti-P53ab were obtained by serial dilution in 10 % v/v serum/ PBS solution and were used as the positive control samples. BSA (1.0 %) diluted in the same matrix was used as the negative control sample. **Figure 4.15** shows (a) the fluorescence emission spectra (b) dose-response curve and (c) analytical calibration curve of the detection system with changes in the concentrations of anti-P53ab ranging from $1.5 \text{ pg}.\text{ml}^{-1}$ to $100.0 \text{ ng}.\text{ml}^{-1}$. The result shows that the sensing approach could distinguish between the positive and the negative control samples. The average fluorescent signal recorded for the negative control samples was found to be 15.03 ± 1.71 for $n = 6$. The value is significantly lower than the fluorescence intensity found for the lowest concentration of anti-P53ab ($1.5 \text{ pg}.\text{ml}^{-1}$) which was 164.80 ± 1.71 for $n = 6$. This indicated that the MB-P53ag/BSA and FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe based immunobiosensor platform shows the high capability to differentiate between serum samples of patients expressing anti-P53ab and a healthy individual. The dose-response curve, **Figure 4.15 (b)** depicted that the fluorescence intensity increases gradually with increasing anti-P53ab.

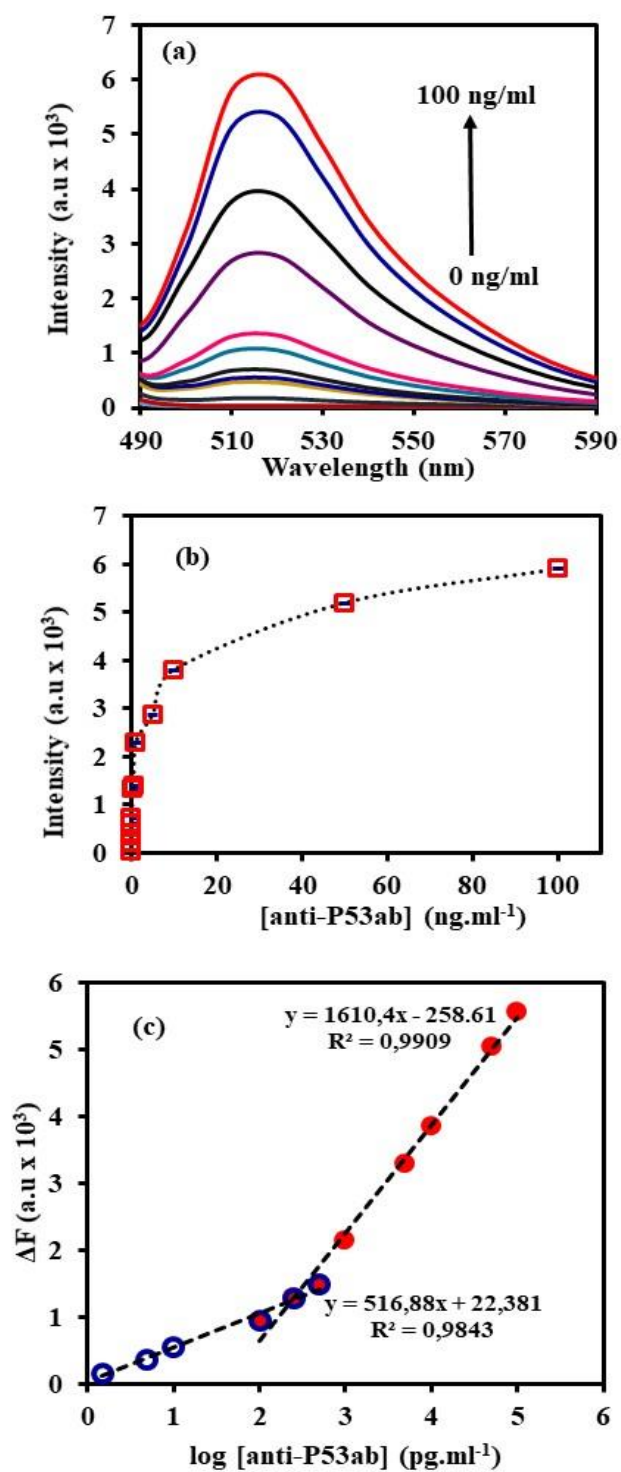


Figure 4.15: (a) Fluorescence spectra of different concentration of anti-P53ab (1.5 pg.ml⁻¹ to 100.0 ng.ml⁻¹) and corresponding (b) dose-response curve, and (c) analytical calibration curves.

The calibration curve was constructed by plotting the difference in the fluorescence intensity of the positive and negative controls ($F_i - F_o$) against the logarithm of the anti-P53ab concentrations, **Figure 4.15 (c)**. Two linear dynamic ranges were obtained. At lower concentrations, the linear range was from 1.5 pg.ml⁻¹ to 500.0 pg.ml⁻¹, and at higher concentrations it was 500.0 pg.ml⁻¹ to 100.0 ng.ml⁻¹. The regression equations for the two linear range were given by **Equation (4.6)** and **(4.7)** for lower and higher concentrations; respectively.

$$\Delta F_i = 516.88 \log [\text{anti-P53ab}] + 22.38, R^2 = 0.984 \quad (4.6)$$

$$\Delta F_i = 1610 \log [\text{anti-P53ab}] - 258.61, R^2 = 0.991 \quad (4.7)$$

The LoD and LoQ were calculated from the standard deviation of the negative control ($\sigma = 1.71$, $n = 6$) as previously described. The LoD and LoQ were obtained to be 9.92 fg.ml⁻¹ and 33.21 fg.ml⁻¹, respectively from the sensitivity of **Equation (4.6)**. The LoD and LoQ calculated from the sensitivity of **Equation (4.7)** were 3.20 fg.ml⁻¹ and 10.62 fg.ml⁻¹, respectively.

The analytical performance of the magnetic bead-based immunobiosensor was compared with previously reported methods. **Table 1.8** summarises the comparison of the analytical performance characteristics of different anti-P53ab sensing systems. The obtained results showed that the MB-P53ag/BSA and FITC@SiNP-APTES-PEI-anti-IgG nanobioprobe sensing platform gave a good analytical performance for the detection of anti-P53ab in serum. The LoDs of the magnetic-bead (MB-P53ag/BSA) based sensing platform (3.20 fg.ml⁻¹ and 9.92 fg.ml⁻¹) were lower than the microwell plate-based (MTP-P53ag/BSA) sensing platform (42.0 pg.ml⁻¹) discussed in **section 4.6**. The linear range of the MB-P53ag/BSA sensing platform (15.0 pg.ml⁻¹ to 100.0 ng.ml⁻¹) was wider than that of the MTP-P53ag/BSA (100.0 pg.ml⁻¹ to 20.0 pg.ml⁻¹). Also, the LoDs of the developed FITC@SiNP-APTES-PEI-anti-IgG

based immunobiosensors was lower than that of the label-free EIS based immunobiosensor (0.103 ng.ml⁻¹) reported in above.

Table 4.3. Comparison of the analytical performance with some related works

Sensing interface	Detection probe	Detection Techniques	Linear range (ng.ml ⁻¹)	LoD	Ref.
Pentadentate peptide	Label free	EIS	-	10.0 fg.ml ⁻¹	[95]
MP-P53ag/BSA	anti-IgG-HRP	colorimetric	1.56 -100	0.390 ng.ml ⁻¹	[141]
GCE-RGO/Au-P53ag	Label-free	SQWV	0.001 - 0.1	88.0 fg.ml ⁻¹	[166]
SiO ₂ QC-APTES-GA-P53ag	Anti-IgG	QCM-D	-	-	[263]
AuSiW-MUA-P53ag	Label-free	cantilever	20 - 2000	20.0 ng.ml ⁻¹	[323]
Au@NPFe ₂ O ₃ NC-P53ag	anti-IgG-HRP	colorimetric	-	-	[240]
Au-PEA-SA-P53ag/BSA	Label-free	EIS	1 - 1000	0.103 ng.ml ⁻¹	Present work
MTP-P53ag/BSA	FITC@SiNP-APTES-PEI-anti-IgG	Fluorescence	0.1 -20	42.0 pg.ml ⁻¹	Present work
MB-P53ag/BSA	FITC@SiNP-APTES-PEI-anti-IgG	Fluorescence	^a 0.015- 0.5 ^b 0.5 - 100	^a 9.92 fg.ml ⁻¹ ^b 3.30 fg.ml ⁻¹	Present work

The analytical performance was compared with previously reported immunobiosensor for anti-P53 antibody detection. The LoD's of the developed FITC@SiNP-APTES-PEI-anti-IgG-based fluorescence immunobiosensor (42.0 pg.ml⁻¹, 9.92 fg.ml⁻¹ and 3.30 fg.ml⁻¹) were lower than the conventional commercial ELISA (0.390 ng.ml⁻¹), the microcantilever-based immunosensor (20 ng.ml⁻¹) and the magnetic bead microfluidic chips (4.0 ng.ml⁻¹). The LoD was comparable to that reported for the peptide-based impedimetric immunobiosensor (10.0 fg.ml⁻¹), and the Au NP/ERGO hybrid based electrochemical immunobiosensor (88.0 fg.ml⁻¹). The MB-

P53ag/BSA and FITC@SiNP-APTES-PEI-anti-IgG nanoprobe based immunobiosensor reported in this chapter have the following advantages:

- (i) The magnetic bead and FITC@SiNP is low cost, easy to synthesize and environmentally stable,
- (ii) The covalent immobilization of the P53ag and anti-IgG provides high stability and reproducibility,
- (iii) The detection steps are rapid, simple and highly sensitive,
- (iv) The lower detection limit was achieved because of the synergetic combination of the large surface area of the magnetic bead, and
- (v) The high fluorescence efficiency of the FITC@SiNP nanoparticle after dissolution with 0.1 mM NaOH.

4.6. Chapter conclusions

Highly sensitive, low cost and efficient fluorescence-based immunobiosensor based on the FITC@SiNP-APTES-PEI-anti-IgG nanoprobe has been developed to detect anti-P53ab. Two different methodologies involving the use of MBs modified with P53ag (MB-P53ag/BSA), and microtiter plate (MTP-P53ag/BSA) as capture was developed. FITC@SiNP-APTES-PEI-anti-IgG was used as the detection probe in both methodologies. The developed immunobiosensors exhibit an excellent performance to provide an accurate determination of levels of anti-P53ab in serum. The LOD of the magnetic bead-based immunobiosensor (3.30 fg.ml^{-1}) and the microwell plate-based immunobiosensor (42.0 fg.ml^{-1}) were both lower than the conventional ELISA and some of the previously reported anti-P53ab immunobiosensors. This high sensitivity could help determine the ultra-trace level of anti-P53ab at the early stage of carcinogenesis. Furthermore, FITC@SiNP is cheap, environmentally stable, and have enhanced fluorescence emission. Hence the developed immunobiosensor had significant

advantages over the commonly used anti-IgG-HRP-based ELISA systems for the detection of anti-P53ab. The developed immunobiosensor have the complete capability for development into a lab-on-a-chip device for the screening of lung cancer patient in low resource settings.

Chapter 5:

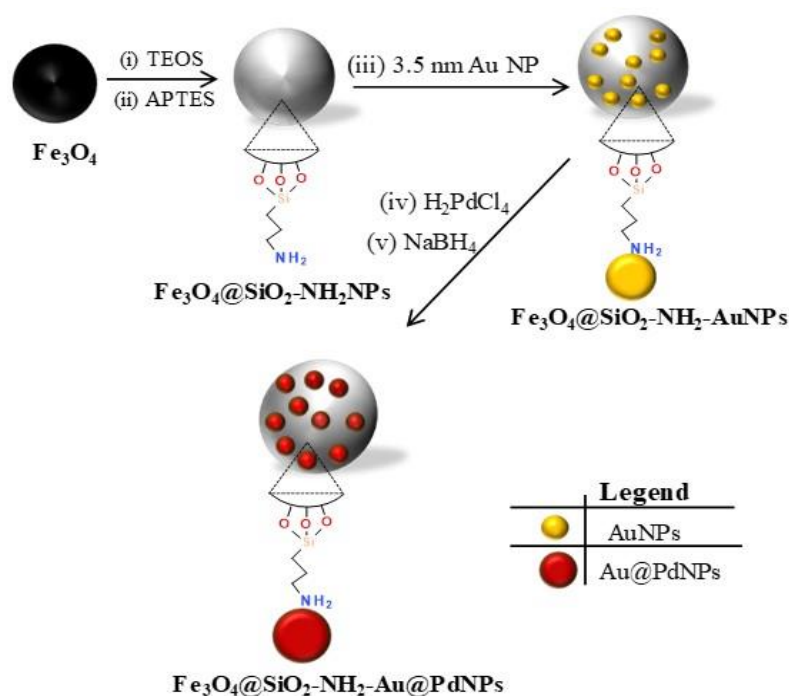
Peroxidase-Like Activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ Nanohybrids

5. Peroxidase-like Activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$

This chapter reports on the synthesis and characterization of a novel nanohybrid material consisting of gold, palladium, and magnetite ($\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$). The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ possesses magnetic properties for ease separation, high thermal stability, catalytic activity, and high surface area. The peroxidase-like activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ nanohybrid was evaluated. The effect of experimental parameters on the peroxidase-like activity was investigated. The steady-state kinetic assay and the mechanism of the peroxidase-like activity were reported.

5.1. Synthesis of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ nanohybrid

Scheme 5.1 shows the schematic illustration for the synthesis of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The synthesis of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ involved three steps. First, highly monodispersed magnetite nanoparticles (Fe_3O_4) were synthesized by thermal decomposition of the iron-oleate complex in 1-octadecene. The oleic acid capped Fe_3O_4 were coated with silica shell using the water-in-oil microemulsion system to form silica-coated magnetite nanoparticles. The mechanism for the ligand exchange of the hydrophobic magnetite nanoparticles by alkoxy silane within the water-in-oil microemulsion systems to obtain hydrophilic magnetite (Fe_3O_4) core-silica shell nanoparticles ($\text{Fe}_3\text{O}_4@\text{SiNP}$) has previously been reported [324,325]. The silica shell was covalently formed on the Fe_3O_4 core via the phase transfer of the oleic acid with hydrolyzed alkoxy groups of TEOS. The transfer of the hydrophilic silanol capped magnetite into the water-in-oil-microemulsion nanoreactor occurs for the subsequent growth of the silica shell. The $\text{Fe}_3\text{O}_4@\text{SiNPs}$ were isolated by breaking the microemulsion via pH adjustment to < 2.0 , periodic cooling in liquid nitrogen, and the addition of acetone. The pH adjustment and cooling with liquid nitrogen prevent the necking and agglomeration of the $\text{Fe}_3\text{O}_4@\text{SiNPs}$ [324]. The $\text{Fe}_3\text{O}_4@\text{SiNPs}$ were modified with APTES to form an amine-functionalized surface that serves as an anchor for gold and palladium.



Scheme 5.1: Schematic representation of the synthesis of $\text{Fe}_3\text{O}_4@\text{SiNP-Au@Pd-APTES}$.

The second step involves the growth of small-sized gold nanoparticles (< 3.5 nm, AuNPs) on the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$. The 3.5 nm gold nanoparticle was dispersed onto the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ support via nitrogen-gold (Au-N) interaction. The color of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ solution changes from dark grey to reddish-brown confirming the formation of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$. The third step is the growth of palladium on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ to form $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The formation of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ was achieved via the seed-mediated method [268,326,327]. The reduction of H_2PdCl_4 using low concentrations of NaBH_4 results in the formation of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The sequential addition of a low concentration of NaBH_4 induces the homogenous reduction of Pd^{2+} on the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$. The homogenous reduction prevents the formation of new Pd nuclei. The < 3.5 nm gold seeds on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ act as the nucleation sites for the Pd^{2+} and results in the formation of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The concentration of H_2PdCl_4 was varied to obtain $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ with different amounts of Pd.

5.2. Characterization of Fe₃O₄@SiNP-APTES-Au@Pd

5.2.1. UV-vis characterization of Fe₃O₄@SiNP-APTES-Au@Pd

Figure 5.1 shows the UV-vis spectra of (i) Fe₃O₄@SiNP, (ii) Au NPs, (iii) Fe₃O₄@SiNP-APTES-Au, and (iv) Fe₃O₄@SiNP-APTES-Au@Pd. The UV-vis absorption spectra of the aqueous dispersion of AuNPs shows surface plasmon resonance (SPR) absorption maximum at 514 nm. This absorption is ascribed to the characteristic SPR absorption of small spherical AuNPs with a size of less than 10 nm [268]. The spectrum of Fe₃O₄@SiNP-APTES shows no bands. The absorption spectrum of the Fe₃O₄@SiNP-APTES-Au shows a weak SPR band centered at 527 nm. This confirms the deposition of the Au nanoparticles on the Fe₃O₄@SiNP-APTES to form Fe₃O₄@SiNP-APTES-Au. The AuNPs peak at 527 nm disappeared after the reduction of Pd²⁺ onto Fe₃O₄@SiNP-APTES-Au to form Fe₃O₄@SiNP-APTES-Au@Pd. The masking of the AuNPs SPR confirms the formation of the palladium shell on the deposited Au seed. The disappearance of the SPR band is consistent with the previous report on bimetallic (Au-M) nanoparticles with Au-core-metallic shell nanostructure [328].

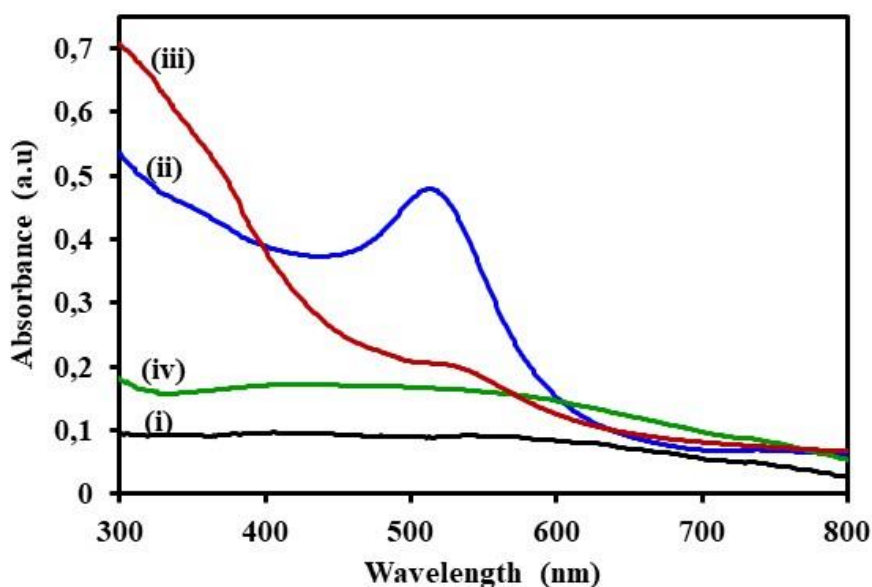


Figure 5.1: UV-vis absorption spectra of (i) Fe₃O₄@SiNP-APTES (ii) 3.5 nm Au NP, (iii) Fe₃O₄@SiNP-APTES-Au, and (iv) Fe₃O₄@SiNP-APTES-Au@Pd aqueous dispersion.

5.2.2. FTIR characterization

The evidence for the chemical bonding interaction and functional groups were obtained by FTIR spectroscopy. **Figure 5.2** shows the FTIR spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4\text{SiNP}$, (iii) $\text{Fe}_3\text{O}_4\text{@SiNP-APTES}$, and (iv) $\text{Fe}_3\text{O}_4\text{SiNP-APTES-Au@Pd}$. The FTIR spectrum of Fe_3O_4 shows aliphatic C-H stretching vibrations at 2916 cm^{-1} and 2846 cm^{-1} and carbonyl stretching vibration at 1710 cm^{-1} . These characteristic bands were attributed to the hydrocarbon backbone of the oleic acid capping on the Fe_3O_4 . The broad peak at 3367 cm^{-1} was due to the O-H stretching vibrations. The bands at 536 cm^{-1} and 444 cm^{-1} correspond to the presence of Fe-O stretching and bending vibrations; respectively. After the coating of the Fe_3O_4 with the silica shell, the carbonyl and C-H stretching vibrations were not observed, **Figure 5.2 (ii)**. This shows that there was a ligand exchange of the oleic acid on the Fe_3O_4 with the hydrolyzed silanol groups within the water-in-oil microemulsion system. This results in the formation of $\text{Fe}_3\text{O}_4\text{@SiNP}$ core-shell nanoparticles.

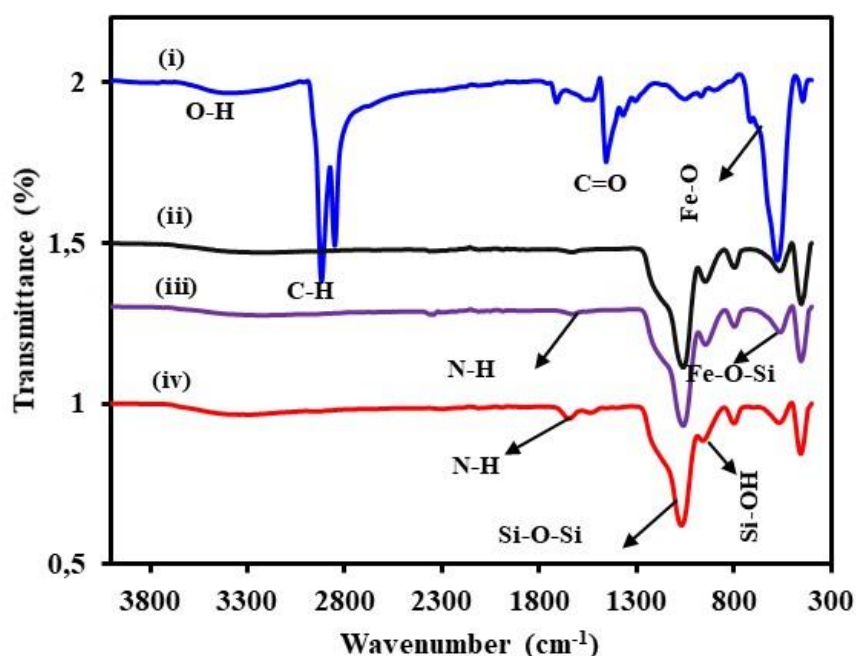


Figure 5.2: FTIR spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4\text{@SiNP}$, (iii) $\text{Fe}_3\text{O}_4\text{@SiNP-APTES}$ and (iv) $\text{Fe}_3\text{O}_4\text{@SiNP-APTES-Au@Pd}$.

The FTIR spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP}$ in **Figure 5.2 (ii)** shows vibrational bands at 1066 cm^{-1} , 797 cm^{-1} , and 951 cm^{-1} . The bands were attributed to the asymmetrical and symmetrical vibration of the Si-O-Si and Si-OH of the silica shell. The intensity of the Fe-O stretching decreases and shifted to 562 cm^{-1} and 452 cm^{-1} . The shift in the bands showed that the silica shell was coated on the surface of Fe_3O_4 by Fe-O-Si bonds. Compared to the FTIR spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP}$, the FTIR spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES shows additional vibrational bands at 1537 cm^{-1} and 16485 cm^{-1} . These bands were attributed to the N-H stretching vibrations. The FTIR spectrum in **Figure 5.2 (iv)**, shows that the intensity of the N-H stretching vibration decreases after the deposition of the Au@Pd on the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES. The decrease in the intensity of N-H stretching vibration was due to the deposition of the Au@Pd on the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES via metal-nitrogen (Au-N) interaction.

5.2.3. Photon correlation spectroscopic characterization (Zeta potential)

Furthermore, the changes in the surface electro-kinetic parameters could be attributed to the dielectric function and composition of the resulting nanoparticle hybrid, which can be measured by the zeta potential. **Figure 5.3** shows the changes in the zeta potential at the different stages of $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd synthesis measured at pH 7.0. The zeta potential values indicate that the Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{SiNP}$ were negatively charged at neutral pH (7.0) with an isoelectric point at pH 4.0. The negative zeta potential values were ascribed to the presence of the hydroxyl and silanol groups on their surfaces. After the modification of the $\text{Fe}_3\text{O}_4@\text{SiNP}$ with APTES, the surface zeta potential at neutral pH increases from -27.2 mV to 31.4 mV and the isoelectric point shifted to pH 10. The changes in the value of the zeta potential to a more positive value (31.4 mV) confirm the grafting of APTES to the $\text{Fe}_3\text{O}_4@\text{SiNP}$ and the presence of amine groups. However, the deposition of the Au@Pd nanoparticles on the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES results in a shift of the zeta potential to a more negative value (-23.2 mV). The potential changes after the deposition of the bimetallic Au@Pd

were attributed to the Au-N interaction and the increase in the electron density due to the metal ions. The net changes in the zeta potential value suggest the successful deposition of Au@Pd bimetallic nanoparticle on the Fe₃O₄@SiNP-APTES. With increasing pH, the surface zeta potential of the Fe₃O₄@SiNP-APTES-Au@Pd became negative due to the combined effect of the metal, capping agents and residual hydroxyl groups on the silica.

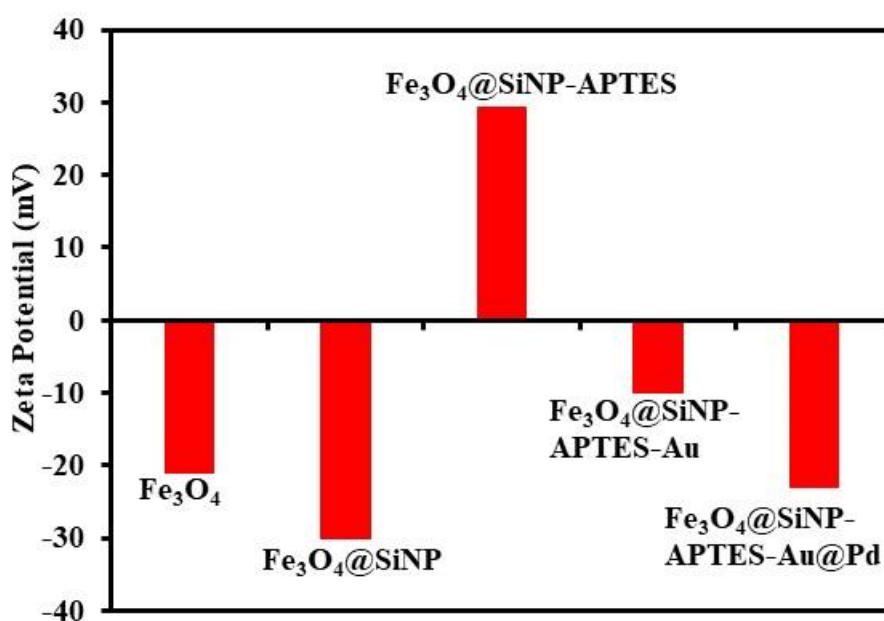


Figure 5.3: Changes in Zeta potential at different steps of the synthesis of Fe₃O₄@SiNP-APTES-Au@Pd measured at pH 7.0.

5.2.4. XRD characterization

Figure 5.4 shows the X-ray diffraction (XRD) pattern of (a) (i) Fe₃O₄, (ii) Fe₃O₄@SiNP-APTES, (iii) Fe₃O₄@SiNP-APTES-Au, (iv) Fe₃O₄@SiNP-APTES-Au@Pd, and (b) Fe₃O₄@SiNP-APTES-Au@Pd synthesized with different ratio of Au to Pd (i) Fe₃O₄@SiNP-APTES-Au₁@Pd₁, (ii) Fe₃O₄@SiNP-APTES-Au₁@Pd₂, (iii) Fe₃O₄@SiNP-APTES-Au₁@Pd₃, and (iv) Fe₃O₄@SiNP-APTES-Au₁@Pd₅. The XRD pattern of Fe₃O₄ in **Figure 5.4 (a) (i)** showed six characteristic diffraction patterns at 2θ of 30.2°, 35.8°, 43.4°, 54.2°, 57.8°, and 62.8°. The diffraction pattern was indexed for Miller Indices (220), (311), (400), (422), (511) and

(440) crystal planes. These are typical of pure magnetite (Fe_3O_4) with a cubic spinel structure having a lattice parameter of 8.375 Å (JCPDS No: 03-065-3107). The high crystallinity of the Fe_3O_4 nanoparticles was evident from the sharp diffraction pattern. The average crystallinity size (diameter, d) calculated according to the Scherer equation based on the XRD pattern of peak 311 as the base peak was approximately 15 nm. There was no evidence of diffraction pattern due to other oxides of iron, confirming the high purity of the synthesized Fe_3O_4 nanoparticle. The XRD diffraction pattern of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ in **Figure 5.4 (a) (ii)** showed that the crystalline diffraction pattern of the Fe_3O_4 core was retained after silica coating. The broad diffraction band at $2\theta < 28^\circ$ corresponds to the amorphous nature of the silica and confirms the coating of the magnetic core with a silica shell.

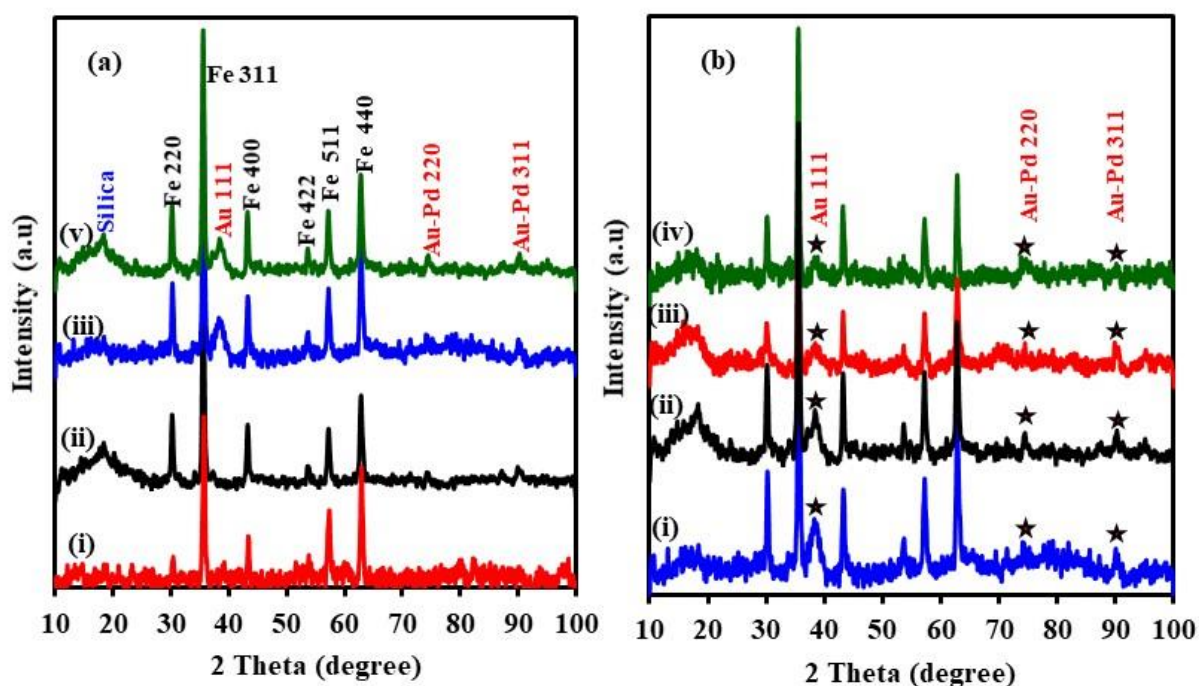


Figure 5.4: XRD pattern of (a) (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, and (b) (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_1$, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_2$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_5$.

The XRD diffractograms of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ showed a new characteristic diffraction pattern at $2\theta = 38.5^\circ$, 68.05° , and 77.8° . The diffraction pattern was indexed for the Miller Indices (111), (220) and (311) planes of face-centered cubic (fcc) structure of crystalline Au and Pd. In **Figure 5.4 (a) (iii) and (iv)**, the broadness of the Au (111) diffraction at 38.5° suggested that the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ consists of a distinct small-sized Au nanoparticle. **Figure 5.4 (a) (iv)** showed that after the deposition of Pd on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, the intensity of the Au (111) peak decreased. The decrease in the intensity of the peaks was due to the absorption of X-rays by the Pd-shell. Hence the signal from the Au core decreased [329]. The decrease in the intensity confirms the formation of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The broadness of the peaks was attributed to the strain induced by the Pd shell on the Au core and the small size of the nanoparticle [330,331]. Furthermore, the XRD pattern of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ synthesized with a different mole ratio of Au to Pd were examined. In **Figure 5.4 (b)**, the diffraction pattern of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ with different ratio of Au to Pd showed a gradual decrease in the intensity of the Au (111) at $2\theta = 38.5^\circ$ with an increase in the composition of palladium. The band almost vanishes at higher Pd concentrations. Also, the intensity of the new peaks at 74.5° , and 90.4° attributed to the (220) and (311) of AuPd increases with increasing concentration of Pd in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The observations from the XRD pattern confirmed that; (i) small-sized bimetallic Au@Pd core-shell nanoparticles were grown on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ to form $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, (ii) the composition of palladium in $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ increases as the concentrations of palladium used for the synthesis increased and (ii) the palladium shell thickness increases with increasing Pd % weight.

5.2.5 Microscopic characterization

Figure 5.5 shows the scanning electron microscope (SEM) micrographs of the powdered samples of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The SEM micrographs show that Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ nanoparticles consisted of nearly smooth spherical nanoparticles. In **Figure 5.5 (iii-iv)**, after the deposition of the Au, and Pd on the surface of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, the Au, and Au@Pd nanoparticles deposition can be observed. The SEM images show that the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ remain discrete nanoparticles and a minimal level of agglomerates was observed.

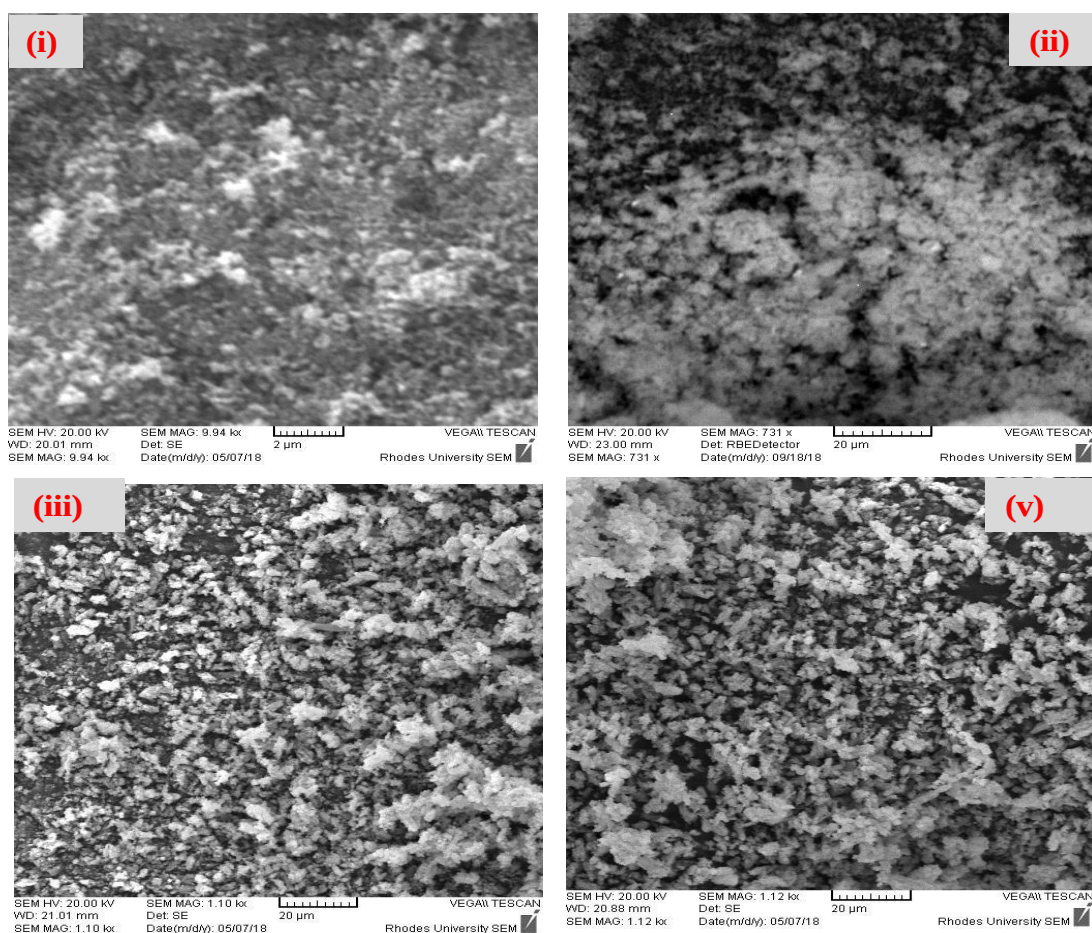


Figure 5.5: SEM images of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$.

The SEM images of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ in **Figure 5.5 (iv)** shows that the particles have a bigger size and appears to be more agglomeration. There were no noticeable morphological changes in the SEM-micrograph of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ prepared from different Au to Pd molar ratio.

The transmission electron microscope (TEM) micrographs were used to confirm the detailed morphology and nanoscale dimensions of the samples. **Figure 5.6** shows the TEM micrograph and corresponding size distribution histograms of (a) AuNPs, (b) Fe_3O_4 , (c) $\text{Fe}_3\text{O}_4@\text{SiNP}$, and (d) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The TEM micrograph shows that the synthesized AuNPs were monodispersed and spherical. The size of the AuNP ranges from 2.6 nm to 4.6 nm and the average particle size was 3.5 ± 0.5 nm. The Fe_3O_4 in **Figure 5.6 (b)** are spherical and highly monodispersed with a narrow size distribution between 13 nm and 17 nm. The average size of Fe_3O_4 was 14 ± 1.0 nm, which corresponds to the size obtained from the XRD (15 nm). The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ has an average size of 40 ± 2.0 nm, indicative of about 13 nm silica thickness around the core Fe_3O_4 nanoparticles, **Figure 5.6 (c)**. From the TEM micrograph, the core-shell was visible with a single Fe_3O_4 core and a silica shell. The TEM micrograph of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ showed agglomeration. This agglomeration was attributed to the high surface energy of the small-sized Au@Pd and attractive van der Waals forces between $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ particles. The TEM micrograph of a single $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, inset **Figure 5.6 (d)** shows the presence of numerous small-sized dark spots which are the Au@Pd nanoparticles. These spots were not observed in the $\text{Fe}_3\text{O}_4@\text{SiNP}$ image. The average diameter of the Au@Pd nanoparticles was 8.0 ± 1.5 nm. This indicated the growth of about 2.5 nm palladium shell thickness around the AuNP core. The images confirmed the successful deposition of Au@Pd and formation of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ nanoparticle.

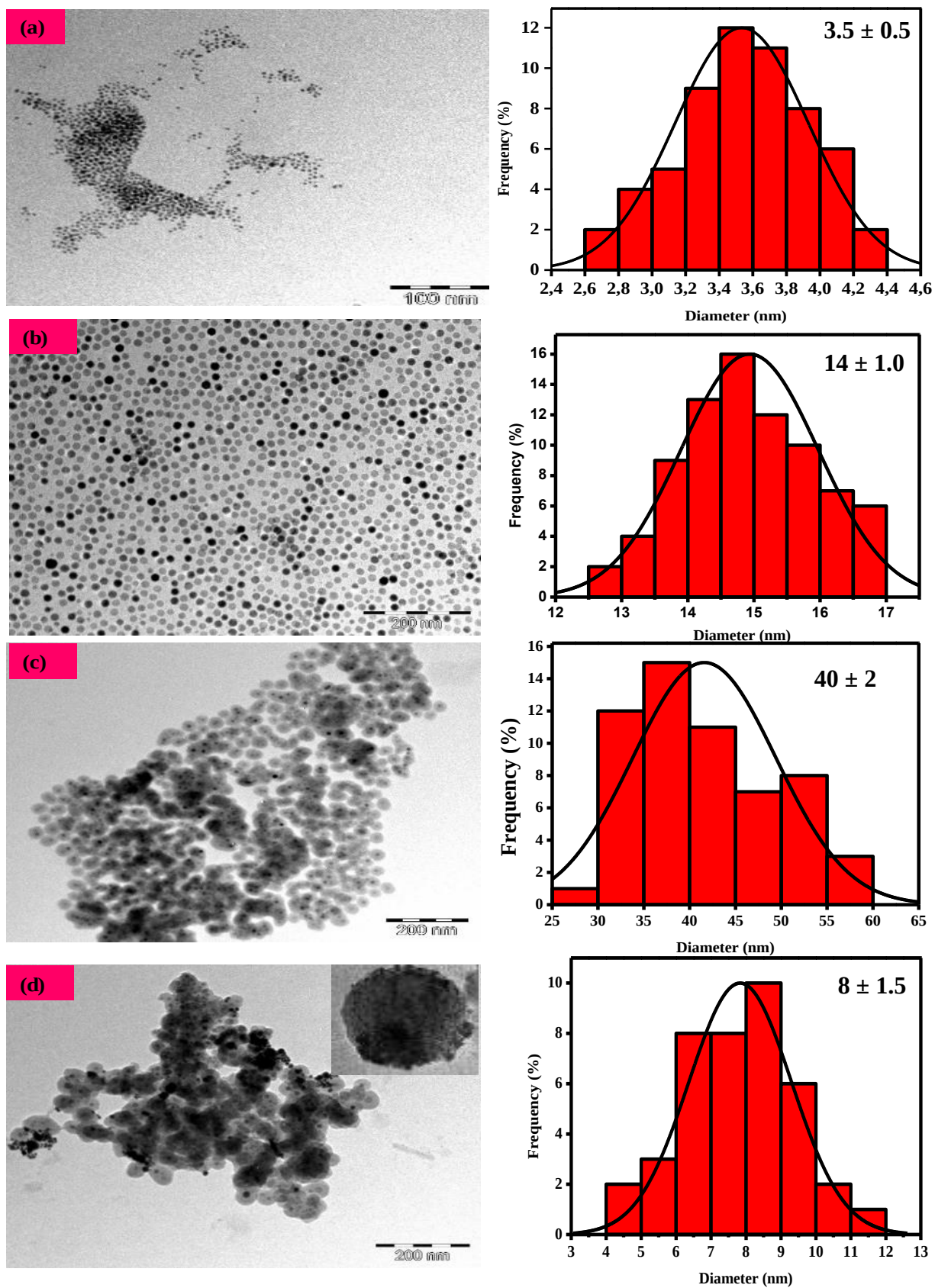


Figure 5.6: TEM micrograph of (a) Au NPs, (b) Fe₃O₄, (c) Fe₃O₄@SiNP, and (d) Fe₃O₄@SiO₂-APTES-Au@Pd.

The samples were subjected to an energy dispersive X-ray spectroscopic (EDS) analysis to confirm the elemental composition. The EDS spectra were obtained from the SEM images in **Figure 5.5**. **Figure 5.7** shows the EDS spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The weight percentage of the elements were summarised in **Table 5.1**. The EDS spectrum of Fe_3O_4 nanoparticles showed the presence of iron, oxygen, and carbon with weight percentages of 66.5 %, 29.5 %, and 4.0 %. The EDS spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP}$ shows the presence of a signal for Fe, O, C, and Si with weight percentages of 45.9 %, 37.2 %, 14.7 %, and 2.2 %; respectively. The presence of the Si signal confirms the coating of the Fe_3O_4 with silica. In **Figure 5.7 (iii)**, the Au signal confirms the successful deposition of AuNPs on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$. The elemental weight percentage composition of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ were 33.3 %, 49.7 %, 12.1 %, 0.7 %, and 4.5 % for Fe, O, Si, C, and Au; respectively. The EDS spectrum in **Figure 5.7 (iv)** substantiated that the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ was constituted of Fe, O, Si, C, Au, and Pd. The existence of Au and Pd confirmed the successful synthesis of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The weight percentages were 30 %, 44.4 %, 14.8 %, 6.2 %, 1.5 %, and 3.1 % for Fe, O, Si, C, Au, and Pd; respectively.

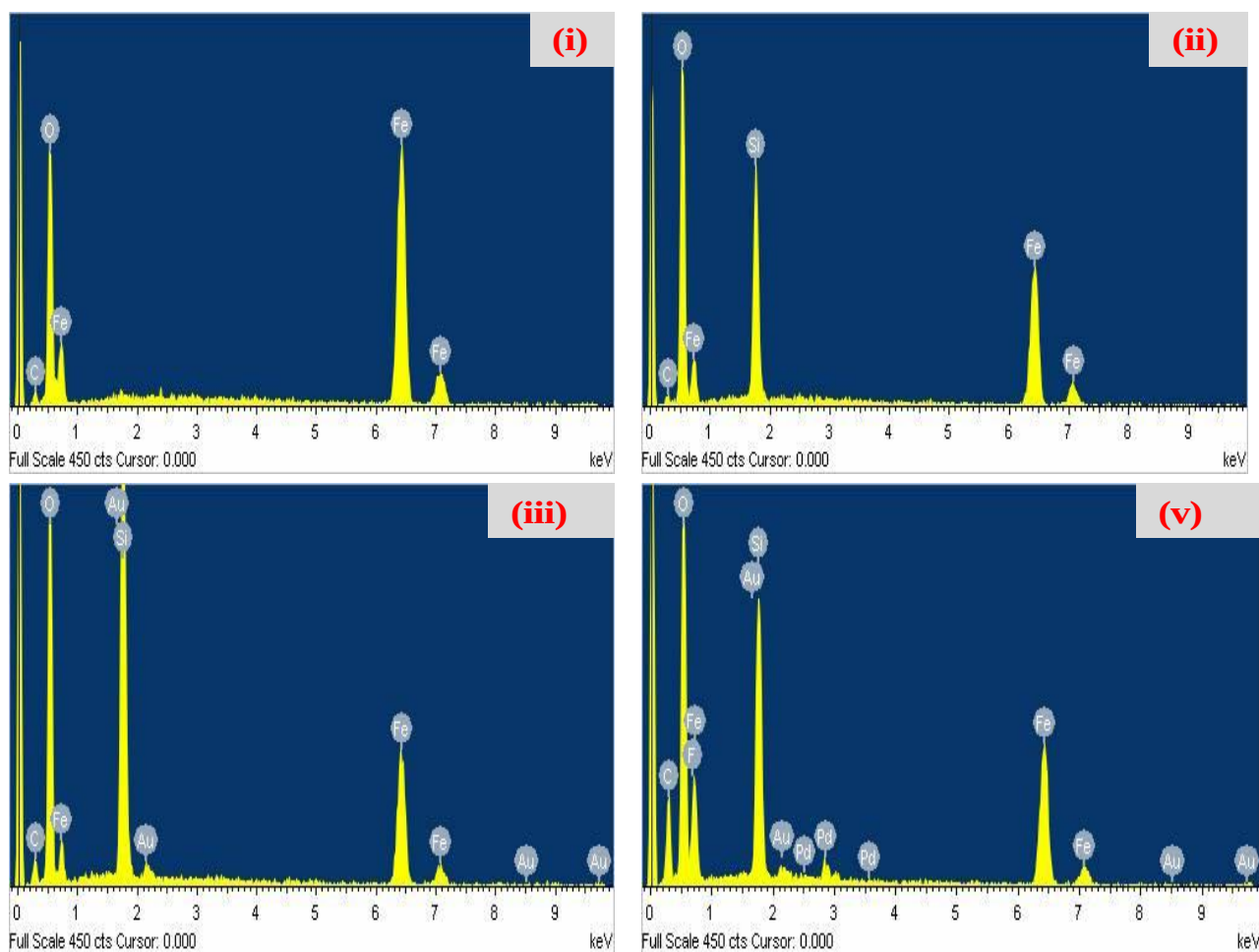


Figure 5.7: EDS spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$.

Table 5.1: Elemental composition (wt. %) obtained from EDS analysis.

Sample	Elemental composition (wt %)						
	Fe	O	Si	C	Au	Pd	Total
Fe_3O_4	66.5	29.5	-	4.0	-	-	100
$\text{Fe}_3\text{O}_4@\text{SiNP}$	45.9	37.2	14.7	2.2	-	-	100
$\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$	33.3	49.6	12.1	0.6	4.4	-	100
$\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$	30.0	44.4	14.8	6.2	1.5	3.1	100

The EDS elemental mapping images were acquired to investigate the distribution of the elements in $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. **Figure 5.8** shows the elemental mapping of Fe, Si, C, Pd and Au of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The EDS elemental mapping images were acquired from the electron image of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ in **Figure 5.8 (i)**. The purple color (assigned to Fe) and the green color (assigned to Si) are uniformly distributed in the sample. This indicated that the Fe and Si were homogeneously distributed within the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The distribution of other elements is not as dense as the Si and Fe. This indicates that Au and Pd were anchored on the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The distribution of the red color (assigned to Pd) and (orange assigned to Au) was uniform indicating the formation of a core-shell Au-Pd structure. The EDS mapping reveals that Au and Pd are homogeneously distributed in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$.

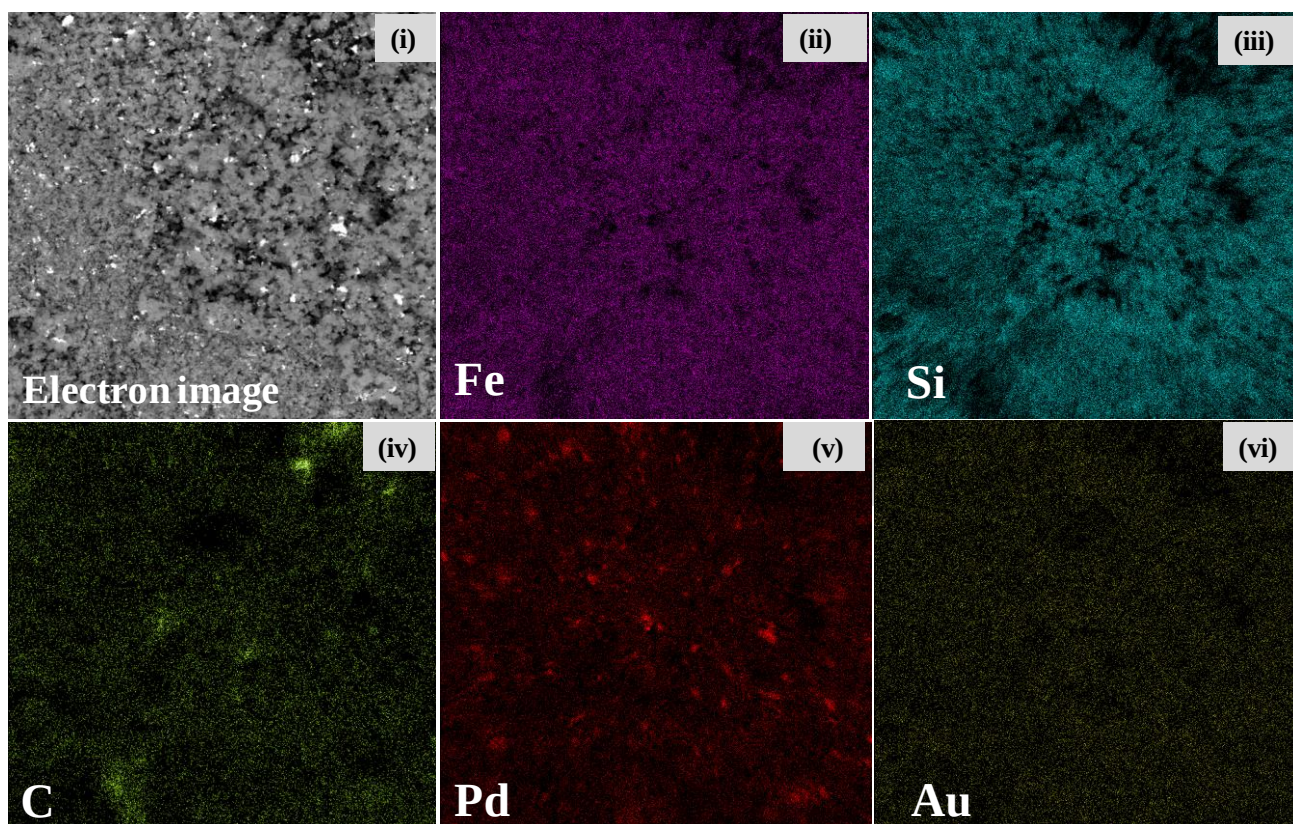


Figure 5.8: (i) SEM image and EDS elemental mapping of the area for (ii) Fe (iii) Si, (iv) C (v) Pd and (vi) Au.

5.2.6. X-ray photoelectron spectroscopic characterization

5.2.6.1. Survey XPS spectra

XPS analysis was used to characterize the various steps in the synthesis of the $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd as shown in **Scheme 5.1**. **Figure 5.9** shows the survey spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd. The spectrum of Fe_3O_4 in **Figure 5.9 (i)** show photoelectron emission at binding energies of 54 eV, 92 eV, 283 eV, 531 eV, 711 eV and 722 eV. These were attributed to the binding energy of Fe 3p, Fe 3s, C 1s, O 1s, Fe 2p³, and Fe 2p¹; respectively. The XPS spectrum confirms the presence of Fe, C, and O in the Fe_3O_4 . The occurrence of carbon in Fe_3O_4 was due to the hydrocarbon backbone of the oleic acid used as the capping agent.

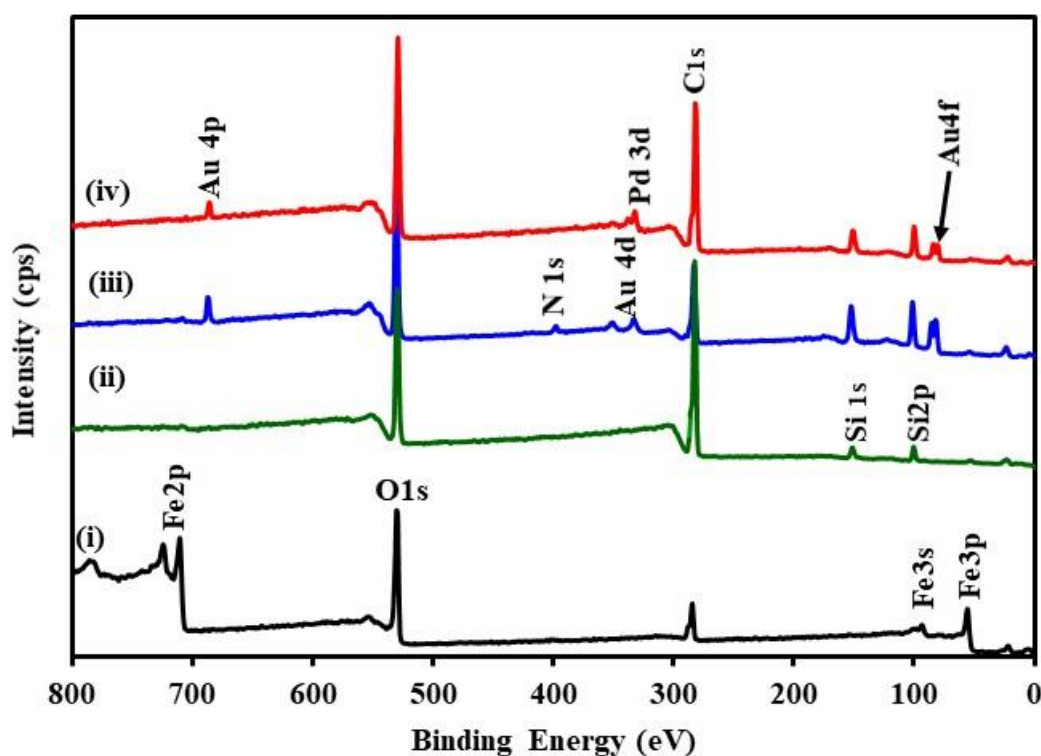


Figure 5.9: XPS survey spectra of (i) Fe_3O_4 , (ii) $\text{Fe}_3\text{O}_4@\text{SiNP}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd.

XPS confirmed the coating of Fe₃O₄ with a silica shell. The survey spectrum of Fe₃O₄@SiNP in **Figure 5.9 (ii)** shows the disappearance of the Fe peaks (Fe 3p, Fe 3s, and Fe 2p). This was attributed to the formation of silica shells around Fe₃O₄ because XPS is a surface-specific technique it can only reach about 10 nm depth of the sample [332]. The attenuation of the X-ray by the silica shell prevents its penetration to the core Fe₃O₄ nanoparticles. As a result, the signals due to Fe 2p were not found in the XPS scan of Fe₃O₄@SiNP, Fe₃O₄@SiNP-APTES-Au and Fe₃O₄@SiNP-APTES-Au@Pd hybrid. Two new peaks at 100 eV and 152 eV in addition to C 1s and O 1s were observed in the spectrum of Fe₃O₄@SiNP. These new peaks were assigned to the binding energy of Si 2p (100 eV) and Si 2s (152 eV). The surface of Fe₃O₄@SiNP is composed of C, O, and Si. The presence of carbon could be attributed to the adsorption of Triton-X used during synthesis. In **Figure 5.9 (iii)**, the survey spectrum of Fe₃O₄@SiNP-APTES-Au showed additional peaks at 82 eV, 331 eV, 686 eV, and 392 eV. The peaks were assigned to Au 4f, Au 4d, Au 4p, and N 1s, respectively. The presence of the N 1s peaks confirmed the successful modification of Fe₃O₄@SiNP with APTES. The Au peaks (Au 4f, Au 4d, and Au 4p) showed that Au NPs were successfully deposited on Fe₃O₄@SiNP-APTES. The appearance of the Pd 3d peak at 333.5 eV in **Figure 5.9 (iv)** confirmed the deposition of Pd onto the Fe₃O₄@SiNP-APTES-Au to form Fe₃O₄@SiNP-APTES-Au@Pd. The elements present on the surface of Fe₃O₄@SiNP-APTES-Au@Pd are Si, Au, C, O, and Pd, which corroborate the EDS elemental mapping results.

The survey spectra were analyzed further to obtain quantitative information. **Table 5.2** shows the elemental composition of the nanoparticles at each stage of Fe₃O₄@SiNP-APTES-Au@Pd synthesis. The atomic percentages (at %) of Fe 2p, C 1s and O 1s were 31.3 %, 30.2 % and 38.5 %, respectively. After the silica shell coating, Fe 2p was not detected due to thick silica coating. The atomic percentages composition of C 1s, O 1s and Si 2p were 71.6 %, 20.5 %, and 7.9 %; respectively. The atomic % composition of O 1s and Si 2p increased to 34.4 % and

17.3 %; respectively for Fe₃O₄@SiNP-APTES-Au. The percentage of nitrogen in Fe₃O₄@SiNP-APTES-Au was 1.7 %, and the composition of Au was 1.1 %. The atomic percentages of Au, Pd, Si, C, and O in Fe₃O₄@SiNP-APTES-Au@Pd determined by XPS was 0.4 %, 0.7 %, 9.4 %, 63.0 %, and 26.5 %; respectively. The atomic composition of the Au 4f in Fe₃O₄@SiNP-APTES-Au was 1.1 %, this value decreased to 0.5 % for the Fe₃O₄@SiNP-APTES-Au@Pd. The atomic % ratio of Au to Pd (Au/Pd) in Fe₃O₄@SiNP-APTES-Au@Pd was 1.6 %. An important feature of the survey spectra in **Figure 5.9** is the changes in the ratio of O 1s to Si 2p (O/Si) ratio. The O/Si ratio of Fe₃O₄@SiNP was 2.6; the value was higher the stoichiometric ratio of 1:2 for SiO₂. The O/Si ratio decreased to 2.0 in Fe₃O₄@SiNP-APTES-Au. The decrease could be attributed to an increase in the composition of Si relative to O after APTES modification and deposition of Au. The O/Si ratio increased from 1.9 to 2.8 for the Fe₃O₄@SiNP-APTES-Au@Pd. The increase in the O/Si ratio could be attributed to the formation of metal oxide on the surface of Fe₃O₄@SiNP-APTES-Au@Pd. The XPS results showed the successful synthesis of the Fe₃O₄@SiNP-APTES-Au@Pd nanohybrid.

Table 5.2: Atomic composition (at %) obtained from XPS analysis.

Sample	Atomic composition (%)							Atomic ratio (%)	
	Fe 2p	C 1s	O 1s	Si 2p	N 1s	Au 4f	Pd 3d	O/Si	Au/Pd
Fe₃O₄	31.3	30.2	38.5	-	-	-	-	-	-
Fe₃O₄@SiNP	-	71.6	20.5	7.9	-	-	-	2.6	-
Fe₃O₄@SiNP-APTES-Au	-	45.6	34.4	17.2	1.7	1.1	-	1.9	-
Fe₃O₄@SiNP-APTES-Au@Pd	-	63.0	26.5	9.4	-	0.4	0.7	2.8	1.8

5.2.6.2. High-resolution XPS studies

The high-resolution XPS spectra were recorded to investigate the metal-matrix interaction, the binding energy of the electrons, and the valence state of the element present in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ hybrid. The high-resolution XPS for the Fe 2p in Fe_3O_4 , and the Si 2p, C 1s, O 1s, N 1s, Au 4f, and Pd 3d present in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ hybrid surface were analyzed. The high-resolution XPS spectrum of Fe 2p in Fe_3O_4 is shown in the supplementary data in **Figure S5.1**. The Fe 2p spectrum of Fe_3O_4 shows that the Fe 2p core levels split into Fe 2p_{3/2} and Fe 2p_{1/2} components due to spin-orbit coupling. The Fe 2p_{3/2} and Fe 2p_{1/2} components are at binding energy (BE) of 711.2 eV and 724.40 eV; respectively. The ΔE of Fe 2p_{3/2} and Fe 2p_{1/2} was 13.2 eV which is comparable to 13.1 eV for pure magnetite [333]. This shows that the Fe_3O_4 was pure magnetite (Fe_3O_4). The Fe 2p_{3/2} peak was the convolution of both Fe^{2+} and Fe^{3+} oxidation state in the Fe_3O_4 . The Fe^{2+} is octahedrally coordinated, and the Fe^{3+} distributed over both octahedral (oct) and tetrahedral oxidation state [334]. The partially filled 3d levels gave rise to complex d-d multiplets splitting [334,335]. The Fe 2p_{3/2} spectrum in **Figure S5.1** was well fitted to multiplet peaks with three main peaks and three satellite peaks for the 2p_{3/2}. After fitting the Fe 2p_{3/2} spectrum, the lowest binding energy at 710.10 eV was attributed to the octahedral Fe^{2+} while the Fe^{3+} was observed at 711.10 eV. The tetrahedral Fe^{2+} was observed at 712.30 eV, and Fe^{3+} was 714.30 eV. The same pattern was repeated for the Fe 2p_{1/2} component. The fittings agree with reported work in the literature [333,335–337]. The peak ratio of the 2p_{3/2} to 2p_{1/2} was found to be 1.63, which was close to the theoretical value of 2. The ratio of the Fe^{2+} to Fe^{3+} was 0.61 for the 2p_{3/2} transition. The ratio was higher than the 0.5 expected from the stoichiometric mole ratio. The high-resolution of Fe 2p spectrum shows that the Fe_3O_4 is pure magnetite, having Fe^{2+} and Fe^{3+} oxidation state. The Fe 2p was not observed after silica coating. Thus further analysis of the Fe 2p after silica coating was not reported.

Figure 5.10 shows the high-resolution spectra of Si 2p (a) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (b) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, (c) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, and O 1s (a') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (b') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (c') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The deconvoluted Si 2p high-resolution spectra gave the chemical environment of the silica coating after each modification step. The XPS spectra of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ surfaces in **Figure 5.10 (a), (b), and (c)**; respectively were fitted into three components with binding energies at 102.2 eV, 103.4, and 105.1 eV. The components at 103.4 eV were associated to the Si-O-Si bonds and Si-OH groups widely present in sol-gel silica materials [338]. The component at 102.2 eV and 105.1 eV were attributable to the presence of Si-C [339] and Fe-O-Si bonds; respectively. The Si-C bond confirmed the grafting of 3-aminopropyl-silane of APTES in the nanohybrid, whereas the Fe-O-Si confirmed the covalent coating of Fe_3O_4 with silica shell.

The chemical state identification of the Si 2p spectra was confirmed by the corresponding spectral region associated with oxygen. The deconvolution of the O 1s high-resolution spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP}$ in **Figure 5.10 (a')** showed three chemical states of oxygen. The intense peak at 532.5 eV (91.3 %) corresponds to the bridging oxygen chemically bonded to Si (Si-O-Si) and non-bridging oxygen bonded to silicon (Si-O). The peak at 530.4 eV (1.4 %) and 535.2 eV (7.3 %) were due to the oxygen of the Fe-O bond and O-H bond of residual silanol groups; respectively. The Si 2p and O 1s XPS confirm the covalent coating of the Fe_3O_4 with the silica shell and APTES modification. **Figure 5.10 (b')** shows that the O 1s spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ was fitted into five components at binding energy of 530.4 eV (2.2 %), 531.7 eV (10.2 %), 532.9 eV (73.1 eV), 531. 3 eV (11.8 %), and 535.4 eV (2.7 %).

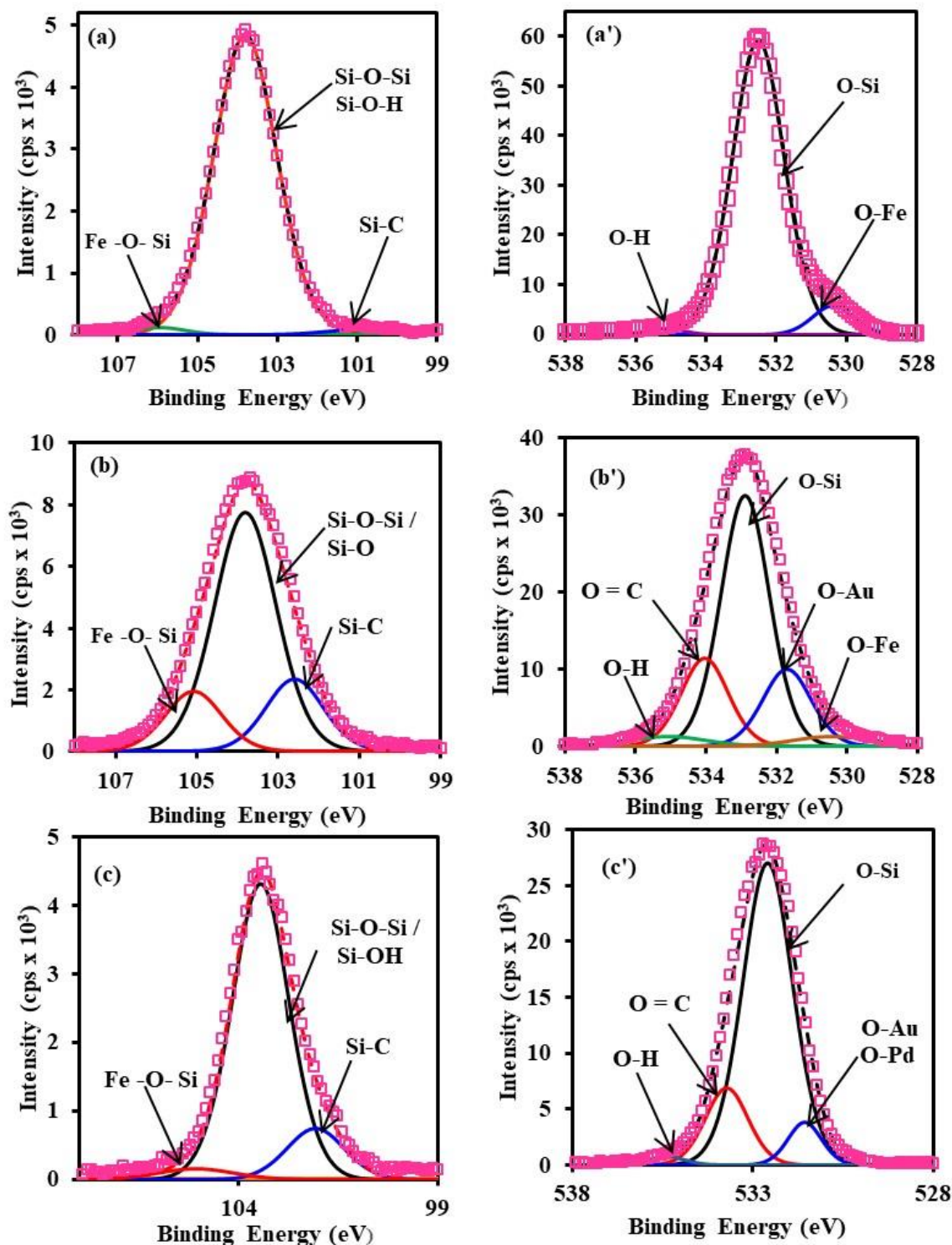


Figure 5.10: The high-resolution spectra of Si 2p (a) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$, (b) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (c) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, and O 1s (a') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ (b') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, and (c') $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$.

The binding energy of oxygen in the silica (O-Si) in Fe₃O₄@SiNP-APTES shifted to higher binding energy by 0.7 eV after the deposition of the Au in Fe₃O₄@SiNP-APTES-Au. Oxygen could interact with the deposited Au NPs via electron transfer to the Au NPs, thus leading to higher O 1s binding energy in Fe₃O₄@SiNP-APTES-Au. The peaks at 531.7 eV were attributed to the oxygen of the Au coordinated (O-Au) due to citrate capping of the Au NPs. The peak at 534.1 eV and 532.4 eV was attributed to the free carboxylic acid moieties (C-O, C = O) and C-OH of the citrate capping on the deposited Au NPs [340]. The O1s spectra of Fe₃O₄@SiNP-APTES-Au@Pd was fitted into four components at 532.0 eV (7.2 %), 532.6 eV (74.0 %), 533.7 eV (16.4 %) and 535.8 eV (2.4 %). The peak at 530.4 eV (O-Fe) and 531.7 eV (COO-Au) in Fe₃O₄@SiNP-APTES-Au were not observed after the deposition of Pd to form Fe₃O₄@SiNP-APTES-Au@Pd. This shows that the displacement of citrate capping on the deposited Au by the Pd. The new component at 533.7 eV in Fe₃O₄@SiNP-APTES-Au@Pd was attributed to oxygen bonded to palladium (Pd-O) from the adsorption of oxygen on the Pd [341]. **Figure 5.11** shows the high-resolution spectra of C 1s (a) Fe₃O₄@SiNP-APTES, (b) Fe₃O₄@SiNP-APTES-Au, (c) Fe₃O₄@SiNP-APTES-Au@Pd, and N 1s (a') Fe₃O₄@SiNP-APTES (b') Fe₃O₄@SiNP-APTES-Au, and (c') Fe₃O₄@SiNP-APTES-Au@Pd. The deconvoluted C 1s spectra show the presence of carbon components in the different chemical environment after each synthesis step. The C 1s spectrum of Fe₃O₄@SiNP-APTES in **Figure 5. 11(a)** was fitted into three components at 284.9 eV, 286.5 eV, and 288.4 eV. The most intense component seen at 284.9 eV was assigned to C-C and C-H bond [342] of unhydrolyzed TEOS and APTES. The components at 286.5 eV were attributed to an amine-linked carbon atom (C-N) of APTES [343]. The third component at 288.4 eV was attributed to the C-O bond of partially hydrolyzed APTES and TEOS. This confirms the successful modification with APTES. These peaks were common at all the stages of the Fe₃O₄@SiNP-APTES-Au@Pd synthesis and were given the same assignment as above.

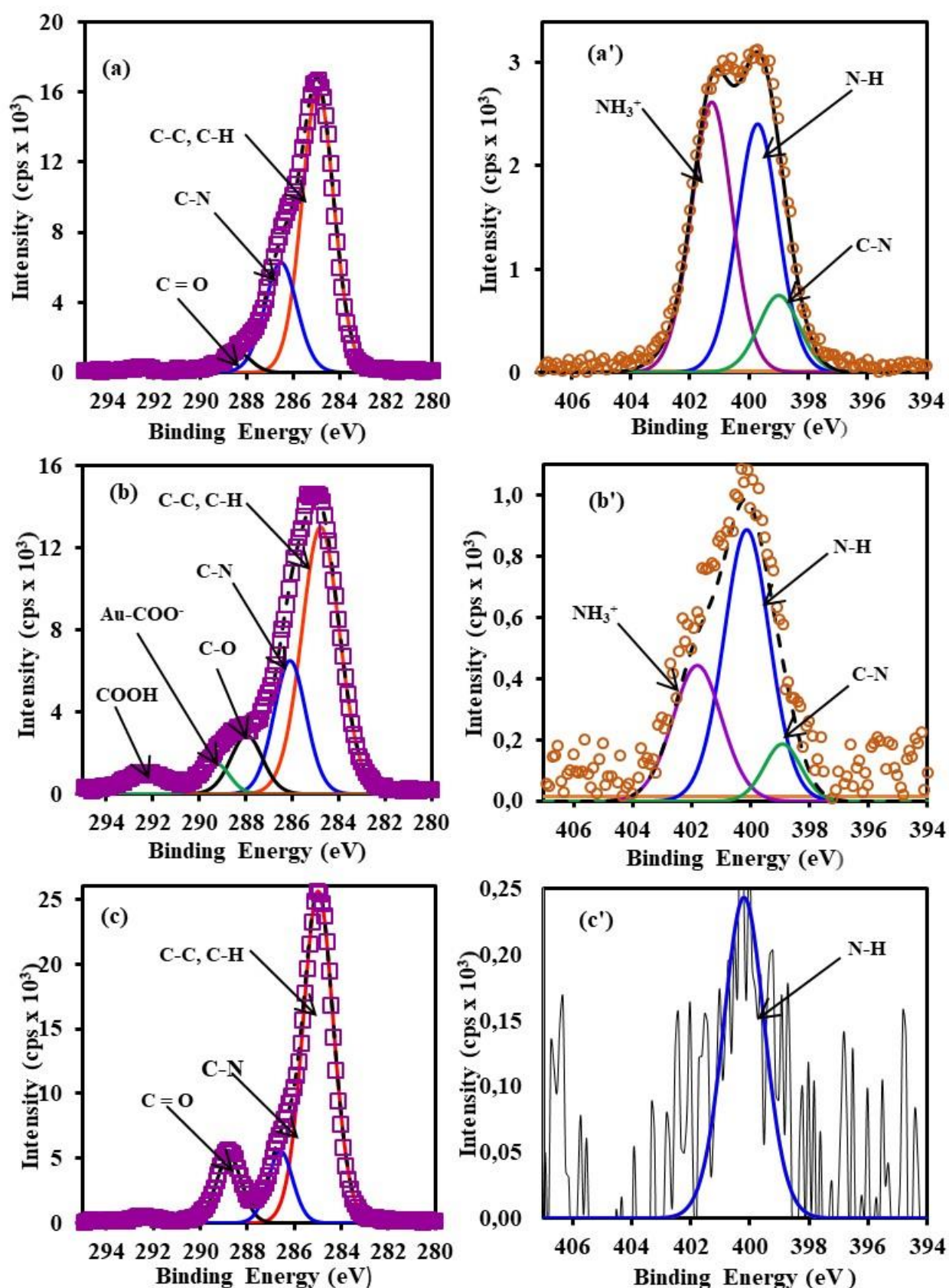


Figure 5.11: The high-resolution spectra of C 1s (a) Fe₃O₄@SiNP-APTES, (b) Fe₃O₄@SiNP-APTES-Au, and (c) Fe₃O₄@SiNP-APTES-Au@Pd, and of N 1s (a') Fe₃O₄@SiNP-APTES (b') Fe₃O₄@SiNP-APTES-Au, and (c') Fe₃O₄@SiNP-APTES-Au@Pd.

The C 1s spectrum of Fe₃O₄@SiNP-APTES-Au in **Figure 5.11 (b)** was deconvoluted into five components centred at 284.9 eV (C-C, C-H, 57.2 %), 286.5 eV (C-N, C-O, 24.1 %), 288.2 eV (C=O, 10.5 %), 289.5 eV (O-C=O, 4.6 %), and 291.5 eV (shake-up, 3.6 %). Thus, the peak at 288.2 eV was assigned to the Au-O of the citrate capping on the deposited AuNPs. The peak at 291.5 eV could be attributed to the shake-up [340]. The C 1s spectrum of Fe₃O₄@SiNP-APTES-Au@Pd in **Figure 5.11 (c)** was fitted into three components which are attributed to the C-C, C-H bonds at 284.9 eV, C-N, C-O at 286.6 eV and C=O at 288.8 eV. The peaks due to the citrate capping (Au-COO⁻) in Fe₃O₄@SiNP-APTES-Au were not observed in Fe₃O₄@SiNP-APTES-Au@Pd. This showed that the palladium shell was formed on the deposited Au NPs following the displacement of the citrate capping as observed in the O 1s spectrum of Fe₃O₄@SiNP-APTES-Au@Pd.

The deconvolution of the N1s spectra of the Fe₃O₄@SiNP-APTES in **Figure 5.11 (a')** was fitted into three components at 399.0 eV (N-H, 11.9 %), 399.7 eV (N-H, 50.9 %), and 401.3 eV (C-N, 37.2 %). The peak at 399.0 eV and 399.7 eV was attributed to the N-H and C-N of free terminal amine groups of APTES. Whereas the peak at the 401.3 eV was attributed to the protonated amine (NH₃⁺) and hydrogen-bonded NH₂. After the deposition of the Au NPs, the deconvoluted N1s spectrum of Fe₃O₄@SiNP-APTES-Au in **Figure 5.11 (b')** was fitted into three components at 398.9 eV (10.0 %), 400.2 eV (58.5 %), and 401.8 eV (31.5 %). The binding energy at 398.9 eV (Au-N) and 400.2 eV (N-H) in Fe₃O₄@SiNP-APTES shifted to 401.8 eV (C-N) for Fe₃O₄@SiNP-APTES-Au. The binding energy of the N 1s was upshifted by 0.5 eV due to the strong electrostatic interaction (charge transfer) between the nitrogen and gold (N-Au). This shows the evidence for the strong Au-N bond in the Fe₃O₄@SiNP-APTES-Au. The N 1s spectrum of Fe₃O₄@SiNP-APTES-Au@Pd in **Figure 5.11 (c')** was fitted into a component at 400.2 eV, which was attributed to the residual N-H groups of APTES on the surface of the Fe₃O₄@SiNP-APTES-Au@Pd.

The high-resolution XPS spectra of Au 4f and Pd 3d in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ were investigated. **Figure 5.12** shows the high-resolution Au 4f spectra of (a) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, (b) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, and (c) Pd 3d spectra of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The deconvoluted Au 4f high-resolution XPS spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ in **Figure 5.12 (a)** was fitted into five components with two pairs of doublets ascribed to the Au 4f_{7/2} and Au 4f_{5/2} with spin-orbit coupling ($\Delta E = 3.7$ eV). The most prominent peak at 83.6 eV (48.9 %) and 87.3 eV (41.9 eV) were assigned to Au4f_{7/2} and Au4f_{5/2} of metallic gold (Au^0). The value of the Au^0 binding energies in $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$ was about 0.4 eV lower than 84.0 for Au4f_{7/2} and 87.7 eV of Au 4f_{5/2} of pristine gold nanoparticle [344,345]. Such a negative shift in binding energy has been reported for active carbon-supported nanosized Au [346]. For $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$, the negative shift in binding energy could be attributed to the electron transfer from the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$ to Au nanoparticles, which cause a decrease in the Au 4f binding energy. The lowered binding energy showed the deposition of AuNPs via strong Au-N bonds. The peak at 85.5 eV (3.54 %) and 89.4 eV (4.4 %) were assigned to Au-N bond and surface Au^+ atom [347]. The Au nanoparticles bonded to nitrogen and interaction with the oxygen on the support results in the Au^{+1} oxidation state [348]. The Au^+ species were reported to results from the interface of the Au nanoparticles in contact with the support ($\text{Fe}_3\text{O}_4@\text{SiNP-APTES}$) [349]. The small peak at 90.9 eV (1.3 %) was attributed to the oxidized form (Au^{3+}), which could be attributed to the formation of gold oxide (Au_2O_3). This was associated with the interaction of the deposited Au with the oxygen on the support.

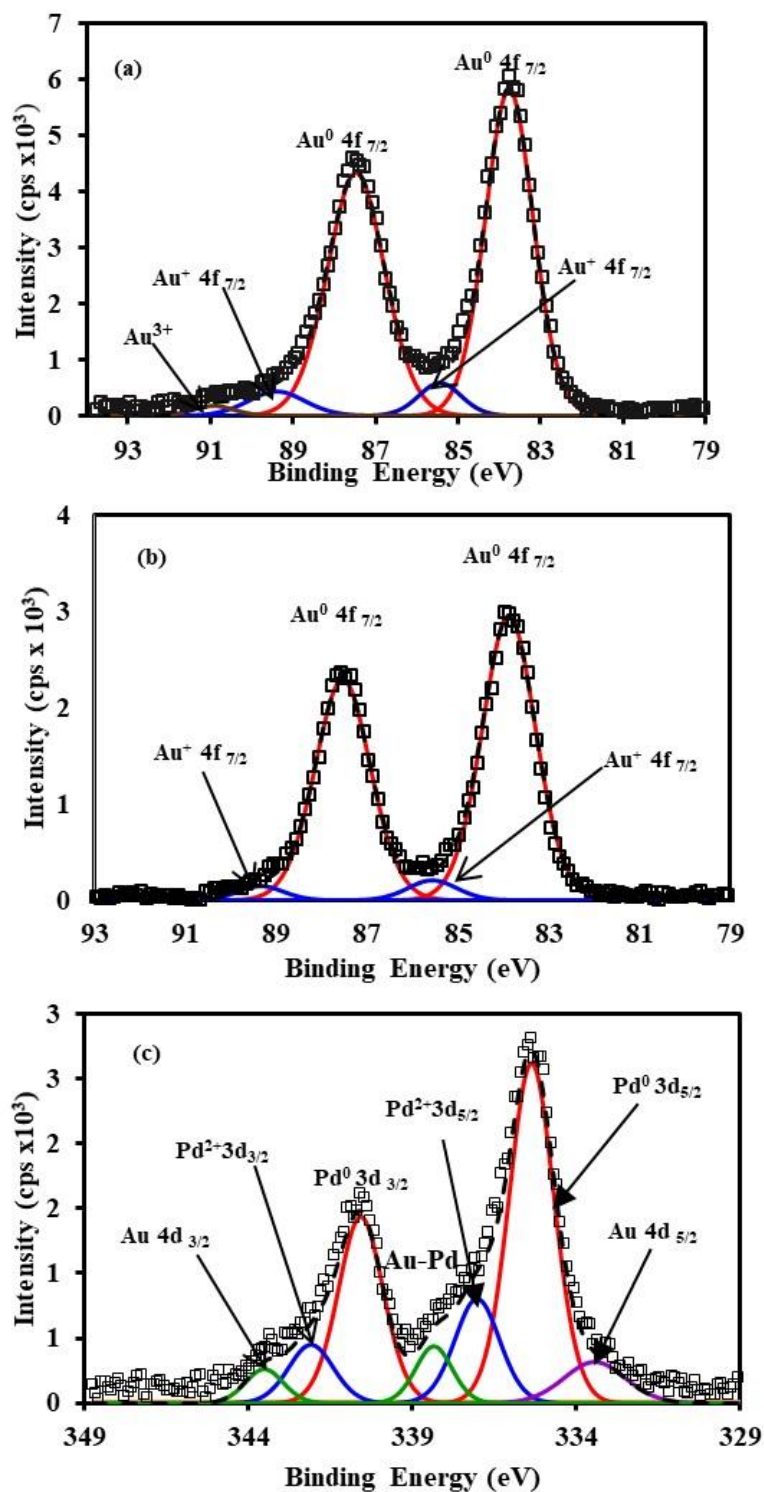


Figure 5.12: High-resolution Au 4f spectra of (a) Fe₃O₄@SiNP-APTES-Au, (b) Fe₃O₄@SiNP-APTES-Au@Pd, and (c) Pd 3d spectra of Fe₃O₄@SiNP-APTES-Au@Pd.

Figure 5.12 (b) shows that the deconvolution of the Au 4f spectrum of Fe₃O₄@SiNP-APTES-Au@Pd was fitted into four components. In comparison to Fe₃O₄@SiNP-APTES-Au, the binding energies of Au 4f in Fe₃O₄@SiNP-APTES-Au@Pd shifted to higher energy. The binding energies of Au4f_{7/2} and Au4f_{5/2} increased from 83.6 eV and 87.3 eV in Fe₃O₄@SiNP-APTES-Au to 84.0 eV (52.1 %) and 87.8 eV (41.0 %) in Fe₃O₄@SiNP-APTES-Au@Pd for Au 4f_{7/2} and Au 4f_{5/2}; respectively. The increase in binding energy is consistent with previous studies on supported bimetallic Au-Pd nanoparticles in the literature [344,348,350]. The shift of the Au 4f peak in Fe₃O₄@SiNP-APTES-Au@Pd to higher binding energy showed that there is a charge transfer process between the Au and the Pd shell. The electronegativity of Au ($\lambda = 2.4$) is larger than Pd ($\lambda = 2.2$). Thus electron flows from the Au to the d-orbital of Pd. The XPS analysis reveals that there is a strong metal to metal interaction (Au-Pd) between the Au-core and the Pd shell. Also, the atomic percentages show that Au exists predominantly in the metallic (Au⁰) in the Fe₃O₄@SiNP-APTES-Au@Pd.

Figure 5.12 (c) shows the deconvoluted spectrum of Pd 3d of Fe₃O₄@SiNP-APTES-Au@Pd. The deconvoluted spectrum of the Pd 3d was fitted into seven components. The binding energy values of Fe₃O₄@SiNP-APTES-Au@Pd are characteristics of the d-orbital of palladium and gold. The main components at binding energies of 335.1 eV (41.5 %) and 340.2 (22.7 %) are assigned to the Pd 3d_{5/2} and Pd 3d_{3/2}; respectively. The separation of the spin components between Pd 3d_{5/2} and Pd 3d_{3/2} was 5.2 eV, which is close to the reported values in the literature [351]. The Pd 3d_{5/2} and Pd 3d_{3/2} were attributed to the existence of metallic palladium nanoparticle with an oxidation state of 0 (Pd⁰) in Fe₃O₄@SiNP-APTES-Au@Pd. However, the binding energies of Pd 3d_{5/2} and Pd 3d_{3/2} in Fe₃O₄@SiNP-APTES-Au@Pd shifted to lower binding energy by 0.7 eV relative to the binding energies at 335.8 eV and 340.8 eV for pure metallic palladium nanoparticles [352]. The lowered binding energy of the Pd 3d further confirms the transfer of an electron from the core-Au NP to Pd shell in the Fe₃O₄@SiNP-

APTES-Au@Pd bimetallic nanoparticles. Thus, a displacement of the Pd binding energy to lower energy values. The doublet spin orbits pair located at 335.25 eV (12.7 %) and 340.3 (7.2 %) are attributed to the Pd^{II} 3d_{5/2} and Pd^{II} 3d_{3/2} of PdO, respectively. The doublet split peak at of 330.9 (6.8 %), and 341.4 (3.5 %) are characteristics of the Au 4d_{5/2} and Au 4d_{3/2} electrons binding energy. The existence of the Au 4d_{5/2} and Au 4d_{3/2} could be ascribed to the formation of a thin palladium shell layer around the Au core. The result suggested that the Fe₃O₄@SiNP-APTES-Au@Pd consists of a metallic Au@Pd core-shell and a PdO surface layer. The analysis of the core-level electron binding energy showed that the binding energy of the Au 4f and Pd 3d electrons in the Fe₃O₄@SiNP-APTES-Au@Pd bimetallic nanoparticles were lower than that of pristine metallic nanoparticles. This showed that there is a synergistic electronic interaction in the Fe₃O₄@SiNP-APTES-Au@Pd nanoparticles. A similar lowering of binding energies has been reported for various metallic hybrid nanoparticles [352–355]. In Fe₃O₄@SiNP-APTES-Au@Pd, the lowering of the Pd 3d binding energy effectively improves the d-electron-orbit cavity of the Pd shell. Hence, Fe₃O₄@SiNP-APTES-Au@Pd possesses an enhanced synergistic catalytic activity. The component positions, their atomic percentages, and assignments for different stages of Fe₃O₄@SiNP-APTES-Au@Pd synthesis were summarized in **Table 5.3**.

The XPS result corroborates other characterization techniques and provides better insight into understanding the characteristics of Fe₃O₄@SiNP-APTES-Au@Pd. From the XPS results, it was evident that; (i) there is an inter-atomic interaction between Au and Pd at the surfaces of the Fe₃O₄@SiNP-APTES-Au@Pd bimetallic nanoparticles, (ii) the surface of the Fe₃O₄@SiNP-APTES-Au@Pd consists of Pd, Au, Si, C, O, and N, which corroborate the result from the FTIR and SEM elemental mapping. (iii) the Fe₃O₄@SiNP-APTES-Au@Pd have a bimetallic Au@Pd core-shell structure as observed in the XRD result. (iv) the surface of the Fe₃O₄@SiNP-APTES-Au@Pd could be easily oxidized by the transfer of electron and hence possesses synergetic redox catalytic activity.

Table 5.3: Summary of the component positions, component percentage and their assignments for different stages of Fe₃O₄@SiNP-APTES-Au@Pd synthesis.

Surfaces	Atoms	Component positions (eV)	Assignments	Component percent (%)
Fe ₃ O ₄ @SiNP-APTES	C 1s	284.9	C-C, C-H, C=C	69.1
		286.5	C-N	27.0
		288.4	C=O	3.9
	O 1s	530.4	O-Fe	1.4
		532.5	O-Si	91.3
		535.2	O-H	7.3
	Si 2p	102.2	Si-C	1.9
		103.4	Si-O-Si, Si-OH	96.5
		105.1	Si-O-Fe	1.6
	N 1s	399.0	NH ₃ ⁺	11.9
399.7		N-C	50.9	
401.3		N-H	37.2	
Fe ₃ O ₄ @SiNP-APTES-Au	C 1s	284.9	C-C, C=C, C-H	57.2
		286.5	C-N, C-O	24.1
		288.2	Au-COO ⁻	10.5
		289.4	O-C=O	4.6
		292.5	COOH	3.6
	O 1s	530.4	O-Fe	2.2
		531.7	O-Au	10.2
		532.9	O-Si	73.1
		534.1	C = O	11.8
		535.2	O-H	2.7
	Si 2p	102.2	Si-C	14.3
		103.4	Si-OH, Si-O-Si	81.7
		105.1	Si-O-Fe	4.0
	N 1s	398.9	NH ₃ ⁺	10.0
		400.2	C-N	58.5
		401.8	H-N	31.5
	Au 4f	83.6, 87.3	Au 4f _{7/2} , Au 4f _{5/2} , (Au ⁰)	48.9, 41.9
85.3, 89.2		Au-N (Au ⁺)	3.5, 4.4	
90.9		Au ₂ O ₃ (Au ³⁺)	1.3	
Fe ₃ O ₄ @SiNP-APTES-Au@Pd	C 1s	284.9	C-C, C=C, C-H	74.4
		286.6	C-N, C-O	12.6
		288.8	C = O	13.0
	O 1s	532.0	O-Au	7.2
		532.6	O-Si	74.0
		533.7	O-Pd	16.4
		535.8	O-H	2.4
	Si 2p	102.1	Si-C	18.5
		103.4	Si-OH, Si-O-Si	66.4
		105.1	Si-O-Fe	15.1
	N 1s	400.2	N-H	100.0
	Au 4f	84.0, 87.8	Au 4f _{7/2} , Au 4f _{5/2} , (Au ⁰)	52.1, 41.0
		85.8, 89.7	Au ⁺ (Au-N)	3.5, 2.4
	Pd 3d	335.1, 340.2	Pd 3d _{5/2} , Pd 3d _{3/2} (Pd ⁰)	41.5, 22.7
335.3, 340.3		Pd ²⁺ 3d _{5/2} , Pd ²⁺ 3d _{3/2}	12.7, 7.2	
330.9, 341.5		Au 4d _{5/2} , Au 4d _{3/2}	6.8, 3.5	

5.3. Evaluation of the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$

The peroxidase-like activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ hybrid was investigated by examining their catalytic action in the oxidation of colorimetric substrates (TMB and OPD) in the presence of H_2O_2 . **Figure 5.13** shows the UV-vis spectra of (a) H_2O_2 + TMB, and (b) H_2O_2 + OPD in the (i) absence, and (ii) presence of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The images of the oxidation color reaction were shown in the inset of **Figure 5.13**. It was verified that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ could catalyze the oxidation of TMB and OPD from the appearance of the blue and yellow colored oxidized products respectively. In contrast, the control experiments without $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ did not produce the observed color changes. Also, it was found that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ or H_2O_2 alone did not produce a significant color change.

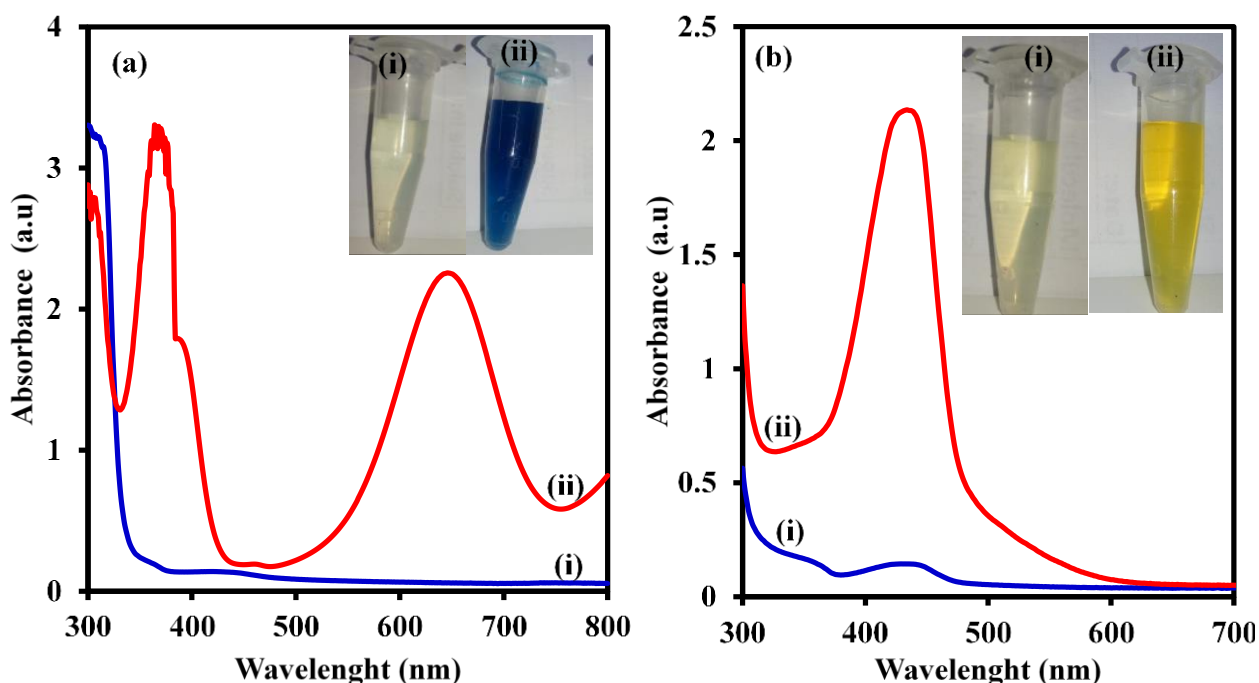


Figure 5.13: UV-vis spectra of (a) (i) H_2O_2 + TMB, (ii) H_2O_2 + TMB + $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$, and (b) (i) H_2O_2 + OPD, (ii) H_2O_2 + OPD + $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$. The corresponding color changes were shown in the inset.

Furthermore, by monitoring the UV-vis absorption, the result in **Figure 5.13** showed that oxidized TMB product with absorption at 652 and 370 nm and OPD with absorption at 450 nm was obtained. The characteristics absorption was not observed in the absence of $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd or H_2O_2 . This shows that H_2O_2 and $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd are required for the efficient oxidation of TMB or OPD. This result confirms that $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd possess an efficient peroxidase-like activity.

The oxidation reaction of TMB in comparison to OPD was very rapid, resulting in faster and deep blue color development within five minutes and also TMB is a non-carcinogenic substrate. Thus TMB was employed as a substrate for further studies. **Figure 5.14** shows the time-dependent peroxidase-like activity of (i) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Pd, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd. The $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES had the lowest catalytic activity. The $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au has a higher activity than $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES.

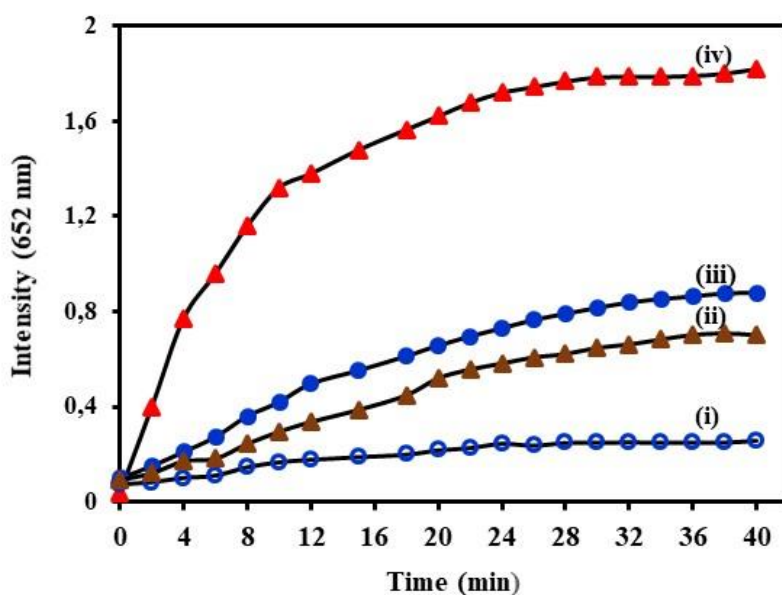


Figure 5.14: Time-dependent peroxidase-like activity of (i) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Pd, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP}$ -APTES-Au@Pd.

The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}@Pd$ had about a 70 % increase in catalytic activity in comparison to $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}$. The enhanced peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}@Pd$ was attributed to the synergistic effect of the metal ions (Pd and Au) onto the nanoparticles. Previous studies have shown that bimetallic nanoparticles with a core-shell type structure exhibit compositional tuneable catalytic activities [258,356]. The peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}@Pd$ synthesized by adding a different ab-initio concentration of Pd was evaluated. **Figure 5.15 (a)** shows the UV-vis absorption spectra of peroxidase-like activity of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_1$, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_2$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_3$, (v) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_5$, and (b) plot of absorption intensity against the ratio of Au to Pd.

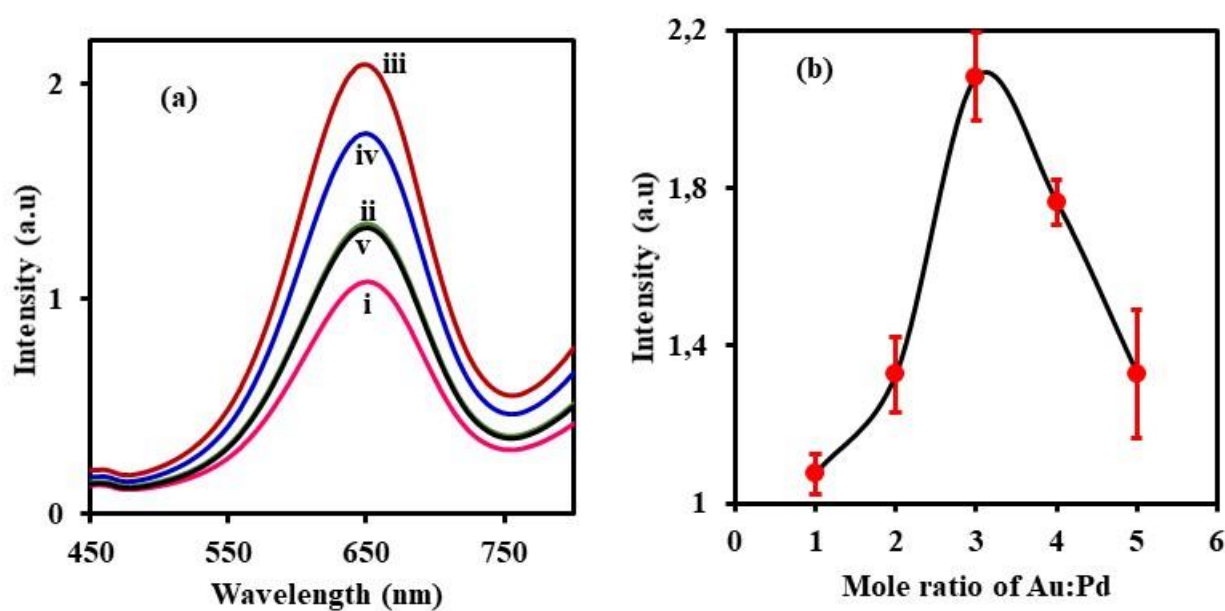


Figure 5.15: (a) UV-vis absorption spectra of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_1$, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_2$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_3$, and (v) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@Pd_5$ and (b) corresponding plot of absorption intensity against Au:Pd.

The absorption intensity increases with increasing mole ratio of Au to Pd up to a mole ratio of 1: 3. This increase was attributed to an increase in the amount of palladium in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}@Pd$. Palladium has higher activity than gold as shown in **Figure 5.15 (iii)**. In **Figure 5.15 (b)** at Au: Pd > 1:3, the absorption intensity decreases gradually. The $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1@\text{Pd}_3$ had the highest absorption intensity and the formation of a deep blue color within five minutes. The blue color was stable for several hours. The result of the investigation led to the selection of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ for subsequent experiments. Leaching test was performed to investigate either the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ originates from the intact $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ or by leached metal ions (Fe^{3+} , Pd^{2+} and Au^{3+}). The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was incubated in a sodium acetate buffer (pH 4.5) for three hours and collected by magnetic separation. The recovered $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ and the leaching solution were evaluated for their peroxidase-like activity. **Figure S5.2** shows the UV-vis absorption spectra of TMB oxidation catalyzed by (i) leaching solution, (ii) recovered $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$, and (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The UV-vis spectrum in **Figure S5.2 (a) (i)** shows that the leaching solution did not catalyze the oxidation of TMB and the catalytic activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ did not decrease significantly after the long-time incubation in the acidic buffer. This indicated that the catalytic activity is attributed to the intact $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ rather than leached metal ions. Furthermore, to investigate the reproducibility of the peroxidase-like activity, six different batches of the $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1@\text{Pd}_3$ were synthesized by consciously keeping all experimental conditions including time, stirring rate, temperature and sequence of reagent addition the same. The peroxidase-like activity of the different batches was evaluated. **Figure S5.2 (b)** shows the absorption intensity at 652 nm for the different batches of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The RSD was 3.5% indicating high reproducibility of the $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1@\text{Pd}_3$ synthesis as well as its peroxidase-like activity.

5.3.1. Optimization of peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$

HRP and peroxidase-mimetic nanomaterials activity are dependent on experimental conditions. These include the buffer pH, temperature, concentration of TMB, and H_2O_2 [357,358]. **Figure 5.16** shows the effect of (a) pH, (b) temperature, (c) concentrations of H_2O_2 , (d) concentration of TMB, and (e) amount of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ on the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiO}_2-\text{Au}_1@\text{Pd}_3$ TMB solutions with different pH values from 1 to 10 was investigated. In **Figure 5.16 (a)**, the catalytic oxidation of TMB was enhanced at weakly acidic pH and was determined to have maximum activity at pH 5.0. The observed trend is consistent with the activity of HRP [359], and other peroxidase mimetics [360–362]. The acid-dependent activity has been ascribed to be limited to low TMB solubility in basic solution. At acidic pH, the protonation of one of the amine groups occurs [363]. At extremely low pH, the protonation of the two amino groups occurs and making the TMB insusceptible to catalytic oxidation. Thus, sodium acetate-acetic acid buffer (pH 5.0) was used for further studies. The effect of temperature on the catalytic activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was investigated from 15 to 80 °C. **Figure 5.16 (b)** shows that the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ peroxidase-like activity increases up to 30 °C and drastically decreases afterward. The effect of H_2O_2 concentrations was investigated in the range of 0.1 to 200 mM. **Figure 5.16 (c)** shows the effect of H_2O_2 concentration on the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. An increase in peroxidase-like activity was observed with an increase in the H_2O_2 concentrations, and the highest activity was at 40 mM. At concentrations greater than 40 mM, the peroxidase activities decrease significantly. The decrease in activity at high H_2O_2 concentrations was attributed to the adsorption of H_2O_2 molecules on the surface of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. This inhibits the interaction of the TMB substrate with the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ and lowers the peroxidase-like activity [357,364].

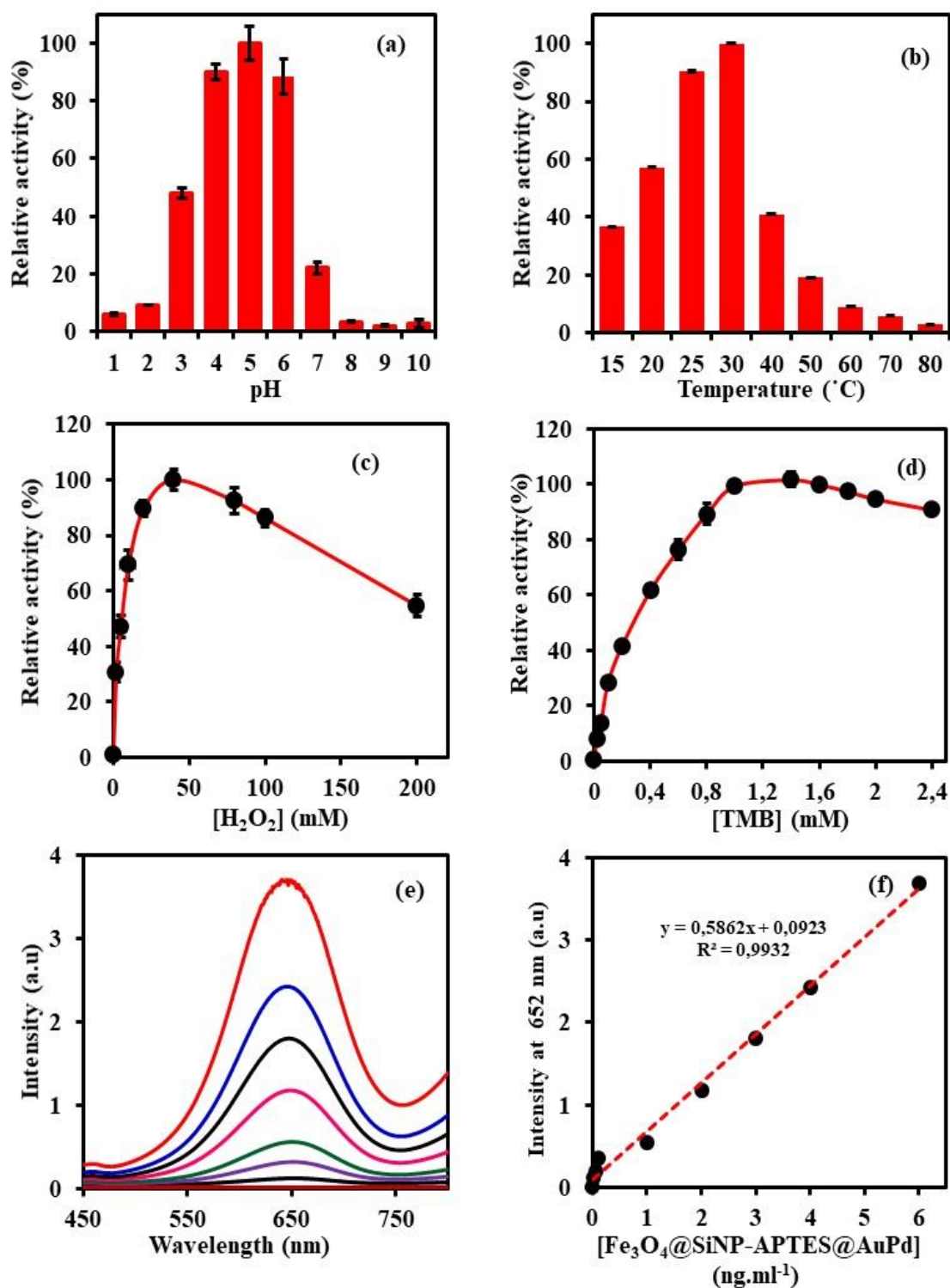


Figure 5.16: Effect of (a) pH, (b) temperature, (c) concentrations of H_2O_2 , (d) concentration of TMB, and (d) amount of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ on the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$, and (f) plot of the change in absorption intensity with the number of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$.

In **Figure 5.16 (d)** maximum peroxidase-like activity was observed at 1.0 mM TMB, and a slight decrease after 1.0 mM was observed. Therefore, 40.0 mM H₂O₂ and 1.0 mM TMB was the best substrate combination for the maximum peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃. The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ concentration dependence peroxidase-like activity was investigated. The absorption intensity at 652 nm increases with increasing concentrations of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ as shown in **Figure 5.16 (e)**. The response curve shows a linear graph with a correlation coefficient of 0.99, **Figure 5.16 (f)**. The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ concentrations dependent response showed that the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ would be an appropriate immunoassay label.

5.3.2. Kinetic studies of the peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃

The peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was further analyzed by determining the apparent steady-state kinetic parameters. The kinetic parameters were determined by changing only the concentration of TMB or only the H₂O₂ concentration. A series of initial rates (V₀) was calculated from the time-dependent absorption value at 652 nm. The obtained absorption values were converted to concentrations using the molar absorption coefficient of oxidized TMB products (39000 M⁻¹.cm⁻¹) [361]. The typical Michaelis-Menten curves were obtained by plotting the initial rates against concentrations of TMB or H₂O₂. The reaction rate at various substrate concentrations was estimated from the slope of the absorption values against time. The Michaelis-Menten parameters (Michaelis-Menten constant, K_m and maximum velocity, V_{max}) were obtained from the Lineweaver-Burk double reciprocal plot ($\frac{1}{V_0}$ vs. $\frac{1}{S}$) using **Equation (5.1)**.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (5.1)$$

Figure 5.17 shows the steady-state kinetic peroxidase-like assay of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ for (a) plot of initial velocity (V_o) versus H₂O₂ concentrations at a fixed TMB concentration of 0.1 mM, (b) Plot of initial velocity versus TMB concentration at a fixed H₂O₂ concentration of 25.0 mM, (c) double reciprocal plot of initial velocity (V_o) versus H₂O₂ concentrations at 3 different fixed TMB concentrations (0.1 mM, 0.2 mM and 0.5 mM), and (d) double reciprocal plot of initial velocity (V_o) versus TMB concentrations at 3 different fixed H₂O₂ concentrations (25.0 mM, 50.0 mM and 100.0 mM). **Figure 5.17 (a) and (b)** shows that with increasing concentrations of TMB and H₂O₂. The initial rate increased and formed a plateau at high TMB and H₂O₂ concentrations. The curve shows that the peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ follows the typical Michaelis-Menten enzymatic behavior.

In **Figure 5.17 (c)-(d)**, the double reciprocal of the initial rate (V_o) versus the substrate concentrations gave an excellent linear relationship. The linear regression equation was given by **Equation (5.2) and (5.3)**.

$$\frac{1}{V_o} = 1.15 \times 10^6 \frac{1}{[\text{TMB}]} + 1.12 \times 10^7, R^2 = 0.994 \quad (5.2)$$

$$\frac{1}{V_o} = 5.25 \times 10^6 \frac{1}{[\text{H}_2\text{O}_2]} + 1.48 \times 10^7, R^2 = 0.994 \quad (5.3)$$

The Michaelis-Menten constant (K_m [H₂O₂], K_m [TMB]), and substrate maximum reaction velocity (V_{max}) were calculated from the slope and intercepts of the corresponding linear graphs. The result of the calculated parameters was summarised in **Table 5.4**. The K_m and V_{max} value of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ with TMB as substrate was 0.090 mM and 8.65 x 10⁻⁸ Ms⁻¹. The corresponding values of K_m and V_{max} with H₂O₂ as substrate was 0.35 mM and 6.75 x 10⁻⁸ Ms⁻¹. The lower the K_m value, the higher the affinity of an enzyme for the substrates. The

K_m value of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ with TMB as substrate (0.090 mM) was about four times lower than that of H_2O_2 as the substrate (0.35 mM).

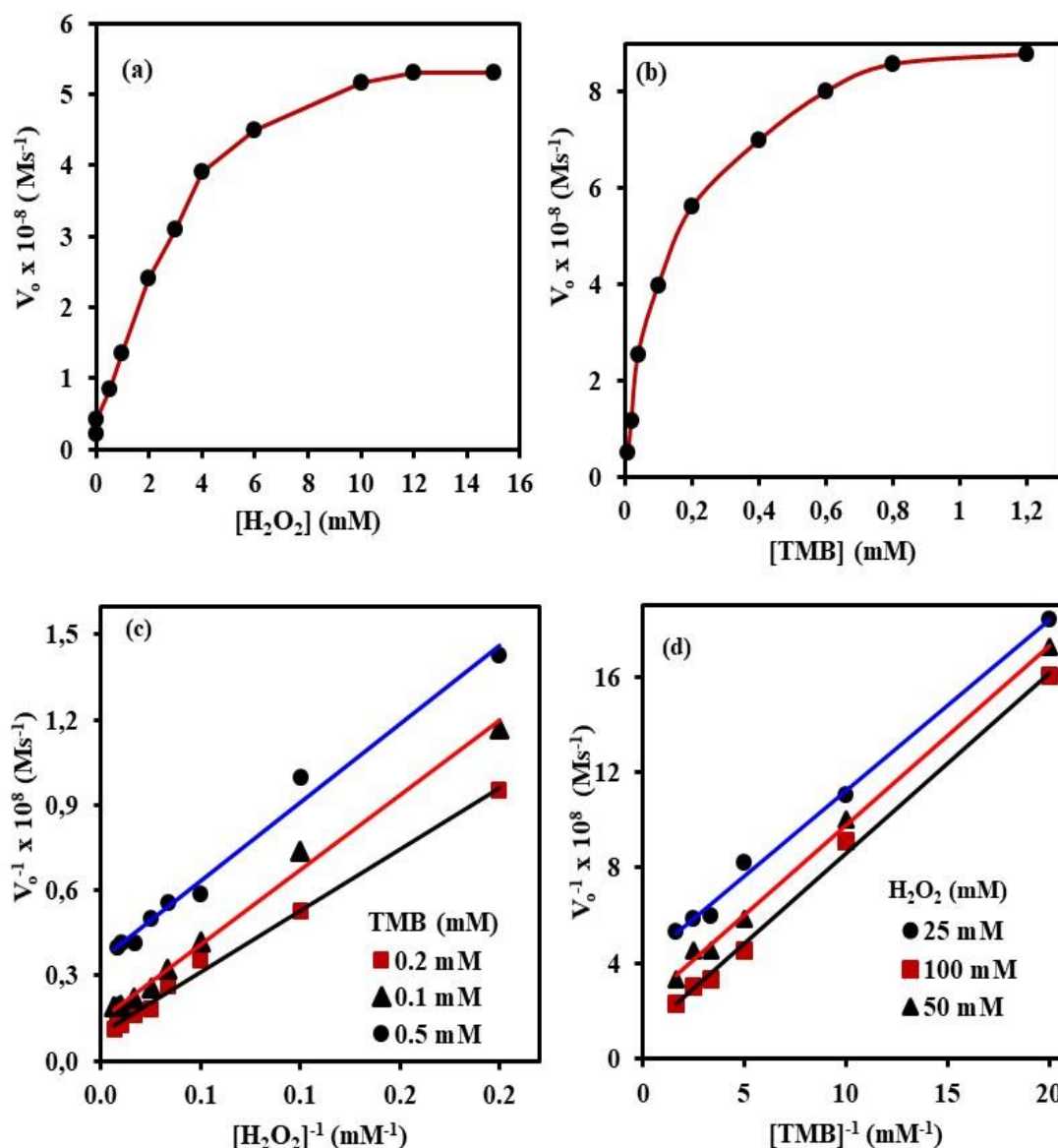


Figure 5.17: Steady-state kinetic assay of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ (a) plot of initial velocity versus H_2O_2 concentration at a fixed TMB, (b) plot of initial velocity versus TMB concentration at a fixed H_2O_2 , (c) double reciprocal plot of initial velocity versus H_2O_2 concentrations at different fixed TMB concentrations, and (d) double reciprocal plot of initial velocity versus TMB concentrations at different fixed H_2O_2 concentrations.

The results showed that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ had a higher affinity for TMB than H_2O_2 . The K_m and $V_{\max}$ values of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ were compared with that of HRP and other reported peroxidase-like nanoparticles. The K_m and $V_{\max}$ values are summarised in **Table 5.4**. The K_m [H_2O_2] and K_m [TMB] of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ were lower than that of HRP. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ has a higher affinity towards TMB and H_2O_2 than HRP. The higher affinity for both substrates may be attributable to the excellent peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. From **Table 5.4**, it can be seen that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ has a higher affinity for TMB and H_2O_2 than some of the previously reported nanoparticles. Also, the $V_{\max}$ of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ with TMB as the substrate ($8.65 \times 10^{-8} \text{ Ms}^{-1}$) and with H_2O_2 as the substrate ($6.78 \times 10^{-8} \text{ Ms}^{-1}$) is comparable to that of HRP ($8.7 \times 10^{-8} \text{ Ms}^{-1}$). The kinetic parameters showed that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ possessed excellent peroxidase-like activity. The double reciprocal plots of the initial steady-state rates of TMB oxidation against the H_2O_2 at different fixed TMB concentrations afforded a set of linear and parallel lines, **Figure 5.17 (c) and (d)**. This is indicative of the ping-pong π - π mechanism (double displacement mechanism). This indicates that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ reacts with the first substrate to release a product and then reacts with the second substrate. This mechanism is similar to that for HRP [365]. The kinetic studies showed that the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ is superior to that of HRP.

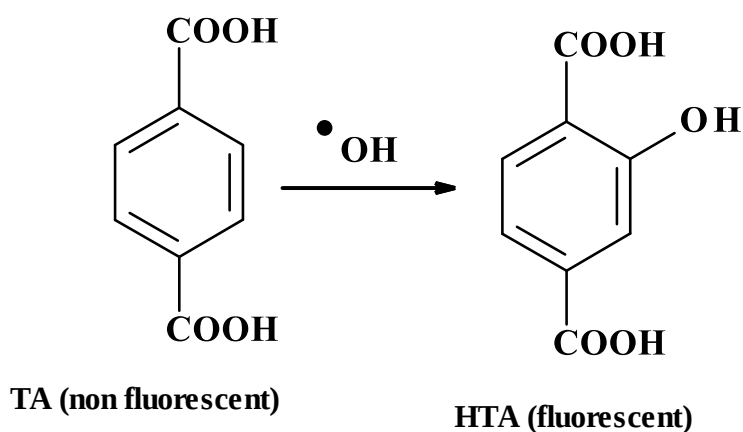
Table 5.4: Comparison of the kinetic parameters of Fe₃O₄@SiO₂-Au₁@Pd₃ with HRP and other nanomaterials.

Enzyme / nanozyme	K _m (mM)		V _{max} (x 10 ⁻⁸ M s ⁻¹)		[Refs]
	H ₂ O ₂	TMB	H ₂ O ₂	TMB	
Fe ₃ O ₄ @SiNP-APTESAu ₁ @Pd ₃	0.35	0.09	6.76	8.65	This work
Pd NPS	0.21	-	0.21	0.00127	[216]
HRP	3.70	0.43	8.71	10.00	[365]
FePt-Au HNPs	0.018	0.46	0.69	24.67	[366]
Fe-MiL-88-NH ₂	2.06	0.28	10.47	7.04	[367]
Ni-MOF nanosheet	6.53	0.36	130	2.49	[368]
Pd/Fe ₃ O ₄ -PEI-RGO	3.66	-	4.01	-	[369]
NiCo ₂ O ₄ MS	9.406	0.14	25.8	2.233	[370]

5.3.3. Mechanism of peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃

The mechanism of peroxidase-like activity of nanomaterials can be divided into three namely, (i) the Fenton's reaction mechanism, (ii) the electron transfer process, and (iii) the oxidase-activation of dissolved oxygen to produce oxidative species [252,371]. Fenton's reaction pathways involve the decomposition of H₂O₂ to produce reactive oxygen species (ROS) such as hydroxyl ([•]OH), superoxide (O₂^{•-}) and peroxy (HO₂[•]) radicals. In order to investigate if the peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ proceeds via Fenton's reaction mechanism, the fluorescence of terephthalic acid (TA) and 5,5-Dimethyl-pyrroline N-Oxide (DMPO) radical trapping experiments were investigated. **Scheme 5.2** shows the reaction of terephthalic acid with hydroxyl radicals to produced 2-hydroxyterephthalic acid. Terephthalic acid is non-fluorescent, but after reacting with hydroxyl radicals, it produces a fluorescent 2-

hydroxyl terephthalic acid (HTA). HTA has a characteristic emission wavelength at 440 nm [365,366,372]. DMPO reacts with $\cdot\text{OH}$ radicals to generate an active spin DMPO/ $\cdot\text{OH}$ adduct signal with characteristics four lines EPR signal with a relative intensity of 1:2:2:1 [372,373].



Scheme 5.2: Scheme is representing the reaction of terephthalic acid with hydroxide radical.

The EPR spin trap and HTA fluorescent experiments were performed on the solution of H_2O_2 +DMPO with UV with or without the addition of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The solution was irradiated with UV light for 10 minutes before EPR, and fluorescence measurement. **Figure 5.18** shows the effect of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ on the formation of hydroxyl radical using (a) terephthalic acid as a fluorescent probe and (b) using DMPO as $\cdot\text{OH}$ spin trap at different concentrations of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ in acetate buffer pH 5.0. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ concentrations were (i) $0.0 \mu\text{g}.\text{ml}^{-1}$, (ii) $5.0 \mu\text{g}.\text{ml}^{-1}$, (iii) $10.0 \mu\text{g}.\text{ml}^{-1}$, (vi) $60.0 \mu\text{g}.\text{ml}^{-1}$, (v) $100.0 \mu\text{g}.\text{ml}^{-1}$, and (vi) $150.0 \mu\text{g}.\text{ml}^{-1}$. The photolysis of H_2O_2 to generates $\cdot\text{OH}$ radicals is well known [372]. In **Figure 5.18 (a)**, the HTA fluorescence and **(b)** DMPO/ $\cdot\text{OH}$ EPR adduct signal decreased with increasing concentrations of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. This indicated that hydroxyl radicals were not generated by the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ did not proceed via the Fenton's reaction mechanism.

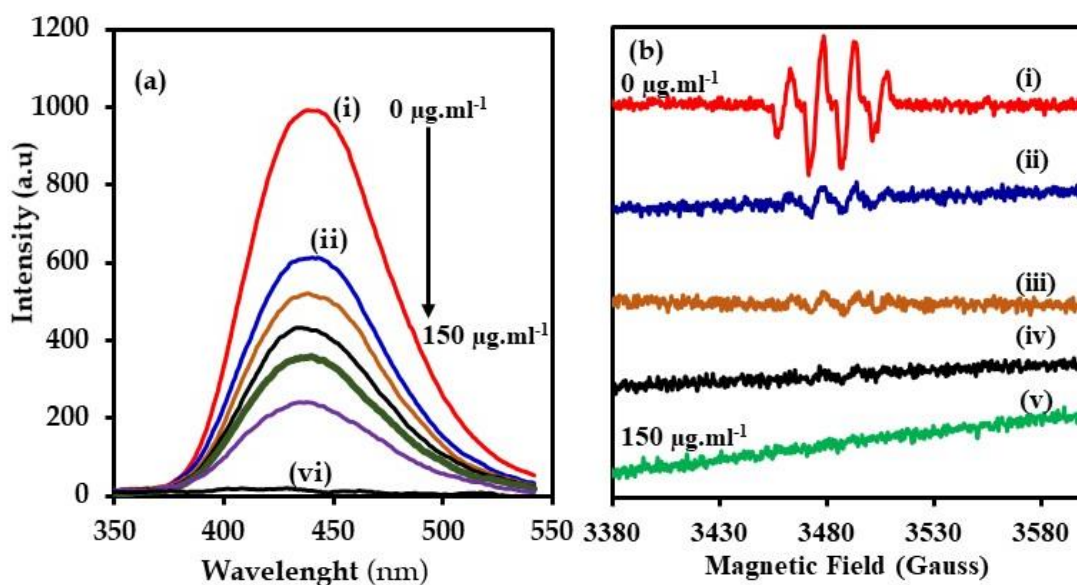


Figure 5.18: Effect of $\text{Fe}_3\text{O}_4@\text{SiNPAPTES-Au}_1@\text{Pd}_3$ on the formation of hydroxyl radical in the presence of H_2O_2 (a) with terephthalic acid as a fluorescence probe and (b) DMPO as $\cdot\text{OH}$ spin trap. (i) $0.0 \mu\text{g.ml}^{-1}$ – (vi) $150.0 \mu\text{g.ml}^{-1}$.

A further experiment was conducted to study oxygen generation as a product of decomposition of H_2O_2 by $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1\text{Pd}_3$. Different concentrations of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was added to a solution of 10.0 mM H_2O_2 in acetate buffer. Air bubbles were observed only in the addition of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The dissolved oxygen was determined using a dissolved oxygen meter (DO meter). **Figure 5.19** shows the effect of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ concentrations on the generation of dissolved oxygen. The dissolved oxygen concentrations increased with increasing concentration of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ up to about $60.0 \mu\text{g.ml}^{-1}$. With the addition of higher concentrations ($> 60.0 \mu\text{g.ml}^{-1}$), the concentrations of DO remained constant, possible due to saturation. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ concentration-dependent increase in dissolved oxygen suggested that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ decomposes H_2O_2 into oxygen and water. This provides evidence that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ possesses catalase-like activity.

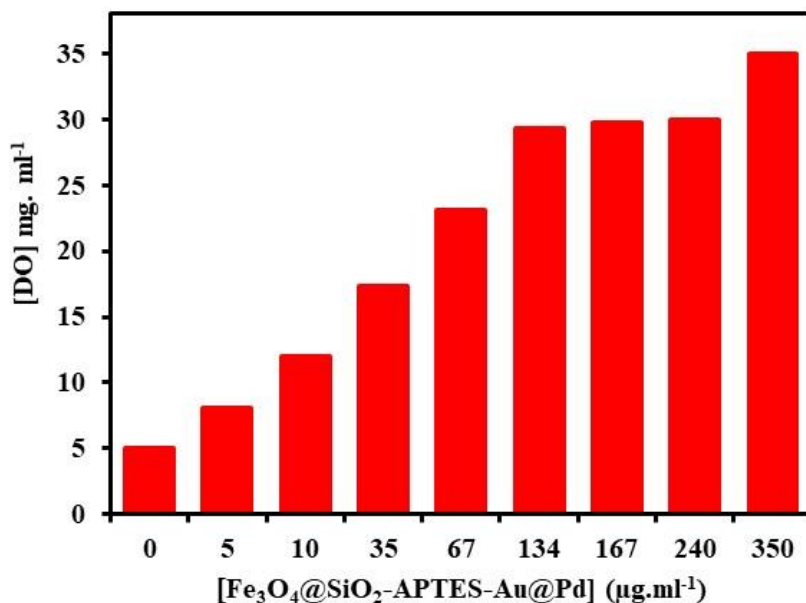


Figure 5.19: The effect of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ Dissolved oxygen measurement in the Fe₃O₄@SiO₂-Au@Pd + H₂O₂ system.

It was reported that horseradish peroxidase isoenzyme C (HRP; ferric) exhibits catalase-like activity [373,374]. The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ shows similar behavior to HRP-C. The HRP-C facilitates the decomposition of hydrogen peroxide to water and oxygen via the electron-transfer mechanism. In the HRP-C catalytic cycle, H₂O₂ is first reduced with the concomitant two-electron oxidation to form an oxidized intermediate, and the oxidized HRP-C is converted back to the natural HRP-C via two successive single electron transfer from reduced substrates molecules [375]. Based on these observations the electrocatalytic behavior of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was investigated. The glassy carbon electrode was modified with the Fe₃O₄@SiNP-APTES-Au₁@Pd₃, and voltammetric and amperometric measurements were recorded in the presence and absence of H₂O₂. **Figure 5.20** shows (a) cyclic voltammograms of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ modified GC electrode in 100 mM sodium acetate buffer (pH 5.0) in the (i) absence and (ii) presence of 10.0 mM H₂O₂ and (b) chronoampereomogram of the modified electrode in 100 mM sodium acetate buffer upon

successive addition of 100 μM H_2O_2 . The CV of the unmodified GC electrode did not show an appreciable reduction current in the absence or presence of H_2O_2 . The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ modified GC electrode showed a reduction peak centered at -0.28 V vs. $\text{Ag}|\text{AgCl}$ with the addition of H_2O_2 into the buffer solution. In **Figure 5.20 (b)** the current response of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ modified GC electrode was higher than that of the bare GC electrodes. The current response increases steadily with increasing concentrations of H_2O_2 . This showed that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ has the ability to facilitate electron transfer between the electrode (electron donor) and H_2O_2 (electron acceptor) in the electrochemical process. This confirmed that $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ possesses an electrocatalytic activity and could mediate electron transfer between the electrode surface and H_2O_2 . Hence, the peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was attributed to an electron transfer process mimicking HRP-C.

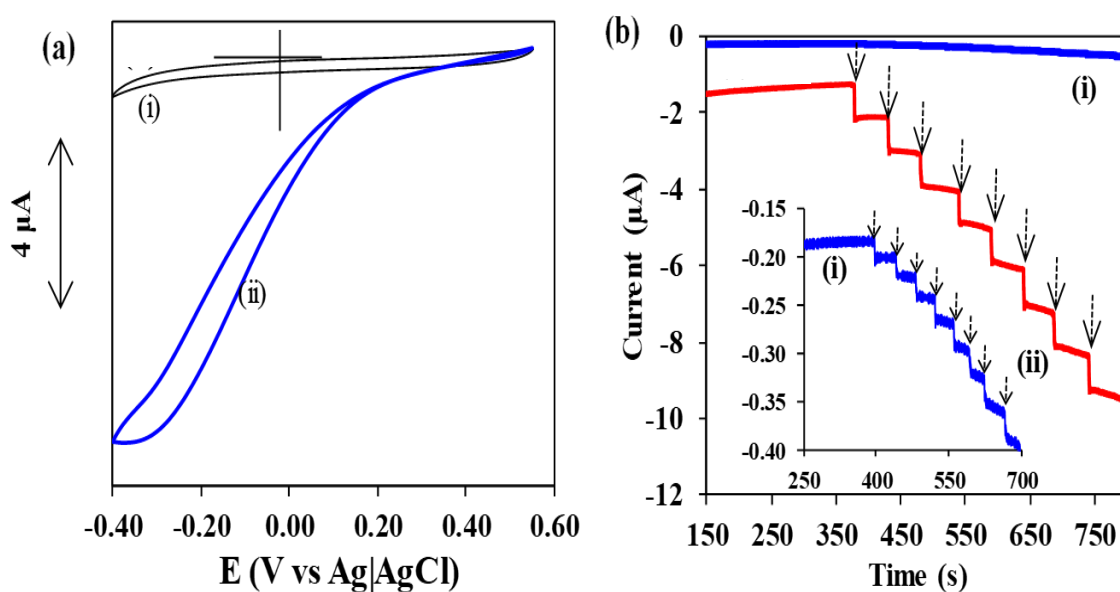
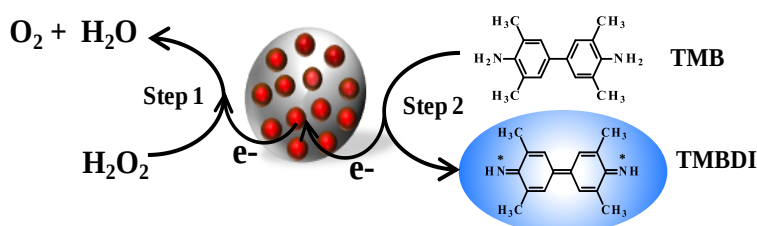


Figure 5.20: (a) Cyclic voltammograms, and (b) amperometric response of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ modified GC electrode in 100 mM sodium acetate buffer (pH 5.0) in the (i) absence, (ii) and presence of H_2O_2 .

The catalytic active sites of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ were the deposited bimetallic $\text{Au}@\text{Pd}$ NPs. The $\text{Fe}_3\text{O}_4@\text{SiNP}$ did not take part in the catalysis of TMB as previously shown in **Figure 5.14**. The silica shell shielded the Fe_3O_4 from interacting with TMB. Therefore the peroxidase-like activity results from the $\text{Au}@\text{Pd}$ active sites on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The XPS results in **section 5.2.6** provide an understanding of the electrocatalytic behavior of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. The binding energy of the Au 4f and Pd 3d was lowered than that of pure metallic Au and Pd nanoparticles. This lowering of energy was due to the lattice-mismatched induced tensile strain effect of the Au core on the Pd-shell [353]. The tensile stress causes an increase in the width of the Pd d-band [376]. The increase alters the energy of the d-band and the overlap changes, although the degree of d-band filling remains the same [356]. The atoms of the $\text{Au}@\text{Pd}$ are stretched, and the average coordination number would decrease. This causes a decrease in the overlap of the palladium d-orbitals, and the bandgap narrowed to maintain the same filling degree due to the closeness of the Fermi level. The d-electrons move up and become unstable. Also, the core Au nanoparticle donates electrons to the 3d-orbital of the Pd shell, resulting in an electron-rich palladium shell. Hence, it is easier to ionize the $\text{Au}@\text{Pd}$ of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ in the presence of an electron acceptor such as H_2O_2 . Electron transfer from the valence band of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ to the lowest unoccupied molecular orbital (LUMO) of H_2O_2 . **Scheme 5.3** shows the proposed mechanism of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ peroxidase-like activity.



Scheme 5.3: Proposed mechanism of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ as peroxidase mimetics.

In **Scheme 5.3**, the proposed mechanism follows a redox-cycles of sequential Au@Pd (oxidized) to Au@Pd (reduced) to Au@Pd (oxidized) cycle. The first step (**step 1**) involves the transfer of an electron from the Au@Pd active sites to the hydrogen peroxide (electron acceptor). This results in the oxidation of the active metal sites and the reduction of H₂O₂ to H₂O and O₂. Simultaneously, TMB donates lone pair electrons of the amino groups to the oxidized Fe₃O₄@SiNP-APTES-Au₁@Pd₃ (**step 2**). The Au@Pd active sites will be reduced, while TMB was oxidized to the blue colored products. The transfer of charges increases the electrochemical potential from the LUMO of H₂O₂ and therefore helps the electron transfer cycle to continue. Therefore, it is reasonable to conclude that the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ possesses a peroxidase-like activity via electron transfer similar to the HRP-C [377], FePt-Au [366], AuNP@CDs [372], MoS₂ nanosheet [371] and Co₃O₄ [377]. This reaction mechanism gave a better understanding of the enhanced peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃ and the steady-state kinetic parameters. The mechanism explains: (i) there is no intermediate radical generation, thus fast colorimetric response (deep within 5 minutes), (ii) high affinity (low K_m) for both H₂O₂ and TMB, (iii) the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ the first react with H₂O₂, to form a reduced intermediate state that reacts with TMB, hence the ping-pong kinetic behavior of the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ peroxidase-like activity.

5.4. Stability of peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃

The stability and environmental conditions for the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ were evaluated. The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ before and after subjecting to various conditions of temperature (4 to 100 °C), pH (2.0 to 12.0), ionic strengths, and long-term storage was investigated. **Figure 5.21** shows the effect of (a) temperature, (b) pH, (c) ionic strength and (d) long-term storage on the peroxidase-like activity of Fe₃O₄@SiNP-APTES-Au₁@Pd₃.

Figure 5.21 (a) shows that there were no significant changes in the peroxidase-like activity of

the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ at varied temperatures from -20 to 100 °C. The same was observed for varied pH conditions from pH 3.0 to pH 12.0 in **Figure 5.21 (b)**. In comparison, HRP loses its catalytic activity at temperatures above 40 °C. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ retains about 95 % of its activity even when subjected to 100 °C. The halogen ions such as chloride ions absorb strongly to palladium and etch gold nanoparticles [378]. This etching effect results in changes in the size and shape of the nanoparticle in buffer saline solution and could affect the catalytic performance of the nanoparticle.

Figure 5.21 (c) shows that the peroxidase-like activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was retained after subjecting to PBS-saline solutions even at high ionic strength. The stability after treatment with a high ionic strength solution could be due to the shielding effect of the palladium shell which prevents etching of the gold nanoparticles. The stability of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ under prolonged storage conditions was also studied. **Figure 5.21 (d)** shows that 98 % of the peroxidase-like activity was retained after storing at room temperature on a laboratory bench for three months. The long-term storage was analyzed every week. The result demonstrated that the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ peroxidase-like activity was stable under very harsh environmental conditions not possible with HRP.

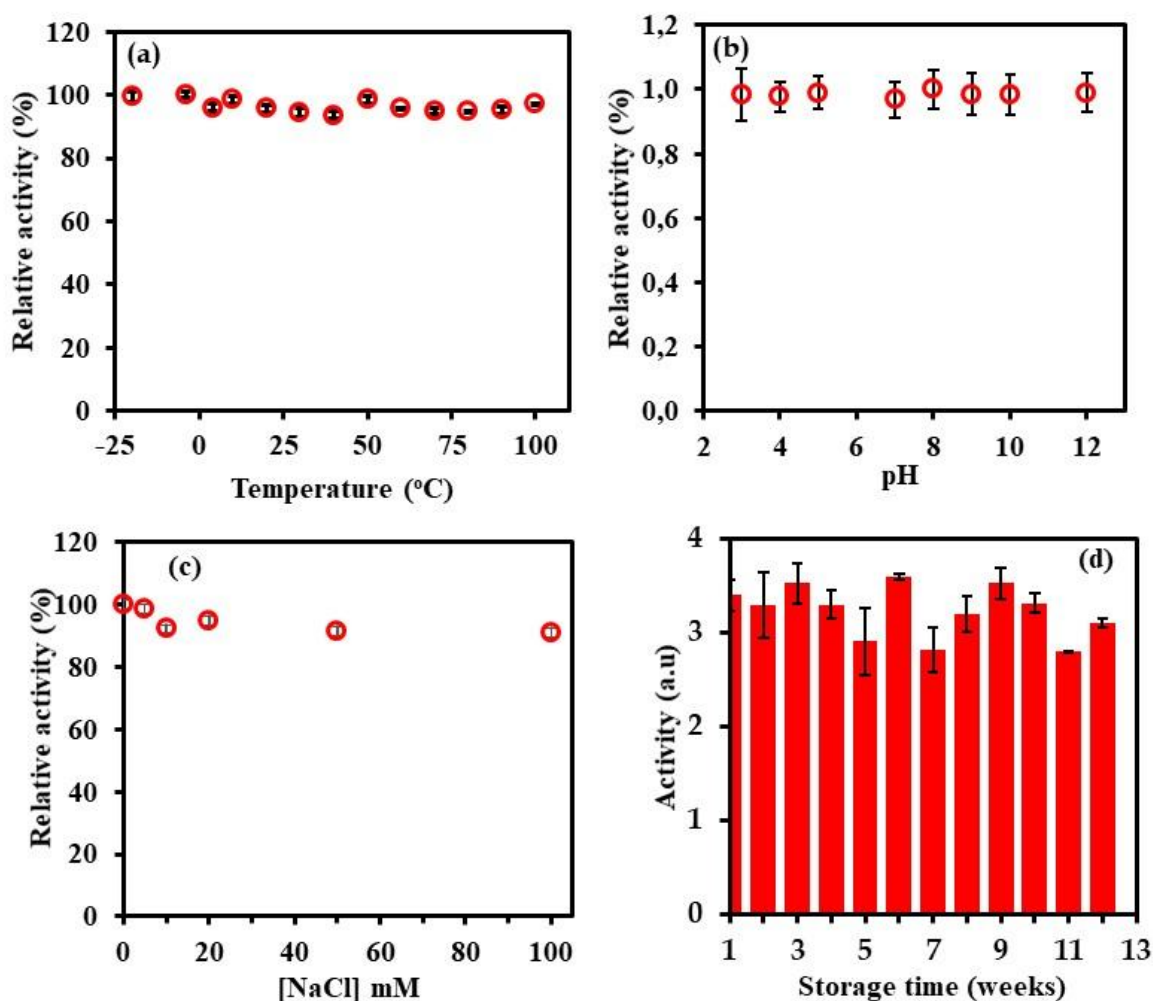


Figure 5.21: The effect of (a) temperature (-20 to 100 $^{\circ}\text{C}$), (b) pH (3.0-12.0), (c) ionic strength NaCl [0.0 -100.0] mM, and (d) long term stability of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$.

5.5. Chapter conclusion

$\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ hybrid nanoparticles were successfully synthesized and characterized. The magnetic property was due to the magnetic Fe_3O_4 core and allowed for magnetic separability. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ possesses intrinsic peroxidase-like, catalase-like and electrocatalytic activity. The peroxidase-like activity of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was attributed to the exposed $\text{Au}@\text{Pd}$ atoms on its surface. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ rapidly catalyzes the oxidation of the TMB in the presence of

H₂O₂ within five minutes. Steady-state kinetic analysis indicated that the peroxidase-like activity was by the Michaelis-Menten kinetics and followed the ping-pong mechanism. In comparison to HRP, Fe₃O₄@SiNP-APTES-Au₁@Pd₃ presents much higher intrinsic peroxidase-like activity and higher affinity towards the substrate TMB and H₂O₂. The Michaelis-Menten constants (K_m) for TMB and H₂O₂ with Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was much lower than that of HRP and some of the previously reported nanomaterials. The peroxidase-like activity with TMB substrate was very rapid and attained maximum activity within 5 minutes. The rapid response was attributed to the fast electron transfer between the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ and H₂O₂. The peroxidase-like mechanism was proposed based on the electron transfer mechanism. In comparison to HRP, the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was stable under harsh environmental conditions. The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ exhibited excellent reproducibility, long term-stability, thermal stability and easy separation using a magnet. These unique properties indicated that Fe₃O₄@SiNP-APTES-Au₁@Pd₃ would be superior in comparison to natural enzymes (HRP) as a label for immunoassay.

CHAPTER 6:

Colorimetric detection of anti-P53ab

6. Colorimetric Detection of anti-P53ab

Based on the excellent peroxidase-like catalytic performance of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ reported in Chapter 5, this chapter reports on the application of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ conjugated to detection antibody (anti-IgG) as a novel-label for anti-P53ab detection. The sensing principle of the developed immunobiosensor was similar to that reported in chapter four. However, fluorescent $\text{FITC}@\text{SiNP-APTES-PEI-anti-IgG}$ and its dissolution by NaOH was replaced by the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd-ProG-anti-IgG}$ and TMB- H_2O_2 substrate. First, the anti-P53ab immunobiosensor was developed in a microtitre ELISA plate. However, the developed immunobiosensor uses an expensive polystyrene microplate, requires large volumes of reagents and a spectrophotometer for quantitative analysis; these requirements limit its application. The design of a paper-based colorimetric immunobiosensor for anti-P53ab detection was investigated to overcome the limitations. The chapter studied the translation and optimization of the $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au@Pd}_3\text{-ProG-anti-IgG}$ microwell plate immunobiosensor onto cellulose-paper platform. Cellulose paper has considerable advantages over other biosensing platforms based on lateral flow in achieving low-cost screening test. This follows the ASSURED criteria (affordable, sensitive, rapid, specific, user-friendly, and robust, equipment-free, and deliverable to end-users) for point-of-care (POC) diagnosis in low-income settings. Paper is an inexpensive and ubiquitous cellulose fiber web with a 3D-microstructure. The detection of anti-P53ab using a smartphone integrated with imaging software for quantitative analysis was also investigated. The advantages of the paper-based immunobiosensor are low cost; miniaturization characterized by a low sample and reagent consumption, easy data acquisition and interpretation without the sophisticated readout devices.

6.1. Bioconjugation of Fe₃O₄@SiO₂-Au₁Pd₃ with anti-IgG antibodies

The Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was conjugated to anti-IgG antibodies for their applications as nanobioprobe (label) for the detection of anti-P53ab. Anti-IgG was conjugated to the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ using four different methods. The selected method has been extensively discussed in **Section 1.6.1, Chapter 1**. The first method was based on the electrostatic attachment. The anti-IgG adsorbed onto the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ surface possibly via the amine functional groups through metal-nitrogen bond (Pd-N) interaction [379,380]. The bioconjugate was referred to as Fe₃O₄@SiNP-APTES-Au₁@Pd₃-anti-IgG_(ads). The second method used the periodate treated anti-IgG as illustrated in **Scheme 1.5**. The periodate treatment allows for the formation of aldehyde functional groups on the glycosylated Fc region of anti-IgG. The aldehyde forms a stable covalent linkage via reductive amination with the amine groups on Fe₃O₄@SiNP-APTES-Au₁@Pd₃. This conjugate was referred to as Fe₃O₄@SiNP-APTES-Au₁@Pd₃-anti-IgG_(PI). The third method utilizes glutaraldehyde as a cross-linker between the amine groups on Fe₃O₄@SiNP-APTES-Au₁@Pd₃ and the amine groups of the anti-IgG to form a stable covalent bond as illustrated in **Scheme 1.3**. The bioconjugate was referred to as Fe₃O₄@SiNP-APTES-Au₁@Pd₃-anti-IgG_(GA). The last method utilizes the conjugation of Protein G (ProG) to Fe₃O₄@SiNP-APTES-Au₁@Pd₃ via glutaraldehyde crosslinking and electrostatic adsorption, this was followed by affinitive adsorption of anti-IgG via Fc-directed immobilization to form Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG bioconjugate as shown in **Scheme 1.4**. Electrostatic adsorption could predominate the covalent bioconjugation approach due to the high affinity of the palladium and gold for amine groups of anti-IgG. The covalent bioconjugate provides additional stability due to the covalent linkage. The detailed bioconjugation procedures were reported in the experimental section, **Chapter 2**.

6.2. Characterization of Fe₃O₄@SiO₂-Au₁Pd₃ with anti-IgG antibodies bioconjugates

6.2.1. Zeta potential analysis of bioconjugates

The conjugation of the anti-IgG to the Fe₃O₄@SiNP-APTES-Au₁@Pd₃ changes the surface electrokinetic properties and hence zeta potential. The zeta potential changes after the electrostatic adsorption and covalent conjugation of anti-IgG was investigated. **Table 6.1** shows the summary of the zeta potential of unmodified and anti-IgG modified Fe₃O₄@SiNP-APTES-Au₁@Pd₃.

Table 6.1. Zeta potential of anti-IgG modified Fe₃O₄@SiNP-APTES-Au₁@Pd₃ conjugates

Nanoparticle	Zeta potential (mV)
Fe ₃ O ₄ @SiNP-APTES-Au ₁ @Pd ₃	-26.7 ± 3.2
Fe ₃ O ₄ @SiNP-APTES-Au ₁ @Pd ₃ -anti-IgG _(ad)	-9.6 ± 0.83
Fe ₃ O ₄ @SiNP-APTES-Au ₁ @Pd ₃ -anti-IgG _(PI)	-3.8 ± 0.92
Fe ₃ O ₄ @SiNP-APTES-Au ₁ @Pd ₃ -ProG-anti-IgG	-2.2 ± 0.73
Fe ₃ O ₄ @SiNP-APTES-Au ₁ @Pd ₃ -anti-IgG _(GA)	-16.7 ± 0.20

PI-periodate; GA-glutaraldehyde, ProG- Protein G. APTES: 3-aminopropyltriethoxysilane

The zeta potential of the unmodified Fe₃O₄@SiNP-APTES-Au₁@Pd₃ was -26.7 ± 3.2 mV. The zeta potential decreased from -26.7 ± 3.2 mV to -9.6 ± 0.8 mV upon the electrostatic adsorption of anti-IgG. After the covalent conjugation of anti-IgG via the periodate method, the zeta potential of Fe₃O₄@SiNP-APTES-Au₁@Pd₃-anti-IgG_(PI) bioconjugate was -3.8 ± 0.92. For the glutaraldehyde cross-linked bioconjugates, the zeta potential was -2.2 ± 0.73 mV for Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG and -16.7 ± 0.20 mV for Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG. The changes in the surface zeta potential confirm the successful formation of the bioconjugates.

6.2.2. Bicinchoninic acid assay (BCA)

A modified BCA assay was performed for the determination of the amount of anti-IgG per milligram of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. In the BCA method, anti-IgG protein induces the reduction of Cu (II) cations to Cu (I), and then the bicinchoninic acid was added for color development. The resultant colorimetric changes and absorption intensity before and after (supernatant concentrations) conjugation of the anti-IgG to $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}$ was measured. The detailed experimental setup and principle of the BCA assay were described in Section 2.5. Figure 6.1 (a) shows the UV-vis absorption spectra of the BCA assay determination of anti-IgG antibody (i) before and (ii-vi) after reaction with $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ via the various conjugation techniques. In Figure 6.1 (a) (i), BCA reacts with anti-IgG to generate a purple colored product with absorption at 567 nm. The initial concentration of anti-IgG before the bioconjugation was $200.0 \mu\text{g.ml}^{-1}$ and the absorption intensity at 567 nm from the BCA assay was 0.76. After conjugation, the absorption intensity drastically decreased due to the lowered concentration of anti-IgG in the supernatant. The anti-IgG concentrations in the supernatant varied with the method adopted for bioconjugation. In Figure 6.1 (a) (ii)-(iii), the absorption of the supernatant was 0.31, 0.28, 0.15 and 0.02 a.u for periodate, glutaraldehyde, electrostatic adsorption, and ProG-anti-IgG; respectively. The decrease in absorption intensity indicated successful conjugation of the anti-IgG to the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ via covalent and electrostatic bonding.

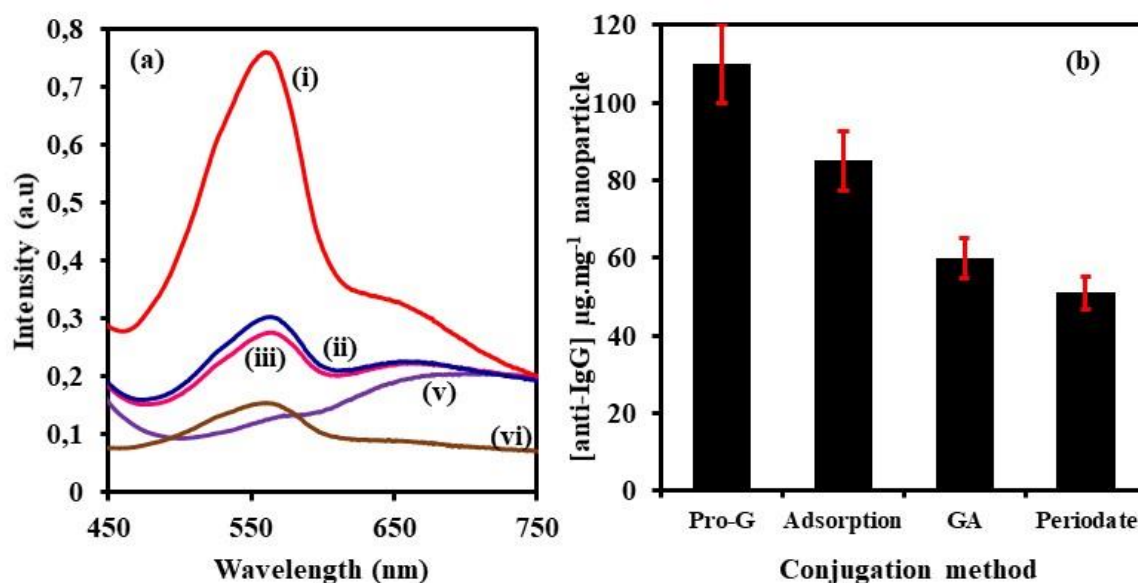


Figure 6.1: (a) BCA assay spectra of (i) anti-IgG (200.0 µg/ml), and supernatant from (ii) periodate method, (iii) glutaraldehyde, (iv) adsorption, and (v) Protein G, and (b) BCA determination of an anti-IgG concentration of the conjugated $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$.

The number of anti-IgG attached to the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was proportional to the differences between pre- and post-attachment. The amount of the unbound anti-IgG was subtracted from the total amount of anti-IgG used for the reaction. The data were expressed as µg of anti-IgG per mg of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$. **Figure 6.1 (b)** shows the concentration of anti-IgG antibody adsorbed or covalently conjugated to the one milligram of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ using different methods. The amount of anti-IgG conjugated to $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ via electrostatic adsorption, periodate method, ProG-anti-IgG binding, and glutaraldehyde crosslinking methods was 85.0 µg, 51.0 µg, 110.0 µg, and 60.0 µg per mg of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$; respectively. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -ProG-anti-IgG had the highest amount of conjugated anti-IgG. This could be attributed to the high and specific affinity of protein-G to the Fc-domain of anti-IgG. Protein G has two Fc-binding domains per molecules and could be responsible for higher conjugation efficiency.

The electrostatic adsorption of anti-IgG onto unmodified $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG had the second-highest conjugation than the covalent attachment. However, the covalent conjugation provides stable conjugates. The periodate method had the lowest conjugation efficiency, but the anti-IgG has the proper orientation that allows for efficient detection of the captured antibody. The orientation was controlled using the glycosylated Fc-region to bind the anti-IgG antibody. Also, protein G was covalently crosslinked via glutaraldehyde to the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ before the oriented capture of anti-IgG, hence forms a stable $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -ProG-anti-IgG bioconjugate.

6.2.3. Effect of anti-IgG conjugation on the peroxidase-like activity

The surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ has active catalytic sites. The effect of surface modification with anti-IgG was investigated. **Figure 6.2** shows the UV-vis absorption spectra for the peroxidase-like activity of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -ProG-anti-IgG, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(ads), (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(PI) and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(GA). The peroxidase-like activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ decreased after the conjugation of anti-IgG. This was attributed to the shielding of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ active sites from the TMB substrate [365]. The reduction might also be due to the Au-N or Pd-N bond formation between the catalytic metal and the amine group of the anti-IgG. The peroxidase-like activity was not significantly affected. **Figure 6.2** shows that peroxidase-like activity does depend on the bioconjugation method. The trend of the peroxidase-like activity of the bioconjugate was as follows $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -ProG-anti-IgG > $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(PI) > $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(ads) > $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -anti-IgG_(GA). The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ -ProG-anti-IgG has the highest peroxidase-like activity even though it has the highest amount of anti-IgG on the surface.

This was followed by the periodate anti-IgG method and the physically adsorbed anti-IgG. This suggested that the orientation of the anti-IgG could influence the peroxidase activity of the bioconjugate.

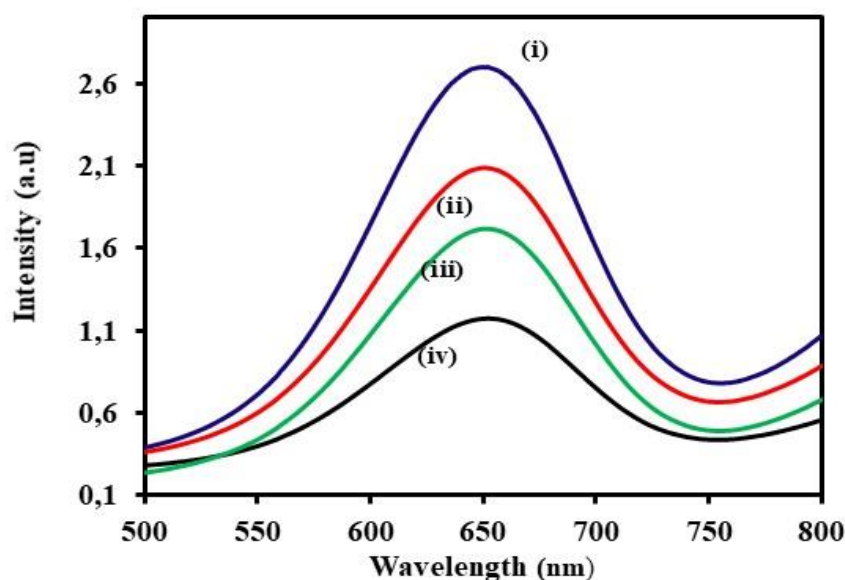


Figure. 6.2: UV-vis absorption spectra for peroxidase-like activity of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$, (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-PI-anti-IgG}$, (iii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-anti-IgG}$, and (iv) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-GA-anti-IgG}$.

Figure 6.3 shows the illustration of the orientation of the anti-IgG on $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ depending on the bioconjugation method. The glutaraldehyde mediated coupling would result in a random orientation with ‘lying-on,’ ‘side-on’ and ‘head-on’ oriented anti-IgG antibodies as shown in **Figure 6.3 (i)**. This would result in the crowding of the anti-IgG antibody on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ surface. Denser surface coverage of anti-IgG blocks the active metal sites and prevents the oxidation of TMB. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ conjugate had the highest peroxidase activity possibly due to the ‘end-on’ monolayer coverage of anti-IgG on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ surface, **Figure 6.3 (ii)**. Also, lower molecular size (21 kDa) Protein G in comparison to the anti-IgG

(150 kDa) were adsorbed electrostatically to the $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1\text{Pd}_3$ during the conjugation. Thus, the interaction of TMB with the active sites is the least inhibited.

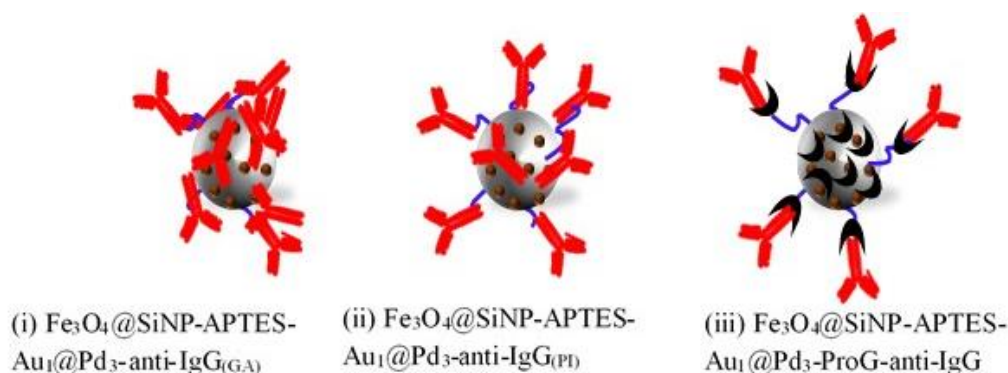


Figure 6.3: Illustration of anti-IgG conjugation and its orientation on $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ via different conjugation methods.

The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ was selected and evaluated for its performance in the development of immunobiosensor for the detection of anti-P53ab. The selection of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ was based on its (i) high peroxidase-like activity, (ii) covalent conjugation of Protein G, (iii) orientated conjugation of anti-IgG, and (iv) the specific affinitive binding of both protein G and anti-IgG for the captured anti-P53ab and would increase the sensitivity of detection.

6.2.4. XPS characterization of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$

Figure 6.4 shows the survey spectra of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ and (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. The survey spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ in **Figure 6.4** (i) shows the presence of binding energies attributed to silicon, gold, palladium, nitrogen, carbon, and oxygen. In comparison to $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$, the XPS spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ in **Figure 6.4** (ii) showed an intense peak at 396 eV, which is attributed to the N1s of protein-G-anti-IgG. The increase in the intensity of N 1s in **Figure 6.4** (ii) showed the successful conjugation of Pro-G-anti-IgG to form $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$.

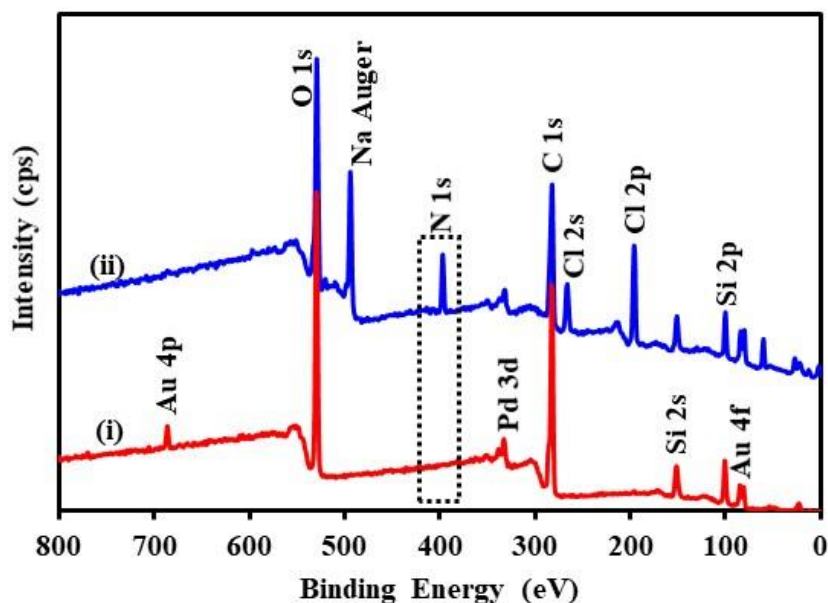


Figure 6.4: The survey spectra of (i) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$, and (ii) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$.

The peak at 495 eV, 265 eV, and 199 eV in **Figure 6.4 (ii)** was attributed to the Na auger, Cl 1s, and Cl 2p; respectively. The emergence of these peaks resulted from the trapping of Na and Cl ions from the PBS buffer (pH 7.4, 137 mM NaCl) used during the preparation of the XPS samples. The presence of gold, silica, and palladium at the surface of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ was evident. The Au 4f, Pd 3p, C1s, O 1s and N 1s high-resolution spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was discussed in **5.2.6, Chapter 5**.

Figure 6.5 shows the high-resolution XPS spectra of (a) C 1s, (b) N 1s, and (c) O 1s of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ bioconjugate. The deconvolution of the C 1s spectrum of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ in **Figure 6.5 (a)**, was fitted into four components at 284.9 eV (C-H, C-C), 286.3 eV (C-N), 288.2 eV (C=O) and 293.2 eV (π - π). The component at 288.2 eV was ascribed to the amide (N-C=O) and the carboxyl (O-C=O) of the Protein G-anti-IgG peptide bonds. The percentage composition of O-C=O, O=C-N was 20.26 %, and the C-N was 25.0 % in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. The components at 286.3 eV were attributed to the C-O and C-N groups of the Pro-

G-anti-IgG and the underlying covalent bond of the peptide of protein-G. The components at 293.2 eV were ascribed to the typical shake up line due to a $\pi - \pi$ transition of the aromatic side chain of phenylamine, tyrosine, and tryptophan of Pro-G and anti-IgG amino acid side chain.

The deconvoluted N 1s spectra of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd-ProG-anti-IgG}$ was fitted into three components at a binding energy of 397.7 eV ($-\text{NH}_2$), 400.10 eV (C-N, N-H) and 402.1 eV (O=C-N). The nitrogen components with the lowest binding energy at 397.7 eV was attributed to $-\text{NH}_2$ from the lysine bond of Protein-G. The peak at 400.1 eV was attributed to the amide bond ($-\text{NH}-$) nitrogen of the ProG-anti-IgG. The peak at 402.0 eV could be attributed $-\text{HN}-\text{C}=\text{O}$ of the amide (peptide) of anti-IgG. The deconvoluted N 1s spectra confirm the immobilization of the ProG-anti-IgG onto the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ via electrostatic adsorption and covalent conjugation. The atomic percentage of components was 93.6 % (CO-NH), 3.7 % (N-H, C-N) and was 2.7 % (N-C). The deconvolution of the O 1s of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd-ProG-anti-IgG}$ was fitted into five components with binding energies at 531.0 eV, 532.5 eV, 533.5 eV, and 533.9 eV, 537.1 eV. The intense peak at 532.5 eV was attributed to the presence of oxygen atoms linked to silicon (Si-O-Si). The peaks at 533.75 eV were attributed to the residual surface silanol groups (Si-OH) and O-H of adsorbed water. The peak at lower energies 531.59 eV could be due to other chemical states of oxygen in the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd-ProG-anti-IgG}$ resulting from the formation of metal oxide (Au-O, Pd-O). The peaks at 535.7 eV and 537.3 eV were assigned to the C = O for the peptide and acidic groups of the ProG-anti-IgG molecules. The percentage atomic composition of the Si-O-Si in $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ was 74.99 % and decreased to 62.3 % after the ProG-anti-IgG conjugation. The decrease was attributed to the conjugation of Pro-G-anti-IgG to $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au@Pd}$ surface. The atomic composition of the C=O and O=C-NH after antibody conjugation was 8.9 % and 18.6 %; respectively. The XPS results show the successful modification of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ with Pro-G-anti-IgG.

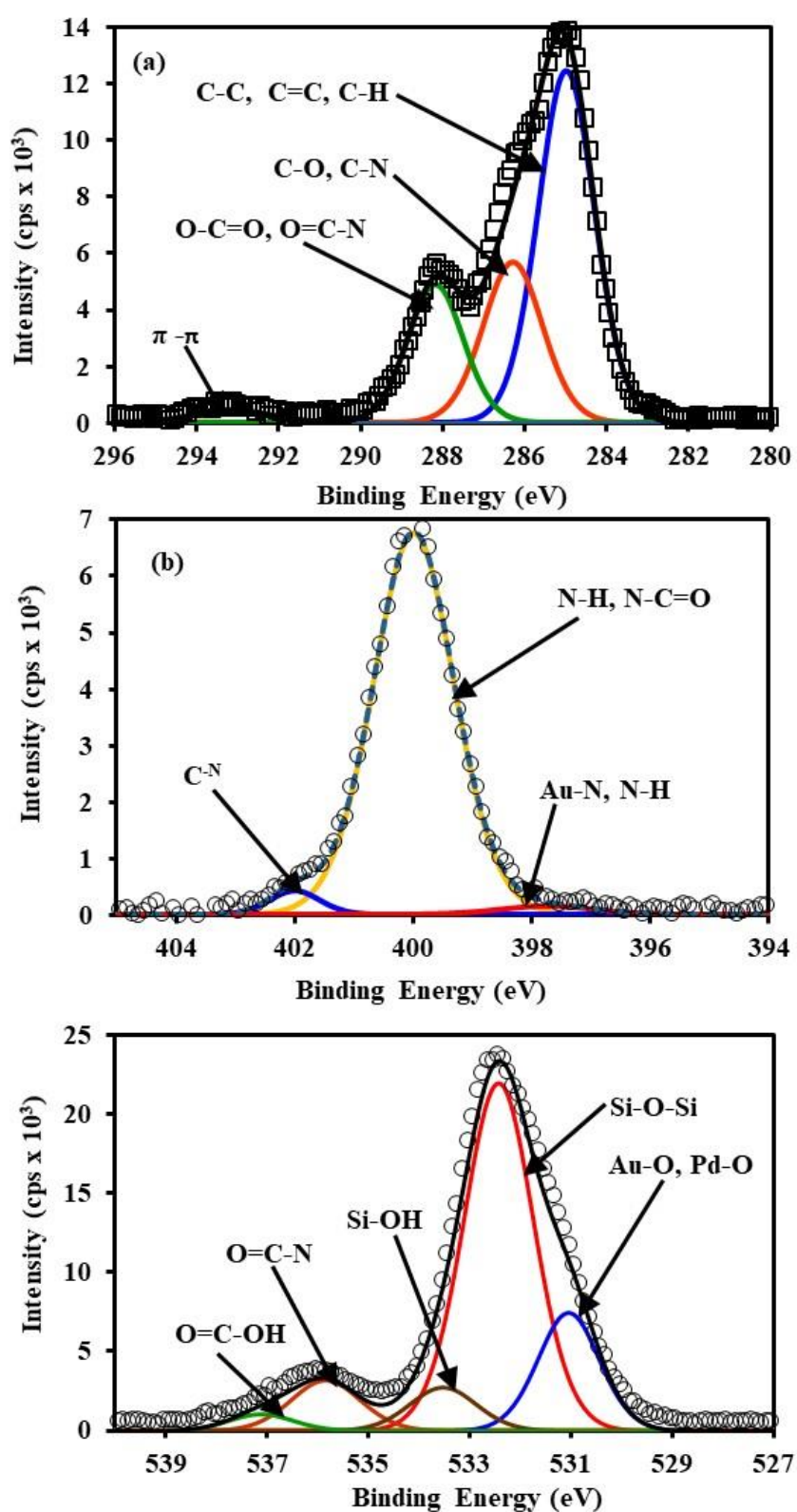


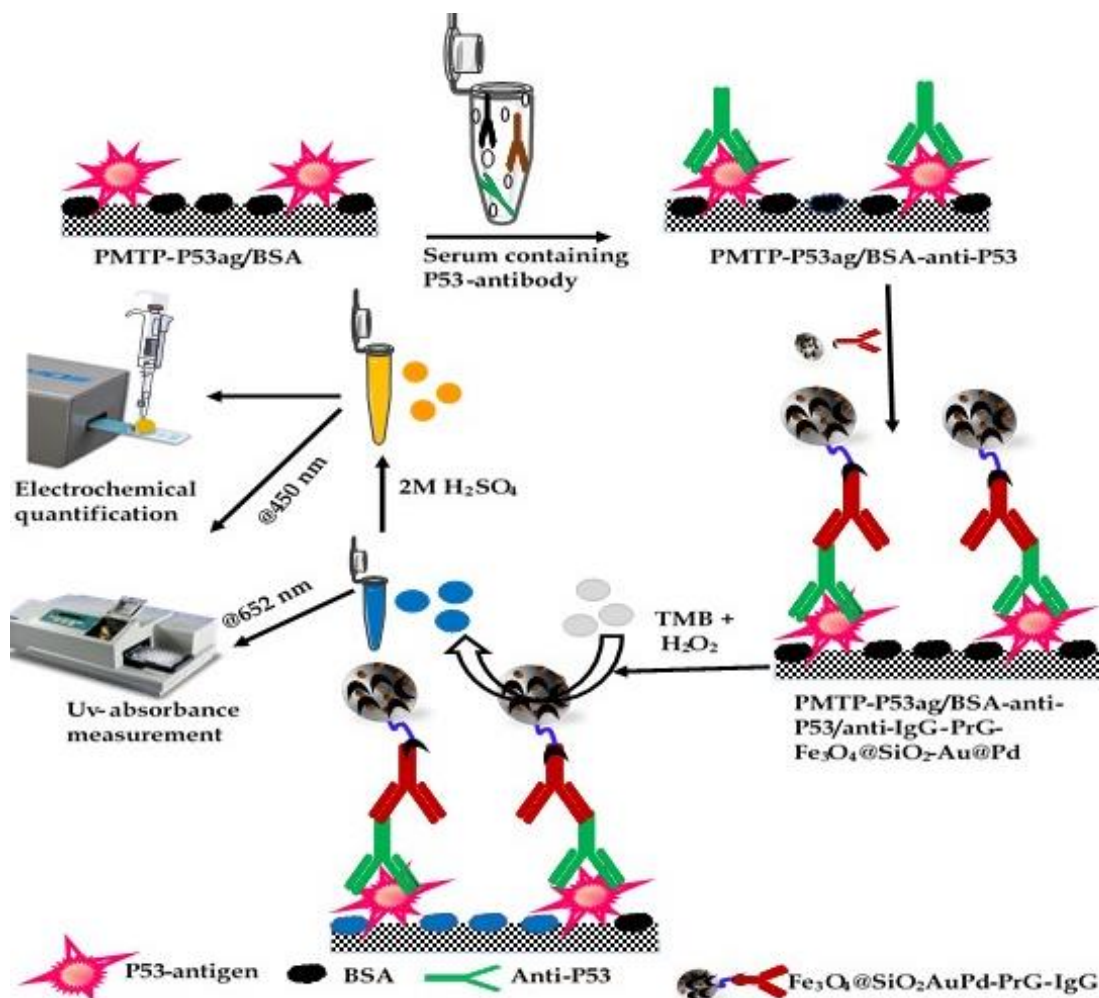
Figure 6.5: High-resolution spectra of (a) C 1s (b) N 1s and (c) O 1s in Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG.

Table 6.2. Summary of components position, component percentage and the assignments of peaks for the unmodified and ant-IgG conjugated Fe₃O₄@SiNP-APTES-Au₁@Pd₃.

Sample	Atom	Component	Position	%
Fe ₃ O ₄ @SiNP-APTES-Au@Pd-ProG-anti-IgG	C 1s	284.9	C-C, C = C, C-H	52.1
		286.3	C-O, C- N	25.0
		288.2	O – C = O, O = C - N	20.3
		293.2	$\pi - \pi^*$	2.6
	N 1s	397.7	N-H, Au-N	3.7
		400.0	N-C = O, O-C=O	93.4
		402.1	N-C	2.7
	O 1s	532.5	Si-O-Si	62.3
		531.1	C-O, metal oxide	18.0
		533.5	Si-OH, O-H	7.4
		535.9	O=C-NH	8.9
		537.1	O-C=O	2.7

6.3. The principle of peroxidase-like detection of anti-P53ab

The principle of the peroxidase-like detection of anti-P53ab was similar to the study in Chapter 4. The difference is the substitution of FITC@SiNP-APTES-PEI-anti-IgG with Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG. The FITC@SiNP-APTES-PEI-anti-IgG utilized fluorescent detection, while Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG utilizes the peroxidase-like activity. **Scheme 6.1** shows the schematic representation of the anti-P53ab peroxidase-like detection principle. P53ag was immobilized on a microtitre plate (PMTP), and BSA was used to block the non-specific binding sites. Serum samples containing anti-P53ab with different concentrations were incubated with the immobilized P53ag.



Scheme 6.1: Schematic representation of peroxidase-like detection of anti-P53ab.

The immobilized P53ag captures the anti-P53ab in the test samples forming a P53ag-anti-P53ab immunocomplex. The captured anti-P53ab interact with the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. The anti-IgG (on the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$) recognizes and binds onto the Fc-domain of the anti-P53abs. This results in an anti-P53ab/anti-IgG capture. The colorimetric substrate ($\text{TMB-H}_2\text{O}_2$) was added, and color development occurred. The intensity of the blue color developed is equivalent to the captured $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. After the addition of the stopping solution ($2\text{M H}_2\text{SO}_4$), the blue colored TMB_{ox} change to a yellow-colored TMB diimine product. The visual analysis or semi-qualitative analysis with Yes or No result could be performed based on

the color change. The comparison of color intensity allows for naked eye readout. For quantitative analysis, the absorption of the developed color was measured at 652 nm and 450 nm for the blue and yellow colored products, respectively. The absorption intensity was proportional to the concentrations of the anti-P53ab in the test samples.

6.3.1. Detection conditions of anti-P53ab

Experimental conditions such as pH, temperature, buffer constituents, concentrations of $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1\text{Pd}_3\text{-PrG-anti-IgG}$ and reaction time were evaluated. The effect of pH, temperature, TMB, and H_2O_2 concentrations and buffer constituent on the catalytic activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ have been optimized and reported in **Section 5.5**. The experimental conditions for the best peroxidase-like activity of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ were in sodium acetate-acetic acid buffer (0.2 M), pH 5.0 at 30 °C and TMB to H_2O_2 mole ratio of 1:40. These conditions were used for the optimization of the other experimental conditions for the detection of anti-P53ab. For quantitative determination of anti-P53ab, the stop solution (H_2SO_4) was added to (i) terminate the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG/TMB-H}_2\text{O}_2$ reaction, (ii) stabilizing the oxidized products for accurate anti-P53ab measurement, and (iii) to convert the TMB oxidized product to yellow derivatives with a 2 to 3-fold increase in the molar absorptivity at 450 nm. The reaction time before the addition of the stop solution was evaluated.

Figure 6.6 shows the plot of absorption intensity against reaction time. The absorption intensity increases with increasing reaction time up to 5 minutes and becomes stable for up to 15 minutes. After 15 minutes a decrease in the absorption intensity was observed. This decrease in absorption could be attributed to the back reaction of the TMB/diimine complex to TMB. Five minutes was selected as the optimum color development time before the addition of the stop solution (2 M, H_2SO_4).

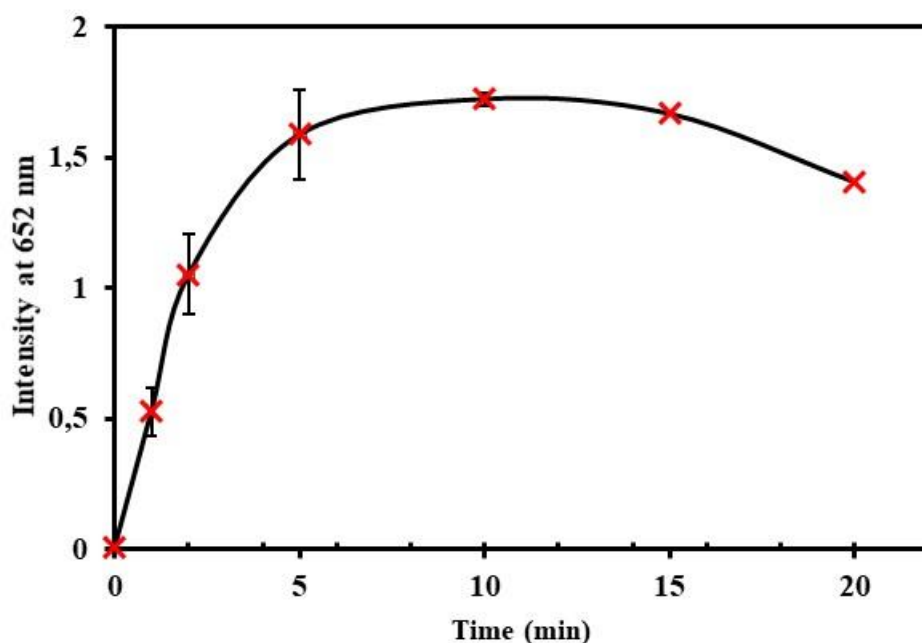


Figure 6.6: Variation of absorption intensity at 652 nm for $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG/TMB-H}_2\text{O}_2$ system with time.

Using the optimal experimental conditions a chessboard titration assay (CBT) was used for the optimization of the working concentration of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ and the incubation time. A stock $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ solution (2.0 mg.ml^{-1}) was diluted serially in PBST buffer (containing 0.05 % BSA) to /10 ($200.0 \text{ }\mu\text{g/ml}$), /20 ($100.0 \text{ }\mu\text{g/ml}$), /100 ($20.0 \text{ }\mu\text{g/ml}$), /1000 ($2.0 \text{ }\mu\text{g/ml}$), /2000 ($1.0 \text{ }\mu\text{g/ml}$) dilution ratio. The concentration of immobilized P53ag was fixed at $10.0 \text{ }\mu\text{g.ml}^{-1}$, and 500.0 ng.ml^{-1} anti-P53ab was detected to optimize the immunobiosensor response.

Table 6.3 shows the results of the CBT with different $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ concentrations. The signal intensity in the positive wells increases as the concentration of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ increases. The increase in signal intensity was due to an increase in the formation of the immunocomplex with $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. The background signal also increases at a high

concentration of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$. The increase in the background signal was due to an increase in the degree of non-specific accumulation of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ to the microtiter plate.

Table 6.3: Chessboard titration assay for the $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-Au}_1\text{Pd}_3\text{-ProG-immunobioprobe}$

Dilution ratio	Absorption @652 nm							S/N
	SW			NC		Average signal intensity ±SD	Average background signal ±SD	
	1	2	3	4	5			
/10	2.843	2.961	3.683	0.972	1.051	3.16 ± 0.45	1.01 ± 0.05	3.1
/20	2.726	2.243	1.836	0.834	0.560	2.27 ± 0.44	0.69 ± 0.19	3.3
/100	1.847	1.549	1.946	0.110	0.135	1.78 ± 0.20	0.12 ± 0.02	14.5
/1000	0.848	1.050	0.796	0.082	0.090	0.90 ± 0.23	0.09 ± 0.01	10.0
/2000	0.342	0.401	0.368	0.052	0.071	0.25 ± 0.03	0.06 ± 0.01	4.2

The stock solution of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ was 2.0 mg.ml^{-1} . The sample wells (SW) are wells feed with anti-P53ab. Negative control (NC) as well without anti-P53ab, signal-to-noise ratio (S/N) is the ratio of the average signal from the SW well to NC.

This results in a low signal-to-noise (S/N) ratio. The S/N ratio of 3.10, 3.30, 14.5, 10.01 and 4.20 was recorded as the dilution factor of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ increases. At high conjugate concentrations of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$, the S/N ratio was low. This will result in false-positive due to the high signal to noise ratio. At low concentrations, the signal is much higher than the noise and this allows for discrimination between the signal and the noise. The optimum high signal to noise ratio was obtained at 100 dilution factor or at a concentration of $20.0 \text{ }\mu\text{g.ml}^{-1}$. The high signal to noise ratio provides a more significant advantage in achieving high specificity, sensitivity and lower

detection limits. The result of the CBT shows that the highest S/N ratio was recorded at a /100 dilution ratio ($20.0 \mu\text{g.ml}^{-1}$). Therefore, the /100 ($20.0 \mu\text{g.ml}^{-1}$, $50.0 \mu\text{l}$) was the optimal concentration of $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ selected to achieve the best analytical performance of the peroxidase-like detection of anti-P53ab and excellent discrimination of signal to noise ratio.

The anti-P53 and $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ incubation time were also evaluated. **Figure 6.7** shows the effect of (a) anti-P53ab and (b) $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ incubation time. In **Figure 6.7 (a)**, the signal increased with an increase in incubation time up to about 40 minutes. A 98 % signal was achieved at 40 minutes and this incubation time was selected as optimum anti-P53ab incubation time. For $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$, the incubation time increased up to 30 minutes, **Figure 6.7 (b)**. The signal decreased afterward. The significant signal output recorded within the first 10 minutes of incubation is associated with the high affinitive binding of the Pro-G-anti-IgG for the Fc-domain of the anti-P53ab. Anti-IgG and Protein-G bind specifically to the anti-P53ab. The optimal $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ incubation time chosen for further investigation was 30 minutes.

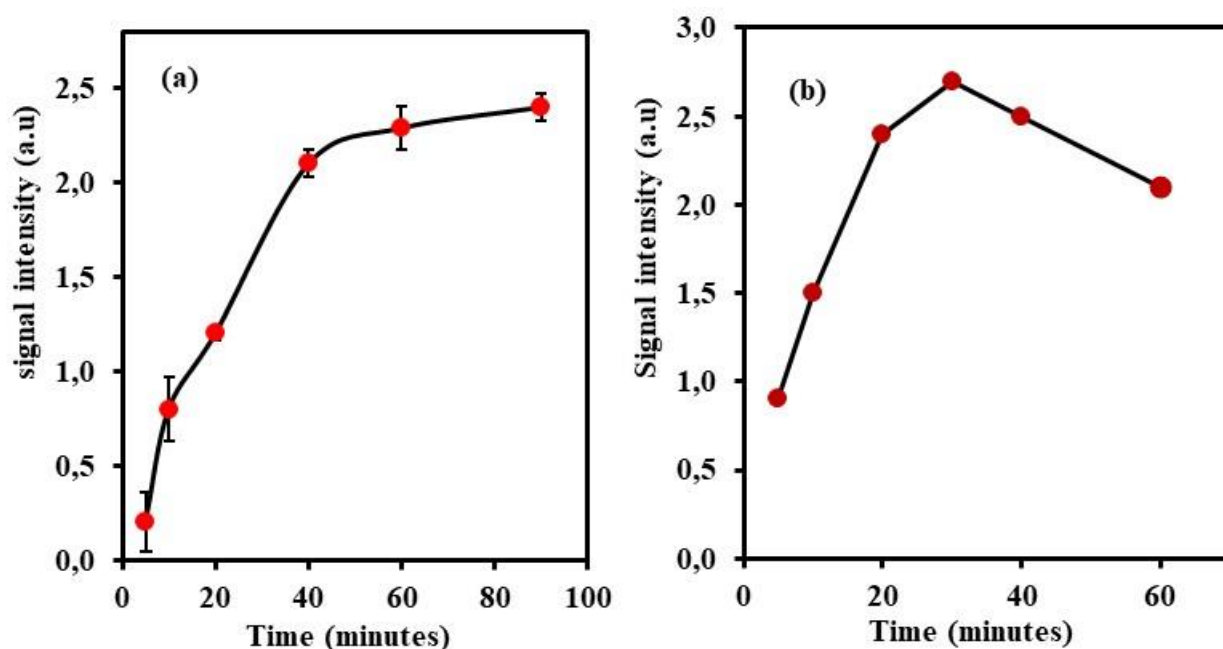


Figure 6.7: Effect of incubation time of (a) anti-P53ab, and (b) Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG on the peroxidase-like detection of anti-P53ab.

6.3.2. Analytical performance of peroxidase-like detection of anti-P53ab

A series of different concentrations of anti-P53ab ranging from 0.200 to 1000.0 ng ml⁻¹ were detected by using Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG as a signal probe. **Table 6.4** shows the experimental setup in a 96 well polystyrene microwell plate. Each well was coated with the same concentration of P53ag (10.0 µg.ml⁻¹) and blocked with BSA to prevent non-specific adsorption. The different concentrations of anti-P53ab were studied in triplicate (n = 3) for each concentration. In Column 1 samples (P1 and M1) are the most concentrated (1000 ng.ml⁻¹) and column 7 (P7 and M7) is the least concentrated (0.20 ng.ml⁻¹). Rows A₁-A₇, B₁-B₇, and C₁-C₇ are used for the detection of the polyclonal anti-P53ab (PAb) while rows F₁-F₇, and G₁-G₇, H₁-H₇ were used for the detection of monoclonal anti-P53ab (Mab). The concentrations of PAbs and MAbs were kept the same in each corresponding column wells. The negative control (NC) wells contain all reagents except the targeted anti-P53ab. The blank

control (B) wells contain the P53ag and blocked with BSA but without anti-P53ab and Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG signal probe. The concentration of the detection probe (Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG) was 20.0 µg/ml. The substrate is acetate buffer (pH 5.0) containing TMB (1.0 mM) and H₂O₂ (40.0 mM).

Table 6.4: The template of the microwell layout for peroxidase-like detection of anti-P53ab

	Positive Test							Blank		Control		
	1	2	3	4	5	6	7	8	9	10	11	12
A	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₇	B	B	NC	NC	NC
B	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₇	B	B	NC	NC	NC
C	P ₁	P ₂	P ₃	P ₄	P ₅	P ₆	P ₇	B	B	NC	NC	NC
D	X	X	X	X	X	X	X	X	X	X	X	X
E	X	X	X	X	X	X	X	X	X	X	X	X
F	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇	B	B	NC	NC	NC
G	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇	B	B	NC	NC	NC
H	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇	B	B	NC	NC	NC

Figure 6.8 shows the color development at each well after the addition of (a) TMB + H₂O₂ substrate and (b) after the addition of 2 M H₂SO₄ stop solution to (a). The positive sample wells with the polyclonal and monoclonal anti-P53ab (row 1-7) showed a dark blue color change after the addition of the TMB/H₂O₂ substrate. The blank samples wells (without Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG) did not show the blue color. The positive and negative control showed blue color development due to the presence of the Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG. The color change in the positive wells confirms the formation of P53ag/anti-P53ab/Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG immunocomplex. The negative control samples (NC) without the targeted anti-P53ab showed a faint blue to colorless color. However, the color intensity of the NC wells can be distinguished from that of the positive test well with the lowest concentration of the anti-P53ab even with the naked eye. The

changes in the appearance of the color from deep blue to light blue with increasing concentration of anti-P53ab (wells A1 to A7). The color inside the wells shows a concentration dependence of the captured anti-P53ab. The variation in the color intensity between the positive and negative samples shows that the immunobiosensor could allow the naked-eye detection of the anti-P53ab. In **Figure 6.8 (b)** the addition of H_2SO_4 results in the stabilization of the TMB oxidation products and the formation of a yellow color product. The intensity of the yellow color increases with an increase in concentrations of anti-P53ab. **Figure 6.9** shows the absorption spectra between 350 nm to 550 nm obtained after the addition of the stopping solution. In **Figure 6.9 (i) and (ix)**, the absorption intensity at 450 nm increases as the concentration of the anti-P53ab increases from 0.20 ng.ml^{-1} to $1.0 \text{ }\mu\text{g.ml}^{-1}$. The increase in the intensity was proportional to the amount of anti-P53ab in the test samples.

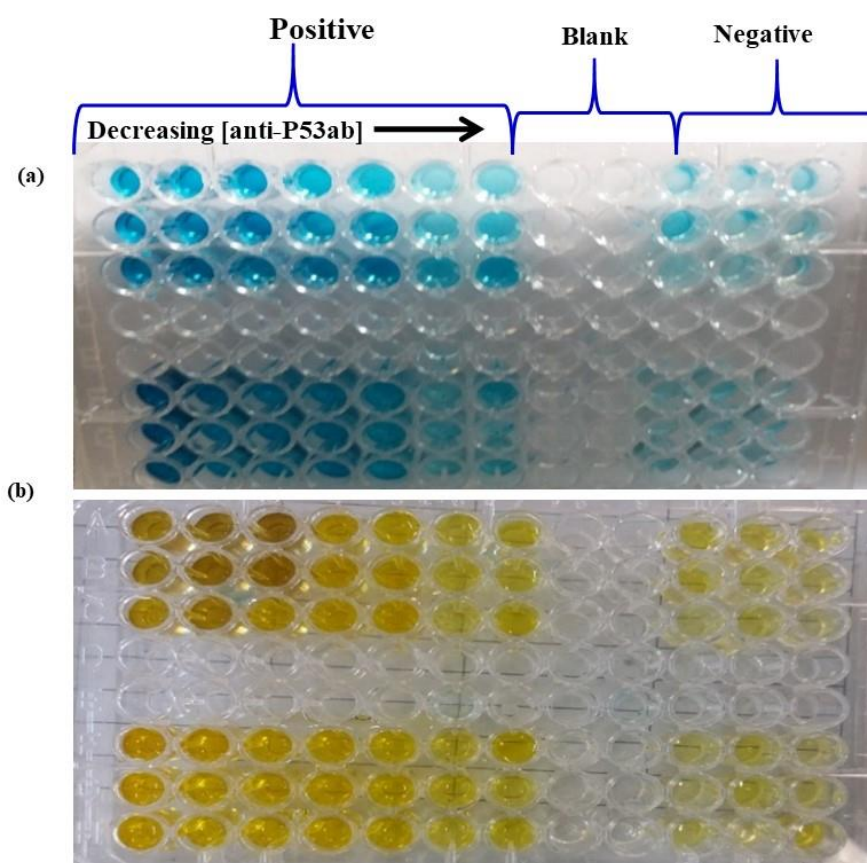


Figure 6.8: Peroxidase-like detection of anti-P53ab after the addition of (a) TMB/ H_2O_2 substrate (b) and after the addition of 0.5 M H_2SO_4 .

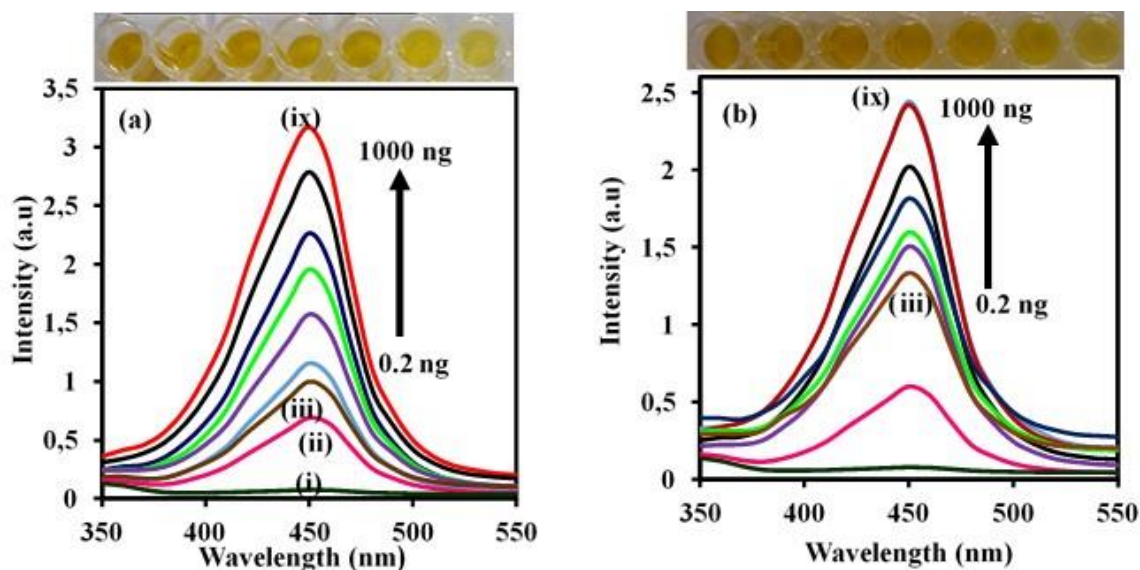


Figure 6.9: UV-Vis absorption spectra of the immunobiosensor as a function of the different concentration of (a) monoclonal and (b) polyclonal antibodies (i) is the blank (ii) the negative control and (ii) anti-P53ab concentrations ranging from 0.2 ng.ml⁻¹ to 1.0 µg ml⁻¹.

6.3.3. Quantitative Analysis of anti-P53ab

For the quantitative colorimetric determination of anti-P53ab, the optical density at 652 nm for the blue colored TMB complex and 450 nm for the yellow-colored TMB diimine products were measured. The mean value of the absorption of the negative control (NC) was subtracted from the mean of the replicate positive test results ($n = 3$) and plotted against the concentration of the anti-P53ab. **Figure 6.10** shows the plot of optical density (OD) at (a) 652 nm and (b) 450 nm against the concentrations of anti-P53ab ranging from 0.20 ng.ml⁻¹ to 1.0 µg ml⁻¹ for (i) monoclonal anti-P53ab and (ii) polyclonal anti-P53ab. The dose-response curve showed that the value of the optical density increases with increasing concentrations of anti-P53ab. Also, it is noticeable from the dose-response curve that the polyclonal anti-P53ab showed higher signal response than the monoclonal anti-P53ab when the absorption measurement at 650 nm was considered. This could be attributed to the polyclonal antibodies interacting with multiple epitopes on the P53ag. After the stop solution was added, the OD values (at 450 nm) were

higher for both polyclonal and the monoclonal anti-P53ab. The higher signal intensity of the OD measurement at 450 nm would result in a higher magnitude of the slope of the calibration curve indicating higher sensitivity. Likewise, HRP- based ELISA also uses the measurement of absorption at 450 nm. Thus, the OD at 450 nm is further considered for the evaluation of the analytical performance of the immunobiosensor. Circulating tumor-associated autoantibodies are secreted from identical B-cell, so they are mostly monoclonal antibody of the IgG subtype. Therefore, the analytical performance of the developed method was based on the detection of monoclonal anti-P53ab and the measurement of OD at 450 nm.

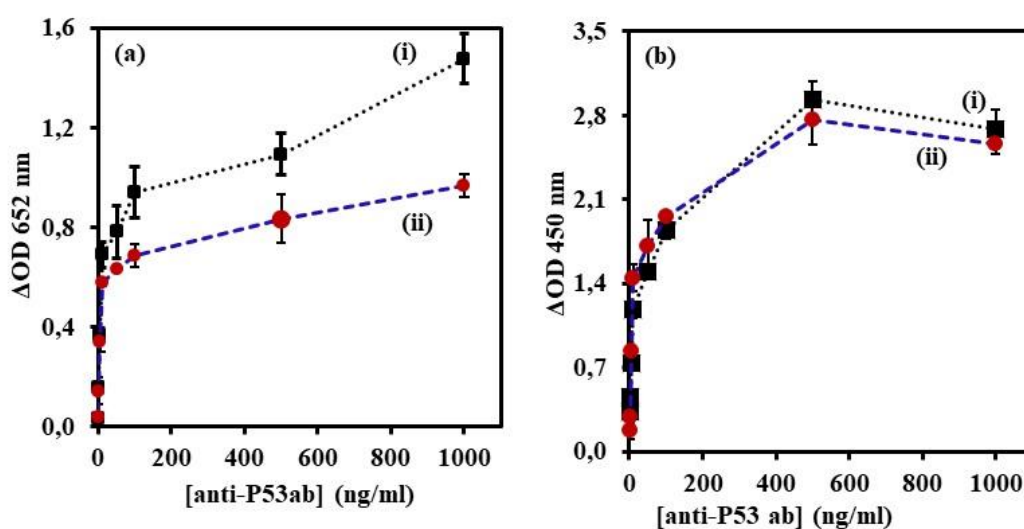


Figure 6.10: Dose-response curve of optical density (OD) at (a) 652 nm and (b) 450 nm against the concentrations of anti-P53ab ranging from $0.2 \text{ ng} \cdot \text{ml}^{-1}$ to $1.0 \mu\text{g} \cdot \text{ml}^{-1}$ for (i) polyclonal anti-P53ab and (ii) monoclonal.

Figure 6.11 shows the calibration curve for the monoclonal anti-P53ab using (a) a linear plot of the change in OD (ΔOD at 450) against the anti-P53ab concentrations in the linear portion of the dose-response curve and (b) using a linear-log plot of ΔOD at 450 against the logarithm of the concentrations of anti-P53ab. The linear-log fit plot linearizes the non-linear portion of the dose-response curve. This increases the linear detection range for the anti-P53ab. In **Figure 6.11 (a)** a linear fit plot of ΔOD against the concentrations of monoclonal anti-P53ab was

obtained between the linear working ranges of 0.2 ng.ml⁻¹ to 10.0 ng.ml⁻¹. The linear regression equation is given by **Equation (6.1)**:

$$\Delta\text{OD at 450 nm} = 0.1298 [\text{anti-P53ab}] + 0.1582 \quad R^2 = 0.998 \quad (6.1)$$

From the linear regression equation (**Equation 6.1**), the LoD and LoQ were estimated to be 110.0 pg.ml⁻¹ and 390.0 pg.ml⁻¹ respectively at a signal to noise ratio of 3 σ /slope and 10 σ /slope respectively ($\sigma = 0.005$, $n = 9$).

In **Figure 6.11 (b)**, the linear-log plot of the logarithm of anti-P53ab concentrations was plotted against the ΔOD . The plot of ΔOD was proportional to the logarithm of the anti-P53ab concentration in the range of 200.0 pg.ml⁻¹ to 100.0 ng.ml⁻¹. The linear regression equation was given by **Equation 6.2**.

$$\Delta\text{OD} = 0.738 \log [\text{anti-P53ab}] - 1.65, \quad R^2 = 0.989 \quad (6.2)$$

The LoD and LoQ were calculated from **Equation 6.2**, and the standard deviation of the negative control samples ($\sigma = 0.005$, $n = 9$) and was found to be 20 fg.ml⁻¹ and 70 fg.ml⁻¹ of anti-P53ab; respectively. The sensitivity, LoD, LoQ and the wider linear range of anti-P53ab detection was improved using the linear-log plot. This was due to the linearization of the non-linear portion of the dose-response curves.

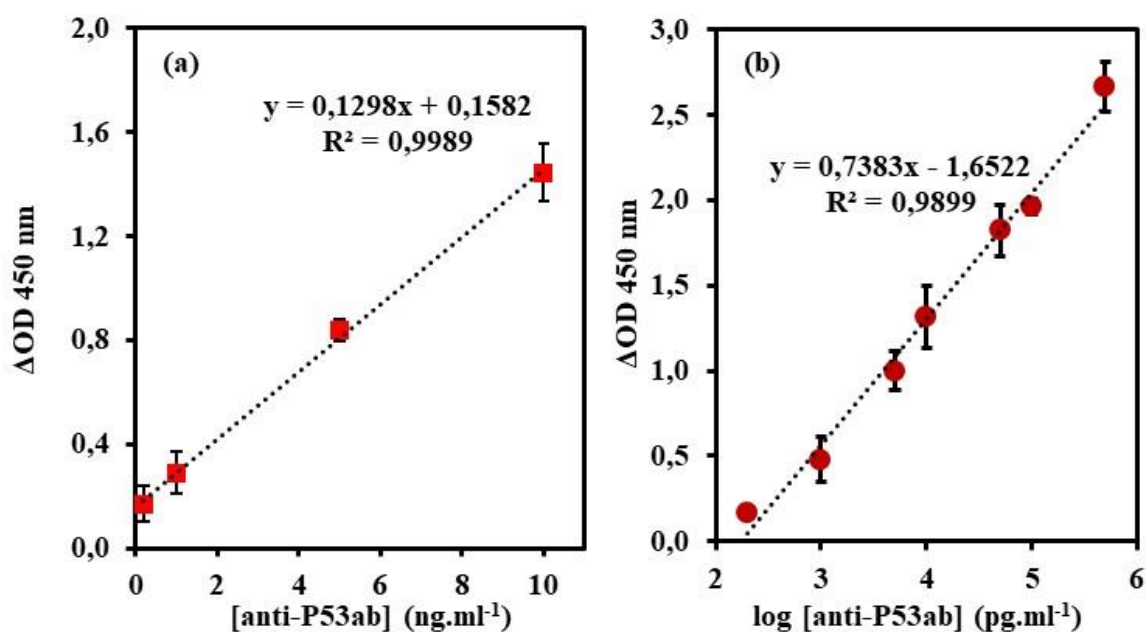


Figure 6.11: Calibration plot of the change in optical density against concentration in the linear range and (d) plot of normalized signal ($\Delta OD/OD_{NC}$) against the logarithm of monoclonal anti-P53ab.

6.3.4. Analysis of anti-P53ab in simulated serum samples

To evaluate the clinical application potential of proposed the $Fe_3O_4@SiNP-APTES-Au_1@Pd_3$ -ProG-anti-IgG mediated immunobiosensor, a recovery test of anti-P53ab in ‘simulated’ serum sample was evaluated. The practical application was evaluated determining the recovery of anti-P53ab in diluted serum samples (1:10). The percentage recovery was calculated using **Equation 6.3**.

$$\% Recovery = \frac{[anti-P53_{detected}]}{[anti-P53_{added}]} \times 100\% \quad (6.3)$$

Table 6.5 shows the recovery studies of 0.2 ng.ml^{-1} , 10.0 ng.ml^{-1} , 50.0 ng.ml^{-1} and 100.0 ng.ml^{-1} of anti-P53ab added into the serum sample. The recovery of the anti-P53ab ranges from 98.5 % to 105.7 % and the RSD ranged from 2.06 % to 3.37 %. These results indicate that the

developed peroxidase-like immunobiosensor has great potential for the detection of anti-P53ab in clinical serum samples.

Table 6.5: Recovery of anti-P53ab from spiked serum samples using the standard addition method.

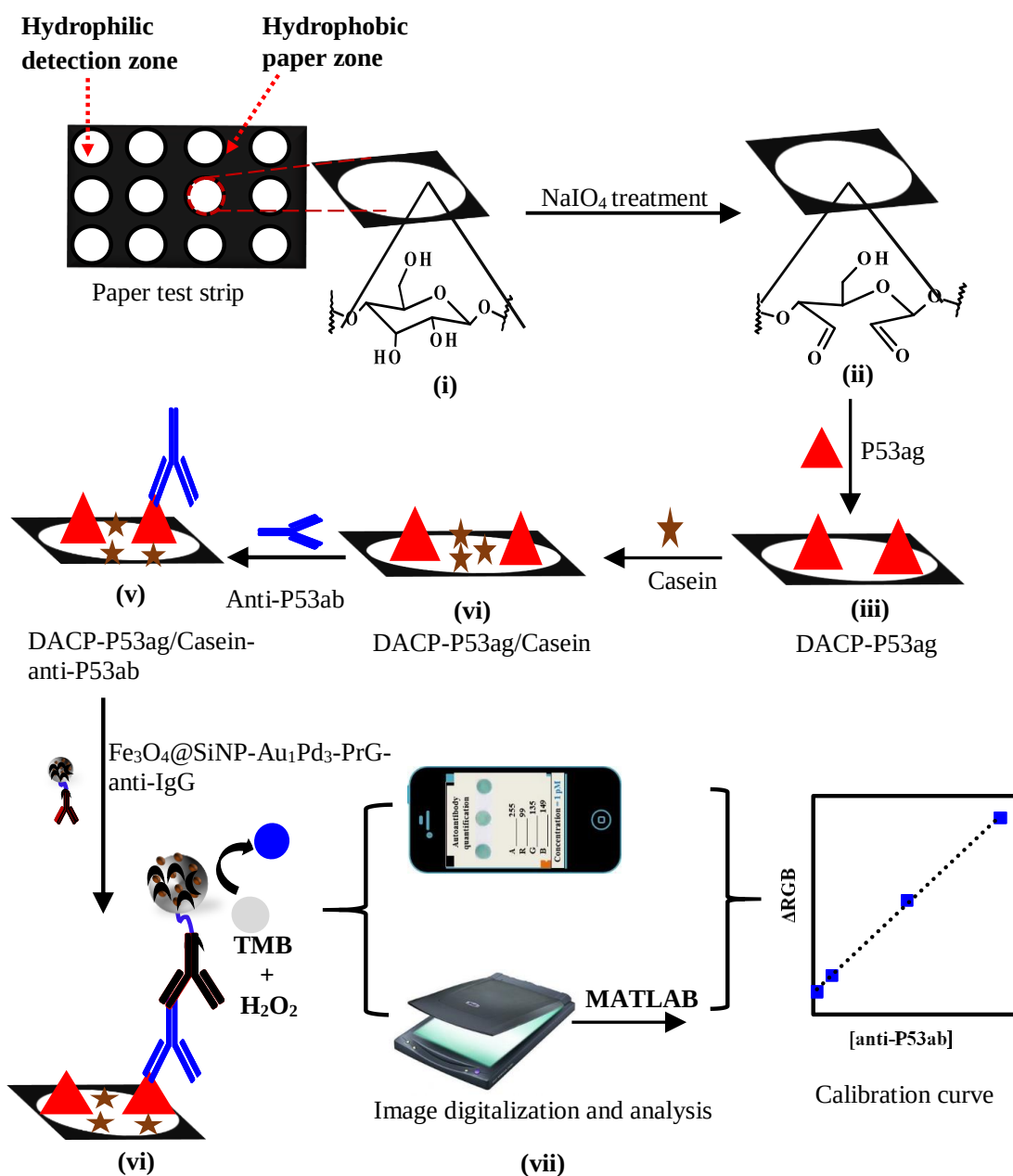
Sample No	Added (ng/ml)	Detected (ng/ml)	RSD (%) (n =3)	Recovery (%)
1	0.2	0.197	3.37	98.5
2	10	10.56	2.31	105.6
3	50	52.4	2.06	104.8
4	100	105.7	2.89	105.7

6.4. Paper-based colorimetric peroxidase-like detection of anti-P53ab

6.4.1. Fabrication of paper discs sensor chips and immobilization of P53 antigen (P53ag)

The fabrication of the paper sensor was done using simple paper cutting and laminating techniques. Work reported by Tenda et al. had inspired the basis of these techniques, [381]. The fabrication is simple, low cost and does not require any sophisticated equipment in the production. The paper sensors were produced using Whatman filter paper No. 1 due to its high wicking rate and permeability [382]. **Scheme 6.2** shows the schematic representation of the fabrication of the paper-based immunobiosensor for the detection of anti-P53ab. The scheme shows (i) paper sensing zone (ii) periodate treated paper zone (iii) immobilization of tumor antigen on paper through aldehyde generated by periodate treatment (iv) blocking with BSA (v) paper zone after incubation with serum autoantibodies solution (vi) incubation with $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ immunobioprobe and color development with TMB/ H_2O_2 substrate, and (vii) image acquisition and analysis using scanner or smartphone readout. The paper was cut into a circular disc of 6 mm using a standard paper puncher. The transparent polyvinyl chloride (PVC) sheets were cut into the desired shape and circular hole

of 4 mm. The paper disc was mounted on the PVC using double-sided tape. The paper disc is hydrophilic and has a small footprint making solvent wicking easy thus enhancing color uniformity.



Scheme 6.2: Schematic representation of paper-based peroxidase-like immunobiosensor for anti-P53ab detection

The sensing area (paper disk) was oxidized with sodium periodate to create reactive dialdehyde groups on the paper sensing area. The dialdehyde cellulose paper (DACP) occurs by the cleavage of the vicinal secondary hydroxyl groups at the C2 and C3 positions of the cellulose paper and subsequent oxidation to aldehyde, **Scheme 6.2 (ii)**. The aldehyde groups react with the amine groups on the P53ag, **Scheme 6.2 (iii)**. Subsequent reduction with sodium cyanoborohydride results in the formation of a covalently bonded antigen onto the paper disc through a secondary amine bond. The paper was treated with BSA to block non-specific adsorption sites. The P53ag modified cellulose paper was referred to as DACP-P53-ag/BSA.

6.5. Characterization of fabricated paper sensor

6.5.1. FTIR characterization of fabricated paper immunobiosensor

The modification of the Whatman cellulose paper with aldehyde groups and immobilization of P53ag was investigated by FTIR. **Figure 6.12** shows FTIR spectra of **(a)** (i) untreated and (ii) periodate treated paper and **(b)** (i) dialdehyde paper and (ii) P53ag modified paper. The untreated Whatman filter paper in **Figure 6.12 (a) (i)** exhibited characteristics IR absorption bands at 3319 cm^{-1} and 1630 cm^{-1} (-OH stretch), 2883 cm^{-1} (-C-H stretch), $1227\text{-}1427\text{ cm}^{-1}$ ($\text{CH}_2\text{-OH}$ bending), $1107\text{-}709\text{ cm}^{-1}$ (-C-O-C glycosidic bond) and 1058 cm^{-1} (pyranose cycles). The FTIR spectrum of periodate treated Whatman paper in **Figure 6.12 (a) (ii)** shows similar bands as the untreated paper. However, the emergence of a new band at 1728 cm^{-1} and an increase in the intensity of the peak at 880 cm^{-1} was evident after the periodate oxidation of the paper. The new band at 1728 cm^{-1} is attributed to the characteristic carbonyl (C=O) stretching vibrations and confirms the successful formation of the dialdehyde cellulose paper (DACP). After the immobilization of P53ag on the DACP filter paper, the intensity of the dialdehyde peak decreased, **Figure 6.12 (b)**. The formation of the covalent bond (Schiff-base) between the aldehyde groups and the amine groups of the P53-antigen was observed. The emergence of the

peak at 1638 cm^{-1} (N=C) and 1554 cm^{-1} (N-H) stretching vibration of the Schiff base. Thus, the P53ag was successfully immobilized onto the cellulose paper.

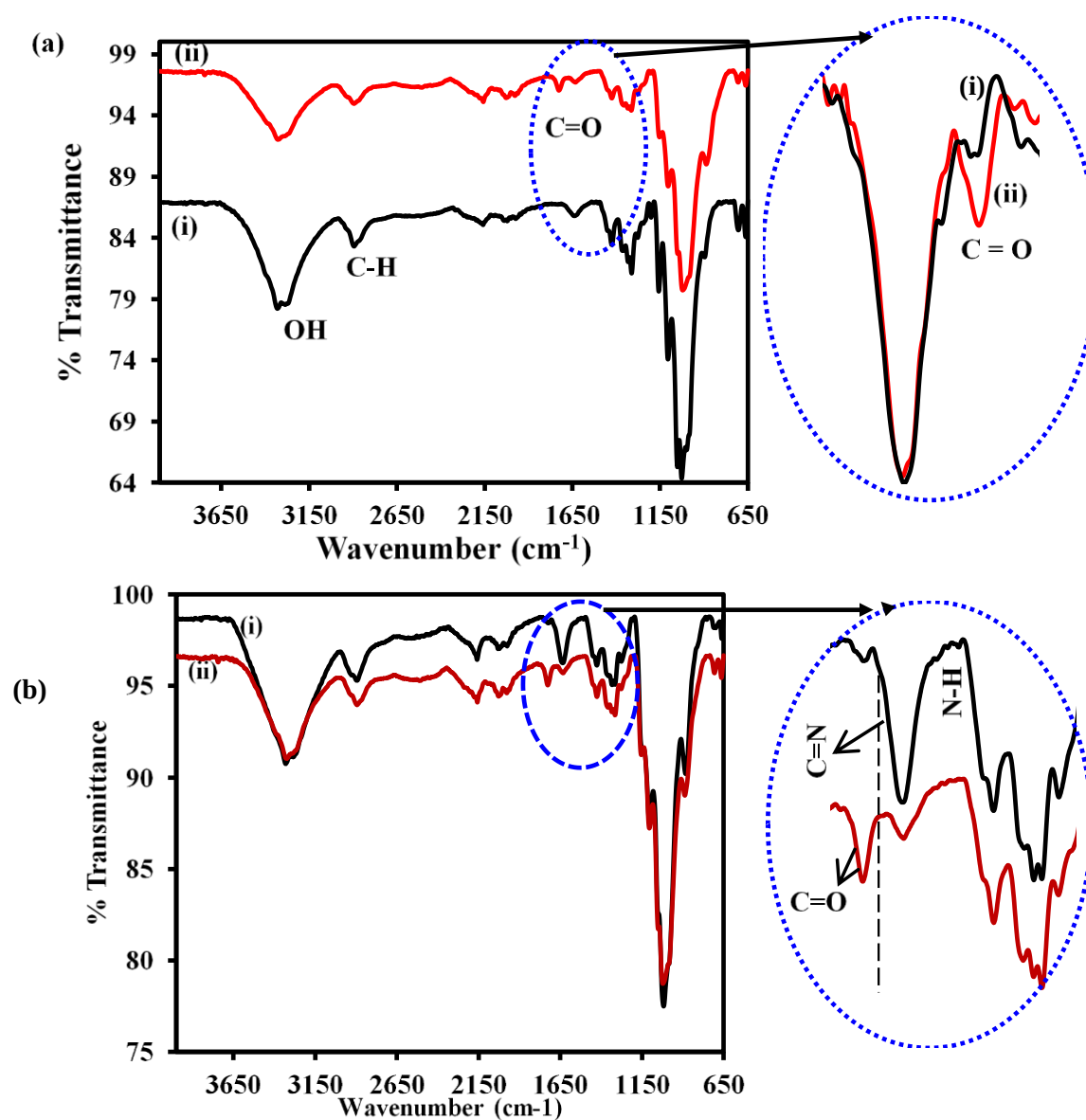


Figure 6.12: (a) FTIR spectra of (i) untreated paper and (ii) periodate treated and (b) FTIR spectra of the paper zone (i) before, and (ii) after tumor-antigen immobilization.

6.5.2. Surface morphology of the fabricated paper immunobiosensor

The surface morphology was investigated before and after the periodate treatment of the paper and after the immobilization of the P53ag. **Figure 6.13.** Shows the SEM micrographs of (a) untreated and (b) periodate treated Whatman filter paper.

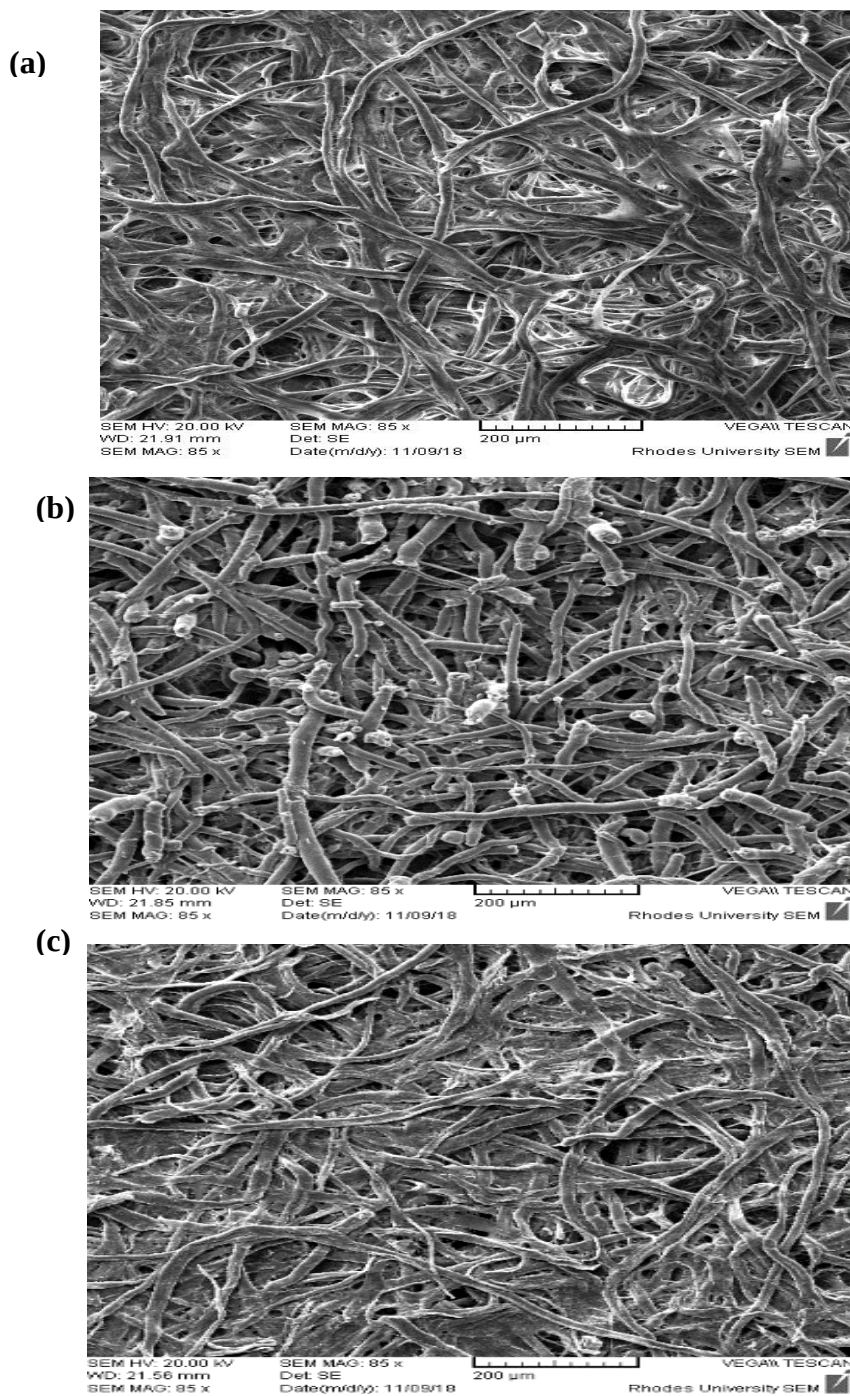


Figure 6.13: SEM images of (a) unmodified Whatman filter paper, (b) periodate treated Whatman filter paper, and (c) P53ag-immobilized cellulose paper.

The SEM image of the unmodified filter paper in **Figure 6.13 (a)** shows a highly interwoven cellulose fiber network with a smooth surface. However, after the periodate treatment, the cellulose fiber network appears like a knit-tube-like structure with a small sting and cracks. The cracks and the thickness of the tube-like bundles increase with increasing periodate concentrations. The SEM image of the P53ag modified paper shows a flattened bundle that is highly interconnected; it could be inferred that the changes in the surface morphology of the paper after the P53ag immobilization was due to the crosslinking of the P53ag to the cellulose fiber network via the dialdehyde groups.

6.3. Optimization of paper-based anti-P53ab detection conditions

The blocking step of the immunobiosensor was optimized to reduce the non-specific binding. The effect of blocking buffer constituents and concentrations were investigated to reduce the non-specific binding. Non-fatty milk protein such as casein and BSA has been used to reduce non-specific adsorption in paper-based immunobiosensors [383–385]. **Figure 6.14** shows the (a) plot of signal response in the positive to negative test zone obtained for different blocking buffer consisting of BSA and milk (1.0 -5.0 %) in PBST buffer. The mean pixel intensity of six replicate for positive and negative test zones is presented with the blue column and red column; respectively. The error bars show the standard deviation. (b) The signal to noise ratio of the different blocking buffer system. The experimental result in **Figure 6.14 (a)** showed that the signal intensity of the negative control sample decreased when the percentage composition of the blocking buffer increases from 1.0 to 5.0 % for both skimmed milk and BSA. The signal output of the sensing zone blocked with milk was lower than that of the BSA blocked zones. However, as shown in **Figure 6.14 (b)**, the 5.0 % non-fat dry milk in PBST buffer blocked zones had a high signal to noise ratio (S/N). This could allow for the detection of the lower concentration of the anti-P53abs. Thus 5.0 % non-fat dry milk in PBST (10 mM, pH 7.5) was used for the blocking of paper immunobiosensor.

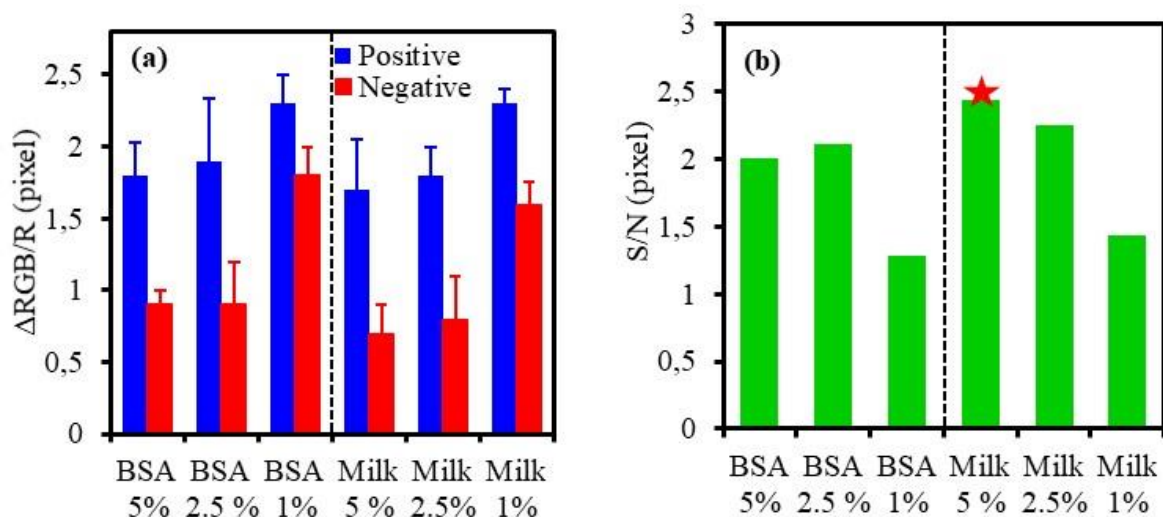


Figure 6.14: (a) plot of signal response in the positive to negative test zone with the different blocking buffer consistent (BSA and milk (1.0 -5.0 %) in PBST buffer), and (b) the signal to noise ratio of the different blocking buffer system. The error bars show the standard deviation.

The effect of reaction time on the $\Delta RGB/R$ pixel intensity was investigated at different intervals after the addition of TMB+H₂O₂ substrate to obtain optimal analytical performance. **Figure 6.15** shows the plot of signal intensity against reaction time after the addition of TMB substrate to the paper zone. The $\Delta RGB/R$ pixel intensity increases with increasing reaction time from 2 to 6 minutes and decreases afterward. For further studies, the optimum color development time selected was 6 minutes before the image was captured. Therefore, the optimum experimental conditions used for the translation of the microwell plate assay to paper-based platform was 50.0 $\mu\text{g}.\text{ml}^{-1}$ (2.0 μl) of P53ag, 20.0 $\mu\text{g}.\text{ml}^{-1}$ (4.0 μl) of Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG, blocking with non-fat dry milk (5.0 %) in PBST (0.1 mM, pH 7.5) and incubated for 10 minutes and the substrate was TMB/H₂O₂ in sodium acetate/acetic acid buffer (0.2 M, pH 5.0).

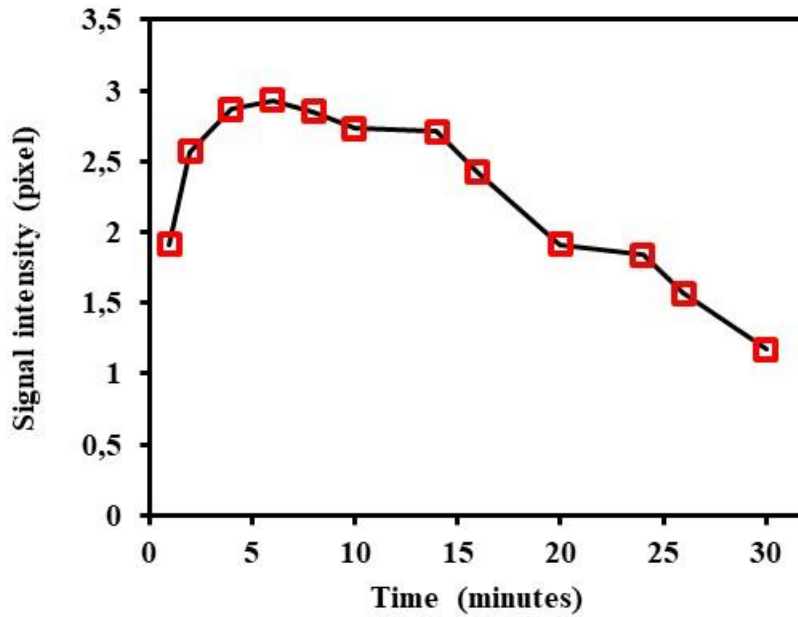


Figure 6.15: Pixel intensity against reaction time after the addition of TMB substrate to the paper zone.

6.4. Immunobiosensor image acquisition and data analysis

The image was captured on an HP color scanner and an Android smartphone. For manual image processing, the captured images were opened in image J (1.52 a). The acquired images were split into red, blue and green. An average pixel value was obtained for each test zone by selecting a constant area (square area of 2500 pixels, the side length of 50 pixels), and the pixel intensity of the selected area was measured. The measured pixel intensity was transferred into an Excel workbook to calculate the ΔRGB values of the favorable test zones relative to the negative control according to **Equation (6.3)**.

$$\Delta RGB = \sqrt{(R_p - R_o)^2 + (B_p - B_o)^2 + (G_p - G_o)^2} \quad (6.3)$$

where R_p , G_p , and B_p represent the pixel intensity of the red, green and blue of the positive samples and R_o , G_o , and B_o is the pixel intensity for the negative control. The manual analysis

of each sensing zone through image J is exceptionally tedious and requires about 10 minutes of processing. With the idea that the immunobiosensor should be user-friendly and suitable for use in limited-resource settings, the image J data processing steps were automated using MATLAB. For automated image processing, the image processing code was written in an M-file for execution in MATLAB 2018b (The MathWorks. INC) equipped with the image processing Toolbox. The written MATLAB code for image processing is shown in **Figure S6.1**. A user selects the file to analyses, and the program automatically split the image into corresponding channels, and the pixel intensity of the test zone was displayed by selecting the test zone area. **Figure 6.16** shows the processed color output obtained from the MATLAB program. The pixel intensity of the red, blue and green channels was obtained, and the average ΔRGB value was calculated using Excel. The average data processing time with the MATLAB processing of the image was less than 60 seconds while the manual Image J processing takes about 10 minutes to analyze each sensor. The result shows that as the pixel intensity of the Red channels increases and could translate to higher response to varying concentrations of the analyte. The preliminary experiments show that the ratio of ΔRGB and blue (B) to red (R) ($\Delta RGB / R$ and B/R) was found to increase with increasing concentrations of anti-P53abs. Thus $\Delta RGB / R$ was used for the monitoring of the signal output.

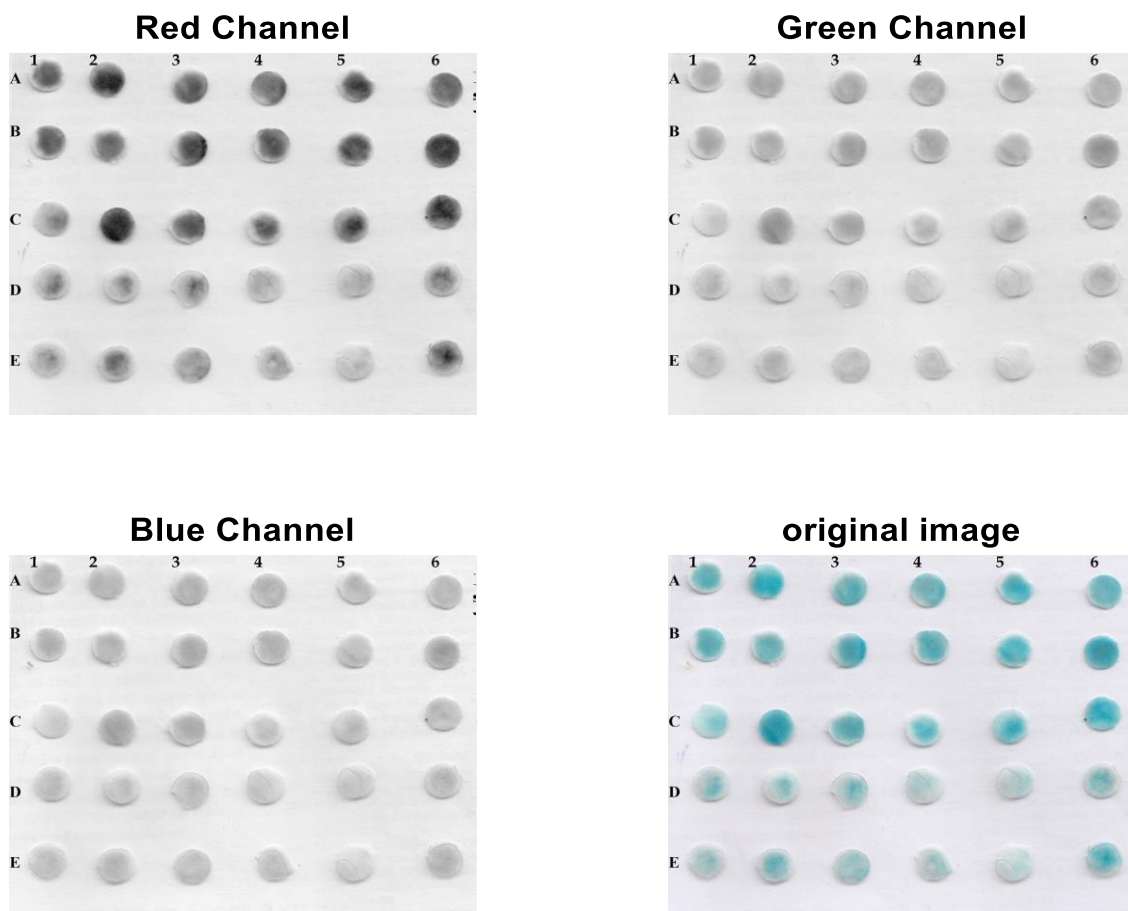


Figure 6.16: Semi-automated MATLAB code processed image output showing the RGB pixel separated into different channels for measurement of pixel intensity.

To further simplify the data acquisition and analysis process, the acquired sensing zones were captured with a cell phone camera under light illumination and analyzed using the cell-phone color picker application developed by Ratonera. The pixel intensity generated using Image J, and the color picker software from the analysis of the same sensing area was compared using the Pearson correlation. **Figure 6.17** shows that the plot of the pixel signal intensity ($\Delta\text{RGB}/R$) obtained with the smartphone color picker software and the manual image J-analysis. The result shows that the two methods show a similar correlation coefficient ($r = 0.96$). Based on the agreement of the two methods the RGB color picker software was used for all further data

analysis. The graph shows the signal output ($\Delta\text{RGB}/R$) for each of the sensing areas of the immunobiosensor ($n=12$) used for the evaluation of optimum color development time.

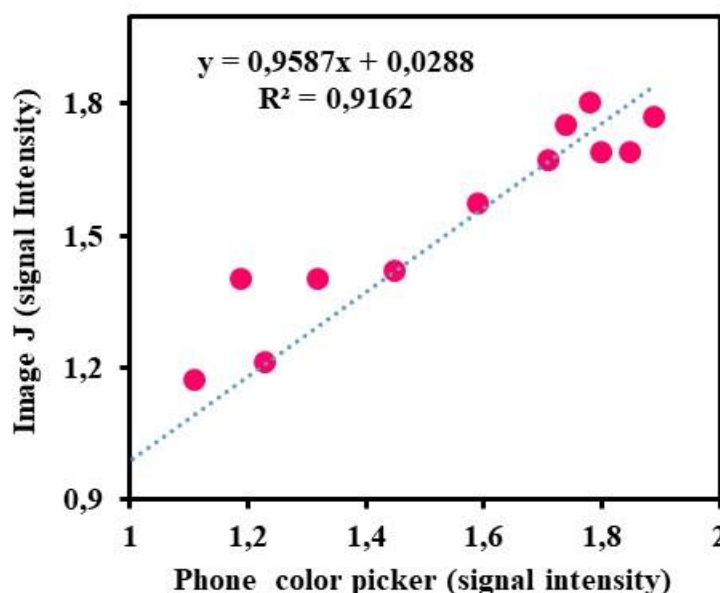


Figure 6.17: Correlation between signal intensity measurements obtained using Image J and smartphone RGB the image picker software.

6.5. Analytical performance of the paper-based immunobiosensor

Under the optimized condition, different concentrations of anti-P53abs were detected using this paper-based immunobiosensor. Increasing the anti-P53abs concentrations resulted in the increase in color intensity which can be observed by the naked eye. The digital image of the sensing area in **Figure 6.18 (a)** was acquired with a smartphone camera, and the image was quantitatively analyzed using the color picker software installed on the phone. **Figure 6.18 (b)** shows the dose-response curve of $\Delta\text{RGB}/R$ against different concentrations of the anti-P53ab from 10 pg.ml^{-1} to 200 ng.ml^{-1} . At lower concentrations, the pixel intensity of $\Delta\text{RGB}/R$ increases with increasing anti-P53ab concentration up to 25.0 ng.ml^{-1} and then levels off afterward. The leveling off at higher concentrations could be attributed to the saturation of the immunobiosensor surface.

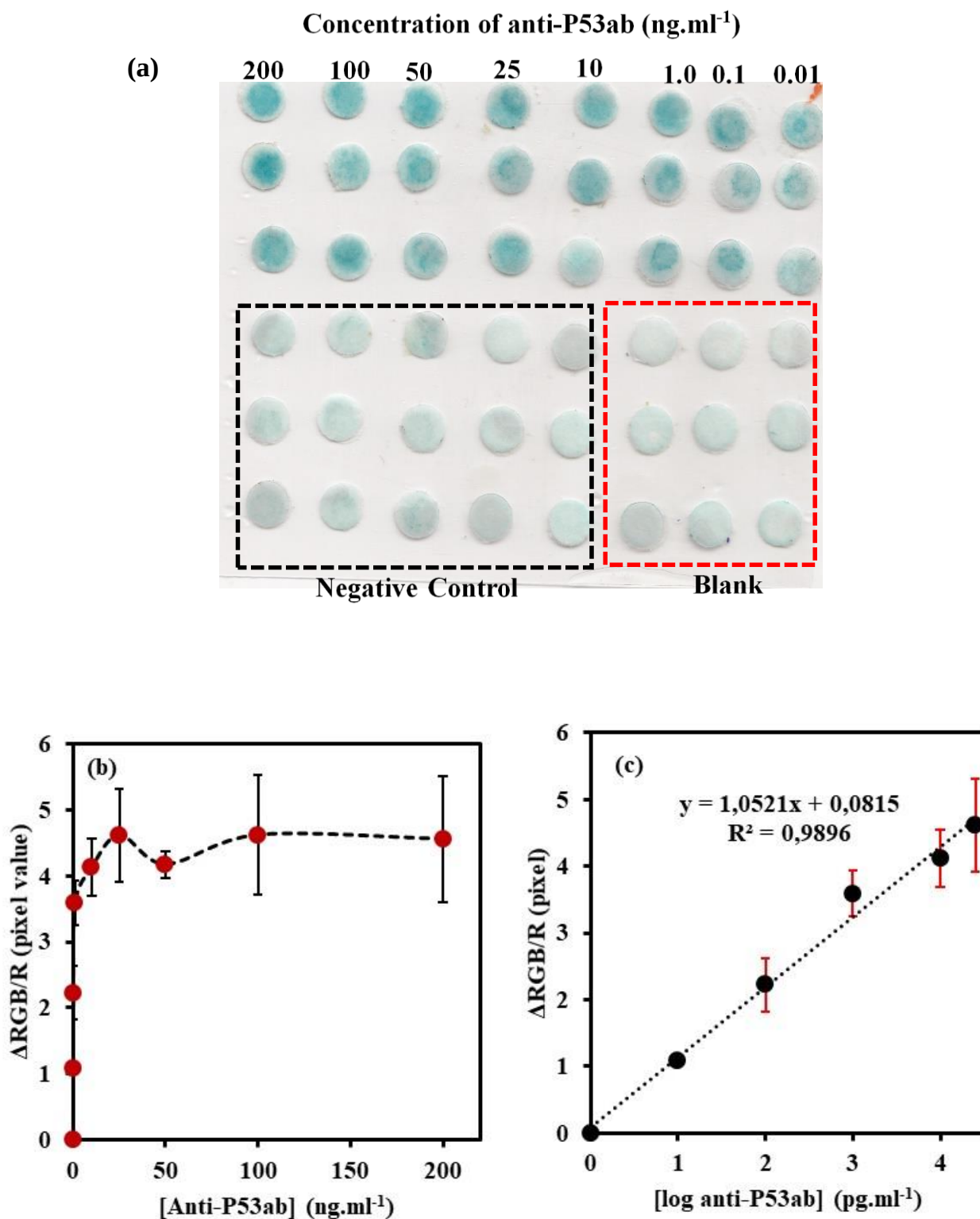


Figure 6.18: Colorimetric response of paper-based peroxidase-like detection of anti-P53ab (a) picture of the sensing area, (b) dose-response curve, and (c) linear calibration curve for detection of anti-P53ab.

Figure 6.18 (c) shows the calibration plot of $\Delta\text{RGB}/R$ values against the logarithms of the concentration of the anti-P53ab from 10.0 pg.ml^{-1} to 25.0 ng.ml^{-1} . The linear regression equation was given by **Equation (6.4)**.

$$\Delta\text{RGB}/R = 1.052 \log [\text{anti-P53ab}] + 0.08, R^2 = 0.989 \quad (6.4)$$

Where $\Delta\text{RGB}/R$ is the pixel intensity ratio of ΔRGB to red ($\Delta\text{RGB}/R$). The sensitivity of paper-based immunobiosensor detection of anti-P53abs was derived from the slope of the linear calibration curve and was found to be 1.05 pixels.ml.pg^{-1} . The LoD and LoQ were calculated based on the $3\sigma/s$ and $10\sigma/s$ criteria. σ is the standard deviation of the signal of the negative control sample signal ($\sigma = 0.2$, $n = 11$). The LoD and LoQ were found to be 63.0 fg.ml^{-1} and 210.0 fg.ml^{-1} respectively. The LoD of the paper-based peroxidase-like immunobiosensor for detection of anti-P53ab was lower than that of the conventional ELISA (0.39 ng.ml^{-1}), the QCM-D (20.0 ng.ml^{-1}) and the microfluidic chip-based detection (4.0 ng.ml^{-1}). The LoD of the paper-based sensor (63.0 fg.ml^{-1}) was higher than the LoD of the immunobiosensor performed in the microwell plate (20.0 fg.ml^{-1}). However, the paper-based sensor employs covalent immobilization of the P53ag which would result in a more stable sensing platform in comparison to the physical adsorption onto the microtiter plate. Also, the paper-based sensor presents an opportunity for low cost and use of a small quantity of samples and reagents.

6.6. Comparison of peroxidase-like detection with other systems

Table 6.6 shows the comparative performance of the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ with other immunobiosensor developed in Chapter 3 and 4, previously reported immunobiosensor, and commercial anti-P53 ELISA. The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ labeled detection had lower detection limits in comparison to the LoD of the labeled-free EIS detection (103.0 pg.ml^{-1}). The $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ peroxidase-like labeled detection of anti-P53ab showed good analytical performance when

compared with the HRP-based labeled detection systems. The limit of detection of the peroxidase-like detection; 20.0 fg.ml⁻¹ and 63.0 fg.ml⁻¹ for the microtiter plate and the paper-based systems; respectively were lower than the HRP-based ELISA test kits (0.390 ng.ml⁻¹), and HRP-anti-IgG based microfluidic chip sensor (4.0 ng.ml⁻¹) [134]. The good analytical performance could be attributed to the superior peroxidase-like activity, stability and high affinitive binding of the Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG in comparison to HRP-based probe. The LoDs were comparable to the AuNP-RGO square wave-based detection (88.0 fg.ml⁻¹) in [166]. In comparison to other methods reported in this thesis, the LoDs of Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-anti-IgG peroxidase-like detection was lower than the fluorescence FITC@SiNP-APTES-anti-IgG developed in the microplate (42.0 pg.ml⁻¹). The LoD was higher than the LoDs of the magnetic bead-fluorescence-based detections (3.30 fg.ml⁻¹ and 10.0 fg.ml⁻¹) reported in Chapter 4.

Table 6.6: Comparison of the analytical performance of Fe₃O₄@SiNP-APTES-Au₁@Pd₃-ProG-IgG peroxidase-like colorimetric detection

Detection Probe	Platform	Techniques	LOD	Linear Range (ng/ml)	Refs.
Fe ₃ O ₄ @SiO ₂ -Au ₁ Pd ₃ -ProG-anti-IgG/TMB	Paper disc	colorimetric	63.0 fg.ml ⁻¹	0.1-25	This work
Fe ₃ O ₄ @SiO ₂ -Au ₁ Pd ₃ -ProG-anti-IgG/TMB	Microwell	colorimetric	20.0 fg.ml ⁻¹	0.2-100	This work
FITC@SiNP-APTES-PEI-anti-IgG	Microwell	Fluorescence	42.0 pg.ml ⁻¹	0.1-20	This work
FITC@SiNP-APTES-PEI-anti-IgG	Magnetic bead	Fluorescence	^a 9.92 fg.ml ⁻¹ ^b 3.30 fg.ml ⁻¹	^a 0.015- 0.5 ^b 0.5 - 100	This work
Label-free	Au Electrode	EIS	0.130 ng.ml ⁻¹	1 - 1000	This work
HRP-anti-IgG	Magnetic bead	colorimetric	4.0 ng/ml	-	[134]
AuNP-GRO	Au Electrode	SQWV	88 fg.ml ⁻¹	0.001-0.1	[166]
HRP-biotin-anti IgG/TMB	Microwell	colorimetric	0.390 ng.ml ⁻¹	1.56-100	BioSource diagnostics
HRP-streptavidin-biotin-anti-IgG/TMB	Microwell	colorimetric	0.470 ng.ml ⁻¹	0.78-50	Wuhan-fine Technologies

6.7. Chapter conclusions

In this chapter, the $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3$ was conjugated to anti-IgG-antibody as a novel immunobioprobe for anti-P53ab detection. The prepared $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ immunobioprobe thoroughly was characterized and evaluated the peroxidase-like activity. It was found to have excellent peroxidase-like activity in comparison to HRP based detection system. The immunobiosensor for anti-P53ab detection was successfully using $\text{Fe}_3\text{O}_4@\text{SiNP-APTES-Au}_1@\text{Pd}_3\text{-ProG-anti-IgG}$ as an immunoprobe. The first immunobiosensor fabricated utilizes the microtiter plate well and peroxidase-like activity. The immunobiosensor yielded a highly sensitive anti-P53ab detection with very low LoD of 20.0 fg.ml^{-1} in the linear range of 0.20 to 100 ng.ml^{-1} . The developed immunobiosensors showed good analytical performance with higher sensitivity, larger linear dynamic range and higher lower limit of detection when compared with the conventional ELISA. The immunobiosensor was also evaluated for the real-time clinical application by the detection of anti-P53ab in spiked serum samples and showed good recovery values ranging from 98.5 to 105.7% with the highest %RSD of 3.37.

The immunobiosensor for the microtiter plate was then transferred into a cellulose paper platform. The developed paper-based anti-P53ab immunobiosensor also exhibited very good analytical performance with high sensitivity. The LoD of paper-based immunobiosensor was 63.0 fg.ml^{-1} at linear range of $0.10 - 25.0 \text{ ng.ml}^{-1}$. The paper-based immunobiosensor utilized the covalent attachment of P53ag as a sensing element and this will result in a robust and stable immunobiosensor. The use of smartphones for data analysis and signal integration is a step towards cheap diagnostics for application in remote and resource-limited settings which is where the designed systems are aimed for application.

CHAPTER 7:

SUMMARY, CONCLUSIONS AND FUTURE OUTLOOK

7. Summary and conclusions

The design of various immunobiosensors for the detection of autoantibodies to human tumor antigen P53 has been developed. The designed immunobiosensors employed methods that could enable low cost, improved sensitivity, higher stability and point-of-care detection of anti-P53ab in low-income settings. Methods based on labeled and labeled-free detection (EIS) transduction techniques have been developed, and their analytical performance evaluated.

The first immunobiosensor investigated was a label-free electrochemical impedance-based immunobiosensor for anti-P53abs. The advantage of the impedance-based immunobiosensor is the ease of miniaturization, simplicity and low-cost due to the non-requirement of additional signal transduction labels. The analytical performance of the label-free impedimetric immunobiosensor developed was comparable to the commercial ELISA kit for anti-P53abs detection. However, the analytical sensitivity performance of the developed impedance-based immunobiosensor was lower when compared to reported immunobiosensor employing signal transduction labels for anti-P53abs. The advantage of the immunobiosensor was the label-free detection which would reduce assay time and cost of detection. Also, the covalent immobilization of P53ag enhances the stability of the immunobiosensor and allows for reusability. Also, the labeled-free detection showed a wider linear range (1.0 to 1000.0 ng.mL⁻¹) in comparison to the linear range of the other detection system reported in; this work, previously in the literature, and commercial anti-P53 detection kits.

Two indirect-labeled transduction methods including colorimetric and fluorescence-based anti-P53ab immunobiosensors were developed. For the fluorescence detection, fluorescent silica nanoparticles were synthesized by covalently overloading FITC into silica nanoparticles. The surface of the fluorescent silica nanoparticles was modified with PEI to achieve covalent and oriented conjugation of the anti-IgG antibody as a novel fluorescent label for anti-P53ab

detection. The fluorescent immunobiosensor employed a microtiter plate and magnetic bead as the P53ag sensing platforms. The enhancement of the fluorescent signal was achieved by the dissolution of the fluorescent silica nanoparticles to release the loaded FITC-molecules and achieved TURN-ON fluorescent emission. The fluorescent silica nanoparticle labeled detection allows for sensitive analytical detection of anti-P53ab with a limit of detection of 42 fg.ml⁻¹ and 3.30 fg.ml⁻¹ for the microplate and magnetic bead-based sensing platform; respectively. The linear range of the magnetic bead fluorescence silica nanoparticles detection was between 0.5 ng.ml⁻¹ to 100.0 ng.ml⁻¹ for anti-P53ab while the linear range of the microtiter plate detection fluorescence sensing was between 0.1 ng.ml⁻¹ to 20.0 ng.ml⁻¹. The magnetic bead-based fluorescent detection simplified the assay and facilitated the analysis time. The magnetic bead-based fluorescent silica immunobiosensor had higher sensitivity than the label-free impedimetric immunobiosensor and the commercial ELISA kit for anti-P53abs detection. The fluorescence immunobiosensor has great potential in the development of a microfluidic test kit for anti-P53ab detection.

The colorimetric immunobiosensor for anti-P53ab detection based on peroxidase-like properties of Fe₃O₄@SiNP-APTES-Au@Pd was investigated. Novel catalytic Fe₃O₄@SiNP-APTES-Au@Pd with enhanced peroxidase-like activity was synthesized and fully characterized. The Fe₃O₄@SiNP-APTES-Au@Pd was conjugated to Protein G and anti-IgG antibodies via covalent interaction and electrostatic adsorption as a new signal label for anti-P53ab detection. Based on the peroxidase-like activity, Fe₃O₄@SiNP-APTES-Au@Pd-ProG-anti-IgG immunobiosensor was developed. The colorimetric immunobiosensors were developed in a microwell titer plate and on cellulose paper platforms. The anti-P53ag was covalently immobilized onto the cellulose paper to achieve higher stability. The Fe₃O₄@SiNP-APTES-Au@Pd-ProG-anti-IgG catalyzed the oxidation of TMB/H₂O₂ into a blue-colored

product. Signal enhancement was achieved by the addition of H_2SO_4 to convert the blue colored product to a yellow product with a 3-fold increase in the absorption intensity at 450 nm.

The quantification of the anti-P53ab was achieved by measuring the absorption intensity at 450 nm. The limit of detection was 20 fg.ml^{-1} of anti-P53ab for the microtiter plate detection. This was lower than the limit of detection for the HRP-based ELISA. The sensing models were translated onto cellulose paper platform to achieve a low-cost paper-based colorimetric detection of anti-P53ab. The analytical performance of the paper-based immunobiosensor for anti-P53abs was superior to that of the label-free impedance immunobiosensor reported in Chapter 3 and other reported methods in the literature. The LoD and sensitivity were comparable to the fluorescent silica nanoparticles based immunobiosensor studied and reported in Chapter 4. However, the paper-based immunobiosensor could provide a better detection platform due to the potential for naked-eye detection and smartphone quantification without the use of sophisticated equipment. The fluorescent and colorimetric based immunobiosensor was successfully translated from a microtiter well plate to a magnetic bead and paper-based platform. This demonstrated the potential of reducing the cost of analysis, simplification of the detection system and reduction of cost to achieve point-of-care screening of anti-P53abs towards lung cancer in low resource settings. The paper-based immunobiosensor gave some additional advantages compared to other systems developed in the thesis. For example (i) the procedure for the capture antigen immobilization to the sensor surface is under 30 minutes, (ii) the response is faster (86 minutes) and (iii) requires a small volume of samples and reagents (2 μL). The other techniques need a higher amount of reagents and take a longer time (15 hours) to run samples. The overarching aim of this research thesis which was to demonstrate the development of immunobiosensor characterized by high sensitivity, selectivity, user-friendly, low cost and deliverable to end-user for detection of anti-P53ab was successfully achieved.

The application of these new technologies could make a real change towards an improved screening for lung cancer in low resource settings.

Future work

Given the results obtained and aiming at the continuation of this research in achieving the realization of a commercial test kit for the detection of anti-P53-abs and other autoantibodies towards lung cancer screening. There are several directions which future research could take to improve the performance of the developed anti-P53-abs immunobiosensors:

- To investigate oriented immobilization of the capture antigen unto the transducer surfaces, this could be achieved using a polyhistidine-tag and biotin-avidin system.
- To investigate strategies to reduce non-specific adsorption which could result in high background signal, this could be tackled by the introduction of polyethylene-glycol and zwitterionic polymers.
- To evaluate the analytical performance of the developed immunobiosensor using another lung cancer-specific autoantibody simple by substituting the capture antigen with specificity for the autoantibody of interest.
- To develop a multiplexed bead and paper-based array immunobiosensor system for the multiplexed detection of autoantibodies detection.
- The analytical performance of the immunobiosensors would need to be tested using real and asymptomatic lung cancer patient's samples for method validation that have been analyzed by other standard approved methods (e.g., low-dose computed tomography, chest X-ray and ELISA). This would evaluate how close the agreement is between the results obtained by the different techniques and the developed immunobiosensors.

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Appendix

Chapter 2

1. Characterization of AEBD

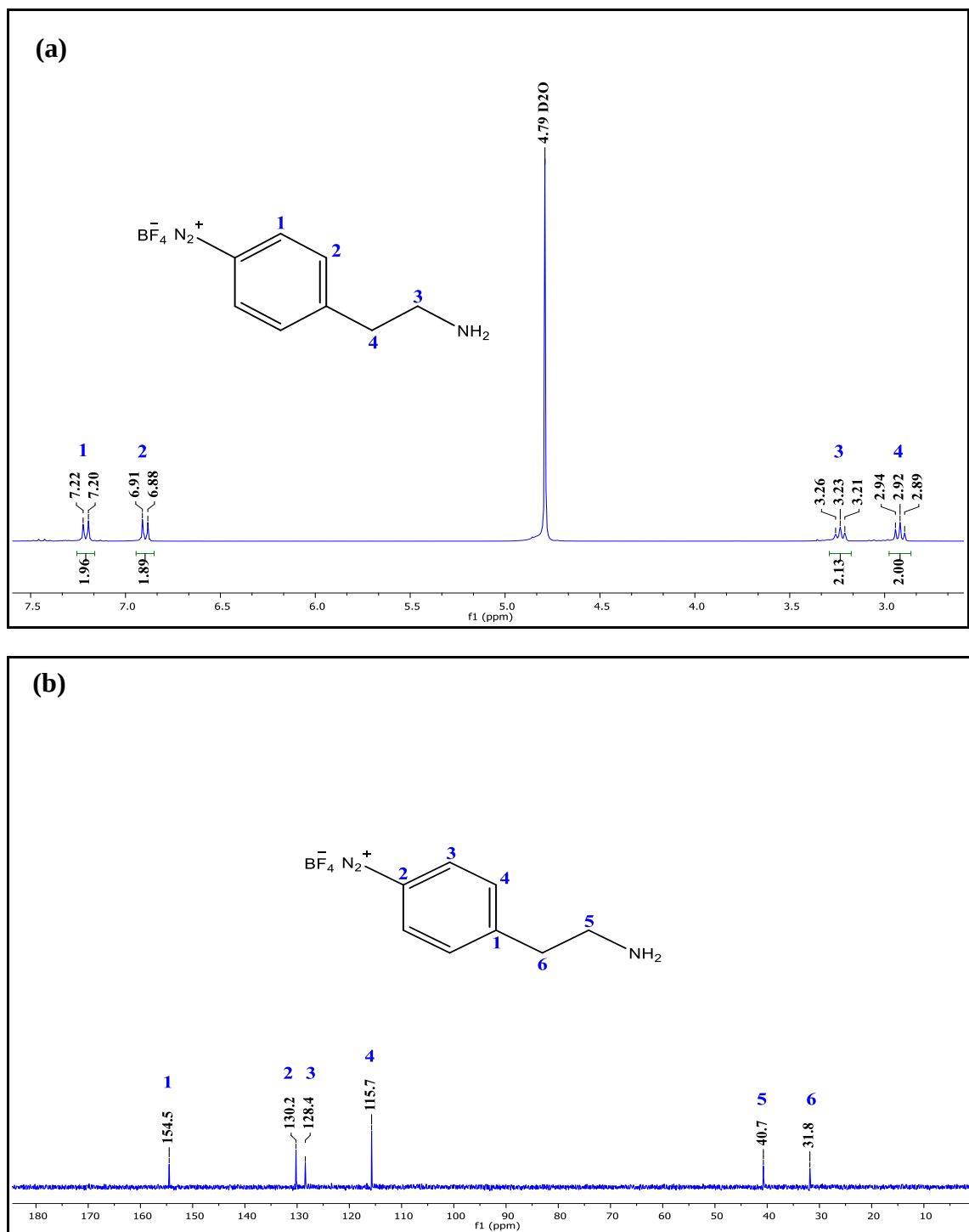


Figure S2.1: (a) ^1H NMR (600 MHz) and (b) ^{13}C NMR (600 MHz) of as-synthesized 4-(2-aminoethyl) benzene diazonium (AEBD).

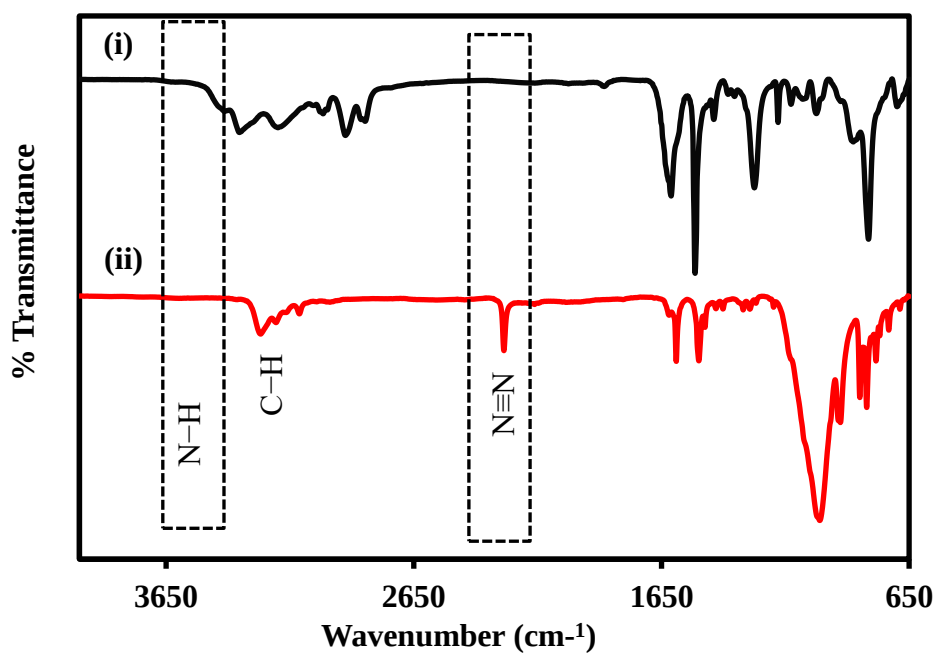


Figure S2.2: FTIR spectra of (a) 4-2-aminoethylaniline (b) as-synthesized 4-(2-aminoethyl) benzene diazonium (AEBD).

Chapter 3 Appendix

2. The CV and EIS Characterization of Immuno-electrode fabrication steps

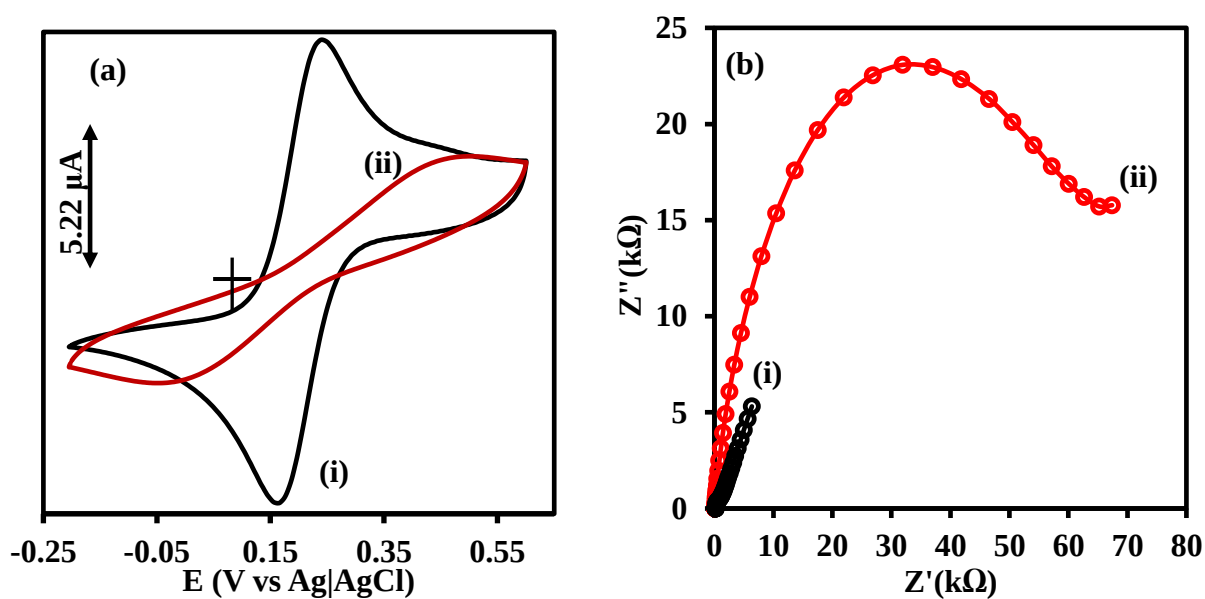


Figure S3.1: (a) Cyclic voltammograms and (b) Nyquist plots of (i) bare Au, and (ii) Au-PEA-SA modified electrode in 2 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution containing 0.1 M KCl.

3. The Plot of redox peak current against scan rate for Au-PEA and Au-PEA-SA-P53ag/BSA

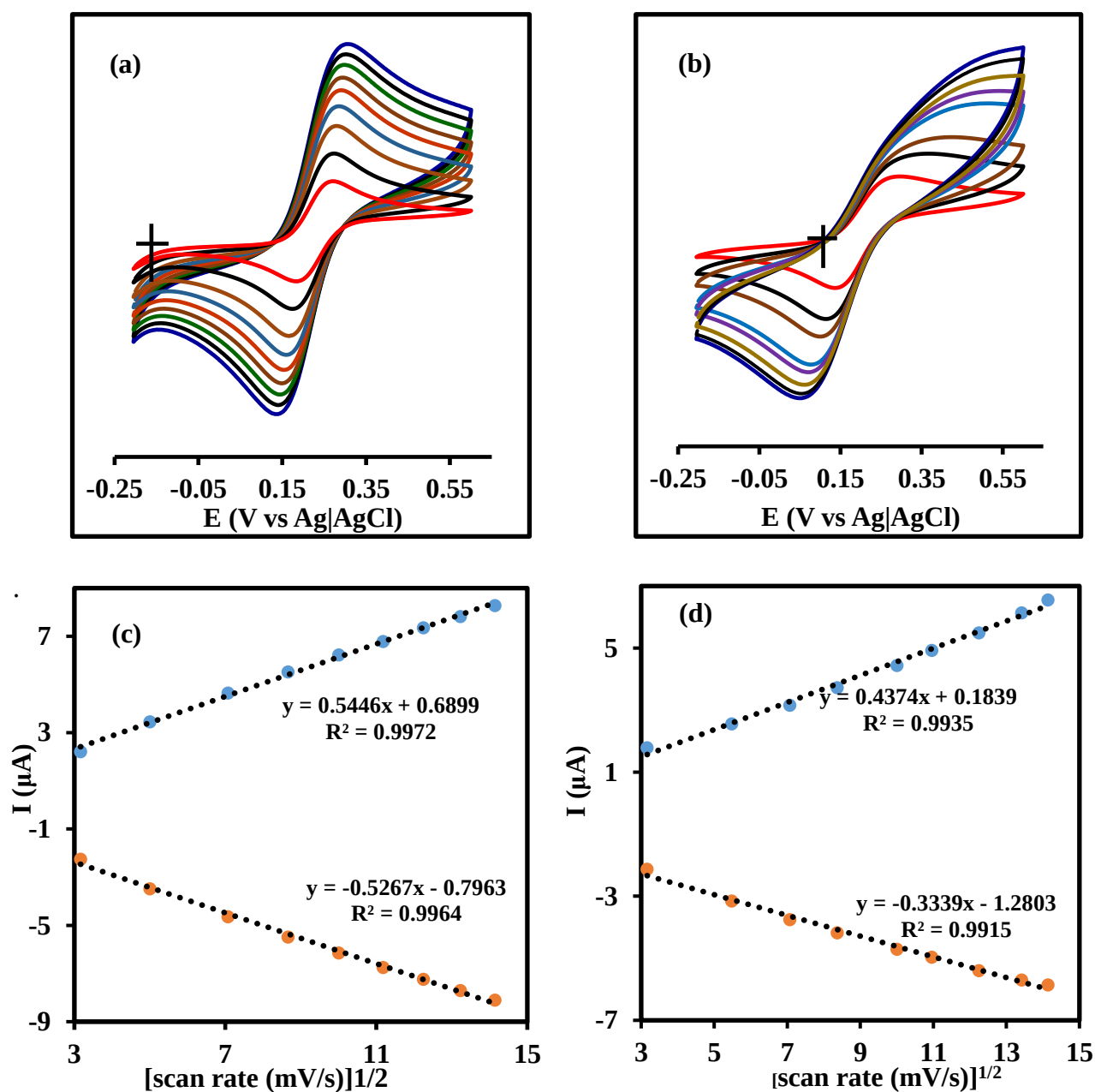


Figure S3.2. The CV of (a) Au-PEA modified electrode, and (b) Au-PEA-SA-P53ag/BSA Immuno-electrode at various scan rates in 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ containing 0.1 M KCl and a corresponding plot of peak current against the square root of scan rate.

3. Calculation of XPS surface thickness estimation

The attenuation of the gold peak is due to the coverage of the gold surface by the organic PEA and protein (P53) and BSA layer. The reduction in the gold peaks can provide an estimate of the thickness of the layer and evidence for successful modification. The attenuation is expressed as shown in equation 1. To obtain the value of the mean free path of the substrate-specific photoelectron (λ) in the organic layer, the Seah and Dench equation (**Equation. S3.1**)

was used
$$\lambda_k = A_n (E_k^2 + B_n E_k^{1/2})^{-1} \quad (\text{S3.1})$$

E_k is the kinetic energy of the photoelectrons in electron Volts (eV). For the Al K_α source, $E_k = 1486.6 - E_n$. For an organic layer, A_n is 49 and B_n is 0.11 mg/m^2 . To convert the λ_k to nm unit the value of λ_k in mg/m^2 is divided by the density of the layer and was assumed to be 1.02 g/cm^3 . Substituting the values into equation SI, for 4f7/2 the λ_k vales were 0.42 mg/m^2 .

Chapter 4-Appendix

1. TEM characterization

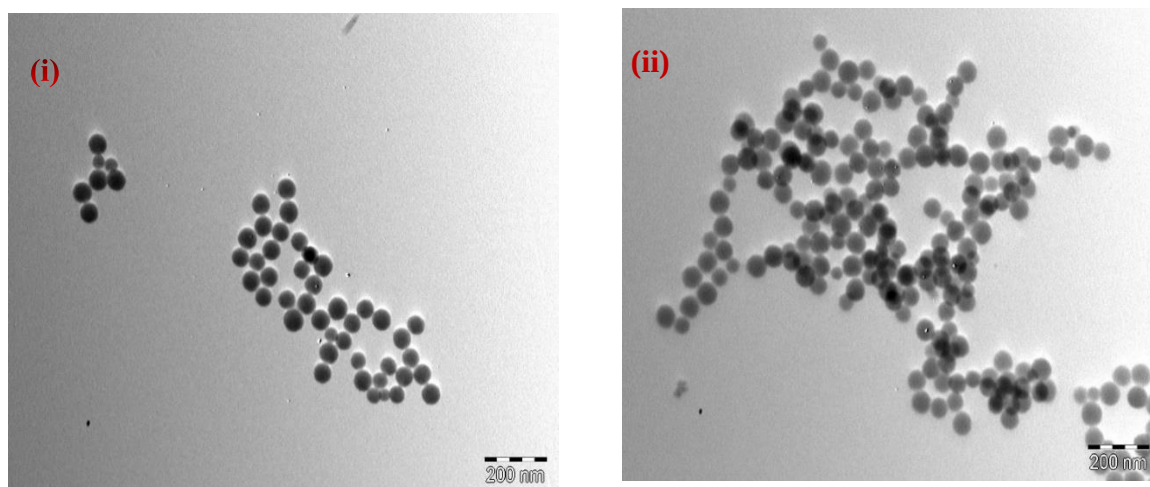


Figure S4.1. TEM micrograph of (i) 4.5 % (ii) 6.5 % FITC-SiNP@SiNP

2. Photophysical characterization.

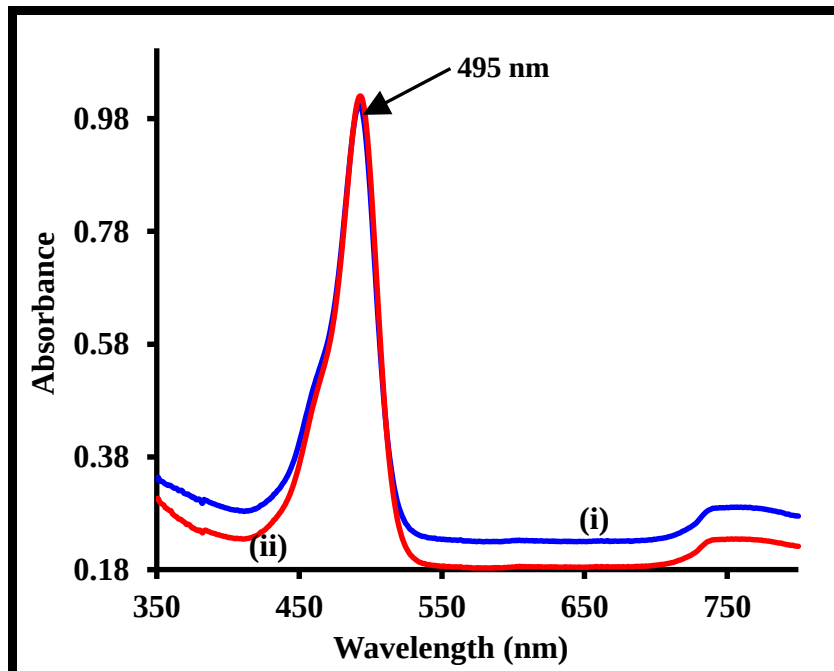


Figure S4.2: UV-vis-absorption spectra of (i) FITC and (ii) FITC-APTES in PBS buffer (pH 7.4)

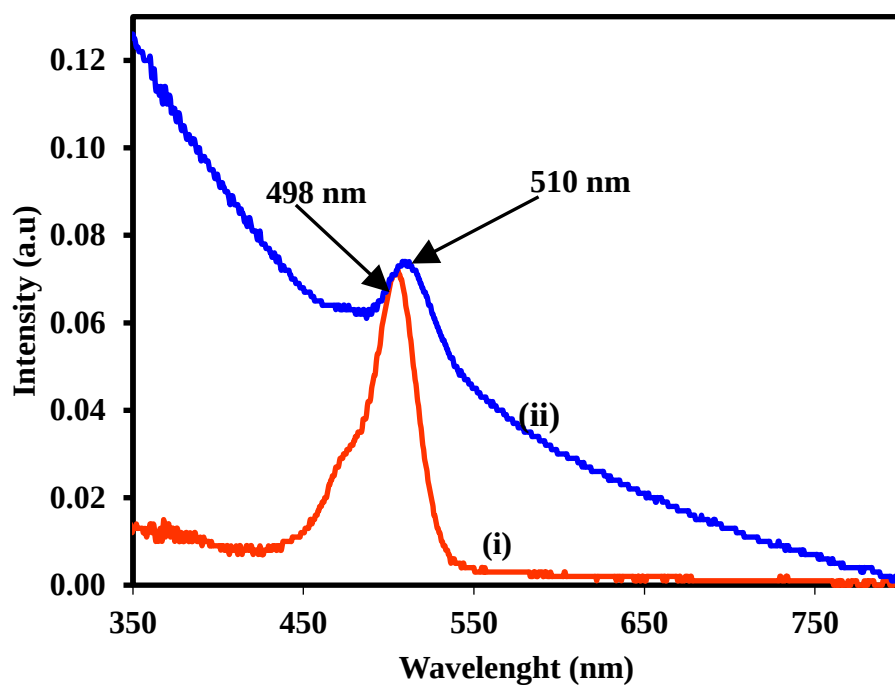


Figure S4.3: UV-Vis absorption of (a) FITC-APTES and (b) FITC@SiNP-APTES-PEI

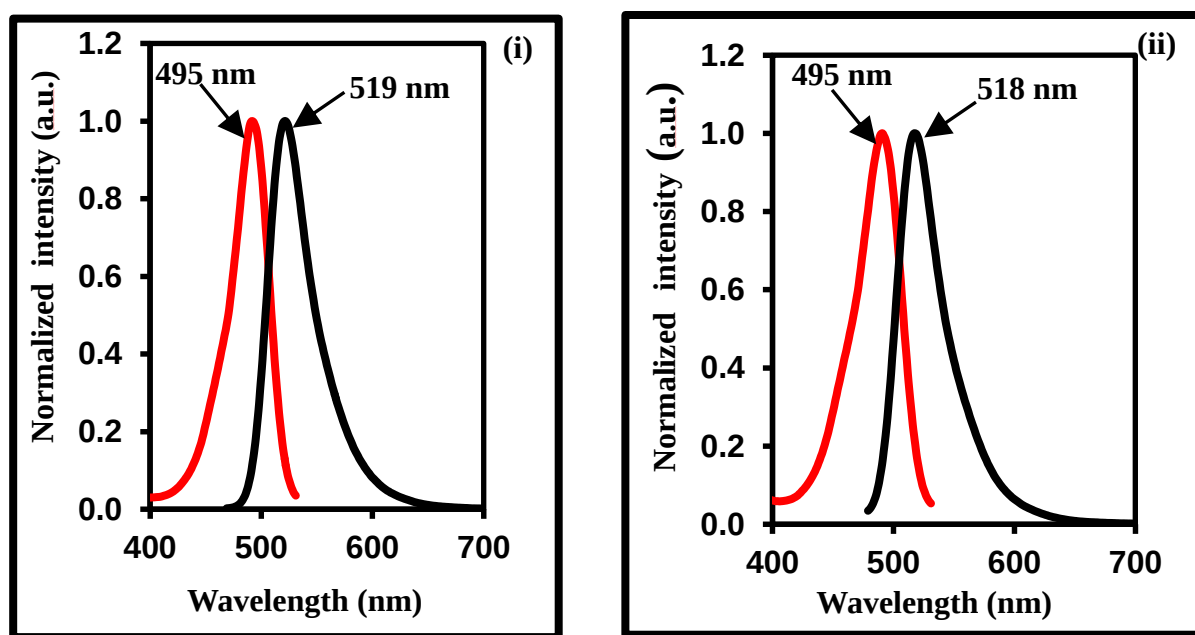


Figure S4.4: Fluorescence results illustrating the excitation (red) and emission (black) of (i) FITC (ii) FITC-APTES.

Chapter 5-Appendix

1. XPS characterization

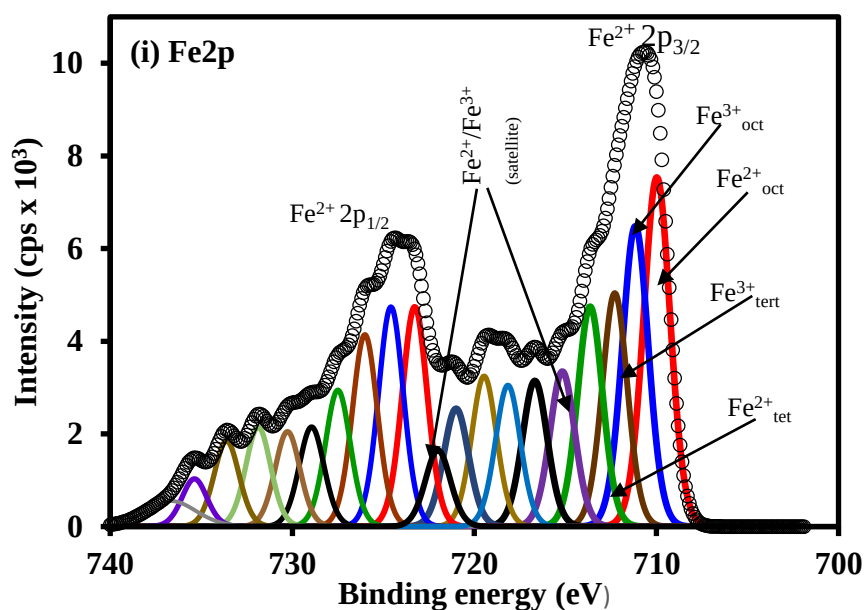


Figure S5.1: High-resolution Fe 2p spectrum of Fe₃O₄

2. Peroxidase like activity of Fe₃O₄@SiNP-APTES-Au@Pd

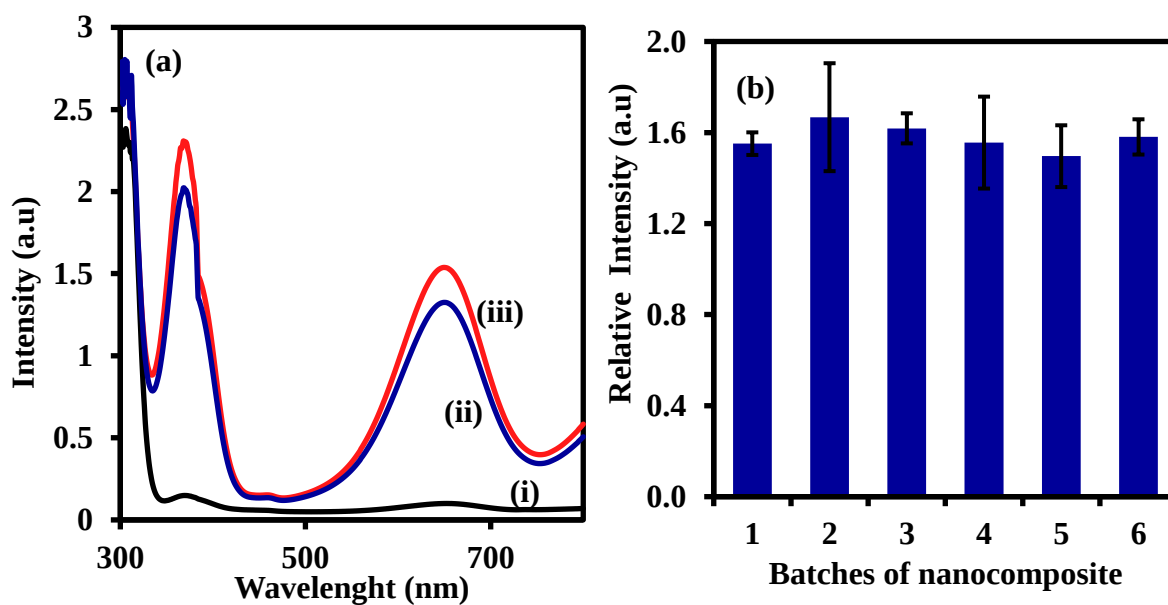


Figure S5.2: UV-vis absorption spectra of (i) leaching solution (ii) Fe₃O₄@SiO₂-Au@Pd before incubation in acid (iii) and after incubation (b) relative catalytic activities of different batches of Fe₃O₄@SiO₂Au₁Pd_{1.95} nanocomposite, RSD (3.5 %).

Chapter 6- Appendix

The MATLAB programing code for the automated analysis of immunobiosensor response

```
Function [signal] = P53-autoantibody
%function [wt]=(RGB_I,mode)
%image analysis algorithm to automatically analyse the mean intensity of
%each well in the sensor strip device
%
% inputs: RGB_I = input image
%         gray_1 =rgb2gray image
%         c_dia = circle diameter for well region-of interest
%         mode = intensity mode
%         -'blue'= Extract blue channel
%         -'red' = Extract red channel
%         -' green' = Extract green channel
% RGB_I = imread ('2020.jpg');
% gray_I =rgb2gray ('RGB_I');
%% RGB color space
rmat=RGB_I(:,:,1);
gmat=RGB_I(:,:,2);
bmat=RGB_I(:,:,3);
rgb2gray=RGB_I

figure
subplot (2,2,1),imshow (rmat);
title ('Red Channel');
subplot (2,2,2),imshow (gmat);
title ('Green Channel');
subplot (2,2,3),imshow (bmat);
title ('Blue Channel');
subplot (2,2,4),imshow (RGB_I)
title ('original image');
impixelinfo
select pixel area on the sensing zone and record intensity for each channel
%end
```