Development of enzymatic assays using a universal detection system based on nanotechnology

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ABSTRACT

Nanotechnology offers the technological potential that can be harnessed to deal with the diagnostic challenges in resource-constrained settings. Optical biosensors based on nanotechnology have being explored for biomedical analysis as they are cheaper and use readily available instruments. Signal transduction has been based on metallic nanoparticles in biosensors for optical detection which are simple, rapid and cost-effective. Gold nanostars (AuNSs) were used as a scaffold to design a universal detection system based on enzyme-guided changes in nanosensors. The detection was based on H_2O_2-mediated growth/shape-alteration of gold nanostars resulting in colorimetric and spectrophometric changes. This detection strategy enabled the fabrication of two oxidase-based biosensors for glucose and cholesterol which were simple in design, sensitive and rapid in detection, and overall high-throughput. Both were colorimetric and utilised a basic entry-level laboratory spectrophotometer plate reader for analysis.

Although a number of synthetic approaches for AuNSs have been reported, the choice of synthesis method depends on a number of experimental parameters and downstream application. Thus, there are still gaps for methods that are appropriate, simple and produce AuNSs suited to their intended purposes. I therefore developed a seedless synthesis strategy for AuNSs that has the advantages of the seeded methods. The method used ascorbic acid as a reducing agent and silver nitrate as an anisotropic growth control assisting agent. AuNSs with multiple branches and diameter of 59 nm were produced. They showed good stability when capped with PVP and modified with an enzyme in relatively strong ionic conditions. I investigated their application in plasmonic sensing by modifying them with glucose oxidase and detection of glucose. The AuNSs were found to be a good scaffold for the enzyme, proved to stable and sensitive as transducers. Thus, the AuNSs showed good promise for further applications in plasmonic biosensing for in vivo biomedical diagnosis.

Gold nanoparticles provide a user-friendly and efficient surface for immobilisation of enzymes and proteins. However, one of the major limitations for the implementation of nanobiosensors in clinical use are related to biofunctionalisation of biorecognition elements such as enzymes and antibodies. I designed a novel approach for enzyme bioconjugation to AuNSs where the nanostars were modified with L-cysteine and covalently bound to N-hydroxysulfosuccinimide (sulfo-NHS) activated intermediate glucose oxidase (GOx) to create a stable and sensitive AuNSs-Cys-GOx bioconjugate complex. This strategy demonstrated potential for increased attachment affinity without protein adsorption onto the AuNSs surface. Good dispersity in buffer

suspension was observed, as well as stability in high ionic environments. Greater sensitivity in the determination of low concentrations of glucose based on plasmonic and colorimetric detection was observed using the AuNSs-Cys-GOx bioconjugates. Such a novel approach for enzyme immobilisation could lead to the production of nanoparticle-enzyme conjugates could be used in nanobiosensors with real clinical samples for biomedical analyses.

Despite the progress made on the design of novel plasmonic colorimetric biosensors there are still significant challenges in their practical application in clinical samples. I optimised and developed a glucose biosensor based on biocatalytic shape-altering of gold nanostars via silver deposition in serum. Improved sensitivity was observed due to nanostars clustering after being functionalised with glucose oxidase (GOx). The biosensor quantified glucose in serum samples with a 1:1000 dilution factor, and colorimetrically distinguished between concentrations. The assay demonstrated good specificity and sensitivity. A rapid assay was developed that could be used for high throughput analyses using either naked eye detection or a basic entry level laboratory spectrophotometry microplate reader. This observation shows the potential for further development of such nanobiosensors that can be validated for practical clinical applications. Future perspectives are on the application of these optimised strategies to other enzyme-based and immunoassays using nanotechnology.

Key terms: biosensors, cholesterol, colorimetric, enzyme-guided growth, glucose, gold nanostars, immobilisation, nanobiosensors, plasmonic, synthesis.

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LIST OF ABBREVIATION

A:	
Ag	Silver
$AgNO_3$	Silver nitrate
ALP	Alkaline phosphatase
Au	Gold
AuNPs	Gold nanoparticles
AuNSs	Gold nanostars
B:	
BSA	Bovine serum albumin
C:	
ChOx	Cholesterol oxidase
CTAB	Cetyltrimethylammonium bromide
Cys	L-cysteine
D:	
ddH_2O	Double distilled water
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTSSP	3 3'-dithiobis(sulfosuccinimidyl propionate)
E:	
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme-linked immunosorbent assays
EM	Electromagnetic wave
G:	
GCxGC-TOFMS	Two dimensional gas chromatography time of flight mass spectrometry
GC-MS	Gas chromatography mass spectrometry
GC-TOFMS	Gas chromatography time of flight mass spectrometry
GOx	Glucose oxidase

H:	
H_2O_2	Hydrogen peroxide
$HAuCl_4$	Hydrogen tetrachloroaurate(III)
hCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HPLC	High-performance liquid chromatography
HR-TEM	High resolution transmission electron microscopy
I:	
INM	Immunophelometry
IUPAC	International Union of Pure and Applied Chemistry
K:	
K_m	Michaelis constant
L:	
LC-MS	Liquid chromatography mass spectrometry
LFA	Lateral-flow assays
LSPR	Localised surface plasmon resonance
LSPR	Localised surface plasmon resonance
M:	
MES	2-(N-morpholino)ethanesulfonic acid
N:	
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBS	Nanobiosensor
NHS	*N*-hydroxysuccinimide

NIR	Near-infrared
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
P:	
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PGA	Poly-γ-glutamic acid
PSA	Prostate specific antigen
PVP	Poly(*N*-vinylpyrrolidone)
Q:	
QDs	Quantum dots
R:	
RI	Refractive index
S:	
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman scattering
SPPs	Surface plasmon polaritons
SPR	Surface plasmon resonance
SPs	Surface Plasmons
T:	
TEM	Transmission electron microscopy
TOAB	Tetraoctylammonium bromide
U:	
UV-vis-NIR spec	Ultraviolet-visible Near Infrared spectroscopy

CHAPTER 1 INTRODUCTION

1.1 Background and motivation

The developing world has unique diagnostic challenges that need technological solutions suited to it. Most of the diagnostic technologies commonly used in the developed world are often poorly suited for use in developing countries (Hauck *et al.*, 2010). There are differences in the disease burdens between the two worlds. For example, infectious diseases are the major source of morbidity and deaths in developing countries compared to the non-infectious, congenital, and life style diseases in the developed ones. Another diagnostic challenge in developing countries is the limited availability of clinical and laboratory facilities (Hauck *et al.*, 2010; Salamanca-Buentello *et al.*, 2005). This poses a biotechnological/engineering challenge to come up with suitable diagnostic devices for the developing world. Ideal diagnostic devices for the developing world need to be cost-effective, portable, point-of-care systems with high sensitivity and specificity (Hauck *et al.*, 2010). Nanotechnology has the technological potential that promises substantial impact on medical diagnosis, especially to deal with the developing world diagnostic challenges (Roszek *et al.*, 2005; Salamanca-Buentello *et al.*, 2005).

Optical biosensors based on nanotechnology have being explored for biomedical analysis as they are cheaper and use uncomplicated instruments. Metallic (silver and gold) nanoparticles have been used as signal transducers in biosensors for detection. These noble metal nanoparticles possess unique properties of high extinction coefficients and strong distance-dependent optics (Huang & El-Sayed, 2010) that have made them candidates for use as probes for disease detection (Alharbi & Al-Sheikh, 2014; Chen *et al.*, 2016; Rizzo *et al.*, 2013; Shinde *et al.*, 2012). They have shown great promise in rapid and robust diagnostics and have enabled the naked-eye colorimetric, fluorometric, chemiluminescent, and electrochemical detection of analytes. They promise to be an alternative to the expensive laboratory equipment that are of limited availability in the developing world (Li & Xu, 2014; Roszek *et al.*, 2005).

Gold nanostars (AuNSs), also called multibranched or star-shaped- gold nanoparticles, are an outstanding platform for near-infrared (NIR) absorption and surface-enhanced Raman scattering (SERS) applications. The high-aspect-ratio branches of the nanostars enable the localisation of low-energy plasmon modes at the tips. This results in a dominant localised surface plasmon resonance (LSPR) band in NIR region which offer good sensitivity for detection (Rodríguez-Lorenzo *et al.*, 2012; Saverot *et al.*, 2016). A number of studies have used AuNSs for the fabrication of colorimetric biosensors because of these unique plasmonic properties. Although much progress has been made in the design of plasmonic nanobiosensors, there are barely any

reports of these being used in biomedical and clinical practice. A number of challenges still remain to be overcome before these innovative technologies could be used in clinical and point-of-care measurements. Aspects such as choice of synthesis methods for AuNSs for appropriate downstream applications; bioconjugation strategies for enhanced stability and optimal function of both the nanoparticles and biomolecules; feasibility of using traditional assay methods with nanoparticle-based detection; and designing strategies to make the nanosensors usable in complex biological sample matrices are some of the motivation for this Ph.D. thesis.

1.2 Structure of thesis

This thesis is a compilation of eight chapters, specifically written to comply with the requirements of the North-West University, Potchefstroom Campus, South Africa, for the *completion of the degree* ***Philosophiae Doctor*** *(Biochemistry) in article format*. Therefore, chapters 4,5,6 and 7 have either been published or submitted for publication in *peer-reviewed* journals and each consists of an introduction, materials and methods, results and discussion, conclusion, and bibliography sections relevant to it. Furthermore, a comprehensive literature review (Chapter 2), aims and objectives (Chapter 3), and general conclusion (Chapter 8) are added in accordance with the stipulated guidelines.

Chapter 1 gives a brief background and motivation for the study. The structure of the thesis and research outputs/publications that emanated from this study are also included here.

Chapter 2 is a literature overview of nanoparticles and enzymes and how they interface in nanobiosensors for biomedical analysis.

Chapter 3 states the aim and objectives developed from the literature survey. It also gives a synopsis of how the remaining chapters address the stated objectives.

Chapter 4 is the feasibility experiment for the use of a universal detection system based on hydrogen peroxide for signal generation using gold nanostars as transducers. This signal generation was optimised for both plasmonic and colorimetric detection using gold nanostars. This was then applied to cholesterol and glucose sensing as a response for simple detection based on nanoplasmonics. This chapter has been submitted for publication in the *South African Journal of Science.*

- Phiri MM, Mulder DW, Vorster BC. 2019 Plasmonic biosensors based on oxidases using gold nanostars with a universal detection. Submitted to *South African Journal of Science* (Manuscript URL: https://www.sajs.co.za/authorDashboard/submission/6389).

Chapter 5 describes a novel synthesis method for seedless gold nanostars with seed-like advantages. The synthesis method is described as well as the gold nanostars produced. The synthesised gold nanostars were applied for glucose sensing as a feasibility for further optimisation. This chapter was *peer-reviewed* and published in *Royal Society Open Science* in collaboration with the Royal Society of Chemistry.

- Phiri MM, Mulder DW, Vorster BC. 2019. Seedless gold nanostars with seed-like advantages for biosensing applications. *R. Soc. open sci*. 6: 181971. http://dx.doi.org/10.1098/rsos.181971

Chapter 6 describes an optimisation strategy for enzyme immobilisation onto gold nanostars that enables optimal stability and function for both the nanoparticles and the enzyme. The aim for this was to have a novel bioconjugation method for attaching enzymes to nanostars that in principle ensured optimal immobilisation and functionality for both particles and enzymes for the fabrication of gold nanostars-enzyme bioconjugates for plasmonic and colorimetric biosensing. This chapter was *peer-reviewed* and has been published in *Royal Society Open Science* in collaboration with the Royal Society of Chemistry.

- Phiri MM, Mulder DW, Mason S, Vorster BC. 2019 Facile immobilisation of glucose oxidase onto gold nanostars with enhanced binding affinity and optimal function. *R. Soc. open sci*. (https://royalsocietypublishing.org/doi/10.1098/rsos.190205)

Chapter 7 is the application, optimisation, and validation of the fabricated gold nanostars-glucose oxidase bioconjugates to glucose in serum. The current difficulties for the use of nanoparticles in biological fluids are outlined and method for optimal sensing by minimising interferences was devised and applied. This chapter was peer reviewed and published in *Biosensors*.

- Phiri, M.M., Mulder, D.W. and Vorster, B.C., 2019. Plasmonic Detection of Glucose in Serum Based on Biocatalytic Shape-Altering of Gold Nanostars. *Biosensors*, *9*(3), p.83.

Chapter 8 gives a general conclusion and discussion to the study and the potential future prospects from the study.

1.3 Outcomes of this study

Besides the outputs in terms of the publications already mentioned, some of materials from this study were presented at academic conferences listed below.

- Phiri MM*, Mulder DW, Mason S, and Vorster BC (24-26 June 2019). Simplified synthesis of nanosensors and facile optimal immobilisation of enzymes for biosensor applications. 10th International Nanomedicine Conference, Sydney, Australia. **Oral Poster Presentation (Best presentation award).**

- Phiri MM, Mulder DW, Vorster BC (August 2018). Rapid Plasmonic Colorimetric Glucose Biosensor via Biocatalytic Enlargement of Gold Nanostars. 20th International Conference on Nanotechnology and Biosensors, Venice, Italy. **Oral Presentation** (Best presentation award).

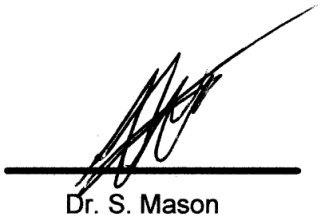
1.4 Author contributions

I conceived, designed, carried out the experiments, data analysis, drafting all the manuscripts and writing of the thesis. Prof B.C. Vorster supervised all aspects of this study. Mrs Danielle Mulder assisted with the study design, planning, execution, drafting and writing of the publication manuscripts. Prof B.C Vorster gave approval of final thesis and research outputs. Dr Shayne Mason assisted with NMR sample analysis and data processing in Chapter 6.

As a co-author, I hereby approve and declare that my role in this study is as indicated above. I hereby give my consent that this work maybe published as part of the PhD thesis of Masauso Moses Phiri.

Prof. B.C. Vorster

Mrs. D.W. Mulder



Dr. S. Mason

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CHAPTER 2 LITERATURE REVIEW

2.1 Nanotechnology in General

Nanotechnology is defined as the design, characterisation, production and application of structures, devices and systems by controlling shape and size at nanometre scale (Hasan, 2015). The nanometre scale is conventionally defined as 1 to 100 nm. Nanotechnology thus deals with at least clusters of atoms that assemble at 1 nm (Hasan, 2015; Sengupta & Sarkar, 2015). Nanotechnology is an emerging field with a technological potential to impact every aspect of human society (Roszek *et al.*, 2005). It is based on the application of nanoscience to practical devices. Nanoscience is an interdisciplinary field that cuts across all vertical sciences such as Chemistry, Biology, Physics, Molecular Biology, Material Science, Biotechnology, Engineering and Surface Science (Farokhzad & Langer, 2006). Thus, the applications of nanotechnology encompass areas such as agriculture, engineering, communication, energy generation or transmission, food manufacturing and/or processing, medicine, etc. Its application to medicine, called nanomedicine, has probably seen the biggest impact of nanotechnology so far (Chen *et al.*, 2016; Farokhzad & Langer, 2006; Kim *et al.*, 2010; Kurbanoglu *et al.*, 2017; Mahato *et al.*, 2016; Roszek *et al.*, 2005; Saha, 2009).

Nanomaterials are objects that have at least one dimension in the nanometre scale (Hasan, 2015; Sengupta & Sarkar, 2015). Some nanomaterials are special because, firstly; the properties of quantum mechanics are the ones that mostly apply as opposed to those of bulk materials (Talapin & Shevchenko, 2016). Secondly; nanomaterials can be fabricated by a process called “bottom-up” whereby nanomaterials are synthesised atom-by-atom in self-assembly to come up with the final product. This enables the manipulation of size, shape, stability and functionality of the produced nanomaterials (Daniel & Astruc, 2004; Saha *et al.*, 2012). Thirdly; nanomaterials have the advantage of large surface-to-volume ratio compared to bulk materials. This enables them to be applied in processes that occur at material surface such as detection and catalysis (Huang & El-Sayed, 2010; Luisa Filipponi, 2010).

2.2 Nanotechnology in Medicine

Nanomedicine is the application of nanotechnology to biomedical and clinical fields (Farokhzad & Langer, 2006; Riehemann *et al.*, 2009b). In a broad sense, nanomedicine is the use of nanometre-sized tools in the process of diagnosing, treating, preventing disease, and for better understanding of the complex underlying pathophysiology. The ultimate goal is to obtain improvements in the quality of life of patients (Kim *et al.*, 2010; Riehemann *et al.*, 2009b; Saha, 2009). This field has the potential to positively impact healthcare at many levels such as:

detection of molecular alterations resulting in disease pathogenesis; diagnosis of disease and imaging; therapeutics and drug delivery; multifunctional systems for combined diagnostics and therapeutics applications (Farokhzad & Langer, 2006). The advances made in the synthesis and characterization of nanomaterials and nanostructures, as well as large financial investments have spawned a vast field of research and applications that can deliver substantial potential benefits to improve healthcare quality and clinical practice (Aznar, 2015; Farokhzad & Langer, 2006).

Clinical diagnostics is essential for effective treatment and successful prevention of diseases (Riehemann *et al.*, 2009b). Nanotechnology applied to medical diagnosis was developed due to the demand for increased sensitivity, selectivity and earlier detection of disease (Alharbi & Al-Sheikh, 2014). Recently, the focus in diagnostics has been the development of novel diagnostic and monitoring devices, such as biosensors and imaging technologies (Aznar, 2015). The need for fast, convenient and cheaper diagnostic tools that can be used for mass screening has been well established. The key drivers for this development have been the cost of medical diagnostic and therapeutic equipment and the current economic conditions, especially in third world countries. The advantage of nanotechnology-based diagnostics lies in the potential for higher sensitivity by using nanostructures compared to the existing methods (Riehemann *et al.*, 2009a). Most of the contribution of nanotechnology to diagnostics has mostly been directed to the design and fabrication of nanosensors and analytical technologies for both *in vivo* (inside the body) and *in vitro* (outside the body) applications (Farokhzad & Langer, 2006; Nazar, 2018).

2.2.1 Nanotechnology in *in vivo* imaging Diagnosis

Significant developments in the use of imaging techniques to identify and monitor diseased tissue *in vivo* have been made in the recent years. Detection of both physiological and pathological changes can be made through visualisation of tissue morphology and cell function (Nazar, 2018). The development and application of sophisticated probes is required for advancements in molecular imaging. This would enable the detection of biological processes at the cellular and molecular level (Key & Leary, 2014; Tay *et al.*, 2015). Nanoparticle probes have significant advantages over single molecule-based contrast agents due to their ability to accumulate at the site of interest and be imaged (Key & Leary, 2014; Schellenberger, 2010). The advantages of these nanoparticle agents are that they allow for brighter, tissue specific imaging to help visualise and aid in the diagnosis of disease at the earliest stages and in some cases even before clinical symptoms disease become apparent. A combination of diagnostic imaging and drug delivery roles have been instituted to enable real-time treatment tracking (Key & Leary, 2014; Nazar, 2018; Schellenberger, 2010).

Several nanoparticles have been used for in vivo diagnostics. The most common among them are quantum dots (QDs), magnetic nanoparticles and gold nanoparticles. (Nazar, 2018). Quantum dots are nanocrystals of inorganic semiconductors that are important as nano-emitters. They are fluorophores that offer significant advantages over the conventionally used fluorescent markers. QDs typically range from 3 – 10 nm and have extraordinary optical properties. QDs are suited for nanodiagnostics because they possess unique optical, chemical and electrical properties, high surface area, good solubility, good biocompatibility, chemical inertness, as well as efficient stability against photo bleaching. QDs have a wide range of applications for molecular diagnostics and genotyping. Luminescent and stable QD bioconjugates have enabled visualisation of cancer cells in living animals. They have also been coated with polyacrylate cap and covalently bond to antibodies for immunofluorescent labelling of breast cancer marker HER2. However, the presence of heavy metals which are toxic in QDs has raised some concerns related to their widespread biological use. Recent developments have seen a number of heavy-metal-free QDs comprising nontoxic elements (Chen *et al.*, 2016; Nazar, 2018).

Magnetic nanoparticles have unique magnetic properties. They have superparamagnetism properties which is a form of magnetism that appears in ferromagnetic or ferrimagnetic nanoparticles. These magnetic nanoparticles have sizes ranging from 5 to 50 nm. The most popular examples are a few types of iron oxide nanoparticles such as Fe_3O_4, α-Fe_2O_3, and γ-Fe_2O_3. These superparamagnetic iron oxide (Fe_3O_4) nanoparticles have been extensively used for bioseparation, biosensing, and magnetic field assisted drug and gene delivery, as well as magnetic therapy of cancer (Chen *et al.*, 2016; Nazar, 2018). Gold nanoparticles (AuNPs) possess distinct properties, both physical and chemical that make them excellent building blocks for manufacturing of novel chemical and biological sensors (Saha *et al.*, 2012). AuNPs are especially effective labels for sensors because of a variety of analytical techniques that can be used to detect them (Nazar, 2018).

2.2.2 Nanotechnology in *in Vitro* Diagnosis

In vitro diagnostics are essential to provide information that could assist in the diagnosis and treatment of disease, test blood supply for safe transfusions, and monitor drug levels in patient (Alharbi & Al-Sheikh, 2014; Shinde *et al.*, 2012). The ideal diagnostic procedure is a non-invasive, quick, and accurate procedure for detection of the biological disease markers for routine screening. This would enable early interventions with appropriate therapeutic regimen (Hauck *et al.*, 2010; Roszek *et al.*, 2005; Salamanca-Buentello *et al.*, 2005). *In vitro* diagnosis is generally based on the analysis of biological fluids such as blood, urine, saliva, cerebrospinal

fluids, etc. The aim is to detect and in some cases quantify specific disease markers (Aznar, 2015; Riehemann *et al.*, 2009b).

Generally, much of the clinical *in vitro* diagnosis is carried out in highly sensitive and specific laboratory assays, which include polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISA), enzyme assays, high-performance liquid chromatography (HPLC), sodium dodecyl sulfate electrophoresis and immunophelometry (INM). Although these conventional methods are quite reliable and highly sensitive, they are laborious, multi-step, time-consuming, cost, and require fully equipped laboratories and specialised trained technicians to carry out the analysis (Aznar, 2015; Lai *et al.*, 2010).

Nanomaterials have been used for the determination of molecules of interest in biological samples (Alharbi & Al-Sheikh, 2014; Janko *et al.*, 2015; Shinde *et al.*, 2012). The reasons for using nanomaterials are to either simplify the readout or improve the sensitivity of the determination (Kim *et al.*, 2010). Gold nanoparticles have been applied for signal enhancement in a standard ELISA where the particles are conjugated with the antibodies. Using minute volumes of blood samples, most reported applications have been shown to offer better sensitivity. Some of the immunosensors showed good reproducibility and stability, enabling batch fabrication (Baptista *et al.*, 2008; Rodriguez-Lorenzo *et al.*, 2012; Xianyu *et al.*, 2014). Nanoparticles are used in lateral-flow assays (LFA) for *in vitro* diagnostics, such as the detection of human chorionic gonadotropin (hCG) in the urine pregnancy test. Gold nanoparticles are also being used in devices for high-throughput genomic detection. This is done without the need for PCR amplification and yet the sensitivity is similar to that of PCR-based assays (Kim *et al.*, 2010).

Simultaneous real-time evaluation of a broad range of disease biomarkers have been enabled via various nanotechnology platforms that have been developed for non-invasive diagnosis. Examples of some of these platforms are the two microtechnological devices; microarray DNA chips and microfluidic systems for lab-on-chip diagnostics. Miniaturisation of these microtechnologies was made possible by the development of photolithography, a technique that allows for lateral resolution in the nanometer range (10 – 100 nm), three orders of magnitude lower that the initial products. This demonstrated the potential capabilities of nanoscaling in biomedical applications. Nanotechnology has also offered the opportunity for single molecule investigations. This opens possibilities for new methods of analysis and detection, such as single-cells analysis. This would help differentiate healthy and tumorous cells. It will also aid in the elimination of the effects between cell types or weak effects of drugs on cells (Riehemann *et al.*, 2009a).

The most promising alternative in clinical diagnosis and monitoring, as opposed to the traditional methods that use high-end laboratory equipment, is the use of biosensor devices. These have enormous potential for having rapid and reliable biomedical analyses. Biosensor platforms can be designed to provide both quantitative and qualitative analytical information. They also have the advantage of using low sample volumes and minimum sample preparations (Aznar, 2015).

2.3 Gold nanoparticles

Gold nanoparticles (AuNPs) are part of the three-dimensional nanomaterials ranging in size between 1–100 nm. AuNPs are the most stable of the metal nanoparticles. They have unique properties such as size-related electronic, magnetic and optical properties and their applications to catalysis and biology (Astruc, 2004). Spherical AuNPs can be obtained with uncomplicated synthetic methods (Nadeau, 2011). They can be synthesised in a straightforward manner and have great stability. They also provide high surface-to-volume ratio with excellent biocompatibility when functionalised with appropriate ligands. The other advantage of these NPs lies in the ability to tune the various properties by varying the size, shape and the chemical environment that surround them. Thus, AuNPs provide a suitable platform for functionalisation with multiple organic and biological ligands for a wide range of applications in selective binding and detection of micro-molecules and biological targets (Saha *et al.*, 2012).

AuNPs are one of the plasmonic nanostructures with a strong localised surface plasmon resonance absorption peak. LSPR is a collective oscillation of free charge carriers at the interface of plasmonic nanomaterials and the surrounding dielectric medium. This is produced when the resonance occurs between the frequency of incident light photons and the natural frequency of surface charge carries (Amendola *et al.*, 2017). The LSPR absorption peak for Au has been observed to maximum in the optical absorption spectrum, in the visible and near-infrared (NIR) regions (Nadeau, 2011). The applicable advantages of metallic structures include their physicochemical stability and low toxicity. They also have extremely strong light absorption properties and strong laser-induced heating. Another such advantage is their absorption peaks which can be manipulated through the variations of shape, size and surface conditions to be in the NIR range where biological tissues have significantly less absorption and scattering (Chen *et al.*, 2016).

2.3.1 Synthesis of gold nanoparticles

Two approaches have been used as preparative methods for spherical AuNPs (Turkevich, 1985). One is called the “top-down” method. This involves the disintegration of bulk metallic gold

by grinding and stabilising the resulting nanosized Au particles by adding colloidal protecting agents. A wide range of nanostructured metal colloids have been produced on a preparative laboratory scale using metal vapor techniques. The use of these techniques is however limited because the operation of the apparatus is demanding and it is difficult to produce narrow particle size distributions (Zhou *et al.*, 2009). The other method is "bottom-up" approach. In this approach, the AuNPs are synthesised by chemical reduction of gold salts using either an appropriate reducing agent, electrochemical pathways, or the controlled decomposition of metastable organometallic compounds (Turkevich, 1985; Zhou *et al.*, 2009).

The two most commonly used methods for the chemical reduction of gold salts for nanoparticle synthesis are the sodium citrate preparation method and the Brust-Schiffrin method for thiol-protected AuNPs as these enable the control of the size, shape, solubility, stability and functionality of the nanoparticles produced (Saha *et al.*, 2012). Of these two reduction methods, the aqueous reduction of gold salt by sodium citrate under stirring is the most commonly used method because of its simplicity (Zhou *et al.*, 2009). The particles synthesised by the citrate method are mostly monodispersed and spherical in shape, especially for those with sizes below 30 nm (Zhou *et al.*, 2009). The particle sizes can be tuned to obtain a desired size by controlling the initial reagent concentrations of the gold salt and the citrate (Turkevich, 1985; Zhou *et al.*, 2009). Citrate has also been replaced by other reducing reagents such as borohydride that is used in a similar manner.

2.3.2 Turkervich-Frens Method

This method is otherwise referred to as the citrate reduction method. It's a method for the synthesis of spherical AuNPs by the reduction of hydrogen tetrachloroaurate(III) ($HAuCl_4$) using trisodium citrate in water. Turkevich introduced this method in 1951 and it was later modified by Frens in 1973 (Alex & Tiwari, 2015). The method requires that the aqueous solution of $HAuCl_4$ is heated to almost boiling when trisodium citrate is added rapidly under vigorous stirring. Wine-red colloidal solution is observed to appear after a few minutes of stirring. Generally, this method synthesises AuNPs with monodispersed sizes around or below 20 nm (Alex & Tiwari, 2015).

The generally proposed mechanism for the stepwise formation of the nanoparticles involves an initial nucleation phase, then followed by growth, and lastly agglomeration (Zhou *et al.*, 2009). The metal salt (Au^{3+}) is reduced to give zerovalent metal atoms (Au^0) in the embryonic nucleation stage. These uncharged metal atoms are unstable in solution and that leads to collisions with other metal atoms, metal ions or clusters to form irreversible seed nuclei which, depending on the strength of the metal-metal bonds, can be below 1 nm. The abundance of

individual Au^0 reduces over time while that of the seed nuclei increases. With time, it becomes more likely that a Au^0 will collide with a nucleate than with another metal ion or atom. This leads to layer-by-layer growth of the particle. In order to stabilise the nanostructured colloidal metals and prevent agglomeration, protective agents are added which achieve this through electrostatic and steric modes. Electrostatic stabilisation is achieved by double layered electrical repulsions between particles, while steric stabilisation is by coordination of sterically bulky organic molecules that act as protective shields on the metallic surface (Nadeau, 2011; Zhou *et al.*, 2009).

Ultimately particle growth is controlled by the concentration of the metal ions and reducing agents, temperature and mixing (Nadeau, 2011; Zhou *et al.*, 2009). Citrate in this method acts both as a reducing and stabilising agent. By manipulating the citrate/Au^{3+} ratio, the size of the AuNPs can be controlled. With relatively lower concentrations of citrate, the AuNPs are incompletely covered, coupled with slow nanoparticle growth kinetics leading to the formation of larger and/or aggregation nanoparticles. On the contrary, as the citrate concentration relatively increases, the fast growth kinetics favours nucleation over growth in particle size and smaller nanoparticles are obtained. AuNPs below the size of 20 nm are generally monodispersed, while those greater than 20 nm are polydispersed (Alex & Tiwari, 2015; Nadeau, 2011). Another factor affecting is the kinetics of nanoparticle growth

The citrate method is generally effective in the synthesis of ≤ 20 nm AuNPs but ineffective in producing larger particles with diameters between 40 to 100 nm. The larger AuNPs synthesised by the citrate reduction have been observed to be polydispersed, low in concentration, low chances of success in synthesising a predetermined particle diameter, and poor repeatability in producing the same mean diameters for two syntheses carried out under identical conditions (Brown & Natan, 1998). To overcome these challenges, a seed-mediated method for the enlargement of colloidal AuNPs was described by Brown and Natan, (1998). The particle size can be enlarged by seed-mediated growth of the small AuNPs under mild condition of reducing agent and gold precursor. Smaller AuNPs are synthesised using the citrate method and then used as seed particles for enlargement by mixing them with gold precursor and reducing agent such as hydroxylamine. In this way, predetermined and controlled larger AuNPs diameters can be produced (Alex & Tiwari, 2015; Brown & Natan, 1998).

2.3.3 Brust-Schiffrin Method

In as early as 1857, Faraday had introduced the synthesis of colloidal AuNPs in a two-phase system. He accomplished this by reducing an aqueous gold salt with phosphorus in carbon disulfide and obtained a ruby coloured aqueous solution of dispersed AuNPs. Initial efforts to

stabilise the AuNPs with alkanethiols by Mulvaney led to a significant breakthrough by Brust and Schiffrin in 1994 (Brust *et al.*, 1994; Saha *et al.*, 2012). Brust and Schiffrin described a two-phase method for the synthesis of AuNPs using strong thiol–gold bonds to protect the particles with thiol ligands (Saha *et al.*, 2012).

The protocol for this method is such that $HAuCl_4$ is transferred from aqueous phase to organic phase (toluene) using tetraoctylammonium bromide (TOAB) which acts as a surfactant. It is then reduced by sodium borohydride ($NaBH_4$) in the presence of dodecanethiol, resulting in a quick change of colour from orange to deep brown in toluene. The AuNPs synthesised in the organic phase have controlled sizes in the range of 1.5 to 5 nm. They are also characterised by superior stability due to the strong thiol–gold covalent bonds and this enables easy handling, characterisation and functionalisation. Tuning of the particle sizes is achieved by varying the reaction conditions, such as gold/thiol ratio, temperature and reduction rate. The AuNPs can be thoroughly dried and later redispersed in organic solvents without any aggregation or loss of stability (Brust *et al.*, 1994; Saha *et al.*, 2012).

2.4 Gold nanostars

Significant advances in the synthesis of AuNPs have enabled the manipulation of nanostructures using various agents thereby readily obtaining other nanoshapes. Different shapes such as nanorods, nanocubes, nanoprisms, nanowires, nanoboxes, nanoshells, triangular, hexagonal shapes, and even nanostars have been produced (Alex & Tiwari, 2015; Nadeau, 2011). Particles with different shapes and sizes produce LSPR signals that can be used to distinguish them (Amendola *et al.*, 2017; Nadeau, 2011). Among the various geometries of gold nanoparticles, gold nanostars (AuNSs), or multi-branched gold nanoparticles, have received much attention in the recent years because of their catalytic activity, molecular detection, and biological applications in immunoassays, dark field imaging of cells and as plasmonic biosensors (Chirico *et al.*, 2015; Maiorano *et al.*, 2011).

The focus of recent research has been to use different morphologies and compositions of nanostructures, such as AuNSs, as a way to tune the LSPR properties of the nanosensors for greater sensitivity (Aldewachi *et al.*, 2018; Rodríguez-Lorenzo *et al.*, 2012). In this light, AuNSs promise to be nanosensors of choice for signal transduction based on the LSPR. The LSPRs spectrum is determined by the nature of the shape of the nanoparticles' width, position, and number of nanostars spikes (Xia & Halas, 2005). A common feature of LSPRs for nanostars is their location at lower energy compared to nanospheres (Amendola *et al.*, 2017). AuNSs have the plasmon band is redshifted and more intense, and typically centred around 650 – 900 nm compared to gold nanospheres with the size of 2–50 nm that show only one plasmon band

centred at about 520 nm (Amendola *et al.*, 2017; Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Saverot *et al.*, 2016). AuNSs, as one of the anisotropic nanocrystals, exhibit higher refractive index sensitivity compared to spherical nanoparticles (Guo *et al.*, 2015) for LSPR sensing based on shape alterations induced by changes in the conditions within the colloidal or detection solution (Langer *et al.*, 2015). Lastly, AuNSs also provided a larger surface areas for enzyme immobilisation with potential for higher load of enzymes per nanoparticle compared to smaller nanospheres (Sapsford *et al.*, 2013).

2.4.1 Synthesis of star-shaped gold nanoparticles

A rough general classification of the synthetic strategies for the production of AuNSs can be split into two main categories: i) the seeded-growth and ii) non-seeded-growth methods (Minati *et al.*, 2014). The choice of one of these synthetic strategies depends on a number of experimental parameters that influence both the nucleation and growth processes in AuNSs synthesis, leading to potential control of the size and degree of branching of the nanoparticles obtained (Guerrero-Martínez *et al.*, 2011). Some of these experimental parameters include; the intrinsic reactivity of the metal precursor, effectiveness of the reducing agent, temperature of reaction and effect of catalysts and additives that can trigger the reaction kinetics of both nucleation and growth (Guerrero-Martínez *et al.*, 2011).

2.4.1.1 The Seeded-growth method

The seeded-growth technique is a popular method for the synthesis of monodispersed gold nanostars. In this method, pre-synthesised seeds (AuNPs) are used as nucleation points where additional material is deposited for growth of the branches (Guerrero-Martínez *et al.*, 2011; Minati *et al.*, 2014). The synthetic process involves reducing $HAuCl_4$ with ascorbic acid (or other reducing agents) on preformed gold seeds in the presence of a surfactant at room temperature. Due to the preferential absorption of the capping agents (surfactants or polymers) on certain facets of the AuNPs, they have been reported to trigger the growth process of the branches by changing the growth rates along specific crystallographic directions. It has also been reported that the addition silver nitrate ($AgNO_3$) at different stages of the growth process of the nanocrystals increases the degree of control of the shape of the gold nanostars produced (Chirico *et al.*, 2015). However, this method has some drawbacks, one of which is the complication caused by the various stabilising agents and surfactant in the post-synthetic cleaning of the nanostars (Minati *et al.*, 2014).

2.4.1.2 The Non-seeded-growth method

The non-seeded-growth method involves the synthesis of gold nanostars by the reduction of gold precursor in the presence of suitable reducing agents and surfactants at room temperature (Chirico *et al.*, 2015). Recent advances in this method have seen the use of "green" chemicals such as *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) as reducing and stabilising agent. In this one-pot synthetic strategy, nuclei evolve to form nanocrystal seeds which get to be bigger particles through the direct addition of metal atoms (Guerrero-Martínez *et al.*, 2011). The presence of piperazine in HEPES was found to be responsible for the formation of the branches in the stars (Xie *et al.*, 2007).

Compared to the seeded-growth strategy, this technique has fewer complications, with the advantage of being completed in one single step and pot. Some protocols are carried out without using surfactants, making the post-synthesis purification of the AuNSs formed less problematic. However, it has a number of disadvantages, one of which is inability to control the dimensions of the resulting nanostars leading to polydispersity in the shapes and sizes of particles produced. Another notable setback is the high sensitivity to changes in the reaction parameters such as reagents concentrations, pH, and temperature, which affects the growth process and reproducibility of the nanocrystals (Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Minati *et al.*, 2014; Saverot *et al.*, 2016; Xie *et al.*, 2007).

Many attempts have been made to optimize the HEPES-mediated method to yield more monodispersed and larger AuNSs (Chandra *et al.*, 2016; Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Xia *et al.*, 2009). For example, a seed-mediated synthetic method using HEPES as a shape-directing agent was reported that produced larger and monodispersed multibranched gold nanoparticles (Maiorano *et al.*, 2011). Saverot and colleagues (Saverot *et al.*, 2016) described a HEPES-mediated seedless AuNSs synthetic method using two-steps to produce larger sized AuNSs. However, setbacks typical of non-seeded synthesis apply to many of these methods, such as high sensitivity to changes in conditions and concentrations of precursor reagents such as pH of the HEPES buffer, temperature, and $HAuCl_4$ concentrations, strongly affects the reproducibility of the synthesized AuNSs (Chirico *et al.*, 2015; Hill, 2015; Minati *et al.*, 2014; Saverot *et al.*, 2016; Xie *et al.*, 2007).

The choice of synthesis method for AuNSs depends on a number of experimental parameters that influence the nucleation and growth processes in colloidal particle synthesis, as well as the downstream applications of the produced nanostars (Guerrero-Martínez *et al.*, 2011). The experimental parameters such as the intrinsic reactivity of the metal precursor, surface stabilisation effect of capping agent, the effectiveness of the reducing agent, the reaction

temperature and, the effects of shape-directing agents, have an effect on the control of the size and degree of branching of the produced nanostars (Chandra *et al.*, 2016; Gasser *et al.*, 2001; Guerrero-Martínez *et al.*, 2011; Kawamura *et al.*, 2009; Sun & Xia, 2002; Xia & Halas, 2005; Xia *et al.*, 2009). AuNSs have been used for different downstream applications such as SERS (Niu *et al.*, 2015; Saverot *et al.*, 2016), in vivo imaging and phototherapy (Liu *et al.*, 2015; Yuan *et al.*, 2012), biosensing (Guo *et al.*, 2016; Langer *et al.*, 2015; Rodriguez-Lorenzo *et al.*, 2012; Tang & Li, 2017), among others. These applications require appropriate synthetic parameters of AuNSs, especially stabilisation agents, for use and responses.

Thus, there is a need for tailor-made AuNSs synthesis method for the intended application. Such a method needs to leverage the advantages of seeded methods such as speed, monodispersity, controlled growth of branches, bigger sizes of nanostars produced for greater LSPR sensitivity, as well as the simplicity and deployment of non-harmful reagents of the seedless method.

2.4.2 Characterisation of gold nanoparticles

It is important to characterise Au nanostructures in a detailed manner before their use in any quantitative experiment. The most commonly used techniques for particle characterisation can be categorised as imaging and non-imaging. Non-imaging techniques include ultraviolet-visible-near infrared spectroscopy (UV-vis-NIR) and dynamic light scattering (DLS), while the imaging methods include transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Buhr *et al.*, 2009). Only when these methods are used collectively do they provide a meaningful and quantitative characterisation of the particles, as they are inadequate in their individual capacities to (Nadeau, 2011).

Among the non-imaging techniques, UV-vis-NIR spectroscopy is useful in providing information on the size distribution of the particles in the sample by studying the optical/plasmonic properties of the nanoparticles which are related to their size and shape. Therefore, it is used to give the size distribution and concentration (in the case of spherical nanoparticles) of the particles using the extinction coefficients. It is usually the first line characterisation method because it is cost effective and readily available (Buhr *et al.*, 2009; Haiss *et al.*, 2007; Nadeau, 2011). DLS is useful in generating a near-ensemble picture of the size distribution of the nanoparticles. The basis of this measurement is single-particle mobility and each individual particle is assumed to be spherical. Caution has been advised when analysing DLS data as statistically weighted distributions are common and can dramatically vary the size of a single sample depending on the weighting factor, these being distributions according to intensity, volume and particle number (Nadeau, 2011). The advantages of these methods are that the

yield statistical results for the distribution of particle size. The major drawbacks, however, are the difficulty in precisely determining the geometries of the particles. This raises the indispensable need for direct imaging if the particle size and shape are to be confirmed, albeit in a non-statistical manner (Buhr *et al.*, 2009).

TEM and SEM are the standard techniques for direct imaging and dimensional measurements of micro- and nanostructures due to their high resolutions and high imaging speeds. TEM achieves a higher lateral resolution than SEM due to electron energies above 100 keV (Buhr *et al.*, 2009). It is used to qualitatively determine or confirm the nanoparticle shape by visualising several particles in the sample. SEM, despite having lower resolutions compared to TEM, provides nanometre resolution that is enough to confirm the shape of nanometre sized particles. Due to high operational costs for TEM, a comparatively inexpensive and easy to operate transmission electron microscope can be achieved using an ordinary SEM with a scanner unit (Buhr *et al.*, 2009; Nadeau, 2011).

2.4.3 Surface Functionalisation of gold nanoparticles

Gold nanoparticles may be subject to changes that may cause agglomeration in biological fluids due to changes in temperature, pH, ionic concentration and the presence of biomolecules (Melby *et al.*, 2017; Nadeau, 2011). It is therefore desirable to have surface functionality for gold nanoparticles for handling purposes and having a functional scaffold for downstream applications (Zhou *et al.*, 2009). This enables particles to ideally remain dispersed and structurally stable in solution and in complex biological systems (Nadeau, 2011).

Four strategies have widely been used for functionalising nanoparticles with stabilising agents. These include the “grafting from” approach; the “grafting to” approach; physisorption; and the post-modification of pre-formed AuNPs (Saha *et al.*, 2012). Not only do surface functionalisation agents give extra stability to particles, they also aid in additional control over their sizes during synthesis (Zhou *et al.*, 2009). Some of the agents commonly used to stabilise AuNPs include poly(*N*-vinylpyrrolidone) (PVP), poly(ethylene glycol) (PEG), chitosan, cetyltrimethylammonium bromide (CTAB), etc (Saha *et al.*, 2012). Coating the particles with polymers confers stability by steric hindrance thereby preventing them from sticking to each other when they collide. Surface modifiers, such as sodium citrate, which are electronically adsorbed are mostly useful during particle synthesis but not as good for imparting enough stability to enable the particles stability in biological systems (Nadeau, 2011).

PEG molecules of different chain lengths and functional groups have been used widely to stabilise both the spherical and star-shaped gold nanoparticles and to attach targeting moieties

(Chirico *et al.*, 2015; Nadeau, 2011). Using PEG-thiols is the best way to absorb PEG onto the AuNPs. The Au–S is a strong bond that creates a stable and viable system in biological matrices. In order to yield higher stability with the Au–S bond, an extra thiol group is added to the linker (PEG-dithiol) whereby there are two Au–S bonds per molecule. Particles stabilised with a PEG-dithiol linker exhibit almost no aggregation and enables easy post-synthetic cleaning with centrifugation without agglomeration. Another advantage of PEG-thiols is the reduction of the toxicity seen in certain sizes and shapes of AuNPs (Chirico *et al.*, 2015; Nadeau, 2011). In an effort to improve the stability of the nanoparticles in acidic and basic pH ranges, extreme temperatures, salt concentrations and in serum, new PEGylated branched polymers have been synthesised (Prencipe *et al.*, 2009). Poly-γ-glutamic acid (PGA) was functionalised with either pyrene moieties or phospholipids to provide robust polymer interactions. These polymers were added to PEG for the purpose of enhancing aqueous solubility and biocompatibility to the nanoparticles. The results reported were that of great stability of the nanoparticles in all the various pH ranges, high temperature overnight and in 50% serum (Prencipe *et al.*, 2009).

Various functionalisation methods of gold nanoparticle systems with biomolecules have been developed for specific target molecules, drug delivery, and biosensing assays. There is need to fine tune the surface modification of nanoparticle that yields a controlled assembly, or enable the attaching of a target moiety to the nanoparticles. AuNSs can be functionalised in a similar way to other nanostructures, however, their high branched shape adds specific characteristics to their surface modification (Chirico *et al.*, 2015). Gold nanoparticles have been functionalised with proteins, such as antibodies and enzymes (Rodriguez-Lorenzo *et al.*, 2012; Xianyu *et al.*, 2014; Zhou *et al.*, 2009). Proteins are commonly attached to particles via the terminus of carboxy-PEG molecules using sulfo-*N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (sulfo-NHS/EDC) coupling chemistries (Nadeau, 2011). This sometimes adds to the stability of the AuNPs, and also confers biocompatible functionalities on to the particles. This enables the particles to have biological interactions or coupling. Such surface modifications of AuNPs with proteins provide biomolecules functionalised nanoparticles that can be used for biomedical applications (Wangoo *et al.*, 2008).

2.5 Nanobiosensors

Functionalised gold nanostructures with suitable biorecognition molecules (such as antibodies and enzymes) have enabled their applications in different areas such as biological sensing and other biomedical applications (Aldewachi *et al.*, 2018; Alex & Tiwari, 2015; Saha *et al.*, 2012; Sapsford *et al.*, 2013). Suitably functionalised gold nanoparticles possess distinct physiochemical properties that make them excellent scaffolds for fabrication of novel and/or

improved biosensors as they have the potential to improve both the biorecognition and signal transduction processes of biosensors (Saha *et al.*, 2012). Gold nanostructures have been exploited for biosensing fabrications due to their straightforward synthesis, high stability when properly functionalised, excellent biocompatibility with high surface-to-volume ratio for ligand functionalisation, possess unique optical properties that can readily be tuned for signal transduction (Aldewachi *et al.*, 2018; Baptista *et al.*, 2008; Saha *et al.*, 2012). Thus, biosensors combined with nanomaterials are a promising gap-filler between advanced detection and routine tests (Chamorro-Garcia & Merkoçi, 2016).

2.5.1 Definition and characteristics

According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is defined as a device that uses specific biochemical reactions mediated by isolated enzymes, immune systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (Thevenot *et al.*, 1999). A nanobiosensor (NBS) is a device that is made up of nanostructured materials and comprises a biological recognition component designed to bind a specific substance, and a physical transducer that can convert the biochemical responses of the analyte into identifiable and quantifiable signals by a detector as illustrated in Figure 2-1(A) (Sepúlveda *et al.*, 2009). A bioreceptor is ideally immobilised to a solid surface where it binds and/or catalyses the molecule of interest. The biorecognition event leads to the production of physiochemical signal that leads to some physical conformational changes to the transducers (nanostructured materials). This results in some semi- or fully quantifiable signal in an easy-read-format (Figure 2-1) (Sepúlveda *et al.*, 2009).

Some essential basic characteristics of a biosensor include a predictable mathematical relationship between the detector response and concentration. This must be high for the detection of target compound in high concentrations in the biological fluids. A biosensor must be selective in the biorecognition of the target molecules of interest. It must also be sensitive to be able to detect the smallest quantities present in the sample. It is also essential that the response time be very short, enabling them to be rapid diagnostic tools (Ali *et al.*, 2017; Mehrotra, 2016; Saha *et al.*, 2012). NBS have been reported to be ultrafast and ultrasensitive analyte detectors. They have shown great promise in rapid and robust diagnostics (Tang, L. 2013; Roszek, B. 2005). They have enabled the naked-eye colorimetric, fluorometric, chemiluminescent, and electrochemical detection of analytes (Li & Xu, 2014).

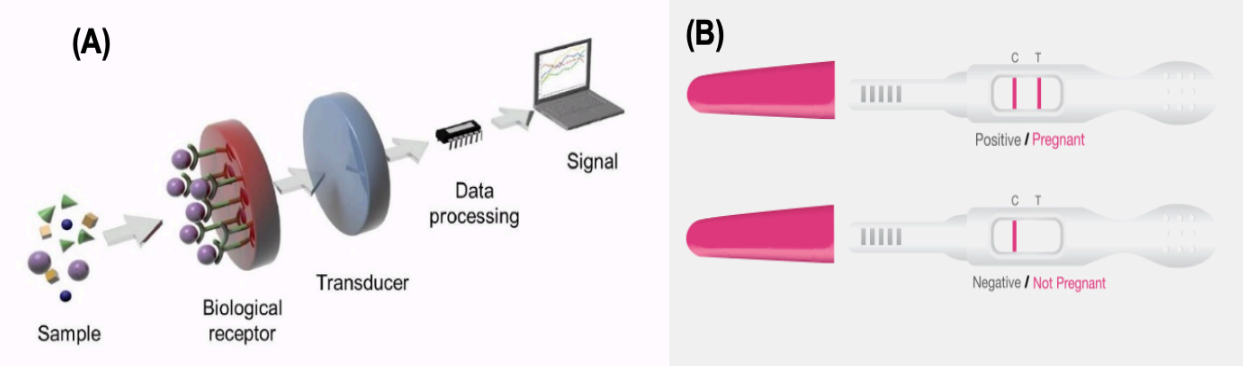


Figure 2-1 (A) Scheme showing the various components of a typical biosensor. (B) A home pregnancy test using gold nanoparticles as colorimetric detection labels. The red strips are gold nanoparticles that are conjugated with complementary DNA base pair to bind hCG.

One excellent example of how nanostructures and their optical properties have been used to solve real world diagnostic need is the home pregnancy test (Alivisatos, 2001; Rojanathanes *et al.*, 2008). The red colour for a positive test result is due to a complementary DNA base pair binding to human gonadotropic hormone (hCG) which is attached to gold nanoparticles which then reflects a red light from the particles (Figure 2-1(B) (Chang *et al.*, 2014; Posthuma-Trumpie *et al.*, 2009; Sepúlveda *et al.*, 2009).

2.5.2 Classifications of nanobiosensors

In the recent years there has been an increase in the number of novel nanomaterials being synthesised and applied in the fabrication of biosensors. These nanobiosensors, which have witnessed an exponential growth, are an integration of various disciplines such as material science, molecular engineering, chemistry and biotechnology, among others. Due to the multidisciplinary nature of research leading to the fabrication of biosensors, a number of biosensors platforms have been developed. These are based on different types of biorecognition molecules and transducers. Therefore, biosensors can generally be classified into two main groups; (i) based on the biorecognition mechanisms or, alternatively, (ii) the physiochemical signal transduction (Aznar, 2015; Kwong, 2000; Mehrotra, 2016; Sepúlveda *et al.*, 2009; Thevenot *et al.*, 1999).

2.5.2.1 Biorecognition mechanisms

Biorecognition molecules interact biochemically with their target molecules based on either an affinity or catalytic nature. Therefore, biosensors can be divided into either affinity or catalytic biosensors as illustrated in Figure 2-2 (Touhami, 2014; Xu *et al.*, 2005).

2.5.2.1.1 Affinity biosensors

The biosensors of this type operate based on the conformational recognition and binding of an analyte by its specific biorecognition molecule. This leads to an equilibrium reaction. The determination of this reaction can be done via the changes in mass, optical or electrochemical variations detected by the transducer (Touhami, 2014). Typically, higher affinity and specificity between an analyte and biorecognition molecule adds to the selectivity and sensitivity of the assay. Biosensors based on immunological reactions between an antigen and its antibody are typical example of affinity assays (Aznar, 2015; D'souza, 2001; Rogers, 2000; Rogers & Mulchandani, 1998). Using this platform, a number of nanomaterials based-biosensors have been developed such as the detection of prostate specific antigen (PSA) and alkaline phosphatase (ALP) (Rodriguez-Lorenzo *et al.*, 2012; Xianyu *et al.*, 2014). Other examples of affinity biosensors are the ones that employ the use of specific interaction between complementary oligonucleotide chains and DNA molecules. Aptamers, which are short single-stranded oligonucleotides that are capable of binding various molecules with high affinity and specificity, are another example of affinity biorecognition molecules in biosensors (Rogers, 2000).

2.5.2.1.2 Catalytic biosensors

In catalytic biosensors, the analyte is bound to a receptor where a biochemical reaction takes place. It is catalysed by a biomolecular receptor that converts a substrate present in the sample into a product. The signal is generated because of the binding and conversion of the substrate of interest into a product which is detected by a transducer. Most of the common biocatalytic molecules are enzymes, microbial organisms, cellular organelles or tissues. Most of the products are detected in the form of proton concentration, release of ammonia, hydrogen peroxide, or oxygen, light or heat emission, etc (Aznar, 2015; Kwong, 2000). Enzyme-based biosensors have shown new properties such as enhanced selectivity, analytical signalling, and sensitivity after being modified by nanoparticles. Nanomaterials enable a large surface area, good electrical conductivity, unique optical and quantum properties akin to nanoscale structures. Hence, these allow for rapid, sensitive, in-time and specific detection of analytes of interest compared to other traditional methods of detection such as chromatography (Chen *et al.*, 2017).

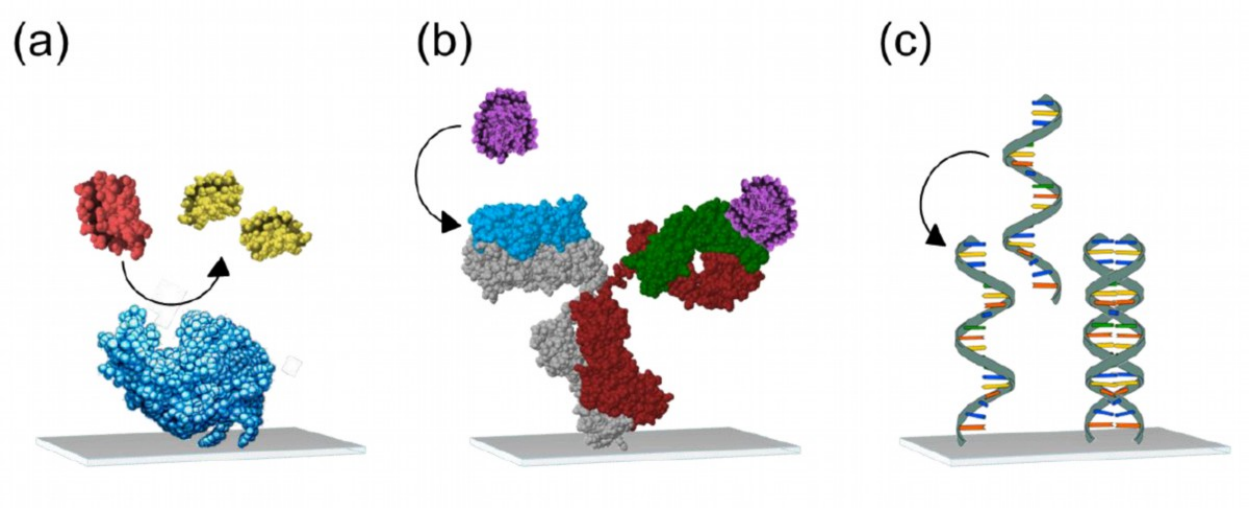


Figure 2-2 Different types of biosensors based on the biorecognition molecules: (a) catalytic (enzymatic biosensor), (b) affinity (immunosensor), (c) affinity (DNA biosensor) (Aznar, 2015).

2.5.2.2 Physiochemical signal transduction

Biosensors are also classified based on the type of signal transduction generation. The transducers can be electrochemical, mechanical or optical as illustrated in Figure 2-3 (Aznar, 2015; Pohanka & Skládal, 2008; Thevenot *et al.*, 1999; Xu *et al.*, 2005).

2.5.2.2.1 Electrochemical biosensors

Electrochemical biosensors were the first scientifically proposed and successfully commercialized among biosensors. In 1962, Clark and Lyons introduced the principle of the first enzyme electrode with glucose oxidase immobilized for the determination of blood glucose from diabetics. They are the most used biosensors in clinical practice as a result of their excellent analytical features, simplicity in fabrication and unique capabilities to be miniaturised. Typically, in these biosensors the transducer measures the electrochemical changes after the interaction between biorecognition layer and the analyte of interest. Native enzymes have been used as biorecognition molecules in the cases where the analyte of interest is an enzyme substrate. Alternatively, enzymes have been used as labels in affinity-based biosensors where they are bound to antibodies, antigens or oligonucleotides with a specific sequence (Pohanka & Skládal, 2008; Xu *et al.*, 2005).

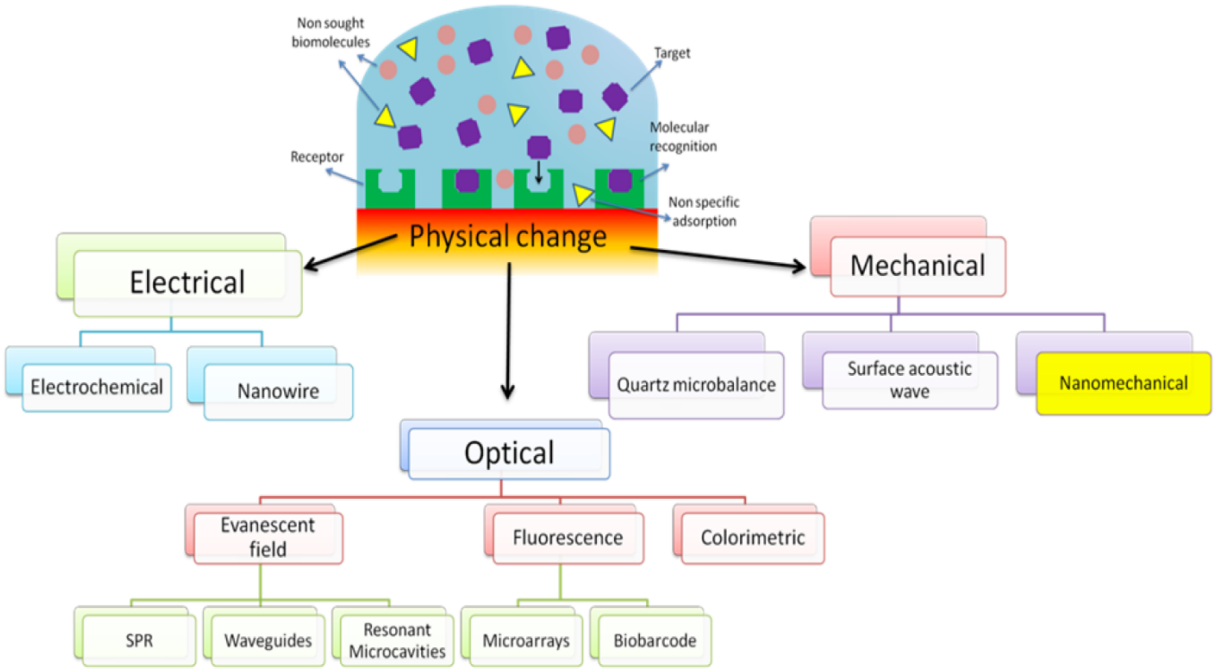


Figure 2-3 Illustration of biosensors divided according to the physiochemical signal transduction.

Based on the mode of operation, electrochemical biosensors can be divided into four main types; potentiometric, amperometric, impedimetric, and conductimetric. Potentiometric biosensors operate on the principle of ions accumulating at the ion-selective membrane interface where a local potential is generated that is detected by the transducer. Amperometric biosensors typically have a working electrode made up of a noble metal or screen-printed layer covered by the biorecognition component. Conversion of the electroactive species generated in the biorecognition layer at the applied potential occurs at the electrode and the resulting electric current is measured. Impedimetric biosensors are based on either impedance or its components resistance and capacitance, while conductimetric sensors on the inverse value of resistance called conductance. With the incorporation of nanomaterials, such as carbon nanotubes, as transducers, electrochemical biosensors have experienced exponential growth. The reason for this is that carbon nanotubes provide a larger surface area, good chemical stability, mechanical strength and is an excellent electrical conductor. It is also easy to fabricate and biofunctionalize with enzymes (Aznar, 2015; Pohanka & Skládal, 2008).

2.5.2.2.2 Mechanical biosensors

Progress in fabrication of micro- and nano-technologies based biosensors are enabling the achievement of increasingly smaller transducers of a mechanical nature. Some of these transducers have micro- and nanosized moving parts whose deformation and vibration are

sensitively altered upon molecular adsorption. One of the most established techniques in mechanical biosensors is the quartz crystal microbalance. The modus operandi of these devices is the measuring of the resonance frequency that is related to the mass changes induced by the interaction between the biorecognition layer immobilised on the crystal surface and the analyte (Tamayo *et al.*, 2013).

The mechanical biosensors that advantageously utilise the nanoscale size for at least one of their dimensions are referred to as nanomechanical biosensors. These are usually shaped like cantilevers. They have been reported to produce outstanding mass resolutions on the verge of single atoms. They can translate biorecognition events into measurable displacements with low mechanical compliance. They have high sensitivity to the mechanical properties of the adsorbed biomolecules since the sizes of biological molecules are within the range of one of the dimensions of the mechanical system. These devices can be used to detect the presence or determine the concentration of a particular analyte in solution; to measure a particular physical property of the analyte for identification and/or classification purposes; or to observe, analyse or characterise a particular biological process (Calleja *et al.*, 2012; Tamayo *et al.*, 2013).

2.5.2.2.3 Optical biosensors

Optical biosensors operate based on the variations in the optical properties of the propagated light to detect the biological interactions. The optical properties measured include light intensity, wavelength, refractive index or polarization (Hill, 2015; Lim & Gao, 2016). Optical biosensors are powerful tools for detection and analysis. They have a wide-range of applications in biomedical and healthcare research, pharmaceutical, environmental monitoring, etc. They are not affected by electromagnetic interferences and have the capability for remote sensing performance and multiplexing in one device (Borisov & Wolfbeis, 2008; Fan *et al.*, 2008).

Generally, optical biosensors can be divided into two main types; fluorescence and evanescent wave sensors. The fluorescence-based detection usually uses optical labels such as dyes or fluorescent molecules which are tagged to biorecognition molecules. The intensity of produced fluorescence indicates both the presence and concentration of a target analyte and its interaction with the biorecognition molecules. Evanescent wave biosensors operate based on the confinement of the electromagnetic wave (EM) in certain dielectric and/or metal which can either be localised or propagating in mode. An evanescent field is formed when part of the EM mode penetrates the external medium. The evanescent field acts as a probe to detect refractive index (RI) changes resulting from a biological interaction, even without the use of a label for sensing as in the fluorescence ones. Thus, these biosensors are also referred to as ‘label-free’ detection biosensors. The RI measured is related only to the sample concentration or surface

density, and not the total sample mass. Detection signal does not scale down with the sample volume. This makes them more advantageous in using ultra small volumes. Examples of evanescent wave biosensors are interferometers, resonators or plasmonic biosensors (Aznar, 2015; Borisov & Wolfbeis, 2008; Fan *et al.*, 2008).

Optical detection approaches have the advantage of being cost-effective with an easy readout format for the detection of clinically relevant molecules for diagnostic purposes (Guo *et al.*, 2016; Radhakumary & Sreenivasan, 2011). Colorimetric methods using gold nanoparticles have other merits; such as rapidity, simplicity and sensitivity (Miao *et al.*, 2017). Naked-eye colorimetric detection is advantageous over fluorescent or chemiluminescent. Colorimetric NBS may not require equipment or trained professionals to interpret a qualitative result. The results can still be quantified using basic flatbed/smartphone scanning technology (Dutta *et al.*, 2016; Sepúlveda *et al.*, 2009) and have potential for technology transfer to other point-of-care devices (Chamorro-Garcia & Merkoçi, 2016; Mahato *et al.*, 2016; Ruppert *et al.*, 2019; Yang *et al.*, 2016). Typical data recording methods such as UV-vis-NIR spectrophotometers are still a viable technology depending on the assay format (Atta *et al.*, 2019; Gao *et al.*, 2017).

Optical approaches are a great need in developing countries where there is limited access to sophisticated expensive laboratory equipment to the bulk of the population (Martinez *et al.*, 2009; Radhakumary & Sreenivasan, 2011). Such simplified and cost-effective procedures have the potential of radically improving the health care delivery in these resource-constrained regions (Hauck *et al.*, 2010; Ruppert *et al.*, 2019; Salamanca-Buentello *et al.*, 2005; Yang *et al.*, 2016).

2.6 Plasmonic Biosensors

Plasmonics is a rapidly growing field in physical optics. It studies the phenomena of electromagnetic waves' interaction with metals and their applications. Plasmonic metallic structures are those that support electromagnetic oscillations at their surfaces known as surface plasmon polaritons. The coupling of optical waves to the free electrons of a metal can give rise to electromagnetic modes named Surface Plasmons (SPs). Excited plasmonic modes at the interface of a noble metal and a dielectric can exhibit an evanescent field that penetrates the surrounding media. The evanescent field is very sensitive to the changes in the refractive index close to the metal surface. This enables them to act as detection probes in plasmonic nanostructures sensing platforms (Amendola *et al.*, 2017; Aznar, 2015; Hill, 2015; Stockman *et al.*, 2018).

Surface plasmons can be grouped into two main types: propagating surface plasmons either referred to as surface plasmon polaritons (SPPs) or Surface plasmon resonance (SPR) and LSPR when excitations of SPs are on sub-wavelength-sized metal nanoparticles (Amendola *et al.*, 2017; Stockman *et al.*, 2018). Hence, plasmonic biosensors can also be classified into two types of sensing platforms: SPR and LSPR biosensors. SPR biosensors are referred to as "gold standards" for characterising biomolecules interactions. Most optical biosensors of this nature are based on SPR changes in thin films. LSPR on the other hand, uses individual inorganic plasmon resonant nanostructures that are significantly smaller than the incident light wavelength and exhibit a collective oscillation of free surface electrons in response to the oscillating electromagnetic field of the light (Hill, 2015; Sepúlveda *et al.*, 2009; Soler *et al.*, 2014).

LSPR sensing platforms are more adaptable to multiplexing and miniaturisation compared to thin film SPR sensing platforms because of their nanoscales materials signal generation. Thus, LSPR plasmonic sensors lend themselves for incorporation into miniaturised lab-on-a-chip or point-of-care biosensor devices (Amendola *et al.*, 2017; Aznar, 2015; Lim & Gao, 2016). They can also be tuned by altering the size, shape and composition of nanoparticles used (Guo *et al.*, 2015). This makes them more advantageous for use in biosensing as the detection system can be tuned to use wavelengths that do not overlap with the spectral features of strongly absorbing naturally occurring biological chromophores, such as haemoglobin. This improves the sensitivity of the detection system in biological samples for target analytes (Hill, 2015).

Park *et al*. demonstrated the promising potential that AuNSs have as nanoprobes for plasmonic biosensing. They observed that the multiple branched tip at the end AuNSs help in concentrating electromagnetic fields more efficiently in comparison to spherical nanoparticles. This enables AuNSs to generate larger plasmonic spectral shifts upon molecular binding. Three key aspects to improve sensitivity in biosensing with AuNSs were suggested, namely (i) covalently coat the surface of AuNSs to maintain particle morphology and its resonance spectra; (ii) cluster the AuNSs to more strongly concentrate electromagnetic energy; and (iii) finding strategies to minimise the interparticle distance in AuNSs clusters. The assay they developed based on these strategies was reported to be simple, fast, robust and easy readout in colorimetric format (Park *et al.*, 2015).

2.6.1 LSPR-based nanobiosensors

Optical sensors have been fabricated using nanostructured metals instead of thin films. These have been expected to offer some advantageous solutions to the unmet needs for label-free and high-throughput analysis in medical diagnosis. Metal nanostructures have attractive optical

properties due to their LSPR (Aldewachi *et al.*, 2018; Saha *et al.*, 2012). Typical plasmonic materials are noble metals, especially silver or gold. Gold has had a preferential advantage over silver due to its higher chemical stability, although it has been noted that silver exhibits more intense and sharper bands than gold. Thus, bringing the two noble metals in signal generation in LSPR-based nanosensors has been observed to increase the sensitivity of the sensors (Guo *et al.*, 2016; Rodriguez-Lorenzo *et al.*, 2012; Sepúlveda *et al.*, 2009). Besides silver or gold, other metals or metal oxide nanostructures such as zinc oxide, aluminium, and copper have been used in the fabrication of plasmonic nanosensors. The drawback for these non-noble metals is their susceptibility to corrosion in aqueous environments and oxidation in air, consequently diminishing their sensitivity to the RI significantly (Guo *et al.*, 2015).

An interaction between a beam of light and metal nanostructures results in part of the incident photons being absorbed and part of it being scattered in different directions. These absorptions and scatterings are enhanced when the LSPR is excited. This makes optical spectroscopy the simplest technique to measure the LSPR on metal nanostructures since it is generally based on extinction or scattering measurements (Amendola *et al.*, 2017; Nadeau, 2016; Saha *et al.*, 2012). Extinction is mostly used in the characterisation of solutions containing a large concentration of nanostructures, e.g. colloidal nanoparticles. Scattering measurements on the other hand present much lower signal-to-noise ratio compared to extinction (Haiss *et al.*, 2007; Khlebtsov, 2008; Liu *et al.*, 2007). Consequently, scattering detection is employed in samples with low density of nanostructures, enabling single nano-objects characterisation optically (Sepúlveda *et al.*, 2009).

Plasmonic nanoparticles are widely used as optical labels in sensors in a similar fashion as fluorophores. This is made possible by the straightforward manner of conjugating biomolecules of interest to gold nanoparticles. The LSPRs of plasmonic nanostructures are superior to those of commonly used fluorophores due to the extreme large scattering cross-sections produced (Hill, 2015). LSPR sensors can be divided in two types based on the origin of LSPR changes. These include sensors that are based on LSPR coupling and refractive index sensors (Figure 2-4) (Guo *et al.*, 2015; Sepúlveda *et al.*, 2009).

2.6.1.1 LSPR coupling

Colloidal aggregation is the basis of label-free colorimetric assays that are extremely sensitive and enable naked-eye visualisation (Sepúlveda *et al.*, 2009). The binding of the target of interest on the surface of plasmonic NPs can induce aggregation of the NPs. This results in inter-particle surface plasmon coupling. The end result of this is a visible colour change of the NP colloidal solution from red to blue (in the case of AuNPs) (Amendola *et al.*, 2017). Numerous

colorimetric sensors based on this strategy have been developed in the last two decades. Their applications have ranged from the detection of metal ions, small organic molecules, proteins, DNA and living cells (Aldewachi *et al.*, 2018; Amendola *et al.*, 2017).

Using this mechanism of LPSR sensing, DNA-mediated aggregation of AuNPs has been used to develop biosensors for DNA genetic mutation detection. This has significant implications in the area of early disease diagnosis, especially cancer (Cairns, 2007). The signal generation approach was via the formation of double-stranded DNA from two single-stranded DNA molecules in the presence of a complementary oligonucleotide of interest. This led to the aggregation of AuNPs and a consequent colour change in the solution. This strategy proved to be selective, sensitive and low-cost for DNA detection, with easy readouts by both naked-eye and using a UV-vis-NIR spectrophotometer (Zhao *et al.*, 2008).

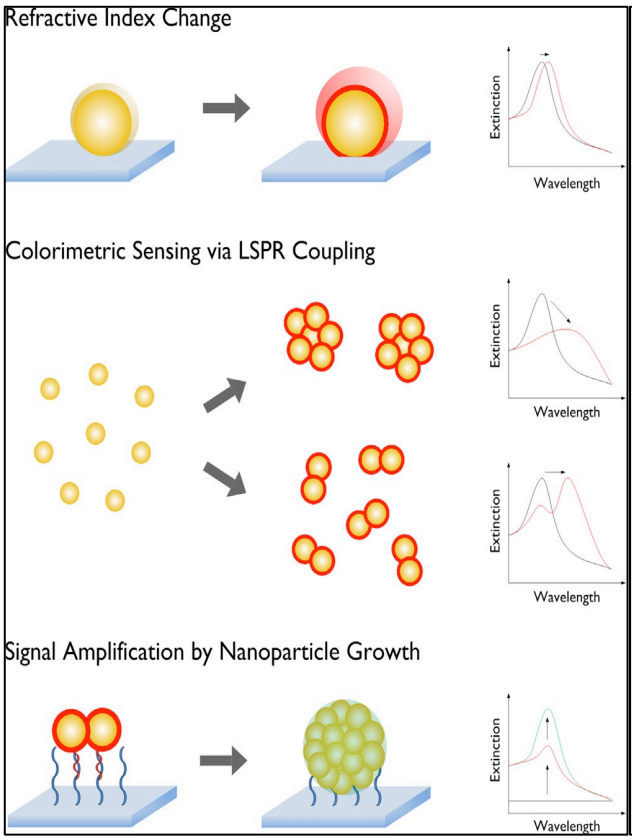
Colorimetric assays for protein and small organic molecules detection of clinical interest have been developed based AuNPs aggregation. For proteins, Au nanostructures have been functionalised with specific antibodies that trigger NPs aggregation upon binding with target antigen. This results in colloidal solution colour change (Amendola *et al.*, 2017). Click chemistry has also been used in a plasmonic based immunoassay to cause aggregation in AuNPs by the reduced Cu (I) (Xianyu *et al.*, 2014). Naked-eye detection of glucose in urine was achieved via immobilised glucose oxidase on AuNPs. On addition of varying concentrations of glucose (from 10 to 100 µL), there was a shift in the LSPR absorption band reflecting the formation of AuNPs aggregates (Radhakumary & Sreenivasan, 2011). Sensitive LSPR coupling-based sensors for the detection of metal ions, such as $Pb^{2+}$and Hg^{2+}, have also been developed. These are reported to be simple, sensitive and rapid in detection based on aggregation of AuNPs upon interaction with these metal ions (Ding *et al.*, 2012; Feng *et al.*, 2018; Gao *et al.*, 2014). Although LSPR coupling through nanoparticle aggregation is the most commonly employed strategy for sensing, it lacks specificity in signal generation as many other factors in solution may cause particle aggregation (Jiang *et al.*, 2010; Radhakumary & Sreenivasan, 2011; Xianyu & Jiang, 2014).

2.6.1.2 LSPR shifts in refractive index

LSPR shift sensing is another strategy for detection and signal enhancement. The basis of the signal generation is the local change in the refractive index of the medium surrounding the NP surface (Amendola *et al.*, 2017). Generally, these sensors are less sensitive compared to the aggregation assays, but they have the advantage of a wide range of functionalisation chemistries that leverage the existing knowledge for conventional LSPR biosensors for greater sensing (Sepúlveda *et al.*, 2009).

The refractive index sensitivity of the plasmonic nanoparticles is largely dependent on their size and shape. Nanospheres or quasi-spherical nanoparticles were the most common constituents of early plasmonic nanosensors. Recent theoretical simulations and experimental data have shown that anisotropic nanoparticles display higher refractive index sensitivity due their high aspect ratios (Aldewachi *et al.*, 2018; Lim & Gao, 2016; Priecel *et al.*, 2016; Rodriguez-Lorenzo *et al.*, 2012). Gold nanorods have turned out to be a favourite in this vein due to the relatively mature synthesis methods and higher yields compared to the anisotropic nanoparticles. Additionally, the typical refractive index shift is approximately 250 nm per unit, compared to ~60 nm of spherical nanoparticles. Other anisotropic nanoparticles investigated for LSPR shift sensing include nanocubes, nanoshells, nanodiscs, nanotriangles, nanopyramids, nanorice, nanoholes, nanocrescents and nanostars (Guo *et al.*, 2015; Li *et al.*, 2014; Rodriguez-Lorenzo *et al.*, 2012; Sepúlveda *et al.*, 2009).

The early tests were performed on the well-known biotin-streptavidin model couple, where biotin was covalently attached to AuNPs already immobilised on a glass surface. The UV-vis-NIR spectrophotometer and a colorimetric end-point assay using an optical scanner were used for detection and quantitation of the biomolecular interactions in real time (Nath & Chilkoti, 2002). Further optimisation of the size of the gold nanoparticles led to an improved detection limit of streptavidin of 0.83 nM (Nath & Chilkoti, 2004). Recent years have seen the advancement of these LSPR sensors by use of gold nanostars and immobilisation of enzymes and/or antibodies for substrate and protein detections (Guo *et al.*, 2016; Rodriguez-Lorenzo *et al.*, 2012; Zhou *et al.*, 2009). Immunoassays developed based on this principle have demonstrated a signal enhancement of several orders of magnitude in comparison to the conventional propagating SPR sensors (Amendola *et al.*, 2017).



Category	Strategies for enhancing sensitivity
Refractive index-based sensing	Composition
	Size
	Shape
	Increased molecular mass
	3D assembled nanostructures
	Plasmonic-molecular resonance coupling Sensing with single nanoparticles
Sensing based on plasmon coupling	Increased aggregate size
	Minimized interparticle distance
	To detect the scattering signals of plasmon coupling
Sensing based on nanoparticle growth	Scanometric array detection coupled with silver enhancement Biocatalytic enlargement of AuNP

Figure 2-4 Illustrations of different strategies to improve the sensitivity of signal generation and amplification in plasmonic nanosensors (Guo *et al.*, 2015).

2.7 Enzymes in Nanobiosensors

Enzyme interaction with nanomaterials has seen considerable progress in less than a decade due to the rapid development of nanotechnology. The demand to bring together nanoparticles and enzymes for applications in biomedicine, biomedical engineering, nanoparticle design, and biosensors, among others, continues to increase. Recently, there has been a surge in enzymatic modification or degradation of nanoparticles, and immobilisation of enzymes on nanoparticles for biosensor applications (Chen *et al.*, 2017; Min & Yoo, 2014). Demand for increased sensitivity and selectivity in nanosensors requires significant interaction between biorecognition molecules and nanoparticles. Nanobiosensors are reputed to be reliable, sensitive, accurate, easy to handle and low cost compared to traditional assay methods. Enzymes, on the other hand, are ideal recognition elements due to their high selectivity and specificity. Therefore, combining the advantageous properties of both enzymes and nanobiosensors makes enzyme-based biosensors ideal for biomedical analysis with great promise (Alharbi & Al-Sheikh, 2014; Hemalatha *et al.*, 2013; Riehemann *et al.*, 2009a).

2.7.1 Nature and kinetics of enzymes

Enzymes are biological catalysts that increase the rate of chemical reactions within living cells without being consumed or permanently altered in the process (Raja *et al.*, 2011). Some enzymes are proteins that require a non-protein component called a cofactor for catalytic activity (Palmer & Bonner, 2007). They usually contain one or more active sites which are specific for particular substrates. The active sites are relatively small compared to the total volume of the whole enzyme structure. They attract the substrate molecules using physical forces such as electrostatic and hydrophobic interactions, hydrogen binding, and van der Waals forces. (Burtis *et al.*, 2012). They are efficient in their catalytic activity as a low concentration of enzymes converts an enormous amount of substrate molecules to products within a short period of time. The mechanism of this catalytic reaction is by increasing the rate at which the equilibrium is established between reactants and products (Raja *et al.*, 2011). Enzymes also direct the reaction to only one pathway in a reaction where several possible pathways are possible (Burtis *et al.*, 2012).

Enzyme kinetics refers to the rate of the enzyme-catalysed reactions. The kinetic description of enzymes helps in understanding how they function (Berg *et al.*, 2002). A number of factors affect the kinetics of enzymes and these include enzyme and substrate concentration, pH, temperature and the presence of inhibitors, activators, prosthetic groups and co-enzymes (Burtis *et al.*, 2012). The reaction rate is generally directly proportional to the concentration of enzyme present in the system. This is used clinically to quantitatively determine enzyme concentration by measuring the rate of reaction in a reaction system (Burtis *et al.*, 2012). When the enzyme concentration in a reaction system is fixed and the substrate concentration is varied, the rate of the reaction is dependent on the concentration of substrate at low values during the first order reaction (Burtis *et al.*, 2012). The rate of reaction of enzyme catalysis is also dependent on pH (Garrett & Grisham, 2010). Enzymes being proteins possess ionisable side chains and prosthetic groups that determine their secondary and tertiary structures and these are affected by pH. This maybe to such an extent that the enzyme experiences irreversible damage at extreme pH values (Burtis *et al.*, 2012). The effects of pH necessitate the use of buffer solutions to control the variation caused by change in pH on the rate of reaction. Temperature affects enzyme-catalysed reactions by increasing the rate with rise in temperature (Garrett & Grisham, 2010). As temperature rises there is a corresponding characteristic increase in enzyme activity. However, at temperatures above 50° and 60°C there is a decline in enzymatic activity due to thermal denaturation of the protein structure of the enzymes at higher temperatures (Garrett & Grisham, 2010).

The rate of reaction of enzyme-catalysed reactions can be decreased or inhibited by inhibitors. Activators on the other hand increase the rate of reaction of the enzyme (Burtis *et al.*, 2012; Garrett & Grisham, 2010). Enzyme inhibition can be both reversible and irreversible (Garrett & Grisham, 2010). Reversible inhibitors are those that bind to the enzyme in noncovalent manner. This implies that the enzyme activity can be fully restored by the removal of the inhibitor from the system of the enzymatic reaction. In contrast, irreversible inhibitors bind covalently to the enzyme leading to a decrease to the concentration of active enzymes in a reaction system (Garrett & Grisham, 2010). Activators are molecules that increase the rates of enzyme-catalysed reaction. The mechanism for this is that the molecules promote the formation of an active state of the enzyme or of other reactants such as the substrate (Burtis *et al.*, 2012). Metal ions form an integral part of the structure for many enzymes. The function of these metals maybe to stabilise the structures of the proteins. Common activating cations are Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, and K^{+}. For anions, mostly it's the monovalent ones that help in the activation of some enzymes such as amylase. Examples of these are Cl^{-}, Br^{-}, and NO_3^{-} (Burtis *et al.*, 2012). Coenzymes, being more complex molecules than activators, affect the rate of reaction in a similar way as the substrate concentration does. It follows a similar Michaelis-Menten pattern of dependence on substrate concentration as they bind momentarily to the enzyme during the course of reaction, similar to how substrates bind (Burtis *et al.*, 2012). Examples of coenzymes are dinucleotides NAD and NADP whose structures are identical except for the additional phosphate group in NADP.

2.7.2 Applications of enzymes in nanobiosensors

Enzymatic nanobiosensors operate on the basis of the relationship between an enzyme and its substrate (Asal *et al.*, 2018). Depending on the analyte of interest, enzymes have been applied in these biosensors as indicators of enzyme activity or concentrations, substrate detection or enzyme inhibition (Asal *et al.*, 2018; Ha & Bhagavan, 2011).

2.7.2.1 Enzymes as biomarkers and label molecules

The rate of an enzyme-catalysed reaction is directly proportional to the concentration of active enzymes present in a system. Therefore, a sensitive and specific way of measuring concentration of enzymes in a sample is based on the rate of reaction under defined and controlled conditions (Nader *et al.*, 2018). Using this principle, a number of nanosensors have been developed. Recently, Guo *et al.*, 2016 report designing a novel biosensor for plasmonic colorimetric detection of ALP. ALP determination was achieved by means of ALP-catalysed dephosphorylation and the controllable silver ion reduction by ascorbic acid in the presence of ascorbic acid 2-phosphate and gold nanostars. ALP catalysed the dephosphorylation of

ascorbic acid 2-phosphate, resulting in ascorbic acid which reduced the silver ions to coat the gold nanostars. The controlled reduction of silver ions was achieved by optimising the concentration of the Ag^+ that was added to the solution (Guo *et al.*, 2016). A lipase activity plasmonic nanosensor was developed based on in situ enzyme-guided growth of gold nanoparticles. Tween 80 was used as a substrate for lipase recognition, as well as a reducing agent of $HAuCl_4$ in situ for AuNPs synthesis. Detection was determined on AuNPs colour changes based on the rate of Au nucleation in the presence or absence of lipase. They report achieving a linear range of 0.025 – 4 mg/mL and detection limit of lipase of 3.47 µg/mL (Tang *et al.*, 2015).

Plasmonic immunoassay nanosensors have also been developed based on the same principle. Xianyu and colleagues reported an effort to come with a plasmonic nanosensor strategy for an ELISA that could directly imposed on the conventional ELISA platform (Xianyu *et al.*, 2014). An enzyme-triggered Cu(I)–catalysed azide/alkyne cycloaddition (CuAAC) was used to integrate the signal generation, signal transduction, and signal amplification simultaneously for plasmonic ELISA. ALP was used to dephosphorylate ascorbic acid 2-phosphate. The desphosphorylated ascorbic acid acted as a reducing agent of Cu(II) to Cu(I) which led to the aggregation of azide/alkyne biofunctionalised gold nanoparticles, resulting in a colour change from red to blue. The nanosensor was reported to be very sensitive, stable, robust and had an easy readout with naked-eye detection compared to the conventional ELISA (Xianyu *et al.*, 2014). Likewise, an ultrasensitive detection of disease biomarkers in plasmonic ELISA is reported (De La Rica & Stevens, 2012). Motivated by lack of affordable methodologies for the detection of ultralow concentrations of disease biomarkers in resource-constrained settings, a signal detection mechanism was devised based the enzyme labelled of an ELISA that controlled the growth of AuNPs resulting in colour changes with distinct tonality. Ultralow concentrations of 1×10^{-18} g/mL for prostate specific antigen (PSA) and HIV-1 capsid antigen p24 were detected in whole serum (De La Rica & Stevens, 2012; Rodríguez-Lorenzo *et al.*, 2012). Other researcher have also reported similar plasmonic nanosensors development where enzyme activity was the basis of detection have been reported (Liu *et al.*, 2014; Liu *et al.*, 2016). A more comprehensive review on nanoparticle-based immunochemical biosensors and assays is given by Farka *et al*., (Farka *et al.*, 2017).

2.7.2.2 Enzymes as biorecognition elements

Enzymes have also been used as analytical reagents for detection and quantification of several substrates and metabolites. Using enzymes for this purpose has the advantage of specificity for the substance being determined. Another advantaged derived from the high specificity for particular substrates is the elimination of the need for sample clean-up or purification. The

analysis can be carried out directly on complex sample matrices such as serum. Substrate determination is either based on equilibrium or kinetic methods (Nader *et al.*, 2018). Equilibrium methods allow a reaction to go to completion so that most of the substrate is converted into a measurable amount of product. Kinetic methods determine the change in substrate concentration over a fixed time interval. The most important reactions for kinetic determination of the substrate concentration are the first-order or pseudo-first-order reactions. It is therefore necessary to have the substrate concentration lower in comparison to the Michaelis constant (K_m) of the enzyme for the reaction to be first-order (Hemalatha *et al.*, 2013; Nader *et al.*, 2018; Palmer & Bonner, 2007; Raja *et al.*, 2011).

Nanosensors have used enzymes as biorecognition components to quantify substrates based on the reaction products (Asal *et al.*, 2018; Das *et al.*, 2016). Enzyme-substrate nanosensors are one of the most reported in literature (Ali *et al.*, 2017). Using this approach, Zayats *et al.*, designed a glucose biosensor using glucose oxidase that generated H_2O_2 which in turn enlarged the AuNPs with the resultant optical signal generation. This was hoped would be a model for further extension to other oxidases to sense their respective substrates (Zayats *et al.*, 2005). The subsequent years after this publication has seen an increase in the number of glucose nanobiosensors with increased sensitivities and lower detection limits (He *et al.*, 2016; Li *et al.*, 2007; Lim *et al.*, 2010; Radhakumary & Sreenivasan, 2011; Sabu *et al.*, 2019; Xia *et al.*, 2013; Xianyu & Jiang, 2014; Xiong *et al.*, 2015; Yi *et al.*, 2013; Zhang *et al.*, 2015). Similar biosensors have been developed for substrates such as cholesterol, galactose, creatinine, and triglycerides, beside others (Arya *et al.*, 2006; Gholivand & Khodadadian, 2014; Hooda *et al.*, 2018; Kumar *et al.*, 2017; Miao *et al.*, 2017; Mohammadi & Khayatian, 2015; Narwal *et al.*, 2018; Saxena *et al.*, 2011; Saxena & Das, 2016; Sharma & Leblanc, 2017; Zhang *et al.*, 2012).

2.7.2.3 Enzymes as means for nanocrystal signal generation

Enzymes have previously been used as biocatalysts for the enlargement of metallic nanoparticles. This led to nanoparticles to be applied as optical labels for the detection of biorecognition events such as antigen-antibody complex formation and enzyme-based biosensors (Guo *et al.*, 2016; Rodríguez-Lorenzo *et al.*, 2012; Zayats *et al.*, 2005). The purpose of enzymes in these different biosensors is to catalyse the reduction of metal ions on the AuNPs and thereby enlarge the metallic NPs. This catalytic enlargement of AuNPs is used for the amplification of the biorecognition signal in biosensors (Zayats *et al.*, 2005). This development of enzyme-catalysed enlargement of AuNPs led to a new sensitive enzyme and enzyme-linked immunoassays and to novel biosensor configurations that employ optical readout signals (Guo *et al.*, 2016; Liu *et al.*, 2014; Rodríguez-Lorenzo *et al.*, 2012; Willner *et al.*, 2006; Xianyu *et al.*, 2014; Zayats *et al.*, 2005).

The enzyme-guided crystal growth of metallic NPs was developed further by Rodríguez-Lorenzo *et al.*, 2012, in their development of a plasmonic sensor with 'inverse sensitivity'. They report using the enzyme glucose oxidase (GOx) to generate H_2O_2 that reduced silver ions around gold nanosensors, in their case, gold nanostars. The gold nanostars were preferred for use due to their high-aspect-ratio spikes that localise the low-energy plasmon mode at their tips, resulting in a dominant LSPR peak in the near-infrared region. The silver ions either formed a silver coating around the nanostars causing a great blue shift in LSPR, or nucleation of silver nanocrystals in solution with a resulting smaller blue shift in the LSPR. The epitaxial growth of nanostars or nucleation of silver nanocrystals was seen to be dependent on the concentration of the GOx. Thus, the lower concentration of the enzyme led to the former and higher concentration to the latter. This phenomenon was applied as a universal tool for biosensing in a classical ELISA. As the concentration of GOx is directly proportional to the concentration of the analyte through immunoreaction, the presence of the target molecule at low concentrations was expected to yield a larger variation of the optical properties of the nanostars by means of the formation of the silver coating. This inverse relationship between concentration and signal generated provided the basis for the development of ultrasensitive sensors for very low concentrations compounds (De La Rica & Stevens, 2012; Rodriguez-Lorenzo *et al.*, 2012).

The setbacks to this, however, are firstly; the formation of the silver nanocrystals in solution leads to inverse response thereby limiting the detectable concentration range of analytes in high concentrations in biological fluids. Secondly; the nanosensor is very sensitive to changes in solution parameters such pH, concentration of precursors and presence of capping agents which have far reaching impacts on the size, morphology, crystallinity and degree of aggregation of nanocrystals. All of these are essential factors that can affect the response of the transducer and limit its practical application (Guo *et al.*, 2016; Rodriguez-Lorenzo *et al.*, 2012). Guo *et al.* reported an optimisation of this that excluded the nucleation of silver nanocrystals reported previously Based on this model, the detection of analytes in a wide range of concentrations was achievable by naked eye (Guo *et al.*, 2016). Optimal immobilisation of enzymes to nanoparticles is another additional factor in improving signal generation via enzyme guided growth of nanoparticles. Yan and co-workers demonstrated how the immobilisation of glucose oxidase onto the nanoparticles for biocatalytic growth of AuNPs enhanced the ferrocene-mediated bioelectrocatalytic oxidation (Yan *et al.*, 2008). Immobilisation is reported to improve the performance, heat stability and functionality of the enzyme (Ahmad & Sardar, 2015; Li *et al.*, 2007; Pandey *et al.*, 2007).

2.7.3 Immobilisation of enzymes in nanobiosensors

For enzyme-based biosensors, gold nanoparticles provide a user-friendly and efficient surface for the immobilising enzymes. Colloidal gold has high biocompatibility and surface energy that enables enzymes to retain their bioactivity, increased enzyme loading and prevents protein denaturation. Enzyme modification with colloidal gold nanoparticles enables the attached enzyme to have more freedom of orientation by providing some distance between the particles and the enzyme, making it less likely to have the active site covered. Nanoparticles also offer a high surface-volume ratio due to their sizes, giving the advantage of more efficient enzyme loading (Putzbach & Ronkainen, 2013). The two main methods of enzyme immobilisation on gold nanoparticles are physical and chemical adsorption. Physical methods employ weak interactions between the matrix and enzyme, while in chemical methods, covalent bonds are formed between the enzyme and matrix (Ahmad & Sardar, 2015).

2.7.3.1 Physical adsorption

This is a quick and simple technique for attaching enzymes to enzymes in biosensors through weak bonds that include electrostatic interactions, van der Waals forces and hydrogen bond interactions. It involves the reduction of Au nanostructures with a negatively charged ligand which insulates them from electrostatic repulsion, thereby offering stability. The resulting negative surface charge of the nanostructure allows positively charged amino acid residues on the enzymes to be electrostatically absorbed on the surface (Figure 2-5). Despite the benefits of speed and simplicity, this method has some disadvantages such as undesirable orientations of enzymes, covering of the active site and decreased functionality (Putzbach & Ronkainen, 2013).

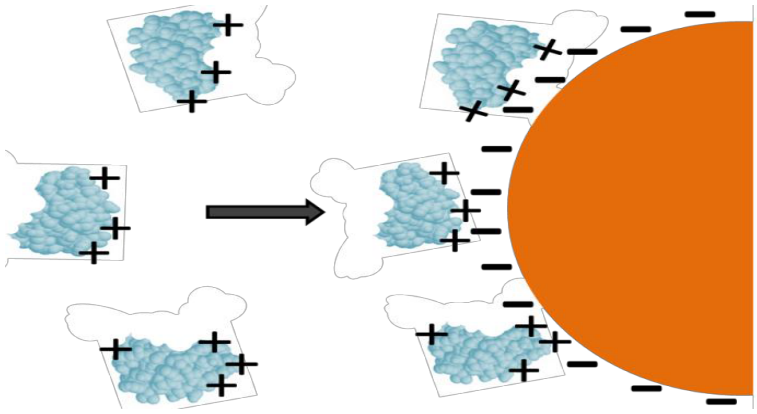


Figure 2-5 Electrostatic adsorption of enzymes directly onto gold nanoparticles (Putzbach & Ronkainen, 2013).

2.7.3.2 Chemical adsorption

This method of enzyme immobilisation involves direct covalent binding between the enzyme and the colloidal gold surface. Direct conjugation of enzymes to nanoparticles for covalent binding utilises the functional groups found on most of the naturally occurring biomolecules. Amino acids residues tend to be targeted for conjugation in proteins and peptides (Ahmad & Sardar, 2015; Sapsford *et al.*, 2013). The ε-amino group on lysine residues on the enzymes is mostly used because those numbers are relatively large and they have a simple chemical derivatization (Putzbach & Ronkainen, 2013). Cysteine has thiol groups that exist as a disulphide which enables efficient attachment to nanoparticles (Muguruma, 2017; Putzbach & Ronkainen, 2013). Others are N-terminal primary amine; the guanidinium group on arginine side chains; the carboxyl groups on aspartic acid, glutamic acid, or the C-terminus. Alternative reaction sites are the hydroxyl groups or aldehydes present in carbohydrates and glycosylated proteins (Sapsford *et al.*, 2013).

Another way of immobilisation of enzymes to colloidal gold nanostructures can be achieved using linker molecules of different lengths. The linker molecule gives the enzyme greater mobility, thereby enhancing its bioactivity as illustrated in Figure 2-6, compared to that of a directly coupled immobilisation (Ahmad & Sardar, 2015). Enzymes may be modified to have reactive groups that are useful for conjugation with appropriately functionalised gold nanoparticles. To accomplish this, homobifunctional or heterobifunctional linker molecules are used to covalently couple some chemical target group on the enzyme and a residue terminal reactive group that can crosslink with the matrix (Hermanson, 2013). Glutaraldehyde is one of the most commonly used homobifunctional crosslinkers that contains an aldehyde group at both ends of a 5-carbon chain. It primarily reacts with amine groups with more than one mechanism of reaction. It can crosslink two molecules with amine groups and form stable bonds (Barbosa *et al.*, 2014; Betancor *et al.*, 2006; Hermanson, 2013). Another most popular crosslinker is (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). It is probably the most frequently used crosslinking agent of all. It is used for conjugating biological substances containing carboxylates and amines. It is mostly used along with *N*-hydroxysulfosuccinimide (NHS) or sulfo-NHS in particle and surface conjugation procedures (Hermanson, 2013). These two crosslinkers account for most of the covalent enzyme-nanogold conjugation procedures encountered in literature (Ahmad & Sardar, 2015; Ding *et al.*, 2015).

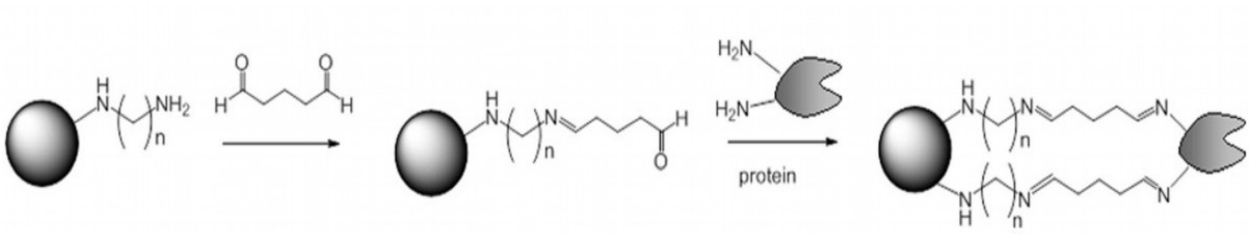


Figure 2-6 Covalent attachment of enzymes to nanoparticles using glutaraldehyde as a linker molecule (Cantone et al., 2013).

The ideal characteristics desired for immobilised enzymes onto nanoparticles are well-delineated by Sapsford and colleagues (Sapsford *et al.*, 2013). Six generalised criteria for attaching a biomolecule to nanoparticles are described that could confer control on the following properties: 1) A high ratio of enzymes per nanoparticle to increase binding and interaction with target analyte. 2) Control over orientation of the enzyme attached to the nanoparticle so that the active site is uncovered. 3) Control over relative separation distance between the enzyme and the nanoparticle. 4) Control over attachment affinity of bioconjugates. 5) Maintenance of optimal function and activity of both the enzyme and nanoparticles. 6) Lastly, the ability to be reproduced in a facile manner with other biomolecules to be immobilised. However, they reported in their comprehensive overview that most reviewed bioconjugation methods failed to meet most of the ideal characteristics (Sapsford *et al.*, 2013). Additionally, one of the main limitations of implementing nanoparticles in clinical use is related to biofunctionalization of biorecognition molecules. There is a need to design and optimise the strategies for enzyme immobilisation to maximise assay sensitivity, selectivity, reproducibility and stability in biological samples. Thus, enzyme orientation control, minimising random chemical modification and non-specific adsorptions during immobilisation on gold nanoparticles are critical (Aznar, 2015; Ding *et al.*, 2015; Guo *et al.*, 2015).

2.8 Literature summary and perspectives

Nanobiosensors are a promising alternative to traditional techniques used in clinical diagnosis. This is even more relevant in developing countries where access to state-of-the-art laboratory equipment is not widespread and yet have high burdens of diseases. Properties such as simplicity and cost-effectiveness in fabrication, sensitivity in signal transduction, as well as an easy optical readout format only utilising basic entry level spectrophotometer make them an attractive option in such settings. However, there is paucity of literature as to the development of nanotechnology-based assays to address some of the diagnostic challenges in Africa. Diagnostic devices for developing countries should meet the prescribed WHO standards which

is ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end-users (Peeling *et al.*, 2006). There is a clear need for continued efforts in the development of miniaturised detection platforms with improved specificity and sensitivities to meet these standards (Abbas *et al.*, 2017; Hauck *et al.*, 2010; Martinez *et al.*, 2009). Nanotechnology and nanobiosensors offer an opportunity and platform for the developing of such diagnostic devices.

The recent trend has been to use anisotropic nanostructures as a way to tune the LSPR properties of the nanosensors for greater sensitivity. For this reason, AuNSs have been seen as the nanosensors of choice for signal transduction. The multi-branched nanoparticles possess excellent optical physiochemical properties for plasmonic sensing. They also provide a larger surface area for enzyme immobilisation with the potential for a higher load of enzymes per nanoparticle compared to smaller nanospheres. Several synthetic approaches for AuNSs has been published and reviewed. However, the choice of synthesis method depends on a number of experimental parameters and downstream application. Thus, there are still gaps for methods that are appropriate, simple and produce AuNSs suited to their intended purposes.

One of the main limitations for nanobiosensors being implemented in clinical use is related to biofunctionalisation of biorecognition molecules. Maintaining the stability of functionalised gold nanoparticles with biomolecules to preserve the functionality of these molecules is a challenge. The robustness of the whole system to analyse biological samples is another issue. Moreover, most of the reviewed bioconjugation strategies fail to meet most, if not any of the characteristics of ideal bioconjugation criteria. There is thus need to design and optimise the strategies for enzyme immobilisation to maximise assay sensitivity, selectivity, reproducibility and stability in biological samples. Parameters such as enzyme orientation control, minimising random chemical modification and non-specific adsorptions during immobilisation on gold nanoparticles need to be critically addressed.

Despite the progress made on the design of novel plasmonic colorimetric biosensors based on biocatalytic enlargement of metallic NPs, a significant challenge remains for their practical application in clinical samples. Biological samples have a complex matrix that affects the physiochemical properties of the nanoparticles in them, resulting in suboptimal signal transduction. Potential for undesirable side reactions and interferences is high in biological samples. Hence the need to optimise the optical NBS to be stable, sensitive and robust enough for detection using clinical samples. The expansion of the modelled catalytic growth of metallic nanoparticles using H_2O_2 for other oxidase-based biosensors that could make a panel of assays remains to be done. For example, there is paucity of literature on optical biosensors with nanoplasmonic detection for cholesterol.

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CHAPTER 3 AIM AND OBJECTIVES WITH STUDY DESIGNS

3.1 Introduction

Nanotechnology is an emerging powerful technology that promises substantial impact on medical diagnosis. It enables the fabrication of optical assays for analysis of bioanalytes that are cheap and use simple instruments. The technology is an alternative to the more expensive approach in which sophisticated instrumentation such as chromatography/mass spectrometry, direct mass spectrometry, DNA-based assays, enzyme activity assays, immunoassays and microfluidics for detection which are expensive and largely inaccessible to the bulk of the population in developing countries. The development of nanotechnology-based assays would offer simple procedures that could be performed in areas without sophisticated instrumentations, bringing radical improvement in the healthcare management in such regions. The need for continued efforts in development of such methods that will be sensitive and robust is well-established.

3.2 Aim and objectives

Therefore, the aim of this study was to develop and optimise nanotechnology-based optical assays with a view to optimise various components of assay fabrication stages for potential clinical diagnostic applications.

The specific objectives to achieve this aim included:

1. Selection of nanosensors that would be sensitive in signal transduction and afford a platform for easy and optimal immobilisation of biorecognition molecules for biosensing applications.

2. Design synthesis strategies that would produce nanosensors of choice in a facile and reproducible manner for application in biosensors.

3. Assessment of a detection system based on shape and size tuning of the gold nanosensors for enzyme related products.

4. Design simple and sensitive biosensor based on nanoparticle detection as a proof of concept for universal detection.

5. Assessment and optimisation of bioconjugation strategies that enhance stability, simplicity, and sensitivity in signal transduction.

6. Design strategies to minimise and prevent interferences in complex sample matrices such as serum.

3.3 Scope and substantiation

It is clear from the literature survey that there is still much room for concerted efforts in research and development of cheaper diagnostic technologies that have the potential to improve the healthcare delivery in resource-constrained settings. The research entity in which these studies were to be conducted (Centre for Human Metabolomics) mainly focuses on small molecule analysis using high-end equipment such as GCxGC-TOFMS, GC-TOFMS, GC-MS, LC-MS, NMR, and others besides. Sadly, much of these modalities are inaccessible to the bulk of the South African and African population. The desire, therefore, has been to design and develop cheaper technologies to help make diagnostic services more accessible even to the poorest populations in the remote areas. Hence, our initiative to explore nanotechnology as a technological medium to pursue this objective.

Nanotechnology when applied to medical diagnostics offers a technological opportunity and platform to develop biosensors for biomedical applications. AuNPs provide an excellent scaffold for the development of optical biosensors due to their simple synthesis, easy functionalisation, and ability to display different colours depending on their size, shape and dispersion state. Using these NPs, optical biosensors can be developed which are more appropriate for resource constraint regions. Such biosensors are easier to fabricate, have easy readout formats such as naked eye detection based on colorimetric changes and optical changes using a UV-vis-NIR spectroscopy. The use of ubiquitous UV-vis-NIR spectrophotometry is advantageous as it is cost-effective, easier to maintain as the sample is never in contact with the analyser and doesn't require special laboratory facilities, or specialised trained personnel to operate. Colorimetric readouts also offer suitable platforms for technology transfer to point-of-care technologies and lateral flow devices for user-friendliness.

Different morphologies and compositions of Au nanostructures have been researched to improve the sensitivity of LSPR-based sensing. Based on the literature review on the comparative advantages between AuNPs and AuNSs for improved sensitivity in biosensing, the multibranched AuNSs were purposely chosen as nanosensors for further development, based on the following reasons; (i) well-established facile methods of synthesis in aqueous nontoxic solutions, yet with room for optimisations, (ii) minor modifications of their morphologies/size would result in changes of their LSPR properties, (iii) easily tuneable LSPR peaks within the visible and NIR spectrum, (iv) a broader range of colour changes based on manipulation of their physiochemical properties, (v) offer a larger biocompatible surface area for attachment of

biological recognition molecules that could lead to higher load of enzymes per NP compared to AuNPs.

Advances in biotechnology and protein engineering have enabled the production of enzymes at fairly acceptable commercial prices. Taking advantage of this, enzymes were used in this research and development of optimal conditions for biosensors. Enzymes, when interfaced with nanomaterials, have been reported to not only act as catalysts or biorecognition components in biosensors, but as means of signal generation through controlled growth of nanoparticles based on the reaction products. Thus, enzyme-guided growth of nanoparticles is a well-established signal generation mechanism that can be generalised for not only enzymatic assays but nanosensor-based ELISAs as well. A favourite type of enzyme for this mechanism are oxidases that generate H_2O_2 that reduces metallic nanoparticles leading to nanocrystal growth. For the purposes of optimising this strategy for further development, as well as investigating the feasibility of using it as a universal detection system for nanosensors, glucose and cholesterol were used as analytes of choice. GOx and glucose were used as a model system that offered a number of advantages such as the availability of more information, a platform for comparison with other literature findings and affordability compared to ChOx and cholesterol, or other oxidase-based analytes like creatinine or triglycerides.

The following chapters are therefore a compilation of articles that present the research carried out and how the aim and objectives within the stated scope were addressed. The order of the articles is deliberate as it follows a set pattern, starting with an investigation on the feasibility of a universal optical detection system based on nanotechnology (Chapter 4), then developing an optimal AuNSs synthesis method (Chapter 5), followed by designing a bioconjugation strategy for ideal AuNSs-enzyme bioconjugates for optimal stability and function (Chapter 6) and finally, optimising the conditions for the application of nanosensors in complex biological matrices (Chapter 7). These articles have either been submitted or accepted for publication in peer-reviewed journals.

CHAPTER 4 PLASMONIC BIOSENSORS BASED ON OXIDASES FUNCTIONALISED GOLD NANOSTARS FOR UNIVERSAL DETECTION STRATEGY

4.1 Preface

The study presented in this chapter is a result of an HEI Seed Fund call by the Technology Innovation Agency (TIA) of South Africa for simple and rapid diagnostic tools at proof-of-concept level. The aim was to develop routine laboratory test using existing chemistries but with nanosensors as detectors as opposed to the typical fluorescent or chemiluminescent sensors. Such sensing would greatly reduce the cost of measurement particularly with regards to the required infrastructure. Therefore, the chapter addressed some of the core objectives of the Ph.D. thesis such as the design of simple and sensitive biosensors based on nanoparticle detection for a universal or generalised detection system. The results of the investigations demonstrate the feasibility of the assays developed for glucose and cholesterol detection based on the use of oxidases functionalised gold nanostars.

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- The author's guidelines of this Journal can be found from this link: https://www.sajs.co.za/files/Guidelines%20for%20Authors_January%202018.pdf

4.2 Abstract

Simple, rapid and cost-effective optical biosensors are a great alternative to high-end laboratory equipment. This is more so in resource-constrained regions of the world where the development of such sensors would radically improve the healthcare delivery systems. A universal detection system based on enzyme-guided changes in gold nanostars is here presented. The detection is based on H_2O_2-mediated growth/shape-alteration of gold nanostars resulting in colorimetric and spectrophometric changes. Based on this detection mechanism, biosensors for two oxidase-based assays, glucose and cholesterol, were constructed. Both were simple in design, sensitive and rapid in detection, and overall high-throughput. Both were colorimetric and utilised a basic

entry-level laboratory spectrophotometer plate reader for analysis. Despite the need for further optimisation, the biosensors provided the feasibility of designing a universal detection platform for a number of biosensors for potential point-of-care use in developing regions.

The significance of the work are (i) the ability to design simple assays based on nanotechnology with the potential for clinical use once optimised and validated; (ii) the presentation of a universal detection system that can be developed for use in enzyme detection and immunoassays; (iii) the use of traditional and time-tested assay chemistries and only modifying the detection system; (iv) the cost-effectiveness of the proposed assays as they use ubiquitous basic laboratory spectrophotometric plate readers for analysis; (v) and lastly, the colorimetric assays are suitable for technology transfer to point-of-care and lateral flow devices, and smartphone detections.

Key words: biosensor, colorimetric, cholesterol, glucose, enzyme-guided growth, gold nanostars

4.3 Introduction

Gold nanoparticles have been used as optical transducers for the detection of biological recognition events in enzyme-based assays, immunoassays, and DNA hybridization biosensors (Hill, 2015; Touhami, 2014). Metallic nanoparticles are used in these applications because of their unique intrinsic physiochemical properties that are altered when size or shape of the particles change (Amendola *et al.*, 2017; Guo *et al.*, 2015; Langer *et al.*, 2015). Nanoparticle signal transduction has also been enabled by the use of enzymes whose products catalyse nanoparticle enlargement thereby generating a signal (Willner *et al.*, 2006). This enzyme-guided growth of gold nanoparticles has been used for the amplification of the biorecognition signal in biosensors (Guo *et al.*, 2016; Rodríguez-Lorenzo *et al.*, 2012; Willner *et al.*, 2006; Yan *et al.*, 2008; Zayats *et al.*, 2005). Different oxidases generate H_2O_2 upon oxidation of their respective substrates in the presence of molecular oxygen. The produced H_2O_2 was observed to reduce $AuCl_4^-$ in the presence of spherical gold nanoparticles (AuNPs) which also acted as catalysts in the reaction (Zayats *et al.*, 2005). Based on this approach a number of optical and electrochemical proof of concept biosensors have been designed (Guo *et al.*, 2016; Rodríguez-Lorenzo *et al.*, 2012; Willner *et al.*, 2006; Yan *et al.*, 2015; Yan *et al.*, 2008; Zayats *et al.*, 2005).

Much has been reported on such biosensor developments for glucose. Despite the progress made on the design of novel plasmonic colorimetric biosensors based on enzyme-guided enlargement of metallic nanoparticles, there still remains a gap in the expansion of the modelled mechanism to other oxidase-based biosensors. Cholesterol, in comparison, has had very few colorimetric biosensors developed for its determination (Arya *et al.*, 2006; Gahlaut *et al.*, 2018;

Narwal *et al.*, 2018). Cholesterol determination has mostly been based on detection strategies that include, among others; electrochemical (Gholivand & Khodadadian, 2014), colorimetric (Miao *et al.*, 2017), electrochemiluminescent (Zhang *et al.*, 2012), and fluorescence (Chang & Ho, 2015). The most frequently used of these strategies is electrochemistry (Arya *et al.*, 2008; Narwal *et al.*, 2018). These biosensors were used to detect cholesterol by measuring O_2 or H_2O_2 produced when the analyte is oxidised by cholesterol oxidase (ChOx). However, determination of H_2O_2 amperometrically requires high anodic potential which typically results in the simultaneous oxidation of other interfering species present in the physiological sample resulting in overestimation of the generated response, poor sensitivity electron conduction with the electrode surface, and other matrix interferences (Saxena & Das, 2016).

As an alternative detection strategy, colorimetric methods using gold nanoparticles have many advantages including being fast, simple, sensitive, and cost-effectiveness (Miao *et al.*, 2017). Gold nanostars (AuNSs) are one of the commonly used morphologies that have been investigated in biosensors (Aldewachi *et al.*, 2018; Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011). AuNSs are multibranched nanostructures with sharp tips that have very interesting plasmonic properties. Minor modifications of their shape enable the tuning of the optical properties resulting in distinct colours derived from different extinction coefficients. They are particularly sensitive as signal transducers because of the surface plasmon effects that can be tuned throughout the visible and near-infrared spectrum (Barbosa *et al.*, 2010; de Puig *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Saverot *et al.*, 2016). AuNSs crystal growth by enzyme-guided silver ions deposition for signal enhancement offers a novel approach for the development of a universal detection system for oxidases based on plasmonic colorimetric readouts that could potentially be a good alternative to other detection modalities. There is paucity of literature on simple and fast optical cholesterol biosensors based on bio-catalytic activity or shape alteration of the AuNSs.

Inspired by the simplicity and relatively low costs of such optical biosensors, and the concept of enzyme-guided crystal growth of nanosensors, the aim of this study was to develop simple and rapid colorimetric biosensors for glucose and cholesterol determination using their respective oxidases with AuNSs as signal transducers. This is an attempt to develop an approach for the biocatalytic tuning of AuNSs as a detection strategy by measuring the oxidation two different analytes catalysed by their respective oxidases. In order to do this, the feasibility of the plasmonic colorimetric assay through the H_2O_2-mediated enlargement of gold nanostars was investigated. This mechanism offered a basis for further development of glucose and cholesterol biosensors based on LSPR shifts caused by changes to the AuNSs.

4.4 Materials and methods

4.4.1 Materials

Hydrochloroauric acid ($HAuCl_4$), trisodium citrate, silver nitrate ($AgNO_3$), ascorbic acid, sodium chloride (NaCl), polyvinylpyrrolidone (PVP) (molecular weight 10000), hydrochloric acid (HCl), glucose, 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6, *N*-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), glucose, Cys and phosphate buffered saline (PBS) at pH 7.4, cholesterol oxidase (ChOx) and glucose oxidase were all purchased from Sigma-Aldrich, South Africa. All glassware was stripped with Aqua Regia prior to use for synthesis. Ultrapure water (ddH_2O) was pre-prepared with a Milli-Q ultra-pure system (18.2 MΩ/cm).

4.4.2 Synthesis of gold nanostars

A modified and optimised synthetic method was followed in the synthesis of the seeded gold nanostars (Bibikova *et al.*, 2015; Yuan *et al.*, 2012). Firstly, 16 nm AuNPs were synthesised using the Turkevich-citrate reduction method (Turkevich *et al.*, 1951). Secondly, AuNS were synthesised in the following manner: 10 mL of 0.25 mM of $HAuCl_4$ dissolved in ddH_2O was acidified by 10 μL of 1N HCl. 5 μL of the synthesised AuNPs were added to the solution as seeds. 50 μL of $AgNO_3$ (10 mM) and 50 μL of AA (100 mM) were added almost on the same spot simultaneously to the contents under mild stirring at room temperature. Within a few seconds, colour change was observed to deep blue from pale yellow. 350 μL of 2.5 mM PVP 10 000 was added to the stirring solution. The solution was left to stir for extra 5 minutes and left to stand overnight at room temperature. It was cleaned up by centrifugation at 3000 *g* for two hours and re-suspended in 2 mL of ddH_20 after removal of the supernatant. It was stored at 4 °C until needed.

4.4.3 Characterizations and instrumentations

UV-vis-NIR spectroscopy analyses were carried out by spectral scanning (400–990 nm) on a HT Synergy (BioTEK) microplate reader. The spherical particles sizes and concentration were determined by the use of the UV-vis-NIR spectroscopy. For size and concentration determination, the methodology of Haiss *et al*. (Haiss *et al.*, 2007) method was followed. The value of the sizes were determined using Tables S-2 and S-3 in the supplementary information of (Haiss *et al.*, 2007). The transmission electron microscopy (TEM) analyses were performed on a Tecnai F20 high-resolution transmission electron microscope (HR-TEM) at an accelerating voltage of 200 kV. Samples for TEM were prepared by applying 20 μL of nanoparticle

suspension onto carbon 200 mesh copper grids (Agar Scientific), followed by drying overnight prior to imaging.

4.4.4 Feasibility of plasmonic colorimetric sensing of hydrogen peroxide

The feasibility of a plasmonic colorimetric assay for cholesterol through bio-catalytic shape alteration of the AuNSs using silver was investigated by several control and several optimisation experiments. Control experiments were carried out in order to find out whether there are colour changes in the presence and absence of the essential components for the cholesterol biosensor. In order to test signal generation by colour change and LSPR shifts, H_2O_2 was added to gold nanostars in PBS buffer (10 mM) at pH 7.4. Silver nitrate was added to initiate growth of the AuNSs in the presence of the NaOH (1M). The optimised conditions were used to establish the relationship between the amount of H_2O_2 produced and the concentration of cholesterol. Lastly, H_2O_2 concentration gradient was carried out using the optimised parameters for the purpose of testing the assay's ability to detect varying concentrations of the analyte. Images and spectral readings were taken.

4.4.5 Plasmonic detection of glucose and cholesterol

The optimised conditions obtained in the feasibility assay were now adapted for glucose sensing using GOx. A glucose concentration gradient was done with various concentrations from 0 – 12 mM. A number of solutions containing 20 µL AuNS, GOx (0.125 mM), in 0.01 M MES buffer had different concentrations of β-D (+) glucose. Likewise, the cholesterol biosensor solution included AuNSs seed solution, PBS (pH 7.4), and ChOx (2 mg/mL) and different concentrations of cholesterol (1.0 – 10 mmol/L). Both were added to on a 96-well plate and incubated for reaction for 30 minutes at 37 °C. After which the detection solution consisting of Ag^+ and NH_3 (3.75 mM) was added to each well. UV-vis-NIR spectra were obtained between 300–900nm for all solutions. Kinetic reads of the reactions were also carried at 560 nm.

4.5 Results and discussion

4.5.1 Gold nanoparticles characterisations

The absorbance spectra of both the AuNPs and the AuNSs are shown in Figure 4-1(A) shows. The synthesized AuNPs with an average particle diameter of 18±2nm had their surface plasmon resonance peak at 520 nm. The colloidal solution had a ruby red colour typical of spherical nanoparticles solutions (top left insert). The UV-vis-NIR spectrum of the AuNSs was characteristic of multbranched or star-shaped nanoparticles due to the broad peak and an increasing LSPR red shift as is observed in Figure 4-1(A). This LSPR band peaked between

660 and 780 nm, in the near-infrared region (Rodriguez-Lorenzo *et al.*, 2012). The colour of the AuNSs solution was blue (Fig 4-1B). These optical properties observed are consistent with experimentally demonstrated and theoretically predicted characteristics of gold nanostars (Maiorano *et al.*, 2011).

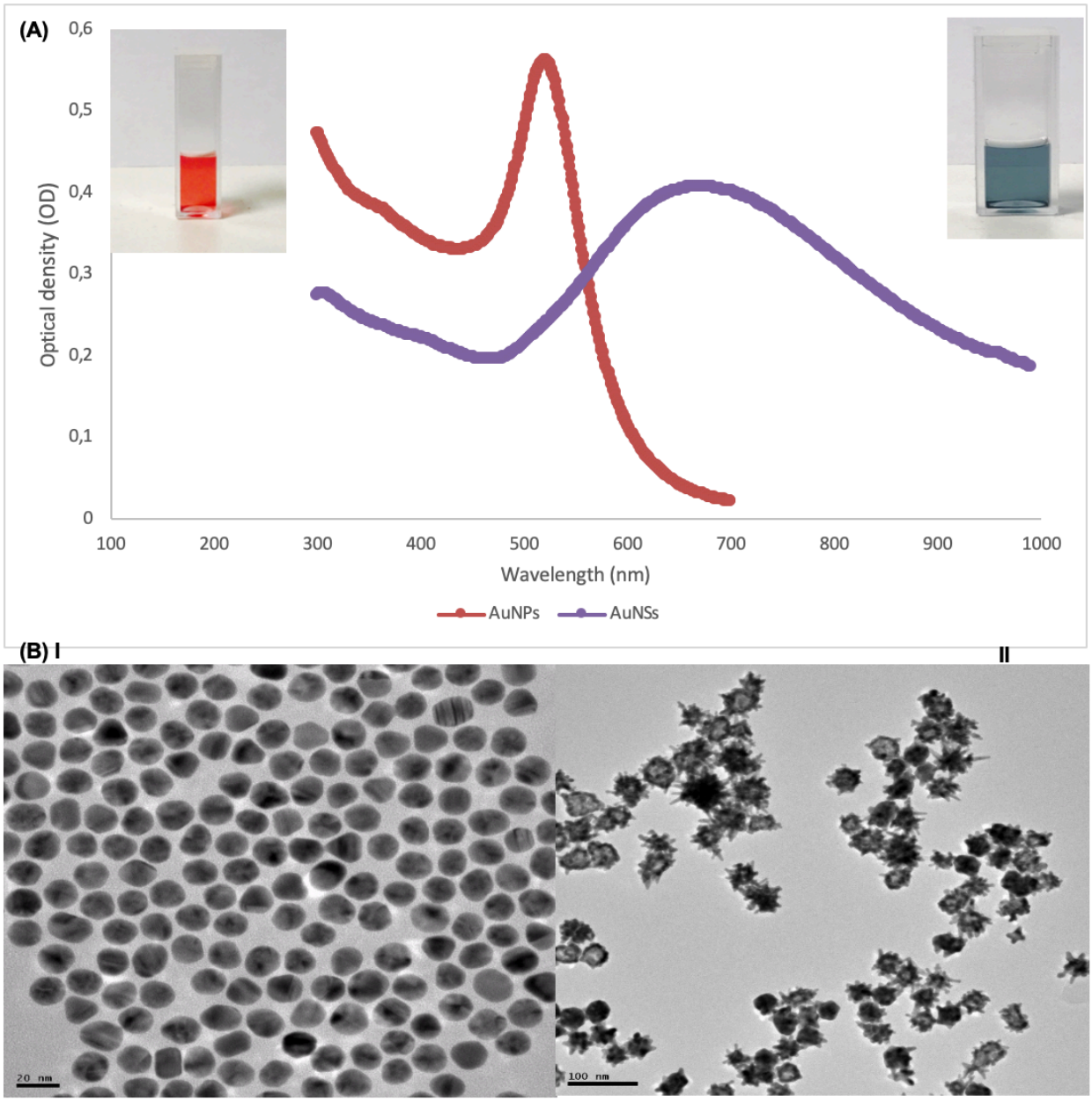


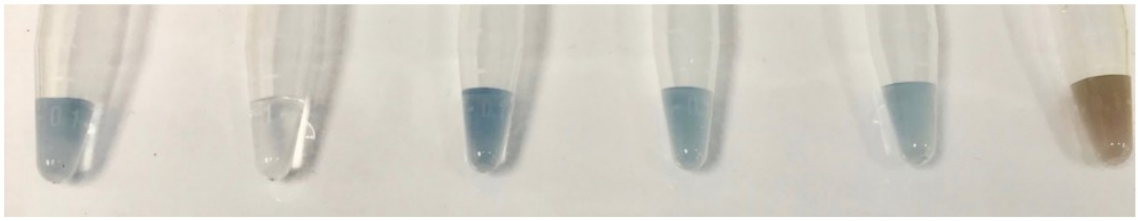
Figure 4-1 (A) UV-vis-NIR absorption spectra of AuNPs and AuNSs. Insert are the solutions of AuNPs in top left corner, and of AuNSs in the top right corner. (B) TEM images of AuNPs (I) and AuNSs (II).

The TEM image in Figure 4-1(B-I) shows that the AuNPs were monodispersed and had a good size and shape control after synthesis. The image further shows that the particles had not

undergone aggregation. The TEM image for AuNSs showed that synthesized stars were multibranched with a size distribution of 60 ± 5 nm. The EDS data showed that the gold nanostars were composed of ~12% silver and the rest was gold. This agreed with the synthetic route of the AuNSs which used silver as a shape directing agent to form the branches on the stars.

4.5.2 Hydrogen peroxide feasibility assay

As shown in Figure 4-2 the colour change in the absence of any of the essential components for the plasmonic colorimetric sensor was not feasible. Without AuNSs, the solution remained colourless. Without the H_2O_2, $AgNO_3$ and addition of base to adjust the pH, only the blue colour derived from the AuNSs was observed. Only in the presence of all the necessary components was there a colour change observed in the tube. This demonstrated that changes in the optical properties of the AuNSs as detectors was dependent on the presence of H_2O_2, and other essential solution constituents.



Buffer	√	√	√	√	√	√
AuNSs	√		√	√	√	√
H_2O_2		√		√	√	√
$AgNO_3$		√	√		√	√
Base		√	√	√		√

Figure 4-2 Control experiments showing the feasibility of plasmonic colorimetric detection based on H_2O_2 sensing.

The effect of increasing H_2O_2 concentrations can be seen in Figure 4-3. The LSPR peaks blue-shifted in response to increase in H_2O_2 concentration. The control AuNSs had their LSPR peak at 783 nm compared to those with the highest concentration of H_2O_2 that was at 492 nm. The blue shift in LSPR peaks was strongly dependent on the concentration of H_2O_2 as was observed by a scatter plot of LSPR absorbance band (Δ λmax) against H_2O_2 concentration. Fitting an ordinary least squares regression model to the data resulted in a coefficient of determination of

R^2 >0.98 implying a predictable linear relationship between Δ λmax and H_2O_2 concentration. The colour changes as the H_2O_2 concentration increased were from blue to purple, ultimately to orange.

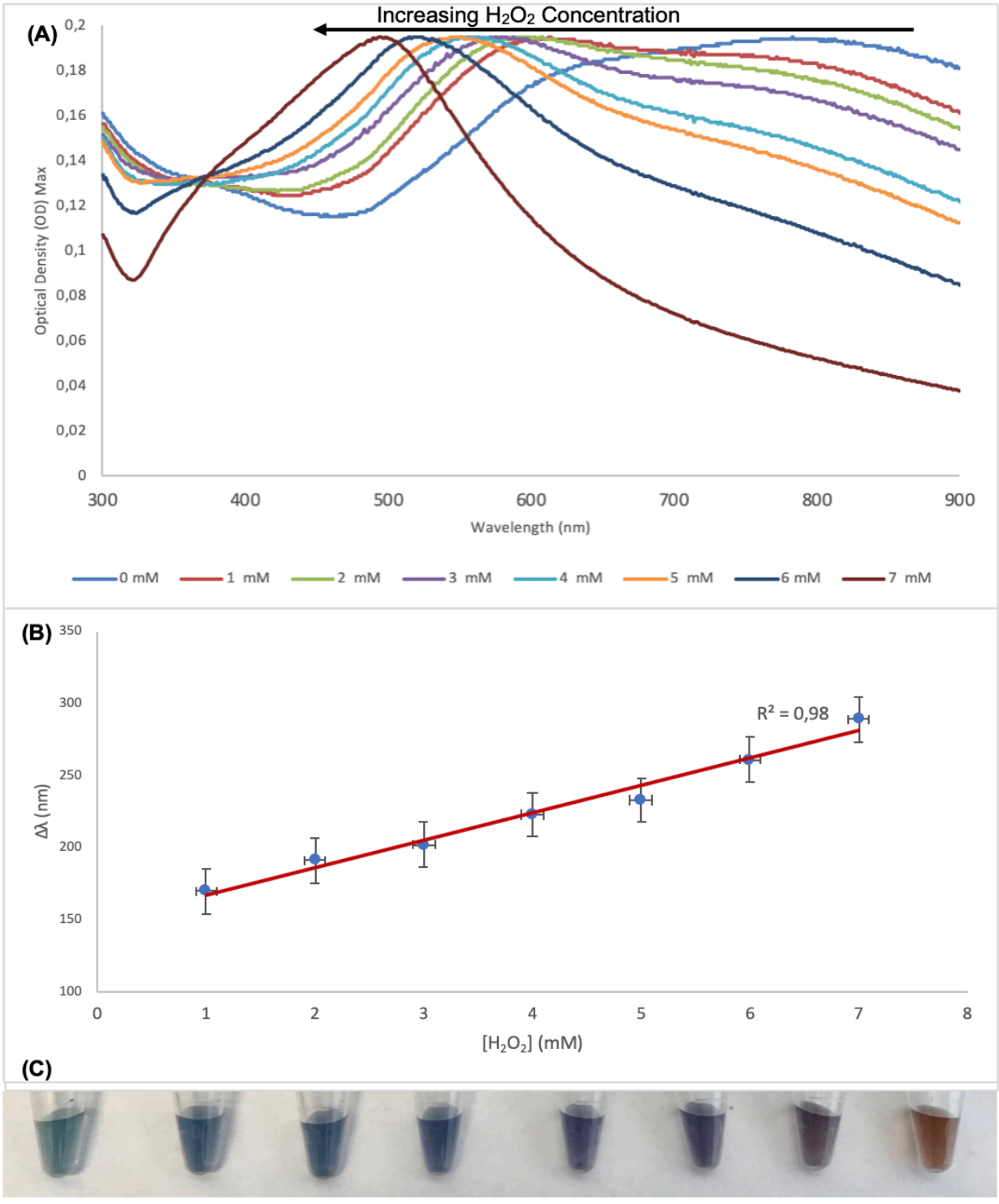


Figure 4-3 H_2O_2 sensing using optimal conditions. (A) UV-spectra of the AuNSs after signal-generation; (B) Blue-shift of the LSPR absorbance band (Δ λmax) as a function of H_2O_2

concentration; (C) photograph showing the corresponding colour changes in H_2O_2 concentration.

Changes in the optical properties observed in the assay were attributed to the morphological changes in the AuNSs. The TEM images in Figure 4-4 shows the changes in morphology of the AuNSs in the presence of 7 mM of H_2O_2. Due to the silver coating, the AuNSs slightly grew in size, and there was change in morphology as the particles become more spherical. Since the optical properties of the nanostructures depends on their sizes and morphologies (Amendola *et al.*, 2017; Fan *et al.*, 2008; Guo *et al.*, 2015; Langer *et al.*, 2015), this explains the colour changes, as well as LSPR peak shifts in the presence of H_2O_2.

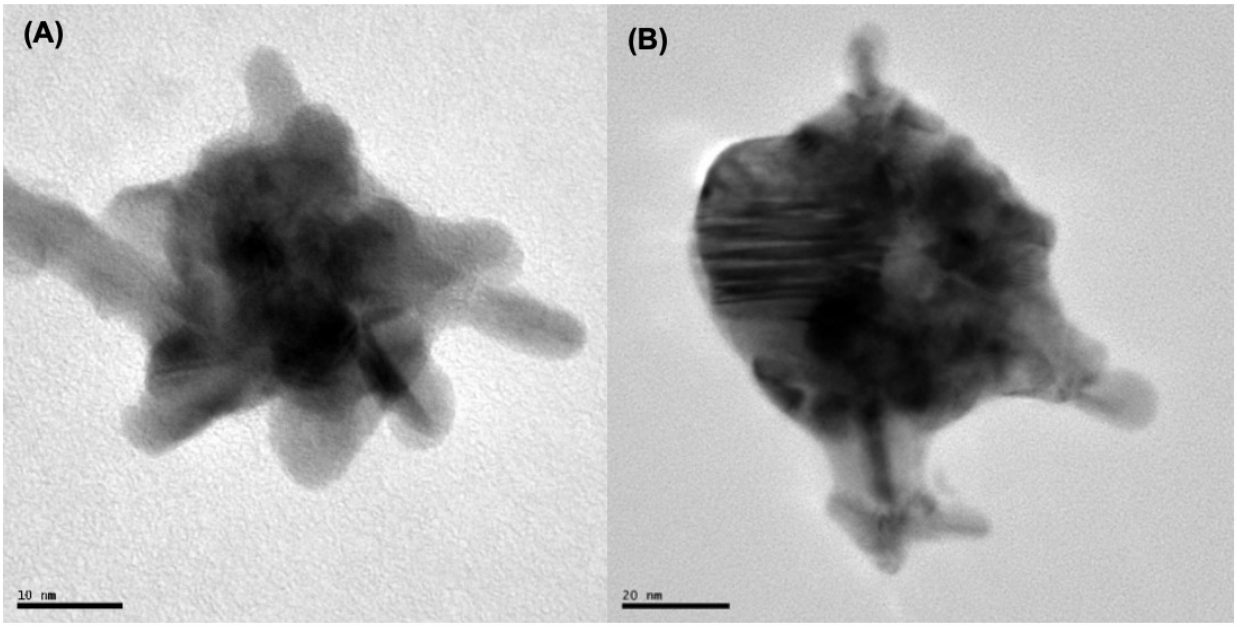
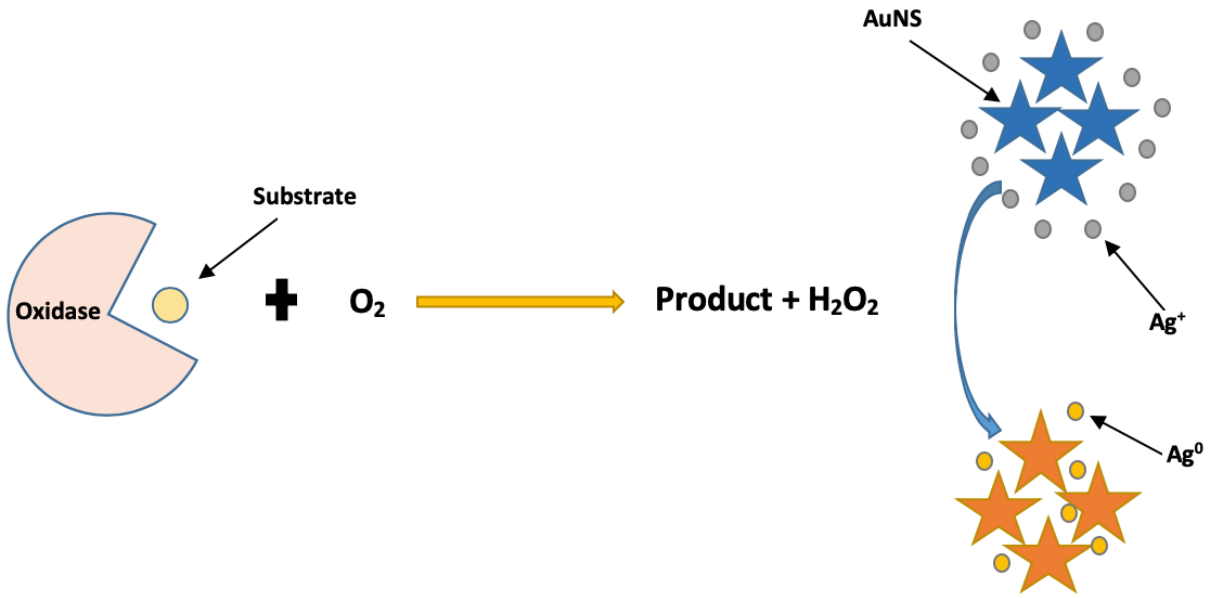


Figure 4-4 TEM image of AuNSs (scale bars, 10 and 20 nm, respectively) before (A) and after (B) signal-generation in H_2O_2 (7 mM).

AuNSs showed great tunability in size, shape and plasmonic optical properties as nanosensors for H_2O_2-mediated signal generation. Enhanced signal generation was achieved by tailoring their LSPR via silver coating after reduction with H_2O_2. This strategy was then investigated as a universal approach for the development of glucose and cholesterol biosensors based on H_2O_2-mediated signal transduction.

4.5.3 Plasmonic detection of glucose and cholesterol



Scheme 4-1 Schematic diagram showing the working principle of the glucose and cholesterol biosensors. The oxidase breaks down the substrate in the presence of oxygen producing H_2O_2 and another by-product. The produced H_2O_2 reduces Ag^+ to Ag^0 which plates on the AuNSs leading to colour changes.

The universal detector system based on generation of H_2O_2 was used for the development of both the glucose and cholesterol biosensors for potential clinical applications. Glucose and cholesterol oxidases were used to oxidise their respective substrates in the presence of molecular O_2 to produce gluconic and cholesterone respectively, and H_2O_2 which reduces Ag^+ to Ag^0, enabling the change in morphology of AuNSs. The process is depicted schematically in Scheme 4-1.

4.5.3.1 Plasmonic detection of glucose

Optimised conditions for the feasibility assay were adopted and applied to glucose sensing using noncovalently attached GOx. Figure 4-5(A) shows the LSPR peak blue-shifts in response to increasing concentration of glucose. Control experiment indicate no signal generation from the AuNSs in the absence of GOx or glucose. The H_2O_2 produced from the oxidation of glucose was directly proportional to the concentration of glucose, thus, increased glucose concentration led to further blue shift of the LSPR band. These observations are similar to those seen in H_2O_2 sensing. Figure 4-5(B) showed change in colour from blue to purple. The changes in colour

could not be distinguished with increases in glucose concentration. However, there was intensification in the purple colour with increase in glucose concentration. The constructed glucose biosensor was based on direct sensitivity of plasmonic colorimetric detection of glucose using AuNSs.

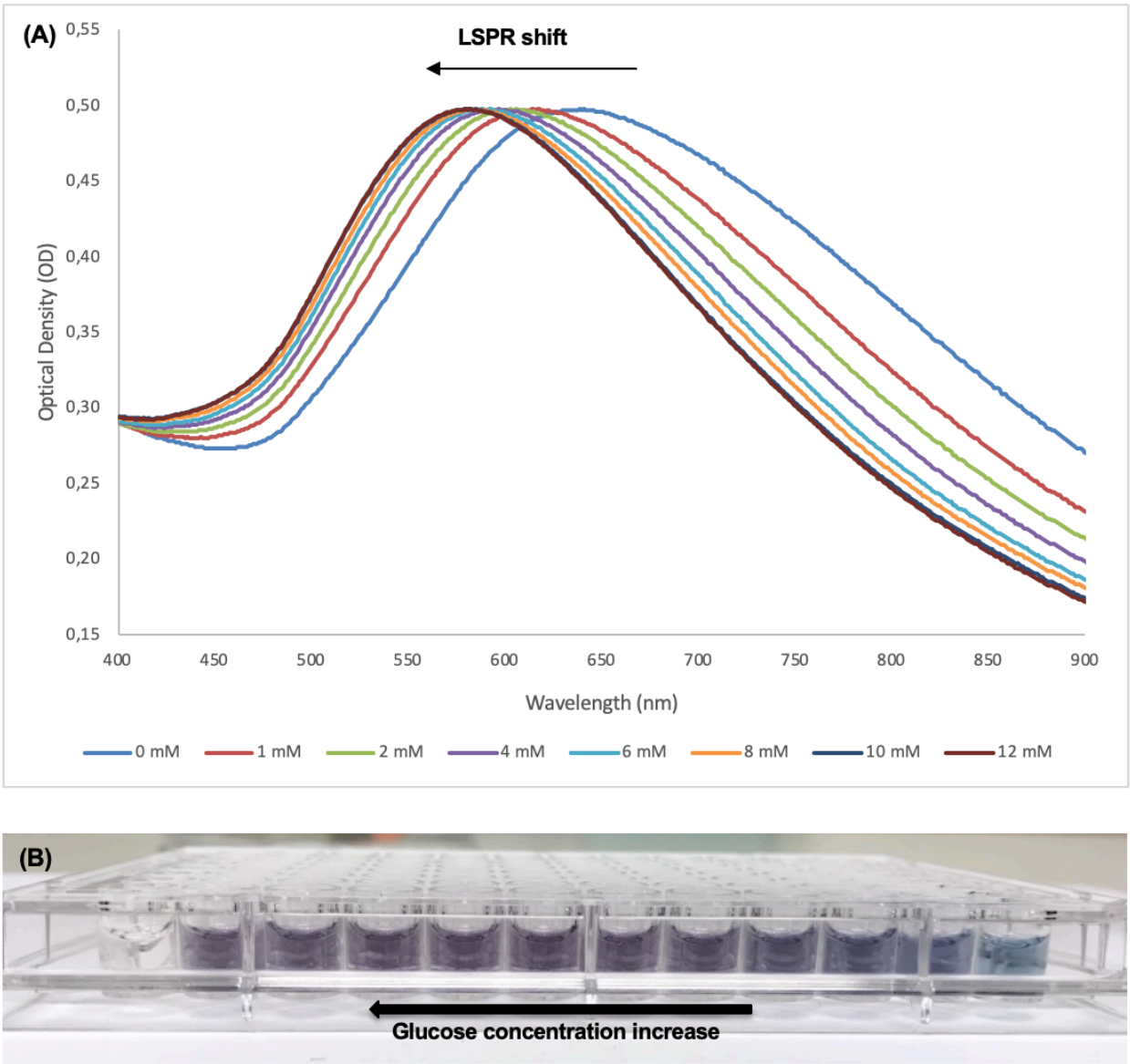


Figure 4-5 (A) Normalised UV-vis-NIR spectral scan (300-900nm) of glucose biosensor with various increasing glucose concentrations; (B) colour of biosensor with various concentrations of glucose.

4.5.3.2 Plasmonic detection of cholesterol

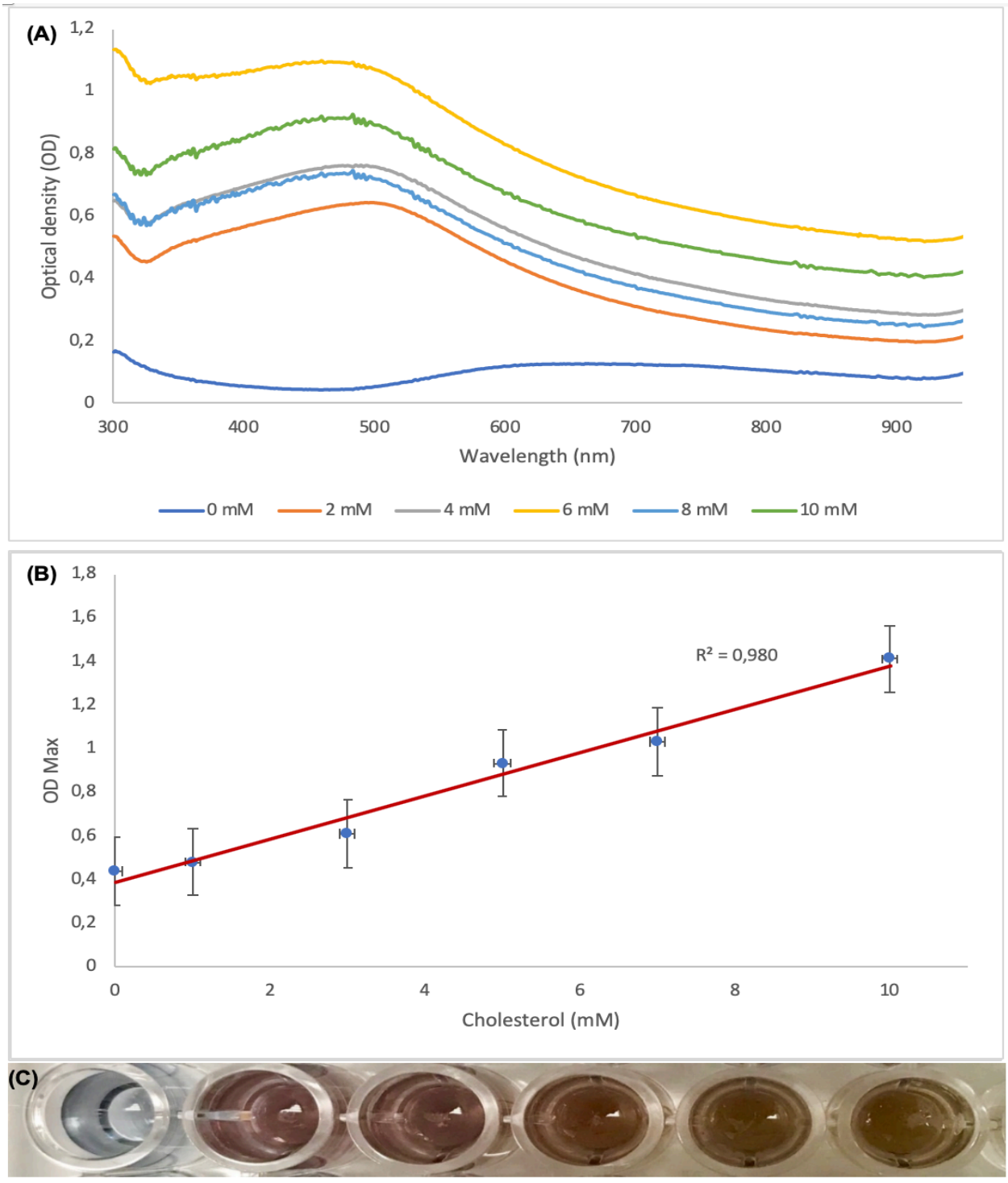


Figure 4-6 (A) UV-vis-NIR spectra (300-900nm) of cholesterol biosensor with various increasing concentrations; (B) Plot of maximum absorbance (λ max) as a function of cholesterol concentration; (C) Colorimetric changes of cholesterol detection according to various increasing concentrations.

A simple cholesterol biosensor was also constructed and Figure 4-6 shows the calibration curve that was derived from the increasing concentrations of cholesterol and maximum absorbance. There was a corresponding increase in absorbance in relation to various increment in cholesterol concentrations. As opposed to the glucose biosensor that was based on LSPR peak shifts, the cholesterol biosensor was particularly based on changes in UV-vis-NIR spectral absorbance due to the growth of AuNSs (Aldewachi *et al.*, 2018). The measurement was based on the mean OD max at λmax. The concentration range chosen was representative of the serum clinical reference ranges of 3–10 mM (Marshall, 2008). The cholesterol biosensor was able to give a linear response (R^2 >0.98) within this concentration range. Colour changes were observed after the incubation of cholesterol with the enzyme and adding the detection solution. The colours changed from blue to orange to orange-yellow and could easily be distinguished from the control. The most plausible explanation for this was growth in the size of the particles that also results in different colours (Amendola *et al.*, 2017; de Puig *et al.*, 2015; Langer *et al.*, 2015).

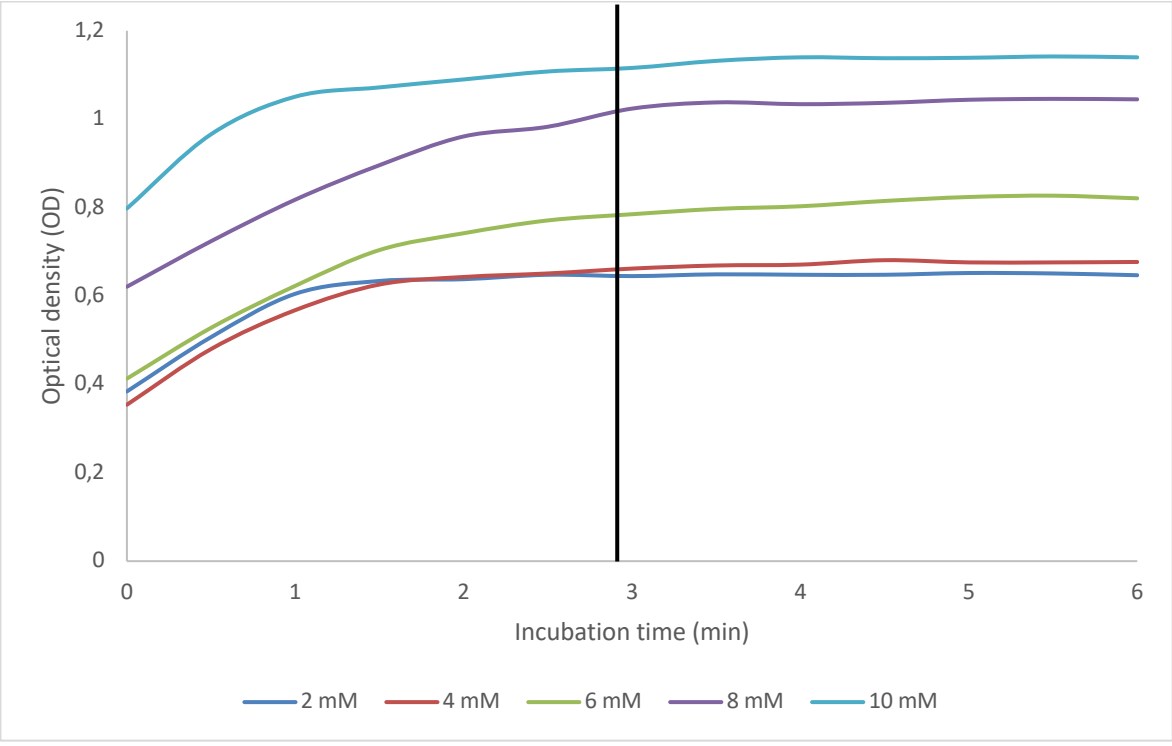


Figure 4-7 An endpoint kinetic read at 450 nm of cholesterol detection using AuNSs. The assay was could detect different concentration levels of cholesterol from very start of the spectral read.

As can be seen from Figure 4-7, the generation of cholesterol signal occurs in less than 3 minutes and the measurement is complete in less than 5 minutes. Thus, the assay was fast and a spectral read with reliable results could be obtained in 5 minutes after incubation and addition of the detection solution. The whole run time from preparation to spectral reading is less than an hour.

4.6 Conclusion

Here H_2O_2-mediated growth of AuNSs for the development of cholesterol and glucose biosensors is reported. AuNSs, which offer tuneable plasmonic properties induced by changes in size, and shape, produced enhanced signal generation which was achieved by modification of their LSPR via silver coating after reduction with H_2O_2. TEM images revealed the change in morphology and enlargement of AuNSs sizes. Changes in particle sizes where commensurate with the optical changes in the nanosensor response which included shifts in the LSPRs, increase in absorption and corresponding colour changes with increase in H_2O_2 concentration. As a result of these optical changes the construction of a biosensor based on H_2O_2-mediated growth of the AuNSs as a universal biosensor especially for oxidases that generate H_2O_2 is possible. This feasibility study has provided the basis for the extension of this principle to the development of glucose and cholesterol biosensors for potential clinical applications. Unattached GOx and ChOx were used to breakdown their respective substrates in the presence of either glucose or cholesterol. The biosensors could be used to determine the concentration of these two analytes within the prescribed clinical ranges for each. Linear ranges were obtained when the maximum absorbance was plotted as a function of substrate concentrations. The biosensors were simple, fast, and with high throughput. From preparation to detection results could be obtained in less than an hour. The biosensors used a basic level spectrophotometer, making them more affordable compared to expensive high-end laboratory equipment. This is an advantage for resource constrained places. Further work to optimise the enzymatic assays based on AuNSs as detectors is currently being undertaken.

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CHAPTER 5 SYNTHESIS OF SEEDLESS GOLD NANOSTARS WITH SEED-LIKE ADVANTAGES FOR BIOSENSING APPLICATIONS

5.1 Preface

This chapter addressed objectives relating to the selection of nanosensors for sensitive signal transduction. The main focus is on a synthesis strategy for producing seedless gold nanostars that could be used in biosensing. Since the synthesis of gold nanostars varies based on experimental parameters and intended application, a synthesis strategy is presented that was tailor-made for the development of the nanotechnology-based detection assays. The motivation for this study, and the synthesis protocols are described. The modification of the nanostars with enzymes are also described and discussed. Finally, the feasibility of plasmonic biosensing using the produced gold nanostars is demonstrated.

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- The author's guidelines of this Journal can be found from this link: https://royalsocietypublishing.org/rsos/for-authors
- The response to the reviews comments can be accessed via this link: https://royalsocietypublishing.org/action/downloadSupplement?doi=10.1098%2Frsos.181971&file=rsos181971_review_history.pdf

5.2 Abstract

Gold nanostars (AuNSs) are seen as promising building blocks for biosensors with potential for easy readouts based on naked-eye and ultraviolet-visible (UV-vis-NIR) spectroscopy detection. We present a seedless synthesis strategy for AuNSs that has the advantages of the seeded methods. The method used ascorbic acid as a reducing agent and silver nitrate as an anisotropic growth control assisting agent. AuNSs with multiple branches and diameter of 59 nm were produced. They showed good stability when capped with PVP and modified with an enzyme in relatively strong ionic conditions. We investigated their application in plasmonic sensing by modifying them with glucose oxidase and detection of glucose. The AuNSs were found to be a good scaffold for the enzyme, proved to be stable and sensitive as transducers.

Thus, the AuNSs showed good promise for further applications in plasmonic biosensing for in biomedical diagnosis.

5.3 Introduction

Significant advances have been made in the synthesis of gold nanoparticles (AuNPs) to an extent that other nanoshapes are readily obtained using appropriate templating agents. Different shapes such as nanorods, nanocubes, nanoprisms, nanowires, nanoboxes, nanoshells, triangular, hexagonal shapes, and even nanostars have been produced(Alex & Tiwari, 2015; Nadeau, 2016). Among the various geometries of gold nanoparticles, gold nanostars (AuNSs), alternatively referred to as nanoflowers(Xie *et al.*, 2008), nanourchins, or multi-branched gold nanoparticles, have received much attention in recent years. This is because they could be deployed in a variety of uses such as catalytic activity, molecular detection, and biological applications in immunoassays, dark field imaging of cells and in plasmonic biosensors(Chirico *et al.*, 2015; de Puig *et al.*, 2015; Li *et al.*, 2013; Maiorano *et al.*, 2011). AuNSs have been shown to be useful building blocks for near-infrared (NIR) absorption and surface-enhanced Raman scattering applications because of their many branches and sharp tips. Due to their high-aspect-ratio spikes that localize the low-energy plasmon mode at their tips, AuNSs give a dominant localized surface plasmon resonance (LSPR) peak in the NIR region. AuNSs are thus an attractive platform for LSPR biosensing application in for diagnostics purposes with potential for easy readouts based on colorimetric and ultraviolet-visible-near infrared (UV-vis-NIR) spectroscopy detection(Guo *et al.*, 2015; Guo *et al.*, 2016; Rodríguez-Lorenzo *et al.*, 2012; Saverot *et al.*, 2016).

The last decade has seen advancements in the various synthesis methods for AuNSs. A rough general classification of the various synthesis strategies falls into two main categories; the seeded-growth and non-seeded-growth methods(Chandra *et al.*, 2016). The seeded-growth strategy is a popular approach for the synthesis of monodispersed gold nanostars. Here pre-synthesized seeds (AuNPs) are used as nucleation points where additional material is deposited for growth of the branches(Guerrero-Martínez *et al.*, 2011; Minati *et al.*, 2014). The synthesis process involves the reduction of hydrochloroauric acid ($HAuCl_4$) with ascorbic acid – or other reducing agents – on preformed gold seeds in the presence of a surfactant at room temperature(Minati *et al.*, 2014). Addition of silver nitrate ($AgNO_3$) at different growth stages of the nanocrystals increases the degree of control over the nanostar shape produced(Chirico *et al.*, 2015). However, this method has some setbacks, one of which is the complication caused by the various stabilizing agents and surfactant in the post-synthesis cleaning of the nanostars(Minati *et al.*, 2014). Recently, a surfactant- and polymer-free shape control synthesis method was proposed that was enabled by unified theoretical framework of nanocrystal

synthesis. Nanostars, among other morphologies, were synthesized with this simple green-chemistry method for catalysis and surface-enhance Raman scattering (Wall *et al.*, 2017).

Recent advances in seedless strategies have seen the use of “green” chemicals such as N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) as reducing and stabilizing agent. In this one-pot synthesis strategy, nuclei evolve to form nanocrystal seeds which get to be bigger particles through the direct addition of metal atoms. The presence of piperazine in HEPES is thought to be responsible for branch formation on the nanocrystals(Chandra *et al.*, 2016; Chandra *et al.*, 2018; Xie *et al.*, 2007). Compared to the seeded-growth strategy, this technique has fewer complications, with the advantage of being completed in one single step and pot. Some protocols are carried out without using surfactants, making the post-synthesis purification of the AuNSs formed less problematic. However, it has a number of disadvantages, one of which is the inability to control the dimensions of the resulting nanostars leading to polydispersity in the shapes and sizes of particles produced. Another notable drawback is their susceptibility to changes in the reaction parameters such as reagents concentrations, pH, and temperature, which affects the growth process and reproducibility of the nanocrystals (Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Minati *et al.*, 2014; Saverot *et al.*, 2016; Xie *et al.*, 2007).

In an attempt to optimize the HEPES-mediated method to yield more monodispersed AuNSs, an in-house method was developed recently in which a specific amount of $AgNO_3$ was added to aid in the growth process of the nanostar branches. It was reported to have yielded less polydispersed AuNSs compared to an earlier reported method (Mulder *et al.*, 2018). However, this method is relatively much slower in comparison to the seeded methods reported. It takes over 30 minutes for AuNSs synthesis to be completed. The size of the AuNSs produced were smaller compared to the seeded ones, with an LSPR peak (which is size dependent) at <630 nm, making them minimally NIR sensitive. Other disadvantages typical of non-seeded synthesis also associated with this approach include susceptibility to changes in the conditions and concentrations of precursor reagents such as pH of the HEPES buffer, temperature, and $HAuCl_4$ concentrations, strongly affects the reproducibility of the synthesized AuNSs(Chirico *et al.*, 2015; Hill, 2015; Minati *et al.*, 2014; Saverot *et al.*, 2016; Xie *et al.*, 2007).

Herein, a seedless one-pot method is reported that leveraged the advantages of the seeded and seedless strategies. Advantages of seeded methods such as rapidity, monodispersity, controlled growth of branches, bigger sizes of nanostars produced for greater LSPR sensitivity, as well as the simplicity and the use of non-harmful reagents of the seedless synthesis method were combined in this method. In this procedure, gold nuclei were reduced with ascorbic acid. $AgNO_3$ was added during synthesis for precise control of the growth of the nanostar branches.

The whole synthesis procedure took approximately 2 minutes to be completed. The stability of the synthesized stars in solution of different ionic strength and pH were investigated. Furthermore, the nanostars solutions were also investigated for plasmonic colorimetric sensing using glucose measurement as a model.

5.4 Materials and Methods

5.4.1 Materials and instrumentations

Hydrochloroauric acid ($HAuCl_4$), glucose oxidase (GOx), trisodium citrate, silver nitrate ($AgNO_3$), ascorbic acid, sodium chloride (NaCl), polyvinylpyrrolidone (PVP) (molecular weight 10000), hydrochloric acid (HCl), glucose, 3 3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP), 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)acid (HEPES) at pH 7.4, Tris-acetate-EDTA (TAE) at pH 8, bovine serum albumin (BSA), ethanolamine and phosphate buffered saline (PBS) at pH 7.4 were all purchased from Sigma-Aldrich, South Africa. All glassware was cleaned with Aqua Regia prior to use for synthesis. Ultrapure water (ddH_2O) was pre-prepared with a Milli-Q ultra-pure system (18.2 MΩ/cm).

UV-vis-NIR spectroscopy analysis was carried out by spectral scanning (300 – 900nm) on a HT Synergy (BioTEK) microplate reader. The transmission electron microscopy (TEM) and energy-dispersive x-ray spectroscopy (EDS) analyses were performed on a Tecnai F20 high resolution transmission electron microscope (HR-TEM) at an accelerating voltage of 200 kV. Samples for TEM were prepared by applying 20 µL of nanoparticle suspension onto carbon 200 mesh Copper grids (Agar Scientific), followed by drying for overnight prior to imaging. The particle-size distribution was estimated by measuring the size of approximately 100 nanoparticles in different grid regions. The EDS analysis was normalized to remove carbon and copper from the total chart as the samples were fixed on carbon-coated copper grids for analysis. Dynamic light scattering (DLS) was used to estimate the hydrodynamic diameter of the AuNSs. It was performed on a Zetasizer Nano (Melvern) in backscatter mode using Zetasizer version 6.20 software in capped polystyrene cuvettes.

Agarose gel electrophoresis of the AuNSs functionalized with PVP with and without GOx was carried out using a Baygene, BG-power, Vacutec electrophoresis gel apparatus. 0.75% agarose gel was prepared by weighing 0.375 g of agarose and dissolved it in 50 mL of TAE in the microwave. The gel was poured into the casting tray and allowed to set. Small aliquots (30 µL; a mixture of 2-parts AuNSs and 1-part glycerol (80%) of the samples, were loaded onto the gel and ran at 40 volts for 45 minutes. Gel images were captured and transferred to the computer.

5.4.2 Gold nanostar synthesis

All experiments were carried out at room temperature unless otherwise stated.

Seedless silver and ascorbic acid assisted nanostars (***seedless-AuNSs***) were synthesized as follows: 10 mL of ddH_2O was acidified with 10 µL of 1M HCl. Following this, 50 µL of 100 mM ascorbic acid was added under mild stirring. 50 µL of 50 mM $HAuCl_4$ was then added to the mixture. Immediately after this, 50 µL of 10mM $AgNO_3$ was added to the solution which resulted in a deep blue colour change within a few seconds. Finally, 500 µL of 2.5 mM PVP was added after which the solution was centrifuged for 90 minutes at 4000*g*. The pellet was then recovered and re-suspended in 1 mL of ddH_20.

For purpose of comparison, two other syntheses of AuNSs were performed; the one via a seeded method (***seeded-AuNSs***), and the other the in-house HEPES-mediated method (***HEPES-AuNSs***). The seeded-AuNSs were synthesized according to previously published methods with minor modification (Bibikova *et al.*, 2015; Yuan *et al.*, 2012). The seeds were synthesized according to the Turkevich-citrate reduction method(Turkevich *et al.*, 1951). Briefly, 0.25 mM $HAuCl_4$ was added to 50 mL ddH_2O and heated to 95°C. Thereafter, 1300 µL 1% trisodium citrate was immediately added to the mixture resulting in seed formation. The seeds were then stored at 4 °C until usage. AuNSs were synthesized by acidifying 10 mL of 0.25 mM $HAuCl_4$ with 10 µL 1M HCl followed by the addition of 5 µL of the seed solution. 50 µL 10 mM $AgNO_3$ and 50 µL 100 mM ascorbic acid were added simultaneously to the solution under mild stirring. Colour change was observed from pale yellow to deep blue. The nanostars were coated with 350 µL 2.5 mM PVP. Sample clean-up was done by centrifugation at 3000*g* for 2 hours and re-suspended in 1 mL ddH_20.

The HEPES-AuNSs were synthesized according to an in-house HEPES-modified method for seedless nanostars(Mulder *et al.*, 2018). In a typical synthesis, 2 mL of 100 mM HEPES was mixed with 3 mL ddH_2O in a 5 mL tube. This was followed by the addition of 20 µL 50 mM $HAuCl_4$ and 4 µL 1 mM $AgNO_3$. The capped tube was inverted a few times to ensure that the reagents were thoroughly mixed. The solution was left to stand for approximately 30 minutes in which the solution changed color from light yellow to slightly purple and finally a deep blue.

5.4.3 Modification of AuNSs with GOx

The seedless-AuNSs were chosen for GOx modification and further investigations. The method of Filbrun *et al,* with minor modifications was followed to attach GOx to the AuNSs with DTSSP (Filbrun *et al.*, 2017). Briefly, AuNSs was suspended in 2 mL PBS to which 100 µL 5 mM DTSSP was added to the solution. After 3 hours' incubation, the sample was centrifuged at

3000*g* for 1 hour to remove excess DTSSP. The sample was then resuspended in 2 mL PBS after which 250 µL of 5 mg/mL GOx was added and left to react for 2 hours. 100 µL of BSA 1 mg/mL and 100 µL of ethanolamine 10 mM were added as blockers and left to react for an extra hour. The solution was centrifuged and the pellet resuspended in 1 mL MES buffer. The GOx-modified seedless-AuNSs were characterized on a UV-vis-NIR spectrophotometer. DLS was used to determine the sizes of the control PVP-coated and GOx-modified seedless-AuNSs. Gel electrophoresis was done to determine the sizes and charges of these AuNSs.

5.4.4 Colloidal stability

The stability of the GOx-modified seedless-AuNSs were investigated in a solution of high ionic strength. The gold nanostars were centrifuged and resuspended in 300 mM NaCl and the UV-vis-NIR spectra was obtained after 2 hours' incubation.

5.4.5 Feasibility of plasmonic sensing using glucose measurement

The GOx-modified seedless-AuNSs were assessed for their ability to be used as plasmonic nanosensors. The mechanism of sensing was the enzyme-guided enlargement of AuNSs through the production of hydrogen peroxide (H_2O_2) from glucose breakdown. 15 µL of 100 mM glucose was added to GOx-modified seedless-AuNSs in 10 mM MES buffer and incubated at 37 °C for 1 hour. 15 µL of a 0.1mM $AgNO_3$ and 40 mM NH_3 solution was added to the mixture (with a total volume of 200 µL) to trigger the reduction of the silver ions (Ag+) on the AuNSs. UV-vis-NIR spectral changes were measured immediately. Furthermore, a range of glucose concentrations were added to the assay solution containing the GOx-modified seedless-AuNSs to investigate their use as signal transducers suitable for analyte detection in a diagnostic assay.

5.5 Results and discussion

5.5.1 Characterisation of the gold nanostars

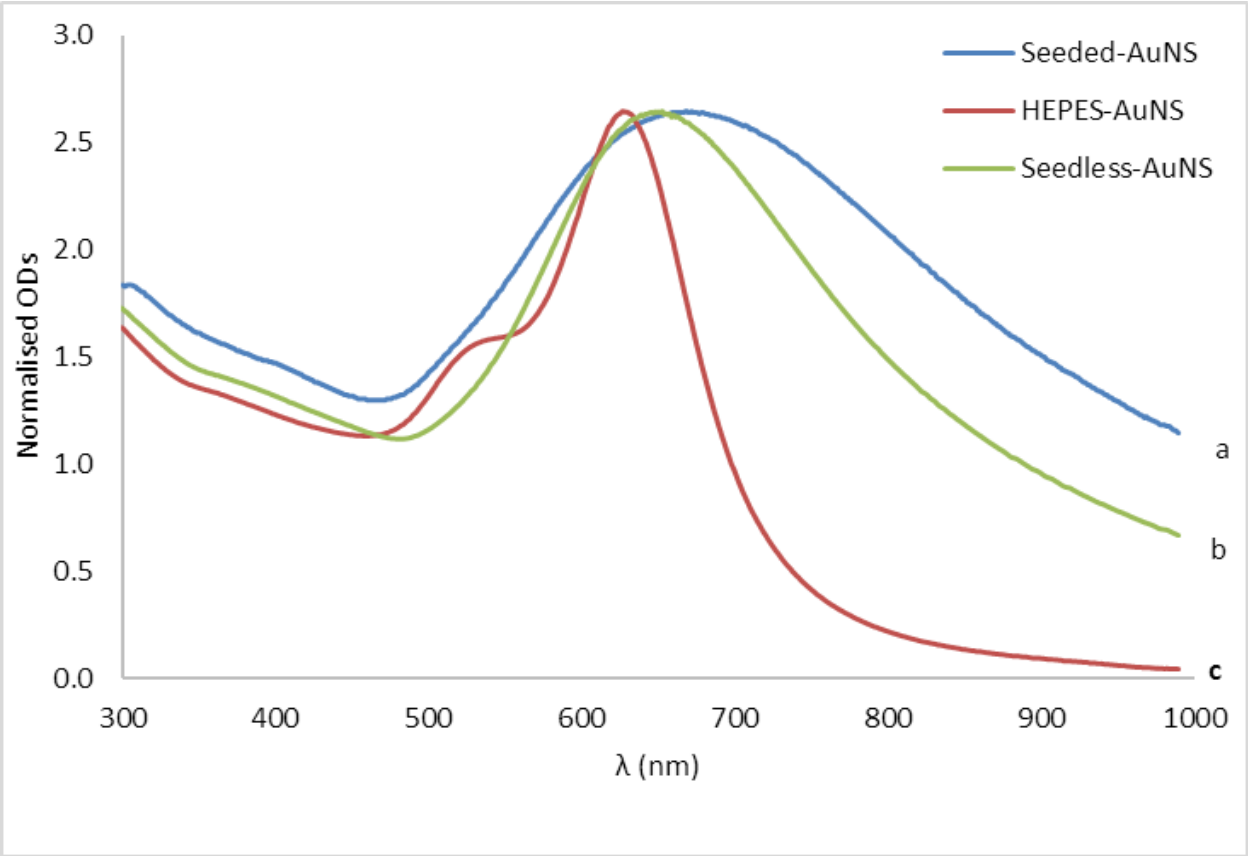


Figure 5-1 Comparison of UV-vis-NIR spectra of seeded-AuNSs (a), seedless-AuNSs (b), HEPES-AuNSs (c).

A comparison of the UV-vis-NIR spectra of the AuNSs synthesized with the three different synthesis methods are shown in Figure 5-1. The spectra observed had typical LSPRs of star-shaped nanoparticles as judged by the longer wavelengths and the broad peaks. Increased aspect ratios of the branches make the longitudinal components of the plasmon band become more intense and red shift relative to the LSPR of spherical particles (Maiorano *et al.*, 2011; Rodríguez-Lorenzo *et al.*, 2012). The maximum absorption of the seeded-AuNSs was at about 668 nm, while the seedless-AuNSs and HEPES-AuNSs absorbed at slightly shorter wavelengths of 653 nm and 627 nm respectively. Particle sizes and aspect ratios of the spikes are known to govern such optical properties (Rodríguez-Lorenzo *et al.*, 2012; Saverot *et al.*, 2016) as observed for these nanostars.

TEM analysis (Figure 5-2) of the synthesized nanostars showed that the seeded-AuNSs (i) were slightly larger at a diameter of 60 ± 5 nm compared to the seedless-AuNSs (ii) and HEPES-AuNSs (iii) with diameters of 59 ± 5 nm and 44 ± 4 nm respectively. The seeded-AuNSs and seedless-AuNSs had sharper tips of increased lengths compared to the HEPES-AuNSs. The newly synthesized seedless-AuNSs were comparable in morphology and size relative to the seeded-AuNSs. The HEPES-AuNSs were uniformly formed with greater size control compared to the both the seeded- and seedless-AuNSs. The representative TEM images of the all the three types of AuNSs synthesized showed that all had a high yield of branched particles with a few spherical morphologies present.

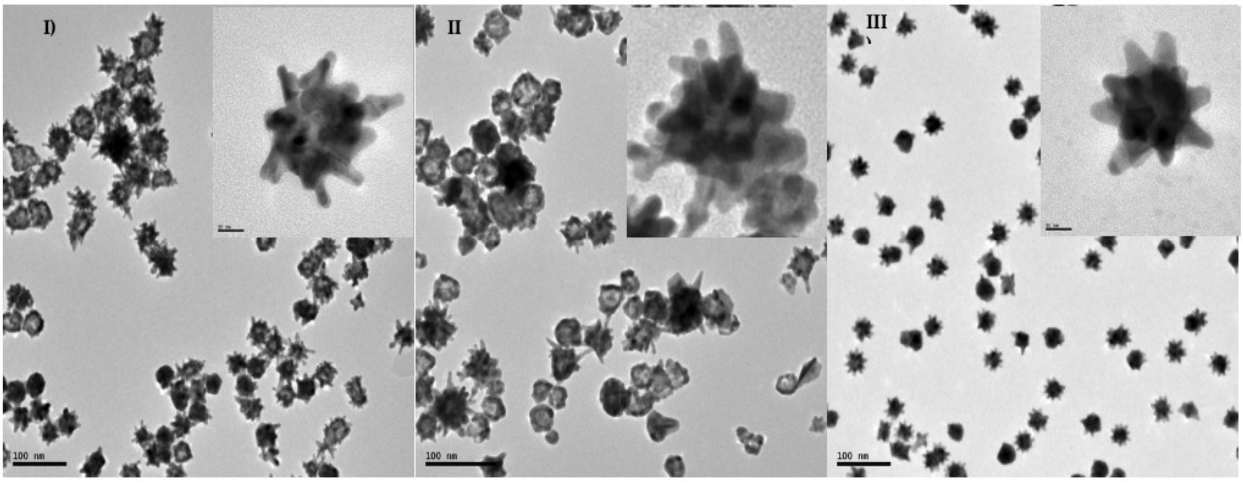


Figure 5-2 TEM images of seeded-AuNSs (i), seedless-AuNSs (ii) and HEPES-AuNSs (iii). The inserts show the magnified TEM images of the respective AuNSs at 10 nm.

This new synthesis method was discovered by changing the order in which the required reagents are added and eliminating the gold colloidal seeds as used in the original method by Yuan *et al* (Yuan *et al.*, 2012). The seeded method followed the procedure of firstly adding colloidal seeds and $HAuCl_4$ to the solution followed by ascorbic acid and $AgNO_3$ which are added simultaneously (Yuan *et al.*, 2012). Whereas, in this new proposed method, ascorbic acid was instead added first soon after lowering the pH of ddH_2O with HCl. Only then was the $HAuCl_4$ added to the mixture, and $AgNO_3$ immediately (10 s) after. As $HAuCl_4$ was in the process of being reduced by ascorbic acid, $AgNO_3$ was added for nanostar formation, without which, nanorods and/or nanospheres are formed (Yuan *et al.*, 2012). The EDS analysis showed that these stars had almost 100% Au with no Ag detected. Thus, it is thought that the main role of silver is to aid the growth of branches on the Au on certain crystallographic facets (Bibikova *et al.*, 2015; Yuan *et al.*, 2012). Addition of a small amount of HCl is thought to promote further red shift in the LSPR of the nanostars. It was observed that even when HCl was not added in the

synthesis procedure, gold nanostars were still formed. Yuan *et al* report that the localised surfaced plasmon resonance of nanostars synthesised at higher pH's such as 7 and 10 were more blue-shifted compared to those synthesised with HCl at pH 3 and 4 (Yuan *et al.*, 2012). They further investigated the effect of lowering the pH by substituting HCl with nitric acid. They observed that the nanostars were not formed at all. This led to them to postulate the Cl- ions could be involved in the formation of the gold nanostars (Yuan *et al.*, 2012). However, the effects of addition or omission of HCl on the size of the nanostars were not investigated in this study.

This new method deals with the problem that was reported by Yuan *et al*., (Yuan *et al.*, 2012) in their attempt to synthesize nanostars without seeds. They report yielding nanostars of diameters >100 nm (Yuan *et al.*, 2012), compared to 59 nm nanostars in this method. This new method also removed the need for careful addition of $AgNO_3$ and ascorbic acid at the same time and fast. In the seeded method reported, if the $AgNO_3$ was added too early, no nanostars are formed due to the precipitation of silver chloride. If the $AgNO_3$ was added too late, the $HAuCl_4$ would have already been reduced to larger gold nanospheres and nanorods. But in this new method, there is a stepwise addition of the reagents. The time for the addition of $AgNO_3$ was observed to be very important in this method. In the experimental protocol, $AgNO_3$, was added about 10 seconds after the addition of $HAuCl_4$. This led to star formation. On the other hand, delay of over 30 seconds in adding of $AgNO_3$ led to the formation of other nanoshapes (Ahmed *et al.*, 2010; Kawamura *et al.*, 2009; Yuan *et al.*, 2012). Without ascorbic acid, the $HAuCl_4$ was not reduced to form any nanostructures. Other studies using the ascorbic acid silver-assisted nanostars recipes adequately report the experimental effects of the various combinations of ascorbic acid in relation to the $HAuCl_4$, as well as adding varying quantities of silver nitrate to the mixture (Ahmed *et al.*, 2010; Kawamura *et al.*, 2009; Yuan *et al.*, 2012). With regard to time factors in the synthesis methods, the seedless-AuNSs took less than 2 minutes for a complete synthesis, whereas the HEPES-mediated reaction took about 30 minutes. For the seeded reaction, the actual star synthesis is comparable in time to the seedless reaction. But when the time for the spherical gold nanoparticles synthesis and maturation is factored in, it took the longest time to be completed of the three strategies.

5.5.2 Modification of AuNSs with GOx

To test the application of seedless-AuNSs as nanosensors, they were covalently modified with GOx using DTSSP. The UV-spectrum of the seedless-AuNSs modified with 6.25×10^{-4} g mL^{-1} GOx was observed to blue-shift relative to control PVP-coated seedless-AuNSs (Figure 5-3(I)). The LSPR of the GOx-modified seedless-AuNSs shifted by 22 nm from 653 to 631 nm. The shift was not accompanied by any broadening of the peak indicating the non-aggregation of the

AuNSs at this point. Gel electrophoresis was used to qualitatively confirm the binding of the enzyme (a: control PVP-AuNSs and b: GOx-AuNSs) to the gold nanostars (Figure 5-3(II)). The gel migration decreased with the GOx-modified seedless-AuNSs compared to the control AuNSs with PVP coating only. DLS analysis (Figure 5-3(III)) confirmed the quantitative growth in hydrodynamic size of the particles functionalized with GOx. These results put together indicate that the LSPR characteristics, size and surface charge of the particles have changed suggesting the attachment of the AuNSs with GOx.

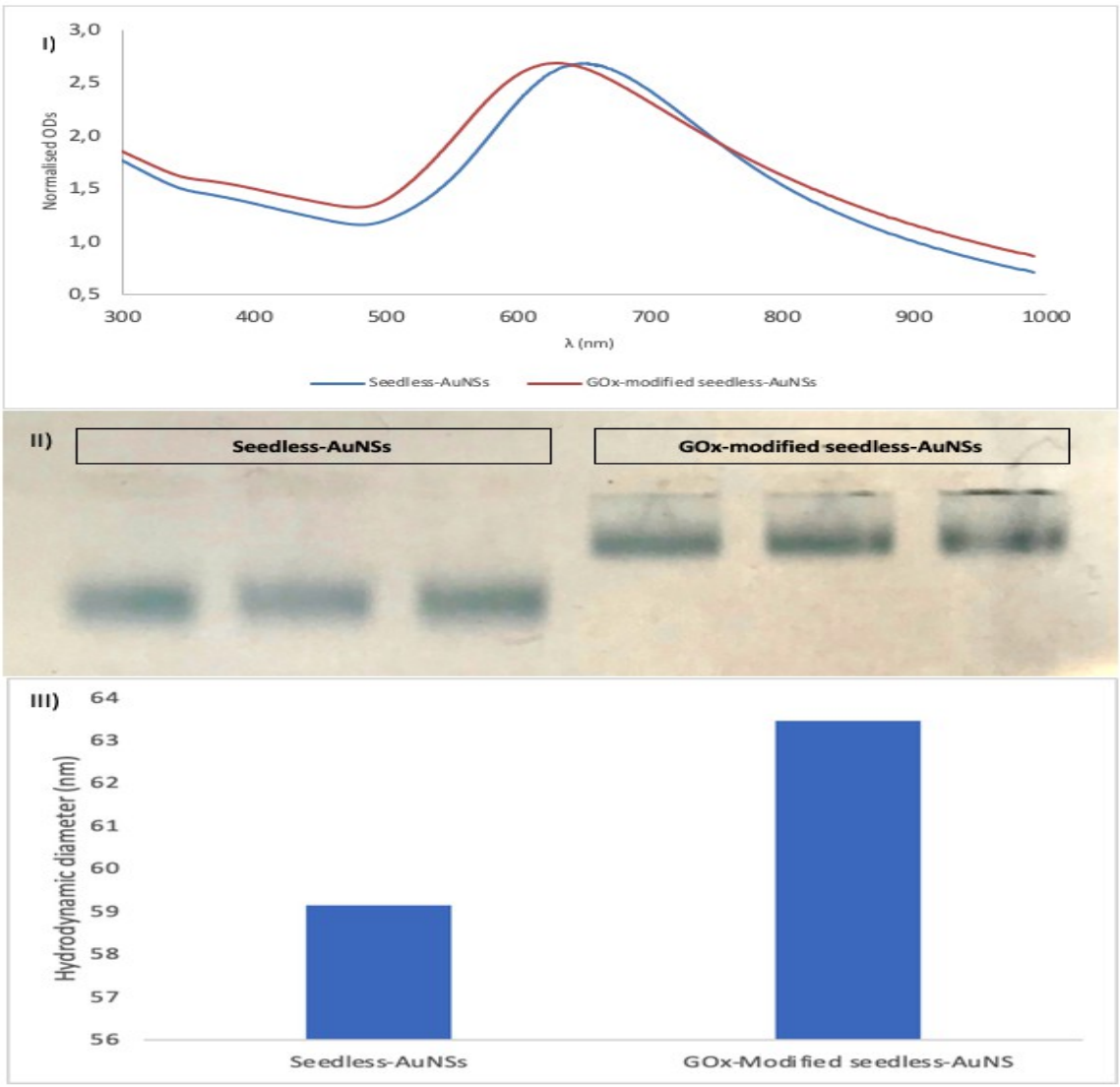


Figure 5-3 Normalised UV-vis-NIR spectra of the control seedless-AuNSs and GOx-modified seedless-AuNSs. (II) Agarose gel electrophoresis of the control nanostars and those modified with GOx. Because of the growth in size, the GOx-modified seedless-AuNSs showed less gel migration compared to the control seedless-AuNSs (III) shows the hydrodynamic diameters of the nanostars before and after modification with GOx.

Application of protein-modified gold nanostars at physiological pH and ionic environment requires that they are stable in solutions containing high concentration of proteins and salts. The stability of the GOx-modified seedless-AuNSs was demonstrated in 0.3 M NaCl at pH 7 (Rodríguez-Lorenzo *et al.*, 2012; Wangoo *et al.*, 2008). The protein modified nanosensors were stable as shown by the lack of variation of the LSPR (Figure 5-4). There was no reduction in absorption or broadening of the peak indicating the integrity of the nanostructures in this environment. This is particularly important because gold nanoparticles can aggregate in solutions with high ionic strength when their surfaces are not adequately coated which will lead to broadening of the peaks and shifts in LSPRs, thereby affecting the reliability of their detections (Rodríguez-Lorenzo *et al.*, 2012).

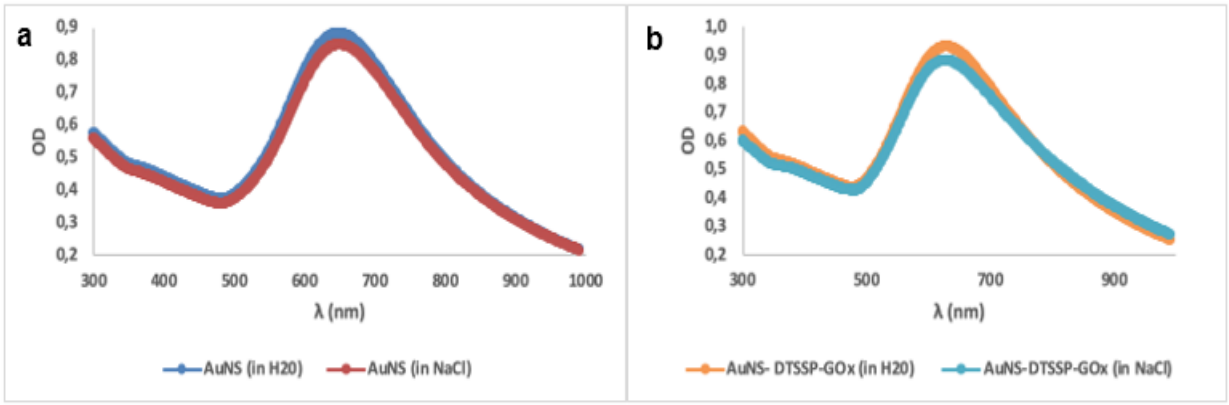
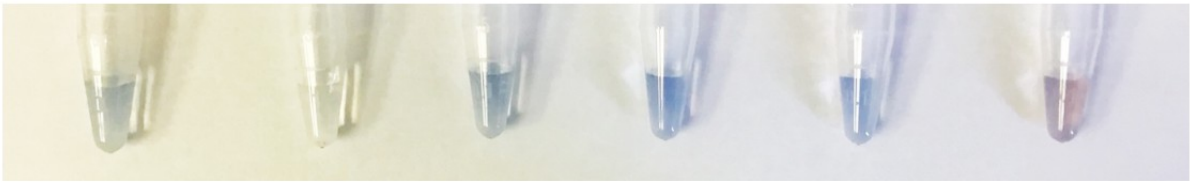


Figure 5-4 Images showing the stability of the stars in a solution of NaCl (0.3M) (a) for control seedless-AuNSs at λmax 650nm and (b) for GOx-modified seedless-AuNSs at λmax 628nm.

5.5.3 Feasibility of Glucose sensing

The feasibility of plasmonic colorimetric sensing using the synthesized seedless-AuNSs was investigated using glucose as a model analyte. Various control experiments were carried out, as shown in Figure 5-5. No appreciable colour changes were observed in the absence of AuNSs, glucose, $AgNO_3$, or base. After an hours' incubation at 40 °C, the only solution that showed significant colour change, as well as a large LSPR shift, was the one that had all the necessary

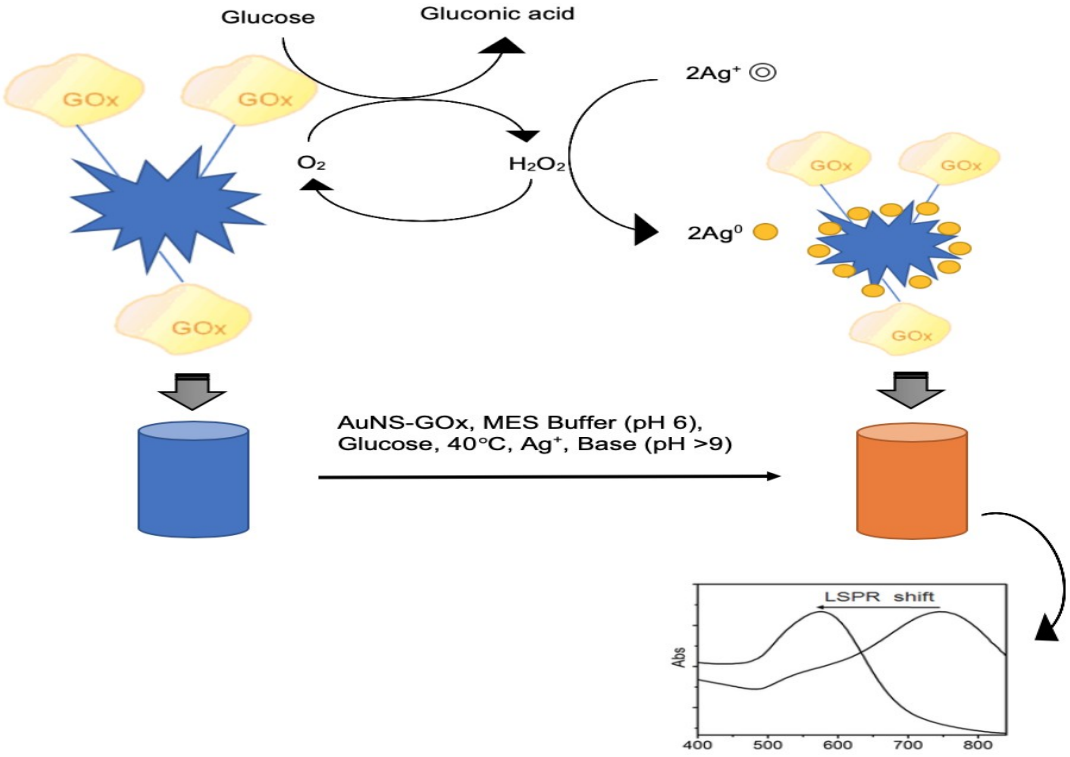
components in it. This observation could be attributed to the enzyme-guided growth of AuNSs, yielding change in colour. The AuNSs growth was induced by the coating of reduced silver on the surface as seeding points leading to epitaxial growth. Prior to this, the silver was reduced by H_2O_2 generated from glucose oxidation by GOx at pH 9. This experiment showed the absolute necessity of all the components to have a significant plasmonic shift and colour change.



AuNS-GOx	√		√	√	√	√
Glucose		√		√	√	√
Ag^+		√	√		√	√
Base		√	√	√		√

Figure 5-5 Feasibility of glucose sensing with GOx-modified nanosensors in glucose solutions of 2.5 mM, Ag+ of 0.1 mM added along with the base to adjust the pH to > 9.

Scheme 5-1 represents the mechanistic aspect of the plasmonic colorimetric strategy for glucose sensing observed in the feasibility experiment.



Scheme 5-1 Schematic representation of plasmonic colorimetric strategy for enzyme-guided growth of Ag^0 on AuNS and LSPR blue-shift.

5.5.4 Glucose detection

Using optimized conditions, this assay system was investigated for the determination of various concentrations of glucose as a proof of concept.

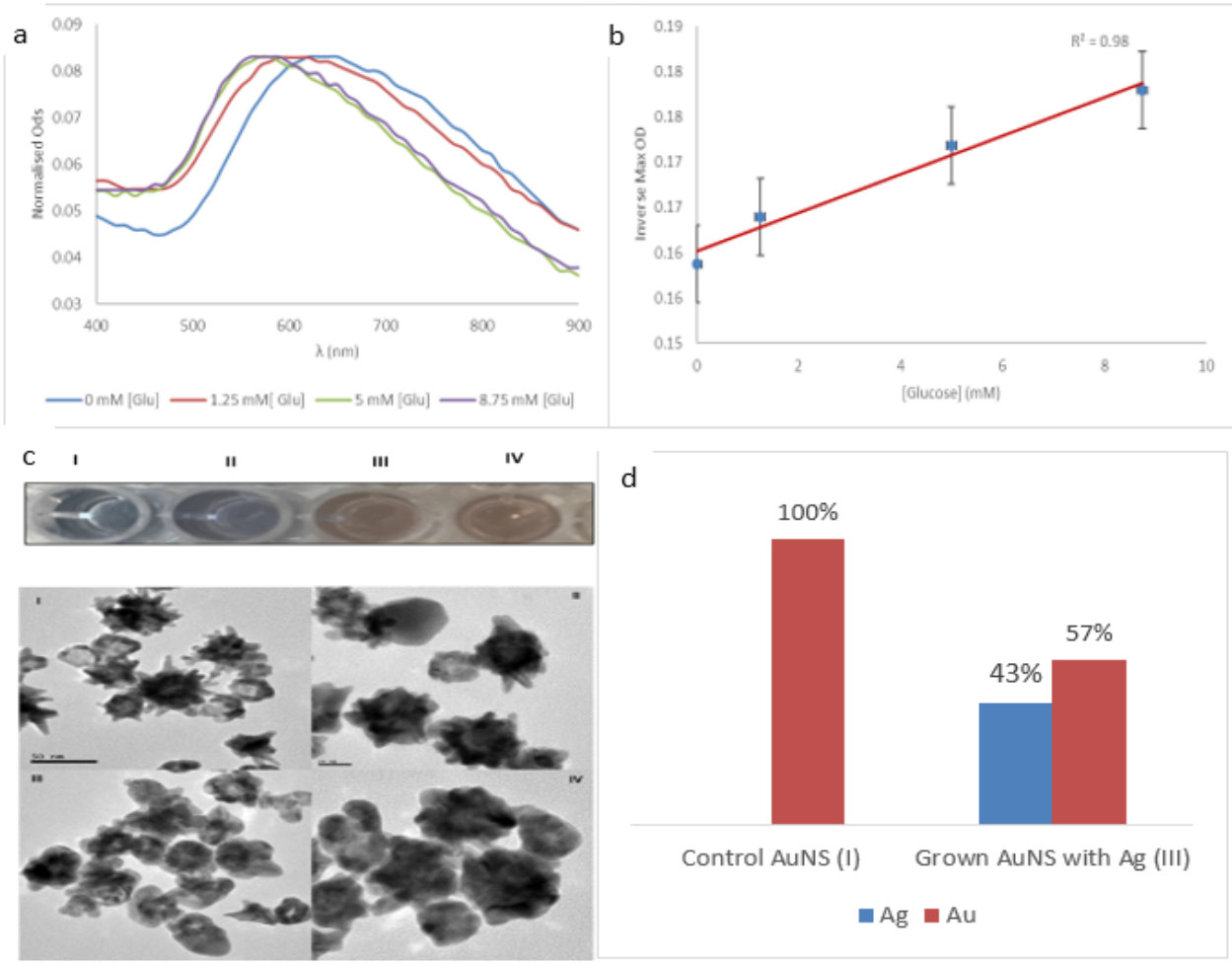


Figure 5-6 (a) Normalized UV-vis-NIR spectra of GOx-modified seedless-AuNSs showing blue-shift on reacting with different concentrations of glucose. (b) Plot of glucose concentration versus inverse maximum absorption. (c) TEM images of the seedless-AuNSs in different analyte concentrations: I) 0 mM, II) 1.25 mM, III) 5 mM and IV) 8.75 mM. (d) EDS analysis of C-I and III of the seedless-AuNSs.

As shown in Figure 5-6a, the LSPR shifted when the glucose concentration was increased from 1.25 to 8.75 mM. This demonstrated the strong dependence of the LSPR shift on the concentration of glucose. The insert in Figure 5-6c shows the colours corresponding to the LSPR shifts. The solutions changed colour from blue in the control sample, to purple and finally to different intensities of orange. This showed the potential of the nanosensors to be applied in assays with both UV-vis-NIR spectrophometric- and naked-eye readouts. The scatter plot of the glucose concentration verses the inverse of the OD max is presented in Figure 5-6b. This suggests a predicable quantifiable relationship of the nanosensors to potentially distinguish between different concentrations of an analyte. The TEM images (Figure 5-6c) show the morphological changes that occurred during the reaction. In the absence of glucose, the

nanostructures remained star-shaped. Conversely, when the glucose was added to various tubes in increasing concentrations, the AuNSs progressively became more spherical in shape. The change in morphology from star to spherical could be attributed to an increase in silver coating on the surface of the nanostars, as illustrated in Scheme 1. To validate the assumption that the change in size was due to increased silver coating, two representative samples (I and III) were analysed for elemental composition. As expected, sample I consisted of Au only while sample III contained a significant amount of silver. The analysis proved the assumption that silver was responsible for the growth or change in morphology of the AuNSs. Therefore, this demonstrated that the nanostars when properly functionalized with enzymes could be conveniently used to detect different amounts of analytes of interest.

5.6 Conclusion

In summary, a surfactant-free seedless one-pot method for AuNSs synthesis that has advantages of both the seeded and seedless strategies was developed. These seedless-AuNSs were simple and rapid to synthesize, with predictable growth of branches. The absorption peak of AuNSs was above 650 nm with a size of approximately 59 nm. This improves sensitivity in blue shift plasmonic LSPR. The AuNSs exhibited good stability when capped with PVP as a stabilizer. When investigated for plasmonic colorimetric sensing using glucose as a model analyte, they exhibited great stability in ionic environments and sensitivity in detection. This suggests that they are suitable transducers for biosensing applications. Further studies are being conducted in our laboratory on the optimal functionalization for further applications in plasmonic LSPR sensing in complex biological matrices.

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CHAPTER 6 FACILE IMMOBILISATION OF GLUCOSE OXIDASE ONTO GOLD NANOSTARS WITH ENHANCED BINDING AFFINITY AND OPTIMUM FUNCTION

6.1 Preface

The main focus of this chapter is to design and optimise the bioconjugation strategies for gold nanostars with enzymes. The motivation for such an undertaking is discussed and the preparation of the bioconjugates is described. The bioconjugates were characterized, and assessed for stability and sensitivity for biosensing applications.

This chapter has been published:

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- The author's guidelines of this Journal can be found from this link: https://royalsocietypublishing.org/rsos/for-authors
- The response to reviewers can be accessed via this link: https://royalsocietypublishing.org/action/downloadSupplement?doi=10.1098%2Frsos.190205&file=rsos190205_review_history.pdf

6.2 Abstract

Gold nanoparticles provide a user-friendly and efficient surface for immobilisation of enzymes and proteins. In this paper, we present a novel approach for enzyme bioconjugation to gold nanostars (AuNSs). AuNSs were modified with L-cysteine (Cys) and covalently bound to N-hydroxysulfosuccinimide (sulfo-NHS) activated intermediate glucose oxidase (GOx) to fabricate a stable and sensitive AuNSs-Cys-GOx bioconjugate complex. Such a strategy has the potential for increased attachment affinity without protein adsorption onto the AuNSs surface. Good dispersity in buffer suspension was observed, as well as stability in high ionic environments. Using the AuNSs-Cys-GOx bioconjugates showed greater sensitivity in the measurement of low concentrations of glucose based on plasmonic and colorimetric detection. Such a novel approach for enzyme immobilisation can lead to AuNSs-Cys-GOx bioconjugate complexes that can be used as catalytic nanodevices in nanobiosensors based on oxidases in biomedical applications.

6.3 Introduction

One of the many advantages that gold nanoparticles offer to biosensors is a user-friendly and efficient surface for immobilising enzymes (Sapsford *et al.*, 2013). The use of gold nanoparticles in biosensors — nanobiosensors — as signal transducers is a promising alternative to traditional detection techniques used in clinical diagnosis. Simplicity and cost-effectiveness in fabrication, sensitivity in signal transduction, as well as easy readout platforms, are some of the excellent properties of nanobiosensors (Guo *et al.*, 2016). Colloidal gold has high biocompatibility and surface energy that enables immobilised enzymes to retain their bioactivity. They also increase enzyme loading as opposed to bulk materials where enzyme adsorption usually leads to protein denaturation and decreased performance (Ahmad & Sardar, 2015; Ding *et al.*, 2015).

An important prerequisite to clinical use of nanoparticles is their surface functionalisation with biorecognition molecules. This functionalisation aids in maintaining the stability of the biorecognition molecules and nanoparticle bioconjugates in biological matrices so as to preserve their functionality (Aznar, 2015; Ding *et al.*, 2015; Guo *et al.*, 2015). Enzyme immobilisation onto colloidal gold nanoparticles enables more freedom of orientation for the attached enzyme with less probability of covering the active site. (Putzbach & Ronkainen, 2013). Furthermore, immobilised enzymes increase in stability to heat and fluctuations in the chemical environment (Arya *et al.*, 2008; Kouassi *et al.*, 2005), although some of their properties such as the Michaelis constant (K_m) or optimum pH value may be changed in the process (Burtis *et al.*, 2012; Ding *et al.*, 2015). Thus, there is need to optimise enzyme immobilisation in order to maximise assay sensitivity, selectivity, reproducibility and stability in biological samples (Aznar, 2015; Ding *et al.*, 2015; Guo *et al.*, 2015).

The two main methods for enzyme immobilisation on gold nanoparticles are physical and chemical adsorption. Physical methods employ weak interactions between the particle surface and enzyme, while in chemical methods covalent bonds are formed between the enzyme and particle surface (Ahmad & Sardar, 2015). Physical adsorption is a quick and simple technique for attaching enzymes in biosensors. Despite the benefits of speed and simplicity that this method offers, there are disadvantages such as undesirable enzyme orientations, covering of active site, and decreased enzyme functionality (Putzbach & Ronkainen, 2013). Interestingly, it is reported that direct adsorption is still being routinely utilised for conjugation of proteins to nanoparticles with reports of better binding activities for antibodies (Blanco-Covián *et al.*, 2017; Filbrun *et al.*, 2017; Lopez *et al.*, 2016).

Chemical adsorption method of enzyme immobilisation, on the other hand, involves direct covalent binding between the enzyme and the colloidal gold surface. These coupling chemistries aid in controlling the orientation of the immobilised protein onto the gold nanoparticles surfaces (Filbrun *et al.*, 2017; Muguruma, 2017; Putzbach & Ronkainen, 2013). Enzyme immobilisation on colloidal gold nanostructures is achieved by the use of crosslinker molecules of different lengths. The linker molecule gives the enzyme greater mobility thereby enhancing its bioactivity, compared to that of a directly coupled immobilisation (Ahmad & Sardar, 2015; Hermanson, 2013a). Enzymes may be modified to have reactive groups that are useful for conjugation with appropriately functionalised gold nanoparticles(Ahmad & Sardar, 2015; Sapsford *et al.*, 2013). To accomplish this, homobifunctional or heterobifunctional linker molecules are used to covalently couple some chemical target group on the enzyme through a terminal reactive group that can crosslink with the supporting surface (Hermanson, 2013a).

Glutaraldehyde is one of the most commonly used homobifunctional crosslinkers that contains an aldehyde group at both ends of a 5-carbon chain. It primarily reacts with amine groups with more than one mechanism of reaction. It is able to crosslink two molecules with amine groups and form stable bonds (Barbosa *et al.*, 2014; Betancor *et al.*, 2006; Hermanson, 2013a). Another most popular crosslinker is (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Li *et al.*, 2007). It is probably the most frequently used crosslinking agent of all. It is used for conjugating biological substances containing carboxylates and amines. It is mostly used along with N-hydroxysulfosuccinimide (NHS) or the water soluble sulfo-NHS and surface conjugation procedures described in the literature (Hermanson, 2013a; Li *et al.*, 2007). These two crosslinkers account for most of the covalent enzyme-nanogold conjugation procedures encountered in literature (Ahmad & Sardar, 2015; Ding *et al.*, 2015).

Six ideal conditions for enzymes immobilisation onto nanoparticles are well delineated by Sapsford and colleagues (Sapsford *et al.*, 2013). These are: 1) A high ratio of enzymes per nanoparticle to increase binding and interaction with target analyte. 2) Control over orientation of the enzyme attached to the nanoparticle so that the active site is uncovered. 3) Control over relative separation distance between the enzyme and the nanoparticle. 4) Control over attachment affinity of bioconjugates. 5) Maintenance of optimum function and activity of both the enzyme and nanoparticles. 6) Lastly, ability to be reproduced in a facile manner with other biomolecules to be immobilised.

Cysteine (Cys) is known to strongly bind to gold surfaces via the thiol group and form self-assembled monolayers (Majzik *et al.*, 2010; Zhao *et al.*, 2013). In a study of the structural and bonding evolution in Cys-gold cluster complexes, the thiol moiety is reported to be a very effective site for interaction with gold nanoparticles in aqueous medium as observed from a

number of techniques, such as: UV-vis-NIR, Fourier-transform infrared, Raman, and ^{1}H NMR spectroscopy (Majzik *et al.*, 2010; Zhao *et al.*, 2013). Gold nanostructures thus functionalised with thiol ligands tend to drastically reduce nonspecific protein adsorption on their surfaces (Li *et al.*, 2007; Lopez-Tobar *et al.*, 2013; Pensa *et al.*, 2012; Tengvall *et al.*, 1992; Zhao *et al.*, 2013). Cys has previously been used together with glutaraldehyde as a support for enzyme immobilisation without adsorption onto the surface with thermal and assay stability (Bezbradica *et al.*, 2014). The disadvantages of using glutaraldehyde for enzyme conjugation reactions are: it is a very hazardous chemical, has a complex reaction mechanism, requires other harmful chemical reagents such as sodium cyanoborohydride, requires high pH of > 9 which may cause nanoparticle aggregation, and the crosslinking is difficult to reproduce and scale up (Barbosa *et al.*, 2014; Betancor *et al.*, 2006; Hermanson, 2013a). Hence there is a need for optimum enzyme immobilisation methods onto gold nanoparticles modified with Cys that can prevent adsorption onto the surface, has thermal and assay stability, are simple to accomplish, use environmentally friendly chemicals, and easily reproducible.

In this study, a bioconjugation approach for attachment of enzymes to gold nanostars (AuNSs) was devised. AuNSs were the nanosensors of choice for signal transduction based on the localised surface plasmon resonance (LSPR). The LSPRs are determined by the shape of the nanoparticles' width, position, and number (Xia & Halas, 2005). A common feature of LSPRs for nanostars is their location at lower energy compared to nanospheres (Amendola *et al.*, 2017). For example, gold nanospheres with the size of 2–50 nm show only one plasmon band centred at about 520 nm, while for nanostars the plasmon band is redshifted and more intense, and typically centred around 650 – 900 nm (Amendola *et al.*, 2017; Chirico *et al.*, 2015; Guerrero-Martínez *et al.*, 2011; Saverot *et al.*, 2016). The current approach has been to use different morphologies and compositions of nanostructures, such as AuNSs, as a way to tune the LSPR properties of the nanosensors (Aldewachi *et al.*, 2018) for greater sensitivity (Rodríguez-Lorenzo *et al.*, 2012). Lastly, AuNSs also provided a larger surface areas for enzyme immobilisation with potential for higher load of enzymes per nanoparticle compared to smaller nanospheres (Sapsford *et al.*, 2013).

The approach described here for enzyme immobilisation to AuNSs brings together the use of Cys and EDC/sulfo NHS to create optimum conjugation that would prevent protein adsorption onto the surface of the particles and offer some relative separation distance between enzyme and nanoparticle. The bioconjugation approach is facile, easily reproducible, used simple chemistries with non-hazardous chemicals, and generated stable and sensitive bioconjugates with increased affinity for attachment without protein adsorption onto the AuNSs surface. This was accomplished by first functionalizing AuNSs with Cys. Secondly, GOx was modified with

EDC/sulfo-NHS to increase the stability and solubility of active esters intermediate, and to increase the conjugation yield. Thirdly and lastly, the cysteine-modified AuNSs (AuNSs-Cys) and NHS-terminated GOx were covalently coupled together to form AuNSs-Cys-GOx bioconjugates. Consequently, stable AuNSs-Cys-GOx bioconjugates were generated as proposed in Scheme 6-1. The AuNSs-Cys-GOx bioconjugates synthesised by this approach were assessed for stability and deployed for glucose detection in a nanobiosensor via an enzymatic assay comparison.

6.4 Materials and Methods

6.4.1 Materials and Instrumentations

Hydrochloroauric acid ($HAuCl_4$), glucose oxidase (GOx), trisodium citrate, silver nitrate ($AgNO_3$), ascorbic acid, sodium chloride (NaCl), polyvinylpyrrolidone (PVP) (molecular weight 10000), hydrochloric acid (HCl), glucose, 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6, *N*-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), Cys and 1x phosphate buffered saline (PBS) at pH 7.4, Pur-A-Lyzer Midi 3500 Dialysis Kit were all purchased from Sigma-Aldrich, South Africa. All glassware was stripped with Aqua Regia prior to use for synthesis. Ultrapure water (ddH_2O) was pre-prepared with a Milli-Q ultra-pure system (18.2 MΩ/cm).

6.4.2 Preparation of AuNSs-Cys-GOx bioconjugates

A recently published method for the synthesis of seedless silver nitrate and ascorbic acid assisted AuNSs by Phiri *et al*. (Phiri *et al.*, 2019) was followed. Briefly, 10 mL of ddH_2O was acidified with 10 µL of 1M HCl. Thereafter, 50 µL of 100 mM ascorbic acid was added under mild stirring. 50 µL of 50 mM $HAuCl_4$ was added to the mixture. Shortly and rapidly (within 30 seconds), 50 µL of 10mM $AgNO_3$ was added to the solution which resulted in a deep blue colour change within a few seconds. Finally, 500 µL of 2.5 mM PVP was added to the mixture. The prepared AuNSs were cleaned-up by centrifugation for 90 minutes at 3000 *g*. The pellet was then recovered and re-suspended in 2 mL of ddH_20. The subsequent AuNSs-Cys were prepared by adding 100 µL of 0.02 mM Cys to 2 mL of PVP-stabilised AuNSs at pH 7 and left to incubate on a rotator for 3 hours. The final mixture of Cys-modified AuNSs was dialysed in 0.8 mL Pur-A-Lyzer tubes using an in-house non-equilibrium dialysis system to remove excess unbound Cys, and re-dispensed in 1 mL of PBS. The chemical modification of enzyme was prepared by adding 250 mM of freshly prepared EDC/sulfo-NHS to 1 mL of GOx (5 mg/mL) in MES buffer (10 mM, pH 6) and allowed to react for 2 hours. The excess EDC/sulfo-NHS molecules were removed by dialysis, as described above, from the modified enzymes. Finally,

the conjugation of the AuNSs-Cys-GOx bioconjugates was accomplished by pipetting 500 μL of EDC/sulfo-NHS-modified enzymes and was added to 2 mL of AuNSs-Cys and incubated overnight in the fridge. Thereafter, the mixture was centrifuged at 3000 *g* for 30 minutes to remove any unbound enzymes. The AuNSs-Cys-GOx bioconjugates were then resuspended in MES buffer and stored at 4°C until usage.

6.4.3 Characterisations and instrumentations

^{1}H-NMR analyses of the samples in fabrication stages was done according to the method by Venter *et al.,* (Venter *et al.*, 2018). 600 μL of samples were measured at 500MHz on a Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse (TXI) ^{1}H[^{15}N,^{13}C] probe head and x, y, z gradient coils. ^{1}H spectra were acquired as 128 transients in 32K data points with a spectral width of 12002 Hz. Fourier transformation and phase and base line correction were done automatically. Software used for NMR processing was Bruker Topspin (V3.5). Bruker AMIX (V3.9.14) was used for metabolite identification (Ellinger *et al.*, 2013). UV-vis-NIR spectroscopy analyses were carried out by spectral scanning (400–990 nm) on a HT Synergy (BioTEK) microplate reader. The transmission electron microscopy (TEM) analyses were performed on a Tecnai F20 high-resolution transmission electron microscope (HR-TEM) at an accelerating voltage of 200 kV. Samples for TEM were prepared by applying 20 μL of nanoparticle suspension onto carbon 200 mesh copper grids (Agar Scientific), followed by drying overnight prior to imaging.

6.4.4 Stability of AuNSs-Cys-GOx bioconjugates

Using a method applied by Rodríguez-Lorenzo and co-workers to demonstrate the stability of the protein-modified AuNSs (Rodríguez-Lorenzo *et al.*, 2012), the stability of the AuNSs-Cys-GOx bioconjugates was investigated in high ionic strength solutions. The AuNSs-Cys-GOx bioconjugates were centrifuged and resuspended in 300 mM NaCl. UV-vis-NIR spectroscopy was used to measure any aggregation that would be evident by shifts in the LSPRs of the AuNSs to longer wavelengths and flattening of the absorption spectra.

6.4.5 Glucose sensing using AuNSs-Cys-GOx bioconjugates

Glucose determination was carried out to test the feasibility of the use AuNSs-Cys-GOx bio-conjugates' as catalytic nanodevices in a nanobiosensor. A previously optimised method for glucose sensing using differently functionalised AuNSs-GOx (Phiri *et al.*, 2019) was followed for the newly prepared AuNSs-Cys-GOx bioconjugates. Briefly, a range of glucose concentration standards (0.2–2 mM) were added to different reaction wells in a 96-well plate. Each 200 μL reaction solution contained 30 μL AuNSs-Cys-GOx bioconjugates, 1 mM MES buffer, and a

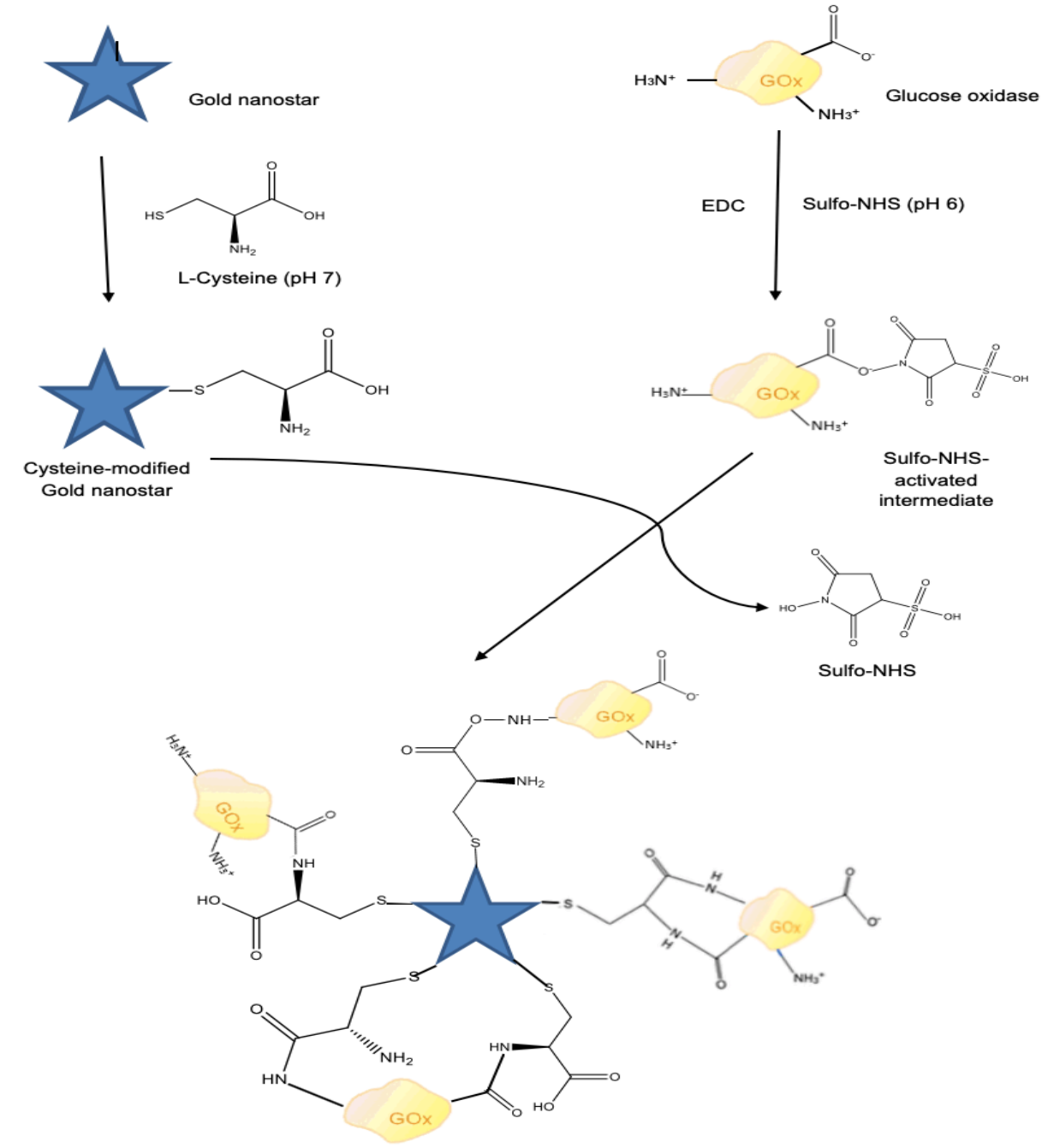
specified concentration of glucose added to it. The mixture was incubated for 1 hour at 37°C after which 12 µL detection solution was added. The detection solution was a combination of 0.1 mM $AgNO_3$ and NH_3 (10 mM)/NaOH (25 mM) equi-volume mixture. Three comparison assays were done to assess if the AuNSs-Cys-GOx bioconjugates offered any advantage in biosensing; 1) AuNSs only without the addition of any GOx to it, as assay controls. 2) AuNSs with 5 µL GOx added to the reaction solutions. 3) Lastly, AuNSs-Cys-GOx bioconjugates in solution as catalytic nanodevices and signal transducers for glucose determination. The detection of glucose was assessed based on shifts in the LSPR peaks on the spectrophotometer and by colour change of the solutions. The mechanism of detection was via biocatalytic enlargement of AuNSs through surface coating of Ag^0 after its reduction by hydrogen peroxide. The hydrogen peroxide itself is a product of the oxidation of glucose in the presence of glucose oxidase and molecular oxygen. These chemical equations depict the reaction:

$$\beta\text{-}D\text{-}Glucose + O_2 + H_2O \xrightarrow{GOx} D\text{-}Gluconic\ acid + H_2O_2$$

$$3H_2O_2 + Ag^+ \xrightarrow{AuNSs} Ag^0 + 3H_2O + \frac{3}{2}O_2$$

6.5 Results and discussion

Scheme 6-1 illustrates and summaries the proposed procedure for the AuNSs-Cys-GOx bioconjugation approach.



Plausible bioconjugations of enzyme to gold nanostar through amide bond formation (not drawn to scale)

Scheme 6-1 A schematic representation of the procedure and plausible covalent attachments of GOx to Cys-modified AuNSs. The gold nanostars are firstly modified by cysteine at pH 7, while the glucose oxidase also reacts with EDC/sulfo-NHS in different tubes. The modified gold nanostars and glucose oxidase are then mixed in a conjugation reaction.

After the removal of excess PVP from the AuNSs, AuNSs-Cys were obtained via ligand exchange reaction at pH 7. At this neutral pH, the AuNSs-Cys system is known to form stable

Au-S structures (Zhao *et al.*, 2013). The thiol bond between Cys and AuNSs leaves a secondary amine and a carboxylate group at the terminal end of the molecules. The enzyme was chemically modified by incubation in EDC/sulfo-NHS in MES buffer at pH 6 to form an active ester before conjugation with an amide- or carboxylate-containing group (Hermanson, 2013b). EDC was used in order to react with a carboxylate group on the enzyme to form an active ester leaving group. Sulfo-NHS was added to the EDC reaction to increase the solubility and stability of the active intermediate, which ultimately reacts with the attacking amine/carboxylate groups from the Cys. The advantage of EDC/sulfo-NHS coupled reactions are that they are highly efficient and tend to increase the yield of conjugation significantly over that obtainable solely with EDC (Hermanson, 2013b). The Cys-modified AuNSs and NHS-terminated GOx could couple in a number of plausible conjugation reactions (Scheme 1) to form amide bond linkages. Thus, a GOx monolayer was covalently immobilised on the surface of AuNSs in such a way as to avoid nonspecific binding of the protein, and to potentially increase both affinity and stability of GOx attachment of the AuNSs (Bezbradica *et al.*, 2014; Hermanson, 2013a; Li *et al.*, 2007; Wade Jr, 2003).

Figure 6-1 ^{1}H-NMR spectra showing discernible peak shifts and splitting as the gold nanostars are conjugated with the glucose oxidase in a stepwise manner: (I) L-Cysteine, (II) Cysteine-modified AuNSs, (III) NHS-terminated glucose oxidase, and (IV) AuNSs-Cys-GOx bioconjugates.

^{1}H NMR spectra of the AuNSs-GOx (Figure 6-1) shows discernible shifts and splitting on the modified molecules compared to pure standards. Spectrum (I) shows free Cys compared to AuNSs-Cys (II). In the region between 3.0 and 4.5 ppm, where representative peaks for Cys exist at 3.25 ppm (CH_2) and 4.10 ppm (CH), the AuNSs-Cys shows that these peaks are drawn together at 3.75 ppm, indicating a shift most likely due to the specific interaction of gold with the sulphur. There is likely an increase in electron density and plausible formation of hydrogen bond between the gold surface bound Cys molecules and the neighbouring bound Cys of the next gold particles, also observed in other studies (Aryal *et al.*, 2006; Leff *et al.*, 1996; Patil *et al.*, 1999). Spectrum (III) shows the peaks for the NHS-terminated GOx. The NHS-terminated GOx's spectrum shows the shifts and different peaks which are due to the esterification of the GOx with EDC/sulfo-NHS — indicating a successful chemical modification of the enzyme. Spectrum (IV) shows the bioconjugation of AuNSs-Cys with NHS-terminated GOx with slightly shifted peaks for the ester-activated enzyme and the AuNSs-Cys, all the three essential molecules in the bioconjugation approach indicating a successful AuNSs-Cys-GOx conjugations. Other recommended spectroscopic techniques such as Fourier transform infrared (FTIR) and C-NMR for structural elucidations employed in conjugation studies (Sapsford *et al.*, 2013), and as done in some studies (Raghu *et al.*, 2007a; Raghu *et al.*, 2007b), were however not carried out in this study due to unavailability.

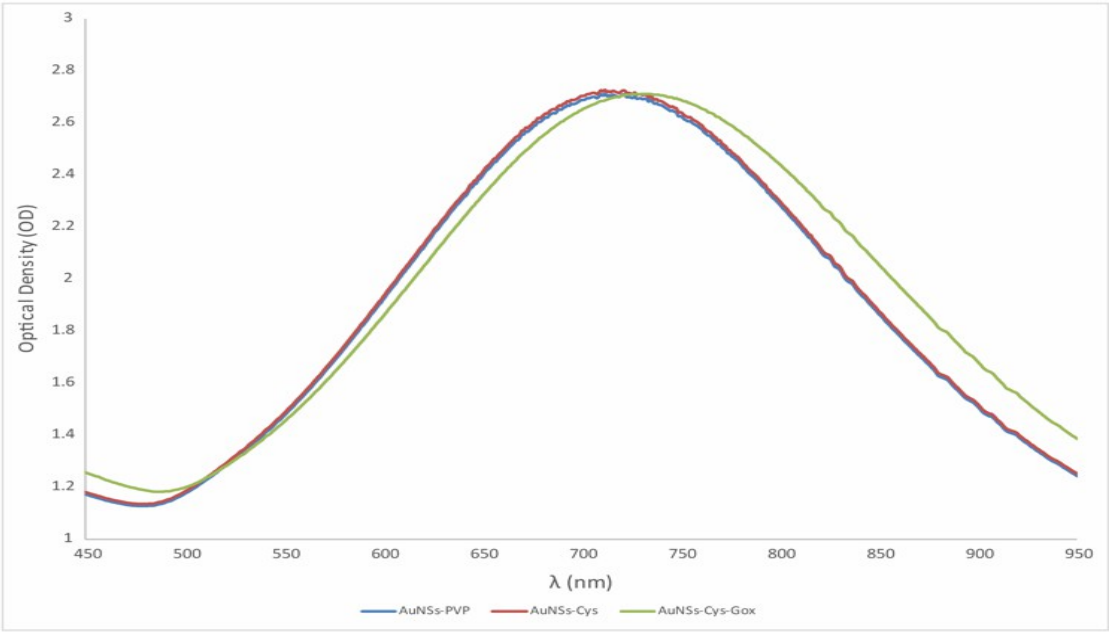


Figure 6-2 Normalized UV-vis-NIR spectra of PVP-stabilized AuNSs, Cysteine-modified AuNSs and AuNSs-Cys-GOx bioconjugates.

Figure 6-2 shows the UV-vis-NIR spectra of AuNSs functionalised with different ligands and their corresponding shifts in LSPR. The AuNSs that were modified with 10^{-6} M Cys had their LSPR at 716 nm compared to 712 nm for the control PVP-stabilised AuNSs. This denotes a slight red shift, probably due to adsorption of Cys molecules on gold surface via the Au–S bond and a so-formed dielectric monolayer of thiol around AuNSs (Li *et al.*, 2007; Nath & Chilkoti, 2002). The LSPR for AuNSs-Cys-GOx bioconjugates was at 732 nm, showing a further red shift by 16 nm due to surface modification with 6.25 e^{-4} gmL^{-1} GOx. The red shift in the GOx-modified AuNSs is plausibly because of changes in the dielectric properties after the attachment of the enzymes to the nanosensors. No broadening of the LPSR spectrum for GOx-modified AuNSs was observed which implied the maintenance of the structural integrity of the AuNSs after conjugation (Li *et al.*, 2007; Xi *et al.*, 2018).

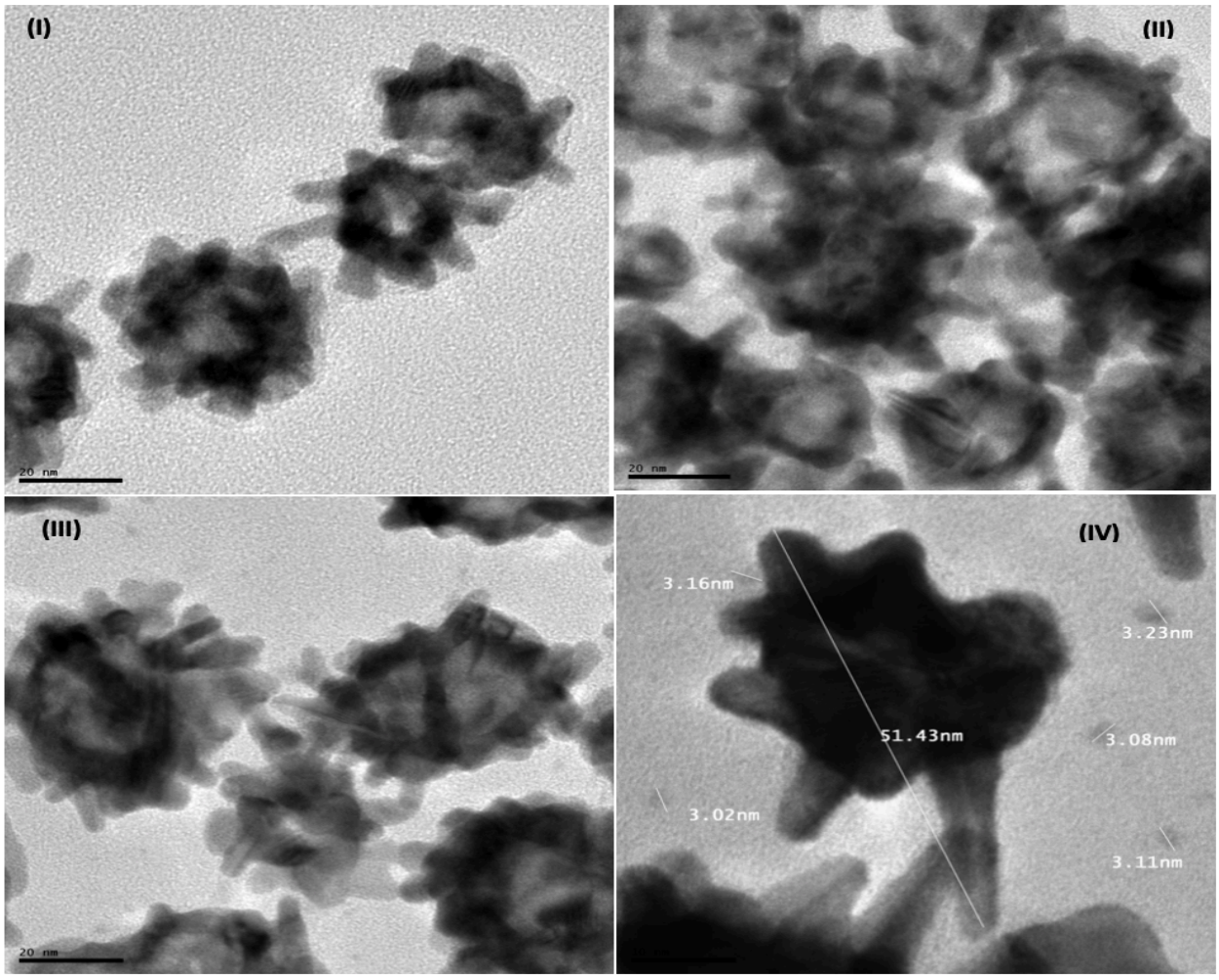


Figure 6-3 HR-TEM images of PVP-stabilized AuNSs (I), Cysteine-modified AuNSs (II), AuNSs-Cys-GOx bioconjugates without staining (III) and AuNSs-Cys-GOx bioconjugates stained by 1% silver nitrate (IV).

The morphology of the differently functionalised AuNSs was characterized by HR-TEM. Figure 6-3(I) shows PVP-stabilised multi-branched gold nanostars. The cysteine-modified AuNSs (Figure 6-3(II)) showed AuNSs agglomeration possibly due to the formation of bonds between the surface bound Cys molecules of adjacent AuNSs-Cys (Aryal *et al.*, 2006). Figure 6-3(III & IV) show the AuNSs-Cys-GOx bioconjugates without and with staining with 1% silver nitrate at different magnifications. The TEM image of AuNSs show good dispersity even after conjugation with GOx (Supplementary information, Fig. 1). The protein layer on the peripheral of the AuNSs surface could not be imaged without staining with 1% silver nitrate due to low electron resistance of protein molecules in HR-TEM examination (Li *et al.*, 2007). However, the staining with silver nitrate enabled the visualisation of some silver nanoparticles around the protein

domain that were formed via reduction by the enzyme GOx (Li *et al.*, 2007). Thus, the enzyme layer was observed as small dark spots of 3.12 ± 0.08 nm around the nanostars particles. Li *et al.*, (Li *et al.*, 2007), reported similar observations in visualising the protein GOx after staining with silver nitrate. The AuNSs-Cys-GOx bioconjugates exhibited structural integrity and good dispersity in solution.

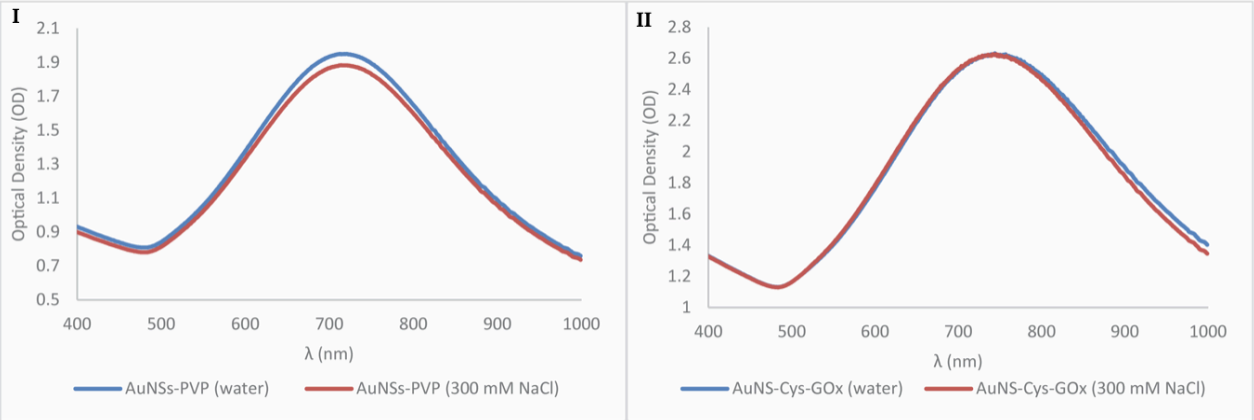


Figure 6-4 Comparison of UV-vis-NIR spectra of (I) PVP-stabilised AuNSs and (II) AuNSs-Cys-GOx bioconjugates in ddH_2O and 300 mM NaCl solutions for ionic stability tests.

The stability of the fabricated AuNSs-Cys-GOx bioconjugates in solution was tested in solution of high ionic concentration. Figure 6-4 shows that there was no variation in the LSPR peaks by broadening and/or flattening of the AuNSs-Cys-GOx curve in salt compared to the one in ddH_2O. In comparison, the PVP-stabilised AuNSs had a slight decline in maximum absorbance by about 3% implying some loss of stability in the salt solution. This demonstrated the extra stability the functionalised proteins added to the AuNSs relative to the PVP-stabilised AuNSs. Application of functionalised nanostars in biological samples requires them to be stable in solutions containing high concentrations of proteins and salts (Wangoo *et al.*, 2008). If the AuNSs are not functionalised sufficiently, they are prone to aggregation in high ionic strength solutions in which the van der Waals attraction is stronger than the steric repulsion provided by the functionalisation molecule (Rodríguez-Lorenzo *et al.*, 2012; Wangoo *et al.*, 2008).

The AuNSs-Cys-GOx bioconjugates were assessed as catalytic nanodevices in the oxidation of glucose and its plasmonic colorimetric sensing. The sensitivity and stability of the bioconjugates in glucose sensing was assessed based on the comparison assays. The mechanism for the sensing was enzyme guided coating of silver ions onto the AuNSs surfaces. Figures 6-5, 6 and 7 show the results of the comparison using differently functionalised AuNSs.

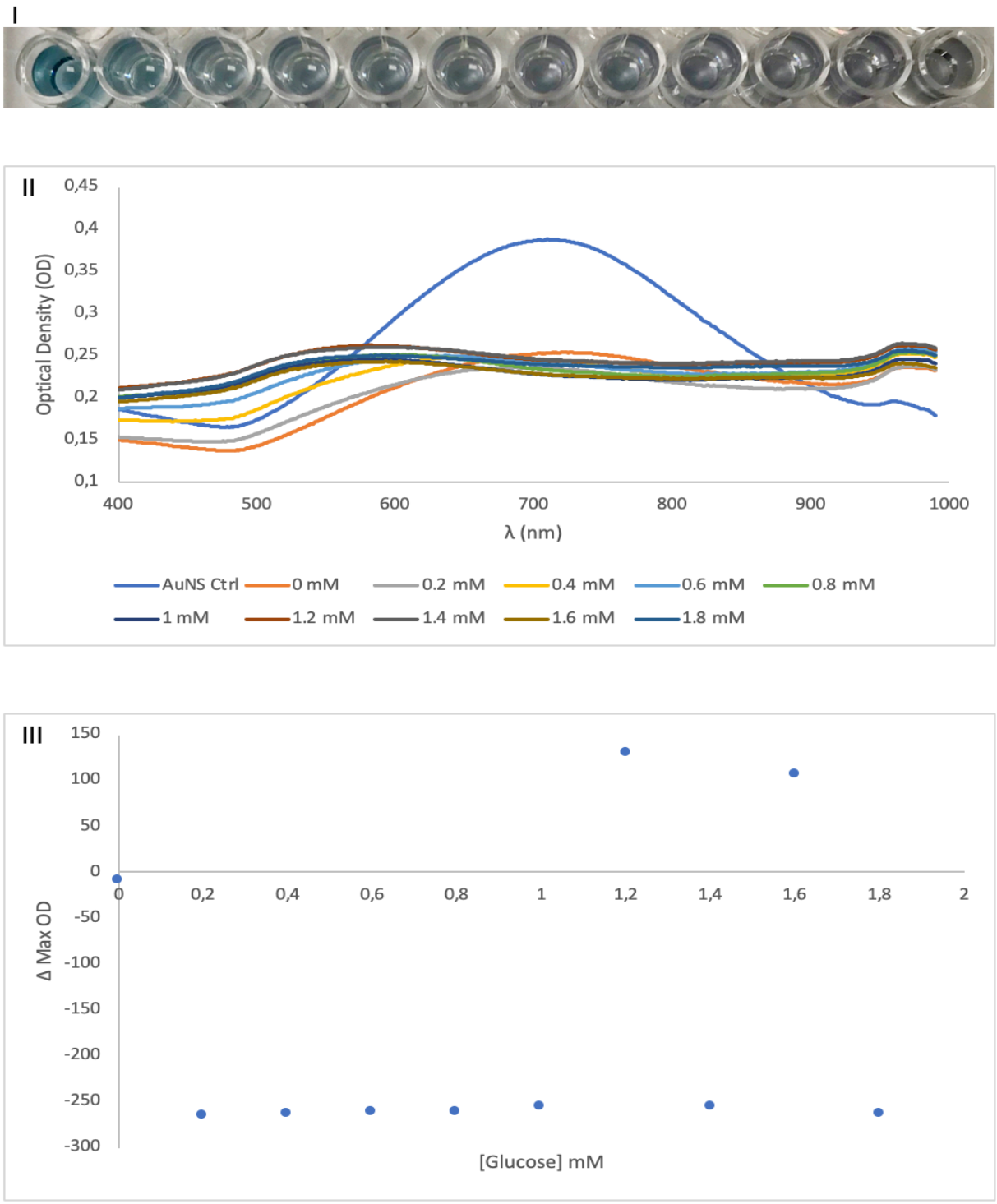
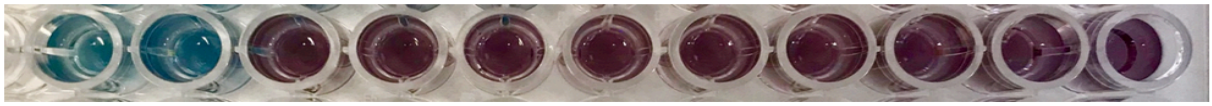


Figure 6-5 Shows (I) the colorimetric photograph, (II) UV-vis-NIR spectra of the mixture of 1 mM MES buffer (pH 6) and PVP-stabilised AuNSs in the presence of varying concentrations of glucose, and (III) a plot of peak shifts vs glucose concentration.

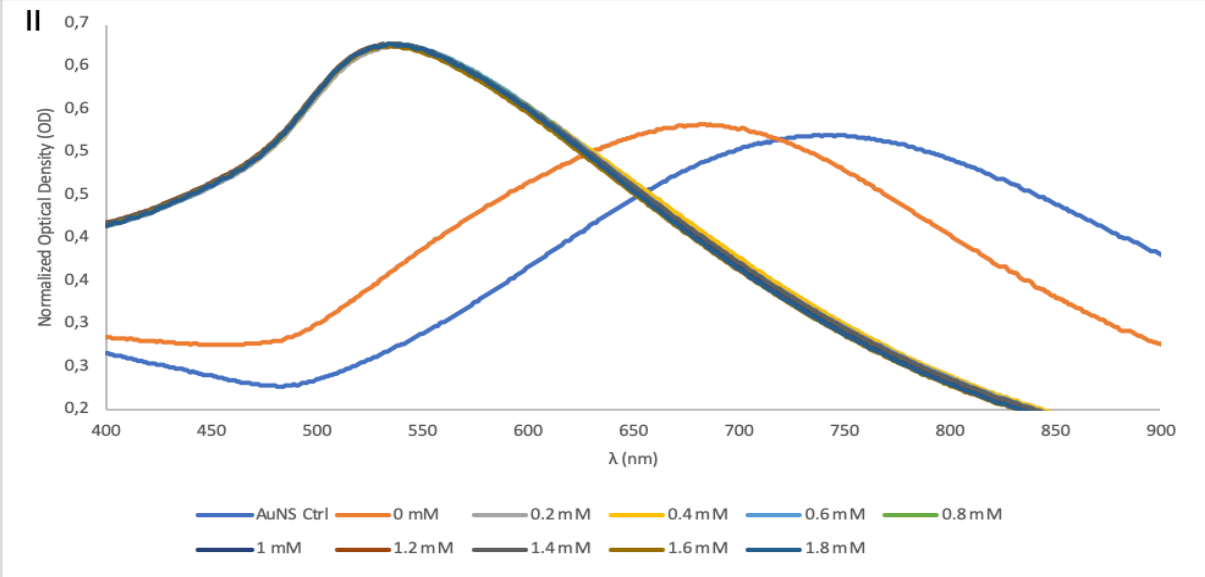
Figure 6-5 shows the results for glucose sensing and detection using AuNSs without any GOx added to the reaction solutions. The AuNSs without any enzyme added to them could not

distinguish between the different concentrations of glucose in solution. In fact, the AuNSs aggregated upon the adjustment of the pH to >9 after the addition of detection solution as shown in the colorimetric photograph. The UV-vis-NIR spectra also confirmed this aggregation by the flattening of the absorption spectra in comparison to the control that had neither glucose nor detection solution added to it. Gold nanoparticles have been reported to act as nanozymes that mimic glucose oxidase in the oxidation of glucose (Gao & Yan, 2016; Tang & Li, 2017). It was clear from the observation made from this particular assay that AuNSs without sufficient functionalisation with glucose oxidase were poor catalytic nanodevices for glucose sensing. Furthermore, poor stability was also observed in the presence of detection solution.

I



II


Normalized Optical Density (OD)
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500
550
600
650
700
750
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850
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λ (nm)
AuNS Ctrl
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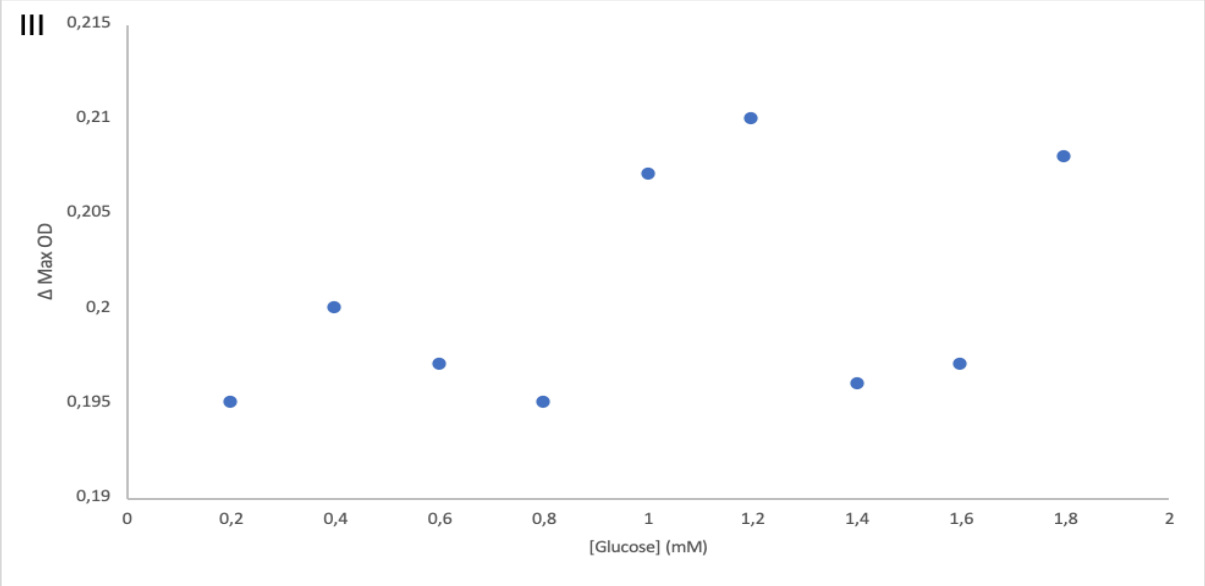
III


Δ Max OD
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0
0,2
0,4
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1
1,2
1,4
1,6
1,8
2
[Glucose] (mM)

Figure 6-6 Shows (I) the colorimetric photograph, (II) Normalised UV-vis-NIR spectra of the mixture of 1 mM MES buffer (pH 6), 5 µL GOx solution, and PVP-stabilised AuNSs in the presence of varying concentrations of glucose, and (III) a plot of peak shifts vs glucose concentration.

Figure 6-6 shows the assay with GOx added to the solutions in the reaction well. In this case, the GOx was attached to the AuNSs via physisorption. In comparison to the one assay without GOx added to it, there was observable colour change in response to the presence of glucose in the solution. The colour changed from blue to purple but the assay could not clearly discriminate between different glucose concentrations. As a result, there was no predictable correlation between signal generated and the varying concentrations of glucose. Physisorption is reportedly a poor method for enzyme immobilisation as it tends to lead to covering of the active site onto the immobilisation surface affecting the biorecognition and oxidation of the target analyte (Putzbach & Ronkainen, 2013; Sapsford *et al.*, 2013). This would explain why there was no discernible differences in the signal generated for different glucose concentrations.

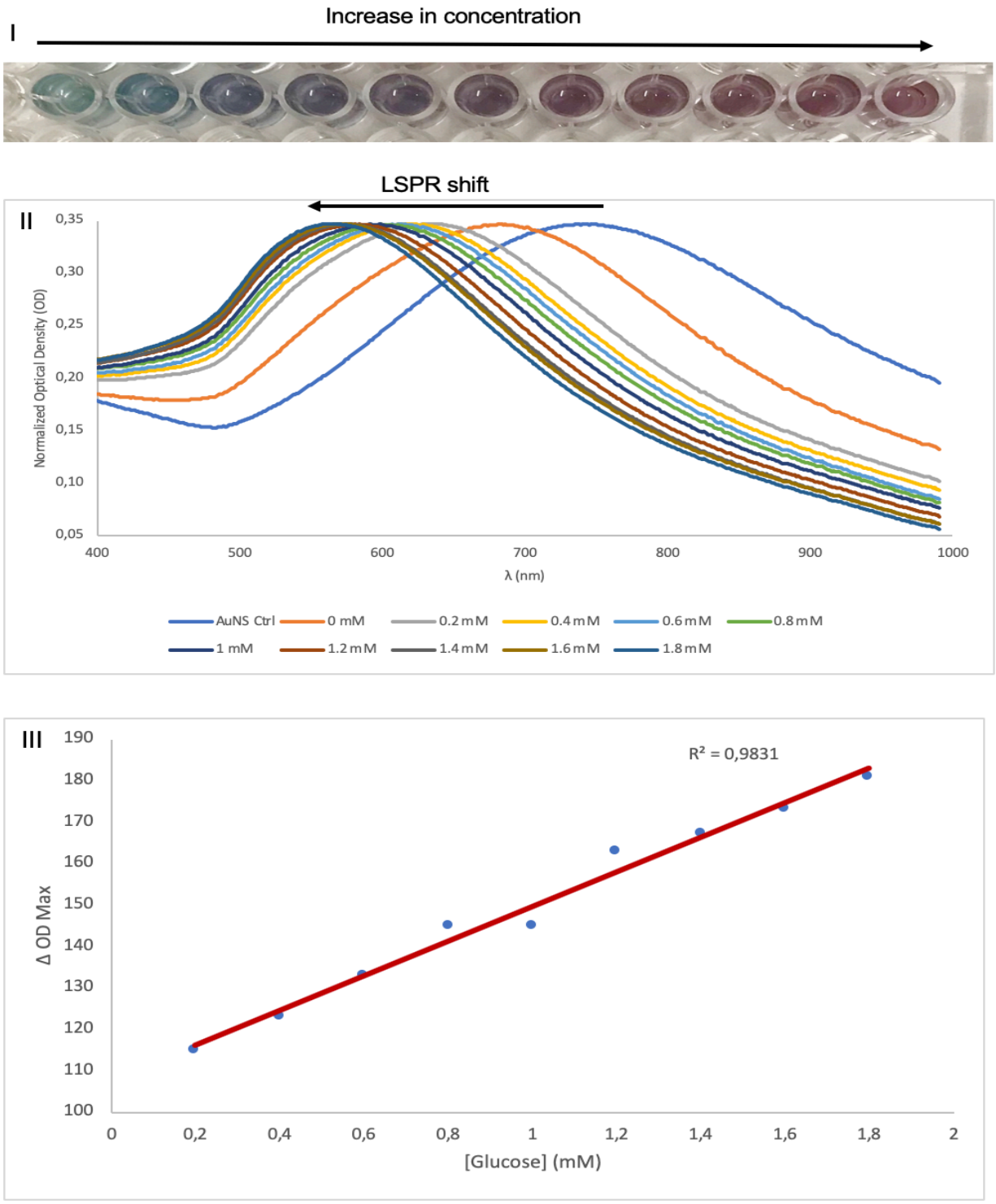

Figure 6-7 Shows the colorimetric photograph of glucose assay, Normalised UV-vis-NIR spectra of the mixture of 1 mM MES buffer (pH 6), 5 µL GOx solution, and AuNSs-Cys-GOx bioconjugates in the presence of varying concentrations of glucose, and a plot of peak shifts vs glucose concentration.

Figure 6-7 shows the glucose assay using the AuNSs-Cys-GOx bioconjugates. The AuNSs-Cys-GOx bioconjugates showed distinguishable differences, both in colour and LSPR peak shifts, in response to different concentrations of glucose. The colours changed from blue to dark blue to purple and pink. The coefficient of determination between signal generated and concentration of glucose demonstrated a good model fit for the detection of glucose (R^2 > 0.98). The linear range was observed to be from 0.2 mM to 1.8 mM glucose. The limit of detection for this method was calculated to be 0.04 mM glucose. This showed that the AuNSs-Cys-GOx bioconjugates fabricated in the proposed approach had potential to be used for detection of low concentrations of analytes with greater sensitivity and stability compared to those of physisorption and bare AuNSs. With further optimisations in the conjugation process and the signal generation procedure, this could be used and extended to other enzymatic and antibody assays that utilise oxidases.

6.6 Conclusion

In summary, a bioconjugation approach for attachment of enzymes to gold nanostars is proposed that is simple and easy to replicate. This approach showed the ability to generate stable and sensitive AuNSs-Cys-GOx bioconjugates. The conjugation procedure potentially increased attachment affinity without protein adsorption onto the AuNSs surface by modifying the AuNSs with Cys. The production of an active ester intermediate on the enzyme using EDC/sulfo-NHS introduced a number of functional groups for covalent binding. The ratio of enzymes per nanoparticle was increased due to the many available functional groups between Cys and the NHS-terminated GOx for covalent attachment. As opposed to only functionalising with EDC plus sulfo-NHS which is a zero-length crosslinker molecule, this approach enabled some relative separation distance between the enzyme and the AuNSs by the use of Cys, thus enabling attachment with the active site uncovered. The method produced AuNSs-Cys-GOx bioconjugates that maintained optimum function and activity for both the GOx and AuNSs. The produced AuNSs-Cys-GOx bioconjugates displayed greater sensitivity and stability in glucose sensing in comparison to the ones where the enzyme was simply added to the reaction well. All this demonstrated the potential the method has to fabricate AuNSs-enzyme bioconjugates for biosensing applications. Further studies are being pursued to replicate this bioconjugation strategy for immobilisation of other oxidases in nanobiosensors.

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CHAPTER 7 OPTIMISATION FOR PLASMONIC DETECTION OF GLUCOSE IN SERUM BASED ON BIOCATALYTIC SHAPE-ALTERING OF GOLD NANOSTARS

7.1 Preface

Design strategies for the use of the nanosensors in complex biological matrix (serum) is reported in this chapter. The reduction of interferences from the sample matrix was optimised. The mechanism for the detection was also elucidated. The optical assay approach reported for the detection of glucose, as a proof-of-concept analyte at low concentrations using optical readout formats allowing for the use of cheap spectrophotometer for analysis, besides the naked eye in very dilute samples. Such an assay format is much needed in resource-constrained regions and provides a model for expansion to other tests such as immunoassays.

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7.2 Abstract

Nanoparticles have been used as signal transducers for optical readouts in biosensors. Optical approaches are cost-effective with easy readout formats for clinical diagnosis. We present a glucose biosensor based on biocatalytic shape-altering of gold nanostars via silver deposition. Improved sensitivity was observed due to nanostars clustering after being functionalised with glucose oxidase (GOx). The biosensor quantified glucose in serum samples with a 1:1000 dilution factor, and colorimetrically distinguished between concentrations. The assay demonstrated good specificity and sensitivity. The fabricated glucose biosensor is a rapid kinetic assay using basic entry level laboratory spectrophotometric microplate reader. Such a biosensor could be very useful in resource-constrained regions without state-of-the-art laboratory equipment. Furthermore, naked-eye detection of glucose makes this a suitable biosensor for technology transfer to other point-of-care devices.

7.3 Introduction

Much interest was sparked in the further development of improved enzyme-based biosensors for glucose monitoring (Ferri *et al.*, 2011) after Clark and Lyons introduced their glucose monitor using glucose oxidase (GOx) and an oxygen electrode (Clark Jr & Lyons, 1962). The measurement of glucose is of major importance as the incidence of diabetes continues to increase due to improved global living standards (Roglic, 2016). Many methods have since been designed for glucose detection, including electrochemistry (Bandodkar *et al.*, 2014; Bihar *et al.*, 2018; Ferri *et al.*, 2011), fluorescence (Hu *et al.*, 2014; Yi *et al.*, 2013), surface-enhanced Raman scattering (SERS) (Hu *et al.*, 2017; Qi *et al.*, 2016), and surface plasmon resonance (Jiang *et al.*, 2010; Radhakumary & Sreenivasan, 2011; Wang *et al.*, 2013; Xianyu & Jiang, 2014).

Electrochemical glucose biosensors are the most widely used clinically (Qi *et al.*, 2016). This approach, however, suffers from a number of disadvantages such as complexity of electrode preparation, lack of stability in signal acquisition, inactivation of electrodes by the generated H_2O_2, relatively high cost, and reproducibility concerns (Bihar *et al.*, 2018; Jacobs *et al.*, 2010; Qi *et al.*, 2016; Xianyu & Jiang, 2014; Zhang *et al.*, 2015). Other detection strategies such as SERS have also been explored owing to its high sensitivity. The method has the ability to provide clear fingerprint information to identify the structures of molecules (Gkogkou *et al.*, 2016; Hu *et al.*, 2017; Qi *et al.*, 2016; Shafer-Peltier *et al.*, 2003; Yonzon *et al.*, 2004). The disadvantage of using this method for glucose measurement is that glucose has an inherent weak Raman activity making it difficult to detect by Raman spectroscopy. Despite efforts for SERS enhancements, the weak surface adsorption ability of glucose produces relatively weak SERS signal (Qi *et al.*, 2016).

Optical detection approaches have the advantage of being cost-effective with an easy readout format (Guo *et al.*, 2016; Radhakumary & Sreenivasan, 2011). A number of optical approaches for glucose sensing based on nanoparticle plasmon resonance have been reported in the last decade (Radhakumary & Sreenivasan, 2011; Sabu *et al.*, 2019). These glucose sensors have been used either as enzyme- or non-enzyme-based assays (Gao *et al.*, 2017; Nguyen *et al.*, 2018; Sabu *et al.*, 2019). Using enzymes has had the advantage of increasing specificity of the assays (Xianyu & Jiang, 2014). GOx, isolated and produced from the fungus *Aspergillus niger,* is the most popularly used enzyme for glucose monitoring due to its very high substrate specificity (Ferri *et al.*, 2011; Xianyu & Jiang, 2014). This enzyme oxidizes glucose in the presence of molecular oxygen producing hydrogen peroxide (H_2O_2) which is used to determine glucose concentration (Ferri *et al.*, 2011; Sabu *et al.*, 2019; Xianyu & Jiang, 2014).

Four main strategies have been applied in optical signal generation using nanoparticles. These include, (i) nanoparticle aggregation; (ii) surface etching; (iii) fluorescence quenching; and (iv) nanocrystal growth (Radhakumary & Sreenivasan, 2011; Rodríguez-Lorenzo *et al.*, 2012; Willner *et al.*, 2006; Xiong *et al.*, 2015; Zayats *et al.*, 2005). Nanoparticle aggregation is the most commonly employed strategy for sensing. The disadvantage with this method however, is that it lacks specificity in signal generation as many other factors in solution may cause particle aggregation (Jiang *et al.*, 2010; Radhakumary & Sreenivasan, 2011; Xianyu & Jiang, 2014). Biocatalytic growth of nanoparticles for signal amplification has been applied for the development of many assays (Guo *et al.*, 2015; Rodríguez-Lorenzo *et al.*, 2012; Xianyu *et al.*, 2014; Yan *et al.*, 2008; Zayats *et al.*, 2005). This mechanism has allowed the tuning of the plasmonic nanoparticle shape and size resulting in different optical properties (Tang & Li, 2017). Nanostructure shape altering mechanism of detection is one of the strategies for enhancing the sensitivity of plasmonic nanosensors. As one of the anisotropic nanocrystals—AuNSs—exhibit higher refractive index sensitivity compared to spherical nanoparticles (Guo *et al.*, 2015). LSPR sensing based on shape alterations induced by an external stimulus is highly sensitive to changes in the conditions within the colloidal or detection solution (Langer *et al.*, 2015). Particle size growth is more sensitive with small sized nanoparticles as they have higher absorption rates compared to larger particles such as AuNSs (Guo *et al.*, 2015).

Most nanoparticle-based glucose biosensors developed were conducted in either buffer systems, urine, or saliva (Ali *et al.*, 2017; Bihar *et al.*, 2018; Cash & Clark, 2010; Cheng *et al.*, 2015; Guo *et al.*, 2015; Mehrotra, 2016; Radhakumary & Sreenivasan, 2011). Yet, venous plasma or serum are the recommended body fluids for clinical glucose diagnosis (Association, 2018; Ladenson *et al.*, 1974; Nader *et al.*, 2018; Zimmet *et al.*, 2016). Some methods have however reported using other biological samples such as spinal cerebral fluids or cell culture media supplemented with foetal bovine serum (Aldewachi *et al.*, 2018; Urban *et al.*, 2016). The complexity of a biological matrix substantially increases the probability of undesirable interferences and side reactions. Therefore, the use of buffered systems is likely to give inaccurate impression about the usability of nanomaterials in clinical diagnostics (Jiang *et al.*, 2010; Melby *et al.*, 2017; Moore *et al.*, 2015; Urban *et al.*, 2016).

Plasma or serum has many proteins and lipoproteins that form a corona around the nanoparticle and changes its physiochemical properties based on the biomaterials coated around it (Aldewachi *et al.*, 2018). This corona affects the effective diameter of the nanoparticles, and increases chances of aggregation. Depending on the identity of the molecule, the biomolecular corona effectively changes the behaviour of the nanoparticles. Metallic nanoparticles may be etched in serum, making the detection in biological samples

difficult (Casals *et al.*, 2010; Cash & Clark, 2010; Hirsch *et al.*, 2014; Jenkins *et al.*, 2015; Kumar *et al.*, 2016; Melby *et al.*, 2017; Monopoli *et al.*, 2011; Moore *et al.*, 2015; Petros & DeSimone, 2010; Salvati *et al.*, 2013; Urban *et al.*, 2016; Verma & Stellacci, 2010; Walkey & Chan, 2012). There is thus need to develop and optimise an optical biosensor that is stable, sensitive and robust enough for detection of clinical samples.

In this work, a sensitive, specific, and rapid optical glucose sensor based on biocatalytic shape-altering of gold nanostars AuNSs is presented. The biosensor was fabricated using the optimum conditions for functionalisation of the seedless AuNSs with GOx for enhanced stability and functionality. The functionalised AuNSs were tested for stability in various fluids and thereafter optimised for glucose sensing in spiked serum samples. Lastly, a number of analytical parameters such as specificity, kinetics, and recovery of the glucose assay were investigated.

7.4 Methods and materials

7.4.1 Materials

Hydrochloroauric acid ($HAuCl_4$), glucose oxidase (GOx), trisodium citrate, silver nitrate ($AgNO_3$), ascorbic acid, sodium chloride (NaCl), polyvinylpyrrolidone (PVP) (molecular weight 10000), hydrochloric acid (HCl), glucose, 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6, *N*-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), sulfo-*N*-Hydroxysuccinimide (sulfo-NHS), glucose, Cysteine (Cys) and phosphate buffered saline (PBS) at pH 7.4 were all purchased from Sigma-Aldrich, South Africa. Ham's F-12K (Kaighn's) cell culture medium used was and supplemented with 10% foetal bovine serum (FBS), which were purchased from ThermoFisher Scientific. Medidrug Basis-line S human blank serum was purchased from Industrial Analytical, South Africa. All glassware was stripped with Aqua Regia prior to use for synthesis. Ultrapure water (ddH_2O) was pre-prepared with a Milli-Q ultra-pure system (18.2 MΩ/cm).

7.4.1 Preparation of AuNSs-Cys-GOx bioconjugates

Synthesis of the PVP-stabilised seedless AuNSs and subsequent GOx bioconjugation were done using recently published methods by Phiri et al (Phiri *et al.*, 2019a; Phiri *et al.*, 2019b). Briefly, 10 mL of ddH_2O was acidified with 10 µL of 1M HCl followed by the addition of 50 µL of 100 mM ascorbic acid under mild stirring. Shortly after the addition of 50 µL of 50 mM $HAuCl_4$ to the mixture, 50 µL of 10mM $AgNO_3$ was rapidly added to the solution which resulted in a deep blue colour change within a few seconds. Finally, 500 µL of 2.5 mM PVP was added to the mixture. Immobilisation of the enzyme onto the gold nanostars were prepared by adding 100 µL of 0.02 mM Cys to 2 mL of PVP-stabilised AuNSs after their clean-up and left to incubate on a

rotator for 3 hours. The chemical modification of enzyme was prepared by adding 250 mM of freshly prepared EDC/sulfo-NHS to 1 mL of GOx (5 mg/mL) in MES buffer (10 mM, pH 6) and allowed to react for 2 hours. Finally, the conjugation of the AuNSs-Cys-GOx bioconjugates was accomplished by pipetting 500 μL of EDC/sulfo-NHS-modified enzymes and adding it to 2 mL of AuNSs-Cys and incubated overnight in the fridge. The mixture was thereafter centrifuged at 3000 *g* for 30 minutes to remove any unbound enzymes. The AuNSs-Cys-GOx bioconjugates were resuspended in MES buffer and stored at 4°C until usage.

7.4.2 Characterizations and instrumentations

UV-vis-NIR spectroscopy analyses were carried out by spectral scanning (400–990 nm) on an HT Synergy (BioTEK) microplate reader. The transmission electron microscopy (TEM) analyses were performed on a Tecnai F20 high-resolution transmission electron microscope (HR-TEM) at an accelerating voltage of 200 kV. Samples for TEM were prepared by applying 20 µL of nanoparticle suspension onto carbon 200 mesh copper grids (Agar Scientific), followed by drying overnight prior to imaging. ImageJ software was used to determine the particles sizes from different grids. ^{1}H-NMR analyses of samples in various fabrication stages was done according to the method by Venter et al., (Venter *et al.*, 2018). Six hundred microliters of samples were measured at 500MHz on a Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse (TXI) ^{1}H[^{15}N,^{13}C] probe head and x, y, z gradient coils. ^{1}H spectra were acquired as 128 transients in 32K data points with a spectral width of 12002 Hz. Fourier transformation and phase and base line correction were done automatically. Software used for NMR processing was Bruker Topspin (V3.5). Bruker AMIX (V3.9.14) was used for metabolite identification (Ellinger *et al.*, 2013).

7.4.3 Stability of AuNSs-Cys-GOx bioconjugates

The stability of the AuNSs-Cys-GOx bioconjugates were tested in MES buffer, blank serum, unsupplemented - and supplemented cell culture solutions. The GOx-modified gold nanostars were centrifuged and resuspended in 200 uL of the above-mentioned fluids. UV-vis-NIR spectroscopy was used to investigate the stability of the bioconjugates and TEM analyses were done to observe the morphology and dispersity of the AuNSs in these fluids.

7.4.4 Enzyme activity assays

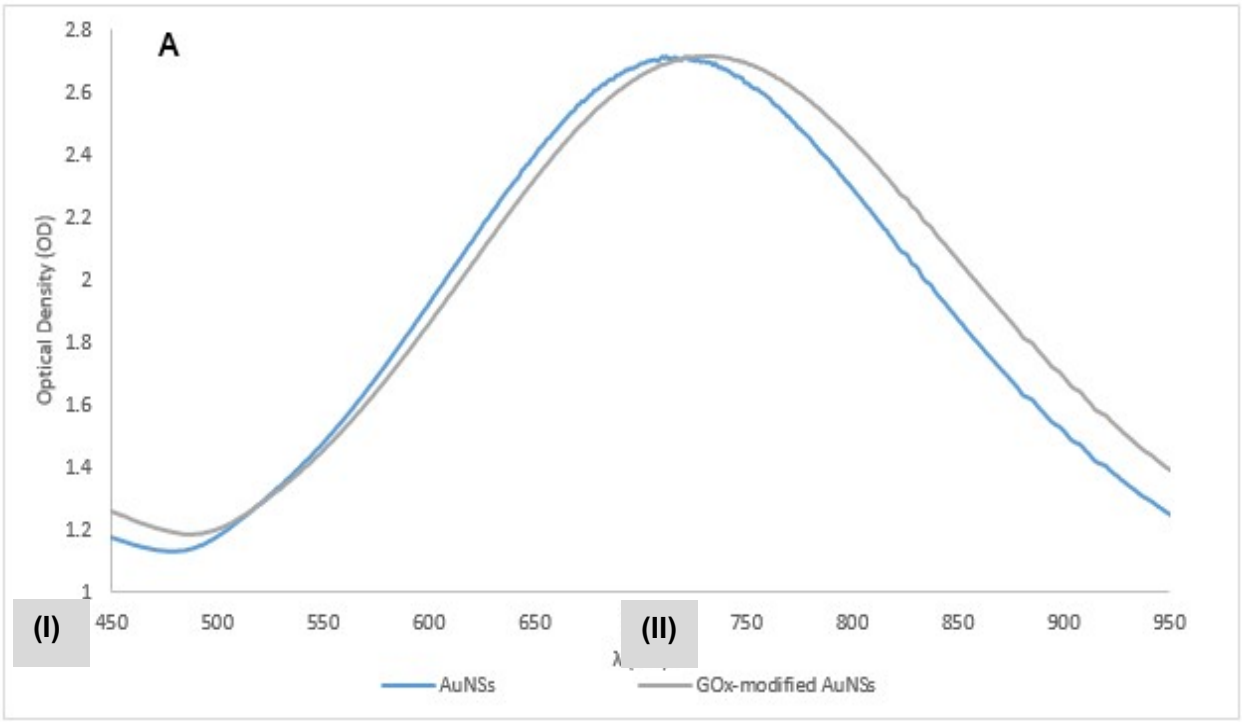
The optimised assay parameters reported in the recent study (Phiri *et al.*, 2019a) were used to compare signal generation for the determination of glucose in serum and MES buffer using AuNSs-Cys-GOx bioconjugates. The detection of glucose was assessed spectrophotometrically based on shifts in the LSPR peaks, and optically by colour changes of the solutions. Kinetic

reads were done at 550 nm to determine the rate of signal generation while after incubation. A number of analytical parameters were evaluated to assess the developed biosensor. Using spiked blank serum with varying concentrations of glucose, the specificity and calibration model were determined. The specificity was determined by spiking the serum with other glucose analogues such as fructose, galactose and sucrose and their signal responses compared under optimum conditions. Additionally, the sensing performance of the biosensor was assessed with serum lipids and cysteine instead of glucose. All experiments were carried out in triplicate.

7.5 Results and discussion

7.5.1 Characterisation of GOx-modified AuNSs

Figure 7-1A shows that UV-vis-NIR spectra of the PVP-stabilised AuNSs had their LSPR peak at 712 nm and those modified by the enzyme at 732 nm. This showed a redshift of 16 nm for the GOx-modified AuNSs bioconjugates from the PVP-stabilised ones. The change in optical properties of the AuNSs was a result of the surface functionalisation with GOx. HR-TEM images in Figure 7-1B showed the morphologies of the PVP-stabilised AuNSs and the enzyme-modified AuNSs. The enzyme layer attached on the peripheral surface of the AuNSs could not be imaged due to low electron resistance of protein molecules in HR-TEM examination (Li *et al.*, 2007). Thus, the HR-TEM was useful for imaging the structural integrity of AuNSs after enzyme attachment. Figure 7-1C shows the 1H-NMR spectra with discernible shifts and splitting on the modified molecules. The cysteine-modified AuNSs showed that these peaks were drawn together at 3.75 ppm, indicating a shift most likely due to the specific interaction of gold with the sulphur, as observed in other studies (Aryal *et al.*, 2006; Leff *et al.*, 1996; Patil *et al.*, 1999). Spectrum (II) showed the bioconjugation of cysteine-modified AuNSs with GOx (using EDC/sulfo-NHS chemistry). The peaks slightly shifted for the ester-activated enzyme covalently coupled to AuNSs-Cys, thereby indicating a successful AuNSs-Cys-GOx conjugation.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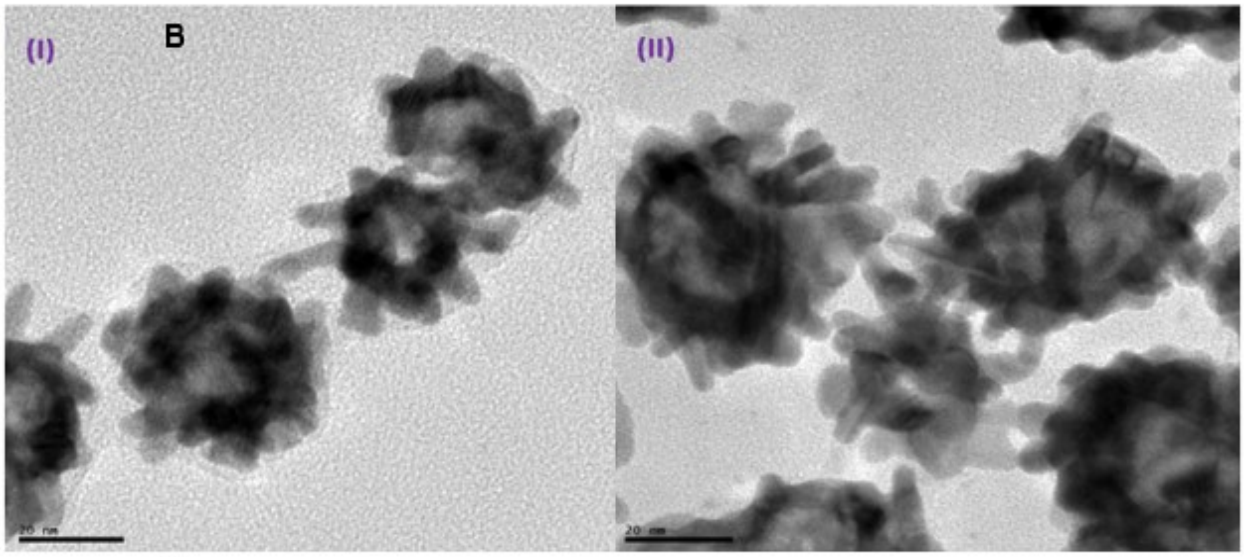
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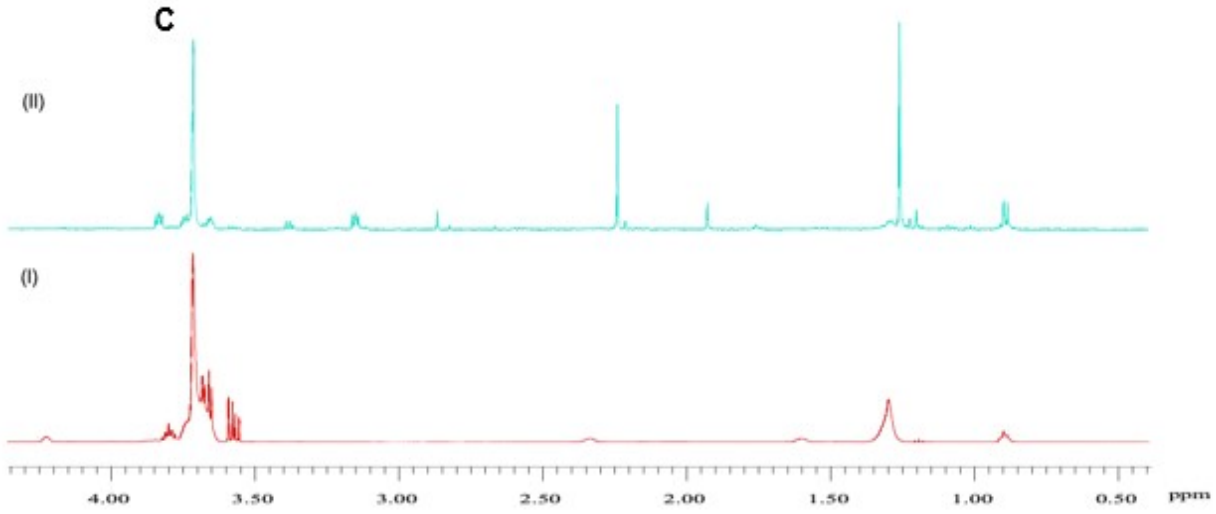
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Figure 7-1 (A) Normalised UV-vis-NIR spectra showing a red shift for the GOx-modified AuNSs. (B) HR-TEM images of (I) PVP-stabilized AuNSs, and (II) GOx modified AuNSs. (C) ^{1}H-NMR spectra shows two peaks with discernible shifts and splitting after the addition of glucose oxidase to cysteine modified AuNSs: (I) Cysteine-modified AuNSs, and GOx-modified AuNSs using cysteine and EDC/sulfo-NHS for bioconjugation.

7.5.2 Stability and characterisation of AuNSs-Cys-GOx bioconjugates in various media

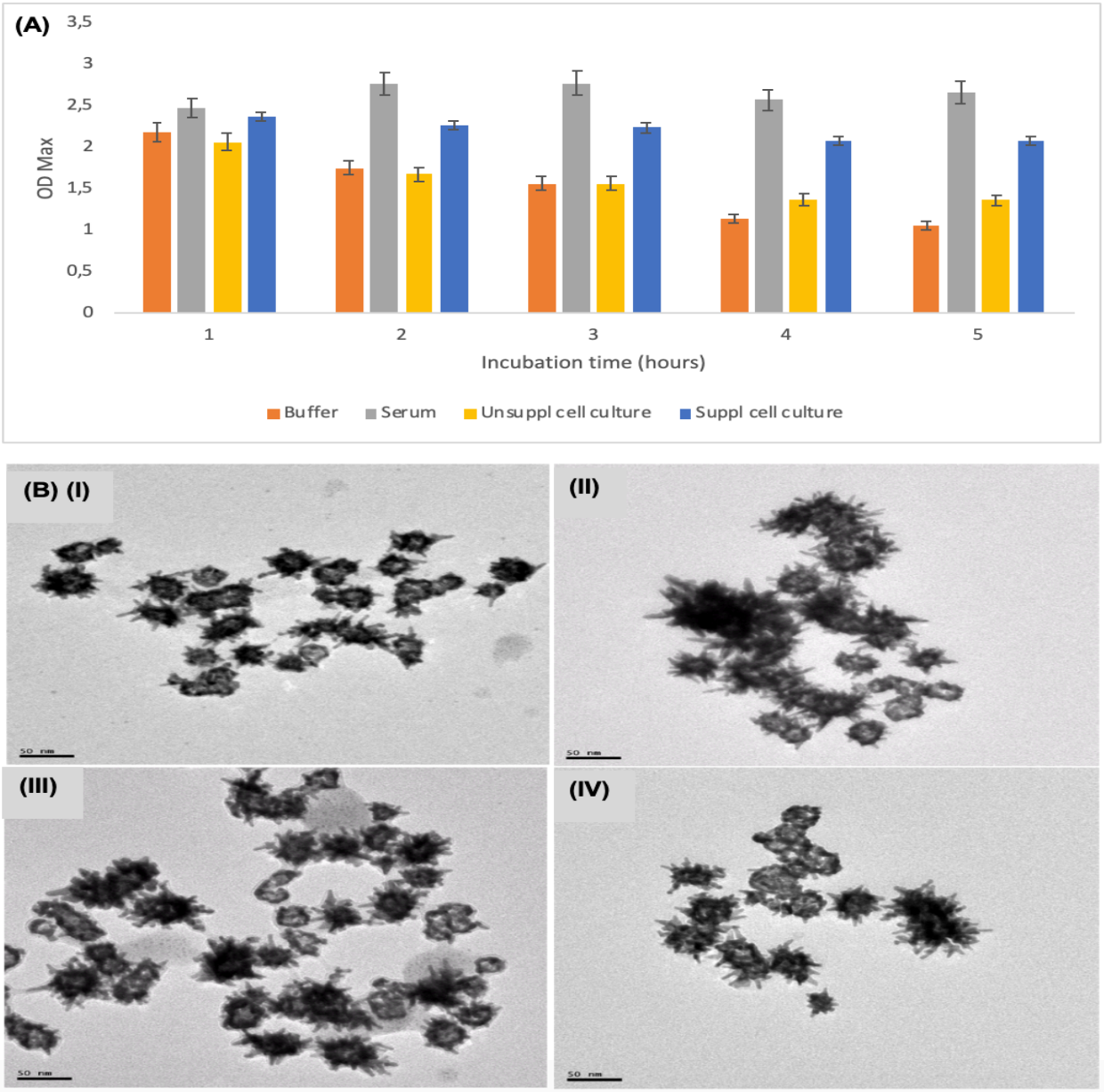


Figure 7-2 (A) The stability of AuNSs-Cys-GOx bioconjugates in different media based on UV-vis-NIR spectroscopy over a 24 hours period. (B) TEM images of the AuNSs-Cys-GOx

bioconjugates in different sample matrices: (I) in buffer; (II) in serum; (III) in unsupplemented cell media; and (IV) in supplemented cell media.

Figure 7-2A shows the stability of the GOx-modified AuNSs in different media based on change in optical densities over time. The maximum absorption of the bioconjugates in MES buffer and un-supplemented cell culture medium were observed to decline with time due to possible aggregation. However, when incubated in serum the maximum optical density increased in the period between 0–2 hours and slightly declined thereafter. The increase in absorption at λmax is probably due to the complexation of unspecified serum proteins (Melby *et al.*, 2017). The UV-vis-NIR spectra of the bioconjugates in serum displayed stability that was slightly greater than those in supplemented cell culture medium. The AuNSs in supplemented cell culture medium showed a steady decline UV-vis-NIR absorption in the period between 0 to 6 hours before stabilising over the remaining hours. This is probably due to the FBS with proteins likely to bind to nanoparticles resulting in relative better stability in media (Hirsch *et al.*, 2013; Moore *et al.*, 2015). Figure 7-2B shows the morphologies of the AuNSs-Cys-GOx bioconjugates in these different matrices. Of note is the agglomeration of the AuNSs in serum (II). Agglomeration was observed for these AuNSs compared to those in the other matrices. A similar observation was made for AuNSs incubated in FBS supplemented cell culture medium (IV). The particle agglomeration observed in these two fluids could be attributed to the changes in surface properties brought about by biomolecules and ionic strength in serum that forms a corona around the nanoparticles (Jenkins *et al.*, 2015; Melby *et al.*, 2017; Urban *et al.*, 2016).

7.5.3 Optimisations of glucose detection conditions in serum

The foregoing observations showed that the AuNSs-Cys-GOx bioconjugates displayed both good stability in serum, as well as nanoparticle clustering. Interestingly, the nanostars clustering was recently reported to be an advantage for improving sensitivity in plasmonic assays (Park *et al.*, 2015). The AuNSs-Cys-GOx bioconjugates thus—characterised by enhanced stability and improved sensitivity—were a fit candidate for biosensor fabrication as nanodevices for detection of glucose in serum. Initial attempts at signal generation using serum sample volumes ≥20 µL proved futile. Despite various attempts to generate a signal, no observable changes in colour were observed. The failure in signal generation of the nanosensors was attributed to the nanoparticle physiochemical properties changes in complex matrices such as serum (Hirsch *et al.*, 2013; Hirsch *et al.*, 2014; Jenkins *et al.*, 2015; Moore *et al.*, 2015).

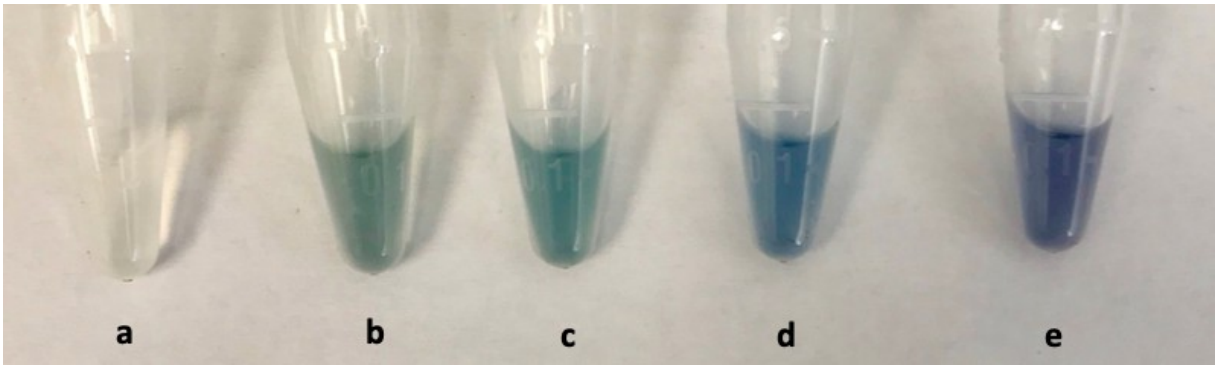
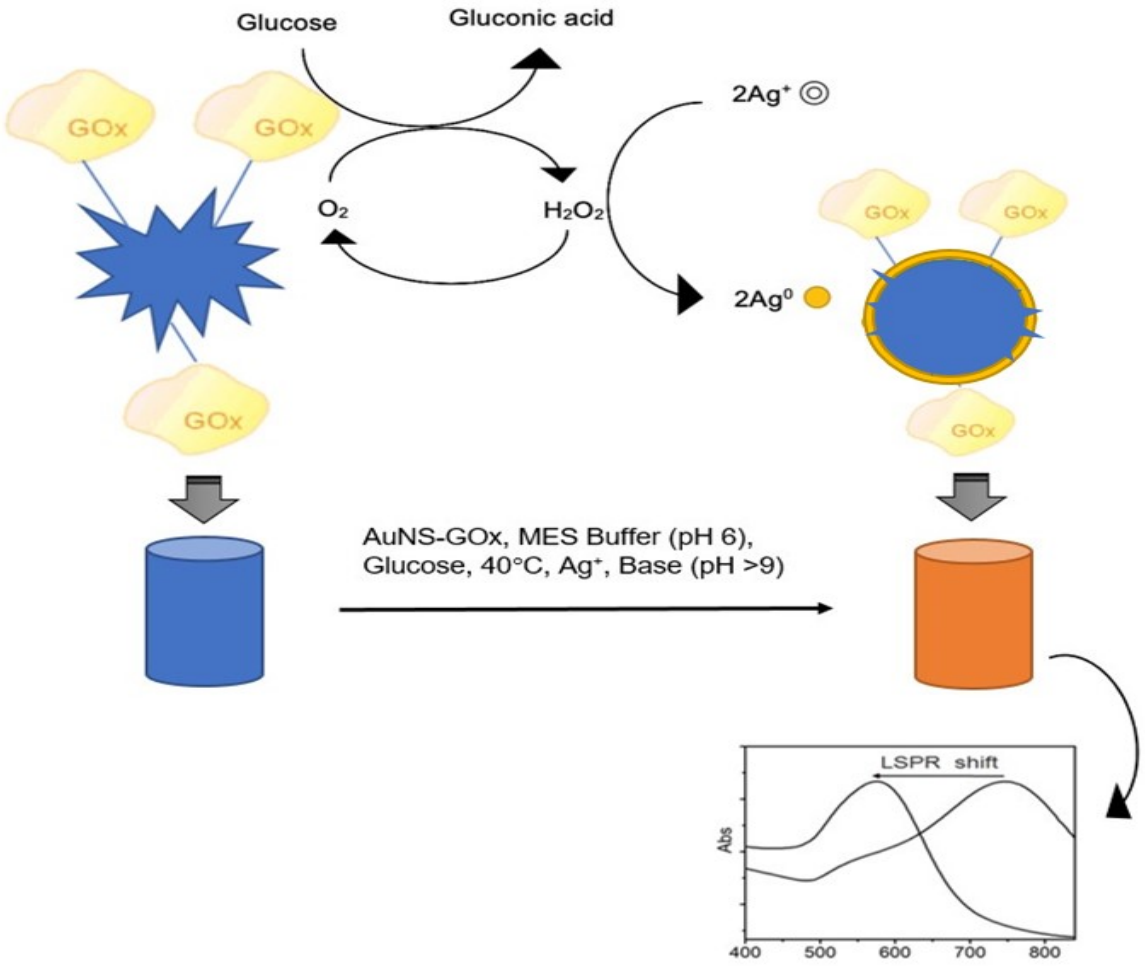


Figure 7-3 Feasibility of glucose detection in serum diluted 100 times using AuNSs as detectors: (a) blank serum; (b) control sample with AuNSs; (c) with 0 mmol/L glucose and detection solution; (d) 0.001 mmol/L glucose; and (e) 0.002 mmol/L glucose.

However, when the sample was serially diluted 1000 times, colour changes were observed as shown in Figure 7-3. The concentrations of $AgNO_3$ in the detection solution was also optimised to a final concentration of 0.25 mM to generate visible colour changes. The biosensor showed ability to differentiate between concentrations of glucose at 1000 times dilution. Sample serial dilution of 1:100–1000 was observed to be optimum for detection of an analyte in serum using the fabricated nanodevices. Such a great sample dilution factor offers the advantages of reducing the effects of complex sample interferences (Rodriguez-Lorenzo *et al.*, 2012), and allows the quantifying of samples whose concentration is very low, and whose volumes are ultra-low. For simplicity's sake and reducing batch variations in experimentations, a dilution factor of 1:100 was chosen for further experiments that enabled the use of 2 µL of serum in a total reaction mixture of 200 µL.

7.5.4 Plasmonic glucose detection by means of AuNSs shape-altering

Scheme 7-1 shows the proposed signal-generation mechanism. The silver ions, reduced by H_2O_2 produced from oxidised glucose, coated around the plasmonic AuNSs resulting in a shape alteration. The extent of the change in AuNSs morphology depended on the concentration of glucose in solution. Thus, nanospheres are potential end-result morphologies of this reaction.



Scheme 7-1 Proposed signal-generation mechanism by means of biocatalytic shape altering of gold nanostars (adapted with permission from Phiri et al (Phiri *et al.*, 2019b)).

Different concentrations of glucose were measured in MES buffer and in serum to observe the influence each fluid had on the AuNSs detection. Figure 7-4A showed that there was a marked difference in the colour change depending on the sample matrix. More varied colours were observed when the measurement was done in MES buffer compared to serum. The colour changed from blue to purple to orange in MES buffer while in serum it was from blue to intensified blue to a deep purple colour. Yet in both matrices there was visible distinctions in colour with increasing concentrations of glucose. This provided an opportunity for this biosensor to be developed and optimised for screening of biological samples with naked eye detection.

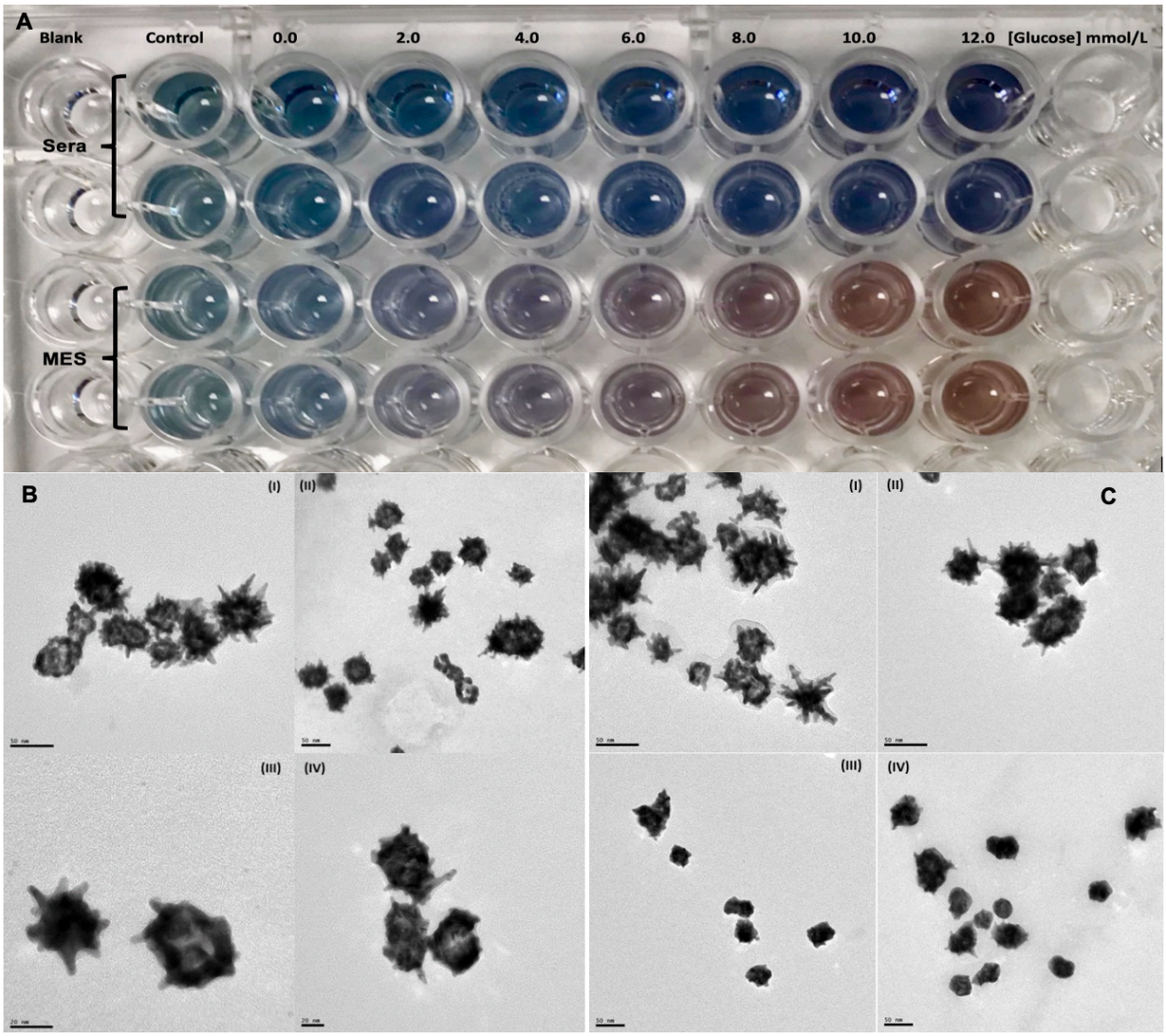


Figure 7-4 (A) Photograph for colour change in the detection solution with glucose concentrations in the AuNSs-Cys-GOx solution for 45 minutes; (B) and (C) TEM images in serum and buffer respectively (I) control, (II) 0, (III) 0.06, (IV) 0.12 mmol/L glucose.

The morphologies of AuNSs corresponding to selected solutions with different glucose concentrations are shown in Figure 7-4B & C. There were clear differences in the change of morphology for AuNSs in serum and MES that explain the degree of the colour changes. In serum, the AuNSs had relatively slight changes in morphology compared to the ones in MES solution. The AuNSs in MES solution became more spherical in morphology due to the silver coating as the glucose concentration increased.

Table 7-1 Changes in AuNSs diameters corresponding to different glucose concentrations in triplicate.

[Glucose]	AuNSs (control)	0 mmol/L	0.06 mmol/L	0.12 mmol/L
Δd (nm) in serum	57.49 ± 6.24	54.28 ± 6.79	54.15 ± 11.42	56.13 ± 9.08
Δd (nm) in buffer	50.88 ± 8.23	50.31 ± 6.67	48.62 ± 3.57	50.14 ± 80.34

From the analysis of the nanoparticle sizes after being coated with Ag^0 as shown in Table 7-1, there wasn't any observable growth in size compared to changes in their morphologies. As opposed to the signal generation mechanism based on growth on nanoparticles by addition of either Ag^+ or $HAuCl^-$ (Gao *et al.*, 2017; Rodríguez-Lorenzo *et al.*, 2012; Yan *et al.*, 2008; Zayats *et al.*, 2005), this was observed to be merely morphology altering.

The LSPR peak shifts of AuNSs in serum in Figure 7-5A showed a total of 80 nm blueshift. In MES solution however, AuNSs showed a clearer and greater shift of 131 nm from the control to the highest glucose concentration as shown in Figure 7-5B. The reason for this observation in the two sample matrices could be due to the interferences caused by proteins and lipoproteins in serum which can affect the analytical performance of the biosensor (Aldewachi *et al.*, 2018). When the LSPR peak shifts were plotted against the increasing concentration of glucose as shown in Figure 7-5A and B inserts, the correlation coefficient (R^2) in both serum and MES were 0.99. This represented a predictable detectable range for glucose concentration with a 100 times dilution of the sample.

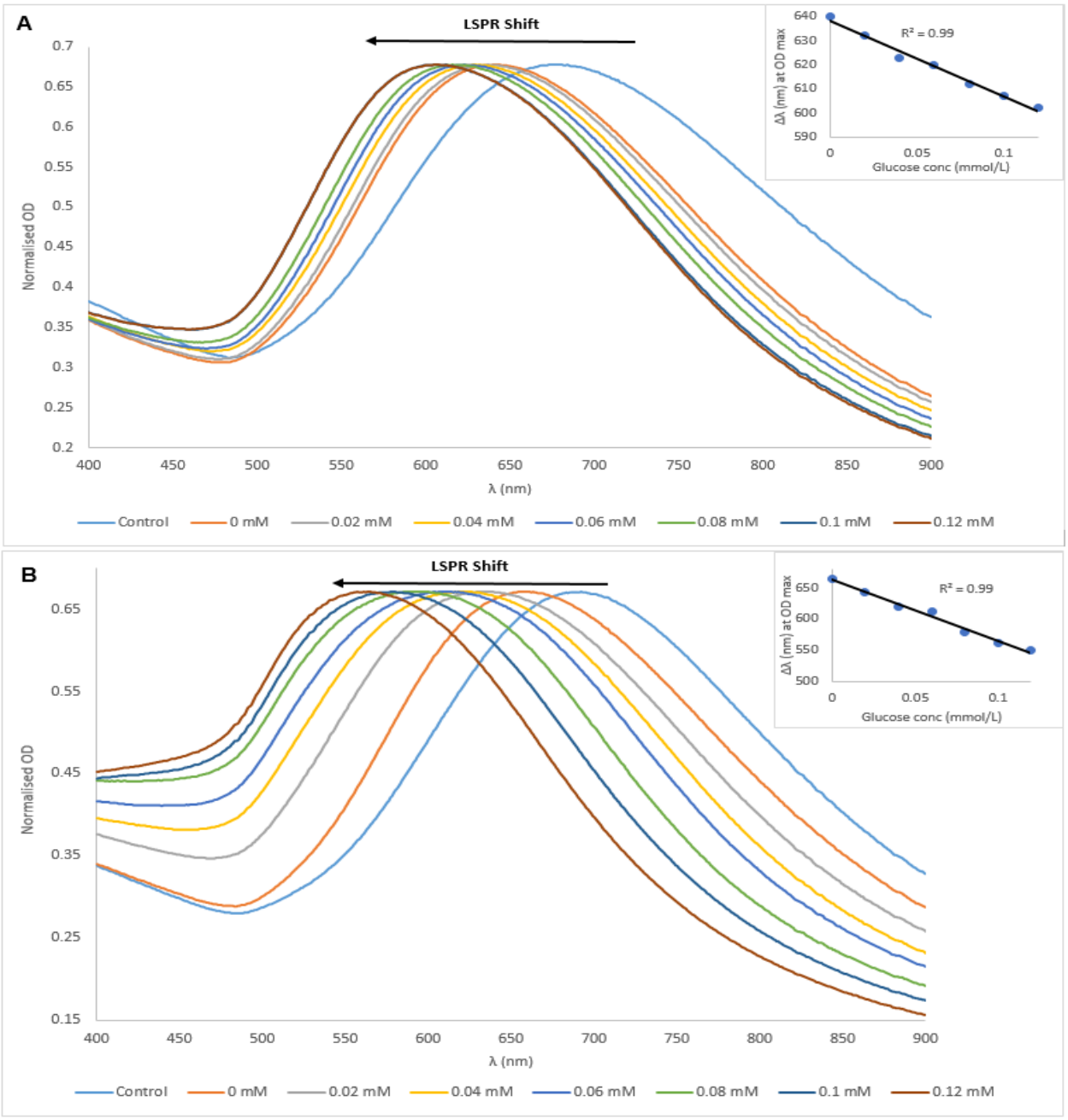


Figure 7-5 UV-vis-NIR spectra of the AuNSs-Cys-GOx bioconjugates and detection solution in the presence of different concentrations of glucose in serum (A) and MES buffer (B); (Inserts) plot of peak shift vs glucose concentration for serum and buffer.

Figure 7-6A shows the kinetic reaction that was monitored 550 nm from the start of incubation with all the optimal reaction conditions with different glucose concentrations. As the reaction proceeded the assay could discriminate between glucose concentrations based on absorbance. The biosensor only required an incubation time of <15 minutes at 37 C for sufficient oxidation of glucose and to generate distinguishable colours and plasmonic shifts for detection. Figure 7-6B

shows signal generation by addition of detection solution after incubation of the reaction mixture at 37 C for 45 minutes. The biosensor was able to generate distinguishable colorimetric and plasmonic signals between glucose concentrations in serum in less than 10 seconds of detection solution addition. Within 5 minutes, the signal generation process was near complete. This demonstrates the rapidity of the biosensor both in incubation time and detection process.

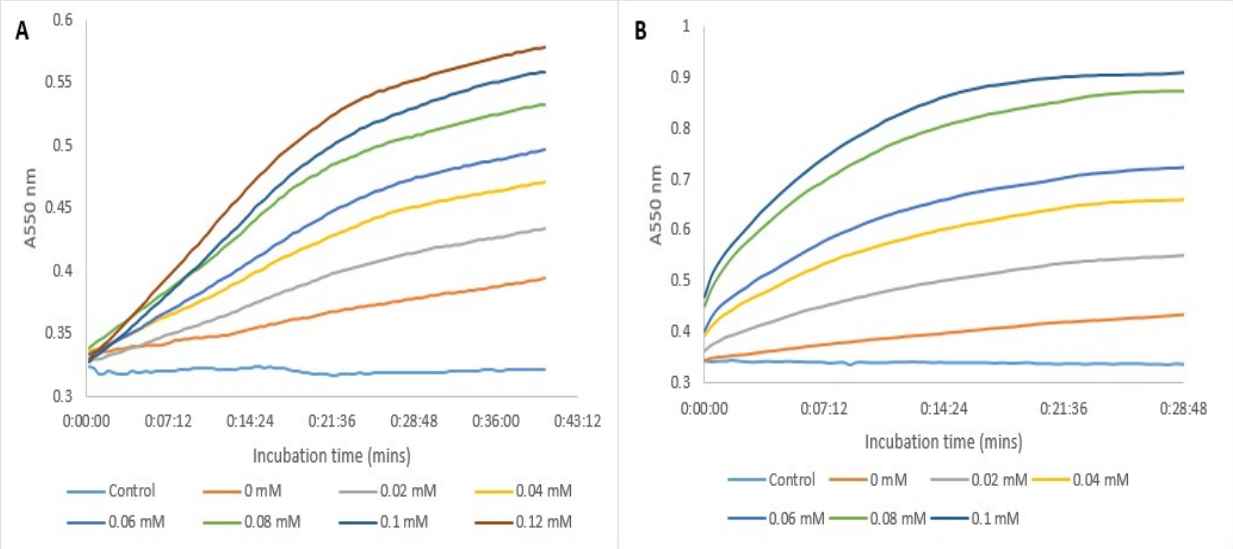


Figure 7-6 (A) Enzyme kinetic process in oxidising glucose and signal generation by deposition of Ag onto AuNSs during incubation at 37 C; (B) kinetic colorimetric detection of glucose after incubation at 37 °C for 45 minutes and addition of detection solution.

7.5.5 Analytical performance of the glucose biosensor

The specificity of the glucose biosensor was investigated by using other saccharides as substrates instead of glucose. Figure 7-7 displays the signal response of the biosensor to these glucose analogues. No significant colour changes or LSPR peak shifts were observed in the presence of other saccharides. The biosensor however, generated a significant response when glucose was the analyte. This observation demonstrated that the signal generation was strongly dependent on the presence of glucose in the reaction solution, and not any other saccharides. The glucose biosensor demonstrated the ability to distinguish presence and absence of glucose within limits of detections in the presence of other structural analogues.

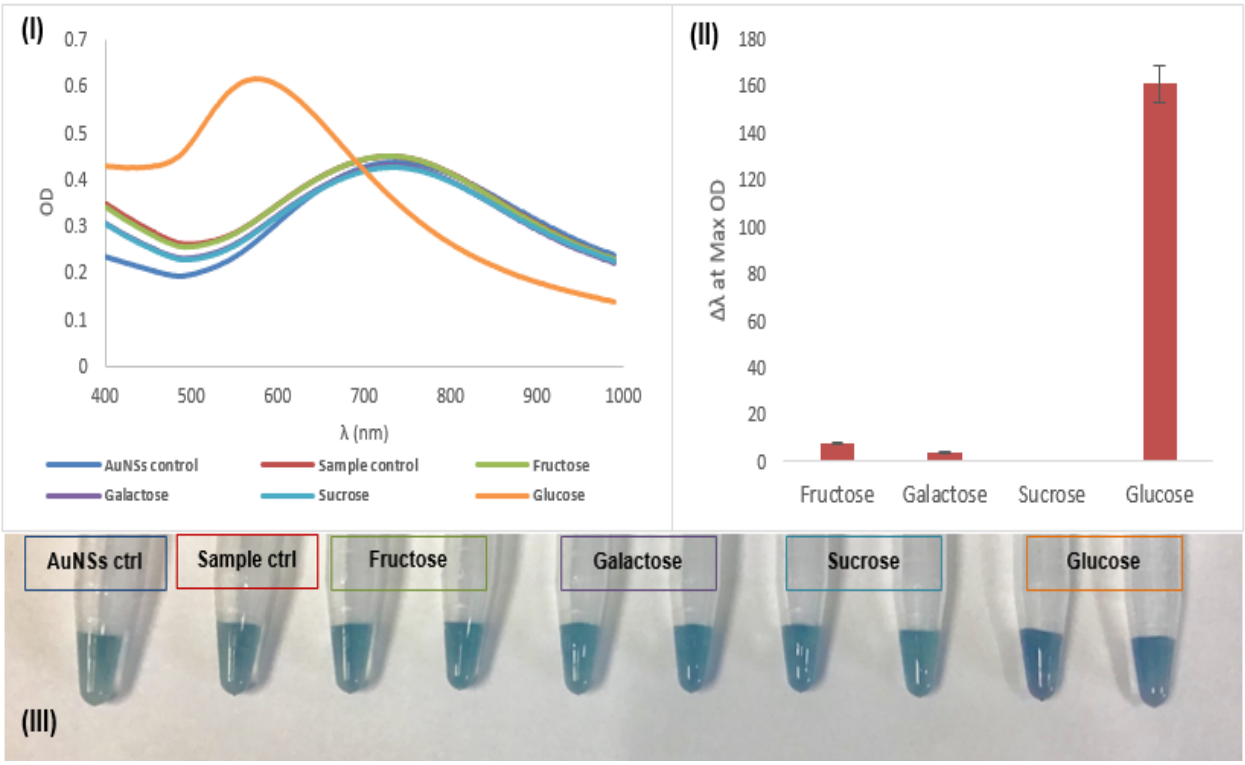


Figure 7-7 (I) UV-vis-NIR spectra of the specificity of the glucose biosensor in the presence of other saccharides at 0.1 mM concentration; (II) Corresponding response in terms of Δλ at OD Max for each saccharide for the glucose biosensor; (III) photograph of the change in colour for the various saccharides.

Furthermore, when L-cysteine and lipids were used as substrates, Figure 7-8A & B showed minimal LSPR peak shifts for these analytes except for glucose. Figure 7-8C shows the colour changes—or lack thereof—of the different analytes. Analytes other than glucose did not produce a change in colour that was significantly different from the blank AuNSs. The biosensor thus demonstrated a specific response for glucose in the presence of potential interferences in serum.

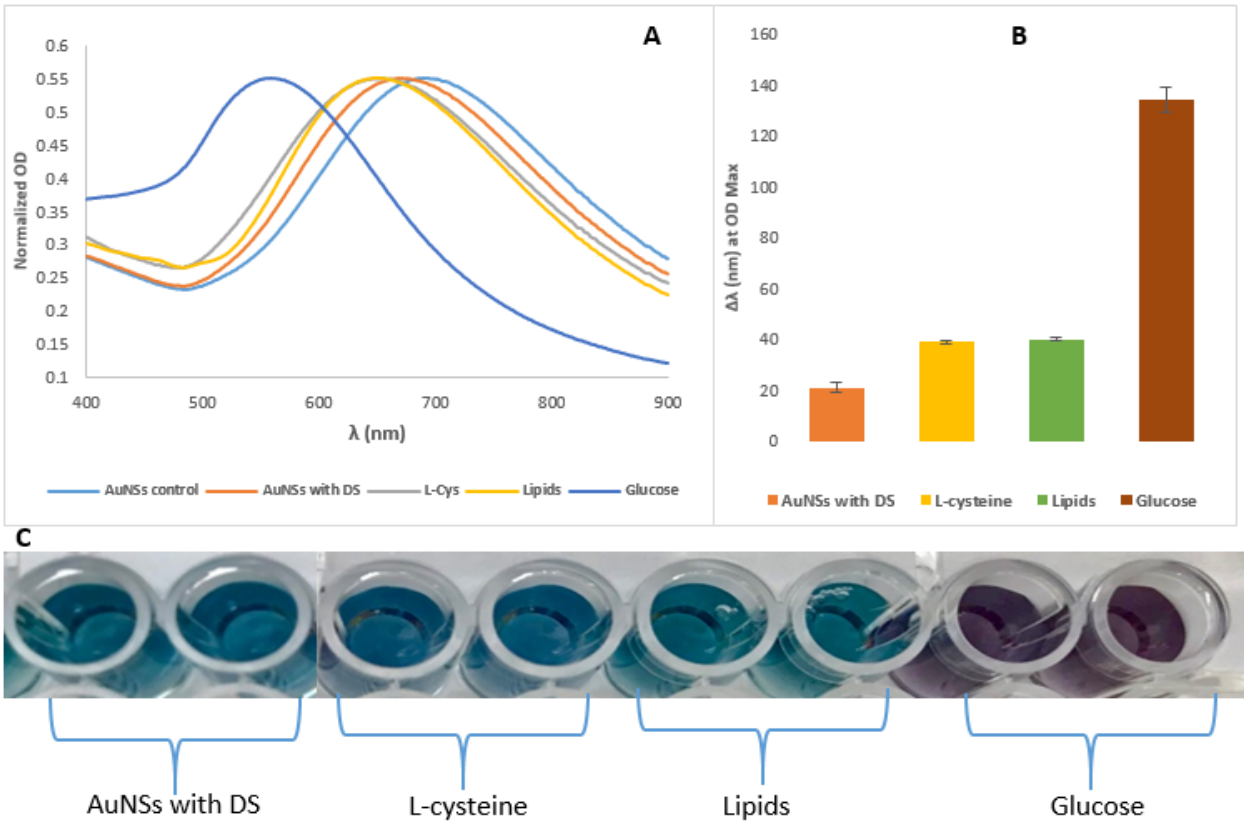


Figure 7-8 (A) UV-vis-NIR spectra of the glucose biosensor's specificity in signal generation in LSPR peak shifts; (B) differences in the magnitude of the LSPR shifts among the compounds all with the concentration of 0.10 mmol/L. The error bars represent the standard deviation of the mean as the reaction was done in triplicates; and (C) photograph for colour changes or its absence in the presence of other analytes when analysed with the AuNSs-Cys-GOx biosensor.

The practical application of the fabricated biosensor was verified by investigating the recoveries of the biosensor by determining different concentration of glucose as listed in Table 7-2. The results showed recovery rates of 97% to 102% for the three concentrations measured in triplicates. These recovery results were high and could meet the needs of actual clinical sample detection and quantification.

Table 7-2 Determination of glucose by the fabricated glucose plasmonic biosensor. The experiments were done in triplicate. The Table shows the results with the standard deviations from the different experimental results.

Concentration (mmol/L)	λ (nm) at OD Max	Concentration determined (mmol/L)	Recovery (%)
0.04	630	0.039 ± 0.004	97
	630		
	633		
0.08	612	0.082 ± 0.006	102
	616		
	612		
0.12	598	0.119 ± 0.001	99
	598		
	598		

7.6 Conclusions

Here presented is a glucose biosensor based on a simple seedless synthesis of gold nanostars, functionalised in a facile way with glucose oxidase for optimal functionality. The assay used AuNSs for greater sensitivity in LSPR peak shifts and colorimetric readouts via biocatalytic altering of their morphologies. Stability in serum and sensitivity in detection was enhanced by nanostar clustering after functionalising with GOx, as well as by the shape altering mechanism of detection. Furthermore, the sample matrix was observed to influence the colorimetric readout of the assay, with MES buffer solution being more pronounced for naked-eye detection. The biosensor was able to quantify glucose in serum diluted 1000 times with the ability to distinguish between different concentrations. Such sensitivity can potentially be applied for measuring samples with volumes such as dried blood spots. The assay demonstrated good specificity in glucose detection. Thus, the fabricated glucose biosensor proved to be a rapid kinetic colorimetric assay that utilises a basic entry level laboratory spectrophotometric microplate reader. Such a biosensor could be very useful in resource-constrained regions of the world without state-of-the-art laboratory equipment. This biosensor is a great candidate for potential clinical diagnosis, research and development applications.

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CHAPTER 8 GENERAL CONCLUSIONS AND FUTURE PROSPECTS

8.1 Introduction

The work presented in this thesis demonstrates the potential of developing biosensors which are simple, selective and sensitive based on nanotechnology detection for application in clinical and biomedical field. Such assays could be the needed solutions for the diagnostic challenges in resource-constrained settings. Progressive advances in nanotechnology and other complementary disciplines such as biochemistry, surface chemistry, and material science has enabled the development of efficient and affordable diagnostic devices as novel solutions that could impact healthcare delivery in the mentioned settings.

The main goal of the study was to develop and optimise strategies for optical assays based on nanotechnology-based detection. Various components of bioassay fabrications were assessed and purposefully optimised to meet the needed requirements of translating these assays into actual clinical use. The assays were developed using nanoplasmonic biosensor platform based on LSPR phenomenon occurring in gold nanostars. Such a strategy offered a simple, selective and rapid detection system for analytes based on both colorimetric changes and UV-vis-NIR spectroscopy. The focus on the different stages of biosensor fabrication such as the selection and synthesis of gold nanostructures, bioconjugation of enzymes for enhanced stability and optimal function, as well assessing the usability of the designed biosensor scaffold in biological matrix enabled an overall improvement in the analytical performance in the fabricated biosensor which was used only as a model.

8.2 Concluding discussions and perspectives

The initial objective of this study was to investigate the feasibility of generalising the detection system for analytes using nanoparticles as signal transducers. Using the recently developed mechanism of enzyme-guided growth of nanoparticles, a cholesterol and glucose biosensors were developed. The method for both involved the use of gold nanostars as nanosensor for their sensitive in LSPR sensing. The well-established seeded methodology of AuNSs synthesis was followed and the enzymes were used without being attached to the nanostars. The results showed that not only could biosensors be constructed with such simplicity, but demonstrated the ability to generalise the detection system using nanotechnology. If nanoplasmonics is to seriously challenge the conventional detection systems such as fluorescence-based enzymatic- and immunoassays, conventional ELISAs, HPLC and others, there is need for a detection system that could be generalised irrespective of the compound of interest.

Cholesterol and glucose were used as model analytes for investigating the universal detection system based on oxidases. Conditions for each analyte of interest had to be optimised, *inter alia,* the pH and buffer whereas the rest of the assay conditions remained the same. Both were detected based on optical changes such as increase in absorbance or shifts in the wavelengths at OD max. In the case of cholesterol, the concentration quantification was based on increase in absorbance. There was an initial blue-shift in LSPR peaks but it could not differentiate various concentrations as predictably as changes in absorbance with increase in concentrations. Glucose, on the other hand, was predictably quantified based on shifts in wavelengths at OD max. Standardising this detection parameter could be achieved by optimising the concentration of detection solutions, as well the appropriate dilution of sample to produce the desired optical changes. Such a generalised detection offers an opportunity for designing a panel of assays for determination of various analytes on a single 96-well plate. This would enable cost-effective simultaneous analysis of a number of analytes of interests.

A novel tailor-made one-pot synthesis method for surfactant-free AuNSs was developed as one of the optimisation steps in the development of nanobiosensors. The synthesis method produced nanostars that leveraged the advantages of the seeded and non-seeded methods. The method was simple, rapid and used nontoxic reagents. The produced PVP-stabilised AuNSs were multibranched that enabled the improvement of plasmonic LSPR-based sensing. When applied for plasmonic sensing using glucose as a model analyte, they exhibited great sensitivity making the suitable nanosensors for further developmental works in nanobiosensors.

Seeing the need to develop and optimise bioconjugation strategies that could meet the ideal characteristics of nanoparticle-enzyme bioconjugates, a facile and easily reproducible approach was devised. The strategy used simple well-established chemistries of gold binding to cysteine using the thiol bond and the modification of the enzyme using EDC/sulfo-NHS. The conjugation procedure enabled control over enzyme orientation, relative separation distance between AuNSs and enzymes, and increased attachment affinity. Optimal function of both the enzymes and AuNSs was observed using the stability studies and well as plasmonic sensing of glucose – as a sample analyte. This method is easily reproducible and could potentially be extended to other enzymes-nanostar conjugations with appropriated modifications and optimisations.

Lack of optimal bioconjugation procedures for developing assays that are reliable and accurate in detection is one of the reasons the majority of the innovative nanoplasmonic biosensors are barely validated for real clinical use. Motivated by the bioconjugation that was developed which potentially produced AuNSs-enzyme bioconjugates with enhances stability and optimal function, attempts were made to apply these in biological matrix using serum. Strategies for minimising the interferences of proteins and lipoproteins in serum were devised. It was observed that when

the sample was not sufficiently diluted, there was signal generated. Optimal conditions in biological matrix were between 100 to 1000 times dilution of sample prior to use for detection. The AuNSs-enzyme bioconjugates displayed great stability in serum and enhanced sensitivity from clustering of nanostars. A shape-altering signal mechanism was optimised that enabled greater sensitivity in detection of glucose in very minute concentrations of 0.001 mmol/L. This is opposed to growth in the size of nanoparticles that is less sensitive. The use of appropriate enzymes – in this case GOx – enabled the biosensor to be specific for glucose only in the presence of other analogous compounds. A rapid assay was developed that could make a high throughput analysing using either naked eye detection or a basic entry level laboratory spectrophotometry microplate reader. This observation demonstrated potential for further development of such nanobiosensors that can be validated for practical clinical applications.

The summarised significance of the work presented are; firstly, the development of simple a seedless synthesis strategy for AuNSs that have the advantages of the seeded methods. Through this contribution, it was demonstrated that there is still room for synthesis methods for AuNSs to produce nanostructures that are appropriate for the downstream applications. Secondly, the design of a biofunctionalisation strategy that produces bioconjugates that are stable and optimal in function for both the nanoparticle and enzyme. The strategy produced AuNSs-enzyme bioconjugates that have control over the relative separation distance between nanoparticle and enzyme, and prevent the physical adsorption of the enzyme onto the nanoparticle. However, one limitation was the inability to experimentally confirm such details of the bioconjugation. Future studies can look at techniques to confirm there and improve on what has been presented. Lastly, the devising of optimisation strategies to minimise interferences of proteins and lipoproteins in serum for optimal and sensitive detection of clinically relevant analytes. The extreme sensitivity of the nanosensors was harnessed by being able to detect analytes diluted a thousand times.

Future perspectives should look on extending these optimised strategies to other enzyme-based and immunoassays using nanotechnology. Using the same principles of nanoplasmonic biosensors, this could be extended to many infectious disease researches to find sensitive, selective and accurate ways to diagnosing these conditions that are plaguing most of the developing countries especially Africa. Using nanotechnology could clearly help to deal with these diagnostic challenges by developing assays that could be transferred to POCT and lateral flow devices.

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