

The suitability of NAD(P)/NAD(P)H as redox agents for gold nanostar shape alteration in colourimetric clinical enzymology

TA Munyayi

 **orcid.org 0000-0001-7872-6133**

Thesis accepted in fulfilment of the requirements for the degree
Doctor of Philosophy in Science with Biochemistry at the North-
West University

Promoter: Prof BC Vorster

Co-promoter: Dr DW Mulder

Co-promoter: Dr EH Conradie

Graduation May 2024

38426544

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Prof. Vorster and the Centre for Human Metabolomics at North-West University for allowing me to pursue my Ph.D.

Coming from a background in chemical engineering, process engineering and polymer engineering, exploring nano-biochemistry frequently left me feeling as if I had bitten off far more than I could chew and swallow. Even after three years, there is still a lot to learn. Due to previous experiences, I had difficulty trusting people in the laboratory at the start of this voyage. Still, as the adventure progressed, **Dr. Mulder** and **Dr. Conradie** demonstrated the power of collaboration and teamwork. I am eternally grateful to these two exceptional gentlewomen.

I am very grateful to **Dr. Mulder**. It did not take me long to realise that Dr. Mulder was right there with me, encouraging me to keep "researching a little deeper" to locate the buried literature gems. Her wry humour, passion, commitment and excitement made even the most difficult circumstances more bearable and meaningful. Her willingness to listen to the great (and sometimes crazy) ideas and theories I had along the way was a major factor in the work's success.

Also, thanks to **Dr. Jordaan** and **Dr. Shuro** for spending many hours in front of the TEM and SEM, waiting to see what the next surprise would be as the gold nanostars led us on this amazing adventure. **Prof. Shayne**, please accept my thanks for your amazing explanations of the NMR data. Furthermore, thanks to all the characterisation labs, this journey would not have been the same without your assistance!

A special thanks to **Mr. Motlatsi Mojahi**, who has provided me with a place to sleep and study, transportation to and from college and free Wi-Fi from the beginning of this journey, right through to the end. During the trying moments, his kind gestures had a significant influence.

I want to express my sincere thanks and deep appreciation to my wife **Lorraine** and son **Kendryque** (age 2), who have been my biggest supporters, continually praying for me and motivating me to keep going. I am particularly grateful to Lorraine for managing both her work responsibilities and single-handedly supporting our son in my absence, enabling me to fully dedicate myself to pursuing my Ph.D. They went to great lengths to ensure that I could realise my dream. I consider myself fortunate to have them in my life.

I also recall the daily inquiries and phone discussions I had with my **Mom** and **Dad** about what I was going to do next and how I was going to conquer each obstacle as well as having my father listen to my explanations and read some of the work to see if it made sense. Knowing they were

comrades made each and everyday challenge more bearable. I am thankful for family and friends who have loved and supported me through the ups and downs, from simple phone calls to assisting with checking on my wife and child. **Dr. Mutsvangwa** and **Eng. Gwaindepi** have my heartfelt gratitude and appreciation for their unwavering support and encouragement.

This entire project would not have been feasible without the incredible support I had received every step of the way.

Science is a beautiful gift to humanity; we should not distort it.

– A.P.J Abdul Kalam

ABSTRACT

Biosensors have proven to be a strong instrument for detecting varied targets such as proteins, DNA, small molecules (disease biomarkers), toxins and metabolites with great sensitivity and specificity.

The fundamental goal of the research presented in this thesis was to evaluate the applicability of NAD(P)/NAD(P)H as redox agents for gold nanostar shape alteration in colourimetric clinical enzymology. Furthermore, our research aimed to develop simple, low-cost, highly sensitive and specific gold nanostar-based colourimetric biosensor platforms for analyte detection as well as to understand how NAD(P)/NAD(P)H affects colourimetric, redox chemistry and morphological changes in gold nanostars.

A seedless 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and silver-mediated method was used to synthesise the gold nanostars. However, the procedure was simple, robust, and repeatable, yielding heteromorphous, monodispersed, multi-branched nanostars measuring $37 \text{ nm} \pm 2 \text{ nm}$ in diameter. The nanostars were found to have varying stabilities in salt, ionic strength and cell culture medium environments after being functionalised with polyethylene glycol (PEG) 8 000, polyethylene oxide (PEO)100 000 and polyvinylpyrrolidone (PVP) 10 000. Our findings show that capping agents influenced gold nanostar stability, functionalisation and colourimetric biosensing capabilities, with PVP capping proving to be the most appropriate option for HEPES-mediated gold nanostar synthesis.

Gold nanostars' anisotropic structure has shown considerable promise in plasmonic colourimetric sensing. When the colourimetric ability of the new nanostars was evaluated, it was discovered that the colourimetric assays for gold nanostars could be tailored for a specific application by using capping agents, enzymes and the detection solution as colourimetric catalysts. The high-energy facets at the terminals of the gold nanostar spikes triggered remodelling to start from there, resulting in the transition from star morphology to quasi-spherical nanoparticles and colourimetric signals. The colourimetric signals obtained were vivid and easily discernible with the naked eye. However, it has been revealed that differences in the morphological properties or the surface nature of gold nanostars strongly affect their plasmonic colourimetric sensing and optical properties.

The protein conjugation and immobilisation approach were also found to have an effect on the analyte detection rate. For optimal concentration-dependent detection findings, apoenzymes were co-immobilised with their coenzymes during biosensor fabrication. The biosensors reacted

to the neo-formed NAD(P)/NAD(P)H after the addition of NAD(P)/NAD(P)H-dependent enzymes galactose dehydrogenase and aldose reductase, resulting in a sufficient colourimetric gradient based on substrate concentration visible to the naked eye and detectable spectrophotometrically. When the same colourimetric assay parameters were used in an ELISA-type assay, the colourimetric variation between different concentrations of glucose oxidase and galactose oxidase enzymes resulted in a sufficient analyte concentration-dependent vivid colourimetric gradient, as expected.

This thesis summarises the most recent studies about the optical and spectroscopic features as well as the effect of NAD(P)/NAD(P)H redox chemistry on gold nanostars. Furthermore, it was found that NAD(P)/NAD(P)H-dependent enzymes could be used in gold nanoparticle-based plasmonic colourimetric assays when hydrogen peroxide-producing enzymes are unavailable. The versatility of technology was demonstrated across a broad spectrum of analytes. We applied it to detect metabolites (glucose and galactose) in a variety of matrices and conditions using plasmonic gold nanostars as both a scaffold and transducer element. The ramifications and scope of these findings have yet to be determined, as the full potential of anisotropic gold nanoparticles is only now being studied. This is critical for the successful development of robust, simple, and low-cost diagnostic equipment for resource-constrained settings. In conclusion, the NAD(P)/NAD(P)H-dependent enzymes immobilised on gold nanostars have the potential to replace costly equipment, reagents and lengthy experiments needed to assess various analytes and related clinical disorders such as diabetes and galactosemia diagnosis.

Keywords

Gold nanostars, Bioassay, Biosensor, Conjugation, Immobilisation, Anisotropic, Plasmonic, Heteromorphic, Apoenzymes, Coenzymes, Gold nanoparticles.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	I
ABSTRACT	III
LIST OF TABLES	XI
LIST OF FIGURES.....	XII
LIST OF SCHEMES	XVI
LIST OF ABBREVIATIONS	XVII
CHAPTER 1 INTRODUCTION	1
1.1 Background.....	2
1.2 Problem statement and Justification	7
1.3 Aim and Objectives	7
1.4 Thesis structure.....	8
1.5 Outcomes of this study	9
1.6 Author contributions	10
CHAPTER 2 LITERATURE REVIEW	11
2.1 Preface	12
2.1.1 Introduction.....	12
2.2 Synthesis of gold nanostars	16
2.2.1 Seeded growth gold nanostars	16
2.2.2 Seedless growth gold nanostars	18

2.3	Surface modification and functionalization of gold nanostars.....	19
2.3.1	Capping agents	19
2.3.2	Types of capping agents.....	20
2.3.3	Polymer-based capping agents.....	21
2.3.3.1	Polyethylene glycol (PEG)	21
2.3.3.2	Polyvinyl alcohol (PVA).....	22
2.3.3.3	Polyvinylpyrrolidone (PVP)	22
2.3.3.4	Chitosan	23
2.3.4	Protein-based capping agents.....	23
2.4	Surface functionality of gold nanostars.....	24
2.5	Gold nanostar-based plasmonic biosensors	25
2.5.1	Biosensors.....	25
2.5.2	Enzyme-based plasmonic gold nanostar biosensors	26
2.5.3	Fundamentals of redox reactions, NAD(P)/NAD(P)H redox couples, and their influence in plasmonic biosensors.....	29
2.5.3.1	Introduction.....	29
2.5.3.2	NAD(P)/NAD(P)H redox couples in biochemical reactions.....	30
2.6	NAD(P)/NAD(P)H redox couples quantification assays	32
2.6.1	Fluorescence assays	32
2.6.2	Colourimetric assays	33
2.6.2.1	Dye-based NAD(P)/NAD(P)H colourimetric assays.....	33
2.6.2.2	Nanoplasmonic-based NAD(P)/NAD(P)H colourimetric assays	34

2.7	Theoretical NAD(P)/NAD(P)H redox reactions with plasmonic gold nanostars	35
2.8	Conclusions and Perspectives	38
CHAPTER 3	STUDY DESIGN.....	40
3.1	Introduction.....	41
3.2	Experimental design.....	41
3.2.1	Aim	41
3.2.2	Objectives.....	41
3.2.2.1	Objective 1: Gold nanostar synthesis, characterisation, optimisation and stability evaluation.	41
3.2.2.2	Objective 2: Comparison of gold nanostar colourimetric biosensors in hydrogen peroxide-free, electron-deficient and electron-sufficient environments	44
3.2.2.3	Objective 3: Theoretical gold nanostar biosensors colourimetric redox chemistry investigation	47
3.3	Scope, limitations, and substantiation.....	48
CHAPTER 4	SYNTHESIS OF GOLD NANOSTARS, CHARACTERISATION AND COLOURIMETRIC ASSESSMENT	52
4.1	Preface	54
4.2	Abstract.....	54
4.3	Introduction.....	54
4.4	Materials and Methods	57
4.4.1	Materials.....	57
4.4.2	Gold nanostar synthesis	57

4.4.3	Gold nanostar capping agents (AuNS–capping agent)	57
4.4.4	Enzyme bioconjugation (AuNS–capping agent–GOx).....	57
4.4.5	Characterisation	58
4.4.6	Stability assessment for capped and AuNS bioconjugates.....	59
4.4.7	Effect of the capping agent on the plasmonic properties of AuNSs in bioassays	59
4.5	Results	59
4.6	Discussion	68
4.7	Conclusions	70
4.8	Acknowledgments	70
4.9	Author contributions	70
CHAPTER 5	GOLD NANOSTAR BIOSENSOR OPTIMISATION	71
5.1	Preface	72
5.2	Abstract.....	72
5.3	Introduction.....	72
5.4	Materials and Methods	74
5.4.1	Reagent and Materials.....	74
5.4.2	Characterisation	75
5.4.2.1	Instrumentation.....	75
5.4.3	Preparation of gold nanostars.....	75
5.4.4	Suitability of NAD, NADPH, and H ₂ O ₂ for redox-mediated change in AuNS morphology.....	76
5.4.5	Preparation of gold nanostar bioconjugates	76

5.4.6	AuNS bioconjugates stability assay	76
5.4.7	AuNS bioconjugates colorimetric assay	77
5.4.8	AuNS bioconjugates specificity assay	77
5.5	Results	78
5.6	Discussion	85
5.7	Conclusion	87
5.8	Acknowledgments	88
5.9	Authors' contributions	88
CHAPTER 6	H₂O₂ MEDIATED PLASMONIC GOLD NANOSTARS – GALACTOSE DEHYDROGENASE QUANTITATIVE GALACTOSE COLOURIMETRIC ASSAY	89
6.1	Preface	91
6.2	Abstract.....	91
6.3	Introduction.....	91
6.3.1	Materials and Methods	93
6.3.1.1	Materials.....	93
6.3.1.2	Instrumentation and Characterisation	94
6.3.1.3	Preparation of Gold Nanostars.....	95
6.3.1.4	Preparation of gold nanostar–galactose dehydrogenase bioconjugates.....	95
6.3.1.5	Preparation of gold nanostar-galactose dehydrogenase biosensor	95
6.3.1.6	Gold nanostar–galactose dehydrogenase biosensor colourimetric assay	96
6.4	Results	97
6.5	Discussion	104

6.6	Conclusions	112
6.7	Author Contributions.....	112
6.8	Acknowledgments	112
CHAPTER 7 THEORETICAL ADIABATIC OXIDATION MATHEMATICAL MODELLING OF GOLD NANOSTARS.....		113
7.1	Preface	114
7.2	Abstract.....	114
7.3	Introduction.....	114
7.3.1	Determination of gold nanostar volume and surface area	116
7.3.2	Theoretical adiabatic oxidation of gold nanostars.....	117
7.4	Discussion	122
7.5	Conclusion	123
CHAPTER 8 CONCLUSIONS AND FUTURE PROSPECTS		124
8.1	Concluding summary	125
8.2	Future prospects	129
8.3	References	130
CHAPTER 9 APPENDECIES		168
9.1	APPENDIX A: SYNTHESIS OF GOLD NANOSTARS, CHARACTERISATION AND COLOURIMETRIC ASSESSMENT	168
9.1.1	Particle size distribution of the AuNS bioconjugates	168
9.1.2	Confirming AuNS auto degeneration in the presence of GOx	168
9.1.3	Confirming AuNS sensitivity to H ₂ O ₂ and colourimetric signal generation	169

LIST OF TABLES

Table 2-1: Qualitative comparison of the seed-mediated versus seed-less synthesis methods of gold nanostars.	19
Table 2-2: Redox half equations and electrode potential values. Extracted from (Haynes, 2002).....	30
Table 4-1: Summary of results: structure–function relationship.....	68
Table 5-1: AuNS bioconjugates quantitative ICP-MS data before and after colourimetric reactions in the water matrix.....	84
Table 6-1: Experimental Procedure for the galactose plasmonic colourimetric detection using AuNS-PVP-GalDH biosensor.....	96

LIST OF FIGURES

Figure 2-1: A schematic representation of the most prevalent gold nanoparticle assemblages and morphologies. Adapted from Freitas de Freitas <i>et al.</i> (2018).	13
Figure 2-2: Schematic diagram of the localized surface plasmon resonance effect.	14
Figure 2-3: Vis-NIR spectra of (a) 30 nm AuNPs, (b) AuNRs in H ₂ O (red) and in CHCl ₃ (green), and (c) AuNSs of different sizes. Reproduced with permission from (Montes & Belén, 2016).....	15
Figure 2-4: Indication of anisotropic nanoparticles syntheses routes and anisotropic morphologies .Reproduced with permission from (Perez-Gonzalez <i>et al.</i> , 2020).....	16
Figure 2-5: Various capping agents routinely employed in nanotechnology. Reproduced with permissions from (Javed <i>et al.</i> , 2020b).	21
Figure 2-6: Biosensor working principle. Adapted from Huang <i>et al.</i> (2021).....	26
Figure 2-7: The hypothesized signal-generation technique using enzyme-guided crystal growth. Adopted from Rodríguez-Lorenzo <i>et al.</i> (2018).	27
Figure 2-8: Iodide-mediated surface etching sensitivity and specificity for HRP detection. a) Iodide-induced concentration-dependent surface etching of AuNS. b) UV-Vis spectra of AuNS etched by varying iodide concentrations. c) AuNS A ₇₈₀ /A ₅₅₀ value etched by iodide varying from 0.7 µM - 0.8 µM. d) Illustration of an HRP-catalyzed iodide consumption reaction that prevents surface etching of AuNS. e) AuNS UV-Vis spectra following iodide consumption catalyzed by varying HRP concentration. f) AuNS colourimetric signals iodide consumption by HRP catalysis (40 ng/mL) and other proteins (800 ng/mL) including ALP, GOx, protease (Prot), BSA, IgG, and streptavidin (S-A). Adopted from Xianyu <i>et al.</i> (2021a).	28
Figure 2-9: Representation of a reduction and oxidation process system.	29
Figure 2-10: Structural difference of the NAD(P)/NAD(P)H redox coenzymes.	31

Figure 2-11: Schematic kinetic mechanism and electron transport in active site of NAD(P)/NAD(P)H dependent enzyme. Reproduced with permission from (Flynn <i>et al.</i> , 1995; Grimshaw <i>et al.</i> , 1990).	32
Figure 2-12: Schematic mechanism of Beutler's test. NADPH chrome fluorescence is used to quantify enzyme activity or deficiencies.	33
Figure 2-13: Structural differences between tetrazolium salt/formazan in the presence of NAD/NADH.	34
Figure 2-14: (a) NADH (0.05 mM) absorption spectra at various intervals after gold nanorods (AuNRs-0.3 nM) were introduced (b) Absorption spectra of 0.05 mM NADH at various periods following the addition of 0.17 mM HAuCl ₂ solution. (c) Absorption spectra of AuNPs (spheres-0.46 nM) at various intervals following the addition of 0.05 mM NADH. (d) NADH oxidation initial reaction rate vs. AuNPs concentration. Adapted from Huang <i>et al.</i> (2005).	37
Figure 3-1: Schematic representation of the auxiliary galactose pathway biosensors.	45
Figure 3-2: Schematic diagram of the suggested colourimetric reaction mechanism.	47
Figure 3-3: Leloir pathway and the endogenous auxiliary pathways for galactose metabolism.....	50
Figure 4-1: (A–C): Effects of capping agents on AuNSs using (A) UV-vis spectrometry, (B) nuclear magnetic resonance (NMR), and (C) gel electrophoresis. (D–F): AuNS capping agents functionalized with glucose oxidase using (D) UV-vis spectrometry, (E) NMR, and (F) gel electrophoresis.	61
Figure 4-2: Representative HR-TEM images of the gold nanostar morphologies: (A) AuNS; (B) AuNS-PVP; (C) AuNS-PEO; (D) AuNS-PEG; (E) AuNS-PVP-GOx; (F) AuNS-PEO-GOx; and (G) AuNS-PEG-GOx.....	63
Figure 4-3: Comparison of UV-vis spectra of capped AuNS stability after 3 h of incubation in (A) salt; (B) urine; (C) serum; and (D) supplemented cell culture media. Storage stability UV-vis spectra at room temperature and 4 °C, respectively, for (E, I) AuNS; (F, J) AuNS-PVP; (G, K) AuNS-PEO; and (H, L) AuNS-PEG.	64

Figure 4-4: UV-vis spectra of stability comparisons for AuNSs bioconjugated with glucose oxidase after 3 h of incubation in (A) salt; (B) urine; (C) serum; and (D) supplemented cell culture media. Storage stability UV-vis spectra at room temperature and 4 °C, respectively, for (E, H) AuNS-PVP-GOx; (F, I) AuNS-PEO-GOx; and (G, J) AuNS-PEG-GOx.	65
Figure 4-5: Glucose-sensing-efficacy-normalized UV-vis spectra and correlation curves: (A, B) AuNS-GOx; (C, D) AuNS-PVP-GOx; (E,F) AuNS-PEO-GOx; and (G,H) AuNS-PEG-GOx.....	67
Figure 5-1: EDS spectra, SEM-EDS elemental mapping profiles, and HR-TEM images for AuNS. (A, C, E) Uncapped AuNS; (B, D, F) PVP capped AuNS.....	78
Figure 5-2: UV-Vis spectra profiles, ¹ H-NMR spectra profiles, electrophoretic profiles, and HR-TEM images for AuNS bioconjugates. (A-D) AuNS-PVP-GalDH; (E-H) AuNS-PVP-AR; (I-L) AuNS-PVP-GalOx.	79
Figure 5-3: UV–Vis spectra of AuNS bioconjugates stability after 3 h incubation in (A) Blank urine; (B) Serum; (C) Supplemented cell culture medium. Short-term storage stability UV-Vis spectra at room temperature and 4 °C in water matrix (D; G) AuNS-PVP-GalDH; (E; H) AuNS-PVP-AR; (F; I) AuNS-PVP-GalOx.	80
Figure 5-4: Preliminary colourimetric UV-Vis profiles for AuNS-PVP in the presence of (A) NAD ⁺ ; (B) NADPH and (C) H ₂ O ₂	81
Figure 5-5: AuNS bioconjugates colourimetric spectra in water and synthetic blood with increasing galactose (A-B) AuNS-PVP-AR; (C-D) AuNS-PVP-GalOx.....	82
Figure 5-6: AuNS bioconjugates TEM images before and after colourimetric reactions in the water matrix.....	83
Figure 5-7: AuNS bioassays analyte specificity test. Glucose was used in place of galactose. (A; D) AuNS-PVP-GalDH; (B; E) AuNS-PVP-AR; (C; F). AuNS-PVP-GalOx.....	85
Figure 6-1: AuNS-PVP-GalDH bioconjugate's (A) UV-Vis spectra profiles; (B) HR-TEM profiles; (C) ¹ H-NMR spectra profiles; (D) electrophoretic profiles; (E) EDS spectra; and (F) SEM-EDS elemental mapping profiles.	98

Figure 6-2: UV-Vis spectra and colourimetric profiles of the feasibility of H_2O_2 in inducing concentration-dependent colourimetric signals for galactose detection in the AuNS-PVP-GalDH assay in the water matrix. The following are the variations in H_2O_2 concentrations: (A) 0.00 mM; (B) 0.05 mM; (C) 0.10 mM; (D) 0.15 mM; (E) 0.20 mM.	99
Figure 6-3: Galactose sensing with AuNS-PVP-GalDH nanosensors of various synthesis batches UV-Vis spectra; (A-C) Water matrix; (D-F) Synthetic blood matrix.	100
Figure 6-4: UV-Vis spectra, colorimetric profiles, and mathematical correlation profiles of mean galactose sensing with AuNS-PVP-GalDH nanosensor. (A-B) Water matrix; Synthetic blood matrix (C-D).	101
Figure 6-5: Galactose sensing with AuNS-PVP-GalDH biosensor HR-TEM images after the biorecognition reaction in the water matrix.	102
Figure 6-6: UV-Vis spectra and colourimetric profiles for pH variations during galactose detection in the water matrix using the AuNS-PVP-GalDH biosensor at different points: (A) Pre-reaction phase; (B) NADH production phase; (C) H_2O_2 introduction phase; (D) Colourimetric signal generation phase.	103
Figure 6-7: UV-Vis spectral profiles for the interaction between NAD(P)H and AuNS over time in a water matrix.	108
Figure 7-1: Interfacial configuration for the core and shell system Au/Au ₂ O where $\alpha = \text{Au}_{(s)}$; $\beta = \text{Au}_2\text{O}_{(s)}$	118
Figure 7-2: EDS spectra and STEM elemental images showing the elemental composition of gold nanostars.	120
Figure 7-3: UV-vis spectra for gold nanostar oxidation transitions at different temperature conditions (A) 4 °C ; (B) Room temperature.	121
Figure 7-4: TEM images showing the oxidation of gold nanostar transformations.	122

LIST OF SCHEMES

Scheme 3-1: Experimental and theoretical design summary schematic diagram.....	48
Scheme 6-1: Proposed signal-generation mechanism based on enzyme-H ₂ O ₂ mediated crystal growth.	106
Scheme 6-2: Proposed colorimetric signal generation and redox chemistry scheme.	110
Scheme 7-1: Theoretical gold nanostar modeling.	116

LIST OF ABBREVIATIONS

A:

Ag ⁺	Silver ions
Au ⁺	Gold ions
AgNO ₃	Silver nitrate
Au	Gold
AuNPs	Gold nanoparticles
AuNS	Gold nanostars
AuNS-GOx	Gold nanostars functionalized with glucose oxidase
AuNS-PVP	Gold nanostars functionalized with polyvinylpyrrolidone
AR	Aldose reductase

C:

CTAB	Cetyltrimethylammonium bromide
CTAC	Cetyltrimethylammonium chloride

D:

DTSSP	3,3'-dithiobis(sulfosuccinimidyl propionate)
-------	--

E:

EDS	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked Immunosorbent assay

F:

FAD	Flavin adenine dinucleotide cofactor
FBS	Foetal bovine serum

G:

GC-MS Gas chromatography-mass spectrometry

GalDH Galactose dehydrogenase

GalOx Galactose oxidase

GOx Glucose oxidase

H:

HAuCl₄ Gold (III) chloride trihydrate (gold salt)

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC High-performance liquid chromatography

HR-TEM High-resolution transmission electron microscopy

I:

ICP-MS Inductively coupled plasma mass spectrometry

L:

LC-MS Liquid chromatography-mass spectrometry

LSPR Localised surface plasmon resonance

N:

NaCl Sodium chloride

NAD⁺ Nicotinamide adenine dinucleotide coenzyme

NADP⁺ Nicotinamide adenine dinucleotide phosphate coenzyme

NADH Reduced nicotinamide adenine dinucleotide coenzyme

NADPH Reduced nicotinamide adenine dinucleotide phosphate coenzyme

NMR Nuclear magnetic resonance

P:

PEG	Polyethylene glycol
pELISA	Plasmonic enzyme-linked Immunosorbent assay
POC	Point of care
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone

T:

TAE buffer	Tris-acetate EDTA buffer
TBE buffer	Tris Borate EDTA buffer
TEM	Transmission electron microscopy
TRIS buffer	Tris-EDTA buffer

S:

SEM	Scanning Electron Microscope
SERS	Surface-enhanced Raman spectroscopy

U:

UV-Vis	Ultraviolet-visible spectrophotometry
--------	---------------------------------------

W:

WHO	World Health Organisation
-----	---------------------------

Units:

%	Percentage
°C	Degrees Celsius
K	Kelvins
L	Litre
µg	Microgram
µL	Microlitre
mL	Millilitre
mg	Milligram
mm	millimolar
M	Molar
n	Moles
nm	nanometre
Nm	nanomolar
OD	Optical density
pH	Potential of Hydrogen
ppm	Parts per million

CHAPTER 1 INTRODUCTION

THE SCIENCE OF TODAY IS THE TECHNOLOGY OF TOMORROW.

– Edward Teller

1.1 Background

Metallic nanoparticles, in particular gold nanoparticles (AuNPs), have piqued the scientific community's interest due to their remarkable physical, chemical and biological properties arising from their "nano" size (Zhang *et al.*, 2009). In general, nanoparticles are particles of any shape having at least one dimension in the 1 – 100 nm range (Guerrero-Martínez *et al.*, 2011). The key arguments for employing gold nanoparticles instead of other metal nanoparticles include the great stability of AuNPs, the ease with which gold surfaces can be chemically modified and their decreased toxicity for biological purposes (Alkilany & Murphy, 2010; Daniel & Astruc, 2004; Eustis & El-Sayed, 2006; Guerrero-Martínez *et al.*, 2011; Javed *et al.*, 2020b; Shipway *et al.*, 2000). The optical effects of colloidal gold, however, evoked the interest of ancient scientists, with colloidal gold being used in Chinese ceramics to sharp reddish colours in 400 BC (Dykman & Khlebtsov, 2012) and in the famous Lycurgus Cup of Roman origin combined with colloidal silver to achieve a dichroic effect in 400 AD (Freestone *et al.*, 2007), or in Persian artwork to obtain lustrous effects in 900 AD (Fermo & Padeletti, 2012). There were little to no applications proposed for AuNPs until the thirteenth century when European alchemists began to propose AuNPs for medical use (Lou-Franco *et al.*, 2020). However, J. A. Chrestien established the feasible potential of AuNPs in treating syphilis patients in the nineteenth century (Chrestien, 1811; Lou-Franco *et al.*, 2020). Following that, John Turkevich published a detailed study of the process of colloidal gold nucleation and growth, paving the way for the wide range of nanoparticle synthesis strategies and applications found today (Turkevich *et al.*, 1951).

It is worth noting that the characteristics of materials at the nanoscale change greatly from those observed in bulk materials due to quantum-size effects (Daniel & Astruc, 2004). Since then, a plethora of research has focused on these properties, whether physical, chemical, or biological, in order to characterise and understand how AuNPs behave (Daniel & Astruc, 2004; Garcia, 2012; Trudel, 2011). AuNPs are being employed in a variety of medical disciplines (drug delivery, immunosensing and biolabeling) and have been shown to improve disease diagnosis, treatment and prevention (Amina & Guo, 2020; Chandra *et al.*, 2010; Howes *et al.*, 2014; Saha *et al.*, 2012; Zhang *et al.*, 2009). The ease of synthesis of AuNPs as well as their distinctive features (particularly their colourimetric and physical properties), enable the easy manipulation and tailoring of sensors for a wide range of targets, including proteins, enzymes, oligonucleotides, metal ions and viruses (Cordeiro *et al.*, 2016; Dykman & Khlebtsov, 2012; Radwan & Azzazy, 2009; Saha *et al.*, 2012; Si *et al.*, 2021). Furthermore, AuNPs can function as a molecular scaffold (a platform for biological component(s) attachment) as well as a signal transducer, increasing sensor sensitivity (Saha *et al.*, 2012). As a modified ELISA approach, AuNPs are also ideal for constructing a 96-well microplate format assay panel with high throughput and sensitivity

(Pallavicini, Cabrini, Cavallaro, *et al.*, 2015; Zhang *et al.*, 2015). However, for practical applications, the characteristics of AuNPs are determined by the synthesis process, size, shape, and surface chemistry (Garcia, 2012; Hammond *et al.*, 2014).

Anisotropic AuNPs, specifically gold nanostars (AuNS), are among the most efficient plasmonic nanomaterials for optical sensing, featuring a solid plasmonic core, which can be a sphere, a rod, or another shape and supports protuberant plasmonic tips (Hao *et al.*, 2004; Liu *et al.*, 2014; Pazos-Perez *et al.*, 2018). The nanostar core acts as a nanoscale antenna, greatly enhancing the excitation cross section and electromagnetic field enhancements of the tip plasmons (Hao *et al.*, 2007b). The protruding arms enhance the electromagnetic field and indirect and direct localised surface plasmon resonance (LSPR) biosensing by adding to the plasmonic contributions and the lightning rod effect (Atta *et al.*, 2016). The tips are thus responsible for the majority of AuNS's plasmonic properties (Pazos-Perez *et al.*, 2018; Rahman *et al.*, 2015; Shao *et al.*, 2012). Furthermore, AuNS exhibit an unequal distribution of localised surface plasmon resonances (LSPRs) across their surface, owing to the high energy facets (protruding arms) and the lower energy facets (core) (Rahman *et al.*, 2015). Controlling the arm density and length of the AuNS architecture without affecting the overall dimensions is a common strategy for tailoring AuNS morphology-directed optical properties (Webb *et al.*, 2014a).

A plethora of research on AuNS-based biosensors revealed that they are extremely sensitive to environmental changes, as seen by rapid LSPR variations in response to a biorecognition event (Moses Phiri *et al.*, 2019; Rodríguez-Lorenzo *et al.*, 2018; Xianyu *et al.*, 2021a). The bulk of these plasmonic colourimetric biosensors use hydrogen peroxide-producing enzymes; the hydrogen peroxide produced biocatalytically will etch/grow the AuNPs, resulting in a plausible LSPR shift (Li, Ma, *et al.*, 2013; Moses Phiri *et al.*, 2019; Xianyu *et al.*, 2021a; Zhang *et al.*, 2018). The LSPR shift is primarily attributed to the change in the surface electron oscillations which may be influenced by the shape, size, dielectric environment, surface coating and nanoparticle-chemical environment interactions (Guo *et al.*, 2016; Mayer & Hafner, 2011; Unser *et al.*, 2015). Furthermore, these variations can be detected spectrophotometrically and by the naked eye through colourimetric signal variations in the AuNP solutions.

Despite AuNS' versatility, there are still aspects of their synthesis that require attention. There are currently no standardised synthesis procedures for ideal monodisperse stars with a sufficient yield of the morphology of interest, well-defined properties and potential scale-up. To understand the underlying behaviour of these nanoparticles, comprehensive experimental, theoretical and computational research are required (Atta *et al.*, 2016; Cristiano *et al.*, 2020; Tsoulos & Fabris, 2018; Tsoulos *et al.*, 2018). In addition to the issues associated with morphological

characteristics, reagent toxicity is a major concern (Sperling *et al.*, 2008). Most published procedures employ environmentally and biologically harmful chemicals such as dimethyl formamide, cetyltrimethylammonium bromide and sodium borohydride (Yang *et al.*, 2015; Zhao *et al.*, 2007). This may have sparked other ecologically friendly nanoparticle production strategies (Adamu usman *et al.*, 2018; Ahmed *et al.*, 2016; Amina & Guo, 2020; Kunoh *et al.*, 2018). These are generally referred to as "green" synthesis methods and the Good's buffers are commonly used in anisotropic AuNS (Chandra *et al.*, 2016; Chen *et al.*, 2010; Jimmy Huang *et al.*, 2020; Xi & Haes, 2019; Xie *et al.*, 2007b).

Two approaches are widely used for the synthesis of anisotropic gold nanostructures: Seeded and seedless. The former relates to chemical synthesis, self-assembly, or molecular manufacturing techniques, such as the nucleation of gold atoms (seeds) from a molecular precursor and their development into colloidal AuNPs. In the seeded AuNS synthesis, gold nanoparticle seeds of various shapes are grown and guided to form branches. These approaches necessitate a number of stages, which may contribute to batch differences as well as post-synthesis purification to eliminate unreacted surfactants (Vinnacombe-Willson *et al.*, 2021; Wang *et al.*, 2022; Wu *et al.*, 2009). The advantages of seeded methods provide extremely monodispersed AuNS with great controlled branch and AuNS size growth, resulting in AuNS with tunable geometries (Kumar *et al.*, 2016; Monsefi *et al.*, 2020; Vinnacombe-Willson *et al.*, 2021; Wang *et al.*, 2022). Most of these high-yield seed-mediated procedures for synthesising AuNS with high dimensional monodispersity use Triton-X, ascorbic acid and AgNO₃ as shape-directing agents (Atta *et al.*, 2019). In contrast, the seedless technique is superior in terms of reagents and reaction time economics (Mulder, 2019; Phiri *et al.*, 2019a; Xie *et al.*, 2007b). The processes are generally uncomplicated, with little to no post-synthesis purification of surplus chemicals (Mulder, 2019; Phiri *et al.*, 2019a). However, because of insufficient precision of reaction parameters such as reagent concentrations, pH and temperature, seedless protocols often produce polydispersed samples (Liu *et al.*, 2019; Phiri *et al.*, 2019a; Xie *et al.*, 2007b; Zhang *et al.*, 2022). However, because various factors are thought to affect the formation of the nanostars, the simplicity of this one-pot approach (two reagents and one solution) can result in considerable batch-to-batch variability (Poklepovich-Caride *et al.*, 2022). Overall, five major parameters (surfactant concentration, Au³⁺ concentration, temperature, pH and stirring) and their secondary interactions affect the growth, shape and optical characteristics of AuNS (Atta *et al.*, 2019; Vinnacombe-Willson *et al.*, 2021; Wu *et al.*, 2009; Zhang *et al.*, 2022).

In this study, 2-[4-(2-hydroxyethyl) piperazinyl] ethanesulfonic acid (HEPES), one of Good's buffers and AgNO₃ were used in a one-pot seedless AuNS synthesis technique. Anisotropic gold nanoparticles with low post-synthesis purification, increased particle stability and a high scaling

potential are formed when HEPES and AgNO_3 are reacted with a solution of HAuCl_4 (Au^{3+} ions) (Chandra *et al.*, 2016; Xie *et al.*, 2007b). HEPES, a zwitterion, aids in the formation and stabilisation of AuNS by acting as a reducing and stabilising agent (Zhang *et al.*, 2022). Through reversible activation of the amine and sulfonate groups in HEPES, HEPES creates sufficient elastic interfacial energy barriers, reducing the possibility of particle agglomeration in solution and electron redistribution decreases its attraction to the AuNS, allowing adsorption to AuNS (Xi & Haes, 2019; Xi *et al.*, 2018). Acidification causes this phenomenon, which is influenced by pH, acid concentration and the protonation state of HEPES (Xi & Haes, 2019). HEPES' piperazine moiety generates nitrogen-centred free radicals that decrease the Au^{3+} ions, making it an excellent weak reducing, capping and shape-directing agent (Araújo-Chaves, 2015; Liu *et al.*, 2014; Xi & Haes, 2019; Zhang *et al.*, 2022). Since Xie *et al.* (2007b) described the use of HEPES for colloidal gold nanostars synthesis (Xie *et al.*, 2007b), a variety of variations have been published, including Saverot and co-workers' two-step technique employing HEPES and the addition of HAuCl_4 for gold nanostar growth and branch length (Saverot *et al.*, 2016). HEPES has minor salt and temperature effects as well as high water solubility, making it useful in cell culture due to its low permeability to cell membranes (Jimmy Huang *et al.*, 2020; Saverot *et al.*, 2016; Xi & Haes, 2019; Xie *et al.*, 2007b). HEPES is noncytotoxic and biocompatible, making HEPES-mediated AuNS more suitable for biological applications (Jimmy Huang *et al.*, 2020; Saverot *et al.*, 2016; Xi & Haes, 2019; Xie *et al.*, 2007b).

AuNS have the most intriguing uses in biology because the surface plasmon resonance of the nanoparticle can be tailored into the tissue transparency window of biological tissue (D'Hollander *et al.*, 2016). Thus, AuNS can be employed as both diagnostic and therapeutic agents, with a variety of diagnostic instruments and techniques including photoacoustic tomography, surface-enhanced Raman spectroscopy, magnetic resonance imaging, positron emission tomography, and X-ray computer tomography (Liu, Yuan, *et al.*, 2015b; Sasidharan *et al.*, 2016). Because of their higher surface-to-volume ratio and contact cross-section, AuNS act more powerfully as a radiation dose enhancer, and research is being performed in cancer treatment (Liu, 2016; Liu, Yuan, Fales, *et al.*, 2015). Furthermore, AuNS tips have an enhanced electromagnetic field that enhances Raman scattering in the region, resulting in a sensitive approach for vivo or in vitro chemical analysis that can identify the presence of specific molecules such as proteins, dendrimers, and nucleotides (Chirico *et al.*, 2015; Hao *et al.*, 2007b; Nalbant Esenturk & Hight Walker, 2009; Pazos-Perez *et al.*, 2018).

The suitability of NAD(P)/NAD(P)H as redox agents in diagnostic applications is crucial in defining the activity of energy-producing pathway-related diseases as well as other NAD(P)/NAD(P)H-driven oxidative stress disorders (Oka *et al.*, 1981; Umrikhina *et al.*, 1990; Ying, 2008). Defects in the equilibrium of these redox couples are linked to a wide range of illnesses, including diabetes, neurological disease, heart disease, and cancer (Ying, 2008). Since the mid-20th century, researchers have investigated the intrinsic fluorescence of NAD(P)/NAD(P)H redox cofactors. Furthermore, while the fluorescence spectra of NAD(P)H are indistinguishable, interpreting the signals coming from their combined fluorescence, typically designated NAD(P)H, can be challenging (Xiao *et al.*, 2018). The measurement of NAD(P)/NAD(P)H is important in biochemistry since it helps to characterise enzyme activity, homeostasis, and metabolism (Xiao *et al.*, 2018). However, present approaches are insufficiently sensitive and/or selective for detecting low levels of NAD(P)/NAD(P)H (Jiang *et al.*, 2020). While nanotechnology approaches for detecting NAD(P)/NAD(P)H, such as the use of AuNPs, are particularly intriguing, their applicability in clinical samples and activity assays is limited (Veskoukis *et al.*, 2018). This work makes a unique contribution by using AuNS, particularly for NAD(P)/NAD(P)H detection in clinical enzymology.

Delayed disease diagnosis is a major problem in developing countries and isolated communities (Boniface *et al.*, 2012; Gaede & Versteeg - Mojanaga, 2011). This is owing to undeveloped people's poor access to and marginalisation of accessible healthcare service distribution when compared to developed communities (Boniface *et al.*, 2012; Gaede & Versteeg - Mojanaga, 2011). Because laboratory infrastructure is limited in underdeveloped areas, point-of-care (POC) diagnostics can aid in the diagnosis and treatment of patients during the same visit, increasing patient health quality (Drain *et al.*, 2014; Giuliano & Grant, 2002; Noah & Ndangili, 2019; St John & Price, 2014). The term "RE-ASSURED" alludes to the World Health Organization's (WHO) point-of-care testing guidelines, which state that tests must be "affordable, sensitive, specific, user-friendly, rapid/robust, equipment-free or minimal and deliverable to those in greatest need" (Cordeiro *et al.*, 2016; Land *et al.*, 2019). Recent nanotechnology advancements have resulted in a broad range of nanosensing systems with distinct properties that are revolutionising molecular diagnostics (Cordeiro *et al.*, 2016; Land *et al.*, 2019; St John & Price, 2014; Syedmoradi *et al.*, 2017). The primary goal of this research was to develop AuNS-based platforms with promising state-of-the-art proofs-of-concept (physical-chemical principles and biomolecular recognition) that are suited for POC development and implementation in resource-constrained areas.

1.2 Problem statement and Justification

NAD(P)/NAD(P)H are universal and ubiquitous redox coenzymes/cofactors that drive unique enzymatic reactions involved in major metabolic pathways. As a result, utilising dehydrogenases and reductases for NAD(P)/NAD(P)H sensing broadens and decentralises the analytical sensing domain, including circumstances when no oxidase enzymes are involved. This strategy can be developed to detect these redox couples for diagnosis of associated clinical disorders.

Anisotropic gold nanostars have a larger extinction coefficient than spheres and, to the best of our knowledge, have never been employed as scaffolds and signal amplifiers for NAD(P)/NAD(P)H plasmonic colourimetric detection. In response to analyte biorecognition events, these anisotropic gold nanostar-based biosensors must generate plausible colourimetric signals with straightforward, rapid, consistent, and reproducible result interpretation.

Motivated by clinical and point-of-care diagnostic applications, gold nanostar-based plasmonic colourimetric detection of NAD(P)/NAD(P)H can meet the ASSURED criteria, hence improving health care delivery in resource-constrained locations. Despite their simple synthesis processes, using environmentally friendly chemicals and producing homogeneous gold nanostars is critical. As a result, gold nanostar-based biosensors have the potential to replace laboratory-based approaches for measuring NAD(P)/NAD(P)H concentrations and enzyme activity, making analyte detection simple and inexpensive.

1.3 Aim and Objectives

This research aimed to assess the suitability of NAD(P)/NAD(P)H as redox agents for gold nanostar shape alteration in colourimetric clinical enzymology. Furthermore, the study sought to create simple, inexpensive, low sample volume, highly sensitive and specific gold nanostar-based colourimetric biosensor platforms for analyte detection as well as to understand how NAD(P)/NAD(P)H affects colourimetric, redox chemistry and morphological changes in gold nanostars. As a result, this study fully investigated the synthesis, functionalisation, characterisation and biosensing properties of gold nanostars.

To achieve this aim, the following **objectives** were implemented:

- 1) To synthesise gold nanostars that are stable in morphology and homogeneity utilising an environmentally friendly protocol. In addition, to determine the optimal capping agent for the gold nanostars synthesised. Furthermore, the gold nanostars were functionalised with glucose oxidase and their biosensor capability in enzymatic assays was assessed in conjunction with the different capping agents i.e., how the capping agents influenced the

colourimetric ability of the enzymatic assays. This could serve as a model for other enzymes that produce hydrogen peroxide.

- 2) The colourimetric parameters of the biosensor produced in Objective 1 were transferred to gold nanostars functionalised with galactose oxidase, NAD(P)/NAD(P)H-dependent galactose dehydrogenase and aldose reductase enzymes. This might be used to evaluate the biosensor capabilities in NAD(P)/NAD(P)H settings by employing galactose oxidase as a hydrogen peroxide-producing enzyme as a reference point for the generation of colourimetric signals.
- 3) To develop a competitive assay that includes hydrogen peroxide utilising the NAD(P)⁺-dependent galactose dehydrogenase assay parameters defined in Objective 2. This could be used to evaluate the colourimetric capabilities of biosensors in NAD(P)H-hydrogen peroxide settings. Furthermore, redox potentials and reaction rates were used to evaluate the theoretical biosensor colourimetric signal production technique.
- 4) To develop a mathematical model of gold nanostar adiabatic oxidation. This might be used to assess the self-degeneration of gold nanostars generated in Objective 1 without capping agents.

1.4 Thesis structure

This thesis is a collection of eight chapters created expressly to meet the criteria of North-West University. It was written in an article format for the completion of the Doctor of Philosophy in Biochemistry degree. Each chapter serves as a foundation and stepping stone for the following chapter; therefore, it includes an introduction, materials and techniques, results and discussion and a conclusion tailored to the chapter.

Chapter 1: This chapter introduces current applications of gold nanoparticles. It also discusses the necessity of anisotropic gold nanostars in point-of-care diagnostic tests. This justifies the use of nanotechnology and clinical diagnostics in the current inquiry.

Chapter 2: This chapter is a detailed overview of gold nanostar synthesis techniques including capping agent selection and biosensor design with a focus on NAD(P)/NAD(P)H plasmonic colourimetric nano-biosensors.

Chapter 3: This chapter describes the project study design and scope.

Chapter 4: This chapter describes a HEPES-mediated seedless synthesis method of anisotropic gold nanostars. It also demonstrates the characterisation and stability of gold nanostars in various conditions using various capping agents. Furthermore, it describes the effects of capping agents on anisotropic gold nanostars.

Chapter 5: This chapter describes the feasibility of NAD(P)/NAD(P)H as redox colourimetric agents employing anisotropic gold nanostars as enzyme scaffolds and galactose as a reference analyte.

Chapter 6: This chapter describes the hydrogen peroxide mediated quantification of NAD(P)H and inverse galactose plasmonic colourimetric sensing using anisotropic gold nanostars.

Chapter 7: This chapter discusses the theoretical modelling of anisotropic gold nanostar adiabatic oxidation.

Chapter 8: This chapter provides a summative conclusion of the study and discusses the study's potential future prospects

1.5 Outcomes of this study

This study resulted in the following publications:

- 1) Munyayi, T.A.; Vorster, B.C.; Mulder, D.W. The Effect of Capping Agents on Gold Nanostar Stability, Functionalization, and Colourimetric Biosensing Capability. *Nanomaterials* **2022**, *12*, 2470. <https://doi.org/10.3390/nano12142470>. Published 2022 (Submitted 15 June 2022).
- 2) Munyayi, T.A.; Mulder, D.W.; Conradie, E.H; Vorster, B.C. Feasibility of NAD(P)/NAD(P)H as redox agents in enzymatic plasmonic gold nanostar assays for galactose quantification. *Royal Society Open Science* **2023**, *10*. <https://doi.org/10.1098/rsos.230825>. Published 2023 (Submitted 15 June 2023).
- 3) Munyayi, T.A.; Mulder, D.W.; Conradie, E.H; Vorster, B.C. Quantitative galactose colourimetric competitive assay based on galactose dehydrogenase and plasmonic gold nanostars. *Biosensors* **2023**, *13*(11), 965; <https://doi.org/10.3390/bios13110965>. Published 2023 (Submitted 08 October 2023).

This research resulted in the following conference contributions:

- 1) Munyayi, T.A.; Vorster, B.C.; Mulder, D.W. The Effect of Capping Agents on Gold Nanostar Stability, Functionalization, and Colourimetric Biosensing Capability. Attendance only. 6th South African Nanoscience and Nanotechnology Summer School, 23 – 25 October 2022, Lord Charles Hotel, Cape Town, South Africa.
- 2) Munyayi, T.A.; Vorster, B.C.; Mulder, D.W. The Effect of Capping Agents on Gold Nanostar Stability, Functionalization, and Colourimetric Biosensing Capability. Poster presentation. 8th International Conference on Nanoscience and Nanotechnology in Africa (NanoAfrica 2022), 26 – 28 October 2022, Lord Charles Hotel, Cape Town, South Africa.

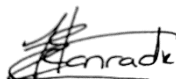
1.6 Author contributions

Mr. T.A Munyayi designed the experiments, performed the laboratory bench work, evaluated the data and produced draft articles for this study. Prof. B.C. Vorster, Dr. D.W. Mulder and Dr. E.H. Conradie were involved in the study's design, data analysis, article reviewing, proofreading and study integrity.

As co-author, I hereby approve and certify that my role in this study, as indicated above, is an accurate portrayal of my contribution and I hereby provide permission for this work to be published as part of Tozivepi Aaron Munyayi' s PhD thesis.



D. W. Mulder



E. H. Conradie



B.C. Vorster

CHAPTER 2 LITERATURE REVIEW

AN OVERVIEW OF THE FUNDAMENTALS OF ANISOTROPIC GOLD NANOSTARS AND THEIR USE AS BIOSENSORS IN THE BIOMEDICAL FIELD

EDUCATION IS ONE THING NO ONE CAN TAKE AWAY FROM YOU.

—Elin Nordegren

2.1 Preface

The following sections of this literature review highlight a brief introduction on gold nanoparticles as a whole, the synthesis of anisotropic gold nanostars with tunable properties and their analytical applications. In addition, a thorough overview of how capping agents influence the surface chemistry, stability, functionalisation and colourimetric signals of conjugated gold nanostars is provided. Finally, a general perspective on several important applications of plasmonic nanoparticles is offered, with special emphasis on in vitro colourimetric sensing of metabolites and NAD(P)/NAD(P)H redox couples.

2.1.1 Introduction

Plasmonic colloidal nanoparticles are distinctive nanostructures capable of showing significant electromagnetic fields, known as localised surface plasmon resonances (LSPRs), at their surface when excited with the right wavelength of irradiated light (Zhao *et al.*, 2008). Aside from colloidal nanoparticle composition, three factors influence the wavelength and intensity of these electromagnetic fields: shape, size and plasmon intercoupling between neighbour particles (Becerril-Castro *et al.*, 2022). Almost every gold nanoparticle shape may have its size readily adjusted. However, when compared to the other two parameters, the effect of size on the wavelength and intensity of the LSPR is minor (Becerril-Castro *et al.*, 2022). Depending on the dielectric environment, geometry, size, composition and particle-particle separation distance, a characteristic absorption band will be seen in a certain region of the electromagnetic spectrum as a result of the LSPR (Li *et al.*, 2009; Marcheselli *et al.*, 2020; Samsuri *et al.*, 2017).

Despite the wide array of existing plasmonic colloidal materials from transition elements, doped semiconductors and metal oxides, chalcogenides and graphene, silver (Ag) and gold (Au) are the predominantly utilised noble metals (Comin & Manna, 2014; Liu & Swihart, 2014; Vilela *et al.*, 2012). Although LSPRs are more efficient in silver, gold nanoparticles (AuNPs) have received special attention due to their superior chemical stability, non-toxicity, inertness, and synthetic accessibility (Dreaden *et al.*, 2012; Eustis & El-Sayed, 2006). The use of nanosized gold colloids may be traced back to illustrious ancient eras such as the Faraday's pioneering colloids and Lycurgus cup era (Faraday, 1857; Freestone *et al.*, 2007). Albeit the preparation of monodisperse gold spherical particles was proverbial since the 20th century; the synthesis of AuNPs with anisotropic morphologies tremendously progressed in the last decade (Fig.2-1) (Gong *et al.*, 2012; Grzelczak *et al.*, 2008; Li, Zhao, *et al.*, 2014; M. Pallares *et al.*, 2019).

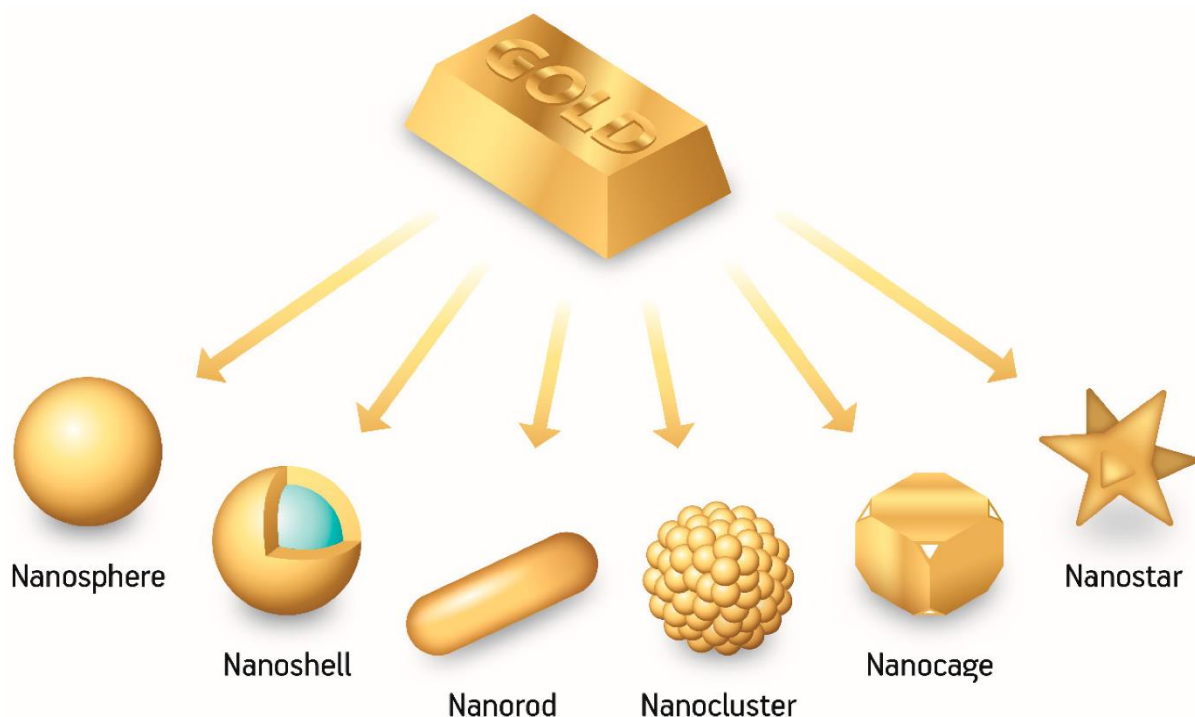


Figure 2-1: A schematic representation of the most prevalent gold nanoparticle assemblages and morphologies. Adapted from Freitas de Freitas *et al.* (2018).

Anisotropic gold nanoparticles (cubes, rods, plates, prisms and stars) have an unequal distribution and hybridisation of the LSPRs on their surface, allowing light concentration in specific places that generally match the tips in their structure (Alvarez-Puebla *et al.*, 2010; Barbosa *et al.*, 2010b). As a result, anisotropic nanoparticles exhibit strong absorption and scattering in the UV-Visible (UV-Vis) range, which confer them with vivid colours that have intrigued scientists for centuries (Link & El-Sayed, 2003).

Michael Faraday pioneered extinction studies on spherical nanoparticles in 1857 and later in 1905, Gustave Mie presented a general solution to Maxwell's equations that construe the extinction of spherical particles of arbitrary size and developed the following equation for irradiation applicable to UV-Vis spectroscopy (Fig.2-2) (Faraday, 1857; Hergert & Wriedt, 2012; Horvath, 2009). Figure 2-2 depicts the metal nanosphere, electron oscillations and the UV-Vis

spectral profile when incident light is irradiated on the nanosphere, resulting in localised surface plasmon resonances.

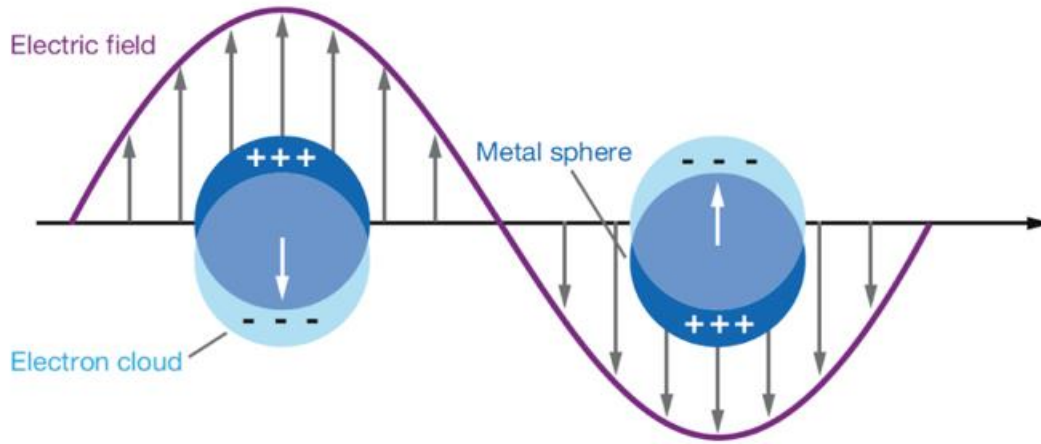


Figure 2-2: Schematic diagram of the localized surface plasmon resonance effect.

$$E(\lambda) = \frac{24\pi N_A a^3 \epsilon_m^{3/2}}{\lambda \ln(10)} \left[\frac{\epsilon_i}{(\epsilon_r + \chi \epsilon_m)^2 + \epsilon_i^2} \right] \quad [1]$$

where $E(\lambda)$ is the extinction (sum of absorption and scattering), N_A is the density of nanoparticles, a is the radius of the sphere, ϵ_m is the dielectric constant of the medium surrounding the sphere, λ is the wavelength of the incident radiation, ϵ_i and ϵ_r are the imaginary and real components of the nanoparticle's dielectric function, respectively, χ is the term that describes the aspect ratio of the nanoparticle ($\chi = 2$ for a sphere). The major determinants of the optical properties of metal nanoparticles is their shape, size and dielectric environment (Kelly *et al.*, 2003).

Figure 2-3 exhibits experimental Vis-NIR spectra of gold nanoparticles of different shapes: gold nanospheres (AuNPs), nanorods (AuNRs) and nanostars (AuNSs) (Montes & Belén, 2016). For AuNRs two plasmon bands are observed, corresponding to longitudinal and transverse plasmon resonance modes and related to light absorption and scattering along the long axis and the short axis of the particles, respectively (Sosa *et al.*, 2003).

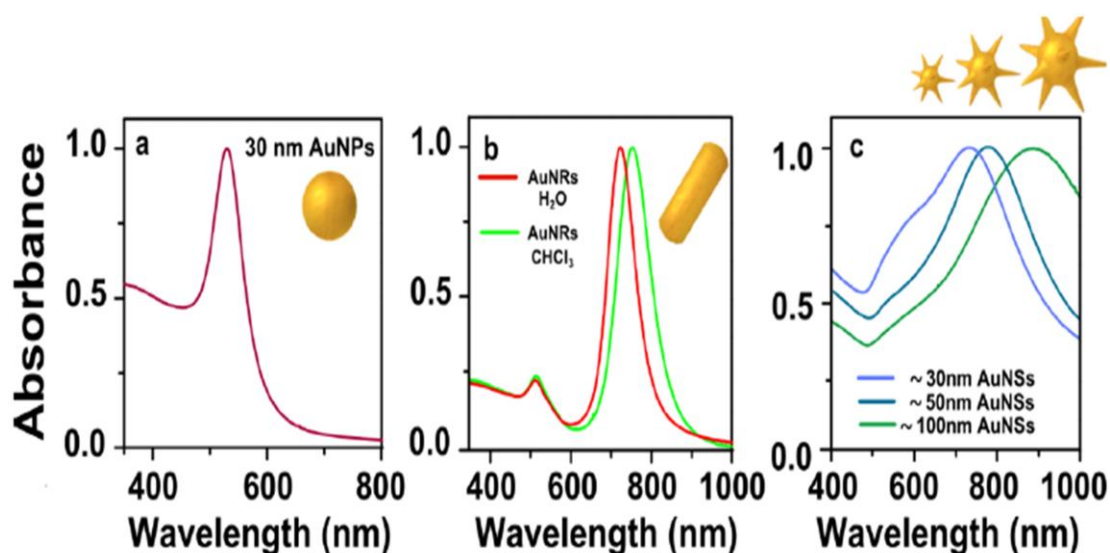


Figure 2-3: Vis-NIR spectra of (a) 30 nm AuNPs, (b) AuNRs in H₂O (red) and in CHCl₃ (green), and (c) AuNSs of different sizes. Reproduced with permission from (Montes & Belén, 2016).

Plasmonic gold nanostars are made consisting of a solid plasmonic core (spherical, rod, or any other shape) that houses protuberant plasmonic spikes (Hao *et al.*, 2007b; Pazos-Perez *et al.*, 2018). Among anisotropic plasmonic nanoparticles, gold nanostars (AuNS) are peculiar as they offer optical tunability by tailoring subtle variations in their geometry (Liu, Yuan, *et al.*, 2015a). While the plasmonic core is significant as an electron antenna that supplies electrons to the tips, the excitation cross section and electromagnetic field enhancements of the tip plasmons are significantly increased (Hao *et al.*, 2007b). Thus, the main plasmonic features of anisotropic AuNS arise from the tips “lightning rod effect”, leading to exceptional sensitivity and signal generation (Pazos-Perez *et al.*, 2018; Rahman *et al.*, 2015; Urbieto *et al.*, 2018). Anisotropic AuNPs are exceptional due to their structural, optical and catalytic properties, which are unique and customarily superior to those of spherical AuNPs (Li, Zhao, *et al.*, 2014). Conclusively, AuNS are a novel sensing nanoplatform applicable in various fields, principally due to their plasmon tunability into the near infrared (NIR) region and their sensitivity to changes in environments (Liu, Yuan, *et al.*, 2015a; Yuan *et al.*, 2013). The ability to manipulate the plasmon band of anisotropic nanoparticles to be in resonance with typical Vis-NIR light sources is a critical property that opens exciting possibilities for biological applications (De Aberasturi *et al.*, 2015; Dreaden *et al.*, 2012). In this regard, this thesis focuses on the use of AuNS as a scaffold and signal-amplifying transducer in the development of plasmonic colourimetric biosensors for clinical enzymology. In addition, the thesis intended to evaluate the suitability of NAD(P)/NAD(P)H as redox agents in plasmonic colourimetric AuNS biosensors.

2.2 Synthesis of gold nanostars

This section focuses on the common synthetic strategies used by researchers to achieve improved shape and size control of anisotropic AuNS by taking into account factors such as shape-directing agents, capping agents, solvent and reactor conditions (Siegel & Baker, 2021). Syntheses of anisotropic AuNS are generally classified as seeded or seedless growth methods (Fig.2-4); with the choice of synthesis method being critical to the parameters of the resultant morphology (Perez-Gonzalez *et al.*, 2020; Siegel & Baker, 2021).

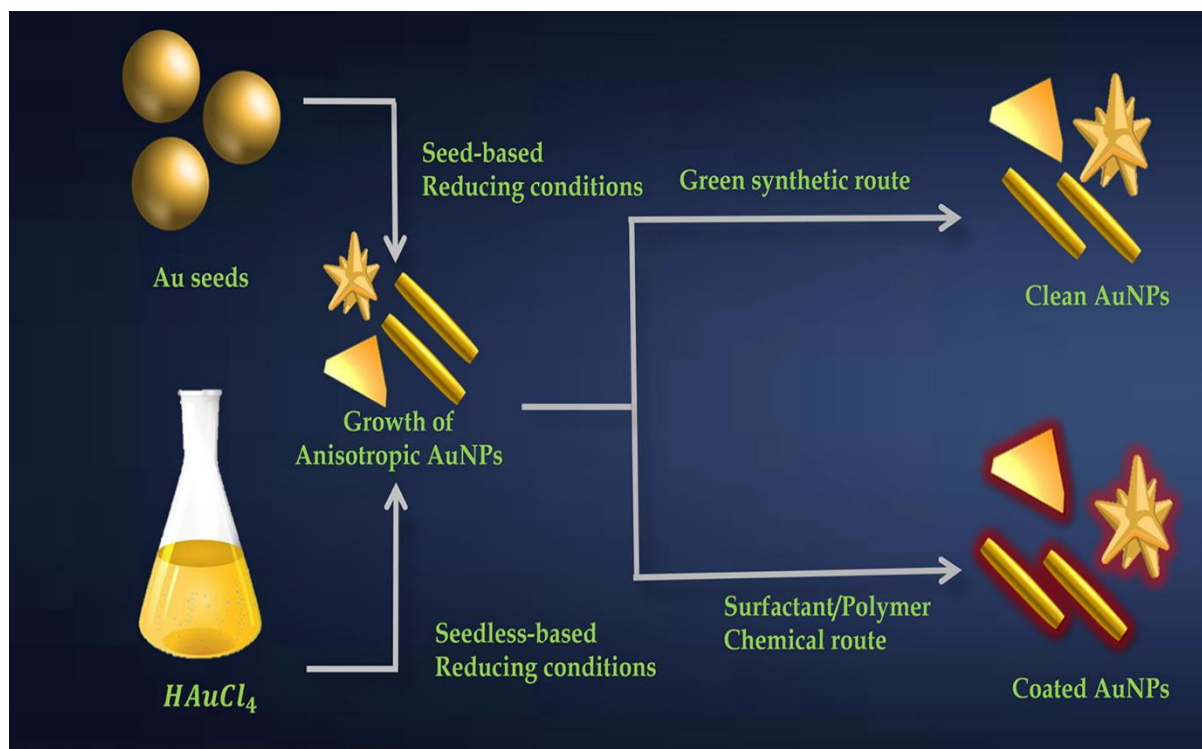


Figure 2-4: Indication of anisotropic nanoparticles syntheses routes and anisotropic morphologies .Reproduced with permission from (Perez-Gonzalez *et al.*, 2020).

2.2.1 Seeded growth gold nanostars

A typical reaction in seed-mediated formation of AuNS involves seed precursors, which are usually spherical AuNPs that act as nucleation centres for the preferential deposition of additional Au⁰ atoms. (Siegel & Baker, 2021). Spherical citrate-stabilised gold nanoparticles can be grown to sizes larger than 100 nm by altering factors such as solution temperature and pH as well as the injected Au³⁺: seed ratio (Bastús *et al.*, 2011; Bigall *et al.*, 2008; Siegel & Baker, 2021). Isotropic growth of branching AuNS can be slowed down by interactions between surfactants or ions with certain crystal faces, resulting in selective deposition of free gold onto unobstructed surfaces (Perrault & Chan, 2009). It has been proposed that the preferential adsorption of capping molecules on specific crystalline facets of the metal seed surface triggers the anisotropic growth

process by changing growth rates along specific crystallographic orientations (Bastús *et al.*, 2011).

The influence of additives such as silver nitrate (AgNO_3) on the growth of AuNS has also been documented, so that a greater degree of control over the nanoparticle star shape can be accomplished by adding silver ions at different stages of the growth process (Chen *et al.*, 2003; Nehl *et al.*, 2006b; Sau & Murphy, 2004; Wu *et al.*, 2009). The use of seed-mediation allows the nucleation and growth processes to be separated in time, allowing for greater control over the shape and size of the emerging AuNS (Bigall *et al.*, 2008; Perrault & Chan, 2009). As a result, as indicated by Wulff's theorem, the crystalline structure of the precursor seed plays a critical role in the ultimate morphology of the AuNS (Li *et al.*, 2016; Miracle-Sole, 2013). Nonetheless, other researchers believe that single or multiple twinned seeds are required to allow branch formation in noble metal particles (Kuo & Huang, 2005; Yuan *et al.*, 2007).

Atta *et al.* (2019) have detailed the synthesis of heteromorphic AuNS (multi- and six-branched AuNS) via single crystalline seed development (Atta *et al.*, 2019). The reduction of tetrachloroauric (III) acid trihydrate (HAuCl_4) by sodium borohydride (NaBH_4) in the presence of surface Triton X-100 at varied concentrations produced single crystalline Au seeds with (111) planes. Finally, the seeds, AgNO_3 and ascorbic acid (AA) were added to a HAuCl_4 growth solution in Triton X-100 with a concentration equivalent to the seed solution, producing the heteromorphic AuNS. De Silva Indrasekara *et al.* (2018) proposed a modified Turkevich approach for the synthesis of surfactant- and capping-agent-free AuNS, indicating that increasing the Au^{3+} : R_{seed} ratio, regardless of seed size, resulted in a branch length: core size drop and branch density drop (De Silva Indrasekara *et al.*, 2018). Chen *et al.* (2003) also reported a seeded procedure for the synthesis of gold multipods (1-4) by employing prefabricated silver plates as seeds in an excess of cetyltrimethylammonium bromide (CTAB) capping agent, which took a significantly lengthy time (1-20 days) to complete (Chen *et al.*, 2003). Barbosa *et al.* (2010b) discovered that adding the seed precursor to the growth fluid prior to full Au^{3+} - Au^+ reduction resulted in greater polydispersity of the emerging branching AuNS due to oxidation of the seeds themselves (Barbosa *et al.*, 2010b). Furthermore, Kuo and Huang developed a similar multistep approach employing tiny gold seeds and sodium dodecyl sulphate (SDS) capping agent, yielding poorly defined heteromorphic anisotropic AuNPs with random branching (Kuo & Huang, 2005). Many researchers have used temperature and pH to synthesize metallic particles with various morphologies. Depciuch *et al.* (2020), produced hollow, porous AuNPs with a broad range of light absorption at have been produced at elevated temperatures of 40-60 °C. pH-dependent synthesis of anisotropic gold nanostructures have also been widely researched and provided outstanding morphological control (Tofanello *et al.*, 2016).

In conclusion, as with most seed-mediated syntheses, precursor concentration, reducing agent concentration, capping agent, reaction time, reaction pH and seed quality are the major anisotropic growth process determinants for AuNS production. Because seed-mediated growth reactions are not one-pot processes, timing the addition of seed to the synthesis is crucial for AuNS production. The use of a gold seed precursor allows for more control over the final morphology of branching AuNS based on seed size and crystalline structure. Furthermore, due to modest heterogeneities in the seed structures, this type of synthesis frequently results in polydispersity in both size and form of the resultant morphologies (Hao *et al.*, 2007a; Kelly *et al.*, 2003; Siegel & Baker, 2021; Webb *et al.*, 2014b).

2.2.2 Seedless growth gold nanostars

In the seedless approach (one-pot synthesis strategy), milder, water-soluble and biocompatible reducing and capping agents are considered to enhance anisotropic AuNS synthesis, performance and application (Xie *et al.*, 2007a). The one-pot, seedless approach is essentially dependent on the reducing agent and surfactant used, with substantial limitations such as shape-directing agents aggressively adsorbing onto the gold surface, restricting use without extra "clean up" of the neo-formed anisotropic AuNS (Barbosa *et al.*, 2010b; Guerrero-Martínez *et al.*, 2010; Siegel & Baker, 2021; Xi & Haes, 2019). The ability to tailor the anisotropic AuNS morphology within seedless syntheses is paramount to their viability in applications.

Xie *et al.* (2007a) suggested a seedless synthetic approach that produces heteromorphic monodispersed AuNS by employing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as a mild reducing and capping agent and AgNO₃ as a shape-directing agent (Xie *et al.*, 2007a). Once passivated by polyvinylpyrrolidone (PVP), the AuNS produced by this approach were multi-branched and roughly 37 ± 2 nm in diameter, with good stability and plasmonic sensing (Xie *et al.*, 2007a). Webb *et al.* (2014a) established the tunability of seedless HEPES-mediated AuNS by varying the concentrations of HAuCl₄ and HEPES as well as the pH of HEPES (Webb & Bardhan, 2014; Webb *et al.*, 2014b). Yuan *et al.* (2012) suggested a seedless silver- and AA technique by rearranging the order of reactant addition in the original seed-based process, which produced monodispersed heteromorphic AuNS (Yuan *et al.*, 2012). Phiri *et al.* (2019b) recently proposed a simple seedless approach using HAuCl₄ and an AA solution at a low pH ($R_{AA} = 2$), allowing for the formation of nucleation sites, after which AgNO₃ was rapidly injected, resulting in heteromorphic anisotropic AuNS formation comparable in terms of size control to that of seeded-growth methods (Phiri *et al.*, 2019b). Hao *et al.* (2004) developed a seedless technique for producing anisotropic AuNPs that exhibited morphological transition at 4 °C storage settings

utilising HAuCl_4 and bis(p-sulfonatophenyl) phenylphosphine dipotassium salt (BSPP) as the stabilizer (Hao *et al.*, 2004).

In conclusion, like with other seedless syntheses, the key anisotropic growth process factors include precursor concentration, reducing/surfactant concentration, capping agent, reaction time and reaction pH. Furthermore, anisotropic growth seedless synthesis techniques are one pot, simple and require only a few stages, with no requirement for premade seed templates for AuNS formation (Saverot *et al.*, 2016; Xie *et al.*, 2007c).

A qualitative comparison of seeded and seedless nanostar approach is shown in Table 2-1 below.

Table 2-1: Qualitative comparison of the seed-mediated versus seed-less synthesis methods of gold nanostars.

Seed-based syntheses	Seed-less syntheses
Greater control over particle size and morphology	Lesser control over particle size and morphology
Not one-pot reactions	One-pot reaction
Defined growth template from precursor seeds	A more sensitive nucleation phase
Often must utilise harsh reagents	Can easily rely on “green” reagents
Relatively insensitive to reaction condition changes	Highly sensitive to reaction condition changes

Conclusively, seeded techniques have an advantage (Table 2-1) because the nucleation phase occurs on a template with predetermined facet composition and crystallinity, whereas seedless approaches use no prefabricated templates (De Silva Indrasekara *et al.*, 2018; Kereselidze *et al.*, 2012b; Xi *et al.*, 2018). Overall, by carefully tailoring the previously mentioned synthetic factors (e.g., pH, reductant concentration/strength), many of the shortcomings of seedless approaches can be averted.

2.3 Surface modification and functionalization of gold nanostars

This section discusses the methods used to modify the surface chemistry, stability, biocompatibility and specificity of gold nanostars by functionalising capping agents and ligand molecules (antibodies, proteins, drugs and biomarkers) to the gold nanostars' surface.

2.3.1 Capping agents

Recent trends in nanoparticle synthesis and surface manipulation, the use of distinctive capping agents, have allowed the preparation of surface-functionalised and monodispersed nanoparticles for various applications (Javed *et al.*, 2020b). Capping agents are molecules that act as

stabilizers, preventing coalescence and steric hindrance hence stabilising the interaction of nanoparticles within the preparation medium (Sidhu *et al.*, 2022). In some situations, capping agents are responsible for modifying the surface chemistry, physico-chemical and biological properties of nanoparticles and colloidal dispersions (Li, Chang, *et al.*, 2013; Restrepo & Villa, 2021). As a result, understanding the interactions between nanoparticles, capping agents and ligands is critical for correlating nanomaterial properties and functionality.

2.3.2 Types of capping agents

Distinct types of capping agents have been utilised in nanoparticles' synthesis including surfactants (Smith & Korgel, 2008), small ligands (Gavia & Shon, 2015), polymers (Baygazieva *et al.*, 2014; Grubbs, 2007), dendrimers (Crooks *et al.*, 2001; Niu & Crooks, 2003), cyclodextrins (Noël *et al.*, 2014) and polysaccharides (Raveendran *et al.*, 2003). Capping agents are categorised into numerous kinds, each with a unique influence that imparts distinct end properties on the nanoparticles, with the overall goal of avoiding coalescence (Campisi *et al.*, 2016b). Besides preventing nanoparticles agglomeration, the modification of metal surface by organic ligands enhances compatibility with another phase or introducing additional functionalities (Campisi *et al.*, 2016a).

Figure 2-5 is representing the various types of capping agents used in nanoparticle synthesis protocols. In addition to the well-known agents, novel biogenic capping agents (proteins and amino acids) in nanoscience are being discovered that enhances the physical properties (size, shape, and surface charge) of the nanoparticles and influence their interactions with the living cells (Sidhu *et al.*, 2022).

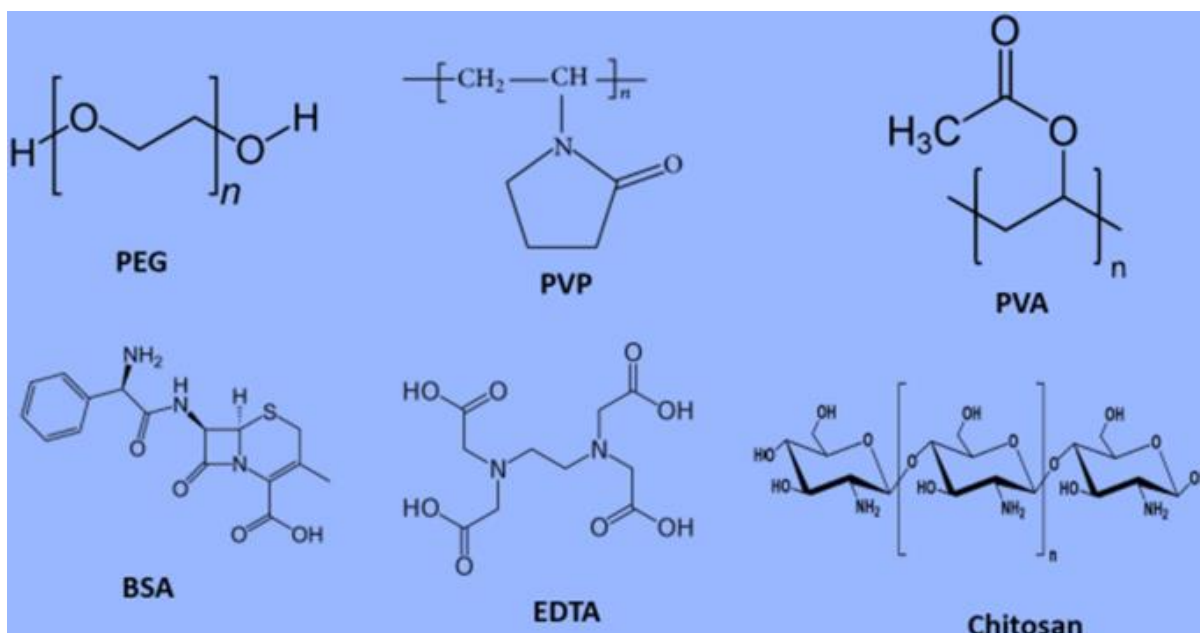


Figure 2-5: Various capping agents routinely employed in nanotechnology. Reproduced with permissions from (Javed *et al.*, 2020b).

2.3.3 Polymer-based capping agents

Polymers are macromolecules formed by the chemical bonding of large numbers of smaller molecules, or repeating units, called monomers (Gad, 2014). Polymers may be naturally found in plants and animals (natural polymers) or may be man-made (synthetic polymers) (Gad, 2014; Grubbs, 2007). Owing to the popularity and versatility of polymeric materials, there are nearly limitless possible combinations of monomers – or oligomers, or prepolymers – that can combine to form a polymer (Gad, 2014). Different polymers have a number of unique physical and chemical properties due to which they find usage in everyday life. The functional groups of a capping polymer stabilise the nanoparticles by steric or electrostatic repulsions and avoid nanoparticle coalescence (Baygazieva *et al.*, 2014; Grubbs, 2007; Shenhar *et al.*, 2005). Among all the strategies for particle solubilisation and stabilisation, ligand exchange methods usually lead to AuNS with limited colloidal stability, while polymer encapsulation appears to be a versatile method and does not depend on the chemical nature of the underlying nanoparticle core (Zhang *et al.*, 2011). The most frequently used polymer capping agents in colloidal dispersions to regulate nanoparticles are as follows:

2.3.3.1 Polyethylene glycol (PEG)

PEG is a biocompatible polymer with structural formula $\text{H}-(\text{O}-\text{CH}_2-\text{CH}_2)_n-\text{OH}$ (Shameli *et al.*, 2012). PEG has been used as a capping agent in nanotechnology extensively for several purposes such as the biomedical field for sustained and targeted drug delivery. The coating of

metal and metal oxide nanoparticles with PEG takes place in wet-chemical synthesis (Rajitha *et al.*, 2021). PEG has been reported in a plethora of studies as a capping agent for metal nanoparticles involving gold (Au), silver (Ag), zinc (Zn) and many others to reduce cytotoxicity, improve stability and biocompatibility associated with metal nanoparticles (Pinzaru *et al.*, 2018; Singletary *et al.*, 2017; Zamora-Justo *et al.*, 2019). Nevertheless, its hydrophilic properties grant a greater water affinity and biodegradability to the polymer (Shameli *et al.*, 2012).

2.3.3.2 Polyvinyl alcohol (PVA)

PVA is one of the high-performance capping agents used in nanotechnology (Aisida *et al.*, 2020; Junaidi *et al.*, 2017). PVA, which is essentially made through hydrolysis of polyvinyl acetate, is a synthetic polymer owning great hydrophilicity, biocompatibility and biodegradability (Amann & Minge, 2012; Jadoun & Jangid, 2019). PVA has been used as a stabilising agent, for shape and size control of Ag nanoparticles for use in various biomedical arenas (Gaaz *et al.*, 2015; Kyrychenko *et al.*, 2017). PVA has been widely employed in biomedical applications for passivating magnetic nanoparticles and improving their dispersion in other materials (Zhang *et al.*, 2019). To improve the optical emission, crystallinity and size dispersion, ZnO nanoparticles capped with PVA were prepared by the sol-gel method (Kundu *et al.*, 2011). Iron oxide or magnetite nanoparticles are most likely to agglomerate due to magnetic forces and it compromises the effectiveness and potential use of these nanoparticles (Hutami Rahayu *et al.*, 2018). Therefore, PVA has been employed as a capping agent and glutaraldehyde as a cross-linker in the fabrication of iron oxide nanoparticles. This has not only prevented the agglomeration but also the oxidation of iron oxide nanoparticles via a two-step method in which first the iron oxide nanoparticles were prepared and then coated and crosslinked with PVA and glutaraldehyde (Hutami Rahayu *et al.*, 2018).

2.3.3.3 Polyvinylpyrrolidone (PVP)

PVP, commonly known as polyvinylpyrrolidone (C₆H₉NO)_n, is a water-soluble polymer formed by the addition polymerization of the N-vinylpyrrolidone monomer (Ajitha *et al.*, 2016). PVP is one of the significant capping agents that have been utilised in nanotechnology to overcome drawbacks associated with conventional methods of preparation of nanoparticles such as their toxicity, size and agglomeration (Ajitha *et al.*, 2016; Safo *et al.*, 2019). Hence, eco-friendly nanosyntheses protocols using PVP as a capping agent around metal nanoparticles such as Iron (Fe), silver (Ag), gold (Au), zinc (Zn), etc. have proved to produce stable nanoparticles (Koczkur *et al.*, 2015; Kumar *et al.*, 2017; Phiri *et al.*, 2019b; Safo *et al.*, 2019). PVP with its exclusive physico-chemical properties like solubility both in water and organic solvents, biocompatibility, chemical stability

and non-toxicity makes it a potential biomaterial in many considerable medical and non-medical purposes (Safo *et al.*, 2019).

2.3.3.4 Chitosan

Chitosan is a co-polymer made up of *N*-acetyl-D-glucosamine and D-glucosamine that is formed through alkaline deacetylation of chitin, which occurs naturally in crustaceans, or hydrolysis of chitin by the enzymatic action of deacetylase (Pellis *et al.*, 2022). In nanoscience, chitosan has been extensively used in the preparation of metal nanoparticles as a stabiliser and promoting wet-chemical synthesis, although the bonding between chitosan and metallic nanoparticles is still a debate (Javed *et al.*, 2020b). For instance, the linkage between platinum (Pt) nanoparticles and chitosan has been suggested to be via amino and secondary hydroxyl groups (Deng *et al.*, 2017). Chitosan has been used as a capping agent to stabilise the colloidal dispersion and to control the morphology and optical properties of metal nanoparticles including gold (Au) , silver (Ag) , copper (Cu), etc. (Cinteza *et al.*, 2018; Franconetti *et al.*, 2019; Jayaramudu *et al.*, 2019). Capping AuNP with chitosan is employed as a temperature manipulation indicator because AuNP changes the colour and intensity of chitosan with storage temperature and duration (Mohan *et al.*, 2019). This difference in colour and intensity is principally owing to the change in shape and size of chitosan capped Au nanoparticles, potentially used for the development of sensors in the food industry (Mohan *et al.*, 2019).

2.3.4 Protein-based capping agents

Proteins are an excellent source of distinctive functional groups that can be utilised in tailoring nanoparticle surface chemistry (Marişca & Leopold, 2019). Charge and hydrophobicity of nanoparticles can be changed by adding particular proteins to their surface (Amina & Guo, 2020; Arib *et al.*, 2021; Liébana & Drago, 2016). Nanoparticles have considerable adsorption capacities due to their large surface area and can bind to proteins (peptides, phospholipids, lipids, nucleic acids) connected to the surface via covalent bonds or adsorption resulting in the formation of a dynamic nanoparticle-protein corona, which alters the stability, functioning and reactivity of nanoparticles in various settings (Saptarshi *et al.*, 2013). A diverse range of proteins are being used in nanoparticle passivation for a variety of applications, owing to the fact that proteins have specific end groups that can impact nanoparticle end use (Guzmán-Soto *et al.*, 2020; Marişca & Leopold, 2019; Wu & Hu, 2007).

2.4 Surface functionality of gold nanostars

Surface modification and functionalisation is a prerequisite for the synthesised nanoparticles as it entails their interaction with the environment and possible downstream application (Sperling & Parak, 2010). These interactions ultimately induce superior colloidal stability, biocompatibility and specificity and may yield a controlled assembly or delivery of nanoparticles to a target by appropriate functional molecules on the particle surface (Dreaden *et al.*, 2012; Sperling & Parak, 2010; Vega *et al.*, 2014). However, because they can be functionalised with one or more proteins that introduce the requisite bio-functionalities, these nanoparticles can behave as multifunctional scaffolds in biological applications. (Conde *et al.*, 2014). Finally, the conjugation technique is directly dependent on a variety of characteristics such as size, surface chemistry and shape as well as the type of ligands and functional groups to be used in functionalisation. (Conde *et al.*, 2014). Furthermore, when evaluating the conjugation approach, it is also important to consider the type of biological molecule and the end application of the nanoparticle conjugate.

Sulphur compounds [thiols ($-SH$) or disulphide groups ($S-S$)] may protect gold surfaces using a weakly bound (physisorbed) form or a strongly bound (chemisorbed) one, often assumed to be $Au(I)$ -thiolate (Häkkinen, 2012; Pakiari & Jamshidi, 2010; Reimers *et al.*, 2016). Experimental and calculated evidence indicated that chemisorbed surface species are actually bound mainly by strong van der Waals (aurophilic-like) forces (Reimers *et al.*, 2016). Gold – Sulphur stable bonds ($Au-S$, $\sim 50 \text{ Kcal mol}^{-1}$) are common anchoring groups utilised for the attachment of other molecules (Love *et al.*, 2005). Other functional ligands such as amines (*i.e.*, dodecylamine or oleyamine) also interact with gold surfaces; withal, the binding forces are comparatively weak (Rao *et al.*, 2019).

A common strategy for improving the colloidal stability of the nanoparticles after synthesis is the so-called “ligand exchange” (Zan *et al.*, 2018). A representative example is the functionalisation of capped AuNS with thiolated ligands (*i.e.* mercaptoundecanoic acid, MUA; mercaptopropionic acid, MPA and 3,3'-Dithiobis(sulfosuccinimidylpropionate) DTSSP) where the surface Au ions form the strong chemisorbed $Au(I)$ -thiolate with the thiolated ligands (Enea *et al.*, 2019; Mulder, Phiri, *et al.*, 2019c; Vega *et al.*, 2014). Therefore, replacement or overcoating of the surfactant bilayer adsorbed on the surface of the nanoparticle by thiols is required to amass biocompatible nanoparticles (Alkilany *et al.*, 2009).

Ligand exchange is also a popular strategy for phase transfer from organic solvents into aqueous solutions (or vice versa) (Yang *et al.*, 2011). In this case, the molecules stabilising the nanoparticles in the starting phase may be overcoated or replaced by other ligands with stronger binding features, inducing the transfer to the second phase with high colloidal stability (Cook *et*

al., 2021). Additionally, different agents can be used to facilitate the phase transfer process. For instance, acetone decreases the surface tension at the interface, while strong acids or bases protonate negatively charged groups (or deprotonate positively charged amino groups), leading to particles with lower surface charge and thus less stable in aqueous media (Lista *et al.*, 2014).

The important role of the modification of the nanoparticle surface in many biological applications is highlighted through the strong effect that surface charge has on both cellular uptake and biodistribution of nanoparticles (He *et al.*, 2010). Positively charged nanostructures have enhanced affinity to negatively charged cell surfaces, thereby being the most likely candidates to cross cellular membranes and enter the cytoplasm of cells, an important aspect to consider in drug delivery applications (Cho *et al.*, 2010). The binding of the target moiety (e.g. antibody, peptide, folate) can occur either directly to the AuNS surface or to the terminal end of an attached polymer chain (Cobley *et al.*, 2011).

Conclusively, surface functionalisation of nanoparticles is a key requisite to provide stability in physiological environments, biocompatibility, impart functionality, amplify sensitivity and create cellular targeting effects, while maintaining the original properties of nanomaterials (Jiang, Win, *et al.*, 2013). Furthermore, protein corona development and ligand-receptor complex formation both enhance nanoparticle stability and functionality (Simonelli *et al.*, 2019).

2.5 Gold nanostar-based plasmonic biosensors

This section describes how plasmonic gold nanostars can be employed in plasmonic-based biosensors as scaffolding, building blocks and signal-amplifying transducers. A comprehensive explanation of plasmonic gold nanostar biosensors in biosensing and theranostics is also presented.

2.5.1 Biosensors

Biosensors are small analytical instruments that use a biological catalyst or receptor in close proximity to an appropriate transducer to detect specific analyte(s) (Belluzo *et al.*, 2008; Nagel *et al.*, 1992). To maximise response, limit interference and simplify reuse, the biological components are tightly incorporated into the physicochemical transducer surface (Damborský *et al.*, 2016b). The transducer turns specific biochemical events into readable signals such as electrical, thermal, or optical signals proportional to the concentration of the analyte of interest, allowing it to be distinguished from structurally related molecules (Fig.2-6) (Prasad, 2014). Even in the presence of complex matrices, a biosensor with a high-specificity biorecognition element may detect analytes directly.

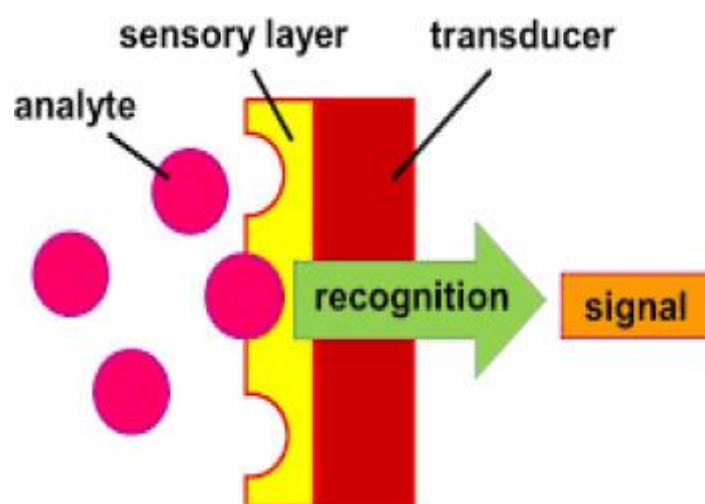


Figure 2-6: Biosensor working principle. Adapted from Huang *et al.* (2021).

Biosensors are typically classified into two categories: one based on biorecognition elements and another based on the type of transducer (Naresh & Lee, 2021). Bio-recognition elements include immunosensors, enzyme sensors and cell/tissue-based sensors and DNA sensors, whereas transducers comprise of electrochemical biosensors, optical biosensors and piezoelectric biosensors (Sawant, 2017). Ultraviolet (UV), gel electrophoretic mobility, surface plasmon resonance (SPR), infrared absorption and fluorescence absorption are some of the principles that optical sensors may employ (Chen & Wang, 2020; Damborský *et al.*, 2016a).

We present a brief overview of the basic principles of operation of plasmonic-based biosensors and their bioanalytical applications. The primary emphasis is on AuNS-based optical biosensors, which are surface plasmon resonance (SPR)-based biosensors that include SPR imaging and localised SPR.

2.5.2 Enzyme-based plasmonic gold nanostar biosensors

Enzyme-based plasmonic biosensors are hybrid biosensors that entail enzymes carry out the biorecognition and the method of transduction is based on optical plasmonic readouts (Bollella & Katz, 2020; Damborský *et al.*, 2016a). The immobilisation and bioconjugation of the enzyme(s) on the nanoparticle surface are the most critical components in enzyme-based biosensors (Ding *et al.*, 2015b; Homaei *et al.*, 2013; Temoçin, 2021). Poor immobilisation and conjugation techniques may impair active site accessibility, stability over time and enzyme functioning (Guzik *et al.*, 2014; Quintanilla *et al.*, 2010b). The major strategies for immobilising enzymes on nanoparticle transducers are adsorption, covalent bonding, crosslinking, encapsulation and entrapment (Mohamad *et al.*, 2015; Siddique, 2021).

Using enzyme-guided crystal growth, Rodríguez-Lorenzo *et al.* (2018) developed a gold nanostar-based plasmonic biosensor with inverse sensitivity (Rodríguez-Lorenzo *et al.*, 2018). In this investigation, glucose and glucose oxidase (GOx) were used as model analytes and enzymes, respectively. The hypothesised signal-generation mechanism involves GOx producing H_2O_2 , which reduces Ag^+ ions to form an Ag^0 coating around the plasmonic AuNS biosensor, causing a substantial blueshift in the LSPR of the AuNS plasmonic band (Fig 2-7). Furthermore, plasmonic colourimetric sensing has a substantial advantage over conventional detection methods in that readouts based on visible colour shifts can be recognised quickly with the naked eye, eliminating the need for complicated apparatus.

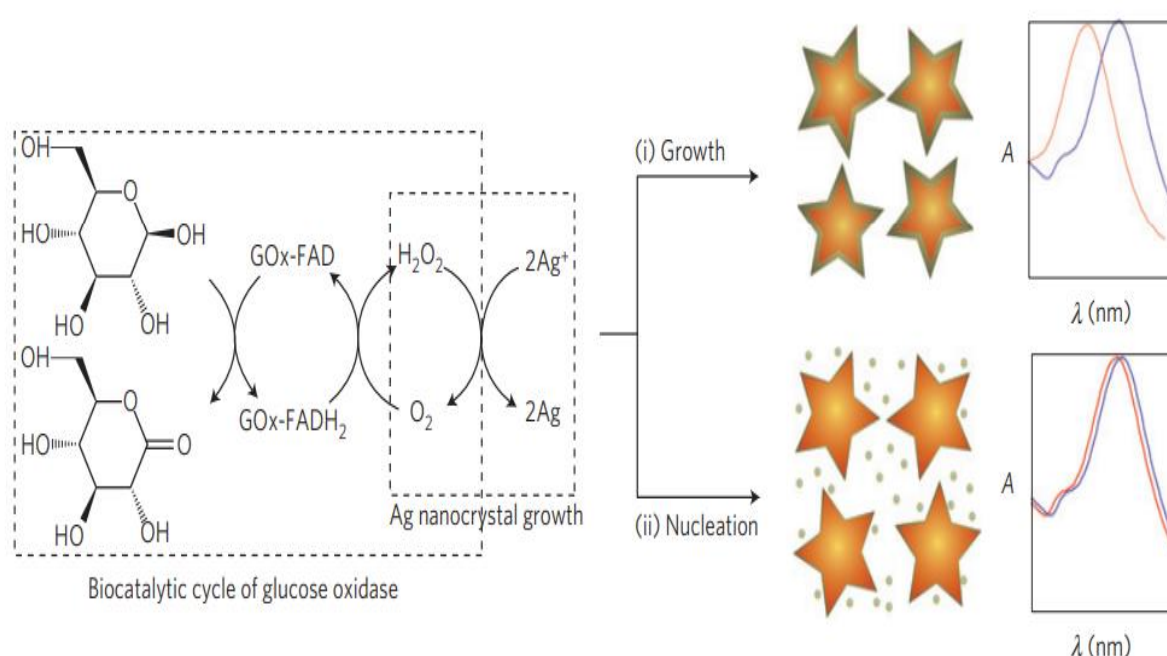


Figure 2-7: The hypothesized signal-generation technique using enzyme-guided crystal growth. Adopted from Rodríguez-Lorenzo *et al.* (2018).

Furthermore, GOx concentration influences the nucleation rate and Ag^0 growth, such that (i) at low GOx concentrations, favours the growth of a conformal Ag^0 coating, causing a large blueshift in the LSPR of the AuNS; and (ii) at high GOx concentrations, favours Ag^0 nucleation, causing a minor variation in the AuNS LSPR. Xianyu *et al.* (2021a) reported the iodide-induced quick and sensitive surface etching of gold nanostars, which they then turned into an adaptable biosensing platform for enzyme-catalysed detections (Xianyu *et al.*, 2021a). Their findings demonstrate that horseradish peroxidase (HRP)-catalysed iodide ion oxidation results in AuNS transformation to spherical AuNPs, resulting in a blue spectral shift in the AuNS LSPR band and enzyme-dependent colourimetric changes in the solution (Fig. 2-8).

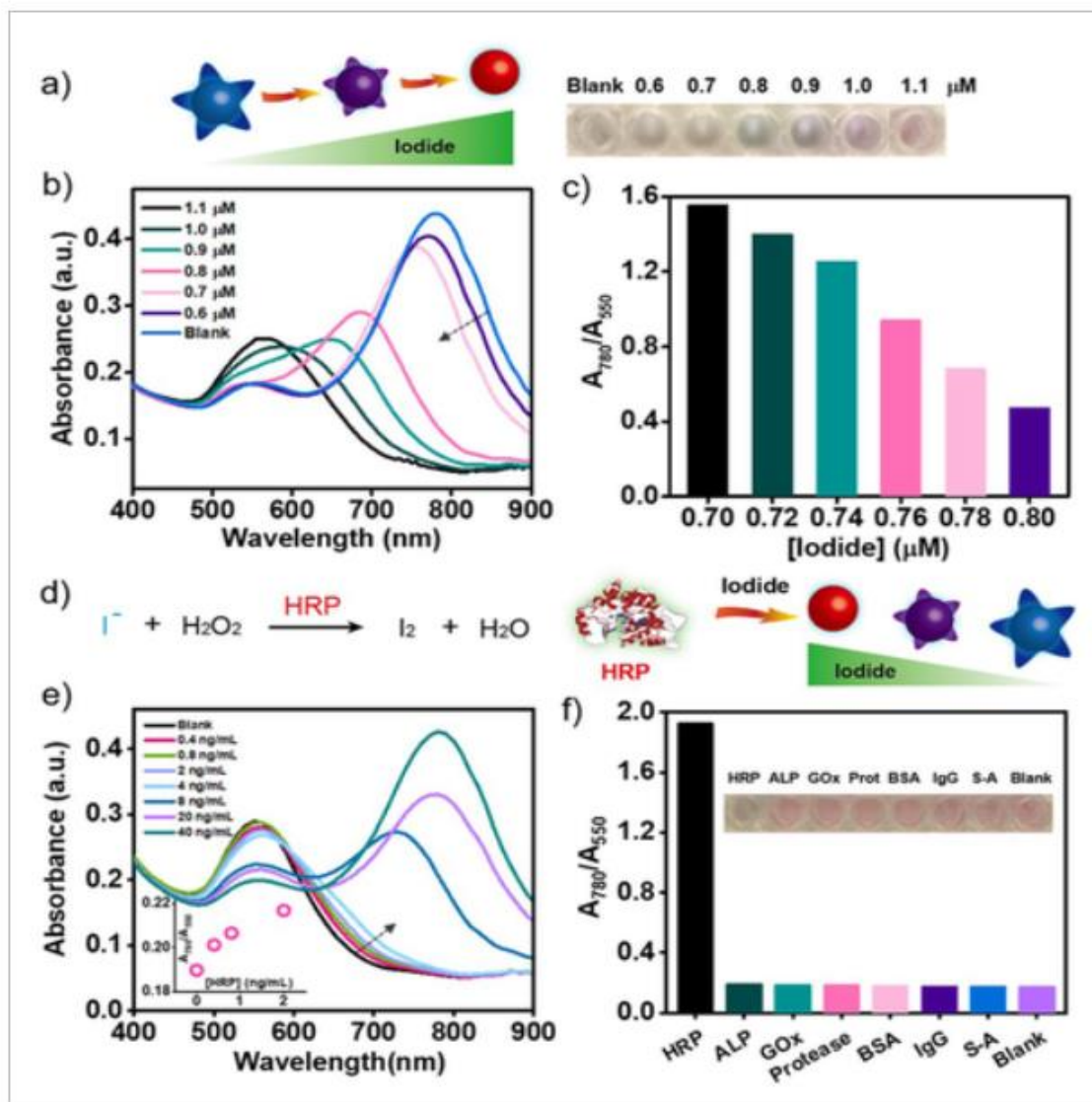


Figure 2-8: Iodide-mediated surface etching sensitivity and specificity for HRP detection. a) Iodide-induced concentration-dependent surface etching of AuNS. b) UV-Vis spectra of AuNS etched by varying iodide concentrations. c) AuNS A_{780}/A_{550} value etched by iodide varying from 0.7 μ M - 0.8 μ M. d) Illustration of an HRP-catalyzed iodide consumption reaction that prevents surface etching of AuNS. e) AuNS UV-Vis spectra following iodide consumption catalyzed by varying HRP concentration. f) AuNS colorimetric signals iodide consumption by HRP catalysis (40 ng/mL) and other proteins (800 ng/mL) including ALP, GOx, protease (Prot), BSA, IgG, and streptavidin (S-A). Adopted from Xianyu *et al.* (2021a).

Furthermore, plasmonic colorimetric sensing has a substantial advantage over conventional detection methods in that readouts based on visible color shifts can be visualised with the naked eye.

2.5.3 Fundamentals of redox reactions, NAD(P)/NAD(P)H redox couples, and their influence in plasmonic biosensors

This section of the literature review is primarily concerned with the fundamentals of redox reactions, NAD(P)/NAD(P)H redox couples and their influence in plasmonic gold nanostar-based biosensors. Using gold nanostars as the biosensor platform, extensive knowledge is available on how neo-formed H_2O_2 from enzymatic processes affects gold nanostar morphology and colourimetric signals. Nonetheless, little is known about how gold nanostars function in the presence of electron-deficient or electron-rich environments as well as competitive inhibition-prone settings and how these environments affect gold nanostar performance. As a result, understanding how NAD(P)/NAD(P)H affect nanobiosensors has the potential to significantly expand the sensing fraternity even in the absence of H_2O_2 -producing enzymes. Furthermore, the feasibility of using NAD(P)/NAD(P)H to drive redox reactions for gold nanoparticle synthesis and colourimetric techniques is evaluated.

2.5.3.1 Introduction

Redox reactions, also known as reduction-oxidation reactions, are chemical processes in which electrons are shuffled from one atom, ion, or molecule to another and the oxidation number of a participating chemical species changes (Cracolice & Peters, 2015; Nelson *et al.*, 2008; Zanello *et al.*, 2019). The notion of redox reactions in biochemistry can be confounding because the transfer might take numerous forms. Electrons and protons are transmitted concurrently, as in the NAD(P)/NAD(P)H redox couples, which transmit two electrons and one proton (H^+) (Sharma *et al.*, 2022). The oxidation and reduction reactions always occur concurrently in redox reactions, as seen in Figure 2-9 below.

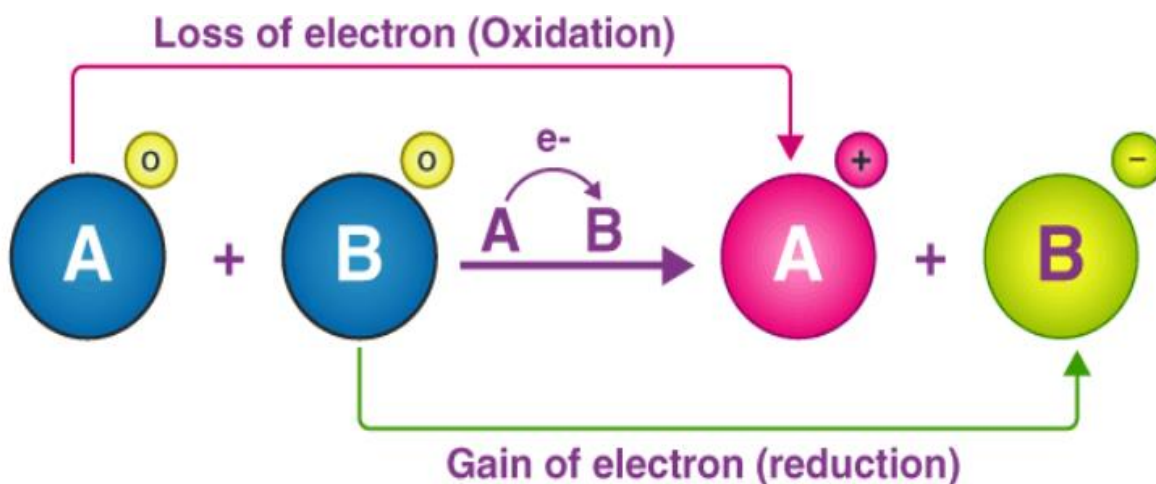


Figure 2-9: Representation of a reduction and oxidation process system.

Any redox process can be divided into two half-reactions, oxidation and reduction, which must be stoichiometrically balanced in order to account for the electrons involved in bond formation with other atoms (Bagotsky, 2005a). The thermodynamic activity of diverse chemical compounds can be assessed using the standard electrode potential E° (Kotz, 1991). However, because there are no methods for measuring its absolute value, the electrode potential of a chemical compound is measured relative to a standard hydrogen electrode (SHE) (Hill & Kolb, 2004). The redox half equations and electrode potential values shown in Table 2-2 are used throughout this section.

Table 2-2: Redox half equations and electrode potential values. Extracted from (Haynes, 2002).

Redox Equation	Electrode Potentials (E°) V
$\text{NAD}^+ + \text{H}^+ + 2e^- \rightleftharpoons \text{NADH}$	-0.32
$\text{NADP}^+ + \text{H}^+ + 2e^- \rightleftharpoons \text{NADPH}$	-0.324
$\text{AuCl}_4^- + 3e^- \rightleftharpoons \text{Au} + 4\text{Cl}^-$ (<i>bulk</i>)	+1.52
$\text{AuCl}_4^- + 3e^- \rightleftharpoons \text{Au} + 4\text{Cl}^-$ (<i>nano</i>)	+1.002
$\text{H}_2\text{O}_2 + 2\text{H}^+ + 2e^- \rightleftharpoons 2\text{H}_2\text{O}$	+1.78
$\text{Ag}^+ + e^- \rightleftharpoons \text{Ag}$	+0.80
$\text{H}_2\text{O}_2 + 2e^- \rightleftharpoons 2\text{OH}^-$	+0.867
$\text{Cu}^{2+} + e^- \rightleftharpoons \text{Cu}^+$	+0.153
$\text{O}_2 + 4\text{H}^+ + 4e^- \rightleftharpoons 2\text{H}_2\text{O}$ (<i>bulk</i>)	+0.401
$\text{O}_2 + 4\text{H}^+ + 4e^- \rightleftharpoons 2\text{H}_2\text{O}$ (<i>nano</i>)	+1.229
$\text{NO}_3^- + 2\text{H}^+ + 2e^- \rightleftharpoons \text{NO}_2^- + \text{H}_2\text{O}$	+0.421
$\Delta G^\circ = -nFE^\circ$ ΔG° : Gibbs free energy change per mole of reaction; E° : standard cell potential n: s of electrons transferred in the reaction; F: Faraday constant ($96485 \text{ Coulombs/mol} \times e^-$)	

2.5.3.2 NAD(P)/NAD(P)H redox couples in biochemical reactions

Nicotinamide adenine dinucleotides are ubiquitous soluble redox coenzymes that undergo reversible oxidation and reduction in all major metabolic pathways (Koju *et al.*, 2022; Xiao *et al.*, 2018). Many pathological disorders have been linked to redox couple imbalances, with the NAD⁺/NADH redox couple regulating cellular energy metabolism (glycolysis and mitochondrial oxidative phosphorylation) and the NADP⁺/NADPH redox couple maintaining redox balance (fatty acid and nucleic acid production) (Koju *et al.*, 2022; Yang & Sauve, 2016; Ying, 2008). NADP⁺ is

structurally identical to NAD⁺, with the exception of an extra 2' phosphate on the adenosine ribose moieties (Fig.2-10).

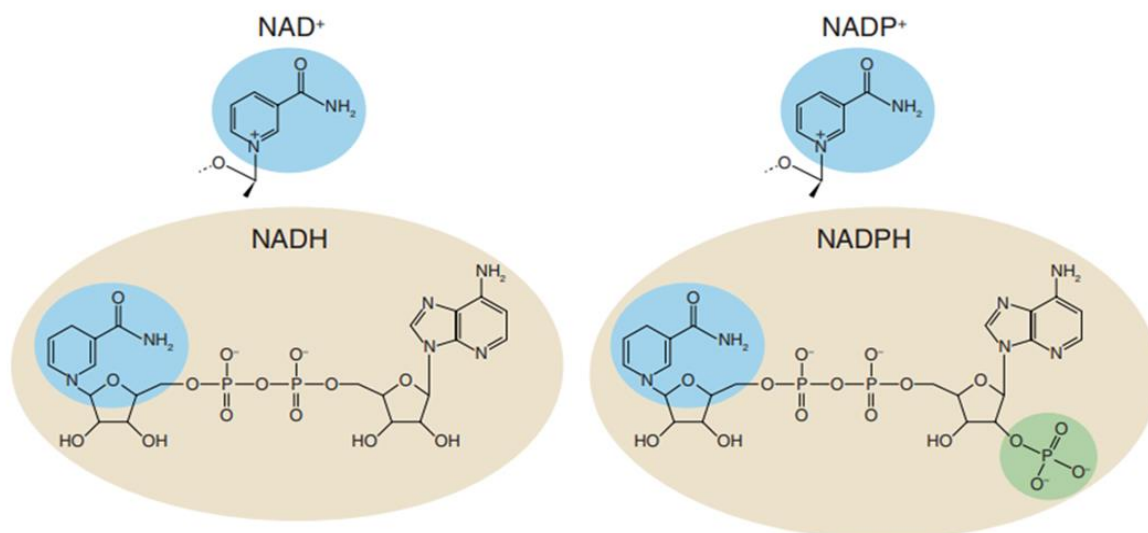


Figure 2-10: Structural difference of the NAD(P)/NAD(P)H redox coenzymes.

The aldo-keto reductases (AKRs) are an enzyme superfamily composed of aldose reductases, aldehyde reductases, hydroxysteroid dehydrogenases and dihydrodiol dehydrogenases that metabolise a diverse variety of substrates in the presence of their coenzymes NAD(P)/NAD(P)H (Jez *et al.*, 1997). AKRs are usually 37 kDa monomers with a (α/β)₈-barrel motif, extensive loops at the back of the barrel that control substrate specificity and a conserved coenzyme binding domain (Penning, 2015). The (α/β)₈ or TIM-barrel motif (triose-phosphate isomerase) is a versatile and adaptable scaffolding with structural modifications required for binding a wide variety of carbonyl substrates (Jez *et al.*, 1997). The binding of the NAD(P)/NAD(P)H coenzyme to the enzyme triggers a massive conformational change, reorienting a loop and effectively locking the coenzyme in place (Borhani *et al.*, 1992). The active site of AKRs is located at the C-terminal face of the barrel and it is optimised for high-affinity interaction with pyridine nucleotides NAD(P)/NAD(P)H in the absence of a canonical Rossmann fold (Barski *et al.*, 2008; Jez *et al.*, 1997). The catalytic mechanism of AKRs involves binding of the substrate to the active-site pocket of the enzyme-coenzyme complex with subsequent hydride transfer from the nicotinamide ring to the carbonyl group (Ehrig *et al.*, 1994). The hydride transfer step is more rate-limiting than proton transfer, while the cofactor exchange and its attendant loop and enzyme conformational changes appear to be the rate-limiting step for the overall enzymatic reaction (Fig.2-11) (Flynn *et al.*, 1995; Grimshaw *et al.*, 1989; Weiner *et al.*, 2009).

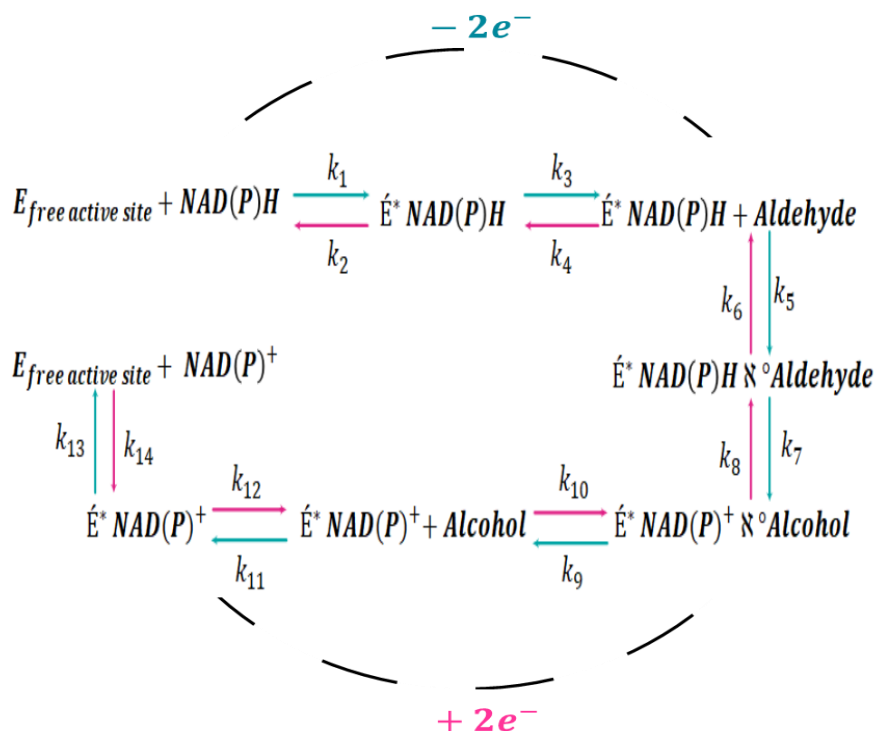


Figure 2-11: Schematic kinetic mechanism and electron transport in active site of NAD(P)/NAD(P)H dependent enzyme. Reproduced with permission from (Flynn *et al.*, 1995; Grimshaw *et al.*, 1990).

These coenzymes attach to a specific enzyme (s) to facilitate electron transport and hence aid in the creation of the enzyme-substrate complexes (Fig.2-11) (Mayr & Nidetzky, 2002). Because the change in redox state is proportional to the original substrate concentration, assessing NAD(P)/NAD(P)H concentration is an indirect method of measuring the required substrate (Mayr & Nidetzky, 2002). The involvement of NAD(P)/NAD(P)H redox couples in electron transport and shuttling activities results in no net nucleotide consumption (Koju *et al.*, 2022).

2.6 NAD(P)/NAD(P)H redox couples quantification assays

There have been numerous methodologies established for quantifying NAD(P)/NAD(P)H, including HPLC, single-cell capillary electrophoresis (CE), enzymatic cycling, fluorescence, colorimetry and chemiluminescence. However, several of these approaches are not only time-consuming, but also need time-consuming sample processing procedures and are costly to operate (Hatam *et al.*, 2017).

2.6.1 Fluorescence assays

The nicotinamide moiety of NAD(P)H absorbs light of wavelength 340 ± 30 nm and emits fluorescence of wavelength 460 ± 50 nm, whereas the related oxidised forms NAD(P)+ are not

fluorescent (De Ruyck *et al.*, 2007; Patterson *et al.*, 2000). Variations in autofluorescence intensity may thus represent changes in [NADH] or [NADPH], which are commonly denoted as NAD(P)H to indicate the unclear origin of the signal (Duchen *et al.*, 2003; Spät & Pitter, 2004). The Beutler test (Figure 2.11), a fluorescence-based test, is the most commonly used dry blood spot diagnostic test for galactosemia (Beutler; Beutler & Baluda, 1966; Fujimoto *et al.*, 2000) .

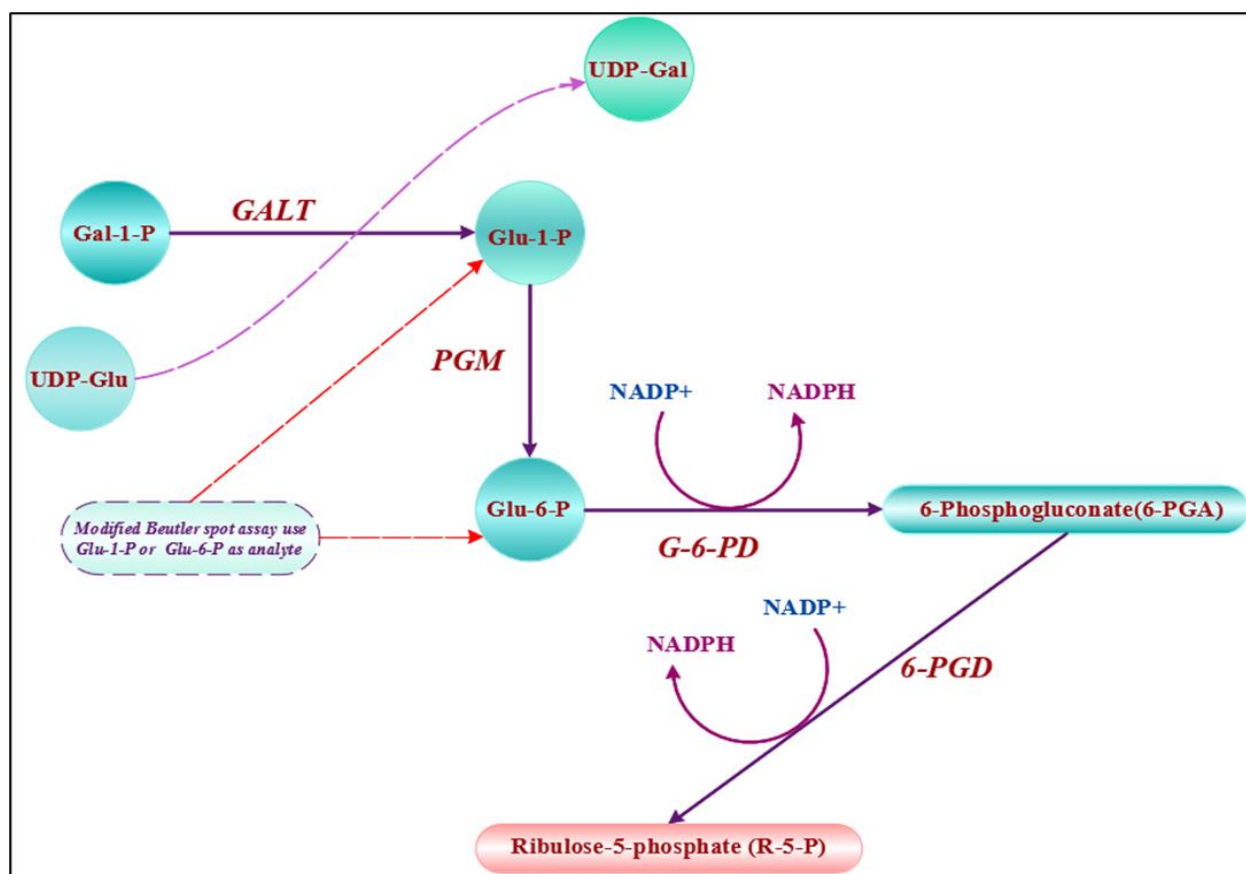


Figure 2-12: Schematic mechanism of Beutler's test. NADPH chrome fluorescence is used to quantify enzyme activity or deficiencies.

The reaction process involves fluorescence detection of the neo-formed NADPH, making Beutler's test an easy and quick assay for GALT and Gal-1-P mass screenings (Misuma *et al.*, 1981).

2.6.2 Colourimetric assays

2.6.2.1 Dye-based NAD(P)/NAD(P)H colourimetric assays

Although the reduction of tetrazolium salts and other organic dyes by NAD(P)H to formazan has been widely used in biological and clinical research, its application is limited due to the formazan product's low solubility and sensitivity (Chamchoy *et al.*, 2019; Tsukatani *et al.*, 2007). Formazan

dyes are synthetic chromogenic compounds produced by the reduction of tetrazolium salts by dehydrogenases and reductases (Fig.2-13). Depending on the initial tetrazolium salt used as the reaction substrate, formazan dyes range in hue from dark blue to deep red to orange (Septisetyani *et al.*, 2014).

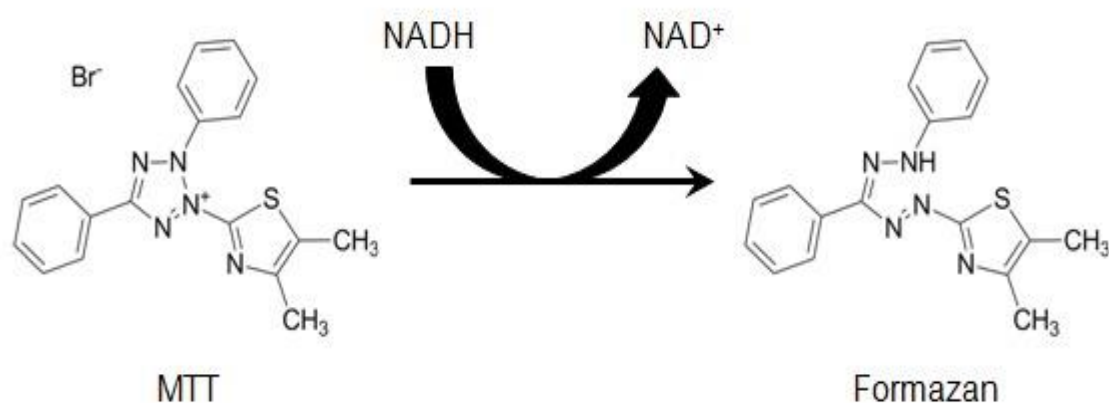


Figure 2-13: Structural differences between tetrazolium salt/formazan in the presence of NAD/NADH.

Polydiacetylenes (PDAs), a conjugated polymer family, have been widely exploited as sensing materials for detecting diverse species due to their distinctive optical features (Chen *et al.*, 2012). The alignment of PDA monomers is sensitive to external stimuli, which results in the release of strain on the backbone, generating a colourimetric change from blue to red and fluorescence amplification (Jiang *et al.*, 2020).

Overall, traditional colourimetric methods based on interactions between organic dye probes and target analytes, on the other hand, are limited by organic dyes' low extinction coefficients (Magaraphan & Joothamongkhon, 2013).

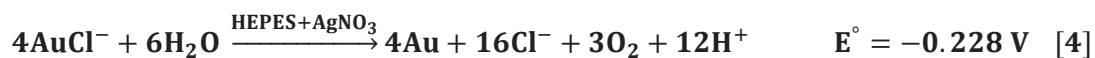
2.6.2.2 Nanoplasmonic-based NAD(P)/NAD(P)H colourimetric assays

Due to the unusually high extinction coefficients of AuNPs attributed by their LSPR, the detection limit of AuNP-based colourimetric assays can be in the nanomolar range (or even lower) (Ghosh & Pal, 2007). Catalytic growth and plasmonic colourimetric readouts for NAD(P)/NAD(P)H-based biocatalysed AuNPs conversions were reported by Xiao *et al.* (2004a). Furthermore, the LSPR band simultaneously redshifted, resulting in red to blue colourimetric signals dependent on the concentration of NAD(P)H in the solution (Xiao *et al.*, 2004a). Boonyuen *et al.* (2020) developed

a controlled reversible assembly of AuNPs in the presence of NADPH for the sensitive colourimetric detection of glucose-6-phosphate dehydrogenase (G6PD) deficiency (Boonyuen *et al.*, 2020). Their findings revealed that AuNPs segregate in the presence of NADPH and aggregate in the absence of NADPH. Finally, the employment of varied AuNP shapes in sensing NAD(P)/NAD(P)H and associated analytes has the potential to diagnose many clinical disorders.

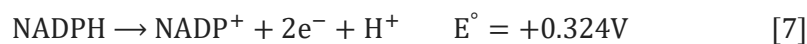
2.7 Theoretical NAD(P)/NAD(P)H redox reactions with plasmonic gold nanostars

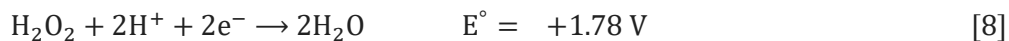
The AuNS synthesis technique was also discussed in redox terms to help interpret the redox chemistry involved when NAD(P)/NAD(P)H interacts with AuNS. HEPES at a certain pH initially reduces the Au (III) salt to Au(I) ions, following this a disproportionation process occurs, yielding thermodynamically unstable Au^0 (Xie *et al.*, 2007b). These activated Au^0 species serve as the nucleation centres and protuberant spikes develop in the presence of $AgNO_3$ (Atta *et al.*, 2019). This seedless growth process produces scattered heteromorphous AuNS of various diameters (Siegel & Baker, 2021).



Here, H^+ enhances the reduction potential of the O_2 half reaction from 0.401 to 1.229 V (vs a normal hydrogen electrode, NHE), while Cl^- acts as complexing agent, which reduces the reduction potential of Au^0/Au^{3+} from 1.52 to 1.002 V (vs NHE) (Long *et al.*, 2014). The electrode potentials of species are highly dependent on environmental factors such as pH value and ligand complexation, which essentially can be understood from the Nernst Equation (Deng *et al.*, 2015; Long *et al.*, 2014). For the case of Cl^- and O_2 , oxygen plays the central role in oxidation while chloride is mainly responsible for corrosion and potential reduction (Long *et al.*, 2014).

Theoretical thermodynamics predicts that NAD(P)⁺ and H_2O_2 spontaneously reacts with Au^0 (AuNS) as follows using typical reduction potentials and half-cell reactions (Table 2-2):





Thermodynamically viable and spontaneous redox reactions show that NAD(P)⁺ and H₂O₂ can trigger morphological changes in AuNS (Equations 9 and 10). The Au-NAD(P)H redox reaction, on the other hand, is not thermodynamically viable; however, Huang *et al.* (2005) suggested that in the presence of oxygen, AuNPs (rods, spheres) can react with NAD(P)H, resulting in spectrophotometrically detectable morphological changes (Fig.2-14) (Huang *et al.*, 2005). It was demonstrated in their study that AuNPs promoted the oxidation reaction of the biofluorophore NADH to NAD⁺ with a visible decrease in absorption intensity at 340 nm and a rise in absorption intensity at 260 nm over time. The shift in the surface plasmon absorption band of AuNPs suggests that NADH is surface-catalysed by the AuNPs, with surface-bound O₂ interacting with NADH to produce NAD⁺. Furthermore, the process was fluorometrically recorded, demonstrating that the conversion reaction causes the fluorescent intensity of NADH to be quenched because NAD⁺ is not fluorescent (Huang *et al.*, 2005).

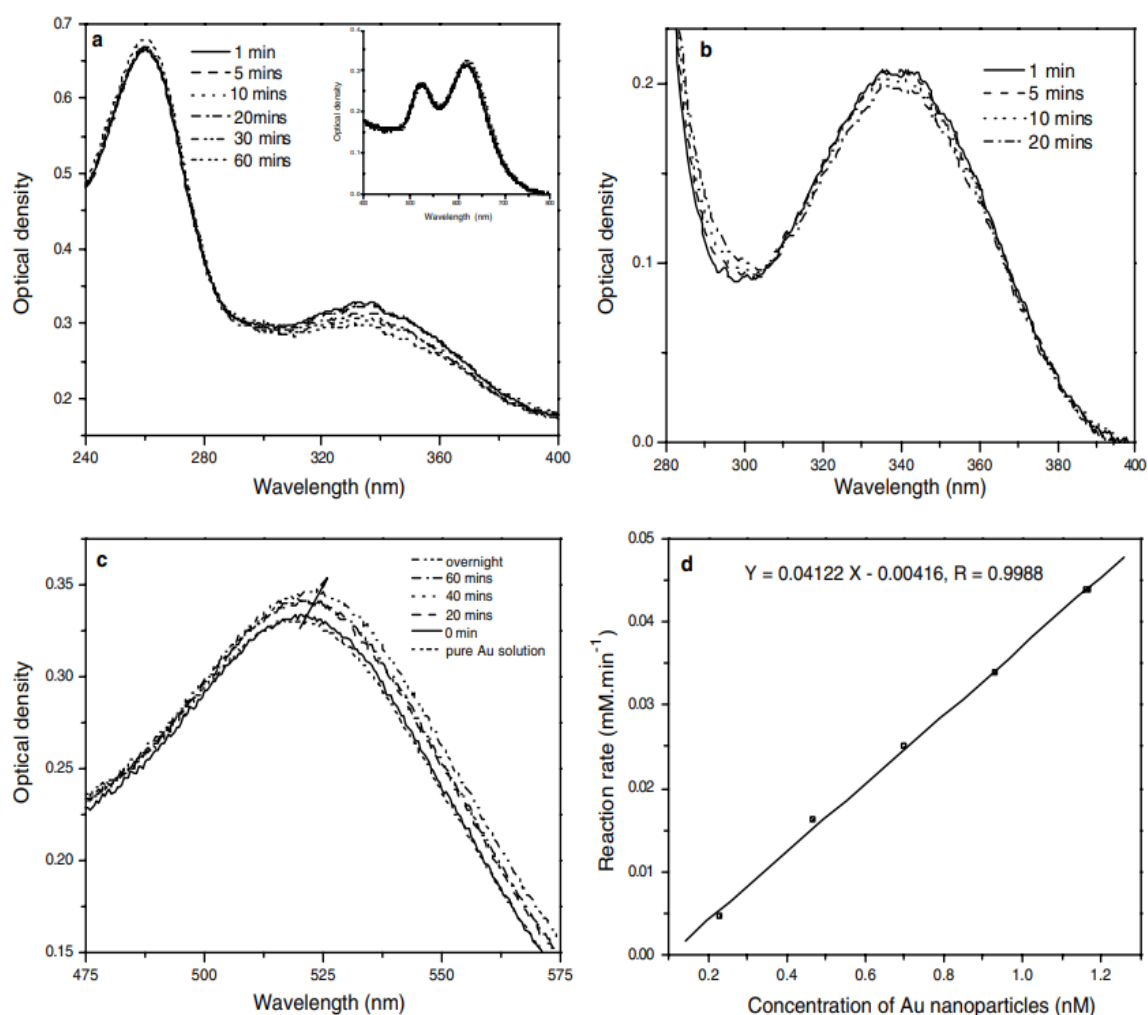


Figure 2-14: (a) NADH (0.05 mM) absorption spectra at various intervals after gold nanorods (AuNRs-0.3 nM) were introduced (b) Absorption spectra of 0.05 mM NADH at various periods following the addition of 0.17 mM HAuCl₂ solution. (c) Absorption spectra of AuNPs (spheres-0.46 nM) at various intervals following the addition of 0.05 mM NADH. (d) NADH oxidation initial reaction rate vs. AuNPs concentration. Adapted from Huang *et al.* (2005).

Hietzschold *et al.* (2019) and Xiao *et al.* (2004b) on the other hand, proposed that NAD(P)H can facilitate the formation of AgNPs and AuNPs in the presence of AgNO₃ and AuCl₄⁻ in the presence of reducing agents (Hietzschold *et al.*, 2019; Xiao *et al.*, 2004b). In this regard, NAD(P)H functions as a moderate reducing agent, resulting in the formation of AgNPs and AuNPs. Xi and colleagues investigated the post-synthesis effects of HEPES, a zwitterion reducing and capping agent in HEPES-mediated anisotropic AuNS syntheses (Xi & Haes, 2019). They clarified how HEPES interacts with AuNS after synthesis and how acidification causes remodelling of AuNS morphology and the effect is reliant on both pH and acid content as well as the protonation state of HEPES (Xi & Haes, 2019).

2.8 Conclusions and Perspectives

According to the literature review, gold nanostars are of interest among the various morphologies because they exhibit (i) excellent tunable LSPR; (ii) strong electric field and surface-enhanced Raman spectroscopy (SERS) enhancement factors among nanoparticle shapes; and (iii) sensitive optical responses for local refractive index sensing. There have been few fundamental investigations on plasmonic AuNS and it is unclear how capping agents affect colourimetric readouts, stability, specificity and functionalisation. Furthermore, the bulk of plasmonic AuNS-based biosensors described in the literature use oxidoreductase enzymes, specifically H_2O_2 -producing oxidase enzymes.

This literature review revealed that nanoparticles act differently than bulk materials of the same composition (Roduner, 2006). This phenomenon could be explained by differences in their physico-chemical characteristics (bulk density, size, surface area and shape) as well as quantum effects (Ghosh, 2015; Kumar, 2016). Furthermore, because gold is chemically inert, it cannot react with NAD(P)H utilising bulk redox chemistry. In nano redox chemistry, on the other hand, NAD(P)H can thermodynamically react with AuNPs, causing concentration-dependent morphological changes in AuNPs. In addition, as Huang *et al.* (2005) indicated, the presence of AuNPs surface-bound etchants (O_2 , halide ions, etc.) might influence NAD(P)H reaction kinetics and redox chemistry (Huang *et al.*, 2005). Furthermore, it remains elusive how plasmonic AuNS-based biosensors perform in H_2O_2 -free environments, electron-rich (reductive) and electron-deficient (oxidative) environments. However, limited efforts have been devoted to conceptualising the fundamental surface, redox and electrochemistry governing plasmonic AuNS with their environment and their efficacy in different environments (H_2O_2 -free environments, reductive and oxidative environments). Some knowledge gaps remain in the literature, with the prominent ones being:

- 1) The difficulty to synthesise standard AuNS with controlled shape, size, and defect distribution in a reproducible manner.
- 2) The availability of standard redox chemistry for AuNS synthesis, functionalisation, and performance in a variety of environments.
- 3) The need to fully characterise electronic, electrostatic, optical, and geometric contributions on plasmonic AuNS behaviour.
- 4) The complexity of real experimental conditions compared to model experiments.

To bridge these knowledge gaps, a comprehensive multidisciplinary approach to tailoring and enhancing fundamental understanding of nanoparticle reaction kinetics, redox processes, thermodynamic properties and surface chemistry is required. To summarise, the selection of synthesis techniques and appropriate capping ligand(s) should be a major consideration in the design of bioassays and AuNS biosensors.

CHAPTER 3 STUDY DESIGN

A MAN'S MIND, STRETCHED BY NEW IDEAS, MAY NEVER RETURN TO ITS ORIGINAL DIMENSIONS.

– Oliver Wendell Holmes Jr

3.1 Introduction

Nanotechnology is a relatively new and rapidly developing science where materials in the nanoscale range are employed to serve as delivery therapeutic agents, imaging agents and medical diagnostic tools. The development of nanotechnology-based assays would ease the diagnosis of diseases in remote areas and empower the healthcare delivery systems at large. Nanotechnology serves as a potentially green, cost-effective and simplified instrument fabrication route for bioanalytes analysis. Therefore, the synthesis, colourimetric and optimisation of readable results of the bioassays in relation to the biorecognition event is the nucleus of this research.

3.2 Experimental design

3.2.1 Aim

This research aimed to assess the suitability of NAD(P)/NAD(P)H as redox agents for gold nanostar shape alteration in colourimetric clinical enzymology. Furthermore, the study investigated and attempted to comprehend the redox chemistry underlying the principles of gold nanoparticle colourimetric reactions in order to tailor the reactions for clinical enzymology.

3.2.2 Objectives

3.2.2.1 Objective 1: Gold nanostar synthesis, characterisation, optimisation and stability evaluation.

This objective was achieved by applying a HEPES-mediated synthesis protocol of gold nanostars as described by Mulder, Phiri, Jordaan, *et al.* (2019), characterising them (size, shape and charge) by standard methods, functionalising them with capping agents and enzyme(s) commonly used in gold nanoparticle work (Chirico *et al.*, 2015; Javed *et al.*, 2020b; Liu *et al.*, 2014; Miao *et al.*, 2011; Restrepo & Villa, 2021; Stankus *et al.*, 2011). ISO standard methods (UV-Vis, TEM and naked-eye inspections) were employed for gold nanostar characterisation throughout this work. Characterisation of these nanostars was critical to ensure reproducibility and consistency with those synthesised by Mulder, Phiri, Jordaan, *et al.* (2019), as this research builds on earlier research and the outcomes must be comparable (Mulder, Phiri, Jordaan, *et al.*, 2019). Furthermore, the nanostars were studied for physico-chemical parameters such as structure size, oxidation state, morphology, electrochemistry, composition and optical characteristics (Clogston, 2021; Raval *et al.*, 2019).

Although substantial work has previously been done on these gold nanostars, the effects of different capping agents on these nanostars have not yet been investigated. In this objective, we investigated two essential gold nanostar surface chemistry properties. Phase 1 looked into how different capping agents affected gold nanostar stability and hydrogen peroxide colourimetric detection ability. Phase 2 looked at how different successful capping agents from Phase 1 affected enzyme functionalisation and the colourimetric biosensing ability of the final nanobiosensors.

Phase 1: Capping agents selection criteria and hydrogen peroxide colourimetric sensing ability

A wide range of capping agents typically employed in nanoparticle synthesis activities are evaluated for their capacity to successfully passivate the freshly synthesised gold nanostars from Objective 1 (Gulati *et al.*, 2018; Javed *et al.*, 2022; Javed *et al.*, 2020b; Pedroso-Santana & Fleitas-Salazar, 2023). Additionally, studies were done to determine if capping agents affected gold nanostar stability, functionalisation and colourimetric sensing biosensing capabilities.

Influence of capping agents on gold nanostar matrix and storage stability

Different capping agents were used to passivate the optimised gold nanostars during synthesis. The capped gold nanostars' stability was assessed for storage conditions (room temperature and 4 °C) and in different matrix conditions (synthetic urine, supplemented medium, high ionic strength and synthetic serum) typically found in biosensor applications. Optimal concentrations of capping agents were selected based on the relative change in the longitudinal resonance maximum absorbance value (OD_{max}) of gold nanostars using UV-Vis spectrophotometer, limited morphological degradation using transmission electron microscopy (TEM) and by visual colourimetric changes of the colloidal solution. Furthermore, capping agents that produced a decline in the OD_{max} of the gold nanostar of more than 30% were declared unstable using UV-Vis spectroscopy (Blessy *et al.*, 2014b). A vertical (hyperchromic and hypochromic) shift in the OD_{max} intensity of gold nanostars on UV-vis spectroscopy implies a change in gold nanostar size, while a horizontal (hypsochromic and bathochromic) shift in the absorption intensity on UV-vis spectroscopy indicates a change in gold nanostar morphology (Siegel & Baker, 2021). Successful capping agents that met these criteria for passivating gold nanostars were used throughout this study.

Influence of capping agents on gold nanostar hydrogen peroxide sensing

Objective 1 involves exposing successfully capped gold nanostars to detect varying concentrations of synthetic hydrogen peroxide. The sensing performance of capped nanostars was improved by optimising experimental conditions (matrix, pH, buffer detection solution and AuNS concentration). TEM images, spectrum shifts, colourimetric signal generation rate and colourimetric vividness were compared for the degree of morphological gold nanostar alterations to determine capping agent efficacy and their effects on gold nanostar biosensing capabilities.

Phase 2: Pilot study – Capping agents' influence on gold nanostar biosensor stability, functionalisation and colourimetric biosensing capability

Successfully capped gold nanostars from Objective 1 were functionalised with enzymes, coenzymes and other proteins to enhance their stability, functionality and specificity (Ding *et al.*, 2015a; Guo *et al.*, 2015). The thiol-based compatibilizer 3'30-dithiobis sulfosuccinimidyl propionate (DTSSP) was used for all protein functionalisation throughout this study. Thiol groups strongly bind to gold via covalent interactions due to the high affinity of gold and sulphur and the ease of conjugating other materials inspired us to adopt DTSSP (Mahato *et al.*, 2019; Zong *et al.*, 2017). Furthermore, different immobilisation techniques may be utilised to bind proteins of choice to the gold nanostars (Decker *et al.*, 2020; Walper *et al.*, 2015). For this study, direct protein and co-immobilisation of proteins were used where applicable.

As proof of concept for Objective 1, successfully capped gold nanostars were functionalised with glucose oxidase (GOx) as a reference enzyme and glucose as a reference analyte, employing Rodríguez-Lorenzo *et al.* (2012) colourimetric strategy (Rodríguez-Lorenzo *et al.*, 2012). The colourimetric response of the biosensors was evaluated to determine whether enzyme orientation and functionalisation affected gold nanostar biosensing capabilities using DTSSP-free GOx and DTSSP functionalised GOx-differently capped gold nanostars from Phase 1 (Chirico *et al.*, 2015; Howes *et al.*, 2014; Phiri *et al.*, 2019a). The biosensors would indirectly monitor glucose concentrations based on plasmonic and morphological changes in gold nanostar in the presence of neo-formed H₂O₂. Concentration-dependent colourimetric signals were obtained by tailoring and optimising experimental conditions (buffer selection, AuNS characteristics, concentrations of H₂O₂, AgNO₃, NaOH and pH).

3.2.2.2 Objective 2: Comparison of gold nanostar colourimetric biosensors in hydrogen peroxide-free, electron-deficient and electron-sufficient environments

As a preliminary step, we simply exposed gold nanostars from Objective 1 to H_2O_2 and NAD(P)/NAD(P)(H) to demonstrate the principle before continuing to develop enzyme assays. The proof of principle is most significant for determining how the concentrations of H_2O_2 and redox agents (and presumably the rate at which they are formed) affect the colourimetric, spectrophotometric and morphological changes in AuNS (Zhang *et al.*, 2018).

Overall, the goal of Objective 2 was to use enzyme assays to evaluate how gold nanostar biosensors performed in hydrogen peroxide-free, electron-deficient (oxidative) and electron-sufficient (reductive) environments. Using the auxiliary pathway of galactose metabolism as shown below (Fig.3-1), three biosensors were developed with one producing H_2O_2 (Control) and the other two producing NAD(P)/NAD(P)H (Experimental). The effects of neo-formed H_2O_2 on colourimetric, spectrophotometric and morphological changes in gold nanoparticle biosensors have long been researched (Chang *et al.*, 2019; Minati *et al.*, 2014b; Phiri *et al.*, 2019a). This research was motivated by the fact that very little is known about how NAD(P)/NAD(P)H affects gold nanoparticle biosensors. To investigate these phenomena, gold nanostar biosensors were used in the colourimetric detection of galactose as a model analyte in the presence of galactose oxidase (GalOx), galactose dehydrogenase (GalDH) and aldose reductase (AR). Furthermore, the fundamental redox chemistry and the influence of NAD(P)/NAD(P)H on colourimetric, spectrophotometric and morphological changes in gold nanostar biosensors were established.

Suitability of NAD(P)/NAD(P)H as redox agents in enzymatic plasmonic gold nanostar assays for galactose quantification

The goal of Objective 2 was to investigate the use of NAD(P)/NAD(P)H as redox agents in colourimetric, spectrophotometric and morphological modifications in AuNS utilising colourimetric parameters defined in Objective 1. To carry out this experiment, three biosensors were created that used the same substrate and reaction conditions but different conjugated enzymes, as shown below (Fig 3-1). The selection of enzymes was motivated especially by the requirement for speedy, simple and inexpensive galactosemia screening in resource-constrained regions as well as to improve galactosemia diagnosis. Furthermore, because NAD(P)/NAD(P)H are universal cofactors/coenzymes, their successful use as redox agents in colourimetric, spectrophotometric and morphological alterations in AuNS might broaden the biosensing fraternity to other clinical disorders.

Gold nanostar colourimetric biosensors fundamental analysis

As described in Objective 2, three biosensors were constructed that used the same substrate (galactose) and reaction parameters but different conjugated enzymes. The oxidase biosensor served as the control because substantial study on H_2O_2 and gold nanoparticles was conducted in the literature and in Objective 1. Using colourimetric criteria stated in Objective 1, ISO standard procedures (UV-Vis, TEM and naked-eye inspections) were used to assess the applicability of NAD(P)/NAD(P)H as redox agents in colourimetric, spectrophotometric and morphological alterations in AuNS.

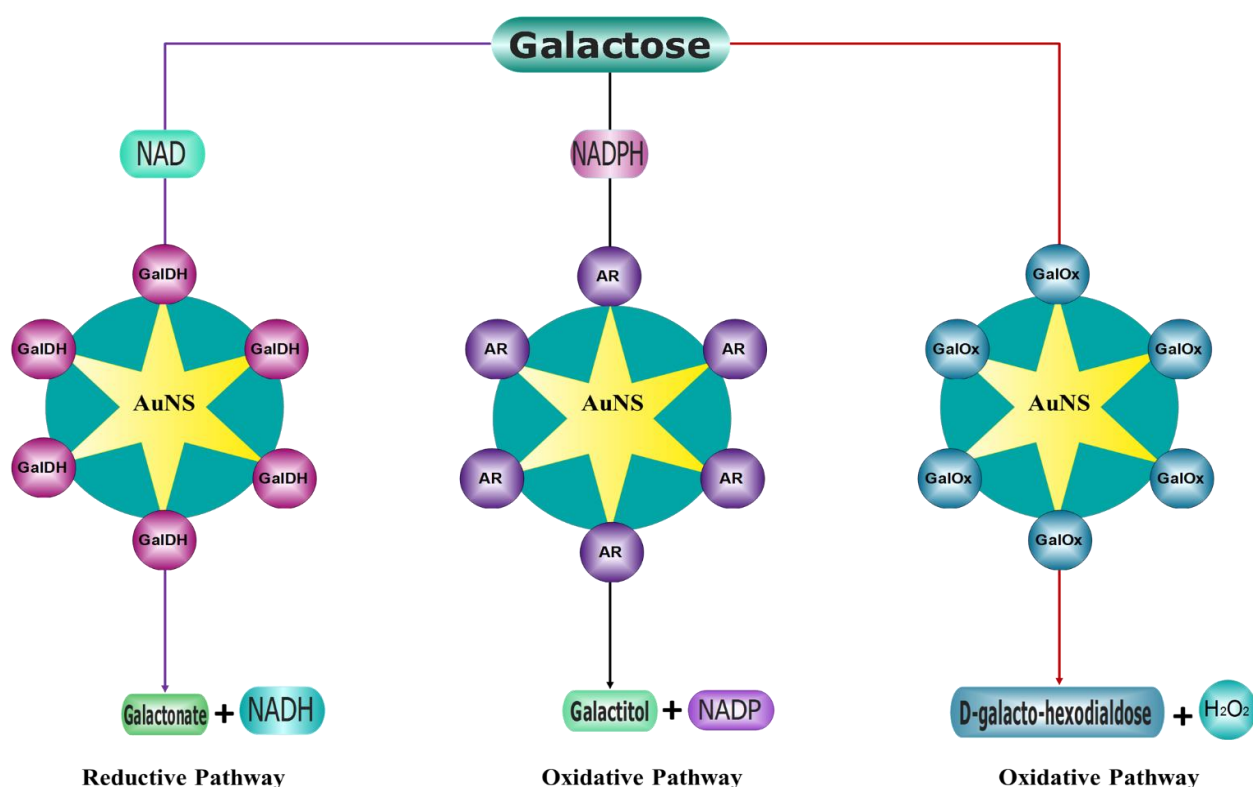


Figure 3-1: Schematic representation of the auxiliary galactose pathway biosensors.

Gold nanostar colourimetric biosensors specificity analysis

The specificities of the enzymes are known (Holden *et al.*, 2003; Leung-Toung *et al.*, 2002; Nagahara, 2011; Pollak *et al.*, 2007) and sugars are known to react with gold nanoparticles, resulting in plausible colourimetric, spectrometric and morphological alterations (Atta *et al.*, 2019; Lang *et al.*, 2014; Rodrigues *et al.*, 2021; Rodríguez-Lorenzo *et al.*, 2012). As a result, the focus of the effort was on demonstrating the preservation of expected enzyme specificities even after passivating and conjugating the gold nanostar scaffold.

To carry out this investigation, three different conjugated biosensors were developed as defined in Objective 2 and a variety of substrates (glucose, maltose, fructose) were used to assess biosensor specificity while maintaining the reaction parameters as defined in Objective 1. Using colourimetric criteria as stated in Objective 1, ISO standard procedures (UV-Vis, TEM and naked-eye inspections) were used to assess the feasibility of NAD(P)/NAD(P)H as redox agents in colourimetric, spectrophotometric and morphological alterations in AuNS.

Gold nanostar colourimetric biosensors matrix specificity analysis

To carry out this investigation, three distinct conjugated biosensors were developed as defined in Objective 2 and a variety of matrices (blank urine, foetal bovine serum, synthetic blood) were used to evaluate biosensor target analyte (s) selectivity efficacy and degree of interference in complex matrices using the colourimetric parameters as established in Objective 1. In order to accomplish this, ISO standard procedures (UV-Vis, TEM and naked-eye inspections) were utilised to assess the application and performance of NAD(P)/NAD(P)H as redox agents in colourimetric, spectrophotometric and morphological changes in AuNS using colourimetric criteria defined in Objective 1.

Comparative gold nanostar biosensors functionality assessment

A comparison study was conducted to evaluate the performance of the reductase and dehydrogenase biosensors in comparison to the oxidase biosensor (Control) as well as how the dehydrogenase biosensor compared to the reductase biosensor and vice versa. To achieve this, ISO standard techniques (UV-Vis, TEM and naked-eye inspections) were used to evaluate the applicability and performance of NAD(P)/NAD(P)H as redox agents in colourimetric, spectrophotometric and morphological changes in AuNS using colourimetric parameters as defined in Objectives 1 and 2. Figure 3-2 demonstrates probable colourimetric and morphological changes in AuNS during the biorecognition process.

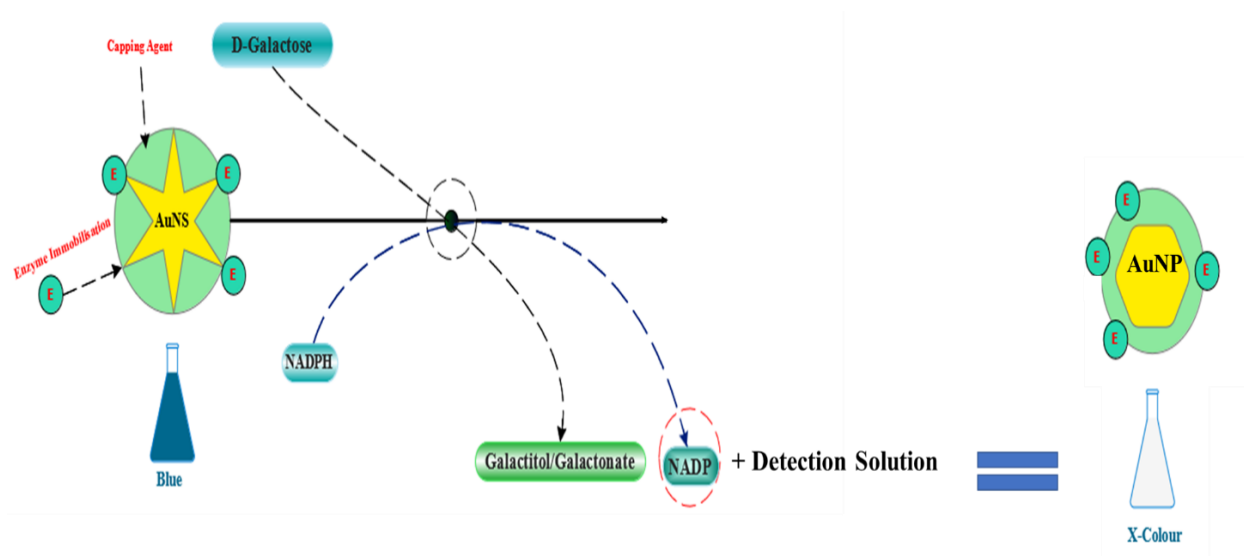


Figure 3-2: Schematic diagram of the suggested colourimetric reaction mechanism.

3.2.2.3 Objective 3: Theoretical gold nanostar biosensors colourimetric redox chemistry investigation

Gold nanostar redox potentials and reaction feasibility study

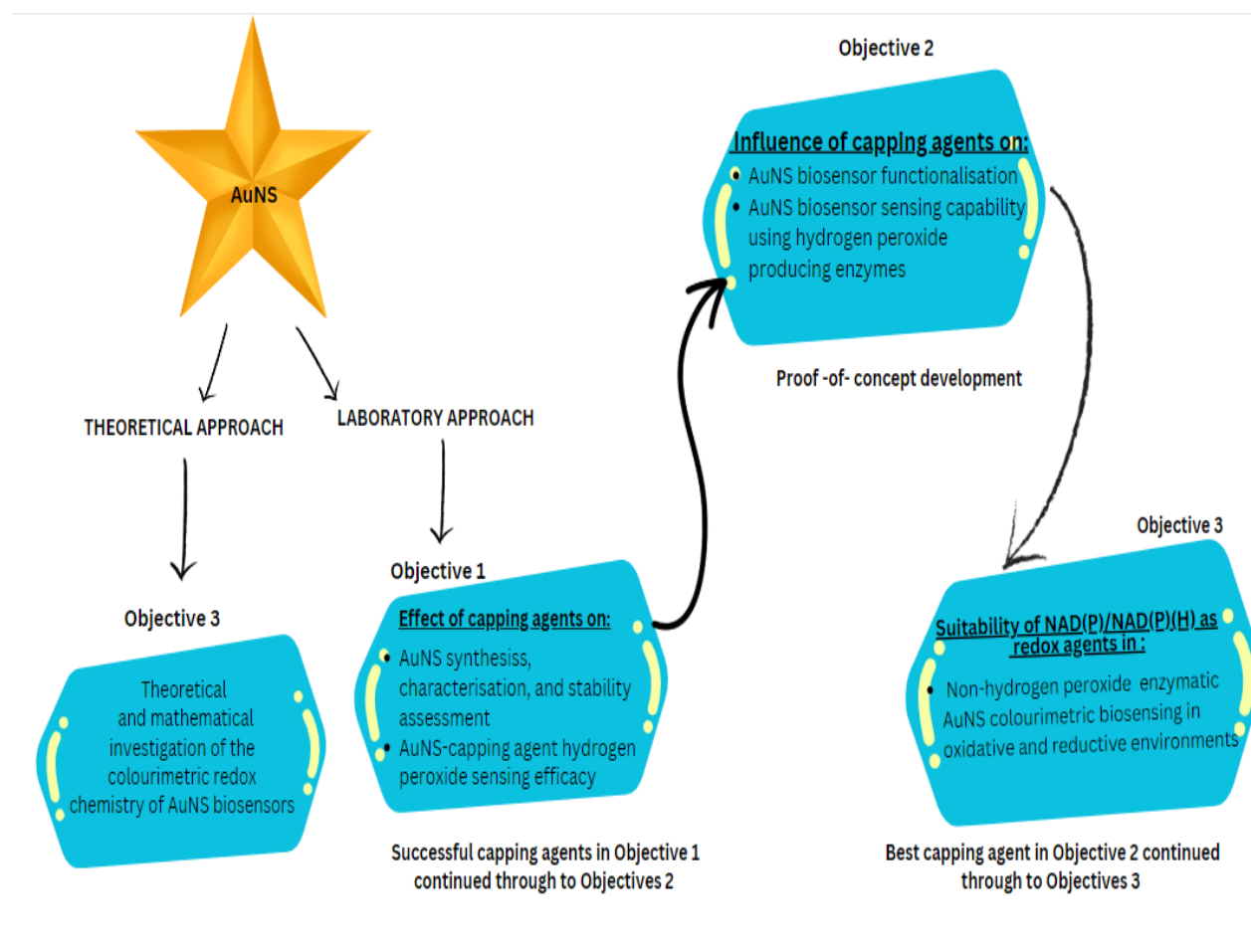
An in-depth understanding of how the redox potentials and thermodynamic properties of NAD(P)/NAD(P)H affect the feasibility of colourimetric reactions in enzyme-immobilised nanoparticles was determined. This was achieved by following the redox reactions from first principles using redox potentials and thermodynamic properties (Gibbs energy) to ascertain feasibility. Theoretical knowledge of how NAD(P)/NAD(P)H redox couples and H_2O_2 can influence the plasmonic colourimetric sensing and morphological changes of AuNS via redox potentials and chemistry are provided.

Gold nanostar mathematical modelling

Mathematical modelling was done to fully conceptualise the redox reactions in situ and other probable side reactions that might be attributed to colourimetric signals and gold nanostar morphological changes. A theoretical mathematical model was developed to describe gold nanoparticle growth or etching as well as the likely primary drivers for either of the two unique events during biorecognition processes using plasmonic gold nanostars redox chemistry.

Adiabatic gold nanostar oxidation

A theoretical mathematical model was constructed to better comprehend adiabatic oxidation, the stability of gold nanostars and the effect of capping agents on gold nanostars. Furthermore, Scheme 3-1 depicts the overall summary of experimental and theoretical design of the thesis.



Scheme 3-1: Experimental and theoretical design summary schematic diagram.

3.3 Scope, limitations, and substantiation

The study sought to explore the underlying working principles of gold nanostars and how NAD(P)/NAD(P)H might influence gold nanostars in colourimetric reactions from a redox reactions' standpoint. Other related techniques, such as liquid chromatography-mass spectrometry (LC-MS), fluorescence, high-performance liquid chromatography (HPLC), bioluminescence and cyclic voltammetry, could be used to investigate the influence of NAD(P)/NAD(P)H in gold nanostars (Chen *et al.*, 2021; Duffield *et al.*, 2021; Jiang, Zhao, *et al.*, 2013; Jiang *et al.*, 2020). However, these techniques have limitations, including a demand for

expertise, expensive reagents and consumables and it is often time-consuming. As a result of the necessity for a simple, fast and effective method of validating and diagnosing metabolic disorders (classic galactosemia), we used gold nanostars as a scaffold and signal-amplifying transducer. However, a recent renaissance in fundamental gold nanostar research has substantially enhanced our understanding of their production, functionalisation, characteristics and hence their applicability biological and clinical applications (Seaberg *et al.*, 2021). Furthermore, a path forward is proposed based on their outstanding intrinsic properties, ease of synthesis and straightforward result interpretation, with the goal of enabling the effective integration of these gold nanostars in the rapid screening and diagnosis of clinical metabolic disorders.

There are various synthetic approaches for gold nanostars; we chose one and utilised it as a model throughout the investigation. This was due to the fact that we had discovered in the literature review that gold nanostars from different gold nanostar synthesis protocols do not always yield the same outcomes in the same chemical/biological environment (Daenzer & Fridovich-Keil, 2017; Seaberg *et al.*, 2023). Therefore, the HEPES and AgNO₃ mediated seedless technique was used for synthesis because it involves fewer stages and employs more environmentally friendly reagents (Mulder, 2019; Xie *et al.*, 2007b). Furthermore, because it is not necessarily generalisable to other similar components, a specific enzyme(s) and functionalisation technique was used and during AuNS synthesis, HEPES was employed as a mild reducing and capping agent, whereas AgNO₃ was used as a precise shape-directing agent (Atta *et al.*, 2019).

Our selection of this pathway as a model was motivated by our understanding of the Leloir and auxiliary pathways, given that three distinct enzymes metabolise the same substrate (galactose), yielding NAD(P)/NAD(P)H, H₂O₂ and other sugar-based by-products (Holden *et al.*, 2003; Srivastava & Beutler, 1969). Because the enzymes work on the same analyte and produce different products and redox conditions, comparing the functionality of the biosensors was simple.

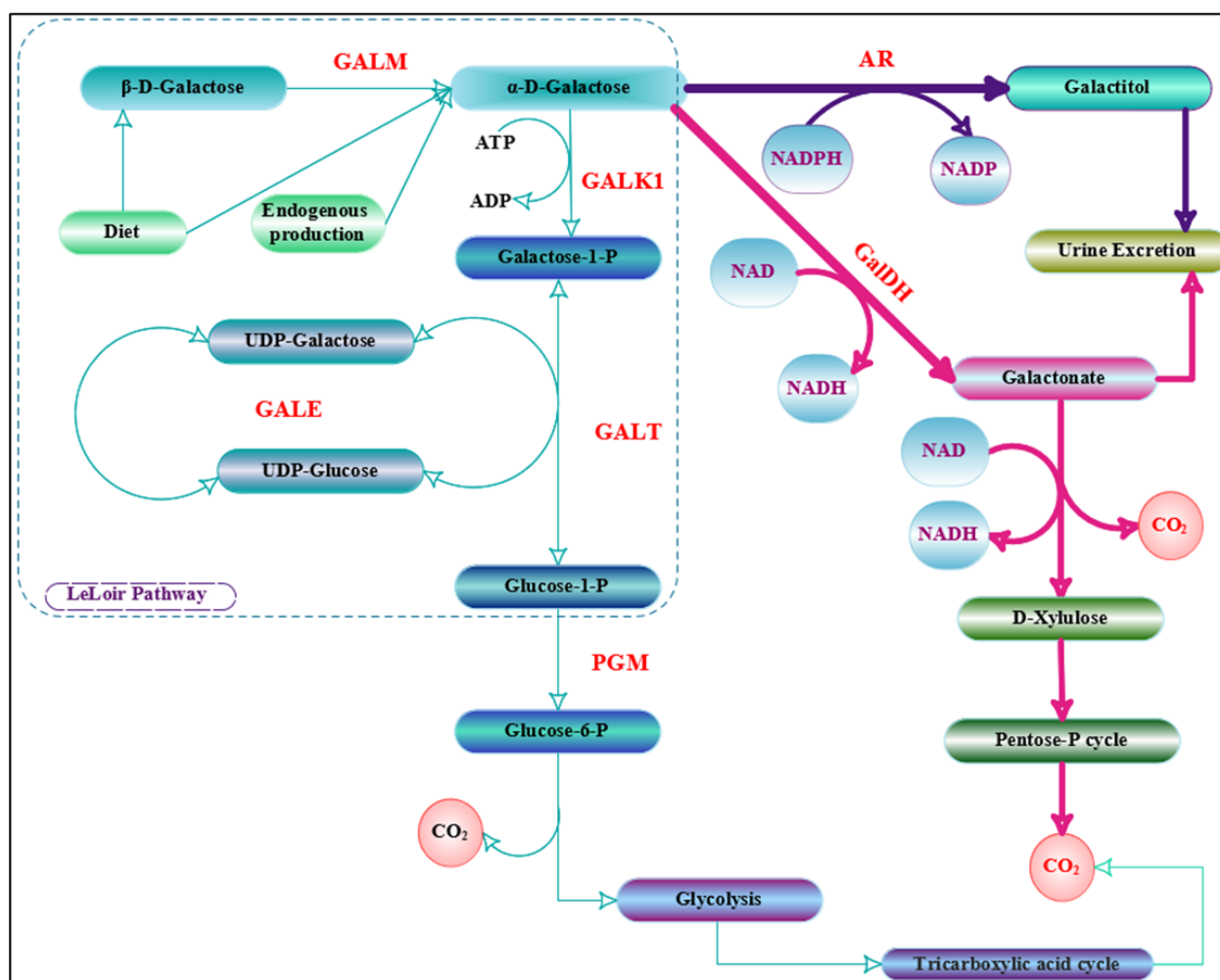


Figure 3-3: Leloir pathway and the endogenous auxiliary pathways for galactose metabolism.

The auxiliary pathways of galactose metabolism were investigated in this study employing NAD(P)/NAD(P)H-dependent enzymes (GalDH and AR) (Figure 3-3) and a reference enzyme GalOx. The enzyme concentrations required to create adequate H_2O_2 for spectrophotometric and colourimetric studies in functionalised AuNS were determined largely using GOx and GalOx. After biocatalytic interactions with target analytes, the by-product H_2O_2 of GOx and GalOx is known to preferentially etch or grow AuNPs depending on the conditions (Chang *et al.*, 2019; Long *et al.*, 2020; Zhang *et al.*, 2018). This basic knowledge ensured that, when we functionalised an H_2O_2 -producing oxidoreductase enzyme to our AuNS, we could get a colourimetric result. As a result, we functionalised GalOx to our AuNS to aid in troubleshooting if a colourimetric result was not obtained. The technical feasibility of transferring and extending the sensing system to non- H_2O_2 -producing oxidoreductases such as NAD(P)/NAD(P)H-dependent (GalDH and AR) was investigated.

NAD(P)/NAD(P)H are universal and ubiquitous redox coenzymes/cofactors involved in metabolism, cellular redox status and fatty acid and nucleic acid production (Xiao *et al.*, 2018). As a result, plasmonic colourimetric sensing of NAD(P)/NAD(P)H redox couples may lead to the straightforward diagnosis of a variety of clinical disorders, including galactosemia, diabetes, cardiovascular disease, neurodegenerative illness and cancer. When the devised techniques showed that AuNS might be used as a biosensor for both non-hydrogen peroxide enzyme-mediated assays and hydrogen peroxide enzyme-mediated assays, AuNS's versatility in colourimetric sensing was substantially enhanced.

CHAPTER 4 SYNTHESIS OF GOLD NANOSTARS, CHARACTERISATION AND COLOURIMETRIC ASSESSMENT

AN INVESTMENT IN KNOWLEDGE PAYS THE BEST INTEREST.

– Benjamin Franklin



nanomaterials

an Open Access Journal by MDPI



CERTIFICATE OF ACCEPTANCE



Certificate of acceptance for the manuscript (**nanomaterials-1795964**) titled:
The effect of capping agents on gold nanostar stability, functionalization, and
colorimetric biosensing capability.

Authored by:

Tozivepi Aaron Munyayi; Barend Christiaan Vorster; Danielle Wingrove Mulder

has been accepted in *Nanomaterials* (ISSN 2079-4991) on 11 July 2022



Academic Open Access Publishing
since 1996

Basel, July 2022

4.1 Preface

This chapter fulfilled Objective 1 from Chapter 3, which was to synthesise, characterise and evaluate the stability and colourimetric signal production of variably capped gold nanostars. Gold nanostars were synthesised and demonstrated to be homogenous with diverse protuberant arm diameters. ISO-recommended procedures were used to assess the authenticity of the gold nanostar morphology. The gold nanostars were discovered to have varying stabilities in bioassay settings based on the capping agent employed to passivate them post-synthesis stage. This chapter was submitted to, accepted by and published in the Journal of Nanomaterials by MDPI (<https://doi.org/10.3390/nano12142470>). Minor changes were made from the published version, based on examiners' comments and supplementary information is included in Appendix A.

4.2 Abstract

Capping agents (organic ligands, polymers and surfactants) are pivotal for stabilising nanoparticles; however, they may influence the surface chemistry as well as the physico-chemical and biological characteristics, of gold nanostar (AuNS)-based biosensors. In this study, we proved that various capping agents affected capped and bioconjugated AuNS stability, functionality, biocatalysis and colourimetric readouts. Capped and bioconjugated AuNSs were applied as localised surface plasmon resonance (LSPR)-based H₂O₂ sensors using glucose oxidase (Gox) as a model enzyme. Furthermore, our analyses revealed that the choice of capping agent influenced the properties of the AuNSs, their stability and their downstream applications. Our analyses provide new insights into factors governing the choice of capping agents for gold nanostars and their influences on downstream applications with conjugated enzymes in confined environments.

Keywords: capping agents; biosensor; colourimetric; gold nanostar; physico-chemical

4.3 Introduction

Gold nanoparticles (AuNPs) are attractive for use in nanomedicine due to their facile synthetic methods, sensitivity, specificity, possibilities for tailoring their charge, hydrophilicity and functionality through surface chemistries (Hu *et al.*, 2015; Javed *et al.*, 2020a; Zhu *et al.*, 2021). AuNPs come in a variety of morphologies that are tuneable for biomedical purposes. A single nanomorphology may be used in therapy, diagnostics, or even theranostics (Pedrosa *et al.*, 2015). An example of this includes the use of nanorods as drug delivery carriers and as cell-imaging and photothermal therapies for cancer (Zong *et al.*, 2021). Due to their inert nature and biocompatibility, gold nanoshells are used mainly in drug delivery, gene delivery, photothermal

therapy, cancer cell targeting and treatment and medical biosensing (Singhana *et al.*, 2014). Gold nanostars are best-suited for biosensing applications due to their strong localised surface plasmon resonance (LSPR), which maximises near the sharp tips of the particles upon excitation from a selected electromagnetic frequency (Pazos-Perez *et al.*, 2018).

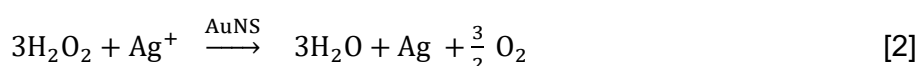
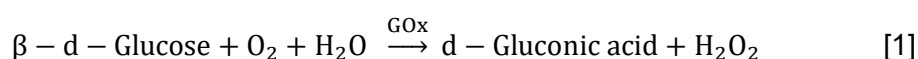
Gold nanostars (AuNSs) are an auspicious morphology with arms protruding from a more or less spherical core. These arms enhance plasmonic reactions based on the lightning rod effect, which has the highest enhancement factors in localised surface plasmon resonance (LSPR) biosensing (Atta *et al.*, 2019; Fukuoka & Tanabe, 2019). Surface plasmon resonance can be tailored by controlling a nanostar's arm density, length and physicochemical surface structure without altering the overall dimensions (Mulder, 2019; Pazos-Perez *et al.*, 2018). This ultimately redefines the limits of AuNS detection by presenting a signal-generating mechanism that induces a more significant signal when the analyte is less concentrated (Mulder, 2019; Rodríguez-Lorenzo *et al.*, 2012). In biological applications, aqueous synthesis is commonly used, although in some instances, AuNSs may be synthesised in organic media (Minati *et al.*, 2014a; Pallavicini, Cabrini & Borzenkov, 2015). This helps to achieve better control over the physical and chemical properties and homogeneity of the colloidal solution (Pallavicini, Cabrini & Borzenkov, 2015). Changes in essential parameters, such as the synthesis method, capping agents, pH, temperature, concentration and environment, greatly influence the quality and quantity of the synthesised AuNSs as well as their characterisation and applications (Patra & Baek, 2014).

Capping agents are usually utilised as stabilisers when creating AuNSs, with these agents being involved in modulating surface chemistry, morphology and size distribution as well as preventing coalescence and aiding in the formation of complex structures with metallic ions in precursor salts (Campisi *et al.*, 2016c; Li, Chang, *et al.*, 2013; Niu & Li, 2014). Capping agents influence nanoparticle shapes, while the capping agent length, size, ω -functionalities and chemical nature influence the shelf-life and colloidal stability of AuNSs in dispersions (Kanelidis & Kraus, 2017). Capping agents play a key role in altering the surface charge and also act as bioconjugation scaffolds (Barman *et al.*, 2022; Neouze & Schubert, 2008; Tonga *et al.*, 2014). Capping agents - enzyme conjugates - are essential considerations in biosensor preparations. In many cases, biocompatible capping agents are commonly used (Ahmed & Narain, 2014a; Hu *et al.*, 2015; Mansur *et al.*, 2011; Wang *et al.*, 2019; Zhu *et al.*, 2021). Capping agents may improve enzyme stability, shield enzymes from undesired interactions and prolong shelf-life (Ahmed & Narain, 2014a; Faghihnejad *et al.*, 2014; Liébana & Drago, 2016). Such shielding effects rely on the physicochemical properties of the capping agents, such as the hydrophilicity, chain length and architecture (Ahmed & Narain, 2014b; Faghihnejad *et al.*, 2014; Hermanson, 1996; Ibrahim *et al.*, 2019; Liébana & Drago, 2016). Capping agents can be categorised broadly as natural or synthetic

surfactants, small ligands, polymers, dendrimers, cyclodextrins, or polysaccharides (Ibrahim *et al.*, 2019; Javed *et al.*, 2020b; Kemal *et al.*, 2008). Polymeric capping agents are usually very effective due to the entropic effect associated with multiple bindings and the steric stabilisation provided by the macromolecule (Gulati *et al.*, 2018; Ibrahim *et al.*, 2019; Kemal *et al.*, 2008). Commonly used polymers are polyvinylpyrrolidone, polyvinyl acetate, polyethylene glycol (either modified or derivatives), poly(acrylic acid) (either modified or not with additional binding agents such as amines), copolymers based on poly(maleic acid) and polyethyleneimine (Dumur *et al.*, 2011; Gulati *et al.*, 2018; Ibrahim *et al.*, 2019; Javed *et al.*, 2020b).

In principle, a strongly adsorbing capping agent provides better colloidal stabilisation to AuNSs, but in some instances, it is necessary to use a weaker capping agent in the synthesis depending on the downstream applications (Hola *et al.*, 2015; Javed *et al.*, 2020b; Poblete & Comer, 2019). The adsorption of capping agents on AuNS surfaces can be through chemisorption, electrostatic interaction, or hydrophobic interaction (Montes & Belén, 2016). However, there may be a pressing need for using eco-friendly (green) capping agents to secure the biological system and the environment, as some are non-biodegradable and toxic (Sidhu *et al.*, 2022; Virkutyte & Varma, 2011).

The following study is conducted based on the ambiguity of why researchers choose specific capping agents over others. A one-pot, silver-assisted nanostar synthesis method using 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) is the synthesis method of choice. Silver nitrate is used as a shape-directing agent to assist the HEPES in obtaining monodispersed, multibranched AuNSs. The effects of various capping agents on AuNS stability are investigated in environmental conditions used in biosensing work. Additionally, the capping agents' influences on the bioconjugate and enzyme activity using glucose oxidase (GOx) as a model enzyme are evaluated. Furthermore, how the capping agents influence the colourimetric potential of the AuNS bioconjugates is assessed based on hydrogen peroxide detection. The biocatalytic growth of AuNS is the mechanism of detection through surface-coating with silver (Ag) after its reduction with hydrogen peroxide, as proposed by the following equations (Atta *et al.*, 2019; Pinheiro *et al.*, 2020):



4.4 Materials and Methods

4.4.1 Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), gold (III) chloride hydrate ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$), glucose oxidase (GOx), hydrogen peroxide (H_2O_2), silver nitrate (AgNO_3), polyethylene glycol (PEG 8000), polyethylene oxide (PEO 100,000), sodium chloride (NaCl), polyvinylpyrrolidone (PVP 10,000), cetyltrimethylammonium bromide (CTAB), 11-mercaptoundecanoic acid (MUA), polyethylenimine (PEI), trisodium citrate, gelatin chitosan, glucose, 3'-30-dithiobis sulfosuccinimidyl propionate (DTSSP) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Ham's F-12K (Kaighn's) cell culture medium supplemented with 10% foetal bovine serum was purchased from Thermo Fisher Scientific (Johannesburg, South Africa). Blank urine was purchased from Industrial Analytical (Pty) Ltd. Lastly, ultrapure water (ddH_2O) was pre-prepared with a Milli-Q ultrapure system ($18.2 \text{ M}\Omega \text{ cm}^{-1}$).

4.4.2 Gold nanostar synthesis

The nanostars were synthesised according to Xie et al. and Mulder et al. (Mulder, 2019; Xie *et al.*, 2007b). Briefly, 3 mL H_2O was added to 2 mL 100 mM HEPES buffer (pH 7.4), followed by the addition of 20 μL 50 mM $\text{HAuCl}_4 \cdot \text{H}_2\text{O}$ and, lastly, 4 μL 1 mM AgNO_3 . The solution turned blue after gentle end-to-end inversion mixing and incubation at room temperature for 30 min.

4.4.3 Gold nanostar capping agents (AuNS–capping agent)

Capping of the nanostars was achieved by adding ranges of PEO, PVP, PEG, CTAB, MUA, PEI, trisodium citrate, gelatin and chitosan concentrations, followed by salt stress testing as outlined below in 4.4.6. Optimal concentrations were selected based on lack of, or limited degradation, after visual and spectrophotometric assessment. The nanostars were incubated for one hour for each of the selected concentrations, after which they were centrifuged at $4180 \times g$ for 35 min. Following this, the soft pellets were washed and resuspended in 500 μL ddH_2O . The clean-up process was repeated twice.

4.4.4 Enzyme bioconjugation (AuNS–capping agent–GOx)

The PVP-, PEO- and PEG-capped AuNSs were bioconjugated with glucose oxidase (GOx) for further investigation. This was to determine if the capping agent affected the enzyme bioconjugation process and biosensor colourimetric reactions. A method suggested by Filbrun *et al.* (2017) with minor modifications was followed to attach GOx to the AuNSs using DTSSP

(Filbrun *et al.*, 2017). Briefly, capped AuNSs were synthesised as described above but were resuspended in 500 μ L 100 mM HEPES (pH 6.9) as opposed to ddH₂O in the final step. Four batches of 500 μ L each were then pooled, making a total volume of 2 mL. An amount of 100 μ L 5 mM DTSSP was then added to the solution, followed by 150 μ L 2 mg/mL GOx. The solution was then left in the fridge for 2 h, allowing for AuNS–GOx attachment. Excess DTSSP and GOx were removed through 2 cycles of centrifugation at 4180 $\times$ *g* for 35 min and resuspension in 500 μ L ddH₂O.

The stability of the GOx-bioconjugated AuNSs (AuNS-GOx, AuNS-PVP-GOx, AuNS-PEO-GOx and AuNS-PEG-GOx) was then confirmed using a stress testing protocol, as described below.

4.4.5 Characterisation

Characterisation of the nanostars was performed with spectroscopy and high-resolution transmission electron microscopy (HR-TEM) analyses (JEOL, Freising, Germany). Spectral scanning (400–900 nm) was conducted with an HT Synergy (BioTEK) microplate reader (Agilent Technologies, Santa Clara, CA, USA). High-resolution transmission electron microscopy (HR-TEM) analysis was performed with a Tecnai F20 transmission electron microscope at an acceleration voltage of 200 kV (JEOL, Freising, Germany). The capped AuNS samples were spotted onto copper grids (Agar Scientific), followed by air-drying before imaging, whereas the AuNS bioconjugates needed staining for enzyme visualisation. This was performed by spotting the samples onto copper grids, staining them with 1% silver nitrate solution and allowing them to air-dry before imaging (Atta *et al.*, 2019). The particle core diameters and arm counts were estimated by averaging a total count of one hundred AuNSs in different grid regions using ImageJ software (ImageJ bundled with 64-bit Java 1.8.0_172, University of Wisconsin at Madison, Madison, Wisconsin, USA).

In addition, successful PVP-, PEO- and PEG-capping and enzyme bioconjugation of the nanostars were demonstrated with nuclear magnetic resonance (NMR) and agarose gel electrophoresis. NMR was performed at 500 MHz with a Bruker Avance III HD NMR spectrometer equipped with a triple resonance inverse (TXI) 1H (15N, 13C) probe head (Bruker, Billerica, MA, USA). Agarose gel electrophoresis of capped and AuNS bioconjugates was carried out using a Baygene BG-power Vacutec electrophoresis gel apparatus (Vacutec, Johannesburg, South Africa). The electrophoresis was conducted using 0.5% agarose and 0.5 $\times$ Tris borate EDTA buffer (TBE buffer) at pH 8. The samples were prepared using 32 μ L samples of nanostars mixed with 4 μ L 80% glycerol and were run at 40 V for 45 min. Gels of AuNS bioconjugates were stained with 25 mL 0.25% (w/v) Coomassie blue for 4 h, followed by rinsing with a destaining solution

(90% w/v isopropanol and 10% w/v glacial acetic acid), after which the protein bands were visible. Gels were kept in ddH₂O until the gel images were captured and transferred to a computer.

4.4.6 Stability assessment for capped and AuNS bioconjugates

The stability during storage and various possible analytical assay matrices of capped and AuNS bioconjugates were assessed. The storage conditions were at both room temperature and 4 °C over 96 h. The analytical assay conditions assessed included various environments (salt, urine, serum and supplemented cell culture medium) at room temperature for 3 h. A change in the LSPR band OD_{max} of greater than 30% was considered unstable using UV-vis (BioTEK , Agilent Technologies , Santa Clara, CA 95051, USA) (Blessy *et al.*, 2014a).

4.4.7 Effect of the capping agent on the plasmonic properties of AuNSs in bioassays

The effect of the capping agents on the plasmonic properties of AuNSs when used as chemical sensors in bioassays was assessed first by the incubation of capped AuNSs with hydrogen peroxide, followed by unconjugated and conjugated GOx-generated hydrogen peroxide assays.

For the hydrogen peroxide assay, reagents were added and pipette-mixed in the following order: 20 µL 10 mM Tris buffer (pH = 8), 15 µL capped AuNSs, 0–20 µL 50 mM hydrogen peroxide in increments of 2 µL, 2 µL 10 mM silver nitrate and 20 µL 500 mM NaOH. As an initial step, a volume of deionised water was added that ensured a final volume of 200 µL. The reagents were incubated for 5 min at room temperature before spectrophotometric measurement (Mulder, Phiri, *et al.*, 2019b).

The unconjugated GOx assay was performed as above, but the hydrogen peroxide was replaced with 5 µL 2 mg/mL GOx and 0–20 µL 1.65 mM glucose in increments of 5 µL, followed by 15 min of incubation at 37 °C. Then, a detection solution consisting of 2 µL 10 mM silver nitrate and 20 µL 500 mM NaOH was added and incubated for another 1 min, after which spectral readings were obtained (Mulder, Phiri, *et al.*, 2019b). The conjugated GOx assay was performed as above, with the exception that the GOx addition was omitted and 0–20 µL 2 mM glucose was added in increments of 5 µL.

4.5 Results

Even though spherical nanoparticles have been capped with CTAB, MUA, PEI, trisodium citrate, gelatin and chitosan, attempts to reproduce this led to degradation of the AuNSs (Kumar *et al.*, 2019; Seoudi, 2011). These agents were subsequently excluded from further investigation. The

optimal concentrations of PVP, PEO and PEG solutions for capping 5 mL AuNS suspensions were 600 μ L 25 mM PVP, 250 μ L 0.25 mM PEO and 500 μ L 35 mM PEG, respectively (data not shown).

The UV-vis spectral scans, NMR spectra and electrophoretic migration of capped (left column) and bioconjugated (right column) AuNSs are presented in Figure 4-1. The absorbance spectra (A and D) showed a small shoulder peak at around 545 nm and a more intense, red-shifted, prominent peak at approximately 635 nm. AuNS and AuNS-PEO achieved the best spectral resolutions of the two LSPRs, followed by AuNS-PVP and AuNS-PEG. A mild loss in spectral resolution was observed for all the GOx-bioconjugated nanostars except uncapped AuNS-GOx, which was almost identical to AuNS. Furthermore, the AuNS-PVP-GOx bioconjugate exhibited the best spectral resolution relative to other AuNS-Capping agent-GOx combinations. The change in the ^1H -NMR spectra when compared to the AuNS spectrum hinted toward the presence of the capping agents (B) and GOx (E) in the samples and agrees with previously reported results (Mulder, Phiri, *et al.*, 2019b; Mulder, 2019). The change in the electrophoretic pattern of the capped AuNSs compared to AuNS (C) as well as between the capped AuNSs and GOx-bioconjugated AuNS (F), indicated a change in the charge or size of the nanostars after capping and bioconjugation. The absence of the GOx band (F) in the bioconjugated AuNS lanes suggested that GOx was successfully conjugated to nanostars. All the nanostars migrated toward the anode, with migration distance being indirectly proportional to capping agent polydispersity. The presence of a five-membered ring moiety and a long-chain structure in PVP probably limited its migration in comparison to the linear chemical structures of PEO and PEG. As expected, the attachment of GOx to nanostars decreased migration when compared to unattached GOx.

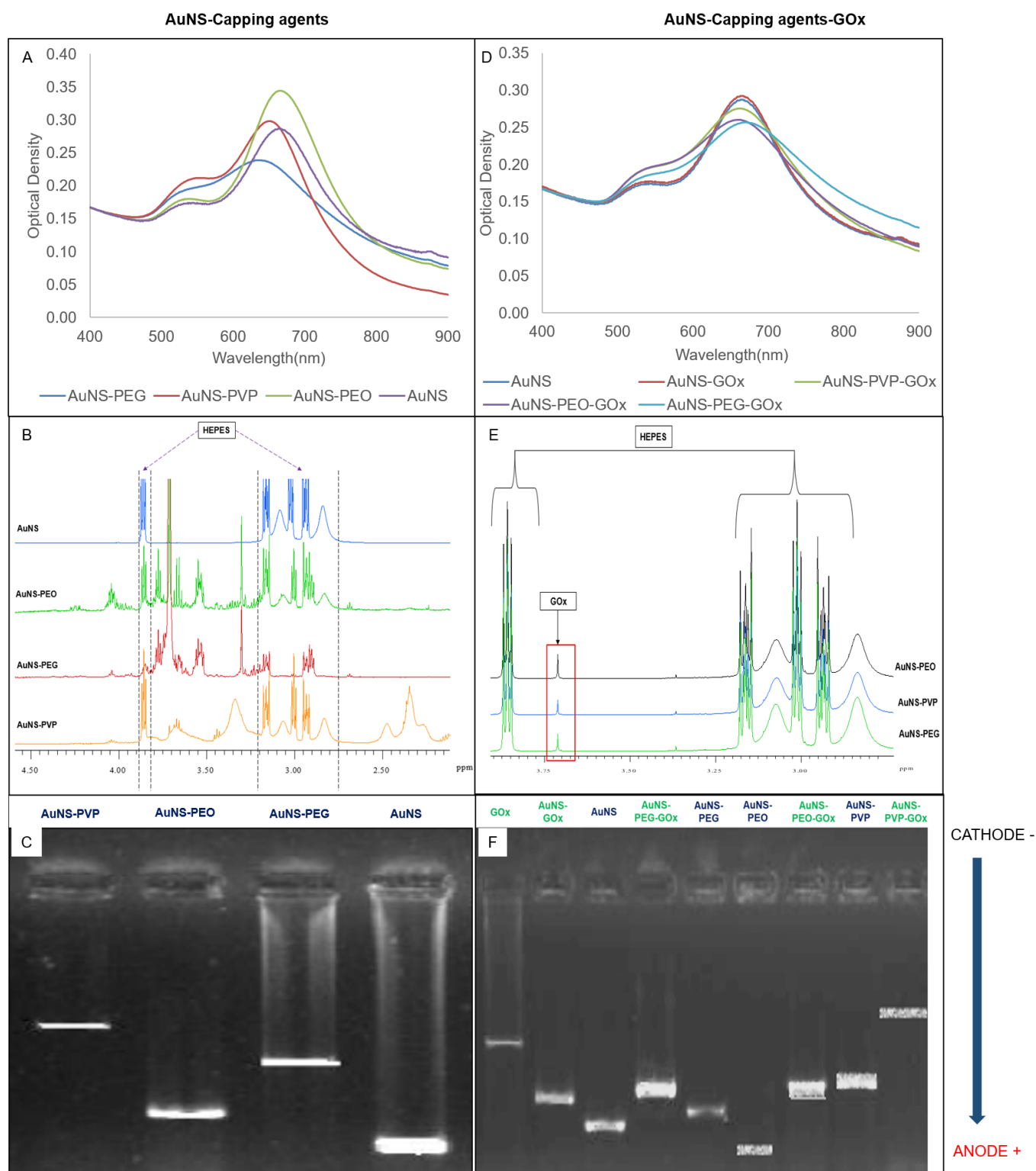


Figure 4-1: (A–C): Effects of capping agents on AuNSs using (A) UV-vis spectrometry, (B) nuclear magnetic resonance (NMR), and (C) gel electrophoresis. (D–F): AuNS capping agents functionalized with glucose oxidase using (D) UV-vis spectrometry, (E) NMR, and (F) gel electrophoresis.

TEM images of capped (top row) and bioconjugated (bottom row) AuNSs are presented in Figure 4-2. AuNS (Figure 4-2A), AuNS-PVP (Figure 4-2B) and AuNS-PEG (Figure 4-2D) particles were moderately monodispersed, with an estimated average size ranging from 40 to 42 nm. Most AuNS-PEO particles (Figure 4-2C) were cleaved during the capping process, with resultant tripod or tetrapod appearances and an average diameter of 25 nm. AuNS-PVP had a lower tendency toward aggregation and most nanostars had a spiked morphology with 12 arms, of which approximately nine protruded around the nanostar and three protruded outwards. When comparing the AuNS bioconjugates, AuNS-PVP-GOx (Figure 4-2E) exhibited better monodispersity, with a larger percentage of nanostars having a spikey morphology relative to AuNS-PEO-GOx (Figure 4-2F) and AuNS-PEG-GOx (Figure 4.2G). Unexpectedly, a protein layer could not be visualised after 1% silver nitrate staining of AuNS-PVP-Gox despite ample evidence of successful functionalisation, as presented in Figure 4-1. The layer was, however, visible when AuNS-PEO-GOx and AuNS-PEG-GOx were stained and it was visualised as small dark spots of approximately 3.0 nm around the nanostar bioconjugates, which is in line with previously reported observations (Li *et al.*, 2007; Moses Phiri *et al.*, 2019). The size distribution plots for the AuNS-PVP-Gox, AuNS-PEO-GOx and AuNS-PEG-GOx are contained in the Supplemental Materials (Figure 4-S1). The AuNS-GOx sample contained mostly aggregated spheres. These nanoparticles were not stable, as was evident from changes in the absorbance spectrum and colour of the solution within 30 min after conjugation (Figure 4-S2).

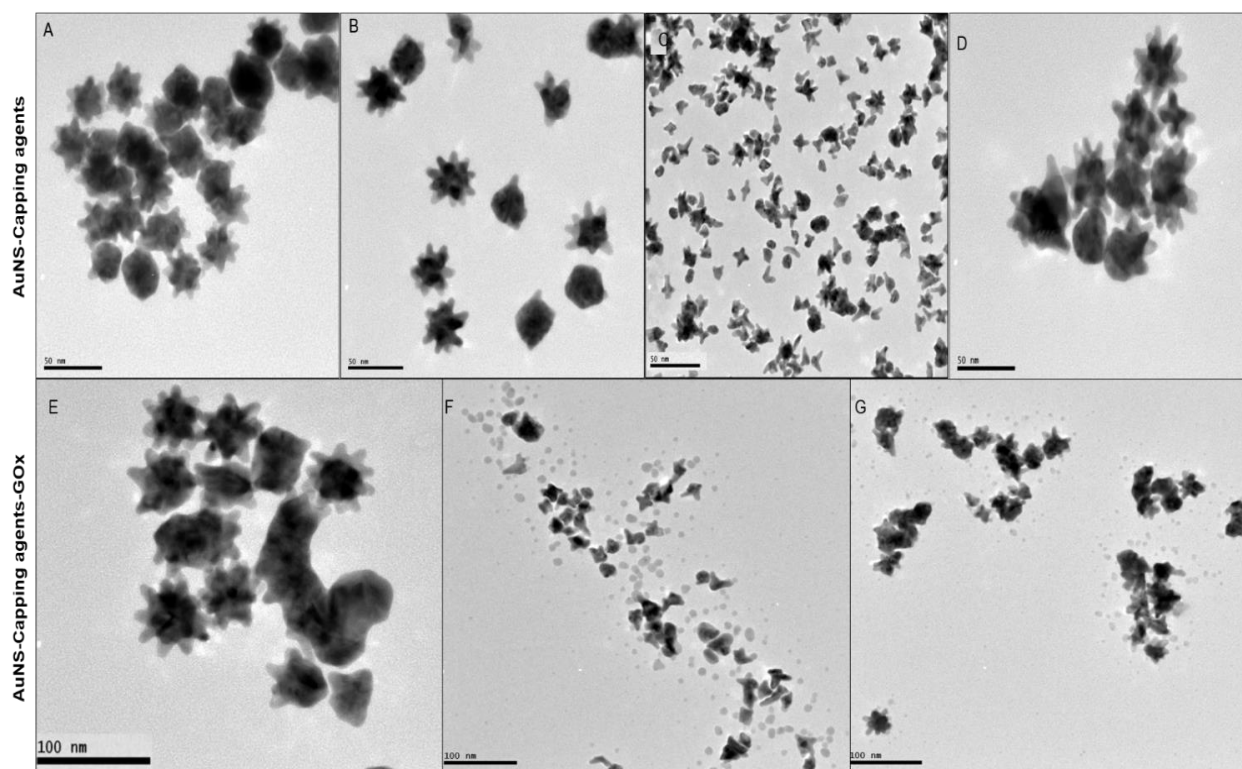


Figure 4-2: Representative HR-TEM images of the gold nanostar morphologies: (A) AuNS; (B) AuNS-PVP; (C) AuNS-PEO; (D) AuNS-PEG; (E) AuNS-PVP-GOx; (F) AuNS-PEO-GOx; and (G) AuNS-PEG-GOx.

The stability of the nanostars in various matrices (left column) and the short-term stability at both room temperature and 4 °C (second and third column) are presented in Figure 4-3. AuNS-PEO and AuNS-PVP maintained stability in all the tested matrices (Figure 4-3A–D), with a slight blue-shift in the spectrum relative to controls in some instances. AuNS-PEG was stable in the salt only, while AuNS was unstable in all the matrices, as shown by a relative change in the longitudinal resonance OD_{max} of greater than 30% after 3 h of incubation. AuNS-PVP and, to a mildly lesser extent, AuNS-PEO were stable for 96 h at both room temperature and 4 °C (Figure 4-3F, J–G, K). Uncapped AuNSs exhibited stability loss at room temperature, as seen by the blue-shift in the longitudinal LSPR peak as well as in the visible colour change within 48 h of synthesis (Figure 4-3E). It did maintain stability at 4 °C over the 96 h period (Figure 4-3I), which was in contrast to AuNS-PEG, which was only stable up to 72 h at both room temperature and 4 °C (Figure 4-3H, L).

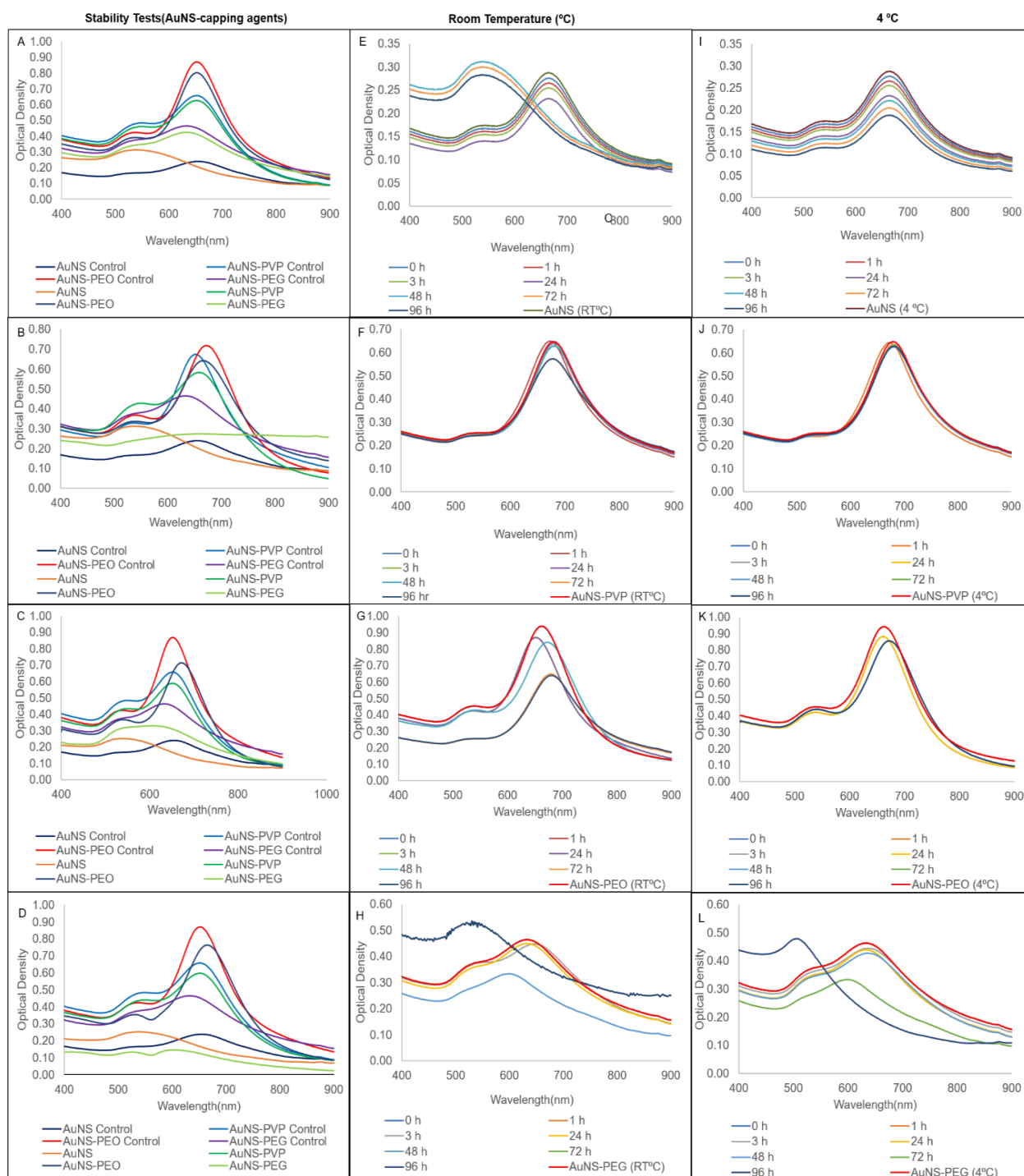


Figure 4-3: Comparison of UV-vis spectra of capped AuNS stability after 3 h of incubation in (A) salt; (B) urine; (C) serum; and (D) supplemented cell culture media. Storage stability UV-vis spectra at room temperature and 4 °C, respectively, for (E, I) AuNS; (F, J) AuNS-PVP; (G, K) AuNS-PEO; and (H, L) AuNS-PEG.

In comparison, the AuNS bioconjugates had minimal loss of stability, demonstrating that enzyme conjugation could induce extra stability in the AuNSs (Figure 4-4A–D). Contrarily, conjugation had less of an effect on short-term stability, with AuNS-PVP-GOx being the most stable, followed by AuNS-PEO-GOx and AuNS-PEG-GOx. Therefore, the results suggested that the choice of

capping agents affects the shelf-life, stability and structural integrity of nanoparticles in suspension. Overall, 4 °C was the optimal storage condition for the AuNS–capping agent and AuNS–capping agent–GOx combinations.

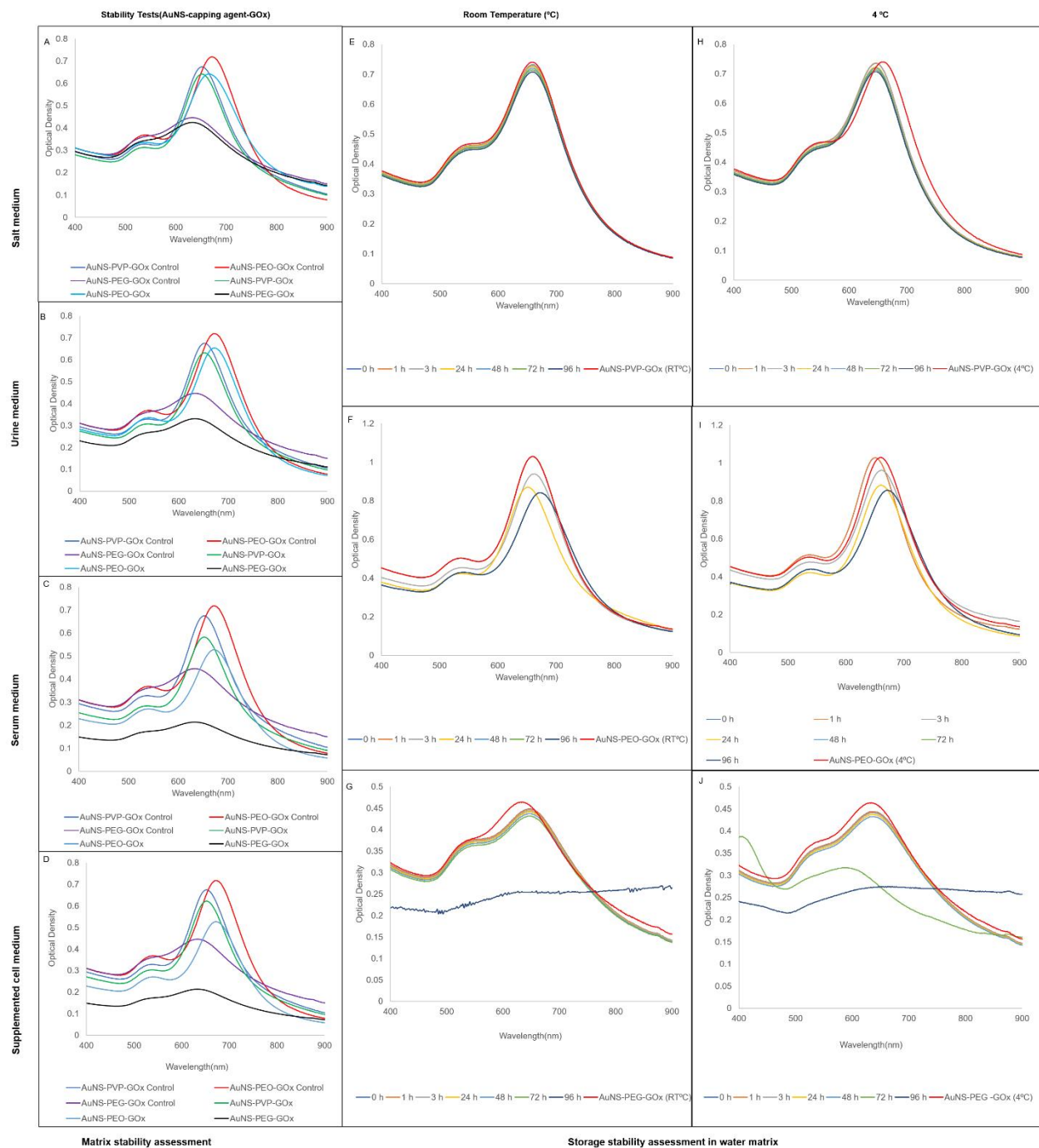


Figure 4-4: UV-vis spectra of stability comparisons for AuNSs bioconjugated with glucose oxidase after 3 h of incubation in (A) salt; (B) urine; (C) serum; and (D) supplemented cell culture media. Storage stability UV-vis spectra at room temperature and 4 °C, respectively, for (E, H) AuNS-PVP-GOx; (F, I) AuNS-PEO-GOx; and (G, J) AuNS-PEG-GOx.

A conjugated GOx assay was used to assess the efficacy of the various bioconjugates to act as chemical sensors of biocatalytically produced hydrogen peroxide. The results of the assay are presented in Figure 4-5. The results of the hydrogen peroxide and unconjugated GOx assays are presented in the Supplemental Materials, Appendix A (Figures 4-S3 and 4-S4). As expected from the stability studies, AuNS-GOx was an inefficient sensor of hydrogen peroxide, as evident from the lack of correlation between the glucose concentration and the spectral shift (Figure 4-5B). The longitudinal LSPRs of AuNS-PVP-GOx (Figure 4-5C) and AuNS-PEG-GOx (Figure 4-5G) blue-shifted, with the degree of the shift correlating to the added glucose concentration. Only one peak was observed in the AuNS-PEO-GOx assay that was also blue-shifted with increasing glucose concentrations (Figure 4-5E). AuNS-PEO-GOx and AuNS-PEG-GOx had an immediate colour change when the detection solution was added, including a reaction at 0 mM glucose. AuNS-PVP-GOx maintained its initial colour at 0 mM glucose and needed 5 min of incubation for visual and spectrophotometric changes to develop at increasing glucose concentrations. AuNS-PVP-GOx outperformed both AuNS-PEO-GOx and AuNS-PEG-GOx as a chemical sensor of biocatalytically produced hydrogen peroxide, as evident from its R^2 , which approached one. The colour of AuNS-PVP-GOx was also the most vivid and changed from blue to purple, whereas the other two capping agents produced mostly varying shades of purple.

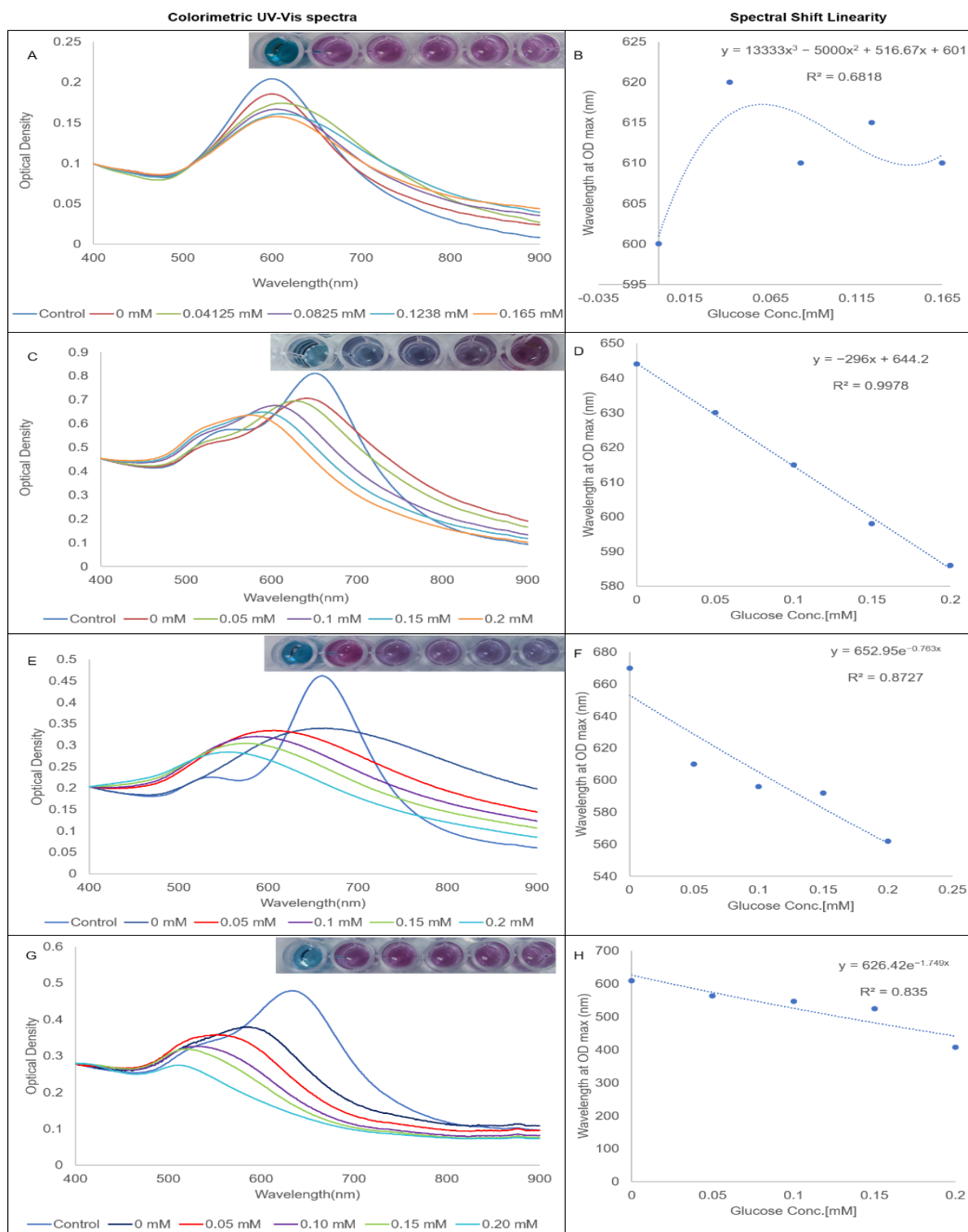
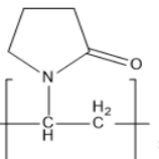
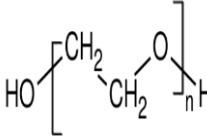
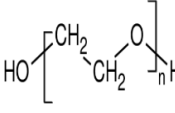


Table 4-1: Summary of results: structure–function relationship.

Capping Agent	Structure	Side group	Stability of capped AuNS and bioconjugates	Colourimetric Biosensing	Suggested Down Stream Applications
PVP		1. Bulk 2. Low segmental mobility 3. Low hydrogen bond-forming capacity	Short Term: Excellent Long Term: Excellent	1. 5 min response time 2. Distinguishable colours by the naked eye 3. Steady reaction	1. Colourimetric biosensing
Mw: 10 000	$(C_6H_9NO)_n$				
PEO		1. Linear 2. High segmental mobility 3. High hydrogen bond-forming capacity	Short Term: Moderate Long Term: Poor	1. Immediate response time 2. Indistinguishable colours by the naked eye 3. Unstable reaction	1. Confirmatory narcotics-drugs testing 2. Confirmatory rapid test kit
Mw: 100 000	$(-CH_2CH_2O-)_n$				
PEG		1. Linear 2. High segmental mobility 3. High hydrogen bond-forming capacity	Short Term: Moderate Long Term: Poor	1. Immediate response time 2. Indistinguishable colours by the naked eye 3. Unstable reaction	1. Confirmatory narcotics-drugs testing 2. Confirmatory rapid test kit
Mw: 8 000	$H(OCH_2CH_2)_nOH$				

4.6 Discussion

In summary, a plethora of the literature has focused on the synthesis of capped AuNSs. However, limited efforts have been devoted to conceptualising the role played by capping agents in controlling the interface of AuNSs with their environment and a clear structure–function relationship remains elusive. In this study, AuNSs synthesised through the chemical reduction of gold salt by HEPES showed traces of HEPES (a zwitterion) on the 1H -NMR spectra (Figure 4-1B,E). The AuNSs had two localised surface plasmon resonance (LSPR) modes with peaks at approximately 545 nm and 635 nm, corresponding to the plasmonic resonance of the core (transverse resonance) and branches (longitudinal resonance), respectively, as has been previously reviewed in the literature (Barbosa *et al.*, 2010a; Hao *et al.*, 2007b; Xu *et al.*, 2018). HEPES, which was present in all the 1H -NMR spectra, has previously been reported as both a shape-directing and a strong capping agent in nanostar synthesis and is likely incorporated within the nanostar lattice or bound to the surface (Liu *et al.*, 2014; Xie *et al.*, 2007b). The interactions between HEPES functional groups (sulfonate, amine and hydroxyl) and AuNSs can be ion- or dipole-induced dipoles. These molecular interactions are heavily influenced by pH fluctuations, electron density and capping agent binding chemistries, as revealed by Xi *et al.* (Xi & Haes, 2019). Quintanilla *et al.* suggested that the larger the capping agent's molecular weight, the more

catalytically active and stable the polycrystalline nanoparticles, which is in line with our findings (Quintanilla *et al.*, 2010a). In addition to this, our results indicated that molecular weight and chemical moieties influenced nanoparticle stability and homogeneity. PVP, PEO and PEG are vinyl polymers with different side groups. The larger side groups on PVP could reduce segmental mobility and hydrogen bond density near the interface and lead to lower adhesion energy, less fragility and degradation, hence increasing biosensor stability, a phenomenon previously reported by Lucius *et al.* (Lucius *et al.*, 2016). Strong hydrogen bonds at the interface influenced major chemical changes, such as chain oxidation and chain scission, leading to a reduction in the molecular weight and degree of polymerisation of a polymer, as in the case of PEO- and PEG-capped AuNS.

The use of PVP as a capping agent in the GOx bioassay resulted in vivid colour changes in response to varied hydrogen peroxide concentrations for both bioconjugated and capped particles despite its slower response time. The PEO and PEG bioconjugates produced fast results, regardless of their instability relative to the PVP bioconjugate and their colours resulted in a change in colour intensity as opposed to a colour range. Our results demonstrated that tailoring (capping) polymer functionality could improve bioconjugate activity and minimise biosensor instability. These observations suggested that the choice of capping agent has major implications in laboratory-based and point-of-care testing diagnostic applications.

Capping agents introduce a protective layer on the AuNS surface, hence introducing two major interfaces, namely the AuNS–capping agent interface and the AuNS–capping agent–protein interface. There is no clear demarcation of these interfaces and they seem intermixed in a transition zone, the AuNS–ligand interface. Similar surface chemistry analyses have been revealed by Grubbs *et al.* and Campisi *et al.* (Campisi *et al.*, 2016c; Grubbs, 2007). Electronic changes at the AuNS–ligand interphase unavoidably affect the catalytic behaviour of capped nanoparticles and bioconjugates, introducing either a poisoning or promoting effect. A similar effect was revealed by Tsunoyama *et al.* for PVP-capped Au nanoparticles (Tsunoyama *et al.*, 2009). The inherent complexity of AuNS–capping agent–protein interactions also contribute to the challenge of deducing general conclusions on the best choice of a capping agent. Overall, the choices of a synthesis method and a suitable capping agent should be key considerations in bioassay design, seeing that they affect the stability, surface chemistry, catalytic response and selectivity of AuNS sensors.

4.7 Conclusions

The AuNSs resulting from this facile and repeatable HEPES-mediated, one-pot synthesis protocol, were approximately 38 ± 4 nm in diameter, multibranched, monodispersed and homogeneous to a great extent. The contribution of our work is two-fold. Firstly, we demonstrated that capping agents affected AuNS stability, functionalisation and colourimetric biosensing capabilities. For polymeric capping agents, we revealed that fewer hydrogen-bond-forming groups in a polymer side group could reduce the hydrogen bond density near the interface, hence increasing biosensor stability. Secondly, we showed that the confinement effects induced by free surfaces and substrates in supported polymer-capped and conjugated biosensors could improve the bioconjugate activity and biosensor stability. Conclusively, PVP capping proved to be optimal for HEPES-mediated AuNS synthesis and the fabricated glucose biosensor proved to be a rapid colourimetric assay that may be a great candidate for potential clinical diagnosis, research and development applications useful in resource-constrained regions.

4.8 Acknowledgments

We would like to acknowledge Anine Jordaan, an electron microscopy specialist from the electron microscopy unit at Potchefstroom campus, NWU, for capturing TEM images of the gold nanoparticles as well as Christian De Wet Van Zyl, an NMR specialist in the NWU biochemistry department, for conducting the NMR experiments.

4.9 Author contributions

T.A.M. carried out all the experimental work, analysed the data and wrote the manuscript. B.C.V. analysed the data and edited the manuscript and D.W.M. designed the experiment, analysed the data and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

CHAPTER 5 GOLD NANOSTAR BIOSENSOR OPTIMISATION

**FEASIBILITY OF NAD(P)/NAD(P)H AS REDOX AGENTS IN ENZYMATIC
PLASMONIC GOLD NANOSTAR ASSAYS FOR GALACTOSE
QUANTIFICATION**

SCIENCE IS MAGIC THAT WORKS.

– Kurt Vonnegut

5.1 Preface

This chapter fulfilled Chapter 3's Objective 2, which was to investigate the appropriateness of NAD(P)/NAD(P)H as colourimetric redox agents in gold nanostar biosensors. This chapter also covers the fundamentals of how gold nanostars react in hydrogen peroxide-free, oxidative and reductive environments. This chapter was submitted, accepted and published in the Journal of Royal Society of Chemistry Open Science (<https://doi.org/10.1098/rsos.230825>). Minor changes were made from the published version, based on examiners' comments.

5.2 Abstract

Plasmonic colourimetric sensors have emerged as powerful analytical tools in biochemistry due to their localised surface plasmon resonance (LSPR) extinction in the visible range. Here, we describe the feasibility of NAD(P)/NAD(P)H as redox agents in enzymatic plasmonic gold nanostar (AuNS) assays for galactose quantification using three model enzymes, galactose dehydrogenase (GalDH), aldose reductase (AR), and galactose oxidase (GalOx) immobilised separately on PVP-capped AuNS scaffolds. These highly specific, sensitive and selective bioassays induce the transformation of AuNS into quasi-spherical nanoparticles during the biorecognition of galactose in water and synthetic blood matrices. As a result, utilising our inexpensive and simple AuNS plasmon bioassays, the presence of galactose may be detected spectrophotometrically and by the naked eye.

Keywords: Gold nanostars, Biosensor, Immobilised, Biorecognition, Bioassay, Redox agents

5.3 Introduction

Nicotinamide adenine dinucleotide (NAD⁺), phosphorylated Nicotinamide adenine dinucleotide (NADP⁺) and their respective reduced forms, NAD(P)H are charge carriers in numerous cellular reactions (Broderick, 2001; Rucker & Chohanadisai, 2016). NAD(P)/NAD(P)H redox couples are ubiquitous cofactors utilised by many oxidoreductase enzymes and their nicotinamide region is responsible for reversible redox processes (Gopalan *et al.*, 2009). Oxidoreductases are a family of enzymes that catalyse redox reactions and the shuttle of electrons from a donor (reductant) to an acceptor (oxidant) molecule in the presence of cofactors (Baron *et al.*, 1994). The nicotinamide moiety of NAD(P)H absorbs light at 340 ± 30 nm and emits blue fluorescence at 460 ± 50 nm. In contrast, the corresponding oxidised forms NAD(P)⁺ are not fluorescent and absorb at 260 nm (De Ruyck *et al.*, 2007; Patterson *et al.*, 2000). NAD(P)/NAD(P)H redox couples, which are by-products of oxidoreductase enzymes, are essential biomarkers in analytical biochemistry for the direct or indirect measurement of diverse substrates (Cantó & Auwerx, 2011). Thus, assessing

the participation of the NAD(P)/NAD(P)H redox couples in metabolism may aid in diagnosing pathological disorders *in vitro* and *in vivo*.

In 1957, NADH's first fluorescence spectrophotometric detection was published (Duysens & Ames, 1957). Beutler devised an endogenous fluorescence-based approach in 1964, recognising that NAD(P)H might be used as an endpoint signal in galactose screening protocols (Beutler & Baluda, 1966; Beutler *et al.*, 1964; Beutler *et al.*, 1965; Beutler *et al.*, 1967). This technical advancement paved the way for using NAD(P)H to reduce/oxidise dyes to monitor enzyme(s) activity, resulting in colourimetric, spectrophotometric and fluorescence assays (Chamchoy *et al.*, 2019; Jiang *et al.*, 2020; Veskokoukis *et al.*, 2018).

Subsequently, simple micro-fluorimetric assays were developed with the potential to simultaneously quantify galactose and galactose-1-phosphate using a cascade of enzymes (Diepenbrock *et al.*, 1992; Fujimura *et al.*, 1981; Orfanos *et al.*, 1986). Other techniques including high-performance liquid chromatography (HPLC), bacterial micro-assay, spectrophotometry and tandem mass spectrometry (MS/MS) have also been utilised for galactose concentration quantification with more sensitivity and precision than enzyme-based methods (Frings & Pardue, 1964; Jeong *et al.*, 2008; Ning & Segal, 2000). However, these methods and techniques require expertise and are costly; hence, they are unfit for rapid screening in resource-constrained areas.

The Leloir pathway is the premier route for galactose metabolism (Holden *et al.*, 2003; Maxwell *et al.*, 1962; Sears, 2020). However, in case of failure, auxiliary pathways can be activated involving distinct enzymes (Srivastava & Beutler, 1969). In auxiliary galactose metabolism pathways, galactose dehydrogenase (GalDH; EC 1.1.1.48) catalyses the dehydrogenation reaction of galactose in the presence of NAD⁺, producing D-galactose-1,5-lactone and NADH (Hjelm & Tengström, 1968). Aldose reductase (AR; EC 1.1.1.21) is a cytosolic NADPH-dependent enzyme that catalyses the reduction of galactose to galactitol and NADP⁺ (Borhani *et al.*, 1992). Galactose oxidase (GalOx; EC 1.1.3.9) is a metalloenzyme that catalyses the oxidation of galactose to galacto-hexodialdose and hydrogen peroxide with strict C-6 primary hydroxyl group regioselectivity (Eisink *et al.*, 2017; Hjelm & Tengström, 1968). Their biochemical properties such as efficiency, specificity and good biodegradability make them fit for rapid enzymatic determination of blood galactose (Sandhya Rani, 2020).

During the last decade, significant development of oxidoreductase-based diagnostic test kits and innovative biosensor designs has been witnessed for various analytical and clinical applications (Baluta *et al.*, 2017; May, 1999). Recently, gold nanoparticles such as gold nanostars (AuNS) have been used as signal transducers and scaffolds in enzymatic-based colourimetric assays (Chirico *et al.*, 2015). These methods are usually based on tunable localised surface plasmon

resonance (LSPR) and colloidal stability in the absence or presence of an analyte resulting in different colours (Kumar, 2018). Extensive research has been published on electrochemical NAD(P)/NAD(P)H biosensors functionalised with varying materials of nanocomposite and oxidoreductase enzymes (Aydoğdu Tığ, 2017; Govindhan *et al.*, 2015; Jin *et al.*, 2023; Li, Lu, *et al.*, 2013; Liao *et al.*, 2008; Nesakumar *et al.*, 2013). To the best of our knowledge, limited research outputs have been reported for the colourimetric detection of NAD(P)/NAD(P)H using plasmonic gold nanoparticles immobilised with hydrogenase or reductase enzymes.

Here, we describe the feasibility of NAD(P)/NAD(P)H as redox agents in enzymatic plasmonic gold nanostar assays for galactose quantification. Three model enzymes, GalDH, AR and GalOx, were immobilised separately on PVP-capped AuNS scaffolds. Upon the biorecognition of galactose, the AuNS acts as a signal transducer concomitantly producing rapid and quantitative colourimetric signals detectable spectrophotometrically and by the naked eye. This study contributes to the feasibility of using NAD(P)/NAD(P)H as redox agents in the development of simpler, faster and less expensive colourimetric assays suitable for resource-constrained areas.

5.4 Materials and Methods

5.4.1 Reagent and Materials

The following reagents were used to synthesise, functionalise and conjugate gold nanostars: Gold (III) chloride hydrate ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$, 99.9 %), silver nitrate (AgNO_3 , 99.9%), HEPES ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$, 99.5%), sodium chloride (NaCl , 99.0%), polyvinylpyrrolidone (PVP 10 000 ($\text{C}_6\text{H}_9\text{NO}$)_n, 99.0%), galactose ($\text{C}_6\text{H}_{12}\text{O}_6$, 99.0 %), glucose ($\text{C}_6\text{H}_{12}\text{O}_6$, 99.0 %), 3'-30-dithiobis sulfosuccinimidyl propionate (DTSSP, $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_{14}\text{S}_4\text{Na}_2$), sodium hydroxide (NaOH , 98.0%), hydrogen peroxide (H_2O_2 , 30% w/w), Tris buffer ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$, 99.7%), galactose dehydrogenase (GalDH, ~80 U/mg protein, 95.0%), aldose reductase (AR, 1 mg/mL, 95.0%), galactose oxidase (GalOx, 3000 U/g, 98.0%), reduced nicotinamide adenine dinucleotide phosphate (NADPH, $\text{C}_{21}\text{H}_{26}\text{N}_7\text{Na}_4\text{O}_{17}\text{P}_3 \cdot x\text{H}_2\text{O}$, 97.0%), reduced nicotinamide adenine dinucleotide (NADH, $\text{C}_{21}\text{H}_{27}\text{N}_7\text{Na}_2\text{O}_{14}\text{P}_2 \cdot x\text{H}_2\text{O}$, 97%) and nicotinamide adenine dinucleotide hydrate (NAD, $\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{14}\text{P}_2 \cdot x\text{H}_2\text{O}$, 97.0%) were purchased from Sigma-Aldrich (Johannesburg, South Africa) and were utilised without any purification. Supplemented foetal bovine serum (Ham's F-12K) was purchased from Thermo Fisher Scientific (Johannesburg, South Africa). Blank urine was purchased from Industrial Analytical (Johannesburg, South Africa). Lastly, ultrapure water (ddH_2O , $18.2 \text{ M}\Omega \text{ cm}^{-1}$) was used for preparing all solutions from the high-purity chemicals and reagents.

5.4.2 Characterisation

5.4.2.1 Instrumentation

All spectra for the research were obtained using an HT Synergy microplate spectrophotometer scanned at 400–900 nm using Gen5.1 software (BioTEK, Agilent Technologies, Santa Clara, CA, USA). The morphology of the functionalised nanostars was determined with high-resolution transmission electron microscopy (HR-TEM-Tecnai F20, JEOL, Freising, Germany) at an acceleration voltage of 200 kV. HR-TEM images were captured using air-dried spotted copper grids (Agar Scientific). ImageJ software (University of Wisconsin at Madison, Madison, WI, USA) was used to measure average particle core diameter and arm counts on a sample size of 100 particles. The chemical compositions of the samples were determined qualitatively using scanning electron microscopy and energy-dispersive x-ray spectroscopy (SEM-EDS, Bruker, Billerica, MA, USA). The elemental concentrations in the samples were quantified using inductively coupled plasma mass spectrometry (ICP-MS, Spectro AMETEK, Inc, Germany). SEM-EDS and ICP-MS were used to quantify and confirm the chemical makeup of the samples precisely. Inductively coupled plasma mass spectrometry (ICP-MS) was utilised to quantify elements at trace levels in the samples. The NMR was performed at 500 MHz on a Bruker Avance III HD NMR spectrometer (Bruker, Billerica, MA, USA) to illustrate the presence of the capping agent and enzyme(s) after AuNS functionalisation. The characterisation of the functionalised AuNS was determined using a Vacutec electrophoresis gel apparatus (Vacutec, Johannesburg, South Africa). The electrophoresis was done using 0.5 % agarose and 0.5 x Tris borate EDTA buffer (TBE buffer) at pH 8. The samples were prepared using 20 μ L AuNS mixed with 4 μ L, 80 % glycerol and ran at 40 V for 45 min. 0.25 % (w/v) coomassie blue staining for 4h with subsequent destaining with (90% w/v isopropanol + 10 % w/v glacial acetic acid) was used to ascertain successful bioconjugation as seen by the protein bands on the gels. The gels were kept in ddH₂O (Millipore, 18.2 Ω M cm⁻¹) for quality imaging using the Bio-Rad imaging system (Bio-Rad Laboratories, CA, USA). NMR and gel electrophoresis demonstrated that the capping and enzyme(s) were present after functionalisation and attached to the samples.

5.4.3 Preparation of gold nanostars

PVP-capped AuNS were synthesised utilising the one-pot HEPES and silver-mediated protocol as proposed in our previous work with minor tailoring (Munyayi *et al.*, 2022). Succinctly, 2 mL 100 mM HEPES buffer (pH 7.4) was added to 3 mL deionised water (Millipore, 18.2 Ω M cm⁻¹), followed by 20 μ L 50 mM gold (III) chloride hydrate (HAuCl₄·xH₂O) and 4 μ L 1 mM silver nitrate (AgNO₃). The screw cap tubes were rotator mixed for 5 min and incubated at room temperature for 25 min

until the solution turned blue. The nanostars were then capped with 600 μL 2.5 mM PVP and the tubes were rotator mixed for 5 min and incubated for 1 h at room temperature. The capped AuNS samples suspension was cleaned up twice by centrifugation for 35 min at 2170 $\times g$ and was resuspended in 500 μL distilled water.

5.4.4 Suitability of NAD, NADPH, and H_2O_2 for redox-mediated change in AuNS morphology

The initial phase of the assays assessed the capacity of NAD⁺, NADPH and H_2O_2 to induce redox-mediated colourimetric and UV-Vis spectral changes to PVP-capped AuNS. This phase was evaluated using a redox potential-based model. The reagents were pipette mixed in water to ensure a final volume of 200 μL in the following strict order for optimum results: 20 μL 10 mM Tris buffer (pH 8.4) followed by the addition of 15 μL AuNS-PVP. In each assay, 5 μL increments until 20 μL of 50 mM H_2O_2 and 8 mM NAD⁺/NAD(P)H were pipette mixed respectively. Finally, the assays were incubated for 5 min at room temperature before adding the detection solution (2 μL 10 mM AgNO_3 + 20 μL 150 mM NaOH) and UV-Vis spectral measurements were taken immediately.

5.4.5 Preparation of gold nanostar bioconjugates

The capped AuNS samples suspension was cleaned up twice by centrifugation for 35 min at 2170 $\times g$ and was resuspended in 500 μL 100 mM HEPES (pH 6.9) after removal of the supernatant. 100 μL 5 mM freshly made up DTSSP crosslinker was added to 3 sets of 2 mL resuspended capped AuNS and the tubes were rotator mixed for 5 min and incubated for 30 min at room temperature. Following the incubation step, 100 μL 8 mM NAD/NADPH was complexed with 150 μL 2 mg/mL GalDH/AR respectively and 150 μL 0.5 mg/mL GalOx was added to the DTSSP functionalised AuNS (in each tube respectfully) and left to incubate at 4 $^\circ\text{C}$ for 2 h for enzyme and cofactor conjugation and immobilisation (Walper *et al.*, 2015). The bioconjugates were cleaned up twice by centrifugation for 35 min at 2170 $\times g$ and were resuspended in 500 μL ddH₂O (Millipore, 18.2 $\Omega\text{M cm}^{-1}$) and stored at 4 $^\circ\text{C}$.

5.4.6 AuNS bioconjugates stability assay

The stability of AuNS bioconjugates was assessed in synthetic blank urine, supplemented cell culture medium and serum flocculation assays at room temperature for 3 h. Spectral readouts for a 1:1 solution of matrices and AuNS bioconjugates were captured and analysed every 5 min for 3 h. Because the AuNS bioconjugates are utilised as biosensors, their stability in matrices for longer periods of time is less important because typical assay formats' incubation time is less than

3 h. Overall, two assessments are made here: (1) storage stability in the water matrix at 4 °C and room temperature for 96 h and (2) short-term stability during the analytical procedure in different matrices for 3 h. A blue UV-Vis spectral shift and a shortening of the spectral curve, as shown by the loss and shift of the AuNS bioconjugate's longitudinal peak, were regarded as indicative of instability (Zhang *et al.*, 2018).

5.4.7 AuNS bioconjugates colorimetric assay

In this phase, the ability of biocatalytically produced NAD(P)/NAD(P)H and H₂O₂ to induce redox-mediated colourimetric and UV-Vis spectrum modifications in AuNS bioconjugates was evaluated. To achieve the best results, the reagents were pipette mixed in water to a final volume of 200 µL in the specific order listed below: 15 µL 10 mM Tris buffer (pH 8.4) was added, followed by 20 µL AuNS bioconjugate. In each assay, 0 – 30 µL 2 mM galactose was added in 5 µL increments, followed by a 15 min incubation at 37 °C. Finally, the detection solution (2 µL 10 mM AgNO₃ + 20 µL 150 mM NaOH) was added to the assays, followed by a 1–5 min incubation at 37 °C before spectral readings measurement. Different incubation durations following the addition of the detection solution demonstrate how quickly the assay generates plausible colourimetric readings.

The final phase of the assays assessed the efficacy of the bioconjugated AuNS in colourimetric signal generation in a synthetic blood matrix. The synthetic blood matrix constituted a 1:1000 (water: synthetic blood) ratio spiked with 2 mM galactose and 8 mM NAD⁺/NAD(P)H for AuNS-PVP-GalDH and AuNS-PVP-AR bioconjugates. For the AuNS-PVP-GalOx bioconjugate exclude the coenzymes.

5.4.8 AuNS bioconjugates specificity assay

The efficacy of the AuNS bioconjugates was investigated through analyte specificity assays, galactose was replaced by its epimer glucose. 2 mM glucose solution was utilised for the analyte specificity tests. The assays were optimised and pipette mixed chronologically as the galactose assays. Finally, as the other assays described above, UV-Vis spectral measurements were acquired.

5.5 Results

In the present study, HEPES-mediated PVP-capped AuNS were synthesised. The synthesised nanoparticles were characterised and used in NAD(P)+/NAD(P)H and H₂O₂ coupled redox-mediated colourimetric assays.

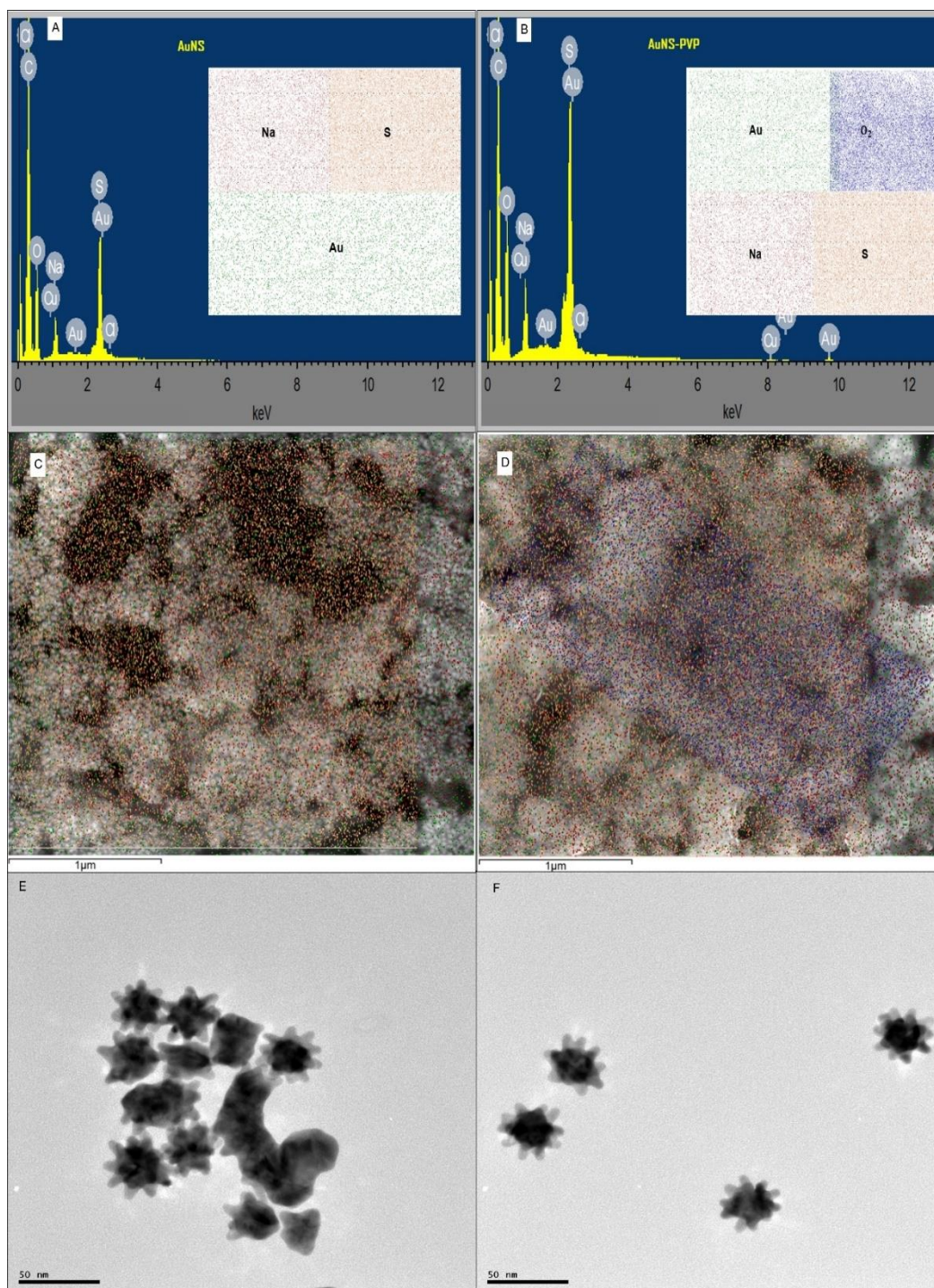


Figure 5-1: EDS spectra, SEM-EDS elemental mapping profiles, and HR-TEM images for AuNS. (A, C, E) Uncapped AuNS; (B, D, F) PVP capped AuNS.

Figure 5-1 shows the elemental composition, mapping and HR-TEM images of AuNS. The AuNS were mainly composed of gold, sulphur and carbon and were moderately monodispersed with a 10-armed morphology and a diameter of approximately 40 – 42 nm.

UV-Vis and ^1H -NMR-spectral profiles, along with electrophoresis and HR-TEM images of GalDH, AR and GalOx AuNS bioconjugates, are presented in Figure 5-2. Two distinct absorbance peaks, corresponding to the longitudinal (~ 690 nm) and transverse plasmon resonance modes (~ 540 nm) are observed on the UV-Vis spectra (Fig.5-2A, E and I) (Hao *et al.*, 2007b).

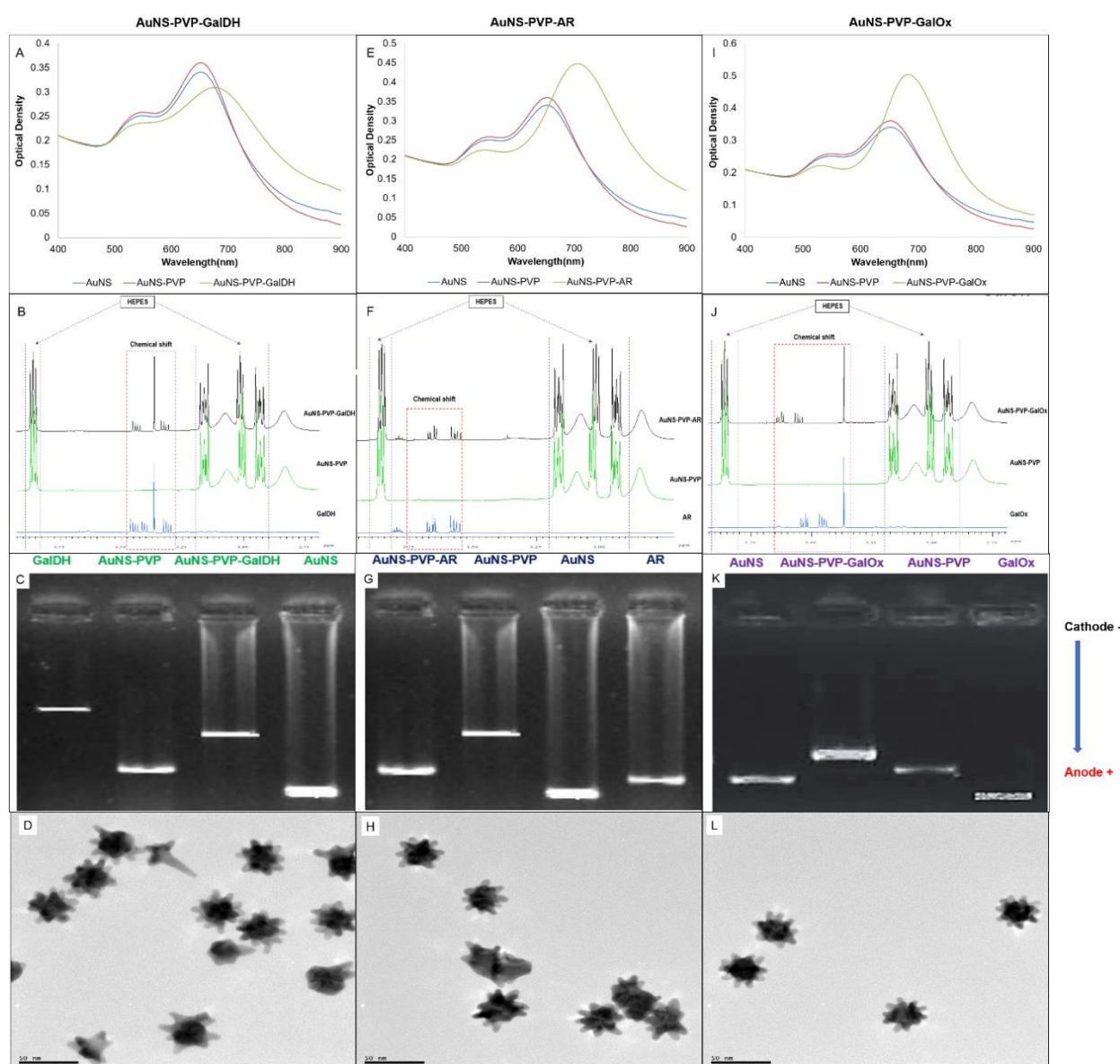


Figure 5-2: UV-Vis spectra profiles, ^1H -NMR spectra profiles, electrophoretic profiles, and HR-TEM images for AuNS bioconjugates. (A-D) AuNS-PVP-GalDH; (E-H) AuNS-PVP-AR; (I-L) AuNS-PVP-GalOx.

The ^1H -NMR spectra confirm the presence of the respective enzymes after bioconjugation Fig.5-2(B, F, J). Complimentarily, the electrophoretic migration patterns suggest successful capping and enzyme bioconjugation to the AuNS and not its mere presence in the sample (Fig.5-2(C, G, K). HR-TEM images in Figure 5-2 (D, H, L) show that AuNS maintain their shape after conjugation while exhibiting slightly different dispersions.

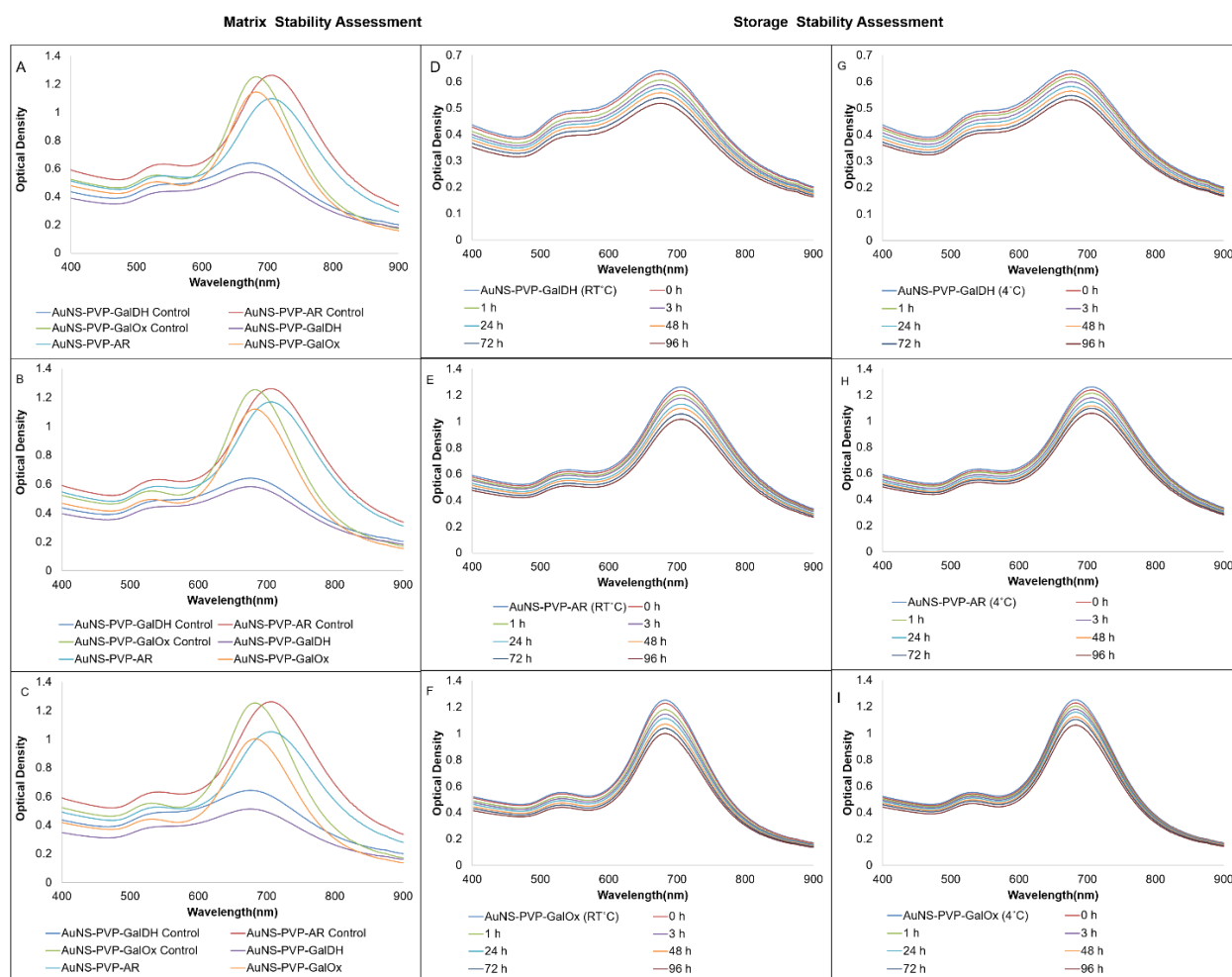


Figure 5-3: UV-Vis spectra of AuNS bioconjugates stability after 3 h incubation in (A) Blank urine; (B) Serum; (C) Supplemented cell culture medium. Short-term storage stability UV-Vis spectra at room temperature and 4 °C in water matrix (D; G) AuNS-PVP-GalDH; (E; H) AuNS-PVP-AR; (F; I) AuNS-PVP-GalOx.

An assessment of the matrix and short-term storage stability of the AuNS bioconjugates was required prior to its use in colourimetric assays, the results of which are presented in Figure 5.3. The AuNS bioconjugates were stable in all matrix environments with a marginal spectral blue shift while maintaining the characteristic AuNS profiles (Fig.5-3A-C). Likewise, the AuNS bioconjugates were stable under short-term storage stability conditions for 96 h and overall, storage at 4 °C was optimal. (Fig.5-3 D-I). These results show that AuNS bioconjugates exhibit adequate stability in a variety of matrices as well as good short-term storage stability in water

which was considered to be sufficient for use in the redox-mediated colourimetric assays that follow.

Figure 5-4 depicts the preliminary colourimetric assays illustrating a direct correlation between NAD(P)/NAD(P)H and H₂O₂ concentrations and AuNS absorbance. The UV-Vis spectral readings and colourimetric signals show that NAD(P)/NAD(P)H and H₂O₂ cause concentration-dependent changes in the AuNS absorbance pattern, which most likely correlates to the nanostar transition from an anisotropic to quasi-spherical shape.

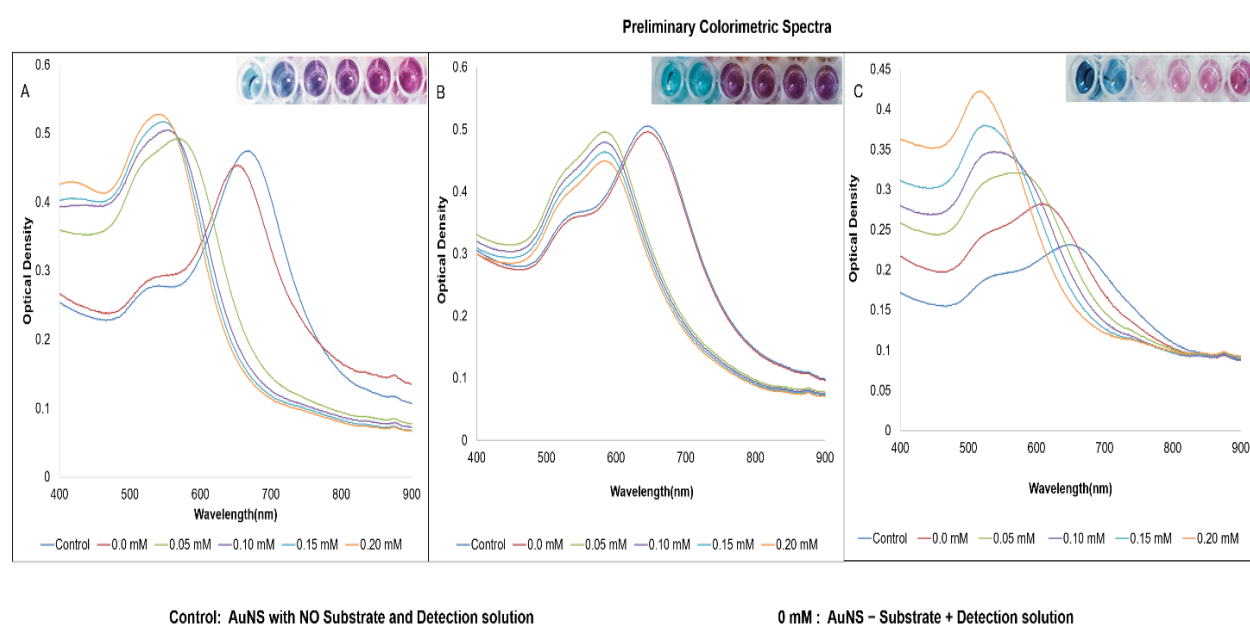
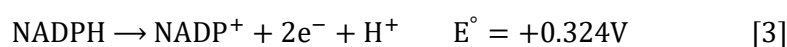
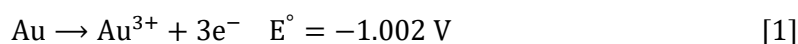


Figure 5-4: Preliminary colourimetric UV-Vis profiles for AuNS-PVP in the presence of (A) NAD⁺; (B) NADPH and (C) H₂O₂.

Thermodynamics predicts that NAD(P)⁺ and H₂O₂ will spontaneously react with Au. Using standard reduction potentials and half-cell reactions, we have theoretically described how NAD⁺ and H₂O₂ induce colourimetric and morphological transformations in AuNS. The standard redox potentials for the NAD(P)⁺ /NAD(P)H and AuCl₄⁻/Au pairs are -0.32 V, -0.324 V and +1.002 V, respectively (Bagotsky, 2005b; Sánchez-Iglesias *et al.*, 2021; Xiao *et al.*, 2004b).





The redox reactions are thermodynamically feasible and spontaneous, suggesting that NAD^+ and H_2O_2 can induce morphological changes in AuNS. In contrast, the Au-NADPH redox reaction is not thermodynamically feasible; however, Huang *et.al.* proposed that in the presence of oxygen, Au nanoparticles can react with NAD(P)H, causing morphological changes visible spectrophotometrically (Huang *et al.*, 2005). In contrast, Hietzschold *et.al* and Xiao *et.al.* proposed that NAD(P)H can facilitate the growth of silver and gold nanoparticles respectively in the presence of AgNO_3 and AuCl_4^- in the presence of reducing agents (Hietzschold *et al.*, 2019; Xiao *et al.*, 2004b).

Figures 5-5 and 5-6 depict the colourimetric assay results of AuNS bioconjugates in water and synthetic blood matrix. In both cases, increasing galactose concentrations cause a blue shift of varying magnitudes, followed by the transition of AuNS into quasi-spherical nanoparticles as shown in the UV-Vis spectra profiles and TEM images in the water matrix.

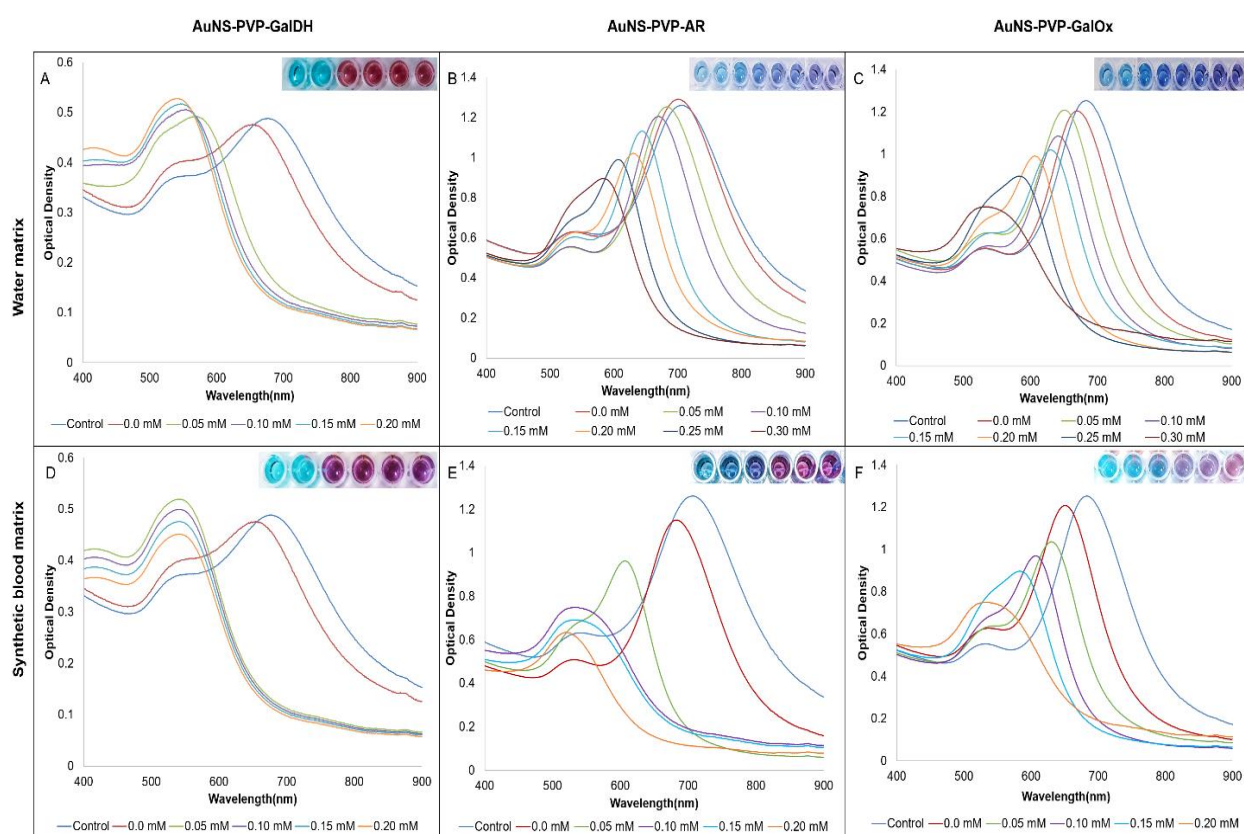


Figure 5-5: AuNS bioconjugates colourimetric spectra in water and synthetic blood with increasing galactose (A-B) AuNS-PVP-AR; (C-D) AuNS-PVP-GalOx.

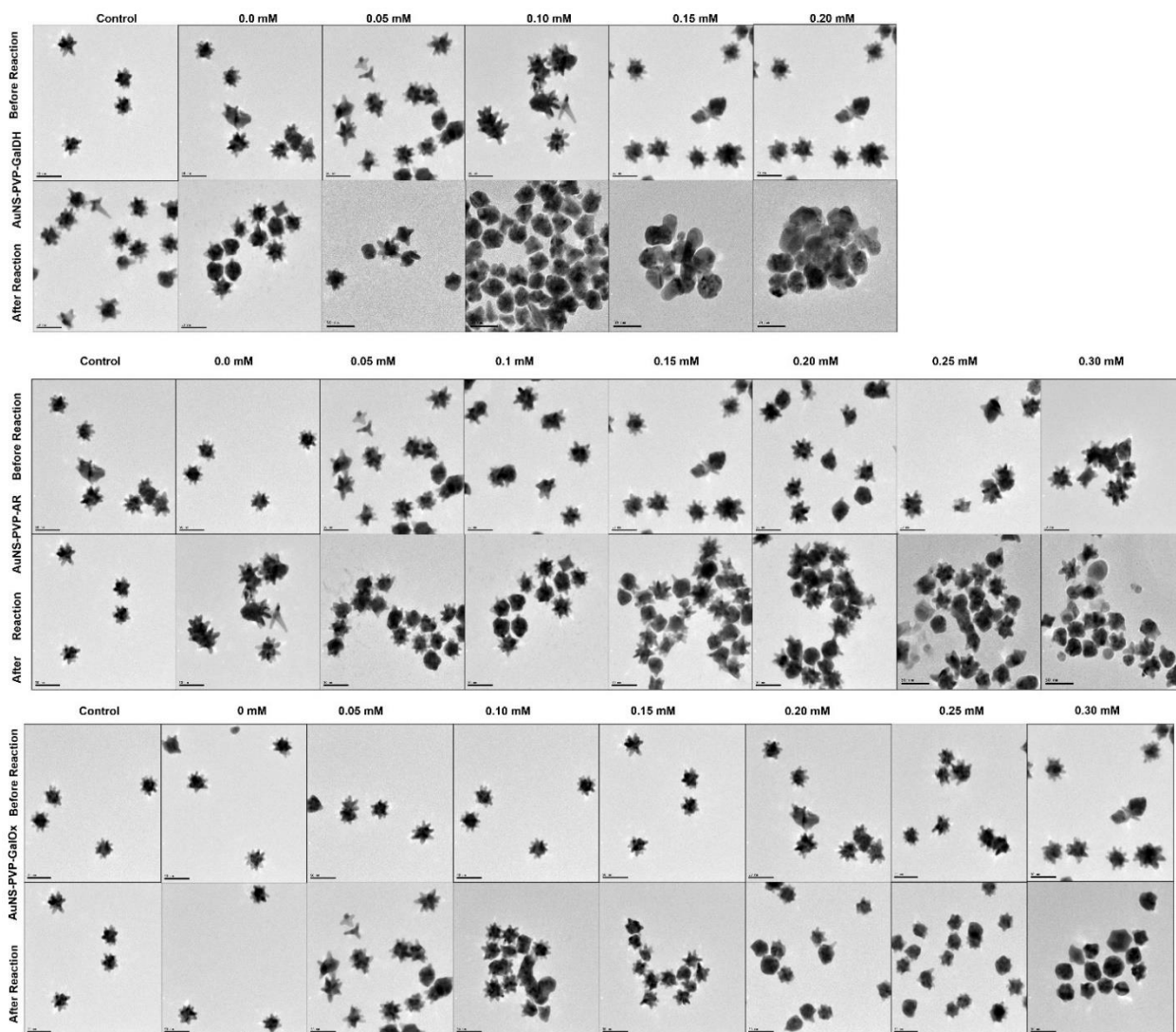


Figure 5-6: AuNS bioconjugates TEM images before and after colourimetric reactions in the water matrix.

The AuNS-PVP-AR assay required a 5 min incubation, whereas the AuNS-PVP-GalOx assay required a 1 min incubation to generate plausible colourimetric signals after adding the detection solution. In contrast, after adding the detection solution in all matrices, the AuNS-PVP-GalDH assay showed an instantaneous change to a wine-red colour. The visually observed colour changes, UV-Vis spectral profiles and HR-TEM images suggest that the AuNS-PVP-GalDH assay has a narrow quantification range in all matrices, in comparison to broader ranges observed for the AuNS-PVP-AR and AuNS-PVP-GalOx assays.

Interestingly, quantitative ICP-MS data (Table 5-1) before and after the reaction reveal that the quantities of Ag and Au remained nearly constant while the quantity of Na increased. This suggests that contrary to previous studies, NaOH participates in our colourimetric assays and that the quasi-spherical gold nanoparticles are not silver-plated, although the presence of Na in the

lattice was not confirmed (Mulder, Phiri, *et al.*, 2019b; Rodríguez-Lorenzo *et al.*, 2012). Overall, the AuNS-PVP-GalDH assay has the potential to be used qualitatively, whilst the AuNS-PVP-AR and AuNS-PVP-GalOx assays could be used semi-quantitatively.

Table 5-1: AuNS bioconjugates quantitative ICP-MS data before and after colourimetric reactions in the water matrix.

Elements	AuNS-PVP-GalDH	AuNS-PVP-GalDH	Fold change
	Before Reaction [ppm]	After Reaction [ppm]	
23 Na	4.6	37.5	8.24
107 Ag	16.9	17.3	1.02
197 Au	39.1	39.8	1.02
Elements	AuNS-PVP-AR	AuNS-PVP-AR	Fold change
	Before Reaction [ppm]	After Reaction [ppm]	
23 Na	4.2	37.5	8.96
107 Ag	16.4	17.3	1.05
197 Au	40.2	39.8	0.99
Elements	AuNS-PVP-GalOx	AuNS-PVP-GalOx	Fold change
	Before Reaction [ppm]	After Reaction [ppm]	
23 Na	4.4	37.5	8.61
107 Ag	16.3	17.3	1.06
197 Au	39.7	39.8	1.00

GalDH and GalOx are galactose specific, whereas AR has a broad specificity for various aldoses and a high K_m for glucose and galactose (Blachnitzky *et al.*, 1974; Gabbay & Tze, 1972; Testa & Meyer, 1996; Whittaker & Whittaker, 2000). Nevertheless, gold nanoparticles can act as a catalyst and can oxidise glucose (Comotti *et al.*, 2004; Lang *et al.*, 2014; Zheng *et al.*, 2011). It was therefore necessary to test the specificity of the assays and in particular the possible interference of glucose. The AuNS-PVP-AR assay UV-Vis spectra are blue-shifted in a concentration-dependent manner with increased glucose concentration, changing the nanostar morphology to quasi-spherical particles in water and synthetic blood matrices (Figure 5-7 B; E). In contrast, when exposed to glucose, the AuNS-PVP-GalDH and AuNS-PVP-GalOx assays showed no reaction or colour changes, indicating the usual blue shift in the spectrum induced by the detection solution

and that the assays were galactose specific as expected (Figure 5.7 A; C) (Blachnitzky *et al.*, 1974; Whittaker & Whittaker, 2000).

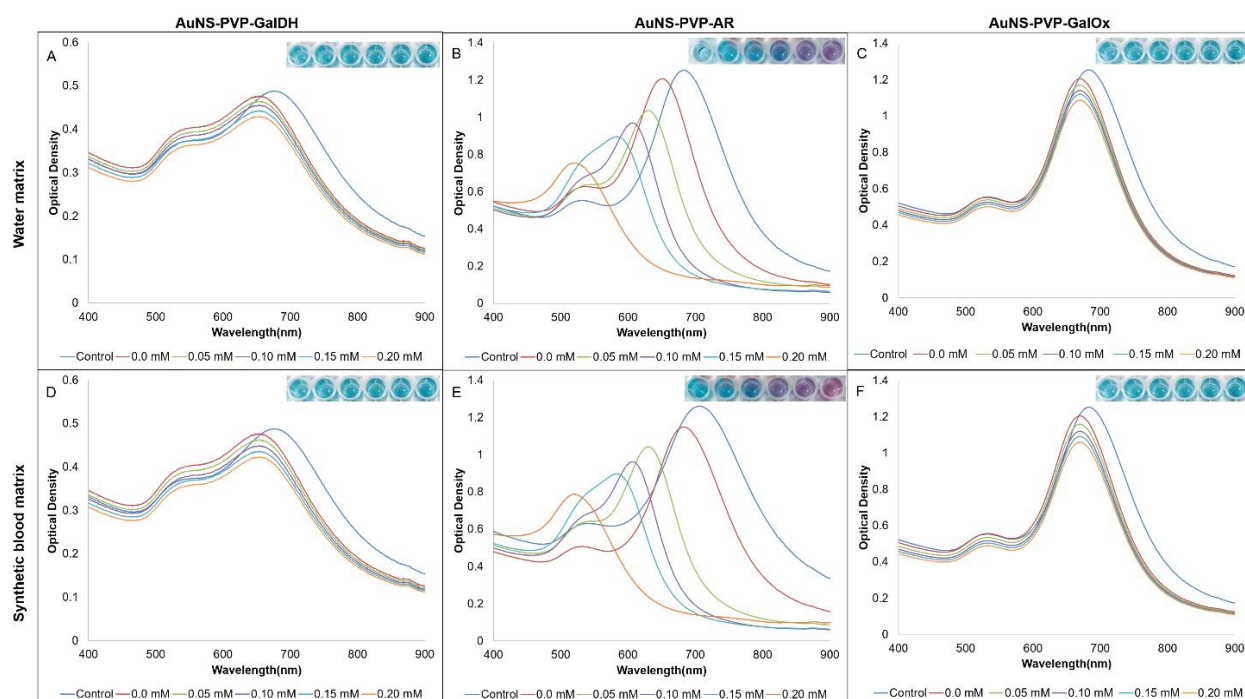


Figure 5-7: AuNS bioassays analyte specificity test. Glucose was used in place of galactose. (A; D) AuNS-PVP-GalDH; (B; E) AuNS-PVP-AR; (C; F). AuNS-PVP-GalOx.

These results show that the proposed catalytic activity of the gold nanoparticles does not change the specificity of the AuNS-PVP-AR bioassay.

5.6 Discussion

Anisotropic AuNS were synthesised using a HEPES-mediated seedless one-pot synthesis procedure. HEPES acted as a reducing, shape-directing and stabilising agent in this technique, resulting in heteromorphic nanostars of various diameters (Fig 5-1. A) (Sajitha *et al.*, 2015; Xie *et al.*, 2007c). Numerous studies have revealed that HEPES has a strong affinity for gold, which could be attributable to the sulfonate groups in its chemical structure (Jimmy Huang *et al.*, 2020; Xi & Haes, 2019; Xie *et al.*, 2007c). This could explain its existence in the AuNS lattice post-synthesis, as evidenced by elemental analyses (Fig 5-1. A-B) and NMR analyses (Fig 5-2. B, F, J), both of which are consistent with prior studies. The anisotropic AuNS synthesised were characterised by the combination of two distinct UV-Vis absorbance peaks, which corresponded to the transverse plasmon resonance mode (plasmonic solid core) and the longitudinal plasmon resonance (plasmonic protuberant spikes) (Hao *et al.*, 2007b).

GalDH and AR are dimeric apoenzymes with a $(\beta/\alpha)_8$ barrel that allows NAD(P)⁺/NAD(P)H to bind and form apoenzyme-coenzyme complexes (Ikeda *et al.*, 1975; Penning, 2015; Vargas *et al.*, 2022). NAD(P)⁺/NAD(P)H operate as conformation primers, stabilising the apoenzyme-coenzyme complex's oligomeric catalytically active structure (Chen & Zhang, 2012; Penning, 2015). The significance of NAD(P)⁺/NAD(P)H coenzymes in the structure and activity of apoenzymes encouraged us to co-immobilise the apoenzyme-coenzyme complexes to the AuNS during the AuNS bioassay synthesis. Furthermore, as shown in Figures 5-1 and 5-2, PVP capping caused slight modifications to the AuNS, the nanostar lattice structure was predominantly composed of gold and HEPES and enzymes were successfully immobilised to the nanostars.

The bioassays' analyte plasmonic colourimetric sensing efficacy was investigated. The AuNS-PVP-GalDH assay produced an instantaneous colourimetric signal. In contrast, the AuNS-PVP-AR and AuNS-PVP-GalOx assays produced nearly comparable spectrum changes in water and synthetic whole blood matrices (Fig. 5-5). NADH, the reaction product of the AuNS-PVP-GalDH assay, is a robust competitive inhibitor of GalDH and also inhibits AuNS dissolution; this could explain the development of an instantaneous colourimetric signals, which is consistent with Liang *et al.*'s findings (Cuatrecasas & Segal, 1966; Liang *et al.*, 2015). The assays, however, were based on the blue-shifted absorption band of nanostar (690 nm) to quasi-spherical nanoparticles (540 nm) upon NAD(P)⁺/NADPH and H₂O₂ biocatalytic reactions (Fig.5-5). When employing synthetic blood, the bioassays had a potential drawback because the bulk of blood components have substantial absorption in the same range as the AuNPs (Fig.5-5 D-F) (Bansal *et al.*, 2020; Li, Lin, *et al.*, 2014; Shao *et al.*, 2016). Nonetheless, AuNS are highly sensitive, with an extinction coefficient greater than that of blood components, hence matrix dilution and a blank subtraction procedure were applied to facilitate result interpretation and analysis (De Puig *et al.*, 2015; Zhao *et al.*, 2017).

The transformation in size, shape and dielectric environment of the AuNS bioassays resulted in plausible plasmon band shifts and colourimetric signals (Fig.5-5). Colourimetric signal generation in our bioassays could be pH-driven, enzyme-mediated, or analyte-mediated, resulting in spectrum shifts and colourimetric changes (Zhang *et al.*, 2018). Overall, the lack of change in Ag or Au concentrations on ICP-MS data (Table 5-1) indicates no silver or residual gold cations reduction in the solution. As a result, we propose that either the remodelling phenomena or a typical ion exchange caused nanoparticle surface oxidation, followed by NAD(P)/NAD(P)H-mediated redox of Au³⁺ to Au⁰, with morphological and colourimetric changes to the AuNPs.

Several enzymatic and non-enzymatic techniques for detecting NAD(P)⁺/NAD(P)H have recently been developed and published in peer-reviewed journals. These are primarily colourimetric and

electrochemical detections, with some successfully integrated into miniaturised devices. Based on the dissolution of gold nanoparticles, Liang *et.al.* (2015) developed a high-sensitivity paper-based device for the rapid visualisation of NADH (Liang *et al.*, 2015). Jafari *et.al.* (2021) recently suggested a colourimetric paper-based biosensor based on the phenylalanine dehydrogenase (PDH) enzyme for the highly sensitive and selective measurement of phenylalanine (Phe), employing cationic dyes as colourimetric mediators (Jafari *et al.*, 2021). Messina *et.al.* (2023) devised a highly sensitive colourimetric method for the enzymatic detection of Phe in the presence of neo-formed NADH using tris (bipyridine) Ruthenium (II/III) mediator (Messina *et al.*, 2023). Furthermore, Maugeri *et.al.* (2023) established a novel photothermal-contrast approach for Phe detection in human blood utilising PDH, resulting in the creation of in-situ gold nanostructures in the presence of neo-formed NADH and AuCl_4^- (Maugeri *et al.*, 2023).

Our findings support Liang *et.al.*'s (2015) findings that neo-formed NADH inhibits AuNS dissolution. In our bioassays, the AuNS operate as colourimetric and sensing signal amplifiers (Cao *et al.*, 2011), avoiding the need for dyes, in contrast to the biosensor designs proposed by Messina *et.al.* (2023) and Jafari *et.al.* (2021). Furthermore, our understanding of redox chemistry (Eqtn.5) confirms Maugeri *et.al.*'s (2023) findings that neo-formed NADH can react with Au^{3+} to generate gold nanoparticles.

5.7 Conclusion

In conclusion, we report the potential of rapid and sensitive redox-driven galactose bioassays utilising NAD(P)/NAD(P)H and H_2O_2 for tailoring gold nanostar morphology and plasmonic properties. A plausible finding from the AuNS bioassays shows that in the absence of oxidase enzymes, dehydrogenase or reductase enzymes can be utilised instead and NAD(P)/NAD(P)H can be used as redox agents in enzymatic plasmonic gold nanostar assays for analyte(s) quantification. We demonstrated that the AuNS-PVP-AR and AuNS-PVP-GalOx assays were semi-quantitative, whereas the AuNS-PVP-GalDH assay was a qualitative galactose assay. Overall, the enzyme-guided etching of the gold nanostar detected low concentrations of analytes, as shown in Figures 5 and 7 and was supported by UV-Vis spectrum profiles, TEM images, colourimetric data and ICP-MS data. With suitable assay tailoring, NAD(P)/NAD(P)H-dependent enzymes can be used to change gold nanostar morphology and plasmonic properties in complex matrices for the analyte(s) detection. Thus, gold nanostars bioassays hold great promise for application in biomedical diagnostics for resource-constrained areas.

5.8 Acknowledgments

We would like to acknowledge Dr. Innocent Shuro from the Laboratory for Electron Microscopy, North-West University, Potchefstroom, South Africa, for assistance with obtaining the nanoparticle images. We would also like to thank the North-West University's Centre of Human Metabolomics (CHM) and the South African Technology Innovation Agency (TIA) for the funding given in order to carry out this work.

5.9 Authors' contributions

Tozivepi Aaron Munyayi originated the study, formulated the study design, conducted the laboratory work, participated in data analysis and drafted the manuscript. Barend Christiaan Vorster, Engela Helena Conradie and Danielle Wingrove Mulder participated in the design of the study, data analysis and integrity of the manuscript. All authors gave their approval for publication.

**CHAPTER 6 H₂O₂ MEDIATED PLASMONIC GOLD NANOSTARS –
GALACTOSE DEHYDROGENASE QUANTITATIVE GALACTOSE
COLOURIMETRIC ASSAY**

EVERYTHING IS THEORETICALLY IMPOSSIBLE, UNTIL IT IS DONE.

– Robert. A. Heinlein



biosensors

an Open Access Journal by MDPI



CERTIFICATE OF ACCEPTANCE



Certificate of acceptance for the manuscript (**biosensors-2663663**) titled:
Quantitative galactose colorimetric competitive assay based on galactose dehydrogenase and plasmonic gold nanostars

Authored by:

Tozivepi Aaron Munyayi; Danielle Wingrove Mulder; Engela Helena Conradie; Barend Christiaan Vorster

has been accepted in *Biosensors* (ISSN 2079-6374) on 31 October 2023



Academic Open Access Publishing
since 1996

Basel, October 2023

6.1 Preface

This chapter fulfilled Objective 2 of Chapter 3, which was to investigate the appropriateness of NAD(P)/NAD(P)H as colorimetric redox agents and the influence of H₂O₂ in gold nanostar biosensors. This chapter was submitted to MDPI-Biosensors Journal, which approved it and published it (<https://doi.org/10.3390/bios13110965>). Minor changes were made from the published version, based on examiners' comments.

6.2 Abstract

We describe a competitive colourimetric assay that enables rapid and sensitive detection of galactose and reduced nicotinamide adenine dinucleotide (NADH) via colourimetric readouts and demonstrate its usefulness for monitoring NAD⁺-driven enzymatic reactions. We present a sensitive plasmonic sensing approach for assessing galactose concentration and the presence of NADH using galactose dehydrogenase-immobilized gold nanostars (AuNS-PVP-GalDH). The AuNS-PVP-GalDH assay remains turquoise blue in the absence of galactose and NADH; however, as galactose and NADH concentrations grow, the reaction well colour changes to a characteristic red colour in the presence of an alkaline environment and a metal ion catalyst (detection solution). As a result, when galactose is sensed in the presence of H₂O₂, the colored response of the AuNS-PVP-GalDH assay transforms from turquoise blue to light pink, and then to wine red in a concentration-dependent manner discernible to the human eye. This competitive AuNS-PVP-GalDH assay could be a viable analytical tool for rapid and convenient galactose quantification in resource-limited areas.

Keywords: Gold nanostars; Enzymatic; Immobilised; Plasmonic; Colourimetric; Detection solution

6.3 Introduction

Gold nanoparticles (AuNPs), particularly gold nanostars (AuNS), have gained considerable attention in diagnostics due to their unique, size-dependent characteristics, biocompatibility and simplicity of chemical manipulation (Chirico *et al.*, 2015; Li, Ma, *et al.*, 2013; Liu *et al.*, 2014; Liu, Yuan, Fales, *et al.*, 2015; Wen *et al.*, 2023). AuNS display polarisation-dependent scattering and absorption with dual spectral peaks (transverse and longitudinal) highly sensitive to protruding tip eccentricity changes (Atta *et al.*, 2016; Hao *et al.*, 2007b; Hrelescu *et al.*, 2009; Nehl *et al.*, 2006a; Tsoulos *et al.*, 2019). Plasmonic AuNS have extraordinarily high extinction coefficients since their surface plasmon resonance is strongly impacted by their size, shape and morphology (Eustis & El-Sayed, 2006; Xianyu *et al.*, 2021a). (Eustis & El-Sayed, 2006; Xianyu *et al.*, 2021a). AuNS are

extremely sensitive to environmental perturbations, resulting in evident morphological, spectrometric and colourimetric changes (Liu *et al.*, 2014; Shao *et al.*, 2012). These excellent properties may pave the way for the development of nanosensors with simple plasmonic absorbance-based readouts (Zhou *et al.*, 2015).

Nicotinamide adenine dinucleotide (NAD^+) is a redox coenzyme essential for energy metabolism (Covarrubias *et al.*, 2021). As a redox carrier, NAD^+ accepts a hydride ion and shuttles electrons from metabolic activities to generate its reduced form, NADH (Xie *et al.*, 2020). NADH can donate one or two electrons depending on the substrate's Gibbs free energy (Anderson, 1980). Several NAD^+/NADH redox couple sensing approaches have been developed, with the majority requiring sophisticated processes and specialist personnel to interpret the results (Brekasis & Paget, 2003; Chen *et al.*, 2021; Lee *et al.*, 2022; Micheli *et al.*, 1993; Mongeon *et al.*, 2016). Traditional colourimetric methods for detecting NAD^+/NADH are based on the colour reaction of organic dyes, which are quickly oxidised by O_2 (Chamchoy *et al.*, 2019; Hirakawa & Mori, 2021). As a result, establishing a reliable NAD^+/NADH detection technique free of chromogenic substrates is crucial. Plasmonic-based biosensing has been widely employed for on-site or in-field solutions to complement traditional diagnostic procedures due to its versatility, low cost, simple result interpretation and robust reaction times (Divya *et al.*, 2022; Wang *et al.*, 2023). As a result, NAD^+/NADH redox pair plasmonic colourimetric sensing has emerged as a feasible alternative to existing methods (Liang *et al.*, 2015; Weng *et al.*, 2019).

Many plasmonic sensors have been constructed by monitoring the resonant electromagnetic field and being sensitive to changes in the refractive index of the medium around nanoparticles after target molecule (NAD^+/NADH) binding (Roh *et al.*, 2011). Liang *et al.* (2015) recently disclosed a paper-based system for quick and sensitive room-temperature measurement of NADH using a colourimetric readout (Liang *et al.*, 2015). In their work, NADH was found to limit AuNPs dissolution by reducing Au^{3+} to Au^+ , resulting in a red colour that became deeper with increasing NADH concentrations. Using a similar approach, Baymiller *et al.* (2017) explored NADH's ability to produce nanoparticles and discovered that this coenzyme alone is adequate to convert Au^{3+} ions to gold nanoparticles in vitro (Baymiller *et al.*, 2017). At room temperature, this method creates uniformly dispersed spherical plasmonic nanoparticles of varying sizes almost instantly, with NADH concentration regulating both the rate of synthesis and the tonality of the produced gold nanoparticles (AuNPs). Using p-aminophenol (pAP)-catalysed and NADH-mediated production of silver nanoparticles (AgNPs), Liu *et al.* (2021) developed a light-controlled colourimetric assay for the quantification and detection of nitroreductase (NTR) (Liu *et al.*, 2021). The hydrolysis product of p-nitrophenol by NTR in the presence of NADH can be employed as a catalyst to catalyse the reduction of Ag^+ by NADH under light, providing NTR concentration-

dependent colourimetric signals. Furthermore, NAD/NADH are widely employed to quantify substrate-to-product conversion directly or indirectly (Li, Lu, *et al.*, 2013; Maenaka *et al.*, 2012). In contrast to NADH sensing, hydrogen peroxide (H_2O_2) plasmonic sensing has been intensively researched in recent years because H_2O_2 sensing is robust, giving colourimetric and morphological alterations to nanoparticles (Rodríguez-Lorenzo *et al.*, 2012; Teodoro *et al.*, 2019; Wu *et al.*, 2016). When nanoparticles are exposed to H_2O_2 , they undergo catalytic dissolution, resulting in a decrease in the nanoparticle absorption band with increasing H_2O_2 concentration (Lai *et al.*, 2023). Overall, plasmonic biosensors generate colourimetric signals primarily as a result of the aggregation/dispersion degree caused by the addition of Au^{3+} solution or through the etching/growth of pre-made nanoparticles in the presence of an alkaline environment and a metal ions catalyst (detection solution) (Rodríguez-Lorenzo *et al.*, 2012; Zhang *et al.*, 2018).

In this study, rather than measuring NAD^+/NADH , we investigated their suitability as redox agents for gold nanostar shape alteration in colourimetric quantification of varied galactose concentrations. We developed a nanoplasmonic sensor for quantitatively detecting NADH and galactose in diverse matrices using galactose dehydrogenase (GalDH)- NAD^+ co-immobilised AuNS. This paper presents an integration of H_2O_2 -induced surface etching of AuNS and NADH-driven biocatalytic growth of AuNPs in the presence of a detection solution to yield morphological and colourimetric changes. This colourimetric detection system takes advantage of NADH inhibiting AuNS dissolution while H_2O_2 induces AuNS dissolution, cyclic oxidation of NADH and biocatalytic growth of AuNPs (De la Rica & Stevens, 2012; Liang *et al.*, 2015). We also used redox chemistry to investigate how the detection solution affects colourimetric signal generation from both experimental and theoretical perspectives. As a result, this assay has significant potential for in-field analytes and inhibitors screening with the naked eye, with a mechanism that is most likely transferable to other NAD^+ -dependent enzymes.

6.3.1 Materials and Methods

6.3.1.1 Materials

All materials were bought from Sigma-Aldrich unless stated otherwise. The chemicals used were; sodium hydroxide, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$), gold (III) chloride hydrate ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$), silver nitrate (AgNO_3), sodium hydroxide (NaOH), 3'30'-dithiobis sulfosuccinimidyl propionate (DTSSP, $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_{14}\text{S}_4\text{Na}_2$), galactose ($\text{C}_6\text{H}_{12}\text{O}_6$), hydrogen peroxide (H_2O_2), galactose dehydrogenase (GalDH), nicotinamide adenine dinucleotide hydrate (NAD , $\text{C}_{21}\text{H}_{27}\text{N}_7\text{O}_{14}\text{P}_2 \cdot x\text{H}_2\text{O}$), Tris buffer ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$), and polyvinylpyrrolidone (PVP) 10 000. Synthetic dry whole blood was purchased from Thistle QA, Johannesburg, South Africa.

The materials used were; sterile 5 mL screw cap tubes (Ascendis Medical), carbon mesh copper grids (Agar Scientific, Rotherham, UK), and clear, flat-bottomed 96-well plates (Corning).

6.3.1.2 Instrumentation and Characterisation

Scanning electron microscopy and energy-dispersive x-ray spectroscopy (SEM-EDS, Bruker, Billerica, MA, USA) were used to assess the chemical compositions of the samples qualitatively. Inductively coupled plasma mass spectrometry (ICP-MS, Spectro AMETEK, Inc., Kleve, Germany) was used to determine the elemental contents in the samples. SEM-EDS and ICP-MS were utilized to precisely quantify and confirm the chemical composition of the samples.

The gold nanostar and bioconjugate spectrum scanning (400–900 nm) was carried out utilizing 96-well plates and the HT Synergy (BioTEK) microplate reader (Agilent Technologies, Santa Clara, CA, USA).

High-resolution transmission electron microscopy (HR-TEM) was carried out using a Tecnai F20 transmission electron microscope (JEOL, Freising, Bavaria, Germany). The capped gold nanostar samples were imaged after being spotted onto copper grids (Agar Scientific) and air-dried. In contrast, the gold nanostar bioconjugates required 1% silver nitrate solution staining prior to copper grid spotting, air drying, and imaging for protein visualization (Atta *et al.*, 2019).

ImageJ software (bundled with 64-bit Java 1.8.0_172, University of Wisconsin at Madison, Madison, WI, USA) was used to estimate particle core diameters and arm dimensions by averaging a total count of one hundred capped gold nanostars and one hundred gold nanostar bioconjugates in different HR-TEM images.

All nuclear magnetic resonance (NMR) experiments at 500 MHz were performed with a Bruker Avance III HD (NMR) spectrometer equipped with a triple-resonance inverse (TXI) 1H (¹⁵N, ¹³C) probe head (Bruker, Billerica, MA, USA). This was used to confirm the gold nanostars' successful capping and protein immobilization.

The capped gold nanostars and gold nanostar bioconjugates were electrophoresed in agarose gel using a Baygene BG-power Vacutec electrophoresis gel apparatus (Vacutec, Johannesburg, South Africa). To confirm the molecules based on their size and electrical charge, electrophoresis was performed at pH 8 with 0.5% agarose and 0.5 × Tris borate EDTA buffer (TBE buffer). The samples were made by combining 32 µL of nanostars with 4 µL of 80% glycerol and running them at 40 V for 45 min. The gold nanostar bioconjugate gels were stained for 4 h with 25 mL of 0.25% (w/v) Coomassie blue and then rinsed with a destaining solution (90% w/v isopropanol and 10%

w/v glacial acetic acid), revealing the protein bands. Gels were stored in ddH₂O until photos were recorded and uploaded to a computer.

6.3.1.3 Preparation of Gold Nanostars

The gold nanostars (AuNSs) were synthesized using the modified HEPES buffer method described and characterized by our laboratory (Mulder, 2019; Munyayi *et al.*, 2022). Succinctly, 2 mL of 100 mM HEPES buffer (pH 7.4) was added to 3 mL of deionized water (Millipore, Temecula, CA, USA, 18.2 $\Omega\text{M}\cdot\text{cm}^{-1}$), followed by 20 μL of 50 mM gold (III) chloride hydrate ($\text{HAuCl}_4\cdot x\text{H}_2\text{O}$) and 4 μL of 1 mM silver nitrate (AgNO_3). The 5 mL screw cap was mixed via end-to-end tube inversion and incubated for 25 min at room temperature until the solution turned turquoise blue. The nanostars were then capped with 600 μL of 2.5 mM PVP. After inverting the tube several times, it was left to stand at room temperature for 1 h. The capped AuNS sample suspension was cleaned twice via centrifugation for 35 min at 2170 $\times$ g and resuspended in 500 μL of ddH₂O

6.3.1.4 Preparation of gold nanostar–galactose dehydrogenase bioconjugates

The capped AuNS sample suspension was cleaned twice via centrifugation for 35 min at 2170 $\times$ g and resuspended in 500 μL of 100 mM HEPES (pH 6.9). Four batches of 500 μL each were then combined, making for a total volume of 2 mL, and 100 μL of 5 mM DTSSP was then added to the solution, followed by 100 μL of 8 mM NAD⁺ and 150 μL of GalDH 2 mg/mL. The solution was incubated at 4 °C for 2 h to allow enzyme and coenzyme conjugation and immobilization. This co-immobilization was carried out to improve enzyme stability, activity, and selectivity or specificity (Kondrat *et al.*, 2022; Rodrigues *et al.*, 2021; Vargas *et al.*, 2022). Finally, the co-immobilized AuNS (AuNS-PVP-GalDH) was cleaned twice via centrifugation at 2170 $\times$ g for 35 min and then resuspended in 500 μL of ddH₂O (Millipore, 18.2 $\Omega\text{M}\cdot\text{cm}^{-1}$) and stored at 4 °C.

6.3.1.5 Preparation of gold nanostar-galactose dehydrogenase biosensor

The gold nanostars (AuNS) were synthesized using the modified HEPES buffer method described and characterized by our laboratory (Mulder, 2019; Munyayi *et al.*, 2022). Succinctly, 2 mL 100 mM HEPES buffer (pH 7.4) was added to 3 mL deionized water (Millipore, 18.2 $\Omega\text{M}\cdot\text{cm}^{-1}$), followed by 20 μL 50 mM gold (III) chloride trihydrate ($\text{HAuCl}_4\cdot x\text{H}_2\text{O}$) and 4 μL 1 mM silver nitrate (AgNO_3). The 5 mL screw cap was mixed by end-to-end tube inversion and incubated for 25 min at room temperature until the solution turned turquoise blue. The nanostars were then capped with 600 μL 2.5 mM PVP. After inverting the tube several times, it was left to stand at room temperature for 1 h. The capped AuNS sample suspension was cleaned twice by centrifugation for 35 min at 2170 $\times$ g and resuspended in 500 μL of 100 mM HEPES (pH 6.9). Four batches of 500 μL each

were then combined, making a total volume of 2 mL, and 100 μL 5 mM DTSSP was then added to the solution, followed by 100 μL of 8 mM NAD^+ and 150 μL of GalDH 2 mg/mL. The solution was incubated at 4 $^{\circ}\text{C}$ for 2 h to allow enzyme and coenzyme conjugation and immobilization. This co-immobilization was carried out to improve enzyme stability, activity, and selectivity or specificity (Kondrat *et al.*, 2022; Rodrigues *et al.*, 2021; Vargas *et al.*, 2022). Finally, the co-immobilized AuNS (AuNS-PVP-GalDH) was cleaned twice by centrifugation at 2170 $\times g$ for 35 min, then resuspended in 500 μL ddH₂O (Millipore, 18.2 $\Omega\text{M}\cdot\text{cm}^{-1}$), and stored at 4 $^{\circ}\text{C}$.

6.3.1.6 Gold nanostar–galactose dehydrogenase biosensor colourimetric assay

The AuNS-PVP-GalDH plasmonic colourimetric tests were performed using galactose as an analyte and different H₂O₂ concentrations (0 mM, 0.05 mM, 0.10 mM, 0.15 mM, and 0.20 mM). Five distinct experiments were performed (as summarized in Table 1), with everything kept constant and varied H₂O₂ concentrations. The reagents were pipette-mixed in water to a final volume of 200 μL in the following order: Following the addition of 15 μL of 10 mM Tris buffer (pH 8.4) and 20 μL of AuNS-PVP-GalDH to each of the five experiments, different volumes (0 μL , 5 μL , 10 μL , 15 μL , and 20 μL per experiment) of 2 mM galactose were added and pipette-mixed into the assays. The experiments were incubated for 10 min at 37 $^{\circ}\text{C}$ before adding (0 μL , 5 μL , 10 μL , 15 μL , and 20 μL) each H₂O₂ concentration employed. The assays were then incubated for 5 min at 37 $^{\circ}\text{C}$ before adding the detection solution (2 μL 10 mM AgNO₃ + 15 μL 150 mM NaOH). Finally, the assays in each experiment were incubated for 2 min at 37 $^{\circ}\text{C}$ before obtaining UV-Vis spectral readings and colourimetric signals.

Table 6-1: Experimental Procedure for the galactose plasmonic colourimetric detection using AuNS-PVP-GalDH biosensor.

Sample	Control	1	2	3	4	5
Water	165 μL	148	138 μL	128 μL	118 μL	108 μL
10 mM Tris (pH 8.4)	15 μL	15 μL	15 μL	15 μL	15 μL	15 μL
AuNS-PVP-GalDH	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL
2 mM galactose	0 μL	0 μL	5 μL	10 μL	15 μL	20 μL
10 min incubation						
H ₂ O ₂	0 μL	0 μL	5 μL	10 μL	15 μL	20 μL
5 min incubation						
10 mM AgNO ₃	0 μL	2 μL	2 μL	2 μL	2 μL	2 μL
150 mM NaOH	0 μL	15 μL	15 μL	15 μL	15 μL	15 μL
2 min incubation						
Colourimetric signal generation						

A total of 5 mg of synthetic whole blood (powder) was dissolved in 1.5 mL of ddH₂O, and end-to-end inversion was performed to ensure appropriate powder dissolution. In 5 mL screw cap tubes,

several galactose solutions (2 mM, 4 mM, 6 mM, and 8 mM) were produced and nitrogen-dried for 24 h to reduce contamination and over-dilution of the matrix. To guarantee adequate spiking, the dried galactose solution screw cap tubes were diluted with 500 μ L of synthetic blood and inverted end-to-end. After spiking the synthetic blood matrix with galactose, the samples were serially diluted 1000 times for easy spectrophotometric reading acquisition (Phiri *et al.*, 2019c). The specificity and efficacy of the AuNS-PVP-GalDH assay were determined using spiked synthetic whole blood with various amounts of galactose. The detection of galactose was tested spectrophotometrically and visually by detecting colour changes in the solutions after substituting galactose with synthetic-blood-spiked galactose, as stated above. All experiments were carried out in triplicate.

Influence of the Detection Solution on the AuNS-PVP-GalDH Biosensor

The alkaline environment and metal ion catalysts' effects on the AuNS-PVP-GalDH biosensor in the aqueous matrix were examined. Furthermore, by employing different gold nanoparticle sizes, gold nanoparticle nucleation or growth and colourimetric signal generation induced by pH fluctuations over time and metal ion catalysts were investigated (Yazdani *et al.*, 2021).

6.4 Results

Figure 6-1 depicts the UV-vis spectral scans, ^1H -NMR spectra, HR-TEM images, and electrophoretic migration of the AuNS-PVP-GalDH bioconjugate. As shown in Figure 6-1A, the distinctive surface plasmon resonance (SPR) band of AuNS red-shifted after protein addition, resulting in a drop in optical density (OD)_{max} absorbance, whereas PVP capping resulted in a modest rise in OD_{max} relative to the uncapped AuNS absorption band. This is due to the interaction and orientation of the coatings on the AuNS, polydispersity, and the size of the side groups in the proteins and PVP, which cause different shifts in the AuNS absorption band. The HR-TEM images in Figure 6-1B demonstrate that the AuNS-PVP-GalDH complex was fairly monodispersed, with an estimated average size ranging from 28 to 30 nm and an average of eight protuberant spikes on well-developed AuNSs. Subsequently, the electrophoretic migration patterns indicate effective capping and protein bioconjugation to the AuNS rather than just being present in the sample (Figure 6-1C). The elemental composition and mapping images of the AuNS-PVP-GalDH bioconjugate are shown in Figure 6-1E, F. Furthermore, quantitative ICP-MS (results not given) and EDS data showed the presence of gold and other trace metals in the AuNS-PVP-GalDH bioconjugate.

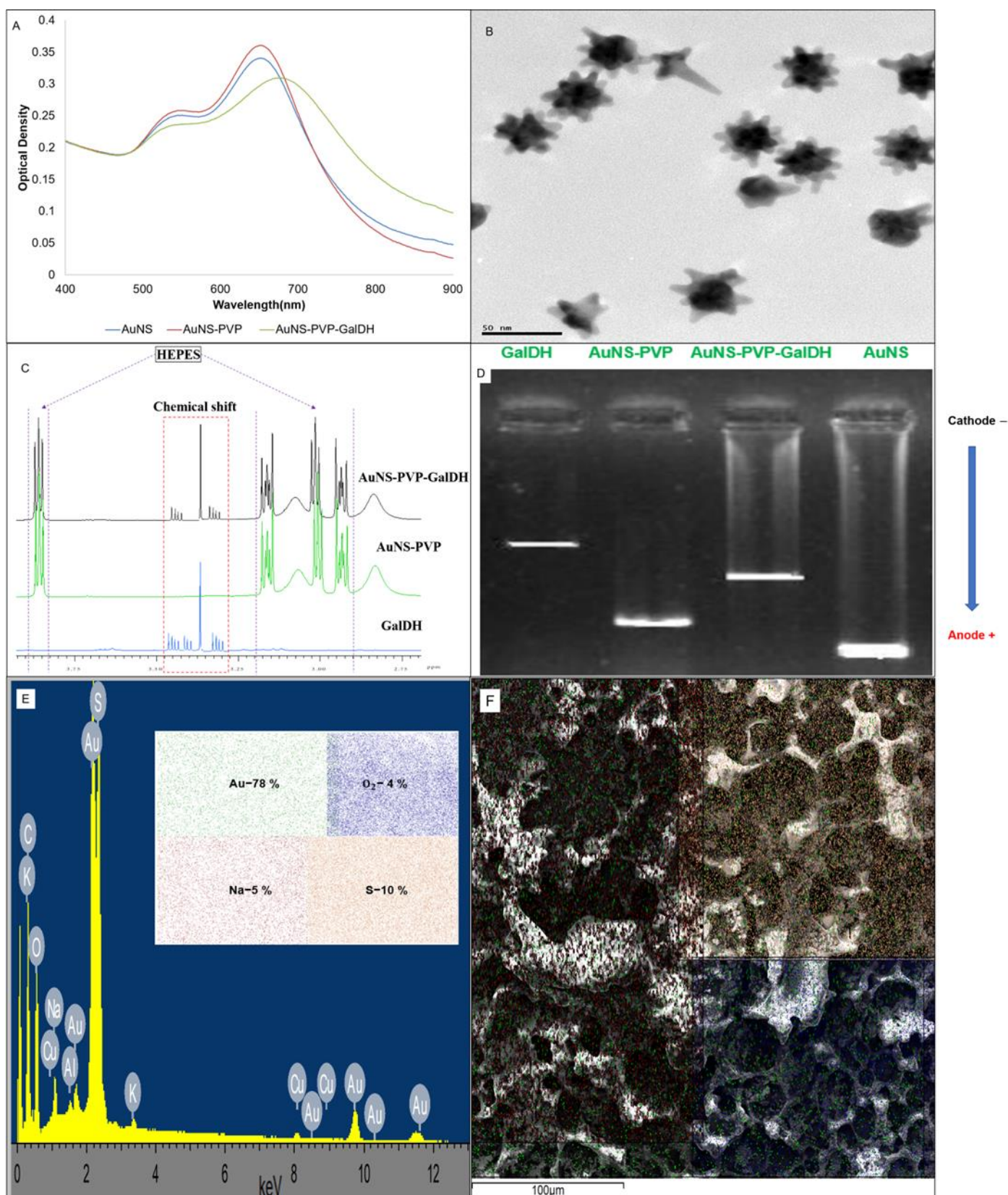


Figure 6-1: AuNS-PVP-GalDH bioconjugate's (A) UV-Vis spectra profiles; (B) HR-TEM profiles; (C) ^1H -NMR spectra profiles; (D) electrophoretic profiles; (E) EDS spectra; and (F) SEM-EDS elemental mapping profiles.

These findings reveal that the AuNS-PVP-GalDH bioconjugates were monodispersed and heterogeneous and that the majority of nanostars had a spiky morphology primarily made up of gold (78%), sulfur (10%), and other trace elements, a result consistent with prior research (Mulder, 2019).

Figure 6-2 depicts the spectrometric and colourimetric results of the AuNS-PVP-GalDH assay in various galactose and H_2O_2 concentrations. When utilizing the H_2O_2 -free assay, the biocatalytic reaction occurred instantaneously, generating a distinct red color, as shown in Figure 6-2A. Nonetheless, the biocatalytic reaction occurred slowly in the 0.05–0.10 mM H_2O_2 concentration range, producing multicolourimetric readings and improved UV-Vis spectral profiles within 2 min of incubation. As shown in Figure 6-2D, E, H_2O_2 rapidly induced AuNS UV-Vis spectral profiles' transformation into spherical AuNP profiles, and the biocatalytic reaction proceeded instantaneously in the 0.15–0.20 mM H_2O_2 concentration range.

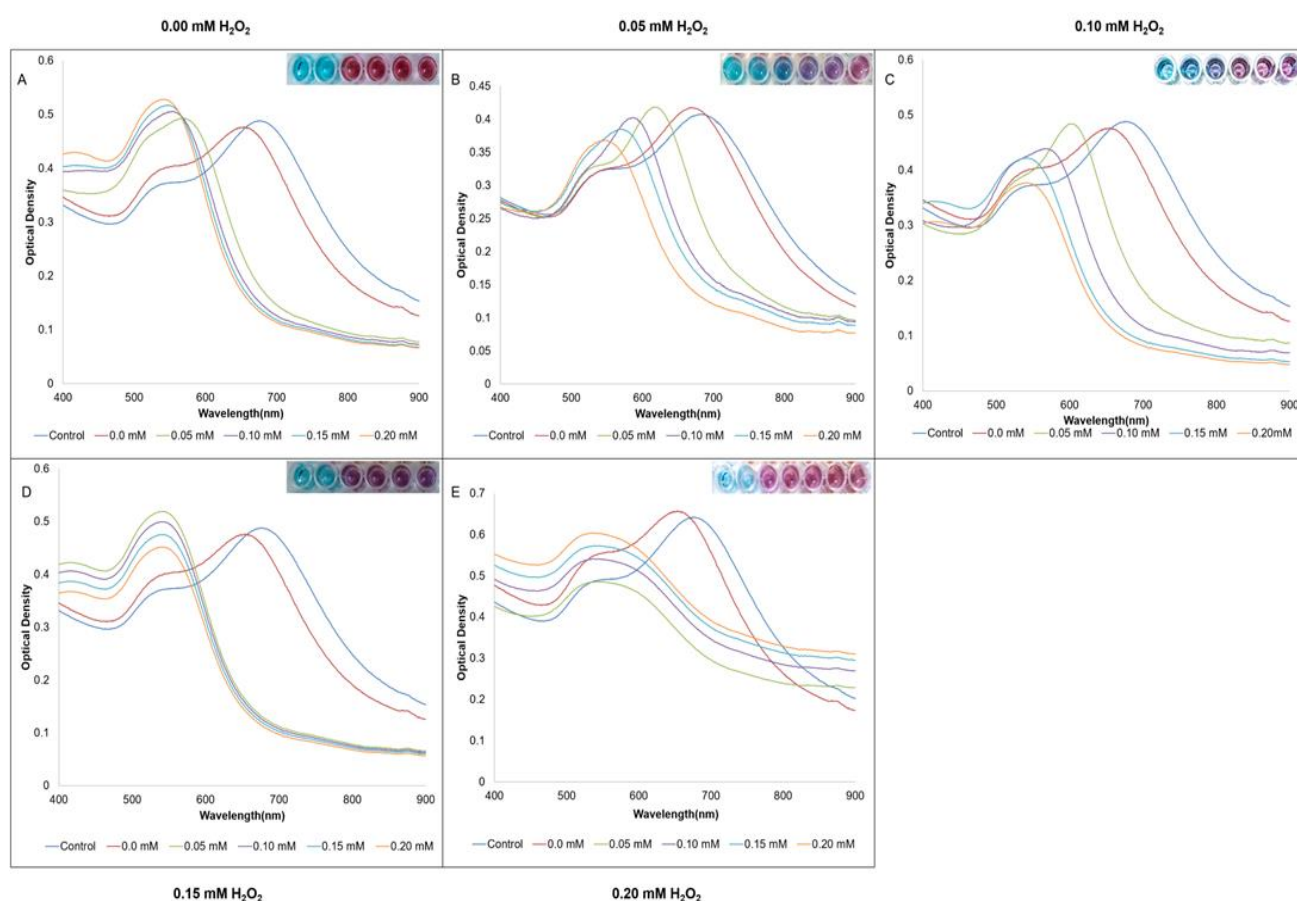


Figure 6-2: UV-Vis spectra and colourimetric profiles of the feasibility of H_2O_2 in inducing concentration-dependent colourimetric signals for galactose detection in the AuNS-PVP-GalDH assay in the water matrix. The following are the variations in H_2O_2 concentrations: (A) 0.00 mM; (B) 0.05 mM; (C) 0.10 mM; (D) 0.15 mM; (E) 0.20 mM.

The results show that concentration-dependent etching of the AuNS-PVP-GalDH assay was most visible when the H_2O_2 concentration was 0.05 mM, indicating that 0.05 mM was the optimal H_2O_2 concentration. Furthermore, the results demonstrate that H_2O_2 concentration affects the rate of colourimetric signal generation and the degree of longitudinal plasmon band (LPB) etching and transverse plasmon mode (TPB) growth.

The results showed that concentration-dependent etching of the AuNS-PVP-GalDH assay was most visible when the H_2O_2 concentration was 0.05 mM, indicating that 0.05 mM was the optimal H_2O_2 concentration. Furthermore, the results demonstrated that H_2O_2 concentration affects the rate of colorimetric signal generation and the degree of longitudinal plasmon band (LPB) etching and transverse plasmon mode (TPB) growth.

In both water and the synthetic whole-blood matrix, different synthetic batches produced nearly identical colourimetric signals in terms of tonality and slightly variable spectrophotometric readouts (Figure 6-3).

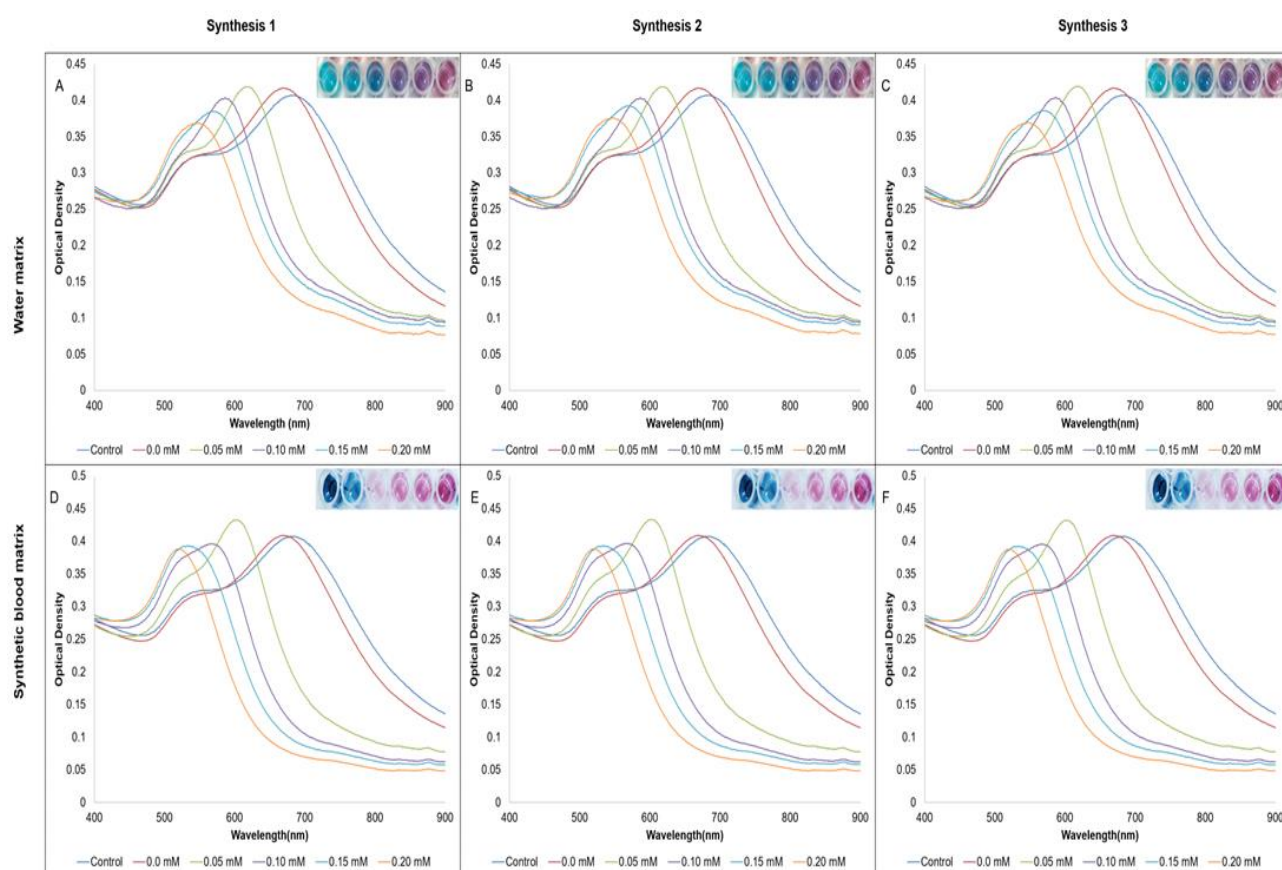


Figure 6-3: Galactose sensing with AuNS-PVP-GalDH nanosensors of various synthesis batches UV-Vis spectra;(A-C) Water matrix; (D-F) Synthetic blood matrix.

As a result, the colourimetric and spectrometric responses of the AuNS-PVP-GalDH bioconjugates for galactose determination were comparable. As a proof of concept, the optimized NADH-H₂O₂-driven assay measured various galactose concentrations in different matrices, yielding multicolourimetric and concentration-dependent spectrum signals. Figure 6-4 depicts the average colourimetric and spectrophotometric signals, as well as mathematical correlations, of several AuNS-PVP-GalDH biosensor syntheses in water and synthetic blood matrices.

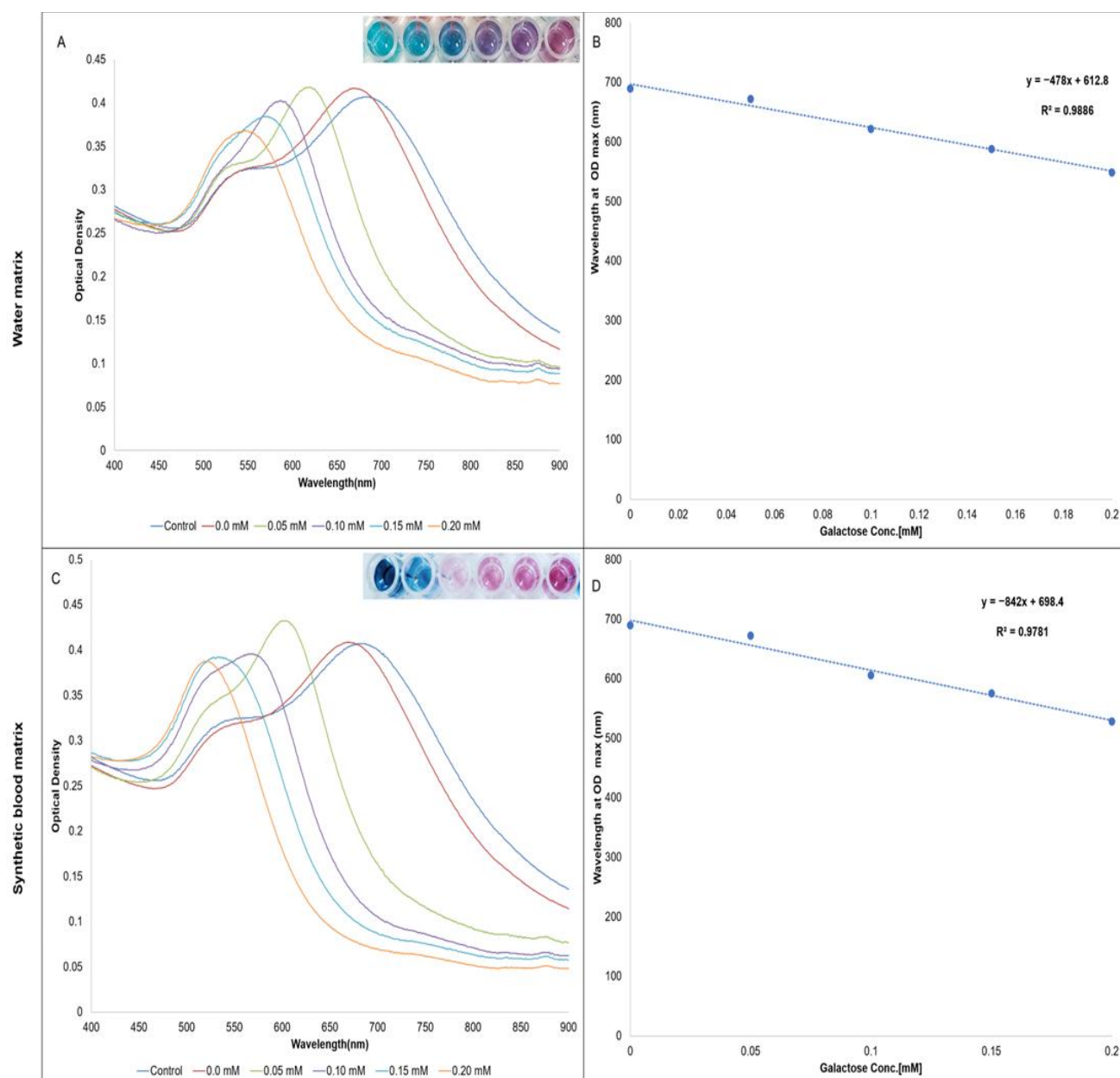


Figure 6-4: UV-Vis spectra, colorimetric profiles, and mathematical correlation profiles of mean galactose sensing with AuNS-PVP-GalDH nanosensor. (A-B) Water matrix; Synthetic blood matrix (C-D).

The AuNS-PVP-GalDH spectrum before the colourimetric biocatalytic reaction exhibited two well-resolved bands centered at about 550 and 700 nm, respectively. In contrast, the LLSPR spectra (700 nm) showed a significant drop in absorbance and a strong blue shift as the galactose concentration increased, and the intensity of the TSPR spectra (550 nm) increased. At a 0.2 mM galactose concentration, single TSPR spectra at 550 nm were detected, demonstrating the full transition of AuNSs to quasi-spherical nanostructures. Furthermore, the subsequent changes in the AuNSs may be visualized as a progressive change in the solution colour from turquoise blue to purplish blue to red pink to wine red in the water matrix and from turquoise blue to red pink to wine red in the synthetic blood matrix with good mathematical correlations (Figure 6-4).

The HR-TEM images of the morphological alterations that occurred during the biorecognition reaction are shown in Figure 6-5. The nanostructures remained star-shaped in the absence of galactose. When galactose concentrations were increased, the AuNSs progressively became more quasi-spherical, with evident core diameter enlargement from approximately 30 to 34 nm.

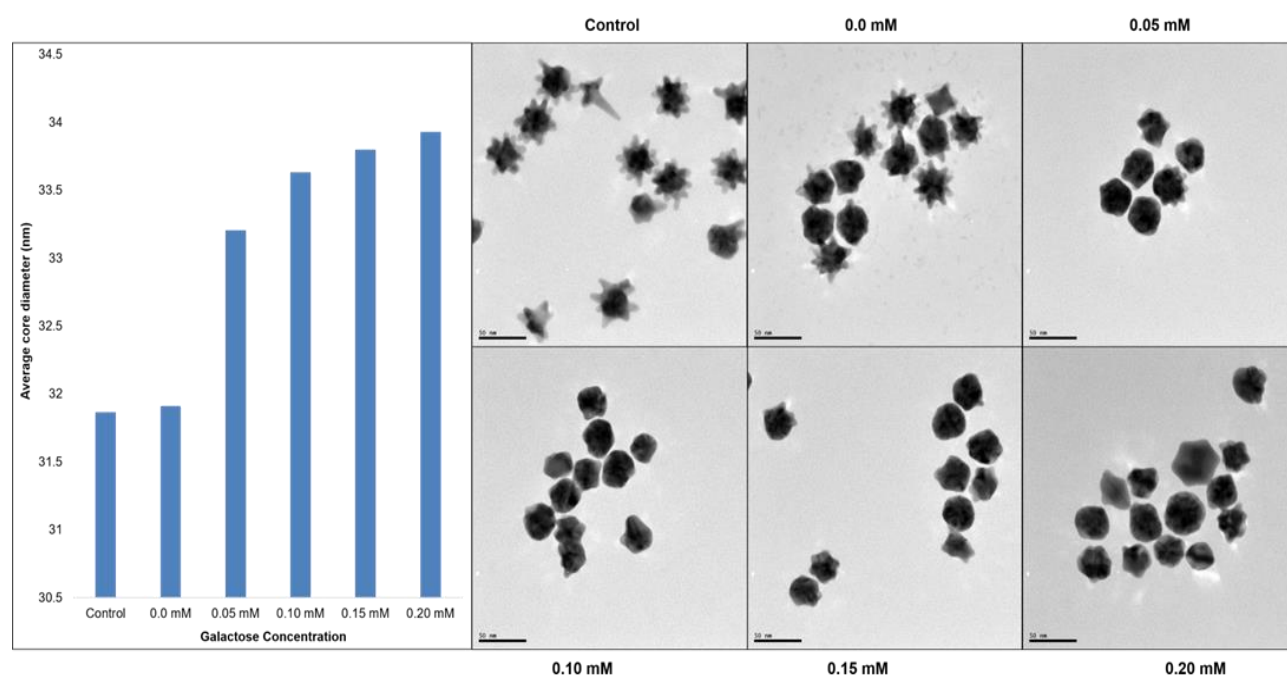


Figure 6-5: Galactose sensing with AuNS-PVP-GalDH biosensor HR-TEM images after the biorecognition reaction in the water matrix.

Overall, the colorimetric biorecognition reaction's representative TEM images revealed a transition from a high yield of branching particles to the existence of high quasi-spherical morphologies.

The UV-Vis spectrum changes and colorimetric signals, as well as the accompanying pH fluctuations, are depicted in Figure 6-6. Even in the presence of varying galactose/NADH and

H₂O₂ concentrations, the bioassays maintained their turquoise blue color with essentially identical spectrum changes as the reaction progressed (Fig.6-6. B-C). The bioassays only produced convincing colorimetric and spectral signals when the detection solution was added (Fig.6-6. D), which is consistent with earlier research (Mulder, Phiri, *et al.*, 2019a; Rodríguez-Lorenzo *et al.*, 2012).

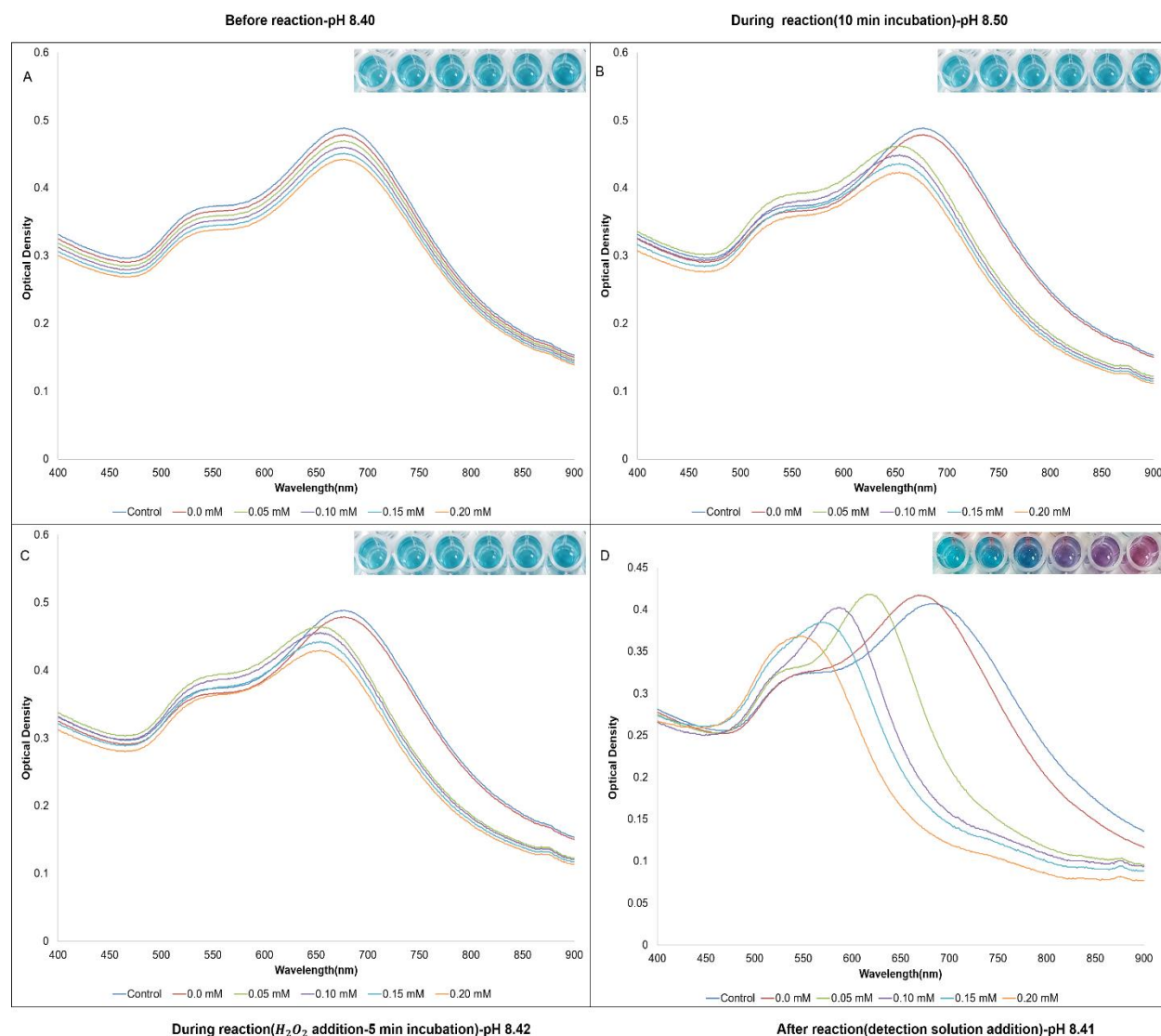


Figure 6-6: UV-Vis spectra and colourimetric profiles for pH variations during galactose detection in the water matrix using the AuNS-PVP-GalDH biosensor at different points: (A) Pre-reaction phase; (B) NADH production phase; (C) H₂O₂ introduction phase; (D) Colourimetric signal generation phase.

The pH increased from 8.4 to 8.5 during the 10 min incubation at 37 °C as the co-enzyme- enzyme complex metabolized the analyte producing NADH and galactonate, and when H₂O₂ was added to the reaction mixture, the pH dropped to 8.42 after the 5 min incubation at 37 °C, and finally, the pH dropped to 8.41 after the addition of the detection solution and the 2 min incubation at 37 °C. Overall, even in the presence of the redox agents NADH and H₂O₂, there was no colourimetric

signal generation in the absence of an alkaline environment and metal ion catalyst in the AuNS-PVP-GalDH biosensor.

6.5 Discussion

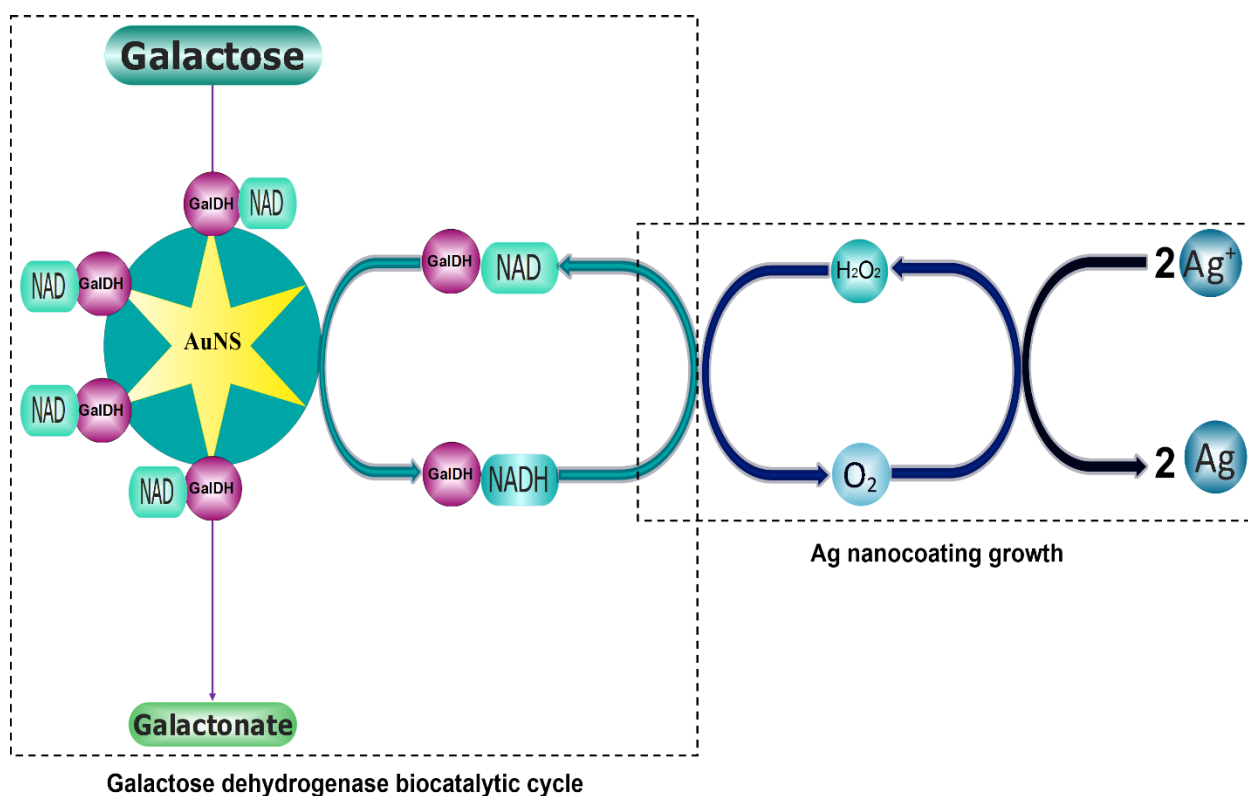
The HEPES-mediated seedless one-pot synthesis protocol was utilized to synthesize anisotropic AuNSs. In this approach, HEPES worked as a mild reducing, stabilizing, and shape-directing agent, resulting in heteromorphic nanostars of varying diameters (Figure 6-1B) (Xie *et al.*, 2007c). Numerous studies have demonstrated that HEPES has a high affinity for gold, which could be attributed to the sulfonate groups that constitute its chemical structure (Xi & Haes, 2019; Xie *et al.*, 2007c). This could account for its existence in the AuNS lattice post-synthesis, as demonstrated by the elemental results (Figure 6-1E, F) and ^1H -NMR analyses (Figure 6-1C), which are consistent with previous research (Chen *et al.*, 2010; Mulder, 2019; Xi & Haes, 2019).

The anisotropic AuNSs were characterized by a hybrid of two distinct UV-Vis absorbance peaks, corresponding to the plasmonic solid core (TLSPR spectra) and the plasmonic protuberant spikes (LLSPR spectra) (Hao *et al.*, 2007b; Khlebtsov, 2010; Nehl *et al.*, 2006a). The AuNS core acts as a nanoscale antenna, greatly enhancing the excitation cross-section and the electromagnetic field of the AuNS spike plasmons (Hao *et al.*, 2007b; Nehl *et al.*, 2006a; Rahman *et al.*, 2015). As a result of its hybrid shape, anisotropic AuNSs have a variety of energy states, with AuNS spikes having a higher energy level than the AuNS core (Hao *et al.*, 2007b; Pazos-Perez *et al.*, 2018; Rahman *et al.*, 2015). The spikes have a higher energy state due to their increased surface area and localization of the electron flux from the core, which creates a strong electric field (lightning rod effect) (Hao *et al.*, 2007b; Nehl *et al.*, 2006a; Rahman *et al.*, 2015).

The designed bioassay has high sensitivity in many matrices and uses a small sample size to get colourimetric findings. Furthermore, bioassays still require significant optimization before they can be used in clinical samples. During the AuNS bioassay synthesis, the relevance of NAD^+ on the structure and activity of GalDH prompted us to co-immobilize GalDH (apoenzyme) and NAD^+ (coenzyme) to the AuNSs. GalDH is a dimer dominated by a $(\beta/\alpha)_8$ barrel that permits NAD^+ to bind and create an apoenzyme–coenzyme complex (Blachnitzky *et al.*, 1974; Ikeda *et al.*, 1975; Vargas *et al.*, 2022). NAD^+ acts as a conformation primer, stabilizing the oligomeric, catalytically active structure of the apoenzyme–coenzyme complex (De Flora *et al.*, 1974; Yagi *et al.*, 1963). The apoenzyme–coenzyme complex, working on the C1 position of the sugar substrate, catalyzes the dehydrogenation of b-D-galactose pyranose to galactonate and NADH (Robinson, 2015; Ueberschär *et al.*, 1974).

The colourimetric technique was divided into two phases: the generation of redox agents from the apoenzyme–coenzyme complex and the activation of morphological change in the AuNS structure by adding the detection solution (Liang *et al.*, 2015). The generation of redox agents on its own is insufficient to induce morphological changes, as shown in Figure 3 and previous studies (Mulder, 2019; Munyayi *et al.*, 2022; Phiri *et al.*, 2019c). In the presence of neo-formed NADH, the bioassay produced instantaneous single-colour change (monocolourimetric) results (Figure 6-2A). Furthermore, in the H₂O₂-free assay (Fig 6-2A), the rate of colourimetric signal generation was instantaneous; this phenomenon might be attributed to pH, temperature, or AuNP catalysis for the oxidative recycling of NADH to NAD⁺ (Huang *et al.*, 2005; Maenaka *et al.*, 2012; Xing *et al.*, 1996). In some instances, the addition of H₂O₂ to the bioassay resulted in better-localized surface plasmonic resonance (LSPR) band shifts and modest colourimetric signal generation rates (Figure 6-2B, C) (Lai *et al.*, 2023; Li, Ma, *et al.*, 2013; Plapp, 2010). As a result, the colourimetric signal generation rates increased as peroxide concentration increased (Figure 6-1D, E).

The assay generates an excess of NADH (reducing agent), and NADH is a probable primary reducing agent for Ag⁺ ions during the formation of the colourimetric signal (Dong *et al.*, 2021). Scheme 6-1 depicts our proposed signal generation technique, with NADH triggering Ag⁺ ions' reduction on AuNSs and low H₂O₂ concentration causing AuNS remodeling into quasi-spherical AuNPs with enlarged core diameters, as supported by the TEM images (Figure 6-3) (Rodríguez-Lorenzo *et al.*, 2012).



Scheme 6-1: Proposed signal-generation mechanism based on enzyme-H₂O₂ mediated crystal growth.

In the presence of the detection solution, however, H₂O₂ has the ability to oxidize both the AuNS and the neo-formed NADH, resulting in plausible morphological and colourimetric changes to AuNSs (Brumaghim *et al.*, 2003; Rodríguez-Lorenzo *et al.*, 2012). In simple terms, GalDH generates NADH, which reduces Ag⁺ ions to grow a Ag coating around the AuNS; furthermore, (i) at low concentrations of NADH and H₂O₂, the nucleation rate is slow, favouring the growth of a surface conformal Ag coating that induces a concentration-dependent blueshift in the AuNS's LSPR and (ii) at high concentrations of NADH and H₂O₂, the fast crystal growth conditions favour Ag nanocrystal nucleation and less Ag is deposited on the AuNS, resulting in monocolourimetric signals (Figure 6-2). The plasmonic AuNS biosensor's signal amplitude is determined by the rate of crystallization, which favors Ag⁰ coating growth on existing nanocrystals (Rodríguez-Lorenzo *et al.*, 2012). To validate this theory, the presence of an increase in the AuNP diameter in solutions containing a AuNS-PVP-GalDH biosensor after the biocatalytic reactions was observed using TEM images and Image-J software bundled with 64-bit Java 1.8.0_172) (Figure6- 4). These findings support the suggested mechanism whereby low H₂O₂ concentrations cause a slow rate of crystallization, which favors the growth of a Ag coating on Au seeds, and NADH is the primary Ag⁺-reducing agent.

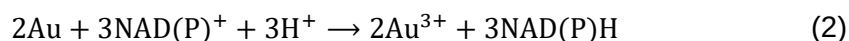
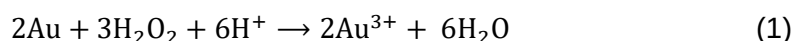
When we compared standard chemistry to experimental work outcomes, we identified anomalies that did not totally support the proposed hypothesis. As a result, we offer theories and explanations for where these discrepancies emerge.

Discrepancy No. 1: gold nanoparticles and NAD(P)H reaction

According to standard redox chemistry, it is thermodynamically impossible for NAD(P)H to react with Au; hence, NAD(P)H has no ability to reduce Au or vice versa. In the presence of gold nanoparticles, however, the AuNS acts as a catalyst in the conversion of NAD(P)H to NAD(P)⁺.

Theory in response to Discrepancy No. 1

Using standard redox chemistry, Au⁰ must be etched or oxidized in the presence of NAD(P)⁺ and H₂O₂ because the reactions are thermodynamically feasible (Equations (1) and (2)).



Nonetheless, we employed spectrophotometry to follow the interaction of different NAD(P)H concentrations (Figure 6-7A ~1 mM; Figure 6-7C ~0.05 mM) with AuNSs during a 24 h period (Figure 6-7). Interestingly, the intensity of the 340 nm NAD(P)H absorption band dropped, while the intensity of the 260 nm NADP⁺ absorption band increased (Figure 6-7A, C), verifying Equation (3). The AuNS spectral band, on the other hand, remained constant (Figure 6-7B,D), hinting that Au⁰ functions as a catalyst for NAD(P)H-to-NAD(P)⁺ oxidation (Equation (3)), a result similar to that found by Huang et al. (2005) (Huang *et al.*, 2005). As a result, this evidence reveals that NAD(P)H is transformed to NAD(P)⁺ on the surface of the AuNS, implying that the AuNS surface-catalyzes NAD(P)H.

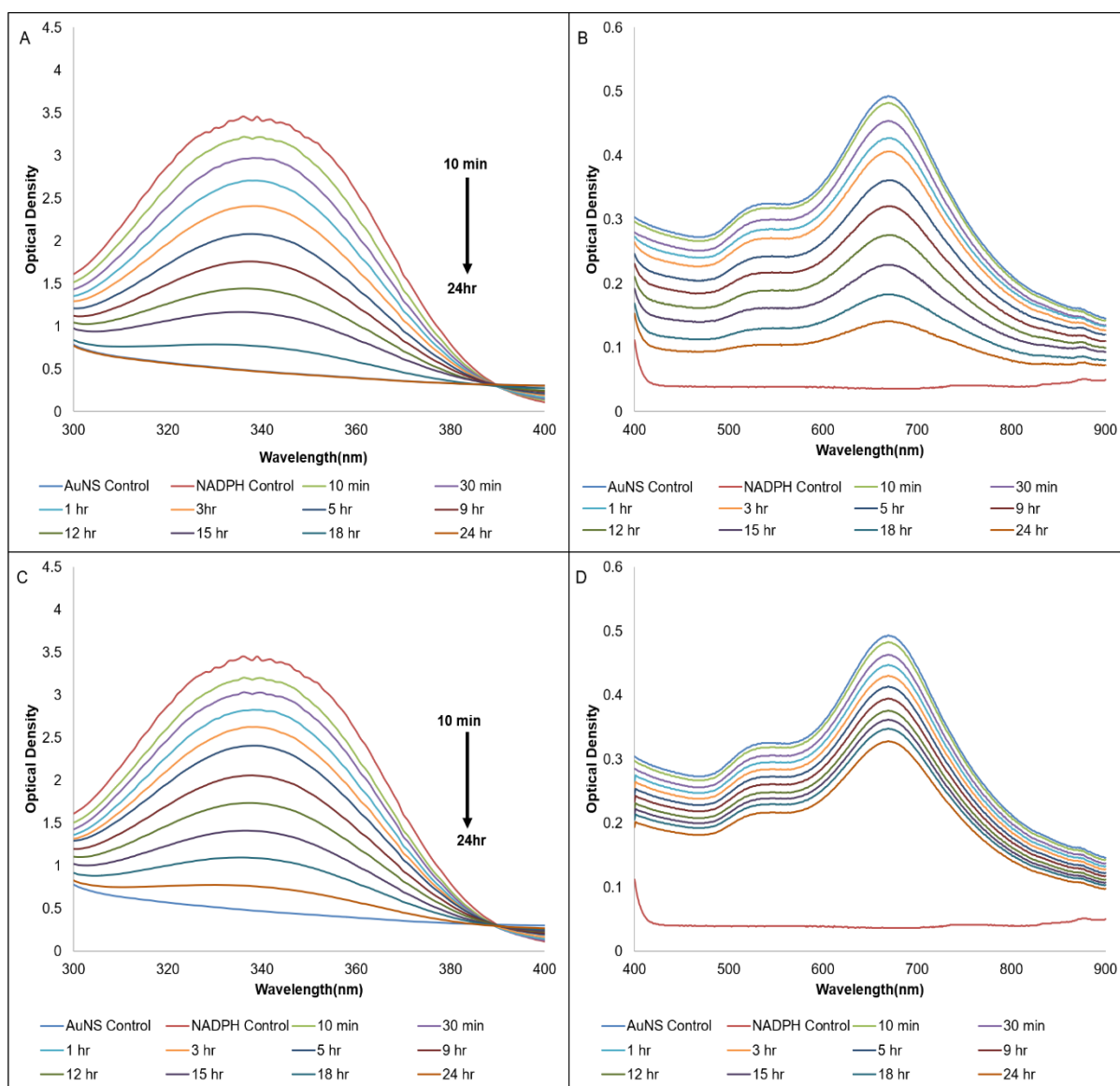


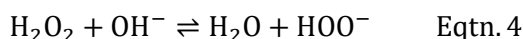
Figure 6-7: UV-Vis spectral profiles for the interaction between NAD(P)H and AuNS over time in a water matrix.

Discrepancy No. 2: Absence of plausible morphological or colourimetric change in AuNS

There is no plausible morphological or colourimetric change with the AuNS in the presence of reducing or oxidizing agents. This is only revealed after the detection solution (a metal ion and a base) is added; in this study, we utilized AgNO_3 and NaOH (Fig.6-6). As a result, the chemistry involved in the generation of colourimetric signals is yet unclear.

Theory in response to Discrepancy No.2

A change in pH, as with many biocatalytic processes, has the potential to terminate the bioreaction. Furthermore, in alkali conditions, NaOH can activate H₂O₂, driving the equilibrium of the acid dissociation (Eqtn.4) and producing hydroperoxide anions (Oraby & Eksteen, 2015).

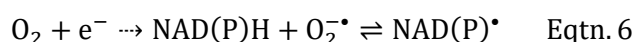


In alkaline settings (pH 8-12) and at low temperatures (37 °C), hydrogen peroxide may quickly form hydroxide radicals (Eqtn.5), which may explain the rapid biorecognition response in some cases and the requirement for alkaline conditions for AuNS signal production and remodeling.

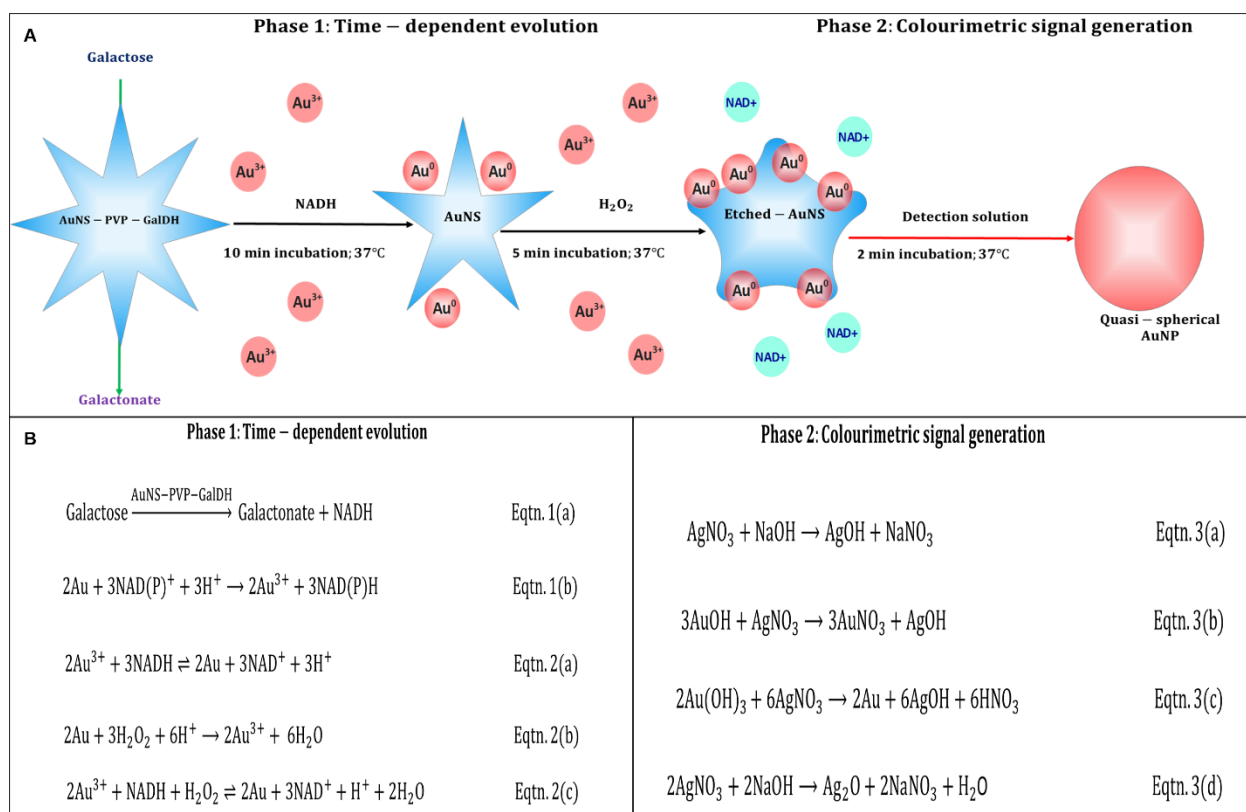


As demonstrated in several of our findings, H₂O₂ is required for successful AuNPs dissolution, and the AuNPs dissolution rate is sensitive to H₂O₂ concentration, which is consistent with prior studies (Eksteen & Oraby, 2015; Nowicka *et al.*, 2010).

NAD(P)H has the potential to cause AuNPs dissolution, as evidenced by some of our findings, and the AuNPs dissolution rate is sensitive to NAD(P)H concentration, which is consistent with earlier studies (Liang *et al.*, 2015). In almost all aerobic systems, the addition of one electron to dioxygen (O₂) nonenzymatically produces the superoxide anion (O₂)⁻, which can radicalize (Kirsch & De Groot, 2001). NAD(P)H can react with oxygen-centered radicals to create NAD(P)^{*} radicals that are stable in alkaline pH (Eqtn.6), and they may be the ones triggering AuNS remodelling and mild dissolution (Farber, 1994; Kirsch & De Groot, 2001; Umrikhina *et al.*, 1990).



Scheme 6-2 displays our thought process prior to spectrophotometrically tracking the reaction and measuring the impact of detection solution on AuNS shape and colorimetrics. Using standard redox chemistry, there must be obvious spectral and colourimetric changes even in the absence of the detection solution in Scheme 6-2; nevertheless, no changes are observed unless the detection solution is present.



Scheme 6-2: Proposed colorimetric signal generation and redox chemistry scheme.

The equations in Scheme 6-2.B above are confirmed by our ICPS-MS data, which shows no overall change in Au or Ag but an increase in Na, which may imply the likelihood of Ag coating growth, as suggested by several researchers, but we did not confirm it (Moses Phiri *et al.*, 2019; Rodríguez-Lorenzo *et al.*, 2012). Furthermore, Ag is a lustrous metal, and it is possible to produce Ag/Ag₂O nanoparticles of various shapes and sizes, plating or alloying with the Au in forming this colourimetric signal (Huang & Xu, 2010). This is likely to have an impact on the plasmonic band and colourimetric signals that we receive in alkaline settings (Guo *et al.*, 2016; Li *et al.*, 2011; Vilela *et al.*, 2012). This could have an impact on the plasmonic band and colourimetric signals that we acquire in alkaline settings. AuNPs, on the other hand, can form hydroxides on their surfaces, which can then react with the detection solution with a reduced likelihood of equations 3(b-c) (Mironov, 2005). In conclusion, we did not fully grasp how nanoparticles preserve their morphological integrity in the presence of oxidising and reducing agents.

Overall, these findings demonstrate that the signal registered by the AuNS biosensor (transducer) together with the physico-chemical properties of the nanosensor, pH, temperature, detection solution concentration, H₂O₂ concentration, presence of capping agents, and presence of contaminants may all impact the generation of the AuNS biosensor colourimetric signals. As a result, the findings suggest that, with appropriate assay tailoring, the AuNS-PVP-GalDH

biosensor can be employed as both a qualitative (Fig.6-2. A) and quantitative (Fig.6-2.B) biosensor.

However, the AuNS-PVP-GaLDH biosensor relied on the blue-shifted absorption band of nanostar (690 nm) to quasi-spherical nanoparticles (540 nm) during NADH and H₂O₂ biocatalytic reactions (Fig.6-2). The bioassay had a potential downside when using a synthetic blood matrix because most synthetic blood components have significant absorption in the same range as the AuNPs (Bansal *et al.*, 2020; Li, Lin, *et al.*, 2014; Shao *et al.*, 2016). In addition to AuNS having a higher extinction coefficient when compared to components, matrix dilution and blank subtraction approaches were also used to improve result interpretation and analysis (De Puig *et al.*, 2015; Zhao *et al.*, 2017).

Weng *et al.* (2019), on the other hand, described a NADH-mediated suppression of H₂O₂-induced gold nanorods etching. The bromine intermediates (Br₃⁻ and Br₂) from cetyltrimethylammonium bromide (CTAB) and 5-bromosalicylic acid (5-BrSA) used to etch gold nanorods were reduced and consumed in the presence of NADH, inhibiting the etching rate and resulting in a slight blue shift of the LPB. Maugeri *et al.* (2023) recently developed a unique photothermal-contrast method for detecting phenylalanine (Phe) in human blood using phenylalanine dehydrogenase (PDH), resulting in the formation of in-situ AuNPs in the presence of neo-formed NADH and AuCl₄⁻. Jafari *et al.* (2021) proposed a colourimetric paper-based biosensor based on the PDH enzyme for highly sensitive and selective quantification of Phe, using neo-formed NADH, cationic dyes and AuNPs as colourimetric mediators.

Our findings indicate that the NADH-H₂O₂-driven competitive assay biocatalytically drives AuNP growth, resulting in concentration-dependent morphological, spectrophotometric and colourimetric changes. H₂O₂-mediated surface etching can adjust the surface plasmon resonance of AuNS by swiftly and sensitively etching the high-energy facets of the AuNS protrusions (Hao *et al.*, 2007b; Pazos-Perez *et al.*, 2018; Rahman *et al.*, 2015; Tsoulos *et al.*, 2017). The NADH-catalysed oxidation of Au³⁺ ions allows for in-situ AuNP development is consistent with the findings of Maugeri *et al.* (2023). The etched AuNS serve as seeds for Au⁰ deposition, eventually leading to the biocatalytic development of larger diameter quasi-spherical AuNPs (Zayats *et al.*, 2005). Furthermore, AuNS served as signal amplifiers and immobilisation scaffold in our biosensor design (Bezuneh *et al.*, 2022; Cao *et al.*, 2011; Ellis *et al.*, 2020), eliminating the necessity for cationic dyes, as suggested by Jafari *et al.* (2021).

6.6 Conclusions

In this study, AuNSs were employed to develop a multicolourimetric NADH biosensor based on simple and sensitive surface etching and the growth of AuNPs. In the presence of NADH, H_2O_2 , and the detection solution, the AuNS-PVP-GalDH biosensor was etched into quasi-spherical nanostructures with increased core diameters. The etching and growth processes resulted in NADH-concentration-dependent colourimetric signals that were visible to the naked eye. Finally, the AuNS-PVP-GalDH assay offers proof of concept for developing a novel galactose plasmonic colourimetric detection method with remarkable sensitivity in simple and complex matrices. In addition, the NADH- H_2O_2 -driven gold nanostar etching system provides a promising practical analytical target detection technique in resource-limited settings.

6.7 Author Contributions

T.A.M. designed the study, carried out the laboratory work, participated in data analysis, and drafted the manuscript. B.C.V., E.H.C., and D.W.M. conceived the study, participated in the design of the study, data analysis, and ensuring the integrity of the manuscript. F.J.S. participated in the redox chemistry interpretation, colorimetric theory development, and overall article review. All authors gave their approval for publication.

6.8 Acknowledgments

We would like to acknowledge Dr. Innocent Shuro from the Laboratory for Electron Microscopy, North-West University, Potchefstroom, South Africa, for assistance with obtaining the nanoparticle images. We would also like to thank the North West University's Centre of Human Metabolomics (CHM) and the South African Technology Innovation Agency (TIA) for the funding given in order to carry out this work.

**CHAPTER 7 THEORETICAL ADIABATIC OXIDATION
MATHEMATICAL MODELLING OF GOLD NANOSTARS.**

PURE MATHEMATICS IS, IN ITS WAY, THE POETRY OF LOGICAL IDEAS.

– Albert Einstein

7.1 Preface

This chapter accomplished Objective 3 of Chapter 3, which required investigating the colourimetric redox chemistry of gold nanostar biosensors theoretically and mathematically. Gold nanostars were produced, uncapped and displayed adiabatic self-oxidation, producing colourimetric changes visible to the naked eye and spectrophotometrically. In addition, this phenomenon prompted the development of a theoretical thermodynamic and mathematical model. This chapter will be submitted to a journal of theoretical computational chemistry.

7.2 Abstract

A theoretical thermodynamic model for uncapped gold nanostar adiabatic atmospheric oxidation was derived from the first principles. The core-shell model was considered because by-products produced from gold nanostar oxidation nano would encapsulate the spherical gold nanoparticles' surface core. Our results exhibit spontaneous oxidation of particles resulting in visible colourimetric changes and longitudinal peak disappearance on gold nanostars using UV-Vis spectroscopy and TEM imaging. Thermodynamically, this surface phenomenon is spontaneous and feasible as denoted by the reaction $4\text{Au}_{(s)} + \text{O}_{2(g)} \rightarrow 2\text{Au}_2\text{O}_{(s)}$ at 4 °C and ambient temperature.

Keywords: Gold nanostars, Adiabatic, Spontaneous, Oxidation, Encapsulate

7.3 Introduction

Plasmonic gold nanoparticles, particularly gold nanostars (AuNS), are unique nanomaterials that exhibit exceptional high extinction coefficients and proficient surface plasmon resonances (SPR) (Eustis & El-Sayed, 2006). AuNS have hybridised localised surface plasmon resonance (LSPR) modes emanating from the solid core and numerous branches leading to unique optical signals (Liu *et al.*, 2014; Tsoulos *et al.*, 2017). AuNS optical properties can be tailored through size, surface chemistry, aggregation state and shape modulation (Deveci, 2021; Liu *et al.*, 2014; Tsoulos *et al.*, 2017). Convenient, reliable and simple LSPR-based AuNS readings can be used in the fabrication of nanosensors by using systematic plasmonic signal adjustment strategies such as aggregation/dispersion, surface etching and isotropic/anisotropic morphological grow (Tang & Li, 2017; Xianyu *et al.*, 2021b; Zhou *et al.*, 2015).

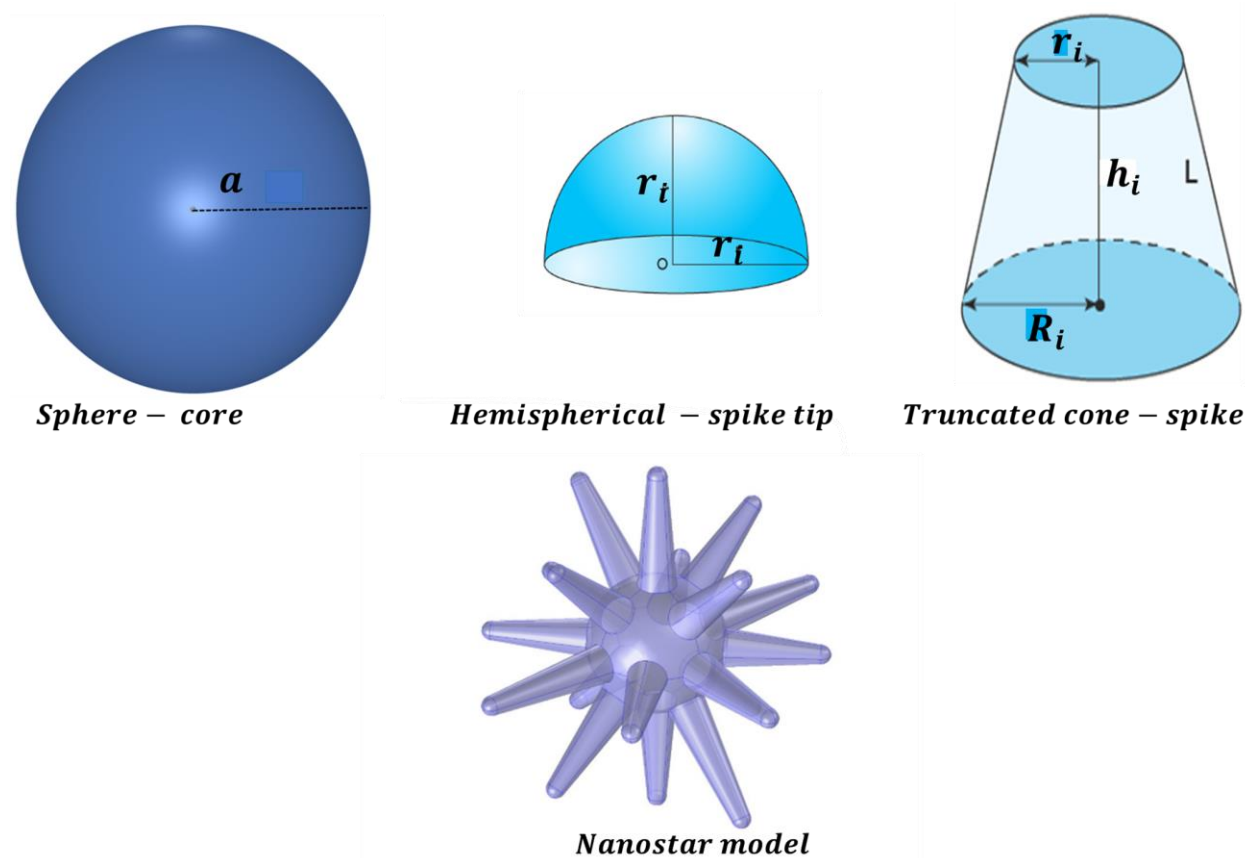
Gold nanostars have the propensity to undergo atmospheric oxidation in alkaline solutions with hypoventilation (Ignaczak *et al.*, 2019; Leitner *et al.*, 2019; Leitner *et al.*, 2020). Our previous stability studies have also shown that AuNS can undergo atmospheric oxidation with subsequent

UV-Vis spectral shifts showing the disappearance of the AuNS arms on the UV-Vis spectra (Munyayi *et al.*, 2022). Using the UV-Vis, EDS, ICP-MS and TEM techniques, it has been hypothesised that the oxidation of AuNS occurs through the disappearance of AuNS arms and concomitant core growth in the presence of surface bound O_2 .

Herein, we present the generic thermodynamic core and shell stochastic oxidation model based on the thermodynamics of $Au_2O_{(s)}$ growth on a plasmonic AuNP core (Fig.7-1) (Cabrera & Mott, 1949; Ramírez *et al.*, 2022). The adiabatic atmospheric oxidation of AuNS has been hypothesised to occur resulting in the formation of Au_2O film which is more metallic than Au_2O_3 (Shi *et al.*, 2007). The adiabatic oxidation of AuNS follows the reaction $4Au_{(s)} + O_{2(g)} \rightarrow 2Au_2O_{(s)}$ and the detailed conditions for AuNS oxidation are cited in our previous publication (Munyayi *et al.*, 2022).

7.3.1 Determination of gold nanostar volume and surface area

As shown in Scheme 7.1, the volumes of AuNSs were computed by modelling the cores as spheres and their spikes as truncated cones with hemispherical tips; subtraction was done to the spherical cap at the intersection of each spike with the core.



Scheme 7-1: Theoretical gold nanostar modeling.

As a result, the volume of each individual AuNS is an integration of three shapes, the volumes of which are as follows:

$$V_{\text{Sphere}} = \frac{4}{3}\pi a^3 \quad [a]; \quad V_{\text{hem.sphere}} = \sum_{t=1}^n \left\{ \frac{2}{3}\pi r_i^3 \right\} \quad [b]$$

Given that the number of spikes on gold nanostars varies, a total of the hemispherical tipped truncated cones volume is given by equation (c):

$$V_{\text{T.cones}} = \sum_{t=1}^n \left\{ \frac{h_i}{3}\pi (R_i^2 + R_i^2 r_i + r_i^2) \right\} [c]$$

Equation (d) gives the spherical cap volume where each spike intersects the core.

$$V_{\text{Int.core-spike}} = \sum_{i=1}^n \left\{ \frac{\pi}{6} \left(a - \sqrt{a^2 - R_i^2} \right) \left[3R_i^2 + \left(a + \sqrt{a^2 - R_i^2} \right)^2 \right] \right\} \quad [d]$$

Overall, the volume of a single AuNS as described by Scheme 7-1 and equations (a-d) yields the primary equation (1).

$$V = \frac{4}{3}\pi a^3 + \sum_{i=1}^n \left\{ \frac{h_i}{3} \pi (R_i^2 + R_i^2 r_i + r_i^2) + \frac{2}{3} \pi r_i^3 - \frac{\pi}{6} \left(a - \sqrt{a^2 - R_i^2} \right) \left[3R_i^2 + \left(a + \sqrt{a^2 - R_i^2} \right)^2 \right] \right\} \quad [1]$$

where a is the radius of the spherical core, R_i is the base radius of a spike, r is the radius of a spike's hemispherical tip, n is the probable number of spikes and h is the distance between R and r . Finally, the surface area of a single AuNS is determined using equation (2), while maintaining the dimensional units as specified above constant.

$$A = 4\pi a^2 \sum_{i=1}^n \left\{ 2\pi a \left(a - \sqrt{a^2 - R_i^2} \right) \right\} + \sum_{i=1}^n \left\{ \pi (r_i + R_i) \sqrt{(R_i - r_i)^2 + h_i^2} + 2\pi r_i^2 \right\} \quad [2]$$

7.3.2 Theoretical adiabatic oxidation of gold nanostars

In thermodynamics, an adiabatic process occurs when there is no heat or mass transfer between the system and its surroundings, thus spontaneous (Bagotsky, 2005c; Boles & Yunus A. Cengel, 2014; Leitner *et al.*, 2020). In the presence of etchants, chemical compounds undergo oxidation, which converts them from an unstable electron configuration to a stable electron configuration (Bagotsky, 2005b; Chernavskii *et al.*, 2007). The protuberant spikes of AuNS are known to be at a higher energy state than the core because the AuNS spikes absorb energy from the excited plasmons and the core working as an electron antenna (Hao *et al.*, 2007b). Thus, because spikes have larger surface energy, the AuNS (oxidation) reshaping begins there (Nehl *et al.*, 2006a; Rahman *et al.*, 2015). As a result, AuNS's anisotropic morphology is susceptible to adiabatic oxidation and the core-shell stochastic adiabatic oxidation reactions are thought to proceed as depicted in Figure.7-1.

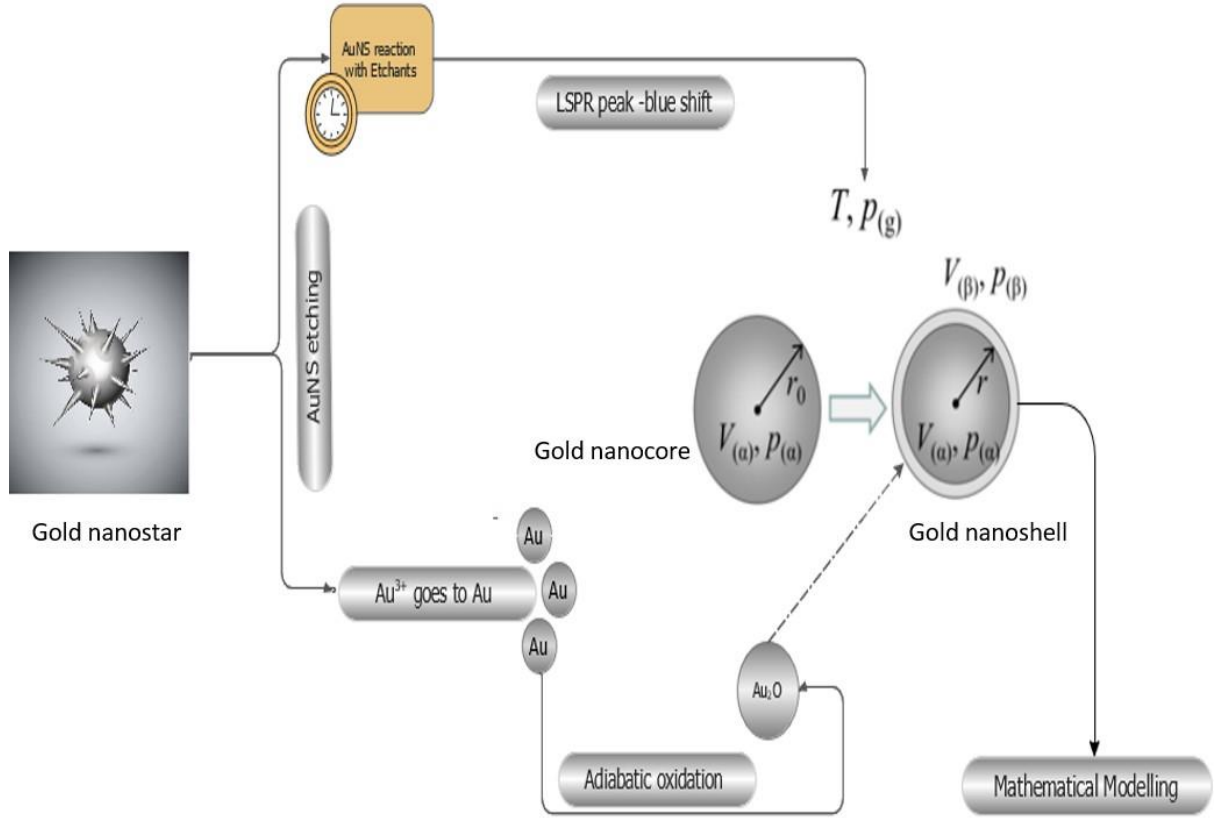
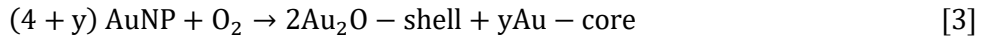


Figure 7-1: Interfacial configuration for the core and shell system Au/Au₂O where $\alpha = \text{Au}_{(s)}$; $\beta = \text{Au}_2\text{O}_{(s)}$.

Utilizing dissolved molecular oxygen (O_2) and HEPES as the primary adiabatic oxidation drivers, O_2 will adiabatically oxidize the AuNS spikes, creating Au^{3+} ions that may be reduced by HEPES to $\text{Au}^0/\text{Au}_2\text{O}$ and deposited on the AuNPs cores. Due to the different interfaces of AuNP and the unoxidized Au core, the adiabatic oxidation reaction is given by equation (3).



The y parameter denotes the amount of unoxidized Au when 2 moles of Au_2O are formed as the nanoparticle shell:

$$y = \frac{4n_{(\text{Au-core})}}{n_{(\text{AuNP})} - n_{(\text{Au-core})}} = \frac{4}{\left(\frac{r_0}{r}\right)^3 - 1} \quad [4]$$

Where r_0 is the initial AuNP radius and r is the AuNP radius at any given time after the adiabatic oxidation process.

Assuming that the bulk properties of AuNP and the Au core are the same, and the equilibrium condition for the oxidation reaction (3) can be expressed as follows:

$$\Delta_r G^{0, \text{AuNP}} = \Delta_r G^{0, \text{bulk}} + \Delta_r G^{\text{surf}} = RT \ln p_{\text{O}_2}^{\text{bulk}} + \Delta_r G^{\text{surf}} = RT \ln p_{\text{O}_2}^{\text{AuNP}} \quad [5]$$

$$\Delta_r G^{\text{surf}} = RT \ln \frac{p_{\text{O}_2}^{\text{AuNP}}}{p_{\text{O}_2}^{\text{bulk}}} \quad [6]$$

where $\Delta_r G$ is the change in Gibbs energy due to the reaction, R is the ideal gas constant (8.3145 J/K·mol), p_{O_2} is the oxygen partial pressure, and T is the absolute reaction temperature. Because the method only works for absolute temperatures, you must first convert the reaction temperature in Celsius ($^{\circ}\text{C}$) to Kelvin (K) or alter equation 6 as follows:

$$\Delta_r G^{\text{surf}} = R [T_{\text{C}} + 273.15] \text{K} \ln \frac{p_{\text{O}_2}^{\text{AuNP}}}{p_{\text{O}_2}^{\text{bulk}}} \quad [6]$$

Furthermore, the final surface morphology profile aspect ratio (γ), for core-shell model 2γ represents the spheres, and influences the surface contribution $\Delta_r G^{\text{surf}}$ conditions as shown in equation (7).

Assumption: At ($r_0 = r_{\text{AuNP}}$; $r = r_{\text{Au-core}}$; $\delta = \text{Au}_2\text{O shell thickness}$)

$$\Delta_r G^{\text{surf}} = y \frac{2\gamma_{(\frac{\text{Au}}{\text{Au}_2\text{O}})} V_{\text{m}(\text{Au})}}{r} + \frac{2\gamma_{(\text{Au}_2\text{O})}}{r + \delta} (y V_{\text{m}(\text{Au})} + 2 V_{\text{m}(\text{Au}_2\text{O})}) - (4 + y) \frac{2\gamma_{(\text{Au})} V_{\text{m}(\text{Au})}}{r_0} \quad [7]$$

Equating equation 6 to equation 7 yields a complimentary equilibrium equation as follows:

$$y \frac{2\gamma_{(\frac{\text{Au}}{\text{Au}_2\text{O}})} V_{\text{m}(\text{Au})}}{r} + \frac{2\gamma_{(\text{Au}_2\text{O})}}{r + \delta} (y V_{\text{m}(\text{Au})} + 2 V_{\text{m}(\text{Au}_2\text{O})}) - (4 + y) \frac{2\gamma_{(\text{Au})} V_{\text{m}(\text{Au})}}{r_0} - RT \ln \frac{p_{\text{O}_2}^{\text{AuNP}}}{p_{\text{O}_2}^{\text{bulk}}} = 0 \quad [8]$$

Using mass balance principles, the dependence of shell thickness (δ) to oxygen pressure at any given initial AuNP radius (r_0), V_{m} is the molar volume, n is the number of moles involved in the reaction and gives rise to the function $F(T, p_{\text{O}_2}^{\text{AuNP}}, r_0, \delta) = 0$.

$$2n_{\text{Au}_2\text{O}} = 2 \frac{(4\pi/3)[(r + \delta)^3 - r^3]}{V_{\text{m}(\text{Au}_2\text{O})}} = n_{\text{Au}}^0 - n_{\text{Au}} = \frac{(4\pi/3)[r_0^3 - r^3]}{V_{\text{m}(\text{Au})}} \quad [9]$$

The expression of Au_2O shell thickness is as follows:

$$\delta = \left[\frac{V_{\text{m}(\text{Au}_2\text{O})}}{2V_{\text{m}(\text{Au})}} r_0^3 + \left(1 - \frac{V_{\text{m}(\text{Au}_2\text{O})}}{2V_{\text{m}(\text{Au})}}\right) r^3 \right]^{1/3} - r \quad [10]$$

Taking into account the isobaric conditions (constant partial pressure p_{O_2}) at bulk equilibrium temperature under adiabatic conditions, equation 5 transforms into:

$$\Delta_r G^{\text{surf}} = R \ln p_{O_2} (T^{\text{AuNP}} - T^{\text{bulk}}) \quad [11]$$

Therefore, substituting equation 8 to equation 11 produces a new equilibrium for the dependence of the shell thickness (δ) on temperature at a given value of r_0 .

$$y \frac{2Y_{\left(\frac{\text{Au}}{\text{Au}_2\text{O}}\right)} V_{\text{m(Au)}}}{r} + \frac{2Y_{\text{(Au}_2\text{O)}}}{r+\delta} (yV_{\text{m(Au)}} + 2V_{\text{m(Au}_2\text{O)}}) - (4+y) \frac{2Y_{\text{(Au)}} V_{\text{m(Au)}}}{r_0} - R \ln p_{O_2} (T^{\text{AuNP}} - T^{\text{bulk}}) = 0 \quad [12]$$

Furthermore, pH and the presence of a reducing agent (HEPES) induce adiabatic AuNS oxidation, indicating that the reducing agent will constantly etch the neo-formed AuNS if they are not passivated (Blessy *et al.*, 2014b; Xi & Haes, 2019).

The AuNS elemental mapping is shown in Figure.7-2, and oxygen is found in samples using EDS and STEM elemental mapping. The stoichiometry of oxygen and HEPES has a considerable influence on the intrinsic properties of AuNS, such as reactivity and inherent stability, shape, and the surface structure of the nanoparticle, as shown in Figure.7-2B.

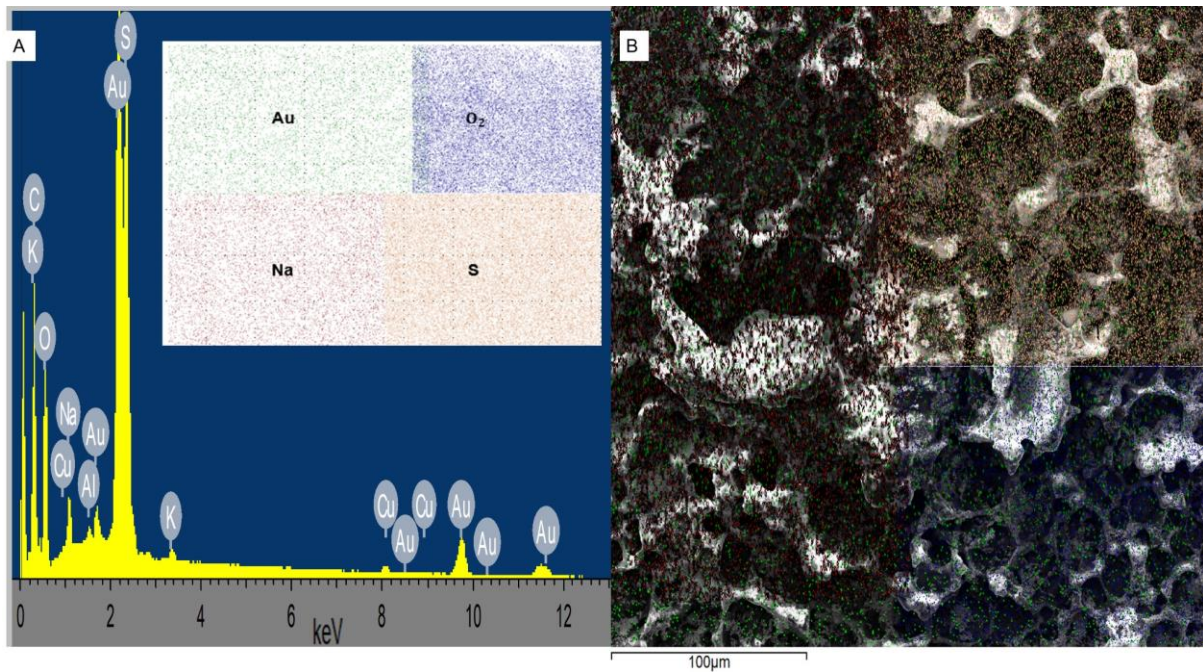


Figure 7-2: EDS spectra and STEM elemental images showing the elemental composition of gold nanostars.

Overall, the key components of HEPES-mediated AuNS are gold and HEPES. Furthermore, the results reveal that dissolved molecular oxygen (O_2) is present throughout the spectrum range,

increasing the likelihood of AuNS oxidation to Au_2O_3 , a conclusion consistent with Shi et al. (Shi et al., 2007). Nonetheless, silver ions (Ag^+) operate as a shape-directing and underpotential deposition (UPD) agent in the synthesis of anisotropic plasmonic gold nanostars (Andreiuk et al., 2022; Kereselidze et al., 2012a; Personick et al., 2011), despite the fact that Ag^0 is absent from EDS elemental findings. The absence of Ag^0 in the spectrum could be attributed to the low concentration used during anisotropic gold nanostar formation (Munyayi et al., 2022), or it could be that Ag^0 causes anisotropic growth in gold nanostars and is not incorporated in the anisotropic gold nanostar lattice (Kereselidze et al., 2012a; Yuan et al., 2007), so it is discarded in supernatant during the washing step of the newly formed anisotropic gold nanostar colloidal solution (Atta et al., 2019; Ye et al., 2022).

Figure. 7-3 depicts the transformations that occur in AuNS under different temperature conditions and the resulting colourimetric and spectral changes during adiabatic oxidation.

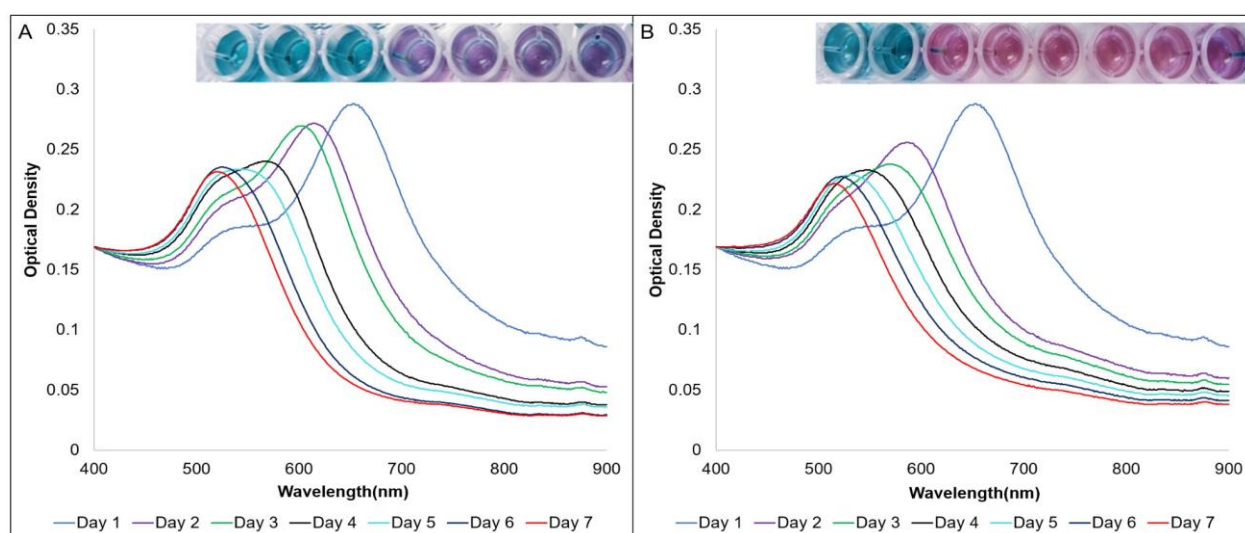


Figure 7-3: UV-vis spectra for gold nanostar oxidation transitions at different temperature conditions (A) 4°C ;(B) Room temperature.

Changes in the physicochemical parameters of AuNS (chemical composition, shape, and size) and changes due to AuNS's limited colloidal stability might produce variations in the extinction spectra.

The substantive result of Equation 8 is shown in Figure.7-4 day 4, showing both the unoxidised AuNS and the oxidised AuNP cores.

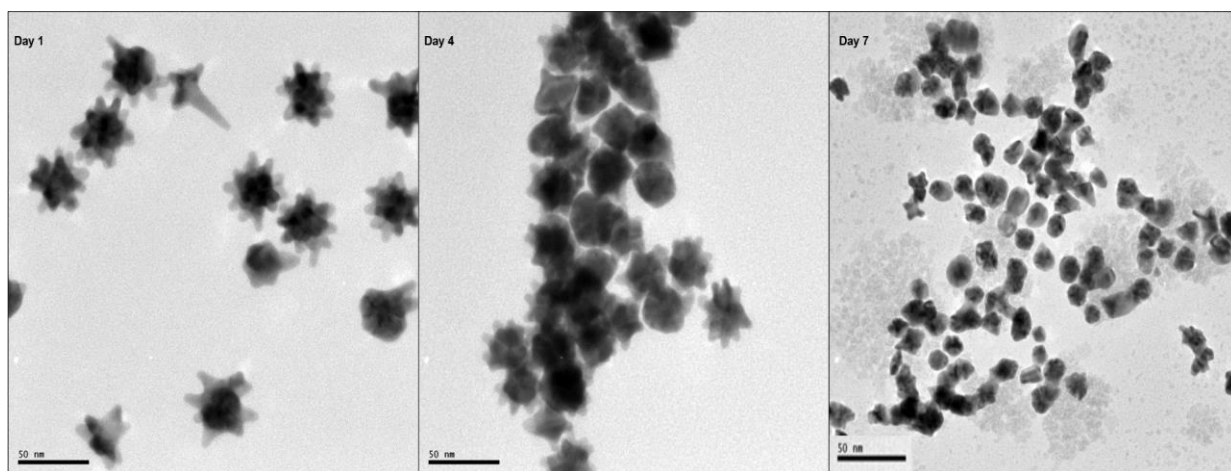


Figure 7-4: TEM images showing the oxidation of gold nanostar transformations.

Overall, AuNS will evolve into quasi-spherical AuNPs, which supports our discussions of the mathematical model and confirms the need to use stable capping agents during AuNS synthesis.

7.4 Discussion

Nanoparticle mathematical modeling has piqued an interest in understanding morphological changes under a variety of situations (McLean & Zhan, 2022). In this study, a nanoparticle mathematical model was used to investigate the performance of gold nanostars during adiabatic oxidation (Iglesias *et al.*, 2021). Theoretical gold nanostar mathematical modeling may play a crucial role in understanding pharmacokinetics, molecular dynamics, and interactions important in exploring drug delivery systems for future cancer treatment (Zong *et al.*, 2021). Thus, we developed a theoretical mathematical model to better understand the morphological evolution of gold nanostars by combining experimental and mathematical data.

The Cabrera-Mott model is pivotal in describing growth kinetics and morphological change induced by nanoparticle oxidation (Ermoline & Dreizin, 2011). Recently, Iglesias *et al.* proposed a thermodynamic theoretical approach to magnetic hyperthermia employing magnetic nanoparticles in non-adiabatic and radiating processes (Iglesias *et al.*, 2021). Their research is similar to ours in that it examines the hybrid regions of nanoscience, thermodynamics, and computational chemistry. Our theoretical thermodynamic model has some limitations as experimental results in literature make the metallic oxide Au_2O more thermodynamically stable in bulk systems at low oxygen pressures than Au_2O_3 (Shi *et al.*, 2007).

Overall, our theoretical hypothesis predicts that surface passivation of AuNS by stable capping agents, ligands and moieties may reduce the propensity for adiabatic oxidation. Furthermore, the

model suggests that the stoichiometry of oxygen and HEPES in the sample lattice may affect the kinetics of adiabatic oxidation.

7.5 Conclusion

This work demonstrated a core-shell adiabatic oxidation thermodynamic model analysis of gold nanostars. Interfacial surface energies between Au and Au/Au₂O greatly influence the thermodynamic kinetics of oxidation. Our findings indicate that oxygen and HEPES can trigger adiabatic oxidation of AuNS, resulting in a blue shift in the UV-Vis spectra and the concomitant production of colourimetric signals visible to the naked eye. Morphologically, the AuNS arms were etched as shown in the UV-Vis spectral profiles and TEM images. Finally, passivating AuNS with ligands can increase stability and reduce etchant adiabatic oxidation post-synthesis.

CHAPTER 8 CONCLUSIONS AND FUTURE PROSPECTS

The important thing is not to stop questioning.

– Albert Einstein

8.1 Concluding summary

This section emphasises and summarises the primary conclusions because each chapter has its own conclusion.

The suitability of NAD(P)/NAD(P)H as redox agents for gold nanostar shape transformation in colourimetric clinical enzymology is described in this thesis. The aim of this research was to devise highly sensitive colourimetric platforms, evaluate the possibilities of employing NAD(P)/NAD(P)H to detect different analytes and validate colourimetric reactions using redox chemistry where possible.

Nicotinamide coenzymes generally work via enzymatic reaction systems to ferry electrons between protein redox centres and the reaction media. NAD(P)/NAD(P)H are typically utilised as redox agents in analytical assays of various systems. They are frequently employed to induce colourimetric, fluorescence and spectrophotometric changes in various scaffolding mediums, including nanoparticles, polymer composites and hydrogels.

The development and commercialisation of diverse analytical assays driven by NAD(P)/NAD(P)H redox coenzymes has been prompted by advancements in nanotechnology, notably the utilisation of gold nanoparticles of various shapes. Because of strong relativistic effects, nano gold has lower coordination numbers and more atoms at the surface and surface atoms are always more reactive, whereas gold atoms in bulk have higher coordination numbers and are chemically inert due to strong suppression by neighbouring gold atoms (Haruta, 2003; Sun *et al.*, 2015). Gold nanoparticles, particularly gold nanostars, have more surface-free energy than bulk gold due to their nanoscale size, giving them larger surface-to-volume ratios and chemical potentials (Chirea, 2013; Chirico *et al.*, 2015; Zhou *et al.*, 2010; Zhou *et al.*, 2011). In terms of chemical reactivity, selectivity and sensitivity to redox agents, anisotropic gold nanostars outperform isotropic gold nanoparticles (Ortiz-Castillo *et al.*, 2020; Siegel & Baker, 2021). Furthermore, gold nanoparticles' stability is dependent on their shape, with anisotropy (the existence of sharp spikes in gold nanostars) promoting instability (Carone *et al.*, 2023).

Surface functionalisation can tailor the stability, shelf-life and functioning of gold nanostars in analytical and diagnostics applications, allowing for the adsorption of capping agents as well as the immobilisation of proteins and other small molecules. Based on the ultimate use of our anisotropic gold nanostars in biosensing applications, as stated in Chapter 4, a range of common capping agents were discovered to ineffectively induce stability to our HEPES-mediated anisotropic gold nanostars. Uncapped anisotropic gold nanostars appear to self-degrade or oxidise, which may be triggered by the HEPES contained in the gold nanostar lattice post-

synthesis, resulting in noticeable colourimetric and morphological changes. The PVP-capped anisotropic gold nanostars retained their colourimetric and morphological stability across a variety of biosensor matrices and storage settings, outperforming the bulk of the studied capping agents in terms of shelf life. Nonetheless, some studies used different gold nanostar synthesis techniques and capping agents such as CTAB, PEG and cysteine, to mention a few. Furthermore, it appears that developing a universal capping agent for anisotropic gold nanostars is impossible because capping agent selection is dependent on the ultimate application and synthetic process of choice. Overall, the anisotropic gold nanostar production method influences the capping agents that can be utilised as well as the shelf life and final application of the anisotropic gold nanostars.

Gold nanoparticles have been found to be sensitive to oxidising and reducing substances such as halides, hydrogen peroxide and NAD(P)/NAD(P)H, leading to the development of plasmonic-based biosensors based on AuNP etching or growth (Zhang *et al.*, 2018). Halides are by far the most thoroughly researched class of monovalent oxidising anion agents. Halides are generally considered to be the most thoroughly researched class of monovalent oxidising anion agents of gold nanoparticles (Langille *et al.*, 2012). The halides' specific adsorption to gold nanoparticles declines in the order $I^- > Br^- > Cl^- > F^-$, illustrating the decrease in solvation energy and dissolution strength of these halides to gold nanoparticles, with I^- being firmly adsorbed and promoting rapid dissolution of gold nanoparticles (Langille *et al.*, 2012; Magnussen, 2002). This could explain why CTAB-induced quick disintegration (oxidation) of our anisotropic gold nanostars (turquoise blue) and transformation into quasi-spherical gold nanoparticles (wine-red) failed to fulfil our capping agent's selection criterion in Chapter 4. Furthermore, in a salt solution (NaCl) strain test, the stability of uncapped and capped gold nanostars was reduced considerably. Xianyu *et al.* (2021a) also devised an iodide-mediated surface etching colourimetric sensing platform by tailoring the surface plasmon resonance of seed-mediated gold nanostars. Thus, morphological and naked-eye colourimetric signals were obtained through quick and sensitive iodide-mediated etching of the gold nanostar protuberant spikes (Xianyu *et al.*, 2021a).

Using GOx-guided crystalline growth, Rodríguez-Lorenzo *et al.* (2012) developed a colourimetric plasmonic seed-mediated gold nanostar biosensor with inverse sensitivity. Neo-formed hydrogen peroxide from GOx reduces silver ions to generate a silver coating surrounding plasmonic gold nanostars. Depending on the GOx concentrations, conformal silver coating growth or nucleation causes varying blue shifts in the LSPR of the gold nanostar, concomitantly providing colourimetric signals (Rodríguez-Lorenzo *et al.*, 2012). Our findings are congruent with those of Rodríguez-Lorenzo *et al.* (2012) when using our HEPES-mediated seedless PVP-capped anisotropic gold nanostar biosensors. In Chapters 4 and 5, we used hydrogen peroxide-producing

oxidoreductases (GOx and GalOx) to produce concentration-dependent colourimetric and morphological changes in plasmonic anisotropic gold nanostar biosensors.

Huang *et al.* (2005) used fluorimetry and spectrophotometry to study the catalytic effect of gold nanoparticles on the surface oxidation of NADH to NAD(+). The fluorescence of NADH was quenched exponentially in the presence of gold nanoparticles, while the absorption intensity of NADH at 340 nm dropped and that of NAD(+) at 260 nm increased as gold nanoparticle concentration grew. As a result, our findings in Chapter 5 are convergent and compatible with Huang *et al.* (2005)'s proposed theory that the conversion of NAD(P)H to NAD(P)(+) occurs on the surface of the gold nanoparticles, implying that NAD(P)H is surface catalysed by the gold nanoparticles. We colourimetrically and spectrophotometrically investigated the effect of synthetic and neo-formed NAD(P)/NAD(P)H on our plasmonic gold nanostars and biosensors in our research. Furthermore, in the presence of neo-formed NAD(P)H, the plasmonic gold nanostars were rapidly transformed into quasi-spherical nanoparticles with a monocolourimetric wine-red colour, whereas in the presence of neo-formed NAD(P)+, the plasmonic gold nanostars were moderately etched in a concentration-dependent manner, yielding multicolourimetric signals. Overall, NAD(P)/NAD(P)H was discovered to produce credible colourimetric and morphological alterations in plasmonic gold nanostars biosensors.

Most plasmonic-based colourimetric assays require an alkaline environment (ammonia, sodium hydroxide and hydroxylamine hydrochloride) and a transition metal ion mostly silver ions to generate the colourimetric signals (Guo *et al.*, 2016; Zeng *et al.*, 2020; Zhang *et al.*, 2018). Other plasmonic colourimetric methods generate gold nanoparticles in enzymatic linked cycles by adding gold (III) solution. De la Rica and Stevens (2013) established a biocatalytic catalase enzymatic system coupled with the development of gold nanoparticles to produce aggregated (blue colloidal solution) or dispersed (red colloidal solution) solutions in the presence or absence of the analyte. Gold ions are reduced in hydrogen peroxide, resulting in well-defined morphology dispersed spherical nanoparticles. When the concentration of hydrogen peroxide falls due to the biocatalytic action of catalase, poorly defined morphology nanoparticle aggregates form and the colloidal solution turns blue. Overall, the hydrogen peroxide concentration influences the degree of aggregation of gold nanoparticles as well as the tonality of the resulting naked-eye colourimetric signals (De la Rica & Stevens, 2013).

Wu *et al.* (2016) changed the surface chemistry of gold nanoparticles to make them hydrogen peroxide responsive by introducing 1-hexanethiol (hydrophobic) and lipoic-phenylboronate (hydrophobic) into the hydrophilic polyethylene glycol capping agent. This means that when exposed to hydrogen peroxide, the gold nanoparticles' surface chemistry changes from

hydrophilic to hydrophobic as the polyethylene glycol detaches from the gold nanoparticles. The gradual dissociation of polyethylene glycol chains from gold nanoparticles diminishes water stability, eventually resulting in gold nanoparticle aggregation and a red-to-blue colourimetric shift in the colloidal solution. Overall, the rate of colourimetric change was concentration-dependent and different hydrogen peroxide concentrations could be identified with the naked eye (Wu *et al.*, 2016).

As a result, if this is the case with capped gold nanoparticles, colourimetric signals can be generated after surface chemistry alteration (detachment of the capping agent) in the presence of oxidising and reducing agents, causing either nanoparticle growth or aggregation (Wu *et al.*, 2016). So, how does one explain the alkaline environment's influence as well as the requirement for a transition metal ion, as indicated by multiple researchers for credible colourimetric signal generation (Guo *et al.*, 2015; Howes *et al.*, 2014; Mulder, Phiri, *et al.*, 2019a; Phiri *et al.*, 2019c; Rodríguez-Lorenzo *et al.*, 2012)?

Furthermore, our findings in Chapter 6 are consistent with Wu *et al.* (2016) in that capped plasmonic gold nanostars retain colourimetric and morphological integrity even in the presence of oxidising and reducing agents. Our findings in Chapter 6 are similarly consistent with Rodríguez-Lorenzo *et al.* (2012), Mulder, Phiri, *et al.* (2019a) and Phiri *et al.* (2019c) in that the generation of plausible colourimetric signals from capped gold nanostars requires silver ions and an alkaline environment.

However, little is known about what causes colourimetric signals in capped gold nanostars and why strong oxidising and reducing chemicals cannot induce rapid aggregation or growth in capped gold nanostars. To understand the basics of plasmonic gold nanoparticles from both a theoretical and experimental standpoint, multidisciplinary study is required. To better comprehend the colourimetric signal and reaction processes of plasmonic nanoparticles in real time, artificial intelligence and computational chemistry modelling technologies must be researched.

In conclusion, we demonstrated that NAD(P)/NAD(P)H are suitable as redox agents in colourimetric clinical enzymology for gold nanostar shape transformation. As a result, NAD(P)/NAD(P)H sensing expands the versatility of gold nanostar biosensor colourimetric assays. Our findings revealed that gold nanostar biosensors are robust in both simple (water) and difficult (serum, urine, blood) matrices, with good spectrophotometric and simple naked-eye colourimetric results interpretation. Conclusively, it was demonstrated that functionalised gold nanostar biosensors have a simple production technique, a short incubation period and rapid-simple result interpretation, making them ideal for clinical diagnosis, research and development applications in resource-limited areas.

8.2 Future prospects

This thesis illustrates the feasibility of NAD(P)/NAD(P)H as redox agents for altering the shape of gold nanostars in colourimetric clinical enzymology. The versatility demonstrated by gold nanostars in this study demonstrated the necessity for more research into the fundamental principles of gold nanostars. This research has merely scratched the surface of the vast potential that gold nanostars have to offer in clinical diagnostic techniques.

In future research, we hope to fully comprehend the redox chemistry involved in gold nanostar synthesis and colourimetric reaction signal generation. Our colourimetric detection platform is easily transferable into a multiplexed, paper-based device, allowing for rapid, low-cost and immediate screening of numerous analytes and clinical conditions. Another possibility for the future is to use these gold nanostar biosensors at a point-of-care application in conjunction with a smartphone-based colourimetric measurement readout. Furthermore, the theoretical model presented in Chapter 7 has the potential to be employed in nanostar computational modelling software development in the future to reduce the complexity of derivations and to promote simple real-time nanostar reaction modelling.

8.3 References

- Adamu usman, I., Abdul Aziz, A. & abu noqta, O. 2018. Application of green synthesis of gold nanoparticles: A review. *Jurnal Teknologi*, 81, 10.11113/jt.v81.11409
- Ahmed, M. & Narain, R. 2014a. Physico-Chemical and Biochemical Properties of Bioconjugates. In. *Chemistry of Bioconjugates*. pp. 431-443.
- Ahmed, M. & Narain, R. 2014b. Applications of Bioconjugates. In. *Chemistry of Bioconjugates*. pp. 444-456.
- Ahmed, S., Annu, Ikram, S. & Yudha S, S. 2016. Biosynthesis of gold nanoparticles: A green approach. *Journal of Photochemistry and Photobiology B: Biology*, 161:141-153. 10.1016/j.jphotobiol.2016.04.034
- Aisida, S.O., Ahmad, I., Zhao, T.-k., Maaza, M. & Ezema, F.I. 2020. Calcination Effect on the Photoluminescence, Optical, Structural, and Magnetic Properties of Polyvinyl Alcohol Doped ZnFe₂O₄ Nanoparticles. *Journal of Macromolecular Science, Part B*, 59(5):295-308. 10.1080/00222348.2020.1713519
- Ajitha, B., Kumar Reddy, Y.A., Reddy, P.S., Jeon, H.-J. & Ahn, C.W. 2016. Role of capping agents in controlling silver nanoparticles size, antibacterial activity and potential application as optical hydrogen peroxide sensor. *RSC Advances*, 6(42):36171-36179. 10.1039/C6RA03766F
- Alkilany, A.M. & Murphy, C.J. 2010. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? *Journal of Nanoparticle Research*, 12(7):2313-2333. 10.1007/s11051-010-9911-8
- Alkilany, A.M., Nagaria, P.K., Hexel, C.R., Shaw, T.J., Murphy, C.J. & Wyatt, M.D. 2009. Cellular uptake and cytotoxicity of gold nanorods: molecular origin of cytotoxicity and surface effects. *Small*, 5(6):701-708. 10.1002/smll.200801546
- Alvarez-Puebla, R., Liz-Marzán, L.M. & García de Abajo, F.J. 2010. Light Concentration at the Nanometer Scale. *The Journal of Physical Chemistry Letters*, 1(16):2428-2434. 10.1021/jz100820m
- Amann, M. & Minge, O. 2012. Biodegradability of Poly(vinyl acetate) and Related Polymers. *Advances in Polymer Science*, 245:137-172. 10.1007/12-2011-153
- Amina, S.J. & Guo, B. 2020. A Review on the Synthesis and Functionalization of Gold Nanoparticles as a Drug Delivery Vehicle. *Int J Nanomedicine*, 15:9823-9857. 10.2147/ijn.S279094
- Anderson, R.F. 1980. Energetics of the one-electron steps in the NAD⁺/NADH redox couple. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 590(2):277-281. 10.1016/0005-2728(80)90032-8

- Andreiuk, B., Nicolson, F., Clark, L.M., Panikkanvalappil, S.R., Kenry, Rashidian, M., ... Kircher, M.F. 2022. Design and synthesis of gold nanostars-based SERS nanotags for bioimaging applications. *Nanotheranostics*, 6(1):10-30. 10.7150/ntno.61244
- Araújo-Chaves, J.C. 2015. Self-assembled complexes of multibranched gold nanoparticles with porphyrins used in photodynamic therapy. In: Érica G. A. Miranda, A.T., Carlos E. de Castro, Fernando C. Giacomelli, Alejandro & Nantes, Z.a.I.L., eds. Conf. on Advances in Bio-Informatics and Environmental Engineering - ICABEE 2015, Rome, Italy. pp. 44-48.
- Arib, C., Spadavecchia, J. & de la Chapelle, M.L. 2021. Enzyme mediated synthesis of hybrid polyedric gold nanoparticles. *Sci Rep*, 11(1):3208. 10.1038/s41598-021-81751-1
- Atta, S., Tsoulos, T.V. & Fabris, L. 2016. Shaping Gold Nanostar Electric Fields for Surface-Enhanced Raman Spectroscopy Enhancement via Silica Coating and Selective Etching. *The Journal of Physical Chemistry C*, 120(37):20749-20758. 10.1021/acs.jpcc.6b01949
- Atta, S., Beetz, M. & Fabris, L. 2019. Understanding the role of AgNO₃ concentration and seed morphology in the achievement of tunable shape control in gold nanostars. *Nanoscale*, 11(6):2946-2958. 10.1039/C8NR07615D
- Aydoğdu Tiğ, G. 2017. Highly sensitive amperometric biosensor for determination of NADH and ethanol based on Au-Ag nanoparticles/poly(L-Cysteine)/reduced graphene oxide nanocomposite. *Talanta*, 175:382-389. 10.1016/j.talanta.2017.07.073
- Bagotsky, V.S. 2005a. Development of Electrochemistry. In. *Fundamentals of Electrochemistry*. 2. Hoboken, New Jersey: John Wiley & Sons. pp. 693-700.
- Bagotsky, V.S. 2005b. Nanoelectrochemistry. In: Leiva, E., ed. *Fundamentals of Electrochemistry*. 2. Hoboken, New Jersey: John Wiley & Sons. pp. 679-691.
- Bagotsky, V.S. 2005c. Thermodynamics of Electrochemical Systems. In. *Fundamentals of Electrochemistry*. 2. Hoboken, New Jersey: John Wiley & Sons. pp. 33-50.
- Baluta, S., Cabaj, J. & Malecha, K. 2017. *Novel oxidoreductase-based sensor for optical neurotransmitter detection*.
- Bansal, S.A., Kumar, V., Karimi, J., Singh, A.P. & Kumar, S. 2020. Role of gold nanoparticles in advanced biomedical applications. *Nanoscale Advances*, 2(9):3764-3787. 10.1039/D0NA00472C
- Barbosa, S., Agrawal, A., Rodríguez-Lorenzo, L., Pastoriza-Santos, I., Alvarez-Puebla, R.A., Kornowski, A., ... Liz-Marzán, L.M. 2010a. Tuning Size and Sensing Properties in Colloidal Gold Nanostars. *Langmuir*, 26(18):14943-14950. 10.1021/la102559e
- Barbosa, S., Agrawal, A., Rodríguez-Lorenzo, L., Pastoriza-Santos, I., Alvarez-Puebla, R.A., Kornowski, A., ... Liz-Marzán, L.M. 2010b. Tuning size and sensing properties in colloidal gold nanostars. *Langmuir*, 26(18):14943-14950. 10.1021/la102559e

Barman, P., Sharma, S. & Saini, A. 2022. Chapter 19 - Improving the functionality of a nanomaterial by biological probes. In: Mahajan, N.M., Saini, A., Raut, N.A. & Dhoble, S.J., eds. *Photophysics and Nanophysics in Therapeutics*: Elsevier. pp. 379-418.

Baron, A.J., Stevens, C., Wilmot, C., Seneviratne, K.D., Blakeley, V., Dooley, D.M., ... McPherson, M.J. 1994. Structure and mechanism of galactose oxidase. The free radical site. *Journal of Biological Chemistry*, 269(40):25095-25105.

Barski, O.A., Tipparaju, S.M. & Bhatnagar, A. 2008. The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab Rev*, 40(4):553-624. 10.1080/03602530802431439

Bastús, N.G., Comenge, J. & Puentes, V. 2011. Kinetically controlled seeded growth synthesis of citrate-stabilized gold nanoparticles of up to 200 nm: size focusing versus Ostwald ripening. *Langmuir*, 27(17):11098-11105. 10.1021/la201938u

Baygazieva, E., Yesmurzayeva, N., Tatykhanova, G., Mun, G., Khutoryanskiy, V. & Kudaibergenov, S. 2014. Polymer Protected Gold Nanoparticles: Synthesis, Characterization and Application in Catalysis. *International Journal of Biology and Chemistry*, 7, 10.26577/2218-7979-2014-7-1-14-23

Baymiller, M., Huang, F. & Rogelj, S. 2017. Rapid one-step synthesis of gold nanoparticles using the ubiquitous coenzyme NADH. *Matters*, 10.19185/matters.201705000007

Becerril-Castro, I.B., Calderon, I., Pazos-Perez, N., Guerrini, L., Schulz, F., Feliu, N., ... Alvarez-Puebla, R.A. 2022. Gold Nanostars: Synthesis, Optical and SERS Analytical Properties. *Analysis & Sensing*, 2(3):e202200005. 10.1002/anse.202200005

Belluzo, M.S., Ribone, M.E. & Lagier, C.M. 2008. Assembling Amperometric Biosensors for Clinical Diagnostics. *Sensors (Basel)*, 8(3):1366-1399. 10.3390/s8031366

Beutler, E. G6PD Deficiency By Ernest Beutler.

Beutler, E. & Baluda, M.C. 1966. A simple spot screening test for galactosemia. *Journal of Laboratory and Clinical Medicine*, 68(1):137-141. 10.5555/uri:pii:0022214366900655

Beutler, E., Baluda, M. & Donnell, G.N. 1964. A New Method For The Detection Of Galactosemia And Its Carrier State. *Journal of Laboratory and Clinical Medicine*, 64:694-705. 10.5555/uri:pii:002221436490037X

Beutler, E., DAY, R., BALUDA, M. & POLK, K. 1965. Screening for Galactosemia Among Mentally Retarded Patients*. *Journal of Intellectual Disability Research*, 9(1):61-68. 10.1111/j.1365-2788.1965.tb00821.x

Beutler, E., Irwin, H.R., Blumenfeld, C.M., Goldenburg, E.W. & Day, R.W. 1967. Field Test of Galactosemia Screening Methods in Newborn Infants. *JAMA*, 199(7):501-503. Date of access: 9/13/2023. 10.1001/jama.1967.03120070113025

- Bezuneh, T.T., Fereja, T.H., Kitte, S.A., Li, H. & Jin, Y. 2022. Gold nanoparticle-based signal amplified electrochemiluminescence for biosensing applications. *Talanta*, 248:123611. 10.1016/j.talanta.2022.123611
- Bigall, N.C., Härtling, T., Klose, M., Simon, P., Eng, L.M. & Eychmüller, A. 2008. Monodisperse platinum nanospheres with adjustable diameters from 10 to 100 nm: synthesis and distinct optical properties. *Nano Lett*, 8(12):4588-4592. 10.1021/nl802901t
- Blachnitzky, E.-O., WENGENMAYER, F. & KURZ, G. 1974. d-Galactose Dehydrogenase from *Pseudomonas fluorescens*. *European Journal of Biochemistry*, 47(2):235-250. 10.1111/j.1432-1033.1974.tb03687.x
- Blessy, M., Patel, R.D., Prajapati, P.N. & Agrawal, Y.K. 2014a. Development of forced degradation and stability indicating studies of drugs-A review. *Journal of pharmaceutical analysis*, 4(3):159-165. 10.1016/j.jpha.2013.09.003
- Blessy, M., Patel, R.D., Prajapati, P.N. & Agrawal, Y.K. 2014b. Development of forced degradation and stability indicating studies of drugs—A review. *Journal of Pharmaceutical Analysis*, 4(3):159-165. 10.1016/j.jpha.2013.09.003
- Boles, M.A. & Yunus A. Cengel, D. 2014. *Thermodynamics: An Engineering Approach*. 4. McGraw-Hill Education.
- Bollella, P. & Katz, E. 2020. Enzyme-Based Biosensors: Tackling Electron Transfer Issues. *Sensors (Basel)*, 20(12), 10.3390/s20123517
- Boniface, R., Moshabela, M., Zulliger, R., Macpherson, P. & Nyasulu, P. 2012. Correlates of Delayed Diagnosis among Human Immunodeficiency Virus-Infected Pulmonary Tuberculosis Suspects in a Rural HIV Clinic, South Africa. *Tuberc Res Treat*, 2012:827148. 10.1155/2012/827148
- Boonyuen, U., Praoparotai, A., Chamchoy, K., Swangsri, T., Warakulwit, C. & Suteewong, T. 2020. Controlled reversible assembly of gold nanoparticles as a new colorimetric and sensitive detection of glucose-6-phosphate dehydrogenase deficiency. *Analytica Chimica Acta*, 1122:61-69. 10.1016/j.aca.2020.05.016
- Borhani, D.W., Harter, T.M. & Petrash, J.M. 1992. The crystal structure of the aldose reductase.NADPH binary complex. *Journal of Biological Chemistry*, 267(34):24841-24847. 10.1016/S0021-9258(18)35840-X
- Brekasis, D. & Paget, M.S. 2003. A novel sensor of NADH/NAD⁺ redox poise in *Streptomyces coelicolor* A3(2). *The EMBO Journal*, 22(18):4856-4865. 10.1093/emboj/cdg453
- Broderick, J.B. 2001. Coenzymes and Cofactors. In. *Encyclopedia of Life Sciences*.
- Brumaghim, J.L., Li, Y., Henle, E. & Linn, S. 2003. Effects of hydrogen peroxide upon nicotinamide nucleotide metabolism in *Escherichia coli*: changes in enzyme levels and

nicotinamide nucleotide pools and studies of the oxidation of NAD(P)H by Fe(III). *Journal of Biological Chemistry*, 278(43):42495-42504. 10.1074/jbc.M306251200

Cabrera, N. & Mott, N.F. 1949. Theory of the oxidation of metals. *Reports on Progress in Physics*, 12(1):163. 10.1088/0034-4885/12/1/308

Campisi, S., Schiavoni, M., Chan-Thaw, C.E. & Villa, A. 2016a. Untangling the Role of the Capping Agent in Nanocatalysis: Recent Advances and Perspectives. *Catalysts*, 6(12):185. 10.3390/catal6120185

Campisi, S., Schiavoni, M., Chan-Thaw, C. & Villa, A. 2016b. Untangling the Role of the Capping Agent in Nanocatalysis: Recent Advances and Perspectives. *Catalysts*, 6:185. 10.3390/catal6120185

Campisi, S., Schiavoni, M., Chan-Thaw, C. & Villa, A. 2016c. Untangling the Role of the Capping Agent in Nanocatalysis: Recent Advances and Perspectives. *Catalysts*, 6(12), 10.3390/catal6120185

Cantó, C. & Auwerx, J. 2011. NAD⁺ as a signaling molecule modulating metabolism. *Cold Spring Harbor Symposia on Quantitative Biology*, 76:291-298. 10.1101/sqb.2012.76.010439

Cao, X., Ye, Y. & Liu, S. 2011. Gold nanoparticle-based signal amplification for biosensing. *Analytical Biochemistry*, 417(1):1-16. 10.1016/j.ab.2011.05.027

Carone, A., Emilsson, S., Mariani, P., Désert, A. & Parola, S. 2023. Gold nanoparticle shape dependence of colloidal stability domains. *Nanoscale Advances*, 5(7):2017-2026. 10.1039/d2na00809b

Chamchoy, K., Pakotiprapha, D., Pumirat, P., Leartsakulpanich, U. & Boonyuen, U. 2019. Application of WST-8 based colorimetric NAD(P)H detection for quantitative dehydrogenase assays. *BMC Biochemistry*, 20(1):4. 10.1186/s12858-019-0108-1

Chandra, D.P., Das, D. & Abdelwahab, A. 2010. Gold nanoparticles in molecular diagnostics and therapeutics. *Digest Journal of Nanomaterials and Biostructures*, 5:363 – 367.

Chandra, K., Culver, K.S.B., Werner, S.E., Lee, R.C. & Odom, T.W. 2016. Manipulating the Anisotropic Structure of Gold Nanostars using Good's Buffers. *Chemistry of Materials*, 28(18):6763-6769. 10.1021/acs.chemmater.6b03242

Chang, C.C., Chen, C.P., Wu, T.H., Yang, C.H., Lin, C.W. & Chen, C.Y. 2019. Gold Nanoparticle-Based Colorimetric Strategies for Chemical and Biological Sensing Applications. *Nanomaterials (Basel)*, 9(6), 10.3390/nano9060861

Chen, C. & Wang, J. 2020. Optical biosensors: an exhaustive and comprehensive review. *Analyst*, 145(5):1605-1628. 10.1039/C9AN01998G

Chen, H., Yu, J., Men, X., Zhang, J., Ding, Z., Jiang, Y., ... Chiu, D.T. 2021. Reversible Ratiometric NADH Sensing Using Semiconducting Polymer Dots. *Angew Chem Int Ed Engl*, 60(21):12007-12012. 10.1002/anie.202100774

- Chen, R., Wu, J., Li, H., Cheng, G., Lu, Z. & Che, C.-M. 2010. Fabrication of gold nanoparticles with different morphologies in HEPES buffer. *Rare Metals*, 29(2):180-186. 10.1007/s12598-010-0031-5
- Chen, S., Wang, Z.L., Ballato, J., Foulger, S.H. & Carroll, D.L. 2003. Monopod, Bipod, Tripod, and Tetrapod Gold Nanocrystals. *Journal of the American Chemical Society*, 125(52):16186-16187. 10.1021/ja038927x
- Chen, W.-D. & Zhang, Y. 2012. Regulation of Aldo–Keto Reductases in Human Diseases. *Frontiers in Pharmacology*, 3, 10.3389/fphar.2012.00035
- Chen, X., Zhou, G., Peng, X. & Yoon, J. 2012. Biosensors and chemosensors based on the optical responses of polydiacetylenes. *Chemical Society Reviews*, 41(13):4610-4630. 10.1039/C2CS35055F
- Chernavskii, P., Peskov, N., Mugtasimov, A. & Lunin, V. 2007. Oxidation of metal nanoparticles: Experiment and model. *Russian Journal of Physical Chemistry B*, 1:394-411. 10.1134/S1990793107040082
- Chirea, M. 2013. Electron Transfer at Gold Nanostar Assemblies: A Study of Shape Stability and Surface Density Influence. *Catalysts*, 3(1):288-309. 10.3390/catal3010288
- Chirico, G., Borzenkov, M. & Pallavicini, P. 2015. Gold Nanostars: Synthesis, Properties and Biomedical Application. *Gold Nanostars*, doi:10.1007/978-3-319-20768-1
- Cho, E.C., Au, L., Zhang, Q. & Xia, Y. 2010. The effects of size, shape, and surface functional group of gold nanostructures on their adsorption and internalization by cells. *Small*, 6(4):517-522. 10.1002/smll.200901622
- Chrestien, J.A. 1811. *De la méthode iatraleptique, ou observations pratiques sur l'efficacité des remèdes administrés par la voie, de l'absorption cutanée dans le traitement de plusieurs maladies internes et externes; et sur un nouveau remède [pour les] maladies vénériennes et lymphatiques (Chez Croullebois: Chez Crochard, Paris, 1811)*. 1. Paris, France: Croullebois.
- Cinteza, L.O., Scomoroscenco, C., Voicu, S.N., Nistor, C.L., Nitu, S.G., Trica, B., ... Petcu, C. 2018. Chitosan-Stabilized Ag Nanoparticles with Superior Biocompatibility and Their Synergistic Antibacterial Effect in Mixtures with Essential Oils. *Nanomaterials (Basel)*, 8(10), 10.3390/nano8100826
- Clogston, J.D. 2021. The importance of nanoparticle physicochemical characterization for immunology research: What we learned and what we still need to understand. *Advanced Drug Delivery Reviews*, 176:113897. 10.1016/j.addr.2021.113897
- Coble, C.M., Chen, J., Cho, E.C., Wang, L.V. & Xia, Y. 2011. Gold nanostructures: a class of multifunctional materials for biomedical applications. *Chemical Society Reviews*, 40(1):44-56. 10.1039/B821763G

- Comin, A. & Manna, L. 2014. New materials for tunable plasmonic colloidal nanocrystals. *Chemical Society Reviews*, 43(11):3957-3975. 10.1039/C3CS60265F
- Comotti, M., Della Pina, C., Matarrese, R. & Rossi, M. 2004. The Catalytic Activity of "Naked" Gold Particles. *Angewandte Chemie International Edition*, 43(43):5812-5815. 10.1002/anie.200460446
- Conde, J., Dias, J.T., Grazú, V., Moros, M., Baptista, P.V. & de la Fuente, J.M. 2014. Revisiting 30 years of biofunctionalization and surface chemistry of inorganic nanoparticles for nanomedicine. *Frontiers of Chemistry*, 2:48. 10.3389/fchem.2014.00048
- Cook, E., Labiento, G. & Chauhan, B.P.S. 2021. Fundamental Methods for the Phase Transfer of Nanoparticles. *Molecules*, 26(20):6170. 10.3390/molecules26206170
- Cordeiro, M., Ferreira Carlos, F., Pedrosa, P., Lopez, A. & Baptista, P.V. 2016. Gold Nanoparticles for Diagnostics: Advances towards Points of Care. *Diagnostics*, 6(4), 10.3390/diagnostics6040043
- Covarrubias, A.J., Perrone, R., Grozio, A. & Verdin, E. 2021. NAD(+) metabolism and its roles in cellular processes during ageing. *Nature Reviews Molecular Cell Biology*, 22(2):119-141. 10.1038/s41580-020-00313-x
- Cracolice, M.S. & Peters, E.I. 2015. *Introductory Chemistry: An Active Learning Approach*. Cengage Learning.
- Cristiano, M.N., Tsoulos, T.V. & Fabris, L. 2020. Quantifying and optimizing photocurrent via optical modeling of gold nanostar-, nanorod-, and dimer-decorated MoS₂ and MoTe₂. *The Journal of Chemical Physics*, 152(1):014705. Date of access: 2022/09/09. 10.1063/1.5127279
- Crooks, R.M., Zhao, M., Sun, L., Chechik, V. & Yeung, L.K. 2001. Dendrimer-encapsulated metal nanoparticles: synthesis, characterization, and applications to catalysis. *Acc Chem Res*, 34(3):181-190. 10.1021/ar000110a
- Cuatrecasas, P. & Segal, S. 1966. Mammalian Galactose Dehydrogenase: II. Properties, Substrate Specificity, And Developmental Changes. *Journal of Biological Chemistry*, 241(24):5910-5918. 10.1016/S0021-9258(18)96357-X
- D'Hollander, A., Mathieu, E., Jans, H., Vande Velde, G., Stakenborg, T., Van Dorpe, P., ... Lagae, L. 2016. Development of nanostars as a biocompatible tumor contrast agent: toward in vivo SERS imaging. *Int J Nanomedicine*, 11:3703-3714. 10.2147/ijn.S91340
- Daenzer, J.M. & Fridovich-Keil, J.L. 2017. Drosophila melanogaster Models of Galactosemia. *Current Topics in Developmental Biology*, 121:377-395. 10.1016/bs.ctdb.2016.07.009
- Damborský, P., Švitel, J. & Katrlík, J. 2016a. Optical biosensors. *Essays of Biochemistry*, 60(1):91-100. 10.1042/ebc20150010
- Damborský, P., Švitel, J. & Katrlík, J. 2016b. Optical biosensors. *Essays in biochemistry*, 60(1):91-100. 10.1042/EBC20150010

Daniel, M.C. & Astruc, D. 2004. Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. *Chemical Reviews*, 104(1):293-346. 10.1021/cr030698+

De Aberasturi, D.J., Serrano-Montes, A.B. & Liz-Marzán, L.M. 2015. Modern Applications of Plasmonic Nanoparticles: From Energy to Health. *Advanced Optical Materials*, 3(5):602-617. 10.1002/adom.201500053

De Flora, A., Morelli, A. & Giuliano, F. 1974. Human erythrocyte glucose 6-phosphate dehydrogenase. Content of bound coenzyme. *Biochemical and Biophysical Research Communications*, 59(1):406-413. 10.1016/s0006-291x(74)80221-4

De la Rica, R. & Stevens, M.M. 2012. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. *Nature Nanotechnology*, 7(12):821-824. 10.1038/nnano.2012.186

De la Rica, R. & Stevens, M.M. 2013. Plasmonic ELISA for the detection of analytes at ultralow concentrations with the naked eye. *Nature Protocols*, 8(9):1759-1764. 10.1038/nprot.2013.085

De Puig, H., Tam, J.O., Yen, C.-W., Gehrke, L. & Hamad-Schifferli, K. 2015. Extinction Coefficient of Gold Nanostars. *The Journal of Physical Chemistry C*, 119(30):17408-17415. 10.1021/acs.jpcc.5b03624

De Ruyck, J., Famerée, M., Wouters, J., Perpète, E.A., Preat, J. & Jacquemin, D. 2007. Towards the understanding of the absorption spectra of NAD(P)H/NAD(P)⁺ as a common indicator of dehydrogenase enzymatic activity. *Chemical Physics Letters*, 450(1):119-122. 10.1016/j.cplett.2007.10.092

De Silva Indrasekara, A.S., Johnson, S.F., Odion, R.A. & Vo-Dinh, T. 2018. Manipulation of the Geometry and Modulation of the Optical Response of Surfactant-Free Gold Nanostars: A Systematic Bottom-Up Synthesis. *ACS Omega*, 3(2):2202-2210. 10.1021/acsomega.7b01700

Decker, E., Bai, C., Nelless, L., Ferrari, E. & Soloviev, M. 2020. Protein Immobilization on Gold Nanoparticles: Quantitative Analysis. In: (Clifton, N.J., ed. *Methods in molecular biology* 2118. pp. 199-211.

Deng, H.-H., Lin, X.-L., Liu, Y.-H., Li, K.-L., Zhuang, Q.-Q., Peng, H.-P., ... Chen, W. 2017. Chitosan-stabilized platinum nanoparticles as effective oxidase mimics for colorimetric detection of acid phosphatase. *Nanoscale*, 9(29):10292-10300. 10.1039/C7NR03399K

Deng, T.-S., van der Hoeven, J.E.S., Yalcin, A.O., Zandbergen, H.W., van Huis, M.A. & van Blaaderen, A. 2015. Oxidative Etching and Metal Overgrowth of Gold Nanorods within Mesoporous Silica Shells. *Chemistry of Materials*, 27(20):7196-7203. 10.1021/acs.chemmater.5b03749

- Depciuch, J., Stec, M., Maximenko, A., Baran, J. & Parlinska-Wojtan, M. 2020. Temperature-controlled synthesis of hollow, porous gold nanoparticles with wide range light absorption. *Journal of Materials Science*, 55(12):5257-5267. 10.1007/s10853-020-04345-8
- Deveci, P. 2021. Synthesis, optical properties and phototherapy applications of gold nanostars. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 99:1-9. 10.1007/s10847-020-01035-5
- Diepenbrock, F., Heckler, R., Schickling, H., Engelhard, T., Bock, D. & Sander, J. 1992. Colorimetric determination of galactose and galactose-1-phosphate from dried blood. *Clinical Biochemistry*, 25(1):37-39. 10.1016/0009-9120(92)80043-G
- Ding, S., Cargill, A.A., Medintz, I.L. & Claussen, J.C. 2015a. Increasing the activity of immobilized enzymes with nanoparticle conjugation. *Current Opinions in Biotechnology*, 34:242-250. 10.1016/j.copbio.2015.04.005
- Ding, S., Cargill, A.A., Medintz, I.L. & Claussen, J.C. 2015b. Increasing the activity of immobilized enzymes with nanoparticle conjugation. *Current Opinion in Biotechnology*, 34:242-250. 10.1016/j.copbio.2015.04.005
- Divya, J., Selvendran, S., Raja, A.S. & Sivasubramanian, A. 2022. Surface plasmon based plasmonic sensors: A review on their past, present and future. *Biosensors and Bioelectronics: X*, 11:100175. 10.1016/j.biosx.2022.100175
- Dong, F., Wu, C., Miao, A.-J. & Pan, K. 2021. Reduction of silver ions to form silver nanoparticles by redox-active organic molecules: coupled impact of the redox state and environmental factors. *Environmental Science: Nano*, 8(1):269-281. 10.1039/D0EN00820F
- Drain, P.K., Hyle, E.P., Noubary, F., Freedberg, K.A., Wilson, D., Bishai, W.R., ... Bassett, I.V. 2014. Diagnostic point-of-care tests in resource-limited settings. *Lancet Infect Dis*, 14(3):239-249. 10.1016/s1473-3099(13)70250-0
- Dreaden, E.C., Alkilany, A.M., Huang, X., Murphy, C.J. & El-Sayed, M.A. 2012. The golden age: gold nanoparticles for biomedicine. *Chemical Society Reviews*, 41(7):2740-2779. 10.1039/C1CS15237H
- Duchen, M.R., Surin, A. & Jacobson, J. 2003. Imaging mitochondrial function in intact cells. *Methods of Enzymology*, 361:353-389. 10.1016/s0076-6879(03)61019-0
- Duffield, C., Lyu, N. & Wang, Y. 2021. Synthesis and characterization of reporter molecules embedded core-shell nanoparticles as SERS nanotags. *Journal of Innovative Optical Health Sciences*, 14, 10.1142/S1793545821410078
- Dumur, F., Guerlin, A., Dumas, E., Bertin, D., Gigmes, D. & Mayer, C.R. 2011. Controlled spontaneous generation of gold nanoparticles assisted by dual reducing and capping agents. *Gold Bulletin*, 44(2):119-137. 10.1007/s13404-011-0018-5

Duysens, L.N.M. & Ames, J. 1957. Fluorescence spectrophotometry of reduced phosphopyridine nucleotide in intact cells in the near-ultraviolet and visible region. *Biochimica et Biophysica Acta*, 24:19-26. 10.1016/0006-3002(57)90141-5

Dykman, L. & Khlebtsov, N. 2012. Gold nanoparticles in biomedical applications: recent advances and perspectives. *Chemical Society Reviews*, 41(6):2256-2282. 10.1039/C1CS15166E

Ehrig, T., Bohren, K.M., Prendergast, F.G. & Gabbay, K.H. 1994. Mechanism of Aldose Reductase Inhibition: Binding of NADP⁺/NADPH and Alrestatin-like Inhibitors. *Biochemistry*, 33(23):7157-7165. 10.1021/bi00189a019

Eisink, N.N., Witte, M.D. & Minnaard, A.J. 2017. Regioselective Carbohydrate Oxidations: A Nuclear Magnetic Resonance (NMR) Study on Selectivity, Rate, and Side-Product Formation. *ACS Catal*, 7(2):1438-1445. 10.1021/acscatal.6b03459

Eksteen, J.J. & Oraby, E.A. 2015. The leaching and adsorption of gold using low concentration amino acids and hydrogen peroxide: Effect of catalytic ions, sulphide minerals and amino acid type. *Minerals Engineering*, 70:36-42. 10.1016/j.mineng.2014.08.020

Ellis, G.A., Dean, S.N., Walper, S.A. & Medintz, I.L. 2020. Quantum Dots and Gold Nanoparticles as Scaffolds for Enzymatic Enhancement: Recent Advances and the Influence of Nanoparticle Size. *Catalysts*, 10(1):83. 10.3390/catal10010083

Enea, M., Araújo, A.M., Almeida, M.P., Soares, M.E., Gonçalves-Monteiro, S., Pinho, P.G., ... Carmo, H. 2019. A Metabolomic Approach for the In Vivo Study of Gold Nanospheres and Nanostars after a Single-Dose Intravenous Administration to Wistar Rats. *Nanomaterials (Basel)*, 9(11), 10.3390/nano9111606

Ermoline, A. & Dreizin, E. 2011. Equations for the Cabrera–Mott kinetics of oxidation for spherical nanoparticles. *Chemical Physics Letters - CHEM PHYS LETT*, 505:47-50. 10.1016/j.cplett.2011.02.022

Eustis, S. & El-Sayed, M.A. 2006. Why gold nanoparticles are more precious than pretty gold: Noble metal surface plasmon resonance and its enhancement of the radiative and nonradiative properties of nanocrystals of different shapes. *Chemical Society Reviews*, 35(3):209-217. 10.1039/B514191E

Faghihnejad, A., Huang, J. & Zeng, H. 2014. Properties and Characterization of Bioconjugates. In. *Chemistry of Bioconjugates*. pp. 415-430.

Faraday, M. 1857. The Bakerian Lecture. Experimental relations of gold (and other metals) to light. *Philosophical Transactions of the Royal Society of London*, 147:145-181. 10.1098/rstl.1857.0011

Farber, J.L. 1994. Mechanisms of cell injury by activated oxygen species. *Environmental Health Perspect*, 102 Suppl 10(Suppl 10):17-24. 10.1289/ehp.94102s1017

Fermo, P. & Padeletti, G. 2012. The use of nano-particles to produce iridescent metallic effects on ancient ceramic objects. *Journal of Nanoscience and Nanotechnology*, 12(11):8764-8769. 10.1166/jnn.2012.6464

Filbrun, S.L., Filbrun, A.B., Lovato, F.L., Oh, S.H., Driskell, E.A. & Driskell, J.D. 2017. Chemical modification of antibodies enables the formation of stable antibody–gold nanoparticle conjugates for biosensing. *Analyst*, 142(23):4456-4467. 10.1039/C7AN01496A

Flynn, T.G., Green, N.C., Bhatia, M.B. & El-Kabbani, O. 1995. Structure and Mechanism of Aldehyde Reductase. In: Weiner, H., Holmes, R.S. & Wermuth, B., eds. *Enzymology and Molecular Biology of Carbonyl Metabolism 5*. Boston, MA: Springer US. pp. 193-201.

Franconetti, A., Carnerero, J.M., Prado-Gotor, R., Cabrera-Escribano, F. & Jaime, C. 2019. Chitosan as a capping agent: Insights on the stabilization of gold nanoparticles. *Carbohydr Polym*, 207:806-814. 10.1016/j.carbpol.2018.12.046

Freestone, I., Meeks, N., Sax, M. & Higgitt, C. 2007. The Lycurgus Cup — A Roman nanotechnology. *Gold Bulletin*, 40(4):270-277. 10.1007/BF03215599

Freitas de Freitas, L., Varca, G.H., Dos Santos Batista, J.G. & Benévolo Lugão, A. 2018. An Overview of the Synthesis of Gold Nanoparticles Using Radiation Technologies. *Nanomaterials*, 8(11), 10.3390/nano8110939

Frings, C.S. & Pardue, H.L. 1964. An Automatic Spectrophotometric Method for the Specific Enzymatic Determination of Galactose in Whole Blood and Plasma. *Analytical Chemistry*, 36(13):2477-2478. 10.1021/ac60219a031

Fujimoto, A., Okano, Y., Miyagi, T., Isshiki, G. & Oura, T. 2000. Quantitative Beutler Test for Newborn Mass Screening of Galactosemia Using a Fluorometric Microplate Reader. *Clinical Chemistry*, 46(6):806-810. Date of access: 5/28/2022. 10.1093/clinchem/46.6.806

Fujimura, Y., Ishii, S., Kawamura, M. & Naruse, H. 1981. Microdetermination of galactose and galactose 1-phosphate in dried blood spots. *Analytical Biochemistry*, 117(1):187-195. 10.1016/0003-2697(81)90709-0

Fukuoka, N. & Tanabe, K. 2019. Lightning-Rod Effect of Plasmonic Field Enhancement on Hydrogen-Absorbing Transition Metals. *Nanomaterials*, 9(9), 10.3390/nano9091235

Gaaz, T.S., Sulong, A.B., Akhtar, M.N., Kadhum, A.A., Mohamad, A.B. & Al-Amiery, A.A. 2015. Properties and Applications of Polyvinyl Alcohol, Halloysite Nanotubes and Their Nanocomposites. *Molecules*, 20(12):22833-22847. 10.3390/molecules201219884

Gabbay, K.H. & Tze, W.J. 1972. Inhibition of glucose-induced release of insulin by aldose reductase inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 69(6):1435-1439. 10.1073/pnas.69.6.1435

- Gad, S.E. 2014. Polymers. In: Wexler, P., ed. *Encyclopedia of Toxicology (Third Edition)*. Oxford: Academic Press. pp. 1045-1050.
- Gaede, B. & Versteeg - Mojanaga, M. 2011. The state of the right to health in rural South Africa. In. pp. 99-106.
- Garcia, M.A. 2012. Surface plasmons in metallic nanoparticles: fundamentals and applications. *Journal of Physics D: Applied Physics*, 45(38):389501. 10.1088/0022-3727/45/38/389501
- Gavia, D.J. & Shon, Y.-S. 2015. Catalytic Properties of Unsupported Palladium Nanoparticle Surfaces Capped with Small Organic Ligands. *ChemCatChem*, 7(6):892-900. 10.1002/cctc.201402865
- Ghosh, C.K. 2015. Quantum Effect on Properties of Nanomaterials. In: Sengupta, A. & Sarkar, C.K., eds. *Introduction to Nano: Basics to Nanoscience and Nanotechnology*. Berlin, Heidelberg: Springer Berlin Heidelberg. pp. 73-111.
- Ghosh, S.K. & Pal, T. 2007. Interparticle Coupling Effect on the Surface Plasmon Resonance of Gold Nanoparticles: From Theory to Applications. *Chemical Reviews*, 107(11):4797-4862. 10.1021/cr0680282
- Giuliano, K. & Grant, M. 2002. Blood Analysis at the Point of Care: Issues in Application for Use in Critically Ill Patients. *AACN clinical issues*, 13:204-220. 10.1097/00044067-200205000-00006
- Gong, J., Li, G. & Tang, Z. 2012. Self-assembly of noble metal nanocrystals: Fabrication, optical property, and application. *Nano Today*, 7(6):564-585. 10.1016/j.nantod.2012.10.008
- Gopalan, A., Ragupathy, D., Kim, H.T., Manesh, K.M. & Lee, K.P. 2009. Pd (core)-Au (shell) nanoparticles catalyzed conversion of NADH to NAD⁺ by UV-vis spectroscopy--a kinetic analysis. *Spectrochim Acta A Mol Biomol Spectrosc*, 74(3):678-684. 10.1016/j.saa.2009.07.022
- Govindhan, M., Amiri, M. & Chen, A. 2015. Au nanoparticle/graphene nanocomposite as a platform for the sensitive detection of NADH in human urine. *Biosensors & bioelectronics*, 66:474-480.
- Grimshaw, C.E., Shahbaz, M. & Putney, C.G. 1990. Mechanistic basis for nonlinear kinetics of aldehyde reduction catalyzed by aldose reductase. *Biochemistry*, 29(42):9947-9955. 10.1021/bi00494a027
- Grimshaw, C.E., Shahbaz, M., Jahangiri, G., Putney, C.G., McKercher, S.R. & Mathur, E.J. 1989. Kinetic and structural effects of activation of bovine kidney aldose reductase. *Biochemistry*, 28(13):5343-5353. 10.1021/bi00439a006
- Grubbs, R.B. 2007. Roles of Polymer Ligands in Nanoparticle Stabilization. *Polymer Reviews*, 47(2):197-215. 10.1080/15583720701271245
- Grzelczak, M., Pérez-Juste, J., Mulvaney, P. & Liz-Marzán, L.M. 2008. Shape control in gold nanoparticle synthesis. *Chemical Society Reviews*, 37(9):1783-1791. 10.1039/B711490G

- Guerrero-Martínez, A., Barbosa, S., Pastoriza-Santos, I. & Liz-Marzán, L.M. 2010. Nanostars shine bright for you Colloidal synthesis, properties and applications of branched metallic nanoparticles. *Current Opinion in Colloid and Interface Science*, 16(2):118-127. 10.1016/j.cocis.2010.12.007
- Guerrero-Martínez, A., Barbosa, S., Pastoriza-Santos, I. & Liz-Marzán, L.M. 2011. Nanostars shine bright for you: Colloidal synthesis, properties and applications of branched metallic nanoparticles. *Current Opinion in Colloid & Interface Science*, 16(2):118-127. 10.1016/j.cocis.2010.12.007
- Gulati, S., Sachdeva, M. & Bhasin, K.K. 2018. Capping agents in nanoparticle synthesis: Surfactant and solvent system. *AIP Conference Proceedings*, 1953(1), Date of access: 9/18/2023. 10.1063/1.5032549
- Guo, L., Jackman, J.A., Yang, H.-H., Chen, P., Cho, N.-J. & Kim, D.-H. 2015. Strategies for enhancing the sensitivity of plasmonic nanosensors. *Nano Today*, 10(2):213-239. 10.1016/j.nantod.2015.02.007
- Guo, Y., Wu, J., Li, J. & Ju, H. 2016. A plasmonic colorimetric strategy for biosensing through enzyme guided growth of silver nanoparticles on gold nanostars. *Biosensors and Bioelectronics*, 78:267-273. 10.1016/j.bios.2015.11.056
- Guzik, U., Hupert-Kocurek, K. & Wojcieszynska, D. 2014. Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. *Molecules*, 19(7):8995-9018. 10.3390/molecules19078995
- Guzmán-Soto, I., Omole, M., Alarcon, E.I. & McTiernan, C.D. 2020. Lipoic acid capped silver nanoparticles: a facile route to covalent protein capping and oxidative stability within biological systems. *RSC Advances*, 10(54):32953-32958. 10.1039/D0RA07080G
- Häkkinen, H. 2012. The gold-sulfur interface at the nanoscale. *Natural Chemistry*, 4(6):443-455. 10.1038/nchem.1352
- Hammond, J.L., Bhalla, N., Rafiee, S.D. & Estrela, P. 2014. Localized surface plasmon resonance as a biosensing platform for developing countries. *Biosensors*, 4(2):172-188. 10.3390/bios4020172
- Hao, E., Bailey, R.C., Schatz, G.C., Hupp, J.T. & Li, S. 2004. Synthesis and Optical Properties of "Branched" Gold Nanocrystals. *Nano Letters*, 4(2):327-330. 10.1021/nl0351542
- Hao, F., Nehl, C.L., Hafner, J.H. & Nordlander, P. 2007a. Plasmon resonances of a gold nanostar. *Nano Lett*, 7(3):729-732. 10.1021/nl062969c
- Hao, F., Nehl, C.L., Hafner, J.H. & Nordlander, P. 2007b. Plasmon Resonances of a Gold Nanostar. *Nano Letters*, 7(3):729-732. 10.1021/nl062969c

- Haruta, M. 2003. When gold is not noble: catalysis by nanoparticles. *Chem Rec*, 3(2):75-87. 10.1002/tcr.10053
- Hatam, N., Askarian, M., Shirvani, S. & Siavashi, E. 2017. Neonatal Screening: Cost-utility Analysis for Galactosemia. *Iran J Public Health*, 46(1):112-119.
- Haynes, R.L.D.a.W.M. 2002. CRC handbook of chemistry and physics; a ready-reference book of chemical and physical data, 83d ed. Vol. 26. Portland: Ringgold Inc.
- He, C., Hu, Y., Yin, L., Tang, C. & Yin, C. 2010. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials*, 31(13):3657-3666. 10.1016/j.biomaterials.2010.01.065
- Hergert, W. & Wriedt, T. 2012. *The Mie theory : basics and applications*. [1 online resource]. Berlin ; Springer. Available from WorldCat.org: 10.1007/978-3-642-28738-1
- Hermanson, G.T. 1996. Enzyme Modification and Conjugation. In: Hermanson, G.T., ed. *Bioconjugate Techniques*. San Diego: Academic Press. pp. 630-638.
- Hietzschold, S., Walter, A., Davis, C., Taylor, A. & Sepunaru, L. 2019. Does Nitrate Reductase Play a Role in Silver Nanoparticle Synthesis? Evidence for NADPH as the Sole Reducing Agent. *ACS Sustainable Chemistry & Engineering*, 7, 10.1021/acssuschemeng.9b00506
- Hill, J.W. & Kolb, D.K. 2004. *Chemistry for changing times*. 10th / Doris K. Kolb ; with special contributions by Steven W. Keller. Upper Saddle River, NJ: Pearson Prentice Hall.
- Hirakawa, K. & Mori, M. 2021. Phenothiazine Dyes Induce NADH Photooxidation through Electron Transfer: Kinetics and the Effect of Copper Ions. *ACS Omega*, 6(12):8630-8636. 10.1021/acsomega.1c00484
- Hjelm, M. & Tengström, B. 1968. Enzymatic determinations of blood galactose with galactose oxidase and galactose dehydrogenase. *Biochemical Medicine*, 2(2):174-176. 10.1016/0006-2944(68)90017-3
- Hola, K., Markova, Z., Zoppellaro, G., Tucek, J. & Zboril, R. 2015. Tailored functionalization of iron oxide nanoparticles for MRI, drug delivery, magnetic separation and immobilization of biosubstances. *Biotechnology Advances*, 33(6 Pt 2):1162-1176. 10.1016/j.biotechadv.2015.02.003
- Holden, H.M., Rayment, I. & Thoden, J.B. 2003. Structure and Function of Enzymes of the Leloir Pathway for Galactose Metabolism*. *Journal of Biological Chemistry*, 278(45):43885-43888. 10.1074/jbc.R300025200
- Homaei, A.A., Sariri, R., Vianello, F. & Stevanato, R. 2013. Enzyme immobilization: an update. *Journal of Chemical Biology*, 6(4):185-205. 10.1007/s12154-013-0102-9

- Horvath, H. 2009. Gustav Mie and the scattering and absorption of light by particles: Historic developments and basics. *Journal of Quantitative Spectroscopy and Radiative Transfer*, 110(11):787-799. 10.1016/j.jqsrt.2009.02.022
- Howes, P.D., Rana, S. & Stevens, M.M. 2014. Plasmonic nanomaterials for biodiagnostics. *Chem Soc Rev*, 43(11):3835-3853. 10.1039/c3cs60346f
- Hrelescu, C., Sau, T.K., Rogach, A.L., Jäckel, F. & Feldmann, J. 2009. Single gold nanostars enhance Raman scattering. *Applied Physics Letters*, 94(15):153113. 10.1063/1.3119642
- Hu, H., Xin, J.H., Hu, H., Wang, X., Miao, D. & Liu, Y. 2015. Synthesis and stabilization of metal nanocatalysts for reduction reactions – a review. *Journal of Materials Chemistry A*, 3(21):11157-11182. 10.1039/C5TA00753D
- Huang, T. & Xu, X.-H.N. 2010. Synthesis and characterization of tunable rainbow colored colloidal silver nanoparticles using single-nanoparticle plasmonic microscopy and spectroscopy. *Journal of Materials Chemistry*, 20(44):9867-9876. 10.1039/C0JM01990A
- Huang, X., Zhu, Y. & Kianfar, E. 2021. Nano Biosensors: Properties, applications and electrochemical techniques. *Journal of Materials Research and Technology*, 12:1649-1672. 10.1016/j.jmrt.2021.03.048
- Huang, X., El-Sayed, I.H., Yi, X. & El-Sayed, M.A. 2005. Gold nanoparticles: Catalyst for the oxidation of NADH to NAD⁺. *Journal of Photochemistry and Photobiology B: Biology*, 81(2):76-83. 10.1016/j.jphotobiol.2005.05.010
- Hutami Rahayu, L.B., Wulandari, I.O., Santjojo, D.H. & Sabarudin, A. 2018. Synthesis and Characterization of Fe₃O₄ Nanoparticles using Polyvinyl Alcohol (PVA) as Capping Agent and Glutaraldehyde (GA) as Crosslinker. *IOP Conference Series: Materials Science and Engineering*, 299,
- Ibrahim, A.M., Alzahrani, H.A.A., Abd El-Latif, M.M. & Selim, M.M. 2019. Influence of different stabilizers on the morphology of gold nanoparticles. *Bulletin of the National Research Centre*, 43(1):33. 10.1186/s42269-019-0070-5
- Iglesias, C.A.M., de Araújo, J.C.R., Xavier, J., Anders, R.L., de Araújo, J.M., da Silva, R.B., ... Bohn, F. 2021. Magnetic nanoparticles hyperthermia in a non-adiabatic and radiating process. *Scientific Reports*, 11(1):11867. 10.1038/s41598-021-91334-9
- Ignaczak, A., Santos, E. & Schmickler, W. 2019. Oxygen reduction reaction on gold in alkaline solutions – The inner or outer sphere mechanisms in the light of recent achievements. *Current Opinion in Electrochemistry*, 14:180-185. 10.1016/j.coelec.2018.07.011
- Ikeda, S., Sumi, Y. & Fukui, S. 1975. Kinetic studies on coenzyme binding and coenzyme dissociation in tryptophanase immobilized on sepharose. *Biochemistry*, 14(7):1464-1470. 10.1021/bi00678a018

Jadoun, S. & Jangid, N. 2019. Polyvinyl Alcohol (PVA) based Nanocomposites for Biomedical and Tissue Engineering Applications. In. pp. 111-126.

Jafari, P., Beigi, S.M., Yousefi, F., Aghabalazadeh, S., Mousavizadegan, M., Hosseini, M., ... Ganjali, M.R. 2021. Colorimetric biosensor for phenylalanine detection based on a paper using gold nanoparticles for phenylketonuria diagnosis. *Microchemical Journal*, 163:105909. 10.1016/j.microc.2020.105909

Javed, R., Sajjad, A., Naz, S., Sajjad, H. & Ao, Q. 2022. Significance of Capping Agents of Colloidal Nanoparticles from the Perspective of Drug and Gene Delivery, Bioimaging, and Biosensing: An Insight. *International Journal of Molecular Science*, 23(18), 10.3390/ijms231810521

Javed, R., Zia, M., Naz, S., Aisida, S.O., Ain, N.U. & Ao, Q. 2020a. Role of capping agents in the application of nanoparticles in biomedicine and environmental remediation: recent trends and future prospects. *J Nanobiotechnology*, 18(1):172. 10.1186/s12951-020-00704-4

Javed, R., Zia, M., Naz, S., Aisida, S.O., Ain, N.u. & Ao, Q. 2020b. Role of capping agents in the application of nanoparticles in biomedicine and environmental remediation: recent trends and future prospects. *Journal of Nanobiotechnology*, 18(1):172. 10.1186/s12951-020-00704-4

Jayaramudu, T., Varaprasad, K., Pyarasani, R.D., Reddy, K.K., Kumar, K.D., Akbari-Fakhrabadi, A., ... Amalraj, J. 2019. Chitosan capped copper oxide/copper nanoparticles encapsulated microbial resistant nanocomposite films. *International Journal of Biological Macromolecules*, 128:499-508. 10.1016/j.ijbiomac.2019.01.145

Jeong, J.-S., Kwon, H.-J., Lee, Y.-M., Yoon, H.-R. & Hong, S.-P. 2008. Galactosemia Screening by Simultaneous Blood Spot Quantification of Galactose and Galactose 1-Phosphate. *Clinical Chemistry*, 54(12):2080-2082. Date of access: 9/13/2023. 10.1373/clinchem.2008.107847

Jez, J.M., Bennett, M.J., Schlegel, B.P., Lewis, M. & Penning, T.M. 1997. Comparative anatomy of the aldo-keto reductase superfamily. *Biochemistry Journal*, 326 (Pt 3)(Pt 3):625-636. 10.1042/bj3260625

Jiang, J., Zhao, S., Huang, Y., Qin, G. & Ye, F. 2013. Highly sensitive immunoassay of carcinoembryonic antigen by capillary electrophoresis with gold nanoparticles amplified chemiluminescence detection. *J Chromatogr A*, 1282:161-166. 10.1016/j.chroma.2013.01.066

Jiang, L., Lei, X., Wang, K., Zhang, Z., Wang, F., Lu, S. & Chen, X. 2020. Colorimetric and fluorometric detection of NADPH using imidazolium functionalized polydiacetylenes with high sensitivity and selectivity. *Dyes and Pigments*, 183:108740. 10.1016/j.dyepig.2020.108740

Jiang, S., Win, K.Y., Liu, S., Teng, C.P., Zheng, Y. & Han, M.-Y. 2013. Surface-functionalized nanoparticles for biosensing and imaging-guided therapeutics. *Nanoscale*, 5(8):3127-3148. 10.1039/C3NR34005H

Jimmy Huang, P.J., Yang, J., Chong, K., Ma, Q., Li, M., Zhang, F., ... Liu, J. 2020. Good's buffers have various affinities to gold nanoparticles regulating fluorescent and colorimetric DNA sensing. *Chem Sci*, 11(26):6795-6804. 10.1039/d0sc01080d

Jin, X., Zhong, M., Zhu, Z., Xie, J., Feng, J., Liu, Y., ... Liu, J. 2023. Application of NAD⁺-Dependent Electrochemical Dehydrogenase Biosensors in Human Physiological Fluids: Opportunities and Challenges. *Journal of Analytical Methods in Chemistry*, 2023:3401001. 10.1155/2023/3401001

Junaidi, J., Triyana, K., Suharyadi, E., Sabarman, H. & Wu, Y. 2017. The Roles of Polyvinyl Alcohol (PVA) as the Capping Agent on the Polyol Method for Synthesizing Silver Nanowires. *Journal of Nano Research*, 49:174-180. 10.4028/www.scientific.net/JNanoR.49.174

Kanelidis, I. & Kraus, T. 2017. The role of ligands in coinage-metal nanoparticles for electronics. *Beilstein Journal of Nanotechnology*, 8:2625 - 2639.

Kelly, K.L., Coronado, E., Zhao, L.L. & Schatz, G.C. 2003. The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment. *The Journal of Physical Chemistry B*, 107(3):668-677. 10.1021/jp026731y

Kemal, L., Jiang, X., Wong, K. & Yu, A. 2008. Experiment and Theoretical Study of Poly(vinyl pyrrolidone)-controlled Gold Nanoparticles. *Journal of Physical Chemistry C - J PHYS CHEM C*, 112, 10.1021/jp803935y

Kereselidze, Z., Romero, V.H., Peralta, X.G. & Santamaria, F. 2012a. Gold nanostar synthesis with a silver seed mediated growth method. *J Vis Exp*, (59), 10.3791/3570

Kereselidze, Z., Romero, V.H., Peralta, X.G. & Santamaria, F. 2012b. Gold Nanostar Synthesis with a Silver Seed Mediated Growth Method. *JoVE*, (59):e3570. 10.3791/3570

Khlebtsov, N. 2010. Anisotropic properties of plasmonic nanoparticles: depolarized light scattering, dichroism, and birefringence. *Journal of Nanophotonics*, 4(1):041587. 10.1117/1.3370232

Kirsch, M. & De Groot, H. 2001. NAD(P)H, a directly operating antioxidant? *The FASEB Journal*, 15(9):1569-1574. 10.1096/fj.00-0823hyp

Koczur, K.M., Mourdikoudis, S., Polavarapu, L. & Skrabalak, S.E. 2015. Polyvinylpyrrolidone (PVP) in nanoparticle synthesis. *Dalton Transactions*, 44(41):17883-17905. 10.1039/C5DT02964C

Koju, N., Qin, Z.H. & Sheng, R. 2022. Reduced nicotinamide adenine dinucleotide phosphate in redox balance and diseases: a friend or foe? *Acta Pharmacol Sin*, 43(8):1889-1904. 10.1038/s41401-021-00838-7

Kondrat, S., Krauss, U. & von Lieres, E. 2022. Enzyme co-localisation: Mechanisms and benefits. *Current Research in Chemical Biology*, 2:100031. 10.1016/j.crchbi.2022.100031

- Kotz, J.C. 1991. *Chemistry & chemical reactivity*. 2nd. Philadelphia: Saunders College Pub. (Saunders golden sunburst series).
- Kumar, A., Das, N., Satija, N.K., Mandrah, K., Roy, S.K. & Rayavarapu, R.G. 2019. A Novel Approach towards Synthesis and Characterization of Non-Cytotoxic Gold Nanoparticles Using Taurine as Capping Agent. *Nanomaterials (Basel, Switzerland)*, 10(1):45. 10.3390/nano10010045
- Kumar, D., Mutreja, I. & Sykes, P. 2016. Seed mediated synthesis of highly mono-dispersed gold nanoparticles in the presence of hydroquinone. *Nanotechnology*, 27(35):355601. 10.1088/0957-4484/27/35/355601
- Kumar, M., Devi, P. & Kumar, A. 2017. Structural analysis of PVP capped silver nanoparticles synthesized at room temperature for optical, electrical and gas sensing properties. *Journal of Materials Science: Materials in Electronics*, 28(6):5014-5020. 10.1007/s10854-016-6157-y
- Kumar, N.a.K., S. 2016. Unique Properties. In. *Essentials in Nanoscience and Nanotechnology*. pp. 326-360.
- Kumar, R. 2018. *Study on Optical Properties and Stability of Gold Nanostars for Localized Surface Plasmon Resonance (LSPR) based Biosensing Applications*. Concordia University: Concordia University.
- Kundu, T., Karak, N., Barik, P. & Saha, S. 2011. Optical properties of ZnO nanoparticles prepared by chemical method using poly (vinylalcohol) (PVA) as capping agent. *IJSCE*, 1:19-24.
- Kunoh, T., Takeda, M., Matsumoto, S., Suzuki, I., Takano, M., Kunoh, H. & Takada, J. 2018. Green Synthesis of Gold Nanoparticles Coupled with Nucleic Acid Oxidation. *ACS Sustainable Chemistry & Engineering*, 6(1):364-373. 10.1021/acssuschemeng.7b02610
- Kuo, C.-H. & Huang, M.H. 2005. Synthesis of Branched Gold Nanocrystals by a Seeding Growth Approach. *Langmuir*, 21(5):2012-2016. 10.1021/la0476332
- Kyrychenko, A., Pasko, D.A. & Kalugin, O.N. 2017. Poly(vinyl alcohol) as a water protecting agent for silver nanoparticles: the role of polymer size and structure. *Physical Chemistry Chemical Physics*, 19(13):8742-8756. 10.1039/C6CP05562A
- Lai, Y., Yu, B., Lin, T. & Hou, L. 2023. Iodide-Mediated Etching of Gold Nanostar for the Multicolor Visual Detection of Hydrogen Peroxide. *Biosensors*, 13(6):585. 10.3390/bios13060585
- Land, K.J., Boeras, D.I., Chen, X.-S., Ramsay, A.R. & Peeling, R.W. 2019. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nature Microbiology*, 4(1):46-54. 10.1038/s41564-018-0295-3
- Lang, N.J., Liu, B. & Liu, J. 2014. Characterization of glucose oxidation by gold nanoparticles using nanoceria. *Journal of Colloid Interface Science*, 428:78-83. 10.1016/j.jcis.2014.04.025

- Langille, M.R., Personick, M.L., Zhang, J. & Mirkin, C.A. 2012. Defining Rules for the Shape Evolution of Gold Nanoparticles. *Journal of the American Chemical Society*, 134(35):14542-14554. 10.1021/ja305245g
- Lee, J.K., Suh, H.N., Yoon, S.H., Lee, K.H., Ahn, S.Y., Kim, H.J. & Kim, S.H. 2022. Non-Destructive Monitoring via Electrochemical NADH Detection in Murine Cells. *Biosensors*, 12(2):107. 10.3390/bios12020107
- Leitner, J., Sedmidubský, D. & Jankovský, O. 2019. Thermodynamic modeling of copper nanoparticles oxidation. *AIP Conference Proceedings*, 2170(1):020007. 10.1063/1.5132726
- Leitner, J., Sedmidubský, D., Lojka, M. & Jankovský, O. 2020. The Effect of Nanosizing on the Oxidation of Partially Oxidized Copper Nanoparticles. *Materials (Basel)*, 13(12), 10.3390/ma13122878
- Leung-Toung, R., Li, W., Tam, T.F. & Karimian, K. 2002. Thiol-dependent enzymes and their inhibitors: a review. *Current Medical Chemistry*, 9(9):979-1002. 10.2174/0929867024606704
- Li, C.-C., Chang, S.-J., Su, F.-J., Lin, S.-W. & Chou, Y.-C. 2013. Effects of capping agents on the dispersion of silver nanoparticles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 419:209-215. 10.1016/j.colsurfa.2012.11.077
- Li, D., He, Q., Cui, Y., Duan, L. & Li, J. 2007. Immobilization of glucose oxidase onto gold nanoparticles with enhanced thermostability. *Biochemical and Biophysical Research Communications*, 355(2):488-493. 10.1016/j.bbrc.2007.01.183
- Li, L., Lu, H. & Deng, L. 2013. A sensitive NADH and ethanol biosensor based on graphene–Au nanorods nanocomposites. *Talanta*, 113:1-6. 10.1016/j.talanta.2013.03.074
- Li, N., Zhao, P. & Astruc, D. 2014. Anisotropic Gold Nanoparticles: Synthesis, Properties, Applications, and Toxicity. *Angewandte Chemie International Edition*, 53(7):1756-1789. 10.1002/anie.201300441
- Li, R., Zhang, X., Dong, H., Li, Q., Shuai, Z. & Hu, W. 2016. Gibbs–Curie–Wulff Theorem in Organic Materials: A Case Study on the Relationship between Surface Energy and Crystal Growth. *Advanced Materials*, 28(8):1697-1702. 10.1002/adma.201504370
- Li, T., Zhu, K., He, S., Xia, X., Liu, S., Wang, Z. & Jiang, X. 2011. Sensitive detection of glucose based on gold nanoparticles assisted silver mirror reaction. *The Analyst*, 136:2893-2896. 10.1039/c1an15256d
- Li, W., Lin, L. & Li, G. 2014. Wavelength selection method based on test analysis of variance: application to oximetry. *Analytical Methods*, 6(4):1082-1089. 10.1039/C3AY41601A
- Li, X., Jiang, L., Zhan, Q., Qian, J. & He, S. 2009. Localized surface plasmon resonance (LSPR) of polyelectrolyte-functionalized gold-nanoparticles for bio-sensing. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 332(2):172-179. 10.1016/j.colsurfa.2008.09.009

- Li, Y., Ma, J. & Ma, Z. 2013. Synthesis of gold nanostars with tunable morphology and their electrochemical application for hydrogen peroxide sensing. *Electrochimica Acta*, 108:435-440. 10.1016/j.electacta.2013.06.141
- Liang, P., Yu, H., Guntupalli, B. & Xiao, Y. 2015. Paper-Based Device for Rapid Visualization of NADH Based on Dissolution of Gold Nanoparticles. *ACS Applied Materials & Interfaces*, 7(27):15023-15030. 10.1021/acsami.5b04104
- Liao, W.Y., Liu, C.C. & Chou, T.C. 2008. Detection of triglyceride using an iridium nano-particle catalyst based amperometric biosensor. *Analyst*, 133(12):1757-1763. 10.1039/b801703d
- Liébana, S. & Drago, G.A. 2016. Bioconjugation and stabilisation of biomolecules in biosensors. *Essays in biochemistry*, 60(1):59-68. 10.1042/EBC20150007
- Link, S. & El-Sayed, M.A. 2003. Optical Properties and Ultrafast Dynamics of Metallic Nanocrystals. *Annual Review of Physical Chemistry*, 54(1):331-366. Date of access: 2022/08/12. 10.1146/annurev.physchem.54.011002.103759
- Lista, M., Liu, D.Z. & Mulvaney, P. 2014. Phase Transfer of Noble Metal Nanoparticles to Organic Solvents. *Langmuir*, 30(8):1932-1938. 10.1021/la404569h
- Liu, B.-W., Huang, P.-C. & Wu, F.-Y. 2021. A novel light-controlled colorimetric detection assay for nitroreductase based on p-aminophenol-catalyzed and NADH-mediated synthesis of silver nanoparticles. *Analytical Methods*, 13(19):2223-2228. 10.1039/D1AY00231G
- Liu, K., He, Z., Curtin, J.F., Byrne, H.J. & Tian, F. 2019. A novel, rapid, seedless, in situ synthesis method of shape and size controllable gold nanoparticles using phosphates. *Scientific Reports*, 9(1):7421. 10.1038/s41598-019-43921-0
- Liu, X.-L., Wang, J.-H., Liang, S., Yang, D.-J., Nan, F., Ding, S.-J., ... Wang, Q.-Q. 2014. Tuning Plasmon Resonance of Gold Nanostars for Enhancements of Nonlinear Optical Response and Raman Scattering. *The Journal of Physical Chemistry C*, 118(18):9659-9664. 10.1021/jp500638u
- Liu, X. & Swihart, M.T. 2014. Heavily-doped colloidal semiconductor and metal oxide nanocrystals: an emerging new class of plasmonic nanomaterials. *Chemical Society Reviews*, 43(11):3908-3920. 10.1039/C3CS60417A
- Liu, Y. 2016. *Multifunctional Gold Nanostars for Cancer Theranostics*. Duke University, USA.
- Liu, Y., Yuan, H., Fales, A.M., Register, J.K. & Vo-Dinh, T. 2015. Multifunctional gold nanostars for molecular imaging and cancer therapy. *Frontiers of Chemistry*, 3:51. 10.3389/fchem.2015.00051
- Liu, Y., Yuan, H., Kersey, F.R., Register, J.K., Parrott, M.C. & Vo-Dinh, T. 2015a. Plasmonic Gold Nanostars for Multi-Modality Sensing and Diagnostics. *Sensors*, 15(2):3706-3720. 10.3390/s150203706

Liu, Y., Yuan, H., Kersey, F.R., Register, J.K., Parrott, M.C. & Vo-Dinh, T. 2015b. Plasmonic gold nanostars for multi-modality sensing and diagnostics. *Sensors (Basel)*, 15(2):3706-3720. 10.3390/s150203706

Long, R., Zhou, S., Wiley, B.J. & Xiong, Y. 2014. Oxidative etching for controlled synthesis of metal nanocrystals: atomic addition and subtraction. *Chemical Society Reviews*, 43(17):6288-6310. 10.1039/C4CS00136B

Long, Y., Shuangshuang, W., Wang, Y., Deng, F. & Ding, T. 2020. Light-Directed Growth/Etching of Gold Nanoparticles via Plasmonic Hot Carriers. *The Journal of Physical Chemistry C*, 2, 10.1021/acs.jpcc.0c04672

Lou-Franco, J., Das, B., Elliott, C. & Cao, C. 2020. Gold Nanozymes: From Concept to Biomedical Applications. *Nanomicro Letters*, 13(1):10. 10.1007/s40820-020-00532-z

Love, J.C., Estroff, L.A., Kriebel, J.K., Nuzzo, R.G. & Whitesides, G.M. 2005. Self-Assembled Monolayers of Thiolates on Metals as a Form of Nanotechnology. *Chemical Reviews*, 105(4):1103-1170. 10.1021/cr0300789

Lucius, M., Falatach, R., McGlone, C., Makaroff, K., Danielson, A., Williams, C., ... Berberich, J.A. 2016. Investigating the Impact of Polymer Functional Groups on the Stability and Activity of Lysozyme–Polymer Conjugates. *Biomacromolecules*, 17(3):1123-1134. 10.1021/acs.biomac.5b01743

M. Pallares, R., Stilson, T., Choo, P., Hu, J. & Odom, T.W. 2019. Using Good's Buffers To Control the Anisotropic Structure and Optical Properties of Spiky Gold Nanoparticles for Refractive Index Sensing. *ACS Applied Nano Materials*, 2(8):5266-5271. 10.1021/acsanm.9b01117

Maenaka, Y., Suenobu, T. & Fukuzumi, S. 2012. Efficient catalytic interconversion between NADH and NAD⁺ accompanied by generation and consumption of hydrogen with a water-soluble iridium complex at ambient pressure and temperature. *American Chemical Society*, 134(1):367-374. 10.1021/ja207785f

Magaraphan, R. & Joothamongkhon, J. 2013. Performance of Dye-Sensitized Solar Cells Using ZnO-Natural Dyes from Sappan Wood, Noni Leaves, Safflower and Black Rice. *Advanced Materials Research*, 658:25-29. 10.4028/www.scientific.net/AMR.658.25

Magnussen, O.M. 2002. Ordered Anion Adlayers on Metal Electrode Surfaces. *Chemical Reviews*, 102(3):679-726. 10.1021/cr000069p

Mahato, K., Nagpal, S., Shah, M.A., Srivastava, A., Maurya, P.K., Roy, S., ... Chandra, P. 2019. Gold nanoparticle surface engineering strategies and their applications in biomedicine and diagnostics. *Biotechnology* 9(2):57. 10.1007/s13205-019-1577-z

Mansur, A., Mansur, H. & González, J. 2011. Enzyme-Polymers Conjugated to Quantum-Dots for Sensing Applications. *Sensors (Basel, Switzerland)*, 11:9951-9972. 10.3390/s111009951

- Marcheselli, J., Chateau, D., Lerouge, F., Baldeck, P., Andraud, C., Parola, S., ... Rivalta, I. 2020. Simulating Plasmon Resonances of Gold Nanoparticles with Bipyramidal Shapes by Boundary Element Methods. *Journal of Chemical Theory and Computation*, 16(6):3807-3815. 10.1021/acs.jctc.0c00269
- Marişca, O.T. & Leopold, N. 2019. Anisotropic Gold Nanoparticle-Cell Interactions Mediated by Collagen. *Materials (Basel, Switzerland)*, 12(7):1131. 10.3390/ma12071131
- Maugeri, L., Messina, M.A., Ruggieri, M. & Petralia, S. 2023. Photothermal-Contrast Method Based on In Situ Gold Nanostructure Formation for Phenylalanine Detection in Human Blood. *ACS Applied Nano Materials*, 6(14):12673-12678. 10.1021/acsanm.3c02651
- Maxwell, E.S., Kurahashi, K. & Kalckar, H.M. 1962. Enzymes of the Leloir pathway. In. *Methods in Enzymology*. 5: Academic Press. pp. 174-189.
- May, S.W. 1999. Applications of oxidoreductases. *Current Opinion in Biotechnology*, 10(4):370-375. 10.1016/S0958-1669(99)80067-6
- Mayer, K.M. & Hafner, J.H. 2011. Localized Surface Plasmon Resonance Sensors. *Chemical Reviews*, 111(6):3828-3857. 10.1021/cr100313v
- Mayr, P. & Nidetzky, B. 2002. Catalytic reaction profile for NADH-dependent reduction of aromatic aldehydes by xylose reductase from *Candida tenuis*. *Biochemistry Journal*, 366(Pt 3):889-899. 10.1042/bj20020080
- McLean, K. & Zhan, W. 2022. Mathematical modelling of nanoparticle-mediated topical drug delivery to skin tissue. *International Journal of Pharmaceutics*, 611:121322.10.1016/j.ijpharm.2021.121322
- Messina, M.A., Maugeri, L., Forte, G., Ruggieri, M. & Petralia, S. 2023. A highly sensitive colorimetric approach based on tris (bipyridine) Ruthenium (II/III) mediator for the enzymatic detection of phenylalanine. *Frontiers in Chemistry*, 11, 10.3389/fchem.2023.1164014
- Miao, X., Wang, T., Chai, F., Zhang, X., Wang, C. & Sun, W. 2011. A facile synthetic route for the preparation of gold nanostars with magnetic cores and their reusable nanohybrid catalytic properties. *Nanoscale*, 3(3):1189-1194. 10.1039/C0NR00704H
- Micheli, V., Simmonds, H.A., Bari, M. & Pompucci, G. 1993. HPLC determination of oxidized and reduced pyridine coenzymes in human erythrocytes. *Clinical Chimie Acta*, 220(1):1-17. 10.1016/0009-8981(93)90002-I
- Minati, L., Benetti, F., Chiappini, A. & Speranza, G. 2014a. One-step synthesis of star-shaped gold nanoparticles. *Colloids and Surfaces A Physicochemical and Engineering Aspects*, 441:623. 10.1016/j.colsurfa.2013.10.025
- Minati, L., Benetti, F., Chiappini, A. & Speranza, G. 2014b. One-step synthesis of star-shaped gold nanoparticles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 441:623-628. 10.1016/j.colsurfa.2013.10.025

- Miracle-Sole, S. 2013. Wulff shape of equilibrium crystals.
- Mironov, I. 2005. Properties of Gold(III) Hydroxide and Aquahydroxogold(III) Complexes in Aqueous Solution. *Russian Journal of Inorganic Chemistry*, 50:1115.
- Misuma, H., Wada, H., Kawakami, M., Ninomiya, H. & Shohmori, T. 1981. Galactose and galactose-1-phosphate spot test for galactosemia screening. *Clinical Chimistry Acta*, 111(1):27-32. 10.1016/0009-8981(81)90416-2
- Mohamad, N.R., Marzuki, N.H., Buang, N.A., Huyop, F. & Wahab, R.A. 2015. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol Biotechnol Equip*, 29(2):205-220. 10.1080/13102818.2015.1008192
- Mohan, C.O., Gunasekaran, S. & Ravishankar, C.N. 2019. Chitosan-capped gold nanoparticles for indicating temperature abuse in frozen stored products. *npj Science of Food*, 3(1):2. 10.1038/s41538-019-0034-z
- Mongeon, R., Venkatachalam, V. & Yellen, G. 2016. Cytosolic NADH-NAD(+) Redox Visualized in Brain Slices by Two-Photon Fluorescence Lifetime Biosensor Imaging. *Antioxid Redox Signal*, 25(10):553-563. 10.1089/ars.2015.6593
- Monsefi, M., Tajerian, T. & Rowan, A. 2020. Size-Controlled Synthesis of Gold Nanostars and Their Characterizations and Plasmon Resonances. *Journal of Nanostructures*, 10(2):198-205. 10.22052/jns.2020.02.001
- Montes, S. & Belén, A. 2016. Gold nanostars : synthesis, stabilization and applications as surface-enhanced Raman scattering tags. In.
- Moses Phiri, M., Wingrove Mulder, D., Mason, S. & Christiaan Vorster, B. 2019. Facile immobilization of glucose oxidase onto gold nanostars with enhanced binding affinity and optimal function. *Royal Society Open Science*, 6(5):190205. 10.1098/rsos.190205
- Mulder, D., Phiri, M. & Vorster, C. 2019a. Tailor-made gold nanostar colorimetric detection determined by morphology change and used as an indirect approach by using hydrogen peroxide to determine glucose concentration. *Sensing and Bio-Sensing Research*, 25:100296. 10.1016/j.sbsr.2019.100296
- Mulder, D., Phiri, M.M. & Vorster, B.C. 2019b. Tailor-made gold nanostar colorimetric detection determined by morphology change and used as an indirect approach by using hydrogen peroxide to determine glucose concentration. *Sensing and Bio-Sensing Research*, 25, 10.1016/j.sbsr.2019.100296
- Mulder, D.W., Phiri, M.M. & Vorster, B.C. 2019c. Gold Nanostar Colorimetric Detection of Fructosyl Valine as a Potential Future Point of Care Biosensor Candidate for Glycated Haemoglobin Detection. *Biosensors*, 9(3):E100.

Date of access: 2019/08//. 10.3390/bios9030100

Mulder, D.W., Phiri, M.M., Jordaan, A. & Vorster, B.C. 2019. Modified HEPES one-pot synthetic strategy for gold nanostars. *Royal Society Open Science*, 6(6):190160. Date of access: 2023/09/18. 10.1098/rsos.190160

Mulder, D.W.P., M. M; Jordaan, A; Vorster, B. C. 2019. Modified HEPES one-pot synthetic strategy for gold nanostars. *Royal Society Open Science*, 6(6):190160. 10.1098/rsos.190160

Munyayi, T.A., Vorster, B.C. & Mulder, D.W. 2022. The Effect of Capping Agents on Gold Nanostar Stability, Functionalization, and Colorimetric Biosensing Capability. *Nanomaterials (Basel)*, 12(14), 10.3390/nano12142470

Nagahara, N. 2011. Catalytic Site Cysteines of Thiol Enzyme: Sulfurtransferases. *Journal of Amino Acids*, 2011:709404. 10.4061/2011/709404

Nagel, B., Dellweg, H. & Gierasch, L.M. 1992. Glossary for chemists of terms used in biotechnology (IUPAC Recommendations 1992). *Pure and Applied Chemistry*, 64(1):143-168. Date of access: 2023-09-08. doi:10.1351/pac199264010143

Nalbant Esenturk, E. & Hight Walker, A.R. 2009. Surface-enhanced Raman scattering spectroscopy via gold nanostars. *Journal of Raman Spectroscopy*, 40(1):86-91. 10.1002/jrs.2084

Naresh, V. & Lee, N. 2021. A Review on Biosensors and Recent Development of Nanostructured Materials-Enabled Biosensors. *Sensors*, 21(4):1109. 10.3390/s21041109

Nehl, C.L., Liao, H. & Hafner, J.H. 2006a. Optical properties of star-shaped gold nanoparticles. *Nano Letters*, 6(4):683-688. 10.1021/nl052409y

Nehl, C.L., Liao, H. & Hafner, J.H. 2006b. Optical Properties of Star-Shaped Gold Nanoparticles. *Nano Letters*, 6(4):683-688. 10.1021/nl052409y

Nelson, D.L., Lehninger, A.L. & Cox, M.M. 2008. *Lehninger Principles of Biochemistry*. 2. Oxford, New York: W.H. Freeman.

Neouze, M.-A. & Schubert, U. 2008. Surface Modification and Functionalization of Metal and Metal Oxide Nanoparticles by Organic Ligands. *Monatshefte für Chemie - Chemical Monthly*, 139(3):183-195. 10.1007/s00706-007-0775-2

Nesakumar, N., Sethuraman, S., Krishnan, U.M. & Rayappan, J.B.B. 2013. Fabrication of lactate biosensor based on lactate dehydrogenase immobilized on cerium oxide nanoparticles. *Journal of Colloid and Interface Science*, 410:158-164. 10.1016/j.jcis.2013.08.009

Ning, C. & Segal, S. 2000. Plasma galactose and galactitol concentration in patients with galactose-1-phosphate uridylyltransferase deficiency galactosemia: Determination by gas chromatography/mass spectrometry. *Metabolism*, 49(11):1460-1466. 10.1053/meta.2000.9512

Niu, Y. & Crooks, R.M. 2003. Dendrimer-encapsulated metal nanoparticles and their applications to catalysis. *Comptes Rendus Chimie*, 6(8):1049-1059. 10.1016/j.crci.2003.08.001

- Niu, Z. & Li, Y. 2014. Removal and Utilization of Capping Agents in Nanocatalysis. *Chemistry of Materials*, 26(1):72-83. 10.1021/cm4022479
- Noah, N.M. & Ndangili, P.M. 2019. Current Trends of Nanobiosensors for Point-of-Care Diagnostics. *Journal of Analytical Methods in Chemistry*, 2019:2179718. 10.1155/2019/2179718
- Noël, S., Léger, B., Ponchel, A., Philippot, K., Denicourt-Nowicki, A., Roucoux, A. & Monflier, E. 2014. Cyclodextrin-based systems for the stabilization of metallic(0) nanoparticles and their versatile applications in catalysis. *Catalysis Today*, 235:20 - 32. 10.1016/j.cattod.2014.03.030
- Nowicka, A.M., Hasse, U., Hermes, M. & Scholz, F. 2010. Hydroxyl radicals attack metallic gold. *Angewandte Chemie International Edition*, 49(6):1061-1063. 10.1002/anie.200906358
- Oka, K., Takahashi, T. & Hori, S.H. 1981. Differential effects of the NADPH/NADP⁺ ratio on the activities of hexose-6-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase. *Biochim Biophys Acta*, 662(2):318-325. 10.1016/0005-2744(81)90045-0
- Oraby, E.A. & Eksteen, J.J. 2015. The leaching of gold, silver and their alloys in alkaline glycine–peroxide solutions and their adsorption on carbon. *Hydrometallurgy*, 152:199-203. 10.1016/j.hydromet.2014.12.015
- Orfanos, A.P., Jinks, D.C. & Guthrie, R. 1986. Microassay for estimation of galactose and galactose-1-phosphate in dried blood specimens. *Clinical Biochemistry*, 19(4):225-228. 10.1016/s0009-9120(86)80031-5
- Ortiz-Castillo, J.E., Gallo-Villanueva, R.C., Madou, M.J. & Perez-Gonzalez, V.H. 2020. Anisotropic gold nanoparticles: A survey of recent synthetic methodologies. *Coordination Chemistry Reviews*, 425:213489. 10.1016/j.ccr.2020.213489
- Pakiari, A.H. & Jamshidi, Z. 2010. Nature and Strength of M–S Bonds (M = Au, Ag, and Cu) in Binary Alloy Gold Clusters. *The Journal of Physical Chemistry A*, 114(34):9212-9221. 10.1021/jp100423b
- Pallavicini, P., Cabrini, E. & Borzenkov, M. 2015. Gold Nanostar Synthesis and Functionalization with Organic Molecules. In. pp. 1-23.
- Pallavicini, P., Cabrini, E., Cavallaro, G., Chirico, G., Collini, M., D'Alfonso, L., ... Taglietti, A. 2015. Gold nanostars coated with neutral and charged polyethylene glycols: A comparative study of in-vitro biocompatibility and of their interaction with SH-SY5Y neuroblastoma cells. *Journal of Inorganic Biochemistry*, 151:123-131. 10.1016/j.jinorgbio.2015.05.002
- Patra, J.K. & Baek, K.-H. 2014. Green Nanobiotechnology: Factors Affecting Synthesis and Characterization Techniques. *Journal of Nanomaterials*, 2014:417305. 10.1155/2014/417305
- Patterson, G.H., Knobel, S.M., Arkhammar, P., Thastrup, O. & Piston, D.W. 2000. Separation of the glucose-stimulated cytoplasmic and mitochondrial NAD(P)H responses in pancreatic islet β cells. *Proceedings of the National Academy of Sciences*, 97(10):5203-5207. 10.1073/pnas.090098797

- Pazos-Perez, N., Guerrini, L. & Alvarez-Puebla, R.A. 2018. Plasmon Tunability of Gold Nanostars at the Tip Apexes. *ACS Omega*, 3(12):17173-17179. 10.1021/acsomega.8b02686
- Pedrosa, P., Vinhas, R., Fernandes, A. & Baptista, P.V. 2015. Gold Nanotheranostics: Proof-of-Concept or Clinical Tool? *Nanomaterials*, 5(4):1853-1879. 10.3390/nano5041853
- Pedroso-Santana, S. & Fleitas-Salazar, N. 2023. The Use of Capping Agents in the Stabilization and Functionalization of Metallic Nanoparticles for Biomedical Applications. *Particle & Particle Systems Characterization*, 40(2):2200146. 10.1002/ppsc.202200146
- Pellis, A., Guebitz, G.M. & Nyanhongo, G.S. 2022. Chitosan: Sources, Processing and Modification Techniques. *Gels*, 8(7), 10.3390/gels8070393
- Penning, T.M. 2015. The aldo-keto reductases (AKRs): Overview. *Chem Biol Interact*, 234:236-246. 10.1016/j.cbi.2014.09.024
- Perez-Gonzalez, V., Ortiz Castillo, J., Gallo-Villanueva, R. & Madou, M. 2020. Anisotropic gold nanoparticles: A survey of recent synthetic methodologies. *Coordination Chemistry Reviews*, 425:213489. 10.1016/j.ccr.2020.213489
- Perrault, S.D. & Chan, W.C. 2009. Synthesis and surface modification of highly monodispersed, spherical gold nanoparticles of 50-200 nm. *American Chemistry Society*, 131(47):17042-17043. 10.1021/ja907069u
- Personick, M.L., Langille, M.R., Zhang, J. & Mirkin, C.A. 2011. Shape control of gold nanoparticles by silver underpotential deposition. *Nano Letters*, 11(8):3394-3398. 10.1021/nl201796s
- Phiri, M., Mulder, D. & Vorster, C. 2019a. Seedless gold nanostars with seed-like advantages for biosensing applications. *Royal Society Open Science*, 6:181971. 10.1098/rsos.181971
- Phiri, M.M., Mulder, D.W. & Vorster, B.C. 2019b. Seedless gold nanostars with seed-like advantages for biosensing applications. *Royal Society Open Science*, 6(2):181971. 10.1098/rsos.181971
- Phiri, M.M., Mulder, D.W. & Vorster, B.C. 2019c. Plasmonic Detection of Glucose in Serum Based on Biocatalytic Shape-Altering of Gold Nanostars. *Biosensors (Basel)*, 9(3), 10.3390/bios9030083
- Pinheiro, T., Ferrao, J., Marques, A.C., Oliveira, M.J., Batra, N.M., Costa, P., ... Fortunato, E. 2020. Paper-Based In-Situ Gold Nanoparticle Synthesis for Colorimetric, Non-Enzymatic Glucose Level Determination. *Nanomaterials (Basel)*, 10(10), 10.3390/nano10102027
- Pinzaru, I., Coricovac, D., Dehelean, C., Moacă, E.A., Mioc, M., Baderca, F., ... Șoica, C. 2018. Stable PEG-coated silver nanoparticles - A comprehensive toxicological profile. *Food Chemistry Toxicology*, 111:546-556. 10.1016/j.fct.2017.11.051
- Plapp, B.V. 2010. Conformational changes and catalysis by alcohol dehydrogenase. *Arch Biochem Biophys*, 493(1):3-12. 10.1016/j.abb.2009.07.001

Poblete, H. & Comer, J. 2019. Chapter 2 - Computational modeling of the adsorption of capping agent biomolecules to inorganic nanoparticles. In: Prieto, J.P. & Béjar, M.G., eds. *Photoactive Inorganic Nanoparticles*: Elsevier. pp. 21-41.

Poklepovich-Caride, S., Oestreicher, V., Mercedes Zalduendo, M., Bordoni, A.V., Soler-Illia, G.J.A.A. & Angelomé, P.C. 2022. A versatile one-pot room temperature approach for the synthesis of gold nanoparticles with multiple sizes and shapes. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 646:128890. 10.1016/j.colsurfa.2022.128890

Pollak, N., Dölle, C. & Ziegler, M. 2007. The power to reduce: pyridine nucleotides--small molecules with a multitude of functions. *Biochem J*, 402(2):205-218. 10.1042/bj20061638

Prasad, S. 2014. Nanobiosensors: the future for diagnosis of disease? *Nanobiosensors in Disease Diagnosis*, 3:1. 10.2147/NDD.S39421

Quintanilla, A., Butselaar-Orthlieb, V.C.L., Kwakernaak, C., Sloof, W.G., Kreutzer, M.T. & Kapteijn, F. 2010a. Weakly bound capping agents on gold nanoparticles in catalysis: Surface poison? *Journal of Catalysis*, 271(1):104-114. 10.1016/j.jcat.2010.02.013

Quintanilla, A., Butselaar-Orthlieb, V.C.L., Kwakernaak, C., Sloof, W.G., Kreutzer, M. & Kapteijn, F. 2010b. Weakly bound capping agents on gold nanoparticles in catalysis: Surface poison? *Journal of Catalysis*, 271:104-114. 10.1016/j.jcat.2010.02.013

Radwan, S.H. & Azzazy, H.M. 2009. Gold nanoparticles for molecular diagnostics. *Expert Rev Mol Diagn*, 9(5):511-524. 10.1586/erm.09.33

Rahman, D.S., Chatterjee, H. & Ghosh, S.K. 2015. Excess Surface Energy at the Tips of Gold Nanospikes: From Experiment to Modeling. *The Journal of Physical Chemistry C*, 119(25):14326-14337. 10.1021/acs.jpcc.5b03944

Rajitha, B., Malla, R.R., Vadde, R., Kasa, P., Prasad, G.L.V., Farran, B., ... Nagaraju, G.P. 2021. Horizons of nanotechnology applications in female specific cancers. *Semin Cancer Biol*, 69:376-390. 10.1016/j.semcancer.2019.07.005

Ramírez, L.P., Bournel, F., Gallet, J.-J., Dudy, L. & Rochet, F. 2022. Testing the Cabrera–Mott Oxidation Model for Aluminum under Realistic Conditions with Near-Ambient Pressure Photoemission. *The Journal of Physical Chemistry C*, 126(5):2517-2530. 10.1021/acs.jpcc.1c09388

Rao, X., Tatoulis, M., Guyon, C., Ognier, S., Chu, C. & Abou Hassan, A. 2019. A Comparison Study of Functional Groups (Amine vs. Thiol) for Immobilizing AuNPs on Zeolite Surface. *Nanomaterials (Basel)*, 9(7), 10.3390/nano9071034

Raval, N., Maheshwari, R., Kalyane, D., Youngren-Ortiz, S.R., Chougule, M.B. & Tekade, R.K. 2019. Importance of Physicochemical Characterization of Nanoparticles in Pharmaceutical Product Development. *Basic Fundamentals of Drug Delivery*,

- Raveendran, P., Fu, J. & Wallen, S.L. 2003. Completely “Green” Synthesis and Stabilization of Metal Nanoparticles. *Journal of the American Chemical Society*, 125(46):13940-13941. 10.1021/ja029267j
- Reimers, J.R., Ford, M.J., Halder, A., Ulstrup, J. & Hush, N.S. 2016. Gold surfaces and nanoparticles are protected by Au(0)π-thiol species and are destroyed when Au(I)π-thiolates form. *Proceedings of the National Academy of Sciences*, 113(11):E1424-E1433. 10.1073/pnas.1600472113
- Restrepo, C.V. & Villa, C.C. 2021. Synthesis of silver nanoparticles, influence of capping agents, and dependence on size and shape: A review. *Environmental Nanotechnology, Monitoring & Management*, 15:100428. 10.1016/j.enmm.2021.100428
- Robinson, P.K. 2015. Enzymes: principles and biotechnological applications. *Essays Biochem*, 59:1-41. 10.1042/bse0590001
- Rodrigues, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R. & Fernandez-Lafuente, R. 2021. Stabilization of enzymes via immobilization: Multipoint covalent attachment and other stabilization strategies. *Biotechnology Advances*, 52:107821. 10.1016/j.biotechadv.2021.107821
- Rodríguez-Lorenzo, L., de la Rica, R., Álvarez-Puebla, R.A., Liz-Marzán, L.M. & Stevens, M.M. 2012. Plasmonic nanosensors with inverse sensitivity by means of enzyme-guided crystal growth. *Nature Materials*, 11(7):604-607. 10.1038/nmat3337
- Rodríguez-Lorenzo, L., de la Rica, R., Álvarez-Puebla, R.A., Liz-Marzán, L.M. & Stevens, M.M. 2018. Addendum: Plasmonic nanosensors with inverse sensitivity by means of enzyme-guided crystal growth. *Nature Materials*, 17(2):205-207. 10.1038/nmat5069
- Roduner, E. 2006. Size matters: why nanomaterials are different. *Chemistry Society Reviews*, 35(7):583-592. 10.1039/b502142c
- Roh, S., Chung, T. & Lee, B. 2011. Overview of the Characteristics of Micro- and Nano-Structured Surface Plasmon Resonance Sensors. *Sensors*, 11(2):1565-1588. 10.3390/s110201565
- Rucker, R.B. & Chohanadisai, W. 2016. Coenzymes and Cofactors. In: Caballero, B., Finglas, P.M. & Toldrá, F., eds. *Encyclopedia of Food and Health*. Oxford: Academic Press. pp. 206-224.
- Safo, I.A., Werheid, M., Dosche, C. & Oezaslan, M. 2019. The role of polyvinylpyrrolidone (PVP) as a capping and structure-directing agent in the formation of Pt nanocubes. *Nanoscale Advances*, 1(8):3095-3106. 10.1039/C9NA00186G
- Saha, K., Agasti, S.S., Kim, C., Li, X. & Rotello, V.M. 2012. Gold nanoparticles in chemical and biological sensing. *Chemical Reviews*, 112(5):2739-2779. 10.1021/cr2001178
- Sajitha, M., Vindhyasarumi, A., Gopi, A. & Yoosaf, K. 2015. Shape controlled synthesis of multi-branched gold nanocrystals through a facile one-pot bifunctional biomolecular approach. *RSC Advances*, 5(119):98318-98324. 10.1039/C5RA19098C

- Samsuri, N.D., Mukhtar, W.M., Abdul Rashid, A.R., Ahmad Dasuki, K. & Awangku Yussuf, A.A.R.H. 2017. Synthesis methods of gold nanoparticles for Localized Surface Plasmon Resonance (LSPR) sensor applications. *EPJ Web Conf.*, 162:01002.
- Sánchez-Iglesias, A., Kruse, J., Chuvilin, A. & Grzelczak, M. 2021. Coupling plasmonic catalysis and nanocrystal growth through cyclic regeneration of NADH. *Nanoscale*, 13(36):15188-15192. 10.1039/D1NR04400A
- Sandhya Rani, G. 2020. Applications of Oxidoreductases. In: Mahmoud Ahmed, M., ed. *Oxidoreductase*. Rijeka: IntechOpen. p Ch. 2.
- Saptarshi, S.R., Duschl, A. & Lopata, A.L. 2013. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. *Journal of Nanobiotechnology*, 11:26. 10.1186/1477-3155-11-26
- Sasidharan, S., Bahadur, D. & Srivastava, R. 2016. Albumin stabilized gold nanostars: a biocompatible nanoplatform for SERS, CT imaging and photothermal therapy of cancer. *RSC Advances*, 6(87):84025-84034. 10.1039/C6RA11405A
- Sau, T.K. & Murphy, C.J. 2004. Room Temperature, High-Yield Synthesis of Multiple Shapes of Gold Nanoparticles in Aqueous Solution. *Journal of the American Chemical Society*, 126(28):8648-8649. 10.1021/ja047846d
- Saverot, S., Geng, X., Leng, W., Vikesland, P.J., Grove, T.Z. & Bickford, L.R. 2016. Facile, tunable, and SERS-enhanced HEPES gold nanostars. *RSC Advances*, 6(35):29669-29673. 10.1039/C6RA00450D
- Sawant, S.N. 2017. 13 - Development of Biosensors From Biopolymer Composites. In: Sadasivuni, K.K., Ponnammma, D., Kim, J., Cabibihan, J.J. & AlMaadeed, M.A., eds. *Biopolymer Composites in Electronics*: Elsevier. pp. 353-383.
- Seaberg, J., Clegg, J.R., Bhattacharya, R. & Mukherjee, P. 2023. Self-Therapeutic Nanomaterials: Applications in Biology and Medicine. *Mater Today (Kidlington)*, 62:190-224. 10.1016/j.mattod.2022.11.007
- Seaberg, J., Montazerian, H., Hossen, M.N., Bhattacharya, R., Khademhosseini, A. & Mukherjee, P. 2021. Hybrid Nanosystems for Biomedical Applications. *ACS Nano*, 15(2):2099-2142. 10.1021/acsnano.0c09382
- Sears, P.S. 2020. Leloir pathway. In: *Catalysis from A to Z*.
- Seoudi, R. 2011. Studies on the Effect of the Capping Materials on the Spherical Gold Nanoparticles Catalytic Activity. *World Journal of Nano Science and Engineering*, 1:51-61. 10.4236/wjnse.2011.12008
- Septisetyani, E.P., Ningrum, R.A., Romadhani, Y., Wisnuwardhani, P.H. & Santoso, A. 2014. Optimization of Sodium Dodecyl Sulphate as a Formazan Solvent And Comparison of 3-(4,5-Dimethylthiazo-2-YL)-2,5-Diphenyltetrazolium Bromide (MTT) Assay With WST-1 Assay in MCF-

- 7 Cells. *Indonesian Journal of Pharmacy*, 25(4):245-254. 10.14499/indonesianjpharm25iss4pp245
- Shameli, K., Ahmad, M.B., Zamanian, A., Sangpour, P., Shabanzadeh, P., Abdollahi, Y. & Zargar, M. 2012. Green biosynthesis of silver nanoparticles using *Curcuma longa* tuber powder. *International Journal of Nanomedicine*, 7:5603-5610. 10.2147/ijn.S36786
- Shao, D., Liu, C., Tsow, F., Yang, Y., Du, Z., Iriya, R., ... Tao, N. 2016. Noncontact Monitoring of Blood Oxygen Saturation Using Camera and Dual-Wavelength Imaging System. *IEEE Trans Biomed Eng*, 63(6):1091-1098. 10.1109/tbme.2015.2481896
- Shao, L., Susha, A.S., Cheung, L.S., Sau, T.K., Rogach, A.L. & Wang, J. 2012. Plasmonic properties of single multispiked gold nanostars: correlating modeling with experiments. *Langmuir*, 28(24):8979-8984. 10.1021/la2048097
- Sharma, V.K., Hutchison, J.M. & Allgeier, A.M. 2022. Redox Biocatalysis: Quantitative Comparisons of Nicotinamide Cofactor Regeneration Methods. *ChemSusChem*, 15(22):e202200888. 10.1002/cssc.202200888
- Shenhar, R., Norsten, T.B. & Rotello, V.M. 2005. Polymer-Mediated Nanoparticle Assembly: Structural Control and Applications. *Advanced Materials*, 17(6):657-669. 10.1002/adma.200401291
- Shi, H., Asahi, R. & Stampfl, C. 2007. Properties of the gold oxides Au₂O₃ and Au₂O: First-principles investigation. *Physical Review B* 75, 10.1103/PhysRevB.75.205125
- Shipway, A.N., Katz, E. & Willner, I. 2000. Nanoparticle arrays on surfaces for electronic, optical, and sensor applications. *Chemistry Europe-ChemPhysChem*, 1(1):18-52. 10.1002/1439-7641(20000804)1:1<18::Aid-cphc18>3.0.Co;2-I
- Si, P., Razmi, N., Nur, O., Solanki, S., Pandey, C.M., Gupta, R.K., ... de la Zerda, A. 2021. Gold nanomaterials for optical biosensing and bioimaging. *Nanoscale Advances*, 3(10):2679-2698. 10.1039/D0NA00961J
- Siddique, N.Z.F.S.F. 2021. Use of nanomaterials for the immobilization of industrially important enzymes. *Journal of Nanotechnology Research*, 11:381-391. 10.26502/jnr.2688-85210023
- Sidhu, A.K., Verma, N. & Kaushal, P. 2022. Role of Biogenic Capping Agents in the Synthesis of Metallic Nanoparticles and Evaluation of Their Therapeutic Potential. *Frontiers in Nanotechnology*, 3, 10.3389/fnano.2021.801620
- Siegel, A.L. & Baker, G.A. 2021. Bespoke nanostars: synthetic strategies, tactics, and uses of tailored branched gold nanoparticles. *Nanoscale Advances*, 3(14):3980-4004. 10.1039/D0NA01057J
- Simonelli, F., Rossi, G. & Monticelli, L. 2019. Role of Ligand Conformation on Nanoparticle-Protein Interactions. *The Journal of Physical Chemistry B*, 123(8):1764-1769. 10.1021/acs.jpcc.8b11204

Singhana, B., Slattery, P., Chen, A., Wallace, M. & Melancon, M.P. 2014. Light-activatable gold nanoshells for drug delivery applications. *AAPS PharmSciTech*, 15(3):741-752. 10.1208/s12249-014-0097-8

Singletery, M., Hagerty, S., Muramoto, S., Daniels, Y., MacCrehan, W.A., Stan, G., ... Vodyanoy, V. 2017. PEGylation of zinc nanoparticles amplifies their ability to enhance olfactory responses to odorant. *PLOS ONE*, 12(12):e0189273. 10.1371/journal.pone.0189273

Smith, D.K. & Korgel, B.A. 2008. The importance of the CTAB surfactant on the colloidal seed-mediated synthesis of gold nanorods. *Langmuir*, 24(3):644-649. 10.1021/la703625a

Sosa, I.O., Noguez, C. & Barrera, R.G. 2003. Optical Properties of Metal Nanoparticles with Arbitrary Shapes. *The Journal of Physical Chemistry B*, 107(26):6269-6275. 10.1021/jp0274076

Spät, A. & Pitter, J.G. 2004. The effect of cytoplasmic Ca²⁺ signal on the redox state of mitochondrial pyridine nucleotides. *Mol Cell Endocrinol*, 215(1-2):115-118. 10.1016/j.mce.2003.11.004

Sperling, R.A. & Parak, W.J. 2010. Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, 368(1915):1333-1383. 10.1098/rsta.2009.0273

Sperling, R.A., Rivera Gil, P., Zhang, F., Zanella, M. & Parak, W.J. 2008. Biological applications of gold nanoparticles. *Chemistry Society Reviews*, 37(9):1896-1908. 10.1039/b712170a

Srivastava, S.K. & Beutler, E. 1969. Auxiliary pathways of galactose metabolism. Identification of reaction products of hexose 6-phosphate dehydrogenase and of "galactose dehydrogenase". *The Journal of Biological Chemistry*, 244(23):6377-6382.

St John, A. & Price, C.P. 2014. Existing and Emerging Technologies for Point-of-Care Testing. *The Clinical Biochemist Reviews*, 35(3):155-167.

Stankus, D.P., Lohse, S.E., Hutchison, J.E. & Nason, J.A. 2011. Interactions between Natural Organic Matter and Gold Nanoparticles Stabilized with Different Organic Capping Agents. *Environmental Science & Technology*, 45(8):3238-3244. 10.1021/es102603p

Sun, K., Kohyama, M., Tanaka, S. & Takeda, S. 2015. Understanding of the activity difference between nanogold and bulk gold by relativistic effects. *Journal of Energy Chemistry*, 24(4):485-489. 10.1016/j.jechem.2015.06.006

Syedmoradi, L., Daneshpour, M., Alvandipour, M., Gomez, F.A., Hajghassem, H. & Omidfar, K. 2017. Point of care testing: The impact of nanotechnology. *Biosensors and Bioelectronics*, 87:373-387. 10.1016/j.bios.2016.08.084

Tang, L. & Li, J. 2017. Plasmon-based colorimetric nanosensors for ultrasensitive molecular diagnostics. *ACS sensors*, 2(7):857-875. 10.1021/acssensors.7b00282

- Temoçin, Z. 2021. Designing of a stable and selective glucose biosensor by glucose oxidase immobilization on glassy carbon electrode sensitive to H₂O₂ via nanofiber interface. *Journal of Applied Electrochemistry*, 51(2):283-293. 10.1007/s10800-020-01502-4
- Teodoro, K.B.R., Migliorini, F.L., Christinelli, W.A. & Correa, D.S. 2019. Detection of hydrogen peroxide (H₂O₂) using a colorimetric sensor based on cellulose nanowhiskers and silver nanoparticles. *Carbohydrate Polymers*, 212:235-241. 10.1016/j.carbpol.2019.02.053
- Testa, B. & Meyer, U.A. 1996. Aldose Reductase Inhibitors as New Antidiabetic Drugs. In: Testa, B. & Meyer, U.A., eds. *Advances in Drug Research*. 27: Academic Press. pp. 171-178.
- Tofanello, A., Miranda, É.G.A., Dias, I.W.R., Lanfredi, A.J.C., Arantes, J.T., Juliano, M.A. & Nantes, I.L. 2016. pH-Dependent Synthesis of Anisotropic Gold Nanostructures by Bioinspired Cysteine-Containing Peptides. *ACS Omega*, 1(3):424-434. 10.1021/acsomega.6b00140
- Tonga, G.Y., Saha, K. & Rotello, V.M. 2014. 25th Anniversary Article: Interfacing Nanoparticles and Biology: New Strategies for Biomedicine. *Advanced Materials*, 26(3):359-370. 10.1002/adma.201303001
- Trudel, S. 2011. Unexpected magnetism in gold nanostructures: making gold even more attractive. *Gold Bulletin*, 44(1):3-13. 10.1007/s13404-010-0002-5
- Tsoulos, T. & Fabris, L. 2018. Interface and Bulk Standing Waves Drive the Coupling of Plasmonic Nanostar Antennas. *The Journal of Physical Chemistry C*, 122, 10.1021/acs.jpcc.8b09263
- Tsoulos, T., Atta, S., Lagos, M., Batson, P., Tsilomelekis, G. & Fabris, L. 2018. *Rational Design of Gold Nanostars with Tailorable Plasmonic Properties*. 1. Cambridge University Press.
- Tsoulos, T.V., Han, L., Weir, J., Xin, H.L. & Fabris, L. 2017. A closer look at the physical and optical properties of gold nanostars: an experimental and computational study. *Nanoscale*, 9(11):3766-3773. 10.1039/C6NR09091E
- Tsoulos, T.V., Atta, S., Lagos, M.J., Beetz, M., Batson, P.E., Tsilomelekis, G. & Fabris, L. 2019. Colloidal plasmonic nanostar antennas with wide range resonance tunability. *Nanoscale*, 11(40):18662-18671. 10.1039/C9NR06533D
- Tsukatani, T., Ide, S., Ono, M. & Matsumoto, K. 2007. New tetrazolium method for phosphatase assay using ascorbic acid 2-phosphate as a substrate. *Talanta*, 73(3):471-475. 10.1016/j.talanta.2007.04.017
- Tsunoyama, H., Ichikuni, N., Sakurai, H. & Tsukuda, T. 2009. Effect of Electronic Structures of Au Clusters Stabilized by Poly(N-vinyl-2-pyrrolidone) on Aerobic Oxidation Catalysis. *Journal of the American Chemical Society*, 131(20):7086-7093. 10.1021/ja810045y
- Turkevich, J., Stevenson, P.C. & Hillier, J. 1951. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discussions of the Faraday Society*, 11(0):55-75. 10.1039/DF9511100055

Ueberschär, K.-H., BLACHNITZKY, E.-O. & KURZ, G. 1974. Reaction Mechanism of d-Galactose Dehydrogenases from *Pseudomonas saccharophila* and *Pseudomonas fluorescens*. *European Journal of Biochemistry*, 48(2):389-405. 10.1111/j.1432-1033.1974.tb03780.x

Umrikhina, A.V., Luganskaya, A.N. & Krasnovsky, A.A. 1990. ESR signals of NADH and NADPH under illumination. *FEBS Letters*, 260(2):294-296. 10.1016/0014-5793(90)80127-5

Unser, S., Bruzas, I., He, J. & Sagle, L. 2015. Localized Surface Plasmon Resonance Biosensing: Current Challenges and Approaches. *Sensors (Basel)*, 15(7):15684-15716. 10.3390/s150715684

Urbieto, M., Barbry, M., Zhang, Y., Koval, P., Sánchez-Portal, D., Zabala, N. & Aizpurua, J. 2018. Atomic-Scale Lightning Rod Effect in Plasmonic Picocavities: A Classical View to a Quantum Effect. *ACS Nano*, 12(1):585-595. 10.1021/acsnano.7b07401

Vargas, J.A., Leonardo, D.A., D'Muniz Pereira, H., Lopes, A.R., Rodriguez, H.N., Cobos, M., ... Garratt, R.C. 2022. Structural Characterization of L-Galactose Dehydrogenase: An Essential Enzyme for Vitamin C Biosynthesis. *Plant Cell Physiology*, 63(8):1140-1155. 10.1093/pcp/pcac090

Vega, M.M., Bonifacio, A., Lughì, V., Marsi, S., Carrato, S. & Sergio, V. 2014. Long-term stability of surfactant-free gold nanostars. *Journal of Nanoparticle Research*, 16(11):2729. 10.1007/s11051-014-2729-z

Veskoukis, A.S., Margaritelis, N.V., Kyparos, A., Paschalis, V. & Nikolaidis, M.G. 2018. Spectrophotometric assays for measuring redox biomarkers in blood and tissues: the NADPH network. *Redox Rep*, 23(1):47-56. 10.1080/13510002.2017.1392695

Vilela, D., González, M.C. & Escarpa, A. 2012. Sensing colorimetric approaches based on gold and silver nanoparticles aggregation: Chemical creativity behind the assay. A review. *Analytica Chimica Acta*, 751:24-43. 10.1016/j.aca.2012.08.043

Vinnacombe-Willson, G.A., Chiang, N., Weiss, P.S., Tolbert, S.H. & Scarabelli, L. 2021. Seeded-Growth Experiment Demonstrating Size- and Shape-Dependence on Gold Nanoparticle–Light Interactions. *Journal of Chemical Education*, 98(2):546-552. 10.1021/acs.jchemed.0c01150

Virkutyte, J. & Varma, R.S. 2011. Green synthesis of metal nanoparticles: Biodegradable polymers and enzymes in stabilization and surface functionalization. *Chemical Science*, 2(5):837-846. 10.1039/C0SC00338G

Walper, S.A., Turner, K.B. & Medintz, I.L. 2015. Enzymatic bioconjugation of nanoparticles: developing specificity and control. *Current Opinion in Biotechnology*, 34:232-241. 10.1016/j.copbio.2015.04.003

Wang, J., Xu, Z. & Kotsifaki, D.G. 2023. Plasmonic and metamaterial biosensors: a game-changer for virus detection. *Sensors & Diagnostics*, 2(3):600-619. 10.1039/D2SD00217E

- Wang, Y., van Asdonk, K. & Zijlstra, P. 2019. A Robust and General Approach to Quantitatively Conjugate Enzymes to Plasmonic Nanoparticles. *Langmuir : the ACS journal of surfaces and colloids*, 35(41):13356-13363. 10.1021/acs.langmuir.9b01879
- Wang, Y., Liu, Y., Wu, Q., Fu, R., Liu, H., Cui, Y., ... He, Y. 2022. Seed-mediated in situ growth of photothermal reagent gold nanostars: Mechanism study and preliminary assay application. *Analytica Chimica Acta*, 1231:340424. 10.1016/j.aca.2022.340424
- Webb, J.A. & Bardhan, R. 2014. Emerging advances in nanomedicine with engineered gold nanostructures. *Nanoscale*, 6(5):2502-2530. 10.1039/C3NR05112A
- Webb, J.A., Erwin, W.R., Zarick, H.F., Aufrecht, J., Manning, H.W., Lang, M.J., ... Bardhan, R. 2014a. Geometry-Dependent Plasmonic Tunability and Photothermal Characteristics of Multibranched Gold Nanoantennas. *Journal of Physical Chemistry C*, 118:3696-3707. 10.1021/jp408793a
- Webb, J.A., Erwin, W.R., Zarick, H.F., Aufrecht, J., Manning, H.W., Lang, M.J., ... Bardhan, R. 2014b. Geometry-Dependent Plasmonic Tunability and Photothermal Characteristics of Multibranched Gold Nanoantennas. *The Journal of Physical Chemistry C*, 118(7):3696-3707. 10.1021/jp408793a
- Weiner, H., Duester, G., Maser, E. & Plapp, B. 2009. Enzymology and molecular biology of carbonyl metabolism. Introduction. *Chem Biol Interact*, 178(1-3):1. 10.1016/j.cbi.2008.11.003
- Wen, X., Ou, L., Cutshaw, G., Uthaman, S., Ou, Y.C., Zhu, T., ... Bardhan, R. 2023. Physicochemical Properties and Route of Systemic Delivery Control the In Vivo Dynamics and Breakdown of Radiolabeled Gold Nanostars. *Small*, 19(29):e2204293. 10.1002/smll.202204293
- Weng, G., Zhao, X., Zhao, J., Li, J., Zhu, J. & Zhao, J. 2019. Nanoplasmonic sensing of NADH by inhibiting the oxidative etching of gold nanorods. *Sensors and Actuators B: Chemical*, 299:126982. 10.1016/j.snb.2019.126982
- Whittaker, M.M. & Whittaker, J.W. 2000. Expression of Recombinant Galactose Oxidase by *Pichia pastoris*. *Protein Expression and Purification*, 20(1):105-111. 10.1006/prep.2000.1287
- Wu, H.-L., Chen, C.-H. & Huang, M.H. 2009. Seed-Mediated Synthesis of Branched Gold Nanocrystals Derived from the Side Growth of Pentagonal Bipyramids and the Formation of Gold Nanostars. *Chemistry of Materials*, 21(1):110-114. 10.1021/cm802257e
- Wu, S., Tan, S.Y., Ang, C.Y., Luo, Z. & Zhao, Y. 2016. Oxidation-triggered aggregation of gold nanoparticles for naked-eye detection of hydrogen peroxide. *Chemical Communications*, 52(17):3508-3511. 10.1039/C5CC09447J
- Wu, Y. & Hu, S. 2007. Biosensors based on direct electron transfer in redox proteins. *Microchimica Acta*, 159:1-17. 10.1007/s00604-007-0749-4
- Xi, W. & Haes, A.J. 2019. Elucidation of HEPES Affinity to and Structure on Gold Nanostars. *Journal of the American Chemical Society*, 141(9):4034-4042. 10.1021/jacs.8b13211

- Xi, W., Phan, H.T. & Haes, A.J. 2018. How to accurately predict solution-phase gold nanostar stability. *Analytical and Bioanalytical Chemistry*, 410(24):6113-6123. 10.1007/s00216-018-1115-6
- Xianyu, Y., Lin, Y., Chen, Q., Belessiotis-Richards, A., Stevens, M.M. & Thomas, M.R. 2021a. Iodide-Mediated Rapid and Sensitive Surface Etching of Gold Nanostars for Biosensing. *Angewandte Chemie International Edition*, 60(18):9891-9896. 10.1002/anie.202017317
- Xianyu, Y., Lin, Y., Chen, Q., Belessiotis-Richards, A., Stevens, M.M. & Thomas, M.R. 2021b. Iodide-Mediated Rapid and Sensitive Surface Etching of Gold Nanostars for Biosensing. *Angewandte Chemie International Edition* 60(18):9891-9896. 10.1002/anie.202017317
- Xiao, W., Wang, R.S., Handy, D.E. & Loscalzo, J. 2018. NAD(H) and NADP(H) Redox Couples and Cellular Energy Metabolism. *Antioxid Redox Signal*, 28(3):251-272. 10.1089/ars.2017.7216
- Xiao, Y., Pavlov, V., Levine, S., Niazov, T., Markovitch, G. & Willner, I. 2004a. Catalytic growth of Au nanoparticles by NAD(P)H cofactors: optical sensors for NAD(P)⁺-dependent biocatalyzed transformations. *Angewandte Chemie International Edition*, 43(34):4519-4522. 10.1002/anie.200460608
- Xiao, Y., Pavlov, V., Levine, S., Niazov, T., Markovitch, G. & Willner, I. 2004b. Catalytic growth of Au nanoparticles by NAD(P)H cofactors: optical sensors for NAD(P)⁺-dependent biocatalyzed transformations. *Angewandte Chemie International Edition* 43(34):4519-4522. 10.1002/anie.200460608
- Xie, J., Lee, J.Y. & Wang, D.I.C. 2007a. Seedless, Surfactantless, High-Yield Synthesis of Branched Gold Nanocrystals in HEPES Buffer Solution. *Chemistry of Materials*, 19(11):2823-2830. 10.1021/cm0700100
- Xie, J., Lee, J.Y. & Wang, D.I.C. 2007b. Seedless, surfactantless, high-yield synthesis of branched gold nanocrystals in HEPES buffer solution. *ACS Chemistry of Materials* 11(19):2823–2830. 10.1021/cm0700100
- Xie, J., Lee, J. & Wang, D. 2007c. Seedless, Surfactantless, High-Yield Synthesis of Branched Gold Nanocrystals in HEPES Buffer Solution. *Chemistry of Materials - CHEM MATER*, 19, 10.1021/cm0700100
- Xie, N., Zhang, L., Gao, W., Huang, C., Huber, P.E., Zhou, X., ... Zou, B. 2020. NAD⁺ metabolism: pathophysiologic mechanisms and therapeutic potential. *Signal Transduction and Targeted Therapy*, 5(1):227. 10.1038/s41392-020-00311-7
- Xing, X., Shao, M. & Liu, C.-C. 1996. Electrochemical oxidation of dihydronicotinamide adenine dinucleotide (NADH) on single crystal gold electrodes. *Journal of Electroanalytical Chemistry*, 406(1):83-90. 10.1016/0022-0728(95)04428-0

Xu, D., Yu, S., Yin, Y., Wang, S., Lin, Q. & Yuan, Z. 2018. Sensitive Colorimetric Hg(2+) Detection via Amalgamation-Mediated Shape Transition of Gold Nanostars. *Frontiers in chemistry*, 6:566-566. 10.3389/fchem.2018.00566

Yagi, K., Ozawa, T. & Ooi, T. 1963. Complex Formation Of Apoenzyme, Coenzyme And Substrate Of D-Amino Acid Oxidase. V. Change In Conformation Of The Protein By Forming A Model Of Enzyme-Substrate Complex. *Biochim Biophys Acta*, 77:20-26. 10.1016/0006-3002(63)90465-7

Yang, J., Lee, J.Y. & Ying, J.Y. 2011. Phase transfer and its applications in nanotechnology. *Chemical Society Reviews*, 40(3):1672-1696. 10.1039/B916790K

Yang, X., Yang, M., Pang, B., Vara, M. & Xia, Y. 2015. Gold Nanomaterials at Work in Biomedicine. *Chemical Reviews*, 115(19):10410-10488. 10.1021/acs.chemrev.5b00193

Yang, Y. & Sauve, A.A. 2016. NAD(+) metabolism: Bioenergetics, signaling and manipulation for therapy. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1864(12):1787-1800. 10.1016/j.bbapap.2016.06.014

Yazdani, S., Daneshkhah, A., Diwate, A., Patel, H., Smith, J., Reul, O., ... Hajrasouliha, A.R. 2021. Model for Gold Nanoparticle Synthesis: Effect of pH and Reaction Time. *ACS Omega*, 6(26):16847-16853. 10.1021/acsomega.1c01418

Ye, Z., Li, C., Celentano, M., Lindley, M., O'Reilly, T., Greer, A.J., ... Bell, S.E.J. 2022. Surfactant-free Synthesis of Spiky Hollow Ag–Au Nanostars with Chemically Exposed Surfaces for Enhanced Catalysis and Single-Particle SERS. *JACS Au*, 2(1):178-187. 10.1021/jacsau.1c00462

Ying, W. 2008. NAD⁺/NADH and NADP⁺/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal*, 10(2):179-206. 10.1089/ars.2007.1672

Yuan, H., Fales, A.M., Khoury, C.G., Liu, J. & Vo-Dinh, T. 2013. Spectral characterization and intracellular detection of Surface-Enhanced Raman Scattering (SERS)-encoded plasmonic gold nanostars. *Journal of Raman Spectroscopy*, 44(2):234-239. 10.1002/jrs.4172

Yuan, H., Khoury, C.G., Hwang, H., Wilson, C.M., Grant, G.A. & Vo-Dinh, T. 2012. Gold nanostars: surfactant-free synthesis, 3D modelling, and two-photon photoluminescence imaging. *Nanotechnology*, 23(7):075102. 10.1088/0957-4484/23/7/075102

Yuan, H., Ma, W., Chen, C., Zhao, J., Liu, J., Zhu, H. & Gao, X. 2007. Shape and SPR Evolution of Thorny Gold Nanoparticles Promoted by Silver Ions. *Chemistry of Materials*, 19(7):1592-1600. 10.1021/cm062046i

Zamora-Justo, J.A., Abrica-González, P., Vázquez-Martínez, G.R., Muñoz-Diosdado, A., Balderas-López, J.A. & Ibáñez-Hernández, M. 2019. Polyethylene Glycol-Coated Gold Nanoparticles as DNA and Atorvastatin Delivery Systems and Cytotoxicity Evaluation. *Journal of Nanomaterials*, 2019:5982047. 10.1155/2019/5982047

Zan, X., Li, Q., Pan, Y., Morris, D.J., Zhang, P., Li, P., ... Zhu, M. 2018. Versatile Ligand-Exchange Method for the Synthesis of Water-Soluble Monodisperse AuAg Nanoclusters for Cancer Therapy. *ACS Applied Nano Materials*, 1(12):6773-6781. 10.1021/acsanm.8b01559

Zanello, P., Nervi, C. & De Biani, F.F. 2019. *Inorganic electrochemistry: theory, practice and application*. Royal Society of Chemistry.

Zayats, M., Baron, R., Popov, I. & Willner, I. 2005. Biocatalytic Growth of Au Nanoparticles: From Mechanistic Aspects to Biosensors Design. *Nano Letters*, 5(1):21-25. 10.1021/nl048547p

Zeng, J., Zhang, Y., Zeng, T., Aleisa, R., Qiu, Z., Chen, Y., ... Yin, Y. 2020. Anisotropic plasmonic nanostructures for colorimetric sensing. *Nano Today*, 32, 10.1016/j.nantod.2020.100855

Zhang, F., Lees, E., Amin, F., Rivera Gil, P., Yang, F., Mulvaney, P. & Parak, W.J. 2011. Polymer-coated nanoparticles: a universal tool for biolabelling experiments. *Small*, 7(22):3113-3127. 10.1002/smll.201100608

Zhang, N., Xu, C., Azer, A. & Liu, H. 2019. Dispersibility and characterization of polyvinyl alcohol-coated magnetic nanoparticles in poly(glycerol sebacate) for biomedical applications. *Journal of Nanoparticle Research*, 21(12):275. 10.1007/s11051-019-4725-9

Zhang, R., Kiessling, F., Lammers, T. & Pallares, R.M. 2022. Unraveling the Seedless Growth of Gold Nanostars through Fractional Factorial Design. *The Journal of Physical Chemistry C*, 126(43):18580-18585. 10.1021/acs.jpcc.2c06396

Zhang, X., Guo, Q. & Cui, D. 2009. Recent advances in nanotechnology applied to biosensors. *Sensors (Basel)*, 9(2):1033-1053. 10.3390/s90201033

Zhang, Z., Chen, Z., Wang, S., Cheng, F. & Chen, L. 2015. Iodine-Mediated Etching of Gold Nanorods for Plasmonic ELISA Based on Colorimetric Detection of Alkaline Phosphatase. *ACS Applied Materials & Interfaces*, 7(50):27639-27645. 10.1021/acsami.5b07344

Zhang, Z., Wang, H., Chen, Z., Wang, X., Choo, J. & Chen, L. 2018. Plasmonic colorimetric sensors based on etching and growth of noble metal nanoparticles: Strategies and applications. *Biosens Bioelectron*, 114:52-65. 10.1016/j.bios.2018.05.015

Zhao, J., Pinchuk, A.O., McMahon, J.M., Li, S., Ausman, L.K., Atkinson, A.L. & Schatz, G.C. 2008. Methods for Describing the Electromagnetic Properties of Silver and Gold Nanoparticles. *Accounts of Chemical Research*, 41(12):1710-1720. 10.1021/ar800028j

Zhao, W., Gonzaga, F., Li, Y. & Brook, M.A. 2007. Highly Stabilized Nucleotide-Capped Small Gold Nanoparticles with Tunable Size. *Advanced Materials*, 19(13):1766-1771. 10.1002/adma.200602449

Zhao, Y., Qiu, L., Sun, Y., Huang, C. & Li, T. 2017. Optimal hemoglobin extinction coefficient data set for near-infrared spectroscopy. *Biomedical Optics Express*, 8(11):5151-5159. 10.1364/boe.8.005151

- Zheng, X., Liu, Q., Jing, C., Li, Y., Li, D., Luo, W., ... Fan, C. 2011. Catalytic gold nanoparticles for nanoplasmonic detection of DNA hybridization. *Angewandte Chemie International Edition English*, 50(50):11994-11998. 10.1002/anie.201105121
- Zhou, W., Gao, X., Liu, D. & Chen, X. 2015. Gold nanoparticles for in vitro diagnostics. *Chemical Reviews*, 115(19):10575-10636. 10.1021/acs.chemrev.5b00100
- Zhou, X., Xu, W., Liu, G., Panda, D. & Chen, P. 2010. Size-Dependent Catalytic Activity and Dynamics of Gold Nanoparticles at the Single-Molecule Level. *Journal of the American Chemical Society*, 132(1):138-146. 10.1021/ja904307n
- Zhou, Z.-Y., Tian, N., Li, J.-T., Broadwell, I. & Sun, S.-G. 2011. Nanomaterials of high surface energy with exceptional properties in catalysis and energy storage. *Chemical Society Reviews*, 40(7):4167-4185. 10.1039/C0CS00176G
- Zhu, W., Wei, Z., Han, C. & Weng, X. 2021. Nanomaterials as Promising Theranostic Tools in Nanomedicine and Their Applications in Clinical Disease Diagnosis and Treatment. *Nanomaterials*, 11(12):3346.
- Zong, J., Cobb, S.L. & Cameron, N.R. 2017. Peptide-functionalized gold nanoparticles: versatile biomaterials for diagnostic and therapeutic applications. *Biomaterials Science*, 5(5):872-886. 10.1039/c7bm00006e
- Zong, Q., Dong, N., Yang, X., Ling, G. & Zhang, P. 2021. Development of gold nanorods for cancer treatment. *Journal of Inorganic Biochemistry*, 220:111458. 10.1016/j.jinorgbio.2021.111458

CHAPTER 9 APPENDECIES

9.1 APPENDIX A: SYNTHESIS OF GOLD NANOSTARS, CHARACTERISATION AND COLOURIMETRIC ASSESSMENT

9.1.1 Particle size distribution of the AuNS bioconjugates

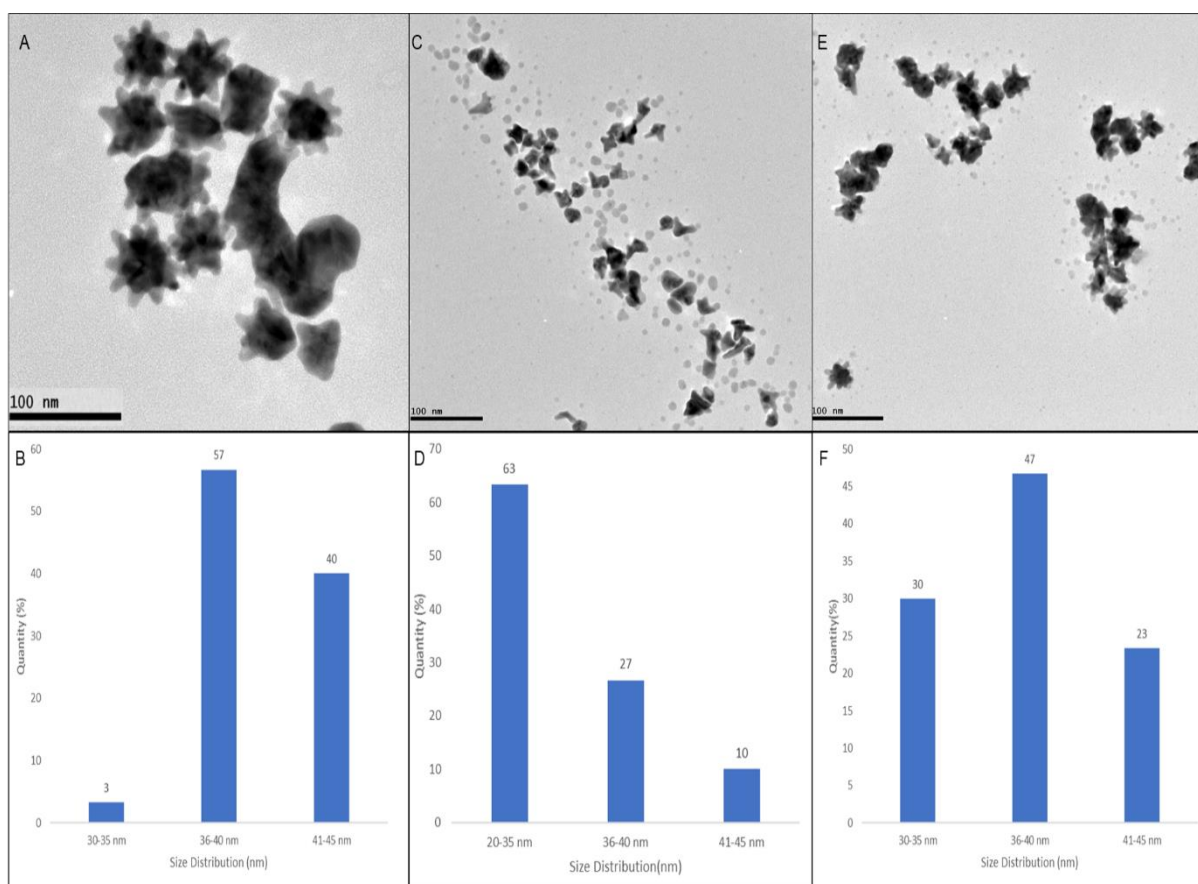


Figure 4-S1. TEM image comparison of bioconjugates and size distribution: (A–B) AuNS-PVP-GOx; (C–D) AuNS-PEO-GOx; and (E–F) AuNS-PEG-GOx.

Of the 100 nanostars analyzed for each sample, a greater percent were 12-armed nanostars, whereas the remainder were more spherical with a few short protruding arms.

9.1.2 Confirming AuNS auto degeneration in the presence of GOx

AuNS-GOx showed morphological change within 30 min of synthesis, as confirmed by 5 min intermittent spectrophotometric readings and suspension colour change visible to the naked eye (see Figure 4S-2A). The TEM image shows the structural and morphological changes in the AuNS-GOx bioconjugate.

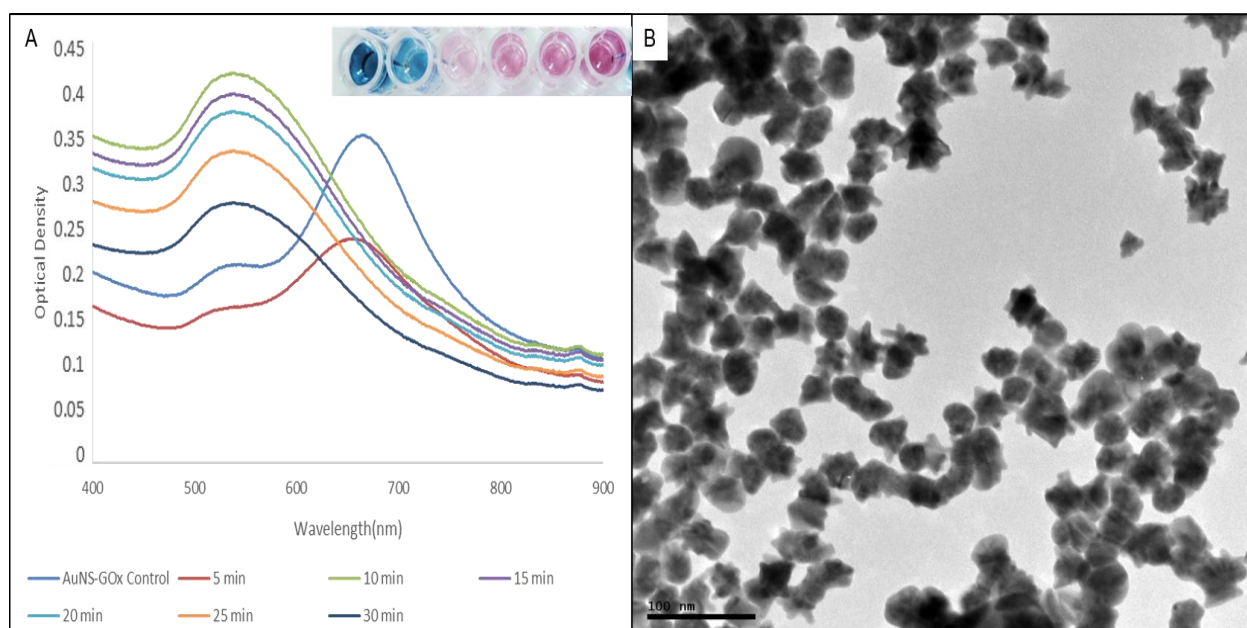


Figure 4-S2. Morphological changes of AuNS-GOx bioconjugate: (A) UV-Vis spectra and (B) TEM image.

9.1.3 Confirming AuNS sensitivity to H_2O_2 and colourimetric signal generation

A pilot colourimetric assay study for the sensitivity of capped AuNSs to various H_2O_2 concentrations was assessed in Tris buffer, as presented in Figure 4-S3.

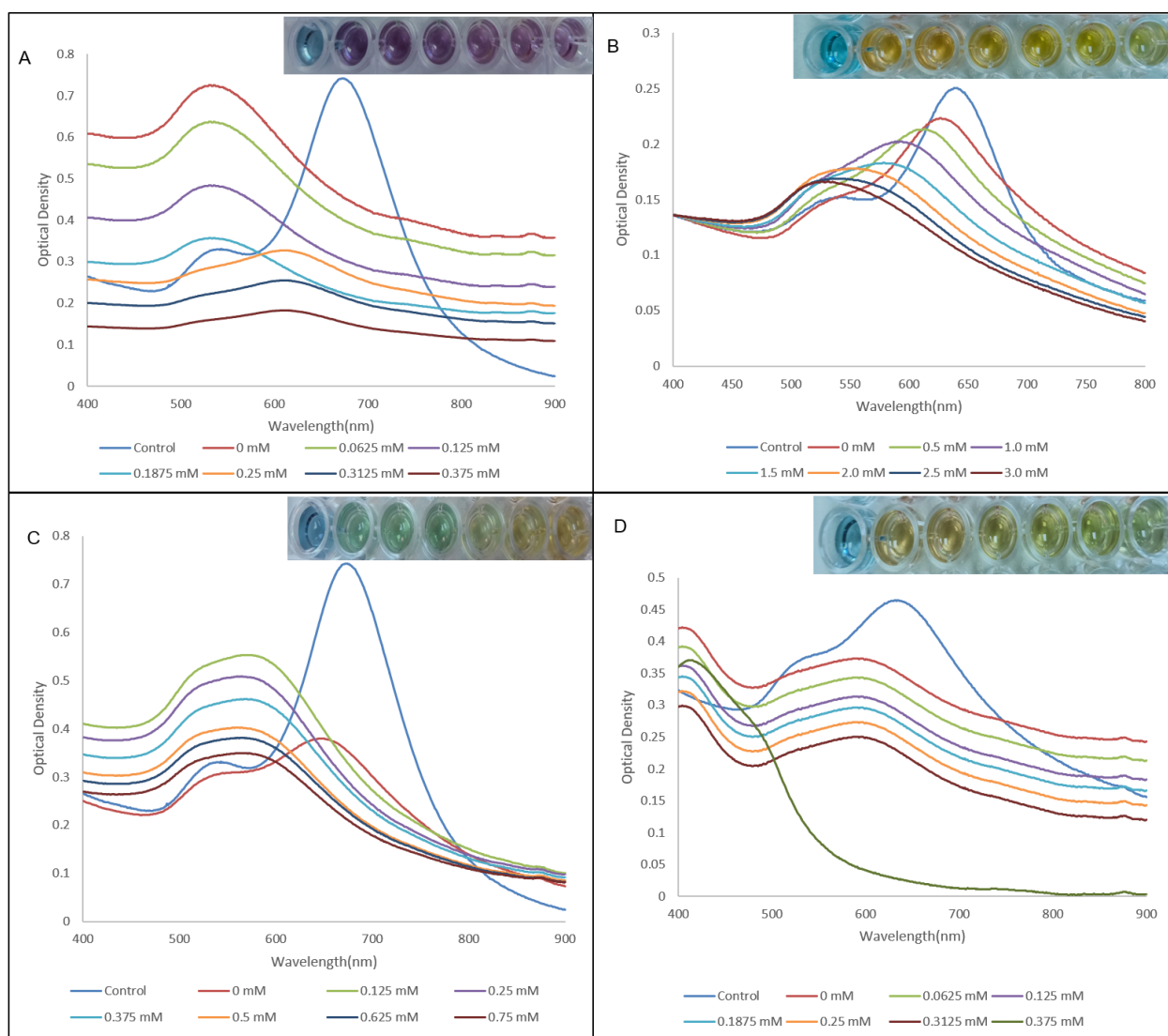


Figure 4-S3. Hydrogen- peroxide- sensing efficacy UV-Vis spectra: (A) AuNS; (B) AuNS-PVP; (C) AuNS-PEO; and (D) AuNS-PEG.

The colour changes observed ranged from deep purple to light purple for AuNS, deep orange to light orange for AuNS-PVP (Fig 4-S3. B), lime green to orange for AuNS-PEO (Fig 4-S3.C), and pale -yellow shades to light green for AuNS-PEG (Fig 4-S3. D). It was noted that capping agents resulted in different colourimetric readouts, and in all the spectra, the longitudinal peak of AuNS was absent, leaving behind the transverse peak. A hypsochromic effect (blue shift) was noted from the UV-Vis spectra relative to the control. The capped AuNS produced excellent colour hues visible to the naked eye. There were no visible colour changes observed when various reaction mixture components were omitted.

Furthermore, it was also found that the spiked GOx procedure produced very similar colours, as confirmed by the inserts (as seen in Figure 4-S4) and the UV-Vis spectra, which showed poor separation.

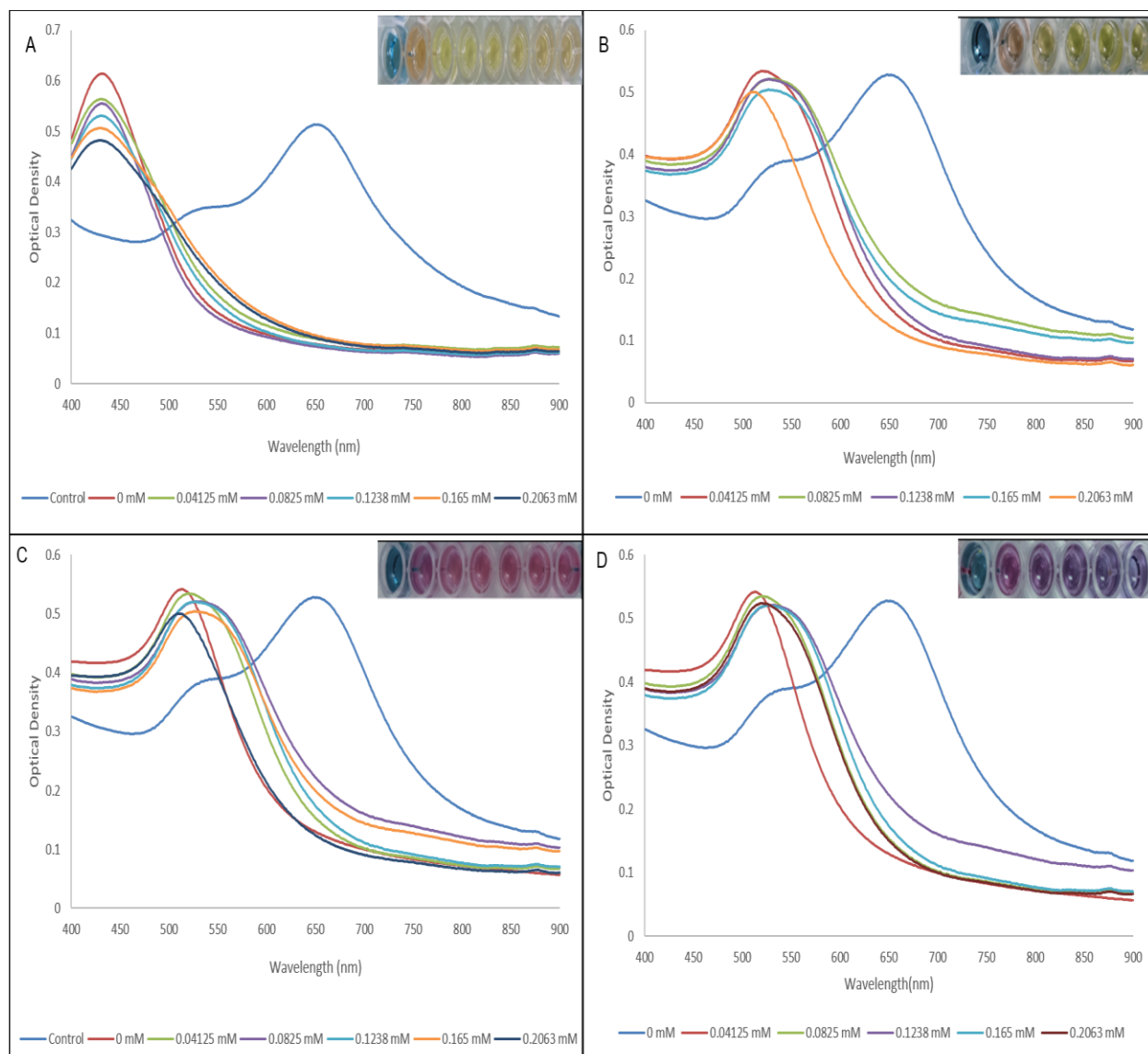


Figure 4-S4. UV-Vis spectra and colourimetric readouts for Gox-spiked AuNSs: (A) AuNS; (B) AuNS-PVP; (C) AuNS-PEO; and (D) AuNS-PEG.

The enzyme orientation on the nanoparticle surface was not controlled. This passive (or physisorption) process should be carefully managed for reproducible conjugates, hence the introduction of DTSSP.