

The Synthesis, Characterisation and Bioapplications of Novel Gold-Zinc Telluride Core-shell Nanoparticles

By

Rekha Dunpall



University of Zululand

April 2016

The Synthesis, Characterisation and Bioapplications of novel
Gold-Zinc Telluride Core-shell nanoparticles

A thesis submitted in fulfillment of the requirement for the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

UNIVERSITY OF ZULULAND

By

Rekha Dunpall

20040184

Supervisor: Prof. N. Revaprasadu

Co-supervisor: Prof. A.R. Opoku

January 2016

SUPERVISOR CONSENT AND DECLARATION

The experimental research presented in this thesis was conducted in the Department of Chemistry, University of Zululand (UZ), Medical Research Council (MRC) Oncology Research Unit and the Biomedical Resource Unit (BRU), University of KwaZulu Natal under the supervision of Prof N. Revaprasadu and co-supervision of Prof. A.R. Opoku.

This study presents interdisciplinary scientific, current and original work by the author. Where use was made by outside resources, appropriate attributions have been cited in the text.

I declare the above statement to be true

Rekha Dunpall

I, Prof. Neerish Revaprasadu, as supervisor of the Ph.D (Biochemistry) research project titled “The Synthesis, Characterisation and Bioapplications of novel Gold-Zinc Telluride Core-shell nanoparticles” hereby consent to the submission of this dissertation.

Prof N. Revaprasadu

I, Prof. Andy Opoku, as co-supervisor of the Ph.D (Biochemistry) research project titled “The Synthesis, Characterisation and Bioapplications of novel Gold-Zinc Telluride Core-shell nanoparticles” hereby consent to the submission of this dissertation.

Prof A.R. Opoku

ACKNOWLEDGEMENTS

I would like to extend my deepest and most sincere gratitude to the following people without whom this work would not have been possible. Prof. N Revaprasadu, my supervisor and mentor, thank you for all the years of support, guidance, patience and motivation. Thank you for affording me the opportunity to develop new skills and techniques within materials chemistry and nanotechnology. Prof A. R Opoku, my co-supervisor and mentor, thank you for all the years of support, guidance, patience and motivation.

I wish to thank my research colleagues from the Nanotechnology Research Group, Department of Chemistry (UZ). I wish to thank the following staff members from the Biomedical Resource Unit (BRU) UKZN (Dr S Singh, Dr L Bester, Mr D Mompe and Ms. Ritta Radebe) for technical support, training and motivation. I wish to thank Dr J Bodenstein, Department of Pharmacology, UKZN, for providing cell culture facilities. I wish to extend my gratitude to Mr. Sanjeev Rambharose, Department of Pharmaceutical Sciences, School of Health Sciences, UKZN, thank you for all the encouragement, technical, training and moral support.

NRF innovation, SARCHi grant and the TIA Seed fund for providing the scholarship and research funds. I am grateful for the funding that has enabled me to complete my research timeously and without financial limitations

To my family, my parents and friends thank you for all the encouragement, love, inspiration and support. I am most grateful to God for providing the opportunity, strength and wisdom to achieve my goals

ABSTRACT

There is a growing demand for the development of innovative nano-drug delivery systems that can both target and improve cancer therapies more effectively than conventional chemotherapy. Novel Au-ZnTe nanoparticles were designed to support biocompatibility features that can be utilised for drug delivery and bio-imaging applications. Au, ZnTe and Au-ZnTe nanoparticles were synthesized and analysed for optical, morphological, crystalline and surface chemistry characterisation. Subsequent to materials characterisation, Au-ZnTe nanoparticles were evaluated for their potential toxicity using *in vitro* and *in vivo* systems. The nanoparticles were surface modified through conjugation with 5-FU and human epidermal growth factor antibody to facilitate targeted anti-cancer drug delivery, followed by the *in vitro* drug efficacy application. The Au-ZnTe nanoparticles displayed core-shell morphology with an average particle size of 7 ± 3.74 nm. The absorption wavelength of Au-ZnTe nanoparticles was dominated by the optical properties of gold and photoluminescence analysis showed that ZnTe dominated the emission properties of Au-ZnTe nanoparticles. Crystalline analysis displayed peaks attributed to both parental materials.

The biosafety and cytotoxicity of these nanoparticles was established using normal human colon, mammary epithelial and cancer cells of breast, prostate and colon origin. Moreover, under certain conditions the particles expressed cytokines in low concentrations and induced an insignificant (20%) cytotoxic response when exposed to human peripheral blood mononuclear cells. Additionally systemic circulation of Au-ZnTe particles displayed no adverse effects in the blood, liver and kidney functions of female Sprague Dawley rats. TEM, FTIR, Zeta potential and optical measurements were performed to confirm the surface conjugation and interaction of 5-FU and EGF

to Au-ZnTe nanoparticles. The *in vitro* anti-cancer therapeutic efficacy study was performed using the MTT cytotoxicity assay on breast cancer cells. The cytotoxicity studies have shown that all components in the 5-FU-EGF-Au-ZnTe nanoparticle formulation work synergistically to attack MCF7 cancer cells displaying 24.74 % increased efficacy than 5-FU at equivalent concentrations. Furthermore receptor-ligand mediated uptake of nano-drug formulations was demonstrated using 5-FU-Au-ZnTe. Several attempts were made to induce and develop a tumour model using Sprague Dawley rats and BALB/c mice. The presence of an external tumour mass was unsuccessful and therefore limited the ability to demonstrate the *in vivo* therapeutic efficacy of 5-FU-EGf-Au-ZnTe nanoparticles. These findings however lay a foundation for future work involving the synthesis and application of biocompatible nanoparticles that can support and improve current medical technologies. This study has generated valuable new knowledge that will help scientists within the field of biotechnology, nanomedicine, biochemistry and materials chemistry, to develop and optimize strategies for more efficient therapeutic application of nanomaterials.

Research Output

1. Patent Publication number: WO2015019297 A1
Title: The Synthesis of Core-Shell Metal-Semiconductor Nanomaterials. Application number: PCT/IB2014/063729
Publication date: Feb 12, 2015
Inventors: Neerish REVAPRASADU, Rekha DUNPALL
Applicant: University Of Zululand

2. Publications **Dunpall R**, Lewis E A, Haigh S, O'Brien P, Revaprasadu N. Synthesis of biocompatible Au–ZnTe core-shell nanoparticles. J. Matter. Chem. B 2015, 2826–2833.
Dunpall R, Mlowe S, Revaprasadu N. Evidence of oriented attachment in the growth of functionalized ZnTe nanoparticles for potential applications in bio-imaging. New J. Chem., 2014, 38, 6002—6007.
Dunpall R, Opoku A.R, Revaprasadu N. Development and characterisation of MCF7 mammary carcinoma xenografts in a non-immunocompromised rat model. Submitted to Tropical Journal of Pharmaceutical Research
Dunpall R, Revaprasadu N. An *in vitro* and *in vivo* bio-interaction responses and biosafety evaluation of novel Au-ZnTe core–shell nanoparticles. Submitted to Toxicology Research
Dunpall R, Revaprasadu N. A current perspective of gold-based core-shell nanoparticles for bio-applications (Review manuscript in preparation)
Dunpall R, Revaprasadu N. The preparation, evaluation and *in vitro* therapeutic application of Surface Conjugated 5-FU-EGF-Au-ZnTe Core-Shell Nanoparticles. (Manuscript in preparation)

3. Conferences **Dunpall R** and Revaprasadu N. (2014), An acute toxicity evaluation study of Au-ZnTe core/shell nanoparticles using

human cancer cell lines and in vivo animal models. MAM-14 International conference 2014. 23-27 November 2014, Johannesburg. South Africa (Oral Presentation, Third Prize)

Dunpall R and Revaprasadu N. (2014), The synthesis, characterisation and in vivo cytotoxicity studies of Au-ZnTe core/shell nanoparticles. NanoAfrica 2014 international conference, 30 March – 2 April 2014. Vanderbijlpark, South Africa. (Oral Presentation)

Dunpall R, Opoku A. R and Revaprasadu N. Synthesis, characterisation and biocompatibility studies of Au-ZnTe core/shell nanoparticles. 2013 Faculty of Science Symposium, University of Zululand (Oral presentation, Second Prize)

Dunpall R, Opoku A. R and Revaprasadu N. Profiling and toxicity studies of Au-ZnTe core/shell nanoparticles. 2014 Faculty of Science Symposium, University of Zululand (Oral presentation, Third Prize)

- | | |
|---------------------------------------|---|
| 4. Laboratory animal science training | IOSP 2014 IUPAC Integrated and organs systems pharmacology, UKZN Westville campus (June-July 2014) |
|---------------------------------------|---|

- | | |
|--------------------|---|
| 5. Special feature | <p>Innovation Bridge</p> <p>South Africa's Premier Technology Showcase and Matchmaking Event, 2 & 3 February 2015</p> <p>CSIR International Convention Centre, Pretoria</p> |
|--------------------|---|

Press article featured in *Public understanding of Biotechnology*

Link: <http://www.pub.ac.za/a-nanoscale-approach-to-combating-cancer/>

Title: A nanoscale approach to combating cancer

List of abbreviations

5-FdUMP	5-Fluoro Deoxyuridine monophosphate
5-FU	5-Fluorouracil
AFM	Atomic force microscopy
AKT	Protein kinase B
ALK Phos	Alkaline phosphatase
ALP	Alkaline phosphate
ALT	Alanine Aminotransferase
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BME	Basement membrane extract
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BRU	Biomedical Resource Unit
CRE	Creatinine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
E-cadherin	Epithelial cadherin
EDX	Energy-dispersive X-ray spectroscopy
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
ERBB2	Receptor tyrosine-protein kinase erbB-2
ERK	Extracellular-signal-regulated kinases
FCS	Fetal calf serum
FDA	Food and Drug Administration
FdUMP	Deoxyfluorouridine monophosphate
FTIR	Fourier transform infrared spectroscopy
FUTP	Fluorouridine triphosphate
G ₁ phase	Gap 1 phase
Gamma-GT	Gamma-glutamyl transpeptidase
GDP	Guanosine diphosphate
GRB2	Growth factor receptor binding protein 2
GTP	Guanine triphosphate
H&E	Hematoxylin and eosin
HAADF	High angle annular dark field imaging
HER2	Human epidermal growth factor receptor 2
HPLC	High-performance liquid chromatography
HRTEM	High resolution transmission electron microscopy
ICP	Inductively coupled plasma
IFN γ	Interferon gamma
IGF	Insulin-like growth factors
IL-1 β	Interleukin-1 Beta
IP	Intraperitoneal
IR	Infrared
IV	Intravenous

LDH	Lactate dehydrogenase
LEDs	Light-emitting diodes
LM	Light microscopy
M phase	Mitotic phase
MAP2K	Mitogen-activated protein kinase kinase
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinase
MEK	Mitogen-activated protein kinase kinase
MEM	Modified eagle medium
MTOR	Mechanistic target of rapamycin
MTORC1	Mechanistic target of rapamycin complex 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MTX	Methotrexate
NCR	National Cancer Registry
NIR	Near-infrared wavelength
NME	New molecular entity
OA	Oriented attachment
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PFOB	Perfluorooctylbromide
PHA	Phytohemagglutinin
PI3 kinase-AKT	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP ₂	Phosphatidylinositol -4, 5-bisphosphate
PIP ₃	Phosphatidylinositol -3, 4, 5-triphosphate
PKB	Protein kinase B
PL	Photoluminescence
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PLT	Platelet
PRAS40	Proline-rich Akt Substrate of 40 kDa
pRb	Retinoblastoma protein
PSF	Pen-strep Fungizone
PTEN	Phosphatase and tensin homolog
QCE	Quantum confinement effect
RAF	Rapidly Accelerated Fibrosarcoma
RBC	Red blood cell
Rheb-GTP	Ras homolog enriched in brain- Guanine triphosphate
RNA	Ribonucleic Acid
S phase	Synthesis phase
SAED	Selected area (electron) diffraction
SD	Standard deviation
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman spectra
SPF	Specific-pathogen free
SPIOs	Superparamagnetic iron oxide
SPR	Surface plasmon resonance
STEM	Scanning transmission electron microscope
TDLU	Terminal duct lobular unit

TEM	Transmission electron microscopy
TNF α	Tumor necrosis factor alpha
TS	Thymidylate synthetase
TSC2	Tuberous Sclerosis Complex 2
UKZN	University of KwaZulu Natal
UV	Ultraviolet
WBC	White blood cell
WST-1	(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)
XEDS	X-ray energy dispersive spectroscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction

Table of contents	Page
Supervisor Consent And Declaration	i
Acknowledgements	ii
Abstract	iii-iv
Research outputs	v-vi
List of Abbreviations	vii-ix
List of Tables	xiv
List of Figures	xv-xvi
Background	xvii
 Chapter 1- Introduction and literature review	 1
1.1 Cancer statistics	1
1.2 Understanding cancer cell biology	1
1.3 The hallmarks in cancer development	4
1.3.1 Self-sufficiency in growth signals	4
1.3.2 Insensitivity of anti-growth signals	5
1.3.3 Escaping apoptosis mechanisms	5
1.3.4 Unlimited replicative potential leading to tumour progression	6
1.3.5 Sustained angiogenesis	6
1.3.6 Tissue invasion and metastasis	7
1.3.7 Control over genome instability	7
1.4 Breast Cancer	7
1.5 The MAPK/ERK pathway	9
1.6 The cell cycle	10
1.7 PI3 kinase pathway	11
1.8. Cancer diagnosis and therapeutics	14
1.9 Chemotherapy	16
1.10 Mechanism of action of 5 Fluorouracil	16
1.11 Strategies to improve or enhance 5FU efficacy using nanotechnology	19
1.12 History of nanotechnology	20
1.13 Nanotechnology within the biological domain	23
1.14 Core-shell nanomaterials	24
1.15 Synthesis of gold based core-shell nanoparticles	27
1.16 Core-shell nanoparticles composed of Au core	30
1.17 Core-shell nanoparticles composed of Au shell	34
1.18 Biomedical applications of Au-based core-shell nanoparticles	36
1.19 Biosafety and nanotoxicology of Au-based core-shell nanoparticles	42
1.20 Statement of research problem	49
1.21 Rationale for developing Au-ZnTe core-shell nanoparticles in bio-applications	49
1.22 Aims and objectives of this study	50-52
References	53-59
 Chapter 2- The synthesis and characterisation of biocompatible ZnTe nanoparticles	 60
2.1 Introduction	61
2.2 Experimental details	66
2.2.1 Materials	66

2.2.2 Synthesis of cysteine capped ZnTe nanoparticles	66
2.2.3 Characterisation of nanoparticles	67
2.2.3.1 Ultra-violet-visible spectroscopy analysis	67
2.2.3.2 Photoluminescence spectroscopy analysis	67
2.2.3.3 Morphological characterisation (TEM, HRTEM and SEM)	68
2.2.3.4 X-ray diffraction analysis	68
2.2.3.5 Surface charge analysis	68
2.2.3.6 Fluorescence of ZnTe nanoparticles in the presence of DNA plasmid pGEMT easy	68
2.2.3.7 Cytotoxicity of ZnTe nanoparticles using MTT assay	69
2.3 Results	70
2.3.1 Optical properties of ZnTe Nanoparticles	70
2.3.2 Morphological characterisation of ZnTe nanoparticles	72
2.3.3 Surface charge of ZnTe nanoparticles	77
2.3.4 Fluorescence properties of ZnTe nanoparticles	78
2.3.5 Cytotoxicity evaluation of ZnTe nanoparticles	78
2.4 Discussion	80
2.5 Conclusions	82
References	83-84

Chapter 3- The Synthesis and characterisation of biocompatible Au-ZnTe core/shell nanoparticles

3.1 Introduction	85
3.2 Experimental	86
3.2.1 Materials	90
3.2.2 Synthesis of gold nanoparticles	90
3.2.3 Synthesis of Au-ZnTe core/shell nanoparticles	90
3.2.4 Characterisation of nanoparticles	91
3.2.4.1 Ultra-violet-visible spectroscopy	91
3.2.4.2 Photoluminescence spectroscopy	91
3.2.4.3 Morphology and elemental mapping	91
3.2.4.4 X-ray diffraction	92
3.2.4.5 Tissue culture media and cell line	92
3.2.4.6 WST-1 cell viability assay	92
3.2.4.7 Cellular uptake of Au-ZnTe core/shell nanoparticles	93
3.2.4.8 Bio-imaging analysis of Au-ZnTe nanoparticles	93
3.3 Results	94
3.3.1 Optical properties of Au-ZnTe Nanoparticles	94
3.3.2 Morphological characterisation of Au-ZnTe nanoparticles	96
3.3.3 Crystalline analysis of Au-ZnTe nanoparticles	98
3.3.4 Preliminary cytotoxicity evaluation of Au-ZnTe nanoparticles	100
3.3.5 Cellular uptake and bio-interaction of Au-ZnTe nanoparticles	101
3.3.6 Bio-imaging potential of Au-ZnTe core-shell nanoparticles	103
3.4 Discussion	105
3.5 Conclusions	110
References	112-113

Chapter 4- The bio-interaction, biosafety and nanotoxicology of Au-ZnTe core-shell nanoparticles

4.1 Introduction	114
4.2 Materials and Methods	115

4.2.1 Ethics Statement	119
4.2.2 Synthesis and characterisation of Au-ZnTe core-shell nanoparticles	120
4.2.3 Cell culture	120
4.2.4 WST-1 cell viability assay	120
4.2.5 Cellular interaction with Au-ZnTe core/shell nanoparticles	121
4.2.6 Human peripheral blood mononuclear cell culture	121
4.2.7 Alamar blue assay	121
4.2.8 Cytokine analysis	122
4.2.9 Animals and conditions	122
4.2.10 Biochemical evaluation of liver and kidney function	123
4.2.11 Histopathology analysis of liver and kidney tissue	124
4.2.12 Statistical analyses	124
4.3 Results	125
4.3.1 Cytotoxicity of Au-ZnTe core-shell nanoparticles using WST-1 assay	125
4.3.2 Cellular interaction and biocompatibility studies using MCF7 cells	125
4.3.3 Immunotoxicity effects of Au-ZnTe core-shell nanoparticles on human PBMC's	127
4.3.4 Effects of Au-ZnTe core-shell nanoparticles on full blood counts in rat models	129
4.3.5 Effects of Au-ZnTe core-shell nanoparticles on liver and kidney function in rat models	129
4.3.6 Histological effects of Au-ZnTe core-shell nanoparticles on liver and kidney organs in Sprague-Dawley rats	131
4.4 Discussion	133
4.5 Conclusion	138
References	139
 Chapter 5- The preparation, evaluation and in vitro application of Surface Conjugated Drug-Antibody Au-ZnTe Core-Shell Nanoparticles	 140
5.1. Introduction	141
5.2 Experimental	147
5.2.1 Materials	147
5.2.2 Synthesis of surface conjugated 5FU-EGF-Au-ZnTe nanoparticles	147
5.2.3 Characterisation of surface conjugated 5FU-EGF-Au-ZnTe nanoparticles	148
5.2.3.1 Transmission electron microscopy	148
5.2.3.2 Ultra-violet-visible spectroscopy	148
5.2.3.3 Photoluminescence spectroscopy	148
5.2.3.4 Fourier Transform Infrared spectroscopy (FTIR)	149
5.2.3.5 Zeta potential	149
5.2.3.6 Cell culture and cytotoxicity	149
5.2.3.7 Infra-red light assisted cytotoxicity	150
5.3 Results	151
5.3.1 Surface conjugation of drug-antibody Au-ZnTe nanoparticles	151
5.3.2 Characterisation 5-FU-EGF-Au-ZnTe nanoparticles	151

5.3.3 <i>In vitro</i> application of 5-FU-EGF-Au-ZnTe nanoparticles	157
5.4 Discussion	162
5.5 Conclusion	168
References	169-170

Chapter 6- Development and characterisation of MCF7 mammary carcinoma xenografts in a non-immunocompromised animal models	171
6.1 Introduction	172
6.2 Experimental	177
6.2.1 Ethics Statement	177
6.2.2 Cell culture	177
6.2.3 Tumour induction using Sprague Dawley rats	177
6.2.4 Tumour induction using BALB/c mice	178
6.2.5 Animals and conditions for tumour induction studies in Sprague Dawley rats	178
6.2.6 Animals and conditions for tumour induction studies in BALB/C mice	179
6.2.7 Hematological evaluation	179
6.2.8 Histopathological evaluation	180
6.2.9 Statistical analyses	180
6.3 Results	181
6.3.1 Cell culture of MCF7 cells	181
6.3.2 Animal observations during the 70 day tumour induction study	181
6.3.3 Hematological analysis of red and white blood cells	182
6.3.4 Histopathology evaluations of tumour site from rat models	183
6.3.5 Tumour development in BALB/c mice	185
6.3.6 Histopathology evaluations of tumour site from mouse models	186
6.4 Discussion	190
6.5 Conclusion	194
References	195-196

Chapter 7- General discussion and conclusion	197
7.1 General discussion	198
7.2 Challenges	199
7.3 Conclusion	200
7.4 Recommendations and future work	202

Appendix A- Publications

Appendix B- Ethics approval certificates

List of Tables	Page
Chapter 1	
Table 1.1 Au-based core-shell nanoparticles: Au core with bimetallic, inorganic and organic or polymer shells	32-33
Table 1.2 Au-based core-shell nanoparticles: Bimetallic, inorganic and organic or polymer core with Au shell	35
Table 1.3. Biosafety and nanotoxicity studies of Au-based core-shell nanoparticles	47-48
Chapter 5	
Table 5.1 Nano-pharmaceutical formulations for improved efficacy of 5-FU	144
Table 5.2 Zeta potential analysis	156
Chapter 6	
Table 6.1 Animal weight gain and tumour observations during the 70 day study	181

List of Figures	Page
Chapter 1	
Figure 1.1 Breast tissue anatomy	8
Figure 1.2 Mitotic phase of the cell cycle	10
Figure 1.3. Signal transduction pathway	12
Figure 1.4 Controlled cell growth in normal cells	13
Figure 1.5 Mechanistic pathway of 5-fluorouracil	18
Figure 1.6 History of nanotechnology	22
Figure 1.7 Comparison of various materials on the nano-metre length scale	24
Figure 1.8 Core-Shell verses Gold (Au) composed Core-Shell nanoparticles	26
Figure 1.9. Morphology of Au-based core-shell nanostructures	29
Figure 1.10 Duel bio-imaging and photothermal ablation therapy of tumour tissue	39
Figure 1.11 Au-SiO ₂ core-shell nanoparticles for anti-cancer drug delivery	41
Figure 1.12 Shows an illustration of the morphology of a eukaryotic cell	45
Chapter 2	
Figure 2.1 Applications of semiconductor nanoparticles	62
Figure 2.2 Band gap properties relative to nanoparticle size	63
Figure 2.3 Optical properties of cysteine passivated ZnTe nanoparticles	71
Figure 2.4 Optical properties of ZnTe at different pH conditions	71
Figure 2.5 TEM characterisation of ZnTe nanoparticles synthesized at varying pH and time intervals	73
Figure 2.6. TEM micrographs of cysteine capped ZnTe nanoparticles	73
Figure 2.7 ZnTe nanoparticles using TEM analysis for pH 4, 2 hour interval	74
Figure 2.8 TEM characterisation of ZnTe nanoparticles	74
Figure 2.9 Oriented attached growth mechanism of ZnTe	75
Figure 2.10 SEM micrograph displaying the surface morphology of ZnTe nanospheres and nanorods	76
Figure 2.11 XRD pattern for cysteine capped ZnTe nanoparticles	77
Figure 2.12 Fluorescence of cysteine capped ZnTe nanoparticles	78
Figure 2.13 Cytotoxicity of ZnTe nanoparticles using MTT assay	79
Chapter 3	
Figure 3.1 Synthesis concept for Au-ZnTe core/shell nanoparticles	89
Figure 3.2 UV properties of Au, ZnTe and Au-ZnTe nanoparticles	95
Figure 3.3. Photoluminescence spectra of Au-ZnTe core/shell nanoparticles	95
Figure 3.4. Morphological characterisation of Au-ZnTe	97
Figure 3.5. Elemental analysis of core-shell structure using HAADF-STEM and EDX spectrum imaging	98
Figure 3.6. XRD pattern of Au-ZnTe, ZnTe and Au nanoparticles	99
Figure 3.7 XPS absorbance of Au-ZnTe nanoparticles	100

Figure 3.8 A. Displays the relative growth of PL45 cells after treatment with Au-ZnTe core/shell nanoparticles	101
Figure 3.9 Cellular uptake of Au-ZnTe nanoparticles	102
Figure 3.10 Bio-imaging potential of Au-ZnTe nanoparticles	104
Chapter 4	
Figure 4.1 Exposure routes of Au-ZnTe nanoparticles	118
Figure 4.2 Cytotoxicity of Au-ZnTe core-shell nanoparticles	126
Figure 4.3. Bio-interaction and cellular uptake of Au-ZnTe nanoparticles	126
Figure 4.4 Immunotoxicity effects of Au-ZnTe core-shell nanoparticles on human peripheral blood mononuclear cells and cytokine expression	128
Figure 4.5 Hematological analysis of animals treated with Au-ZnTe core-shell nanoparticles	129
Figure 4.6 Effects of Au-ZnTe core-shell nanoparticles on liver and kidney function in Sprague-Dawley rats.	130
Figure 4.7 Histological effects of Au-ZnTe core-shell nanoparticles on liver and kidney organs in Sprague-Dawley rats	131
Chapter 5	
Figure 5.1 Comparative FTIR scans of cysteine capped Au-ZnTe nanoparticles and cysteine	152
Figure 5.2 Comparative FTIR scans of 5-FU and antibody-drug conjugated Au-ZnTe nanoparticles	153
Figure 5.3 Optical properties of 5-FU-EGF-Au-ZnTe nanoparticles	154
Figure 5.4 Morphology of 5-FU-EGF-Au-ZnTe	155
Figure 5.5. Cytotoxicity of 5-FU	157
Figure 5.6 <i>In vitro</i> therapeutic efficacy	159
Figure 5.7 <i>In vitro</i> therapeutic efficacy of 5-FU-Au-ZnTe nanoparticles in the absence of human EGF.	160
Figure 5.8 Infrared light assisted anti-cancer therapeutic efficacy	161
Figure 5.9 Receptor-ligand EGF-EGFR mediated cellular uptake and release of 5-FU-EGF-Au-ZnTe nanoparticles	167
Chapter 6	
Figure 6.1 Tumour induction site located on the right thoracic gland	176
Figure 6.2 White and red blood cell	182
Figure 6.3 Differential white blood cell counts	183
Figure 6.4 Histopathology evaluations of tissues for the tumour injection site using light microscopy	184
Figure 6.5 Histopathology of BALB/c mice at day 30	185
Figure 6. 6 Cross-section of mammary tissues from BALB/c mice	186
Figure 6.7 Cross-section of mammary tissues from tumour induced BALB/c mice	187
Figure 6.8 Cross-section of mammary tissues from tumour induced BALB/c mice	188
Figure 6.9 Comparison of control and treated BALB/c mice	189

Background

The application of biocompatible nanomaterials to improve therapeutic efficacy and reduce the harmful side-effects of existing cytotoxic drugs, is an active area of research in the field of cancer therapeutics and diagnostics. This multidisciplinary research project is aimed at developing a novel, water soluble Au-ZnTe core-shell nanomaterial, intended for targeted anti-cancer drug delivery through surface conjugation with human epidermal growth factor and 5 fluorouracil. The structure of the thesis is outlined within the following chapters;

- Chapter 1:** Introduction and literature review, provides a detailed background, motivation, aims and objectives for this study
- Chapter 2:** The synthesis and characterisation of biocompatible ZnTe nanoparticles
- Chapter 3:** The synthesis and characterisation of biocompatible Au-ZnTe core-shell nanoparticles
- Chapter 4:** The Biosafety, bio-interaction and toxicity evaluation of Au-ZnTe core-shell nanoparticles
- Chapter 5:** Synthesis, characterisation and *in vitro* bio-application of surface modified Au-ZnTe with 5-FU and EGF
- Chapter 6:** *In vivo* xenograft application for mammary tumour induction in mice and rat animal models
- Chapter 7:** Discussion and conclusion, links contributions from each chapter to the main aim of the project, summarising the important findings, limitations, challenges and provides recommendations and suggestions for future studies

CHAPTER ONE- Introduction and literature review

1.1 Cancer statistics

International and national cancer registries take high priority in monitoring cancer incidence, mortality and survival rate every year. This epidemiological data assists medical practitioners and researchers to establish vital links between risk factors and specific cancer types [1]. The collective efforts of epidemiologists, medical doctors and scientists are used to improve treatment strategies and disease management [2]. Cancer remains a major health crisis worldwide. Undeniable epidemiology statistics reveal that the incidence and prevalence of cancer cases are increasing exponentially every year. It is currently the second leading cause of deaths in the United States. A sum of 1,658,370 new cancer cases and 589,430 cancer deaths are projected to occur in the US during 2015 [3].

In South Africa, the latest national cancer registry 2005-2008 release, shows that one in every four South Africans will be affected by cancer through diagnosis of family, friends or self [4, 5]. Additionally the South African survival rate is six in every ten cancer patients. Breast and prostate cancers are rated the top cancers diagnosed in South African females and males respectively. It is estimated that in 2008, 1.4 million new cases of breast cancer were reported and 458 000 breast cancer related deaths were reported worldwide [6]. The Cancer Association of South Africa highlights the causes of breast cancers to include inherited oncogenes such as BRCA1 and BRCA2, hormones and unhealthy diets [7].

1.2 Understanding cancer cell biology

In order to thoroughly understand how a normal cell develops into a cancer cell knowledge on the biochemical regulation of cell growth must be established. The human body is composed of trillions of cells. These cells are highly specialised units

that associate to form functional organs. Normal physiological processes enable the cells to grow and divide as it is required. As the cells age or become damaged they are eliminated by programmed cell death, a process known as apoptosis and that cell is replaced by a new cell through a carefully regulated cell growth cycle to generate new cellular components and daughter cells [8].

Cancer is a collective disease that evolves from multiple genetic alterations which result in the uncontrolled growth and metastasis of abnormal or mutated cells [9]. There are more than 100 types of cancers that develop within various organs in the body. Carcinomas are the most common type of cancer which are epithelial in origin. Epithelial cells line the interior and exterior surfaces and cavities of the body. Adenocarcinoma is a cancer that develops in epithelial cells of the breast, colon, and prostate tissues. Other forms of cancers include soft tissue sarcomas, lymphoma, multiple myeloma, melanoma, blood forming tissue leukaemia and central nervous system related tumours [10].

The earliest account of cancer was discovered in Egypt, 3000 BC, through the study of fossilized bone tumours and human mummies [11]. The quest to understand and develop knowledge on cancer stemmed from intense investigations and successive pathological analysis over the 16-19th centuries. During the 1970's oncogenes and tumour suppressor genes were discovered [11, 12]. Oncogenes was correlated to cancer through its ability to causes cells to grow in an uncontrolled fashion. Tumour suppressor genes were classified as normal genes that control cell division, cell death and DNA repair. The understanding of various oncogenes and tumour suppressor genes have increased rapidly, scientists are now able to use these genes as diagnostic screening tools in early discovery of patients who are at higher risk of developing cancers through inherited genomes.

1.3 The hallmarks in cancer development

The genetics of cancer is classified by various classes of genes. Proto-oncogenes is a class of genes encoded for protein biomolecules that catalyse cellular division or inhibit normal cell death [13]. Oncogenes is a class of mutated genes (proto-oncogenes) which encode for proteins responsible in cell division or inhibit normal cell death. Tumour suppressor genes is a class of genes which encode for protein biomolecules that prevent cell division or initiate apoptosis. Understanding the mechanistic pathways involved in cancer development is a key factor in developing innovative and improved anti-cancer/anti-tumour therapies. Physiological and biochemical analysis of all types of human and animal cancer biopsies, animal tumour models and cultured cells has provided evidence that cancer cells share various mechanistic traits that make them different from normal human cells. These characteristics are commonly referred to as the seven hallmarks of cancer;

1.3.1 Self-sufficiency in growth signals

Cancer cells develop autocrine signalling strategies by producing self-growth signals that result in positive feedback signalling toward the activation of cellular growth and differentiation of damaged cells. Truncated versions of epidermal growth factors (EGF) are produced to enable rapid binding to epidermal growth factor receptor sites which initiate activation of the cell cycle [14, 15]. Additionally the expression of cell surface receptor sites within the cellular membrane domains of cancer cells are upregulated to support activation of growth activity without stimulation from mitogenic factors within the biological microenvironment. The cancer cell is able to utilize the surrounding tissue environment, extracellular matrix ligand components, fibroblasts and epithelial cells to support its autocrine signalling features.

1.3.2 Insensitivity of anti-growth signals

Normal cells have prescribed regulatory mechanisms such as anti-growth signals that are used to control cell division and maintain homeostasis. Immobilised inhibitors and soluble growth inhibitors actively bind to transmembrane receptor sites activating a cascade of reactions that halt the cell cycle into the inactive G0 phase [16]. Anti-growth signals activate the retinoblastoma protein (pRb) pathway through hypophosphorylation of pRb, which results in altering the transcription of genes that are essential for active G1 and S phases of mitosis. Cancer cells however are able to escape anti-growth signals by disrupting the retinoblastoma protein (pRb) pathway and develop insensitivity to anti-growth factors by preventing phosphate mediated activation of pRb, resulting in cellular division of cancer cells [14]. Furthermore cancer cells are able to reduce expression of cell adhesion molecules that communicate anti-growth signals whilst simultaneously inducing pro-growth signals that support the active phases of mitosis [15].

1.3.3 Escaping apoptosis mechanisms

The rate of cell death or apoptosis is a distinct feature in the progression of cancer cells. Transmembrane receptor mediated sensors and effector molecules are key components in regulating the apoptosis pathway. Sensors monitor homeostatic conditions within the intra and extracellular environment to determine when a cell should undergo apoptosis. Once the sensor detects abnormalities it is able to signal effector molecules known as caspases that induce cell death by disrupting the cellular membrane and destroying the nuclear and cytoplasmic domains [17]. Identifying over expression of oncogenes or damaged DNA segments trigger the cells apoptotic mechanisms,

enabling the death of cancer cells is a significant homeostatic mechanism in the tissue environment. Cancer cells are able to resist apoptosis through various strategies, such as mutations in the p53 and PTEN tumour suppressor genes and activation of PI3 kinase-AKT kinase pathways or extracellular IGF survival factors [15-17].

1.3.4 Unlimited replicative potential leading to tumour progression

Normal cells are restricted to a limited number of cell divisions before they become senescent, enter a crisis mode and die [16]. This type of cell death is associated with the reduction of chromosomal lengths following successive cellular divisions. Cancer cells are able to multiply in an uncontrolled and unlimited manner to form a mass of tumour cells by upregulating the expression of telomerase enzymes which facilitate the addition of hexanucleotide repeat segments onto the ends of the telomeric DNA, thereby preventing the shortening of the chromosomes and rendering cancer cells unlimited replications commonly referred to immortalisation of cells [14, 15].

1.3.5 Sustained angiogenesis

Cells within tissues are located within 100 µm blood capillary vessel, this feature ensures the supply of oxygen and vital nutrients which is essential for cellular functions. During the growth and development of normal tissue the close locality of a blood supply for cellular functions is strictly regulated through synchronised growth of vasculature [15, 18]. A cancer cell or developing tumour mass is able to activate the angiogenesis process by disrupting the gene transcription factors that encode for the homeostatic balance between angiogenic inhibitors and inducers. The production of tumour vasculature ensures the survival and facilitates metastasis of aggressive tumour cells [14].

1.3.6 Tissue invasion and metastasis

During the progression of a malignant tumour, active cells are able to enter the vasculature and migrate to other organs, where it adheres and starts establishing secondary tumour sites, a process termed metastasis [14, 19]. This process of “cancer spreading” can rapidly invade neighbouring organs and tissues through controlled upregulation of mutated versions of cell adhesion molecules such as E-cadherin [15].

1.3.7 Control over genome instability

The characteristics of cancer cells or malignant tumour masses are maintained through subsequential gene mutations and malfunctions of various regulatory pathways within the cell cycle. Tumour promoting inflammatory conditions, reprogramming energy metabolism to accommodate for the excessive requirements of tumour growth and evasion of immune attacks [18]. The tumour microenvironment plays a key feature in understanding malignant tumour growth and spreading of mutated cells and tumour resistance. Cancer cells develop over-ruling mechanisms to evade carefully regulated homeostatic pathways inherent in normal cells [15].

1.4 Breast Cancer

The human mammary (breast) organs are composed of ducts and lobules that are surrounded by multiple layers of fibrous and adipose tissue, in addition to a complexity of lymph nodes (**Figure 1.1**). The lobules are composed of secretory units called alveoli which produce milk during lactation. The cells that line the lobules are epithelial in origin [20, 21]. In some cases cancer growths develop in the adipose cells within the fibrous tissue of the breast however breast cancer is commonly caused by

mutations within the epithelial cells that line the lobules of the breast tissue hence the term breast carcinoma.

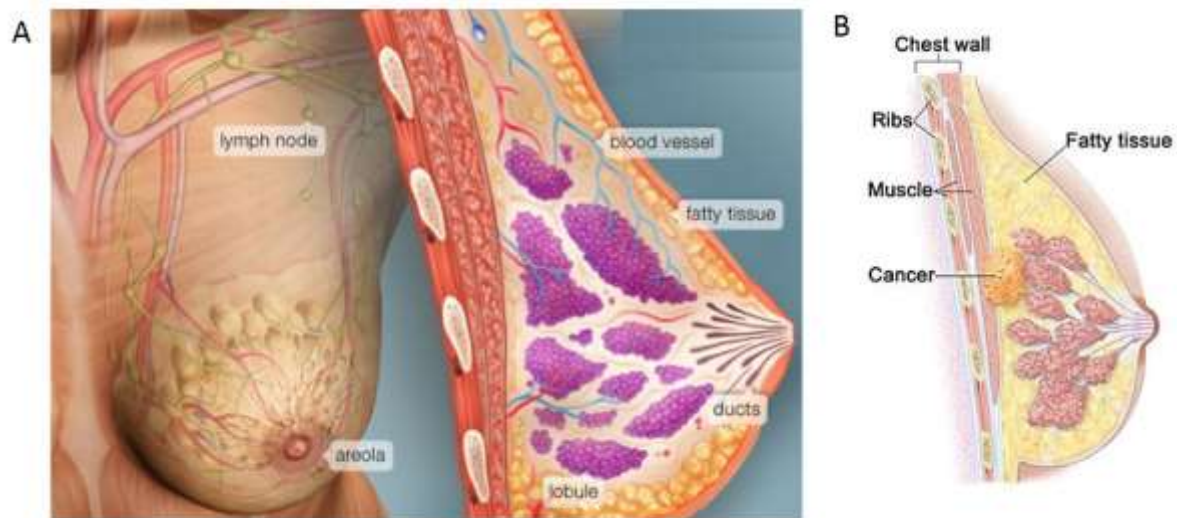


Figure 1.1 Breast tissue anatomy (A) Anatomy of normal breast tissue, illustrating the lymph nodes, lobules and ducts. (B) Anatomy of breast tissue illustrating a cancer growth. Modified from webpage: http://img.wemd.com/dtmcms/live/wemd/consumerassets/site_images/articles/imagearticle_collections/anatomy_pages/493x335_breast.jpg; <http://www.healthnadvise.com/wp-content/uploads/2013/09/breast-cancer.jpg>, 2015.

This type of cancer is due to mutations in the genes that encode for human epidermal growth factor receptor 2 (HER2), also known as receptor tyrosine kinase ERBB2 or ERBB2 [22]. The protein ligand known as epidermal growth factor (EGF) is able to bind to the extracellular region of HER2 and thereby activate a series of cell cycle events through receptor mediated signal transduction. In a normal cell there are two copies of the HER2 gene that originate from the two homologous chromosomes. DNA mutations may lead to amplification in the number of genes that encode for HER2 within the genome. These mutations leads to over-expression of HER2 on the cellular membrane. When the HER2 is over-expressed it allows for excessive EGF binding, which activates a cascade of reactions that stimulate multiple cell divisions through pro-growth signals which can lead to excessive cell proliferation causing breast carcinoma

[23]. There are two common pathways that are activated when EGF binds to HER2 known as the MAPK/ERK and PI3 Kinase pathways.

1.5 The MAPK/ERK pathway

During the binding of EGF to HER2, a conformational change takes place which allows for HER2 receptor dimerization with an adjacent activated HER2. The HER2 structure has an internal tyrosine kinase domain, which is able to catalyse the phosphorylation of tyrosine residues activating the enzyme tyrosine kinase [24]. Growth factor receptor binding protein 2 (GRB2) then binds to the phosphorylated form of tyrosine residues located on the intracellular region of HER2. Another protein known as SOS is able to bind to the tyrosine kinase-GRB2 complex [25]. The activation of the SOS protein is able to catalyse the conversion of a monomeric G protein known as RAS protein from its off-state (RAS is bound to guanine diphosphate, GDP) to its on-state (RAS is bound to guanine triphosphate, GTP). The on-state RAS protein is then able to activate a serine/threonine protein known as RAF through subsequent ATP phosphorylation. This reaction is followed by the phosphorylation of MEK kinase, also known as mitogen activated protein kinase-kinase or MAPKK or MAP2K. The active MEK kinase is then able to activate the next kinase known as MAP kinase, which is also known as mitogen activated protein kinase, microtubule associated protein kinase, MAPK or ERK (extracellular signal regulated kinase). The activated MAP kinase is then able to add phosphate groups onto transcription factors known as MYC or ELK-1 [26]. Activated ELK-1 transcription factor can bind to the promotor region on the genome and alter the affinity of that particular promotor region for RNA polymerase, which can increase or decrease the transcription of a specific gene known as C-FOS. C-FOS is able to bind to another protein called C-JUN to form a FOS-JUN heterodimer transcription

factor that is responsible for moving the cell from interphase (inactive) to an active G₁ phase of the cell cycle [27].

1.6 The cell cycle

The cell cycle is the process in which one cell divides into two identical daughter cells. The cell cycle can be divided into five phases i.e Interphase, Growth 1 phase, Synthesis phase, Growth 2 phase and the mitotic phase. During the interphase (I-phase) the cell is not in the growth phase/ not dividing. The cell receives ligand-receptor mediated (EGF-HER2) signals that stimulate increased transcription factors MYC and FOS-JUN heterodimers that trigger the expression of proteins taking the cell into an active G₁ phase [28].

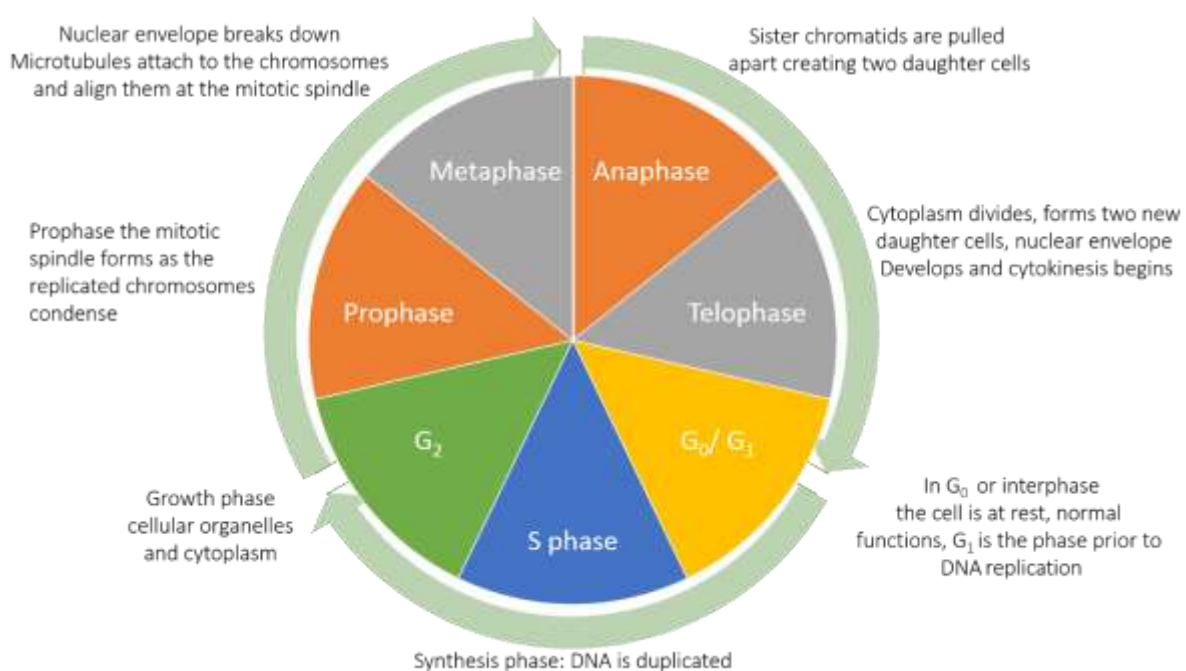


Figure 1.2 Mitotic phase of the cell cycle. Displays the various phases involved in cellular division to produce two identical daughter cells

During the G₁ phase the various proteins involved in organelle functioning and structure are reproduced. The cell then enters into the S phase where the DNA is replicated. The second growth phase G₂ involves more expression of functional proteins and production of machinery that will be used in the mitotic phase. The M

phase is split into two parts i.e. mitosis, which involves the division of the nucleus to produce two identical nuclei containing one copy of all 46 chromosomes, followed by the second part known as cytokinesis which involves the division of the cell to form two daughter cells (**Figure 1.2**) shows a breakdown of the mitotic phase during the active cell cycle [8, 16].

1.7 PI3 kinase pathway

HER2 receptor dimerization with an adjacent activated HER2 is initiated through the binding of EGF on the extracellular domain of HER2. Activation of the HER2 structure catalyses the phosphorylation of tyrosine residues activating the enzyme tyrosine kinase which is then able to activate PI3 kinase also known as PI3K or phosphoinositide-3-kinase. The phospholipids present in the bilayer of the cellular membrane known as phosphatidylinositol -4, 5-bisphosphate (PIP_2) is converted to phosphatidylinositol -3, 4, 5-triphosphate (PIP_3) through the phosphorylated PI3 kinase enzyme [29]. Phosphoinositide dependent kinase 1 is then able to bind to the active PIP_3 , which is followed by the subsequent activation or phosphorylation of AKT kinase also known as Protein kinase B (PKB). AKT kinase is then able to activate MTOR, which is a complex of proteins known as the MTOR complex 1. The MTORC1 protein (Mechanistic target of rapamycin) complex 1 [30]. The MTORC1 is made up of various proteins that associate to form MTOR. AKT kinase interacts with the MTOR complex 1 by phosphorylating the PRAS40 protein, resulting in the cleavage of PRAS40 from the MTORC1. AKT kinase then interacts with the tuberous sclerosis complex TSC2 which upregulates Rheb-GTP. The activated form of Rheb-GTP triggers the MTORC1 and it acts in the same manner as MYC transcription factor in stimulating the G_1 phase of the cell cycle [25, 29].

Illustrated below is the signal transduction pathway triggered by receptor mediated epidermal growth factors which stimulate the cell to undergo division. The cell membrane surface presents many receptors and protein channels that are used to facilitate thousands of cell cycle events such as oxygen, nutrient, ions and other biomolecule intake. As the EGF binds to the cellular receptor a chain of reactions known as signal transduction is set into motion (**Figure 1.3**) causing the cell to proliferate either through the PI3 kinase or MAPK/ERK pathways.

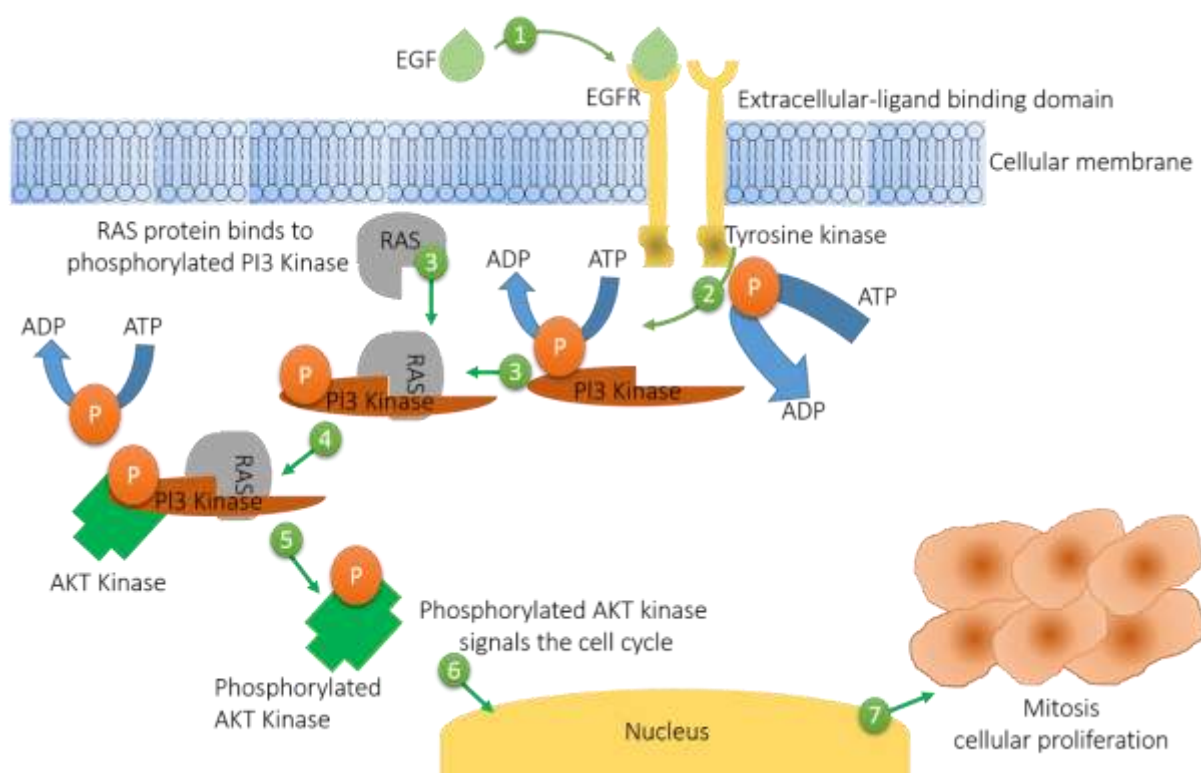


Figure 1.3. Signal transduction pathway- a growth signal activates phosphorylation of tyrosine kinase. This receptor mediated activation cascade activates various kinases until AKT kinase transmits a signal to the nucleus triggering the mitosis cycle.

In addition, although the growth factor is present within the physiological environment, cell division is a highly controlled process. Cells have internal controls that regulate the biochemical pathways that activate cell growth. Many regulatory proteins work simultaneously within a cell to determine whether the cell will undergo cell division or

not. PTEN is a protein that can stop the process of mitosis through the deactivation of phosphorylated PI3 kinase enzyme, PTEN is then able to disrupt a cascade of signal transduction reactions that lead to the phosphorylation of AKT kinase and prevents the enzyme from transmitting signals that cause the cells to enter the G₁ phase of cell division, illustrated in **Figure 1.4**, PTEN control of cell division in a normal cell [31-33].

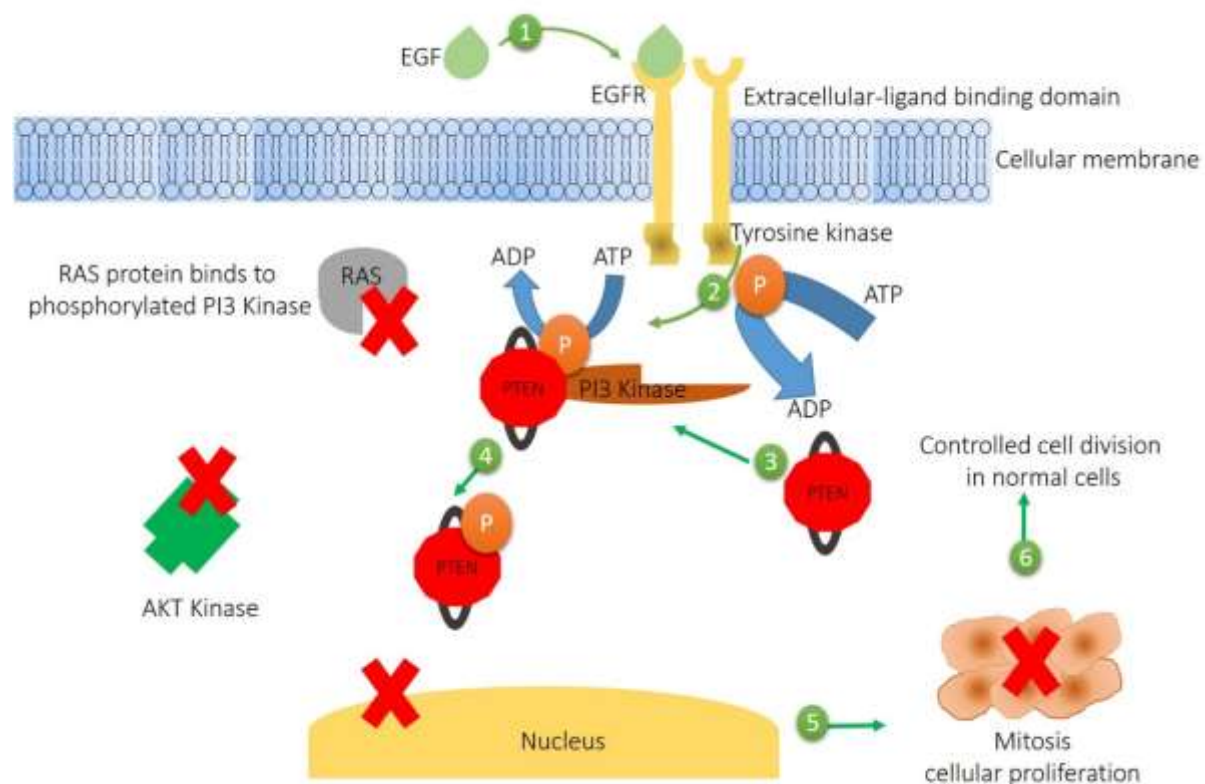


Figure 1.4 Controlled cell growth in normal cells- *PTEN pathway is involved in deactivating the PI3 kinase by removing the phosphate group thereby preventing binding with the RAS protein and halting the cell cycle.*

Under normal conditions a growth factor is required to activate the PI3 kinase pathway, however mutations in these enzymes or proteins that encode for PI3 kinase, RAS protein, AKT kinase and PTEN protein can result in spontaneous activation of mitosis and lead to uncontrolled growth of mutated cells which result in the formation of cancer cells [33]. Additionally in a cancer cell, a mutagen such as harmful carcinogenic chemicals, exposure to UV light, excessive free radical damage, viruses, poor nutrition

or unhealthy lifestyles of tobacco smoking and alcohol consumption may lead to further mutations in cellular DNA. The cell containing the damaged DNA may undergo mitosis irrespective of activation through growth factors or the regular biochemical pathways to form a cancer cell [34].

HER2 amplification in breast epithelial cells can result in activation of the MAPK/ERK or PI3 kinase MTOR pathways which cause excessive cellular proliferation of cells containing mutated genes. A popular monoclonal antibody known as Trastuzumab or Herceptin is able to bind to the extracellular domain of the HER2 and does not induce a conformational change or series of phosphorylation cascades and thereby blocks the EGF from binding to the HER2 [35]. Herceptin acts as a competitive antagonist against the receptor and stops the cell from dividing.

1.8. Cancer diagnosis and therapeutics

The incidence and prevalence of cancer cases worldwide is increasing exponentially every year [1, 3, 36, 37]. Statistics report that breast cancer is the most prevalent amongst females [21]. Cancer develops progressively and is diagnosed according to various stages. These stages are used to describe the severity of the cancer and is based on the size, morphology and physiological activity of the original tumour. Staging assists in prescribing the appropriate treatment regime for the cancer patient, additionally the information is used to provide health care workers, researchers and epidemiologists with valuable insight on cancer development, relevant trends and updated statistics [11]. The development and advancement of cancer is a complex disease, therefore the goal of the treatment is to eliminate the tumour and cure the patient in early stage cancer or like other chronic diseases to manage and control its progression [38, 39]. Cancer tumours can be treated through surgery which involves

the removal of the tumour or cancerous tissues in part or in its entirety. The second treatment regime is radiotherapy, which causes tumour destruction by exposure to harmful radioactive rays. Another common treatment regime is chemotherapy, which involves the administration of systemic cytotoxic drugs to destroy the cancer cells [11]. These therapies can be used in isolation or combination depending on the type and stage of cancer.

Cancer patients are diagnosed and treated according to the stage of cancer they present. **Stage 0:** the early stage of cancer, an In situ neoplasm, also known as situ carcinoma, which refers to an area or collection of abnormal cells within its original location. **Stage I:** The cancer is localised in one part of the body known as a benign tumour which contains an oncogene. The cancer at this stage can be surgically removed **Stage II:** The progression of cancers within a localised area in the body. These type of cancers can be treated by chemotherapy, radiation or surgery. **Stage III:** Is followed by advanced growth of stage II cancers which affect the lymph nodes and can be classified and treated upon diagnosis. This stage of cancer can be treated by chemotherapy radiation or surgery. **Stage IV:** The cancer develops into a malignant tumour and metastasises to other areas within the body. This stage of cancer can be treated by chemotherapy radiation or surgery. Cancer can be treated and managed through various techniques such as radiation, removal of abnormal tissues or through the use of cytotoxic drugs known as chemotherapy. Other forms of treatment involve immunotherapy and targeted therapies involving lasers and light active techniques [38].

1.9 Chemotherapy

The use of cytotoxic drugs to kill cells, control cell proliferation and reduce tumour growth is a common treatment approach in cancer. Chemotherapy was first introduced in 1950's to improve the survival rate and quality of life for millions of patients worldwide and has since evolved into an advanced field of pharmaceuticals and drug development [40]. There are several classes of chemotherapeutic drugs, these drugs are grouped according to the mechanism by which they interact with the various phases of the cell cycle and stop cell growth or cause cell death [41]. Some of these classes of drugs include alkylating agents such as mechlorethamine, altretamine, platinum based drugs such as cisplatin [42]. Anthracyclines are anti-tumour antibiotics such as doxorubicin which works in all phases of the cell cycle [43]. Mitotic inhibitors are plant derived alkaloids that inhibit the M phase of the cell cycle, common mitotic inhibitors include paclitaxel, estramustine and taxanes [44].

Target therapies involve the use of drugs such as imatinib or bortezomib which attack the mutated genes that cause cancer [45]. Antimetabolites drugs function within a cell by competitively inhibiting the enzymes which catalyse the production of nucleotides required during chromosomal replication. This class of drugs are structurally similar to folic acid, purines and pyrimidines [46, 47]. This feature enables antimetabolite drugs to act against cells that are actively replicating DNA and RNA during the S phase of the cell cycle [48]. These drugs include 6-mercaptopurine, capecitabine, hydroxyurea, methotrexate and 5-fluorouracil (5-FU) [49, 50].

1.10 Mechanism of action of 5 Fluorouracil

5 Fluorouracil is an FDA approved, broad spectrum antimetabolite or anti-cancer drug that is used for the treatment of breast, prostate, colon, stomach, pancreas, head and

neck cancers [51]. FDA approval history for all formulations of fluorouracil was published during 1962 to 2012. 5-FU was first FDA approved under the trade name Adrucil® in 1962 as an injectable drug formulation containing fluorouracil as the active ingredient, by the applicant VALEANT, under the chemical type New molecular entity (NME), the marketing status for this drug is currently discontinued [52]. Several modified formulations were approved thereafter, in 2012 an injectable formulation of fluorouracil was FDA approved by the applicant MYLAN LABS LTD, with a current prescription marketing status [53].

The pharmacokinetic information of 5-FU shows that this drug displays 28-100% bioavailability, about 8-12% of the drug is able to bind to protein, the drug has a half-life of 16 minutes and is metabolised in the liver and excreted by the kidney [54]. 5-FU is administered as an injection into the vein intravenous (IV), or as a bolus infusion. The dosage is prescribed depending on the type of cancer, stage of cancer and the patient specific details such as weight, full blood counts, health status, medical history etc. 5-FU commonly causes undesired and adverse side effects such as nausea, suppressed appetite, diarrhoea, unusual bruising, blood in urine and stools, mouth ulcers, fatigue, neurological damage, depression and swelling of the hands and feet. A challenge in improving 5-FU therapeutic efficacy and reducing these harmful side effects is developing mechanisms to direct or target 5-FU to the site of action (tumour tissue) without affecting normal cells and increasing its uptake into the cancer cells [55]. 5-FU is classified as an antimetabolite cytotoxic drug that blocks RNA and DNA synthesis within actively growing cells [56]. 5-FU is able to perform this cytotoxic function because it is structurally similar to uracil, with the exception that it contains a fluorine atom located on the 5th carbon of the pyrimidine ring. 5-FU is a pyrimidine analogue which competitively inhibits the function of the enzyme thymidylate

synthetase [57]. Thymidylate synthetase is the only enzyme responsible for the production of thymine nucleotides, without the formation of thymine, DNA replication stops thereby killing the actively growing cell.

During DNA synthesis Deoxyuridine monophosphate (dUMP) is converted into Deoxythymidine monophosphate (dTMP) nucleotide by thymidylate synthetase (**Figure 1.5**). When the cell is exposed to 5-FU, it is converted into 5-FdUMP, which irreversibly blocks the active site and inhibits the function of thymidylate synthetase [54]. Additionally the phosphorylated form of 5-FU, fluorouridine triphosphate (FUTP) is able to block RNA transcription (**Figure 1.5**)

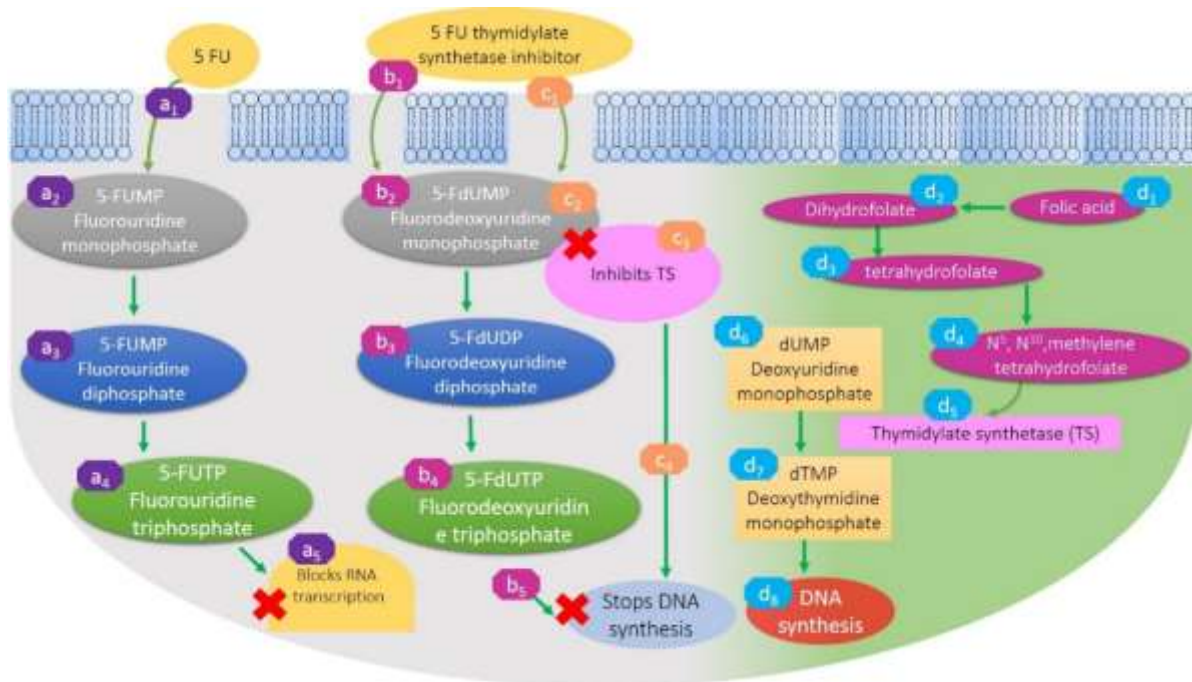


Figure 1.5 Mechanistic pathway of 5-fluorouracil. 5-FU binds to the substrate Deoxyuridine monophosphate (dUMP) to form deoxyfluorouridine monophosphate (FdUMP) and actively inhibits the enzyme thymidylate synthetase (TS). When TS is unable to produce Deoxythymidine monophosphate (dTMP), DNA synthesis is halted. Fluorouridine triphosphate (5-FUTP) actively blocks RNA transcription, both pathways lead to cell death.

1.11 Strategies to improve or enhance 5FU efficacy using nanotechnology

A major drawback of chemotherapeutics such as 5-FU, is that these drugs circulate systemically within the body and have no mechanisms of targeting actively dividing cancer cells. In addition since a small percentage of the drug is bioavailable, larger doses of drug are required to compensate for the active targeting of cancer infected tissues [54, 55]. This means that the circulating drug is able to also act on normal cells which generally divide more rapidly such as the cells that are responsible for hair growth or the epithelial cells that line the gastrointestinal tract. As a result adverse side-effects are observed such as nausea, hair and weight loss, diarrhoea, weakened immunity and opportunistic infections, diseases and sometimes even death [58, 59]. Mechanisms to improve these therapies through cancer cell targeting and increased cellular uptake of the drug is an ongoing aspect of research. The main objective is to improve the pharmacokinetics of existing cytotoxic drugs, to impart new scientific and medical knowledge and to improve patient adherence to therapies that offer greater benefits such as reduced side effects[54, 60].

Literature highlights the strategies employed to enhance and improve the application of 5-FU [54]. The first strategy involves the combination of various classes of chemotherapeutics that act synergistically within an anti-cancer cocktail that can be administered directly to the site of the tumour. The second strategy falls within the domain of nanotechnology, involving the assembly or encapsulation of 5-FU and other chemotherapeutics within biocompatible nanomaterials [61, 62]. Biocompatible nanoparticles can be surface modified through the addition of specific cellular ligands such as growth factors and a combination of chemotherapy drugs to result in improved efficacy of the drug [63-65]. Additionally the use of radioactive isotope nanoparticles can be used to treat tumours, similar to that of brachy therapy used to treat cancer

cells. Another strategy to improve chemotherapy involves the use of nanomaterials with unique optical properties that can be utilized in bio-imaging or localised photothermal therapies that can attack the growing tumour [66-68]. Over the years, researchers have demonstrated a huge demand for nano-based drugs that can improve cancer diagnostics and therapies. In 1995, the first food and drug administration (FDA) approved nano-drug called Doxil® was introduced, this drug was developed through doxorubicin loading within PEGylated nano-liposomes and has been on the market for the last 20 years [69]. FDA Approved paclitaxel albumin-stabilized nanoparticle formulation under the name Abraxane® has been approved for treatment of breast, non-small cell lung and metastatic pancreatic cancers [70]. Furthermore there is an influx of several novel nano-anticancer drugs that are presently in clinical trials [71]. In order to appreciate how these nano-drugs improve conventional therapies an understanding of nanotechnology, the design and synthesis of anti-cancer nano-drugs needs to be described.

1.12 History of nanotechnology

The earliest discovery of nano-inspired materials dates back into the 4th century, during which iron workers and craftsmen were able to generate novel properties from existing resources through the use of heat and the combination of different materials. During this era, dichroic glass was used to make the famous Lycurgus cup which illustrates the optical properties of gold and silver colloidal suspensions, allowing the cup to change colour relative to the direction of light (**Figure 1.6**). Thereafter, throughout the 9-17th century the use of copper, silver and other metallic nanoparticles was applied in the production of ceramics, creating a glowing, glittering effect that swept across Europe and other parts of the world [72]. The design, manipulation and application of gold chloride and other metal oxide nanomaterials became more

common throughout the centuries leading to stained glass windows that adorned European cathedrals [73]. In addition cementite nanowires and carbon nanotubes was used to produce high quality strong swords commonly known as Damascus- saber blades [74] (**Figure 1.6**).

Nano-inspired structures have also existed largely within the natural domains of our animal kingdom [75]. The adhesion properties observed on the feet of a gecko which allow the reptile the ability to travel the walls of ceilings and windows with great ease are due to the microscopic hair-like nanostructures known as setae. The setae branch into thousands of smaller hairs with paddle-shaped ends that are in the size dimension of 200 nm [76]. The increased surface area of these structures utilizes the effect of van der Waals forces, the weak electrical pull between every molecule in the gecko and every molecule in the surface it sticks to. The collective force is so strong that a gecko can carry its entire body weight from a single toe [77]. Scientists have applied the understanding of these adhesion properties using the assembly of carbon nanotubes and other nanomaterials to replicate the gecko setae phenomenon to create adhesives, super-sticky tapes and even a wall-climbing gecko robot [78]. Another fascinating example is the colourful wings of a butterfly that demonstrate how nanostructures interact with light to create different colours. The scales on their wings are composed of various proportions of nanoscale chitin canals, folds, and craters that scatter light in different directions to produce a particular colour, even more interesting is the iridescent property of the wings that causes the colour to change relative to the angle of scattering light [79]. The understanding of the nanostructures observed in nature provide key links in developing reinforced structures, surface modified features, camouflage and robotic devices.



Figure 1.6 History of nanotechnology. The ancient applications of nanomaterials <http://cdn.phys.org/newman/gfx/news/hires/2013/goblettricks.jpg>; http://jameelcentre.ashmolean.org/media/collection/w800/Collections/Single_Objects/EA/EA_P/EA_P_0000/EA_P_59-a-L.jpg; <http://m4.i.pbbase.com/u35/moorlands/upload/23048634.TheMainWindow.jpg>; <https://jasonstravelsdotcom.files.wordpress.com/2012/09/notre-dame-stained-glass-window-paris-france.jpg>; http://41.media.tumblr.com/f763fcbe589dac9af783784b6fee62a6/tumblr_nm7crnPtO1th9k5co3_1280.jpg, 2015

Remarkable discoveries during the passage of history, such as the understanding of optical interactions of ruby colloidal gold solutions, the development of the field emission microscope, semiconductor transistors, fabrication of monodispersed nanomaterials for industrial applications and the development of various analytical instruments or techniques has contributed largely to our understanding of nanoscience [80]. The diverse knowledge of engineering materials that display enhanced or novel properties has led to the modern era of nanotechnology. In 1959, Professor Richard P. Feynman, introduced the revolutionary concept of nanotechnology in his lecture titled, "There's Plenty of Room at the Bottom", which focused on the concept of molecular fabrication of matter on the nano level [81]. Nanotechnology has rapidly evolved into a multidisciplinary science that involves the design, synthesis and applications of new materials through the control of matter on the nanometre-length

scale. Nanoparticles are defined as units of matter with at least one dimension between 1-100 nm [82]. Nanotechnology currently supports a myriad of industrial, computational, engineering, mechanics and biomedical applications [83]. Over the past few decades nanoparticles have gained much scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures [84].

1.13 Nanotechnology within the biological domain

Nanomaterials display unique physicochemical characteristics such as increased surface area to volume ratios, increased reactivity, size, surface chemistry and quantum confinement properties that are not present in their bulk counterparts [85]. In addition they can also be synthesized to have controllable sizes ranging from a few nanometres up to tens of nanometres, which place them at dimensions that are smaller than or comparable to those of a cell (10 – 100 μm), a virus (20 – 450 nm), a protein (5 – 50 nm) or a gene (2 nm wide and 10– 100 nm long) [84] (**Figure 1.7**). It simply means that they can gain access and interact with biological entities of interest. This feature enables nanoparticles to be applied within a wide range of bio-applications such as gene therapy, drug delivery and diagnostics. There are many different nano-structured materials, such as nanospheres, nanocubes, nanorods, nanowires, nanoflowers, nanosheets, nanotubes, quantum dots and core-shell nanoparticles which are categorized according to their optical, electrical, chemical and morphological features [86-88].

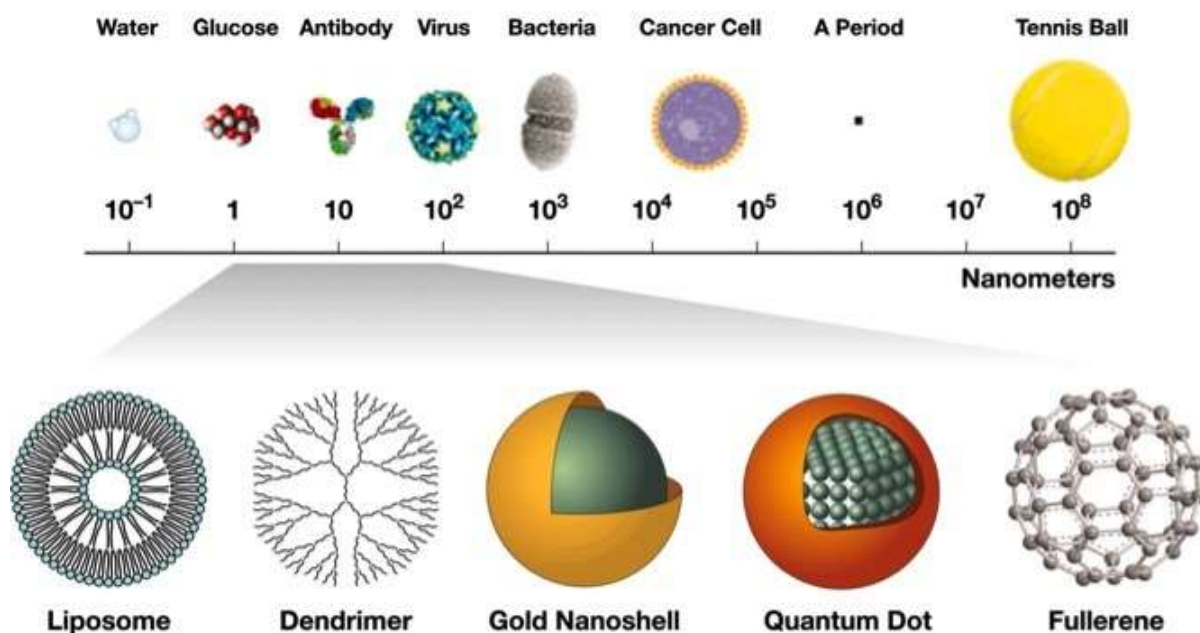


Figure 1.7 Comparison of various materials on the nano-metre length scale (1-100 nm), illustration of the many classes of nanomaterials. <https://ocg.cancer.gov/sites/default/files/Nanotechtop1.jpg>, 2015.

1.14 Core-shell nanomaterials

Over the many decades nanoparticles generated huge scientific interest because of the size dependent properties that are absent in their bulk equivalents [89]. These novel size dependent properties displayed the potential to support a myriad of applications within materials chemistry, engineering, biomedical, pharmaceutical and consumer products [81]. Despite the novel properties and applications of nanomaterials, in recent decades the demand and need for greater advancements within materials chemistry and technology has led to the synthesis of core-shell nanostructures. During the 1980s composite colloidal semiconductor nanoparticles were developed, resulting in superior functional and structural properties over their corresponding parent nanoparticles alone [90, 91]. This observation led to the development of heterogeneous nanomaterials such as multi-layered semiconductor

nanoparticles that was synthesised with the aim of enhancing already existing properties [92]. Core-shell nanoparticles are synthesized by combining two or more different types of nanomaterials [93]. The assembly of core-shell nanoparticles is characterised by a distinct core material that is surrounded by a layer of shell material [94]. Several types of core-shell nanomaterials exist, some are categorized as semiconductor/semiconductor, magnetic/ semiconductor and metal/ semiconductor heterostructures [95-97]. The inherent optical, quantum confinement and electrical properties of the parent nanomaterials can be improved by the design of heterostructured nanomaterials [93, 94]. Work reported by Verma and co-workers displayed enhanced magnetic properties of Fe₃O₄-PLGA core-shell nanoparticles that were surface modified through the addition of anti-cancer drug quercetin, for applications within aerosolized drug delivery for lung cancer [98]. Their study demonstrated the enhanced magnetic properties of the core-shell nanoparticles over conventional Fe₃O₄ nanoparticles and showed increased solubility of the drug when incorporated with the core-shell nanocarrier [98]. The synthesis of Pt-Cu core-shell nanoparticles through polyvinylpyrrolidone or oleylamine/oleic acid stabilizers was used to display improved stability and catalytic activity of nitric oxide reduction, the study further established that the reciprocal core-shell Cu-Pt core-shell displayed high catalytic activity. In another study, improved optical properties were demonstrated through the synthesis of sulphide based core-shell nanoparticles [99]. The optical properties of the MnS-ZnS and PbS-ZnS core-shell heterostructures displayed enhanced blue shifts and increased band gaps when compared to the ZnS nanoparticles alone [100]. Literature shows that core-shell nanoparticles can host a wide range of enhanced features such as changes in the band gap, surface chemistry, photoluminescence or optical, electrical and magnetism properties [94, 101-103]. The

intended application is the primary factor that determines the type of component materials utilised to construct the desired core-shell heterostructure [104-106]. Research trends show that the use of gold nanoparticles in the design of Au-based core-shell nanostructures is increasing. (**Figure 1.8**) illustrates the increase in research publications of core-shell nanostructures verses core-shells composed partly of gold nanoparticles over the last two decades [106-115].

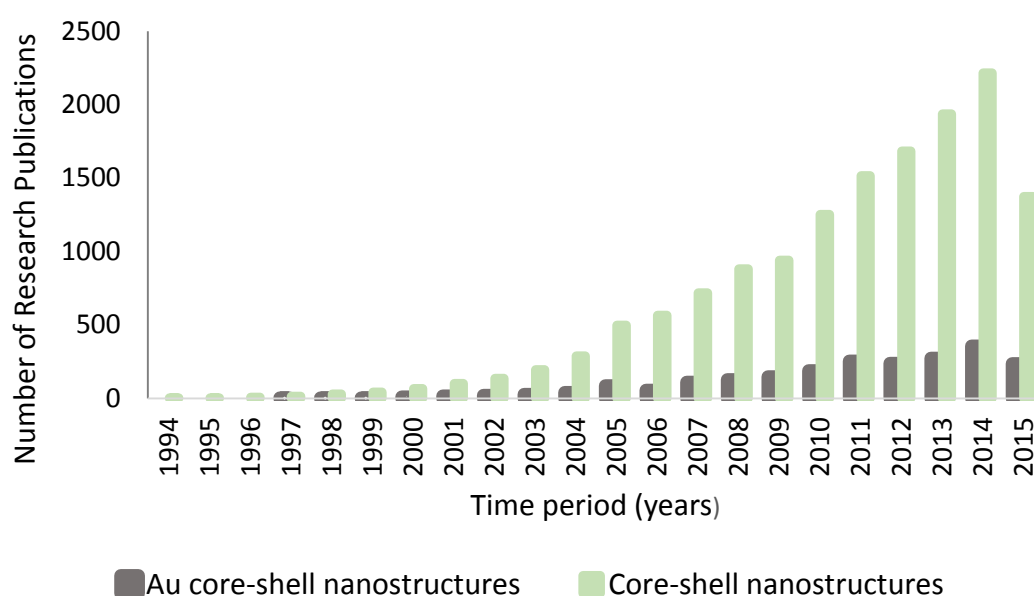


Figure 1.8 Core-Shell versus Gold (Au) composed Core-Shell nanoparticles. Research over the last twenty years indicate a growing niche for the synthesis and applications of core-shell nanomaterials which contain gold as either the core or shell component.

The synthesis, characterisation and applications of gold nanoparticles have been well documented throughout history. Gold nanoparticles are utilised in several applications because they highly stable, non-toxic, easily synthesized in a variety of shapes, sizes and surface chemistry [116-118]. Additionally gold nanoparticles display catalytic, electronic and optical features [119]. Upon stimulation by incident light, gold nanoparticles display unique surface plasmon resonance (SPR) properties that are characterised by the collective oscillation of valence electrons which results in enhanced light scattering and absorption features [120]. The optical properties can be

used as contrasts agents in biomedicine and bio-imaging applications as they do not exhibit photobleaching [121]. These unique optical features enable the rapid conversion of absorbed light into heat resulting in photothermal therapy [122]. The intrinsic properties of gold nanoparticles makes them promising components in the assembly and synthesis of core-shell nanostructures that are multifunctional and support various applications in catalysis, bio-labelling, drug delivery, electron transfer theories, bio-sensing and dye-sensitized solar cells [123].

1.15 Synthesis of gold based core-shell nanoparticles

There are two approaches for the synthesis of nanostructures including core-shell nanomaterials, commonly known as the bottom-up and top-down approach [124]. The bottom-up approach involves the growth of nanostructures through the assembly of building precursors such as atoms or molecules [125]. These building precursors can be created by using chemical synthesis, self-assembly, laser-induced assembly or colloidal aggregation growth techniques. The top-down approach involves the synthesis of nanostructures through the breakdown or reduction of bulk materials [126]. Common top-down processes include mechanical techniques, film deposition, laser-beam processing and lithographic techniques [127, 128]. In the case of core-shell nanoparticles the synthesis usually involves two steps, the synthesis of a core followed by the growth of a shell. These growth techniques can be categorised on the availability of the core material during the synthesis procedure [129]. (I) involves the initial growth of the core followed by the shell particles in situ within the same reaction solution. (II) Involves independent growth of the core or seeding materials followed by the addition of the shell material through the use of appropriate coating or surface modifications. In the first technique the core is synthesized using suitable shape directing reactants or surface capping agents, after the core is formed more reactants

are added to initiate shell growth in situ [94, 130, 131]. In the second technique the core particles are independently synthesized and purified followed by surface modification that facilitates the growth of the shell material [123]. An important aspect during the synthesis of the core-shell nanoparticles is to maintain uniform shape and control of shell thickness coating. These features determine the type of applications the core-shell heterostructure may be used in. Establishing proper control over shell thickness and coating is still a challenge [132]. Researchers observe various difficulties in this step such as incomplete or bare core material, aggregation of core material within the reaction solution, formation of alloy materials, formation of shell materials or secondary nanomaterials and precise control of the reaction rates [94]. The use of appropriate surfactants, temperature and pH are employed to change the surface properties of the core materials to make it more suitable for selective deposition and growth of shell particles [133, 134]. Literature cites several dedicated reviews that cover specific materials composed of Au-based core-shell nanoparticles are reported for SiO₂/Au, Au/TiO₂, Au/polymer, magnetic/Au and Au-Ag core-shells [135-138]. These reports illustrate the diversity of Au-based core-shell nanostructures in scientific research [123]. In an elaborate review by Chaudhuri and Paria, the synthesis, applications and the progress of research advancements of Au-based core-shell nanoparticles was reported [123]. In summarising the findings of their report, the authors demonstrated that early research on Au-based core-shell nanoparticles focused primarily on optimising various synthesis routes, core to shell ratios and core to shell combinations. In optimising the materials chemistry domain scientists were able to correlate size tunable properties that have contributed to the many advancements within applications of Au-based core-shells. The synthesis routes are broadly classified into two methods i.e. wet and dry chemistry. Wet chemistry routes

are further classified as reduction of gold salt, electrochemical synthesis and thermal decomposition and photochemical method. Dry chemistry methods involves the condensation of metal from metal vapour under inert atmospheric conditions commonly known as the laser vaporization/ controlled condensation technique [139-141].

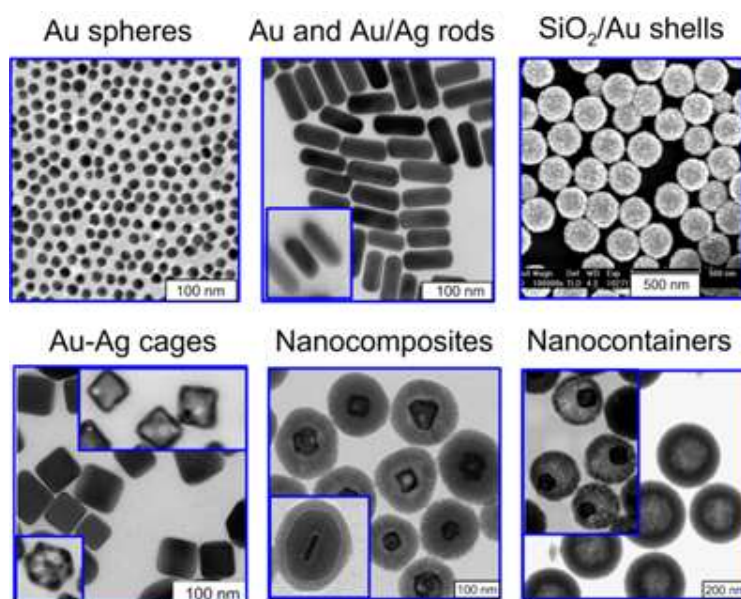


Figure 1.9. Morphology of Au-based core-shell nanostructures. Core-shells display tuneable properties, they can be designed in a variety of shapes, sizes and core-to-shell ratios. (<http://www.thno.org/v03p0167.htm>,2015)

Due to the many advances and improvements in synthesis techniques, it has become possible to produce core-shell nanoparticles in various shapes such as nano disks, nanorods, nanocubes, hexagonal and tubular shapes (**Figure 1.9**). The shape of the core-shell, also known as surface anisotropy effects, contributes to the properties of the heterostructured nanomaterials [94, 123, 141, 142]. It is well demonstrated that gold nanospheres display unique optical properties, however when the gold nanoparticle is in the shape of a nanorods, the surface plasmon resonance properties increase largely. The catalytic activity, electrical, optical and melting points of nanomaterials are shape-dependent [120].

1.16 Core-shell nanoparticles composed of Au core

Au-based core-shell heterostructures can be produced by the assembly of an external shell material. The shell layer can be grouped into three classes i.e. metallic shell (bimetallic), inorganic (oxides or metal chalcogenides) and organic or polymer shells [123]. The synthesis of bimetallic core-shell nanoparticles offer improved catalytic, electronic and optical properties. Au/Ag, Au/Pd and Au/Pt are the most popular bimetallic core-shell nanostructures [115, 143-145]. These core-shell structures are formed because of their close matching of the lattice constant, such that the metal shell atoms epitaxially nucleate and growth on the surface of the Au core or seed material [123, 146]. The shape, size and core to shell ratios are controlled and regulated by experimental parameters such as the size, shape and surface chemistry of the Au seeds, the reducing and shaped directing agents, reactant concentration, reaction media and temperature [147]. The shape and size of the core imparts a direct influence on the passivation of the shell layer. The synthesis of Au core-inorganic shell nanoparticles include TiO_2 and SiO_2 . An advantage of silica coating provides an inert shell material without disturbing the optical properties of Au [103, 148, 149]. Usually silica coated Au-based core-shell nanoparticles are produced by modified Stöber/ sol-gel reactions in the presence of thiol type polymer-capped Au nanoparticles [103]. The shell thickness in this case is controlled by the coating time, the reactant concentration and the use of catalysts. TiO_2 is a catalytic material and when incorporated as a shell material surrounding a Au core, the core-shell is able to exhibit superior catalytic activity for various chemical reactions such as NO_x reduction, CO oxidation and water-gas shift chemical reactions [131, 150, 151]. Other metal oxides such as ZnO, ZrO_2 , Fe_3O_4 , Fe_2O_3 and Cu_2O were used to coat Au cores to enhance or improve size tuneable optical and catalytic properties. Additionally chalcogenide semiconductor-

coated Au core-shell nanostructures have been synthesized for photocatalytic applications [152-154]. This category includes other shell components such as CdSe, Cu₂S, CdS and Ag₂S [112, 155, 156]. The use of organic or polymer shells on Au cores offer improved biocompatibility and surface functionalisation properties such as ligand attachments for biomedical applications in drug delivery and bio-imaging [157-159]. Low density polymers are used to coat Au surfaces for superior dispersibility and reduced aggregation of particles [160, 161]. The growth of Au cores can be stimulated through activation or excitation of the surface plasmon resonance of gold nanospheres causing the growth of Au-CdS nanospheres [162]. Metal-semiconductor core-shell nanoparticles display enhanced optical, electrical and catalytic properties [133, 163]. These properties can be further manipulated for bio-applications through functionalisation of the core-shell surface with biocompatible surface layers [163, 164]. Temperature is the most common parameter in controlling the growth of core-shell nanoparticles [165, 166]. High temperatures are required for thermal decomposition reactions and low temperature methods are used for conversion during polymerisation reactions. Low-temperature methods are used to produce biologically capped, water soluble core-shell nanoparticles. The nucleation, growth and agglomeration or self-assembly stages during the reaction are also influenced by pH, effect of reactant concentration, capping agents and other external forces like electric currents or sound energy [107, 167, 168]. Hydrophilic surfaces that support bio-conjugation in the development of biocompatible core-shell containing gold as the core supports a diverse range of applications (**Table 1.1**). The multicomponent Au-Based core-shell nanoparticles have been tabulated to highlight the class of the heterostructure, combination of nanomaterials, synthesis method and application.

Table 1.1 Au-based core-shell nanoparticles: Au core with bimetallic, inorganic and organic or polymer shells

	Core-Shell component (class)	Reaction method	Properties/ Application	Reference
<i>Bimetallic shell</i>	Au/Ag	Aqueous chemical method	Detection of adenosine	[169]
	Au/Ag	Reduction method	Catalytic	[170]
	Au/Ag	Seeded growth	Food chemistry, bio-sensors in food products	[171]
	Au/Ag ₂ S	Reduction method	Immunosensor	[172]
	Au/Pt	One-step synthesis, green chemistry using Gallic acid as the reducing agent	Catalytic	[173]
	Au/PtAu	Intermediate-template directed method	Catalytic	[115]
	Au/Pd	Reduction method	Catalytic	[174]
	Au/Co	One-step seeded growth	Catalytic	[175]
	Au/Ni	Reduction method	Optical, Catalytic	[110]
	Core-Shell component (class)	Reaction method	Properties/ Application	Reference
<i>Inorganic shell (oxide or metal chalcogenides)</i>	Au/SiO ₂	Stöber processing	Immunosensor	[176]
	Au/LDH (Layered double hydroxides)	Seeded growth	Anti-cancer drug therapy	[177]
	Au/SnO ₂	Seeded growth	Electro-optical switches, redox catalysts	[178]
	Au/TiO ₂	Hydrothermal route	Photocatalytic	[179]

	Au/CdSe	One-pot seeded growth	Photovoltaic, nanoelectronic devices.	[112]
	Au/CdS	Seeded growth	Optics, electronics	[180]
	Au/Cu ₂ S	Wet chemistry route	Optical Photocatalytic	[154]
	Au/GON (graphene oxide nanocolloids)	One-pot synthesis, reduction	Bio-imaging, photodynamic therapy	[181]
	Core-Shell component (class)	Reaction method	Properties/ Application	Reference
	Au/rGO-PEG (pegylated reduced graphene oxide)	Au nanorods seed mediated synthesis, PEGylated GO was conjugated to Au	Photothermal therapy	[182]
	Au/ZnO	Seeded growth, Au reduction via sodium citrate	Optical	[168]
	Au/ZnO	Etching treatment	Bio-sensors	[152]
	Au/ZnS	Chemical precipitation method	Photocatalytic	[183]
	Au/Fe ₃ O ₄		Photocatalytic	
	Au/In ₂ O ₃	Au@carbon template growth	Gas sensing	[184]
	Au/Fe ₂ O ₃	Seeded growth	Catalytic	[185]
<i>Organic/ polymer shell</i>	Au/poly(3-dimethylammonium-1-propyne hydrochloride)	Chemical reduction, bio-conjugation of polymer	Enzyme immobilisation for anti-cancer therapy	[186]

1.17 Core-shell nanoparticles composed of Au shell

Similarly Au-based core-shell heterostructures can be produced by the coating of gold nanoparticles over metal, inorganic and organic cores [123]. In this case the core component is categorised into three classes i.e. metallic core, inorganic core and organic or polymer core (**Table 1.2**). Ag, Fe, Co, Pd and Pt may be used as metallic cores in the synthesis of Au-based core-shell nanoparticles to support various optical, catalytic and magnetic applications [94]. Ag/Au core-shell nanoparticles display great importance in surface-enhanced Raman spectra (SERS) intensity or the shifting of the absorption peaks towards the near-infrared wavelength (NIR) region which facilitates optical imaging processing of biological specimens [187-189]. Similarly the use of super paramagnetic iron oxide, magnetic shell nanoparticles such as (Fe and Co) are used in cellular labelling, hyperthermia and drug delivery applications [190-194]. Au particles have high standard reduction potentials which allows for the easy synthesis of metallic core-Au shell nanoparticles through the galvanic replacement method [195-197]. Coating metallic nanoparticle cores with Au shells enhances the stability and biocompatibility properties of the heterostructure nanomaterial [106, 153]. The use of gold as a shell, supports lower-toxicity and stabilises the core-shell for advanced surface modifications. In some cases the use of a gold shell is then able to support additional layer by layer growth of additional shell materials. The synthesis of inorganic material/ oxide cores and gold shells are challenging due to the difference in surface energies of the materials. However the combination of inorganic cores-Au shell yields core-shell nanostructures that display low toxicity, biocompatibility, low reactivity and favourable binding affinity to amine and thiol terminal groups, these features can be utilised within biomedical applications within cancer therapeutics and diagnostics [62, 158, 198].

Table 1.2 Au-based core-shell nanoparticles: Bimetallic, inorganic and organic or polymer core with Au shell

	Core/Shell component (class)	Reaction method	Properties/ Application	Reference
<i>Bimetallic shell</i>	Ag/Au	Co-reduction method, antibody conjugated through self-assembly monolayer technique	Electrochemical biosensor, rapid detection of <i>E. Coli</i>	[199]
	Ag/Au	Reduction method by leaf extract of <i>Catharanthus roseus</i>	Anti-microbial	[200]
	Pt/Au	Reduction method	Electro-catalyst	[201]
	Pt/Au			
	Pd/Au	Langmuir-Blodgett method	Hydrogen sensors	[202]
	Ni/Au	Sodium citrate reduction method	Magnetic	[203]
	Co/Au	Inert gas condensation method	Biomedical magnetotherapy	[204]
<i>Inorganic shell (oxide or metal chalcogenides)</i>	SiO ₂ /Au	Stöber method	Optical	[205]
	TiO ₂ /Au	Photocatalytic reduction method	Catalytic	[206]
	ZnO/Au	Hydrothermal route	Bio-sensors	[207]
	Fe ₃ O ₄ /Au	Co-precipitation method, alkyne-functionalised folate molecules	Targeted drug delivery	[208]
	Fe ₃ O ₄ /Au	Reduction method, bio-conjugation with folic acid	Anti-cancer drug delivery	[209]
	Fe ₂ O ₃ /Au	Lectin conjugated reduction method	Bio-imaging, tumour targeting	[210]

1.18 Biomedical applications of Au-based core-shell nanoparticles

Modern architectures, such as core-shell nanoparticles have greatly influenced many medical advancements within bio-imaging, bio-sensing, gene delivery, rapid detection of disease, anti-microbial agents and cancer therapeutics [211-213], some of these advances have been highlighted in (**Table 1.3**). These nano heterostructures are cleverly designed to possess multifunctional properties that are favourable over either of their parent materials alone (i.e. the core or shell component).

During the last twenty years of nanotechnology research, much attention was paid to the design and synthesis mechanisms in combining nanomaterials that function as a single entity to produce novel properties [123]. Scientists sought out to understand how two different nanomaterials interact to form a core and shell nanostructure and the role of core to shell ratios in relation to the functional properties it exhibits. Large contributions within the fields of materials chemistry has shown that the experimental conditions such as pH, reaction time, temperature, atmospheric conditions, solvents, reducing, stabilising and structure directing agents facilitates precision and controlled core-to-shell growth [93, 94]. The intended application usually directs the types of materials that may be compatible to form a core-shell heterostructured nanomaterial for specific bio-applications. Other factors include lattice matching, reduction potential and band gap properties of the contributing parental materials [214]. In addition to the favourable size, these core-shell nanoparticles must be biocompatible, water soluble and have surface properties that are able to conjugate or absorb cellular receptors, ligands, peptides, drugs and other biomolecules [164, 215].

These properties can be utilised in a wide range of applications such as stem cells, neurological, diabetes and cardiovascular disorders [216-218]. Recent developments

within bio-applications of core-shell nanoparticles show that these nanomaterials are “new-age smart” architectures that can improve medical technology and diagnostics drastically. Of particular interest to this project are Au-based core-shell nanostructures for bio-applications such as photothermal therapy, bio-imaging, drug delivery and cancer therapeutics. A common strategy of core-shell nanostructures containing gold is the design of gold cores surrounded by a versatile shell material or a gold shell that encapsulates a core material to can improve optical, magnetic or surface chemistry features. Improving these features provides a platform to advance bio-imaging, photothermal and targeted drug delivery for cancer therapeutics [153, 158, 219].

The design and application of gold-based core-shell heterostructures is important in localised photothermal destruction of tumours [122, 220]. Combinations of metallic gold and magnetic nanoparticles are able to exhibit a property known as photothermal ablation therapy which involves the release of localised heat between 42-46 °C resulting in irreversible cellular necrosis [221]. The hyperthermia mechanisms by which multicomponent core-shell nanoparticles function is based on the conversion of laser energy into thermal energy. Core-shell nanoparticles that display strong, tunable NIR (650-1064 nm) absorption, biocompatible surface features and low toxicity are favoured for photothermal ablation therapy [222]. Current research shows that most photothermal nanomaterials contain gold nanostructures either in the form of hollow, caged or care-shell nanomaterials [181].

Additionally Au-based core-shell nanoparticles offer dual functions that can be exploited for their bio-imaging potential when combined with bimetallic or magnetic iron oxide nanoparticles [223]. Work reported by Chen et al, illustrated the synthesis and application mesoporous silica enclosed by gold nanorods that form a core-shell with advanced optical and photothermal properties [224]. The study further showed

improved thermal stability of the gold nanorods and demonstrated cellular uptake and tumour destruction through a unique 2-step sequential intra-particle energy transfer upon two-photon excitation. A combination Au and Ag core-shell nanoparticles conjugated to thiol-capped aptamer molecules were synthesized by the reduction of citrate ions and utilized for *in vitro* photothermal therapy using lung adenocarcinoma (A549) cells [225, 226]. The use of Au-Fe₃O₄ core-shell nanoparticles are extensively researched for *in vivo* photothermal and bio-imaging of tumours [153, 158, 227]. Other reports describe the use of additional chemotherapy drugs and target ligands to enhance the photothermal properties of Au-Fe₃O₄ core-shell combinations [208]. An extensive report by Melancon and co-workers described the mechanisms by which different classes of Au-nanoparticles can be used for photothermal and bio-imaging applications [228, 229]. This report highlights the advances within cancer diagnosis and therapy through the use of multifunctional core-shell nanoparticles [230]. It is worth noting that a class of silica core/gold shell nanoparticle has reached clinical trial status under the trade name AuroLase [231]. In another report, multifunctional core-shell nanocapsules were synthesized via loading perfluorooctylbromide (PFOB) and superparamagnetic iron oxide nanoparticles (SPIOs) into poly(lactic acid) (PLA) nanocapsules, followed by the formation of PEGylated gold nanoshell on the surface [232] (**Figure 1.10**). The multicomponent core-shell was able to provide ultrasound, magnetic resonance imaging and photothermal ablation therapy using a human xenograft tumour model. The study showed that the unique optical features of both core and shell components was used to provide multiply features thereby increasing therapeutic efficacy within the nude mice models. The study further demonstrated by controlling the thickness of the gold shell and the diameter of the core, the plasmon resonance and the resulting optical absorption of gold nanoshells can be tuned to the

NIR region, where the absorption of human tissues is minimal and penetration is optimal [232].

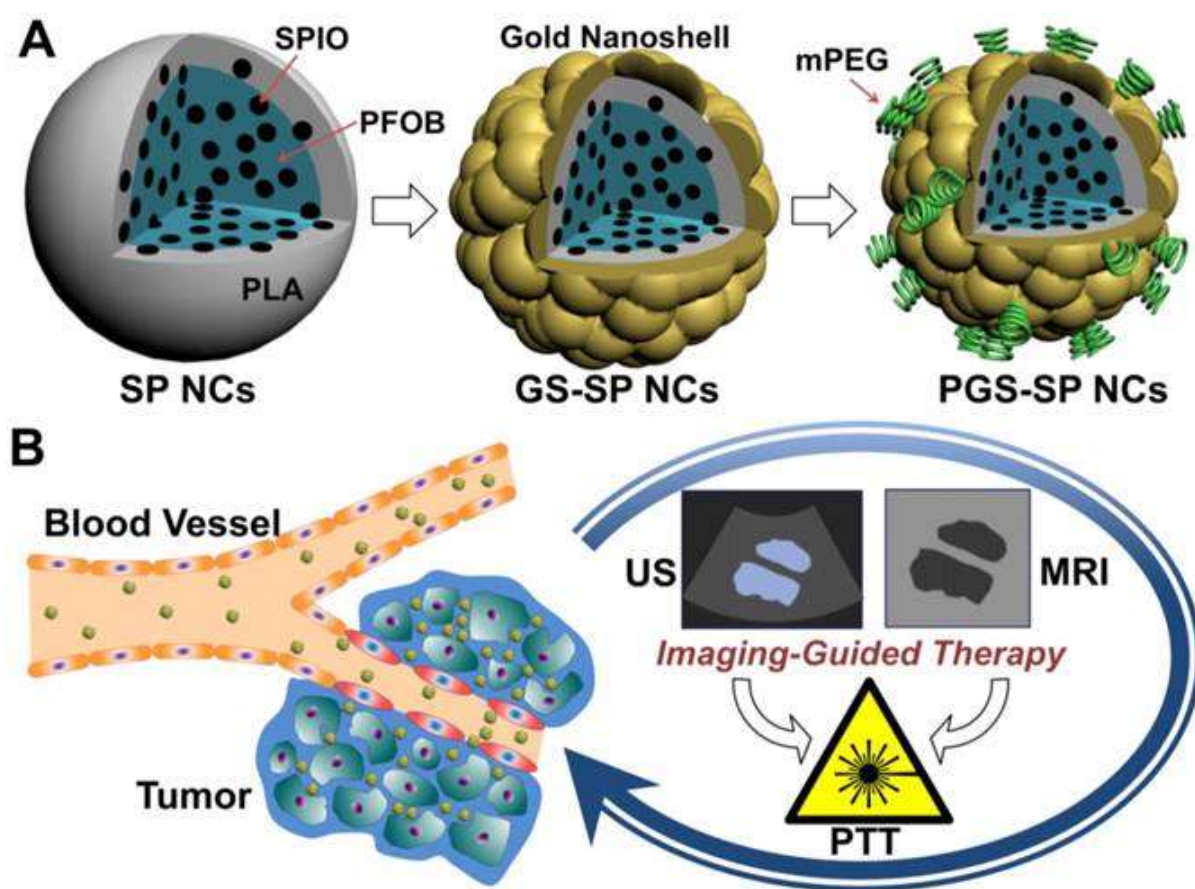


Figure 1.10 *Dual bio-imaging and photothermal ablation therapy of tumour tissue. Superparamagnetic iron oxide- Au nanoshell, for cancer therapeutics.* (<http://www.thno.org/v04p0012.htm>, 2015)

The design and application of gold-silver bimetallic core-shell nanoparticles is important in improving anti-bacterial, anti-microbial and bio-imaging applications [233, 234]. In addition to their unique optical features they can be surface modified to direct applications in food chemistry, bio-sensors and drug delivery [235]. In general gold nanoparticles provide biocompatible, non-toxic vehicles for targeted anti-cancer drug delivery applications [120]. These features can be further improved through combination of multiple nanomaterials, chemotherapy drugs and cell specific ligands. Important factors in developing Au-based core-shell nanoparticles for targeted drug delivery include the stability of the drug modified nanomaterial, the ability to exhibit

sustained, controlled or regulated release of the drug at the target site and improved therapeutic efficacy over conventional drugs [236]. By understanding the underlying mechanisms that present specifically within cancer cells researchers demonstrated the biocompatibility and targeted drug delivery of Fe-Au core-shell nanoparticles conjugated to doxorubicin for in vitro anti-cancer studies [237].

Cancer cells display amplified expression of growth and folate receptors to support its uncontrolled growth, scientists use complementary cellular ligands to drive the delivery core-shells directed to the cancer cells [229, 238-240]. This targeting mechanism is enhanced when injected directly into the tumour site. In a recent reports scientists were able to demonstrate the use of folate ligands and chemotherapy drugs conjugated to iron oxide and gold core-shell nanoparticles, the study demonstrated the enhanced anti-cancer properties of core-shells functionalised with folate or various cellular receptors [240]. In an independent study Au-SiO₂ core-shell nanoparticles were fabricated, the core-shell was surface conjugated to methotrexate (MTX) anti-cancer drug and shown to have superior efficacy over MTX alone [177]. Similarly the assembly of Au-SiO₂ core-shell nanoparticles were designed for multiple applications within cancer therapeutics [241]. Mesoporous silica coated-gold nanorods were used to demonstrate photothermal and chemotherapy therapy in human cell lines. The study also demonstrated localised tumour therapy and illustrated the laser activated properties of core-shell nanoparticles that display optical features within the NIR region (**Figure 1.11**) [241].

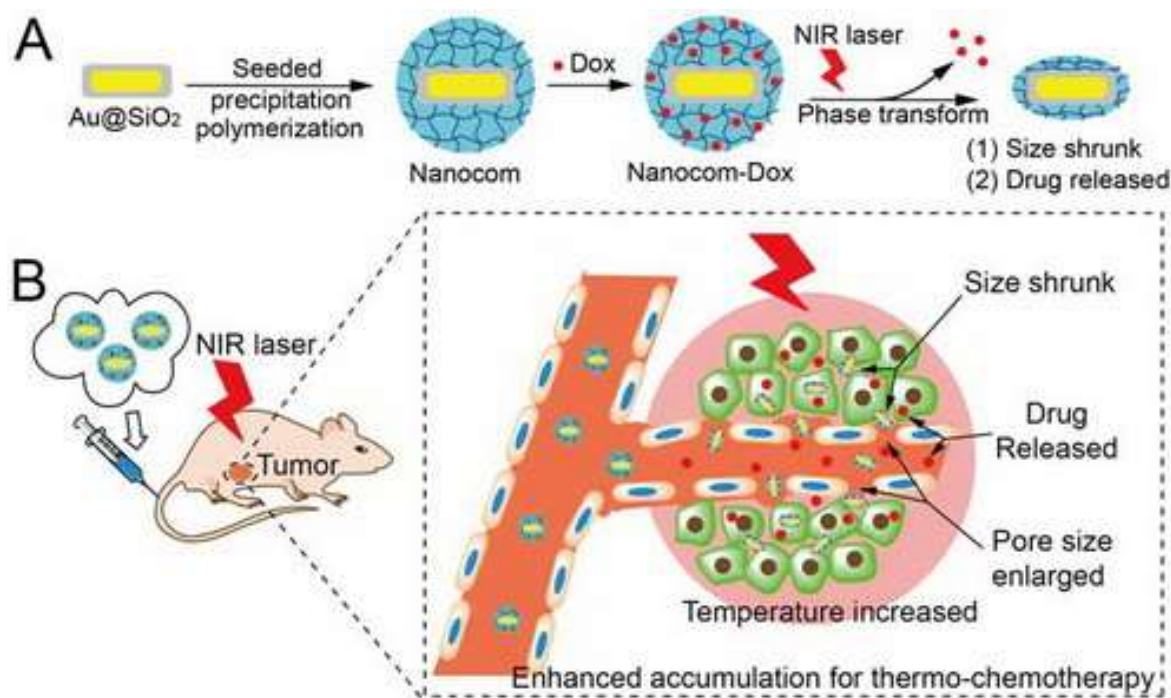


Figure 1.11 Au-SiO₂ core-shell nanoparticles for anti-cancer drug delivery. Improved efficacy of Dox chemotherapy drugs through the design of biocompatible, multifunctional Au-SiO₂ core-shell nanoparticles.

(http://english.nanoctr.cas.cn/pw/201405/t20140529_121529.html, 2015)

The ability of Au-based core-shell nanoparticles to be applied within targeted drug delivery is an ongoing area of research that is largely advancing to downstream clinical trials and pharmaceutical applications. The main goal of developing biocompatible Au-based core-shell nanomaterials is to demonstrate its in vivo applications and so that new improved nano drugs can be approved. Although literature illustrates a huge output of various core-shell nanoparticles, very limited attention is paid to the bio-safety and toxicity of these nanomaterials (**Table 1.3**). Establishing the bio-interaction of core-shell nanoparticles within cellular, animal and environmental systems is a crucial factor in the successful applications and advancement of nanotechnology [242].

1.19 Biosafety and nanotoxicology of Au-based core-shell nanoparticles

Due to increased synthesis and applications of core-shell nanoparticles, toxicology and safety of these nanomaterials are a critical aspect within nanotechnology applications. Toxicology is generally defined as the study of the adverse effects of chemical, physical or biological agents on living organisms, the ecosystem and our living environment. It is based on the 16th century principle that any substance can be toxic if depending on its quantity [243]. Strict regulations are in place to prevent toxic materials from entering consumer and medical products [244]. Most countries have enacted laws and regulations to control the marketing and applications of drugs, vaccines, food additives, pesticides, industrial chemicals, and other substances of potential toxicological risk. Such regulations determine whether the benefits of a particular substance in this case nanomaterials outweigh its risks to human health and the environment. Dose metrics are key components in a nanotoxicology assessment which is based on the measurement of dose against a physical metric of particle mass, total surface area of particles or particle numbers for a well characterised nanomaterial to enable quantitative and qualitative interpretation of data [245]. In essence contact exposure during the production of nanomaterials at the work environment or exposure routes such as inhalation, ingestion, digestion, dermal exposure or administered injection in the case of therapeutic applications allow nanoparticles into a biological system [246]. It travels from the exposure site, circulates systemically through the blood circulatory and lymph system until it is able to interact with organs, tissues, cells and sub-cellular organelles or biomolecules [246]. Exposure to nanoparticles of any kind may lead to mild or severe toxic responses. It is important to conduct a toxicology assessment of the proposed nanoparticles that are intended for bio-applications. Investigating the toxic responses and biochemical interactions between nanoparticles

and biological entities of interest will provide valuable information on therapeutic dosages for future application, in some cases it may even provide evidence that a nanoparticle is too toxic to be considered for bioapplications and in other instances it may provide motivation to further investigate the application potential of certain nanoparticles [246, 247]. Nanotoxicity responses are evaluated by the following parameters

- I. Physical and chemical changes in cellular morphology- The physical features of a cell may be disrupted or denatured during toxic exposures. The characteristic features of the cell membrane, cytosol, internal membrane bound organelles and the nucleus each have vital roles in the cell cycles, should one organelle incur damage or stress it will impact on the entire cellular system and may even lead to apoptosis (programmed cell death).
- II. Innate immunity- Indicates a first line of defence to foreign materials that interact with the biological system, these include responses such as pain, fever, platelet aggregation and inflammation. Molecular markers such as pro-inflammatory cytokines, white blood cells or heat shock proteins are secreted at elevated levels to maintain homeostasis after an initial toxicity response.
- III. Integrated organs and *in vivo* animal systems- indicates a holistic view of the biochemical interactions with nanoparticles, mechanistic pathway interactions, hormone or enzyme release, tissue and organ analysis and clinical observations.

The morphology and physiology of a living cell can provide primary information on the toxicity of nanomaterials [248]. A eukaryotic mammalian cell is a basic unit of life. Cells

grow to form tissue, which differentiate to form functional organs that conform to produce a multifunctional living organism (**Figure 1.12**). Cells typically consist of a cytoplasmic membrane and a cytoplasmic fluid matrix that houses membrane bound cellular organelles. During toxic exposures from foreign agents that enter the biological system many cellular organelles may undergo stress, DNA damage, chemical and physic changes [249]. Each organelle plays unique roles that synergistically contribute to homeostasis and cellular processes. Scientists believe that nucleic acids are responsible for the creation and evolution of life. This is simply because they are the only biomolecules that carry the astonishing potential for self-duplication. The blueprint for an organism is encoded in deoxyribonucleic acid (DNA). These nucleic acids are found within a nucleus of a cell, they serve as the repositories and transmitters of genetic information for every cell [187]. During toxic exposures DNA may undergo mutations that lead to the growth of cancerous cells [16].

The mitochondria is a membrane enclosed organelle that is bean shaped and usually between 0.5 -1 μm in size. This organelle is called the power house of the cell and is responsible for the breakdown and supply of energy known as adenosine triphosphate (ATP). Mitochondria are further involved in cellular signalling, cellular differentiation and cell death. Any stress placed on this organelle would have detrimental effects on the cellular processes and other organelles [250]. Cytoplasmic membranes are protective and structural features that separate the internal cellular environment from the external environment. The cell membrane consists of a phospholipid bilayer that is selectively permeable to ions and organic molecules [251]. The cellular membrane plays a key role in the interaction and entry of nanoparticles, therapeutic drugs and other chemicals. During the production and application of core-shell nanoparticles, different exposure routes inevitably lead the nanoparticles into the blood circulatory

system where they will be transported to the organs, tissues and cells [246]. Once the nanoparticles enter the cell, they are capable of interacting with the cell membrane, cytoplasmic environment, mitochondria, cellular organelles and even the nucleus and DNA molecules. It is therefore extremely necessary to evaluate the interaction between the core/shell nanoparticles and biological entities.

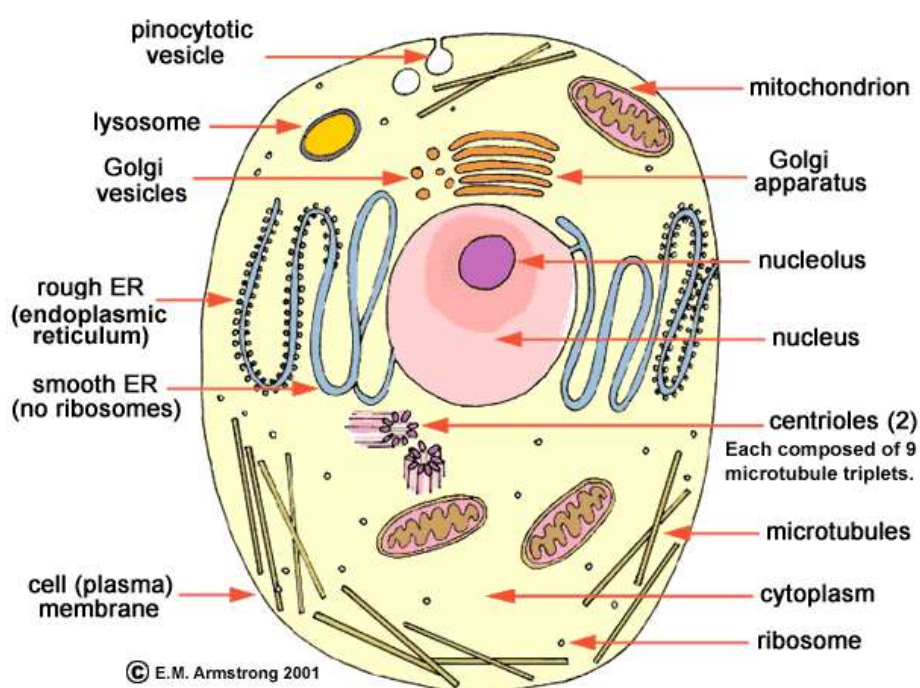


Figure 1.12 Shows an illustration of the morphology of a eukaryotic cell and its functional organelles (<http://waynesword.palomar.edu/images/animal.gif/2013>)

The blood circulatory system serves as a transport and communication mechanism between organs, tissues and cells. It is composed of white blood cells, red blood cells and platelets. The blood system is responsible for transporting nutrients such as amino acids, oxygen, carbon dioxide, hormones, electrolytes and even administered drugs or nanoparticles throughout the living system. The blood system is vital in stabilizing body temperature, pH and maintaining homeostasis [252]. Administered nanomaterials interact directly with blood cells before reaching their target tissues, cells or organelles [253]. Nanotoxicology research is quite diverse, establishing the

bio-safety of core-shell nanoparticles can be performed using cell culture techniques which provides an *in vitro* perspective of how the nanomaterial is interacting with the living cell under control experimental conditions [248]. These cytotoxicity studies can be performed on a variety of human cells from different origin such as normal or cancer, liver, kidney, neural, dermal or pancreas cells. Essentially the cell types are different, they possess different morphological features and organ functions however the baseline cell cycle functions are similar. The advantage of *in vitro* cytotoxicity studies is that the interaction is rapid and can be observed within a few hours to days after exposure to the nanomaterials [249]. Researchers use various microscopy imaging techniques to investigate how the nanoparticles interact with the cell membrane and other sub-cellular organelles. Cellular uptake of core-shell nanoparticles is a key aspect in targeted drug delivery and bio-imaging of cancer cells or tumour tissues. Additionally mechanistic interactions can be investigated using *in vitro* cell culture techniques [254]. Molecular and biochemical tools can be used to track the up or down regulation of proteins, DNA damage, cell signalling and enzyme cascades that result from exposure and bio-interaction with nanomaterials [248]. Additional bio-safety information is studied through the use of animal models. Animal research is a highly regulated platform that is controlled ethical guidelines, animal welfare and human safety protocols. *In vivo* animal models provide critical bio-interaction mechanisms that are not visible in cell culture experiments [255]. During *in vivo* experiments, the animals are treated with nanomaterials. Scientists are then able to evaluate bio-distribution, degradation, homeostasis mechanism, organ interaction and elimination of core-shell nanomaterials [253, 256].

Table 1.3. Biosafety and nanotoxicity studies of Au-based core-shell nanoparticles

Core/shell	Material properties	Method of toxicity/ bio-interaction evaluation	Bio-application	Reference
Fe ₃ O ₄ /Au	10 nm in size, plasmon resonance peak 530-540 nm	Real time assay- interaction with blood serum, tumour suppressor gene	Nanobiosensors for DNA methylation	[257]
Fe ₃ O ₄ /Au	H-PhA (PhA heparin conjugated to Au shell. Plasmon resonance absorption at 521 nm	Cellular uptake and in vitro MTT cytotoxicity assay using A549 cells Tumour xenograft using A549 in nude mice and in vivo imaging	Photodynamic cancer therapy, bio-imaging	[258]
Fe ₃ O ₄ /Au	Thiol-PEG-carboxyl stabilised particles 56,6 ±8,0 nm, conjugated with anti-CD105 antibody.	Bio-interaction, staining and imaging of HUVEC cells, MRI imaging of breast induced tumours in nude mice	MRI imaging of tumour angiogenesis	[164]
Au/ polymer (PSMA) Poly(styrene- <i>alt</i> -maleic acid)	22 nm core size, absorption of aromatic drugs such as doxorubicin	MTT cytotoxicity assay using HeLa cervical cancer cells	Photodynamic and drug therapy	[259]
Au/CuS	4.15 µM of Au/CuS was used in the growth medium	Anti-microbial activity to Bacillus Anthracis cells. Efflux DNA and SEM analysis confirmed cell membrane damage	Photothermal therapeutics and anti-microbial	[220]
Au/Ag	Modified with methoxy-PEG-SH, water soluble,	MTT assay on A549 cells, Broth dilution assay for anti-bacterial evaluation, Au/Ag injection in rats and mice	Bio-imaging, anti-bacterial	[234]

Core/shell	Material properties	Method of toxicity/ bio-interaction evaluation	Bio-application	Reference
Fe/Au	Au-conjugated to PSMA methotrexate 6,4± 09 nm particle size	WST-8 cytotoxicity assay on Hep-G2 cells	Magnetic hyperthermia Anti-cancer therapeutics	[114]
Fe/Au	PEG coated Fe/Au 24± 5 nm particle size	<i>In vitro</i> live/dead cell viability assay using glial and stem cells	Medical imaging, drug delivery	[260]
Au/ZnMoS ₄	PEGylated, water dispersible	In vitro intracellular copper detoxifying agent using Hep-G2 cells	Therapeutics	[261]
Au/SiO ₂	Particle size 36 nm, absorption peak at 533 nm	In vitro detection of glucose and uric acid at different concentrations	Biosensors	[262]
Au ₂ Se/Au	Particle size between 450-600 nm embedded within bovine serum albumin	CellTiter-Glo® Luminescent Cell Viability Assay using Eca-109 cancer cells	Cancer phototherapy	[263]
ZnO/Au	Folate conjugated particles, [113]	MTT cytotoxicity assay using HeLa and A549 cells	Tumour targeting drug delivery	[113]
Au/Ag	Nanorods core/shell particle size 53 ±7 nm in length and 18 ± 2 nm in diameter	Membrane permeability rhodamine 123 dye was used to evaluate mitochondrial damage apoptosis evaluation by FITC-Annexin V kit using mouse fibroblast cells	Anti-microbial	[264]
Au/CdS	Particle size 22 nm, stabilised using sodium citrate	Human urine and serum samples was used to establish bio-sensing of Dopamine	Biosensors	[265]

1.20 Statement of research problem

Cancer is a disease characterised by the uncontrolled proliferation and metastasis of abnormal cells. The prevalence of cancer is increasing exponentially, more than 100 000 South Africans are diagnosed each year. Chemotherapy is a commonly accepted treatment approach which involves the use of cytotoxic drugs that kill both normal and cancer cells. This treatment outcome results in adverse side effects such as hair and weight loss, diarrhoea, vomiting, weak immunity and sometimes even death. Researchers are faced with a challenge of developing novel and viable drug delivery systems that can target cancer cells and deliver multiple functions within the domain of cancer diagnostics and therapeutics. The synthesis of core/shell nanoparticles have the potential to support this application if designed appropriately through the optimum use of chemical synthesis techniques, reaction conditions such as temperature, pH and capping reactant. This research involves the synthesis, characterisation and bioapplications of a novel Au-ZnTe core/shell nanoparticle.

1.21 Rationale for developing Au-ZnTe core-shell nanoparticles in bio-applications

In surveying the literature available on the synthesis of Au-based core-shell nanoparticles it is evident that a combination of gold and zinc tellurium has not been previously reported by any other research team. The proposed combination and synthesis of a gold core and zinc telluride shell is therefore a novel heterostructure. Au nanoparticles generally display immense potential for cancer diagnosis and therapy. These nanoparticles exhibit Surface plasmon resonance (SPR) which results in enhanced light scattering and absorption properties. These unique optical properties of Au nanoparticles enable the rapid conversion of absorbed light into heat resulting

in localized photothermal therapy and destruction of cancer cells. Au nanoparticles are highly stable, nontoxic, easily synthesized in a variety of sizes and biocompatibility and support a wide range of surface bio-conjugation for drug delivery applications. In addition Au nanoparticles can be used as contrast agents in bio-imaging applications as they do not exhibit photobleaching. On the other hand ZnTe nanoparticles also display immense potential in medical applications. ZnTe quantum dots are an attractive II–VI semiconducting material with a direct band gap (bulk) of 2.26 eV. These nanoparticles are commonly utilized for their unique optical and electrical properties in light emitting diodes, solar cells and photodetector applications. They are reported as less toxic when compared to other cadmium based chalcogenides. The use of Au and ZnTe components in a heterostructure is expected to result in enhanced optical properties that is blue shifted from the parent nanomaterials. Increasing the optical properties is an important aspect in bio-imaging and photothermal properties.

1.22 Aims and objectives of this study

The research focus of this study is to develop a biocompatible Au-based core-shell nanoparticle that can be used to enhance the therapeutic efficacy of existing cancer chemotherapeutics by increasing the cellular uptake of the conventional drug and further directing its drug load specifically to active cancer cells, providing a platform of nano-drugs that deliver a therapeutic without causing harmful side-effects. A project of this magnitude requires numerous aims and objectives to fulfil this research focus, however within the scope of this project, the aims and objectives are limited to the following; *the synthesis and characterisation of Au, ZnTe and Au-ZnTe core-shell nanoparticles, biosafety and toxicology evaluation of Au-ZnTe core-shell nanoparticles, synthesis and characterisation of surface modified anti-cancer Au-ZnTe*

core-shell nanoparticles using 5-FU and human EGF, in vivo application of anti-cancer Au-ZnTe core-shell nanoparticles using 5-FU and human EGF, using animal models

In order for these aims to be accomplished, the following objectives were met

a. The synthesis and characterisation of Au, ZnTe and Au-ZnTe core-shell nanoparticles

- The synthesis of the parent materials ZnTe and Au and Au-ZnTe core-shell nanoparticles were established using modifications and adaptations from previously reported methods.
- The properties of the synthesised nanomaterials were characterised for its morphology, crystalline and optical properties using UV, PL, TEM, HRTEM, AFM, XRD, XPS, elemental mapping and surface charge analysis.

b. Biosafety and toxicology evaluation of Au-ZnTe core-shell nanoparticles

- The bio-interaction, toxicity and safety of Au-ZnTe nanoparticles was evaluated using normal human colon, mammary epithelial and cancer cells from breast, prostate and colon origin.
- An *in vitro* immune simulation using blood peripheral mononuclear cells (PBMCs) was used to evaluate the bio-interaction and cytotoxicity of Au-ZnTe core-shell nanoparticles on human immune cells, additionally the expression of inflammatory markers, cytokine expression was measured.
- The biocompatibility, cellular membrane interaction and cellular uptake was established through resin-embedding, sectioning and microscope image techniques.

- The use of *in vivo* rat models for systemic circulation and toxicity effects of Au-ZnTe core-shell nanoparticles was investigated to determine acute toxicity responses and impact of excretory liver and kidney organs.
- The full blood counts and histopathology of liver and kidney organs was evaluated.

c. *Synthesis and characterisation of surface modified anti-cancer Au-ZnTe core-shell nanoparticles using 5-FU and human EGF*

- Au-ZnTe core-shell nanoparticles was used for bio-conjugation/ surface modification of human epidermal growth factor and anti-cancer drug 5 fluorouracil.
- The surface modified Au-ZnTe nanoparticle was characterised using UV, PL, TEM, FTIR and zeta potential analysis.
- The efficacy of the surface modified Au-ZnTe core-shell nanoparticle was investigated using *in vitro* cell culture comparative cytotoxicity studies.

d. *In vivo application of anti-cancer Au-ZnTe core-shell nanoparticles using 5-FU and human EGF, in animal models*

- Human xenograft techniques were used to induce mammary tumours in Sprague-Dawley female rats using MCF7 breast cancer cells.
- Human xenograft techniques were used to induce mammary tumours in Balb/c female mice using MCF7 breast cancer cells
- After tumour growth was established in terms of size, shape and clinical observations the surface modified drug was prepared and used for localised tumour therapy.

References

- Ries, L.A.G., et al., *SEER cancer statistics review, 1975–2005*. Bethesda, MD: National Cancer Institute, 2008: p. 1975-2005.
- Jemal, A., et al., *Global patterns of cancer incidence and mortality rates and trends*. Cancer Epidemiology Biomarkers & Prevention, 2010. **19**(8): p. 1893-1907.
- Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2015*. CA: a cancer journal for clinicians, 2015. **65**(1): p. 5-29.
- Parkin, D.M., et al., *Global cancer statistics, 2002*. CA: a cancer journal for clinicians, 2005. **55**(2): p. 74-108.
- Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008*. International journal of cancer, 2010. **127**(12): p. 2893-2917.
- Vorobiof, D.A., F. Sitas, and G. Vorobiof, *Breast cancer incidence in South Africa*. Journal of clinical oncology, 2001. **19**(suppl 1): p. 125s-127s.
- Hortobagyi, G.N., et al., *The global breast cancer burden: variations in epidemiology and survival*. Clinical breast cancer, 2005. **6**(5): p. 391-401.
- Pardee, A., et al., *Animal cell cycle*. Annual review of biochemistry, 1978. **47**(1): p. 715-750.
- Cella, D.F. and D.S. Tulskey, *Quality of life in cancer: definition, purpose, and method of measurement*. Cancer investigation, 1993. **11**(3): p. 327-336.
- Fearon, K., et al., *Definition and classification of cancer cachexia: an international consensus*. The lancet oncology, 2011. **12**(5): p. 489-495.
- DeVita, V.T. and E. Chu, *A history of cancer chemotherapy*. Cancer research, 2008. **68**(21): p. 8643-8653.
- Lobo, N.A., et al., *The biology of cancer stem cells*. Annu. Rev. Cell Dev. Biol., 2007. **23**: p. 675-699.
- Levine, B., *Cell biology: autophagy and cancer*. Nature, 2007. **446**(7137): p. 745-747.
- Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. cell, 2000. **100**(1): p. 57-70.
- Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. cell, 2011. **144**(5): p. 646-674.
- Blagosklonny, M.V., *Cell immortality and hallmarks of cancer*. Cell Cycle, 2003. **2**(4): p. 295-298.
- Sledge, G. and K. Miller, *Exploiting the hallmarks of cancer: the future conquest of breast cancer*. European Journal of Cancer, 2003. **39**(12): p. 1668-1675.
- Cavallo, F., et al., *2011: the immune hallmarks of cancer*. Cancer Immunology, Immunotherapy, 2011. **60**(3): p. 319-326.
- Liotta, L.A., P.S. Steeg, and W.G. Stetler-Stevenson, *Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation*. Cell, 1991. **64**(2): p. 327-336.
- Wooster, R., et al., *Identification of the breast cancer susceptibility gene BRCA2*. Nature, 1995. **378**(6559): p. 789-792.
- Coughlin, S.S. and D.U. Ekwueme, *Breast cancer as a global health concern*. Cancer epidemiology, 2009. **33**(5): p. 315-318.
- Yarden, Y., *Biology of HER2 and its importance in breast cancer*. Oncology, 2001. **61**(Suppl. 2): p. 1-13.
- Lee-Hoeflich, S.T., et al., *A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy*. Cancer research, 2008. **68**(14): p. 5878-5887.
- Mirzoeva, O.K., et al., *Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition*. Cancer research, 2009. **69**(2): p. 565-572.
- Roberts, P. and C. Der, *Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer*. Oncogene, 2007. **26**(22): p. 3291-3310.
- DeBerardinis, R.J., et al., *The biology of cancer: metabolic reprogramming fuels cell growth and proliferation*. Cell metabolism, 2008. **7**(1): p. 11-20.
- Thompson, N. and J. Lyons, *Recent progress in targeting the Raf/MEK/ERK pathway with inhibitors in cancer drug discovery*. Current opinion in pharmacology, 2005. **5**(4): p. 350-356.
- Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells*. Cell research, 2002. **12**(1): p. 9-18.
- Cantley, L.C., *The phosphoinositide 3-kinase pathway*. Science, 2002. **296**(5573): p. 1655-1657.
- Chang, F., et al., *Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy*. Leukemia, 2003. **17**(3): p. 590-603.
- Downward, J., *Ras signalling and apoptosis*. Current opinion in genetics & development, 1998. **8**(1): p. 49-54.
- Sun, H., et al., *PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3, 4, 5-trisphosphate and Akt/protein kinase B signaling pathway*. Proceedings of the National Academy of Sciences, 1999. **96**(11): p. 6199-6204.
- Chalhoub, N. and S.J. Baker, *PTEN and the PI3-kinase pathway in cancer*. Annual review of pathology, 2009. **4**: p. 127.
- Armstrong, D.K., et al., *Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells*. Cancer research, 1994. **54**(20): p. 5280-5283.
- Scaltriti, M., et al., *Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients*. Proceedings of the National Academy of Sciences, 2011. **108**(9): p. 3761-3766.
- Jemal, A., et al., *Cancer statistics, 2010*. CA: a cancer journal for clinicians, 2010. **60**(5): p. 277-300.
- Group, U.C.S.W., *United States Cancer Statistics: 1999–2011 incidence and mortality web-based report*. Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute, 2014.
- Edge, S.B., et al., *AJCC cancer staging manual*. Vol. 649. 2010: Springer New York.
- Scarpignato, C. and I. Pelosini, *Somatostatin analogs for cancer treatment and diagnosis: an overview*. Chemotherapy, 2001. **47**(Suppl. 2): p. 1-29.
- Vokes, E.E., et al., *Induction chemotherapy followed by concomitant chemoradiotherapy for advanced head and neck cancer: impact on the natural history of the disease*. Journal of clinical oncology, 1995. **13**(4): p. 876-883.
- Heller, J.R., *Cancer chemotherapy, history and present status*. Bulletin of the New York Academy of Medicine, 1962. **38**(5): p. 348.
- Brookes, P., *The early history of the biological alkylating agents, 1918–1968*. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1990. **233**(1): p. 3-14.
- Pai, V.B. and M.C. Nahata, *Cardiotoxicity of chemotherapeutic agents*. Drug safety, 2000. **22**(4): p. 263-302.
- Peterson, J.R. and T.J. Mitchison, *Small molecules, big impact: a history of chemical inhibitors and the*

- cytoskeleton. *Chemistry & biology*, 2002. **9**(12): p. 1275-1285.
45. Dunleavy, K., et al., *Differential efficacy of bortezomib plus chemotherapy within molecular subtypes of diffuse large B-cell lymphoma*. *Blood*, 2009. **113**(24): p. 6069-6076.
46. Weinstein, J.N., et al., *Neural computing in cancer drug development: predicting mechanism of action*. *Science*, 1992. **258**(5081): p. 447-451.
47. Capasso, C. and C.T. Supuran, *Sulfa and trimethoprim-like drugs—antimetabolites acting as carbonic anhydrase, dihydropteroate synthase and dihydrofolate reductase inhibitors*. *Journal of enzyme inhibition and medicinal chemistry*, 2014. **29**(3): p. 379-387.
48. Saint-Pierre, M.D., et al., *Malignant Pleural Mesothelioma Outcomes in the Era of Combined Platinum and Folate Antimetabolite Chemotherapy*. *Lung Cancer International*, 2015. **2015**.
49. Krynetskaia, N., et al., *High mobility group protein B1 is an activator of apoptotic response to antimetabolite drugs*. *Molecular pharmacology*, 2008. **73**(1): p. 260-269.
50. O'Shaughnessy, J., *Extending survival with chemotherapy in metastatic breast cancer*. *The oncologist*, 2005. **10**(Supplement 3): p. 20-29.
51. Mojardin, L., et al., *New insights into the RNA-based mechanism of action of the anticancer drug 5'-fluorouracil in eukaryotic cells*. 2013.
52. Dagher, R., et al., *Accelerated approval of oncology products: a decade of experience*. *Journal of the National Cancer Institute*, 2004. **96**(20): p. 1500-1509.
53. Kruse, G.B., et al., *Analysis of costs associated with administration of intravenous single-drug therapies in metastatic breast cancer in a US population*. *J Manag Care Pharm*, 2008. **14**(9): p. 844-57.
54. Longley, D.B., D.P. Harkin, and P.G. Johnston, *5-fluorouracil: mechanisms of action and clinical strategies*. *Nature Reviews Cancer*, 2003. **3**(5): p. 330-338.
55. Arias, J.L., *Novel strategies to improve the anticancer action of 5-fluorouracil by using drug delivery systems*. *Molecules*, 2008. **13**(10): p. 2340-2369.
56. Pullarkat, S., et al., *Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy*. *The pharmacogenomics journal*, 2001. **1**(1): p. 65-70.
57. Parker, W.B. and Y.C. Cheng, *Metabolism and mechanism of action of 5-fluorouracil*. *Pharmacology & therapeutics*, 1990. **48**(3): p. 381-395.
58. Coates, A., et al., *On the receiving end—patient perception of the side-effects of cancer chemotherapy*. *European Journal of Cancer and Clinical Oncology*, 1983. **19**(2): p. 203-208.
59. Cohen, P., *New role for the mitochondrial peptide humanin: protective agent against chemotherapy-induced side effects*. *Journal of the National Cancer Institute*, 2014: p. dju006.
60. Burish, T.G. and R.A. Jenkins, *Effectiveness of biofeedback and relaxation training in reducing the side effects of cancer chemotherapy*. *Health psychology*, 1992. **11**(1): p. 17.
61. Nair, L., et al., *Biological evaluation of 5-fluorouracil nanoparticles for cancer chemotherapy and its dependence on the carrier, PLGA*. *International journal of nanomedicine*, 2011. **6**: p. 1685.
62. Le, V.-M., et al., *An investigation of antitumor efficiency of novel sustained and targeted 5-fluorouracil nanoparticles*. *European journal of medicinal chemistry*, 2015. **92**: p. 882-889.
63. Cheng, M., et al., *5-Fluorouracil nanoparticles inhibit hepatocellular carcinoma via activation of the p53 pathway in the orthotopic transplant mouse model*. 2012.
64. Brannon-Peppas, L. and J.O. Blanchette, *Nanoparticle and targeted systems for cancer therapy*. *Advanced drug delivery reviews*, 2012. **64**: p. 206-212.
65. Sahu, S. and S. Mohapatra, *Multifunctional magnetic fluorescent hybrid nanoparticles as carriers for the hydrophobic anticancer drug 5-fluorouracil*. *Dalton Transactions*, 2013. **42**(6): p. 2224-2231.
66. Chanda, N., et al., *Radioactive gold nanoparticles in cancer therapy: therapeutic efficacy studies of GA-198 AuNP nanoconstruct in prostate tumor-bearing mice*. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2010. **6**(2): p. 201-209.
67. Wang, C., et al., *Iron oxide@ polypyrrole nanoparticles as a multifunctional drug carrier for remotely controlled cancer therapy with synergistic antitumor effect*. *ACS nano*, 2013. **7**(8): p. 6782-6795.
68. Zhou, M., et al., *Single agent nanoparticle for radiotherapy and radio-photothermal therapy in anaplastic thyroid cancer*. *Biomaterials*, 2015. **57**: p. 41-49.
69. Barenholz, Y.C., *Doxil®—the first FDA-approved nano-drug: lessons learned*. *Journal of Controlled Release*, 2012. **160**(2): p. 117-134.
70. Roy, V., et al., *Phase II trial of weekly nab (nanoparticle albumin-bound)-paclitaxel (nab-paclitaxel)(Abraxane®) in combination with gemcitabine in patients with metastatic breast cancer (N0531)*. *Annals of Oncology*, 2008: p. mdn661.
71. Ali, I., et al., *Advances in nano drugs for cancer chemotherapy*. *Current cancer drug targets*, 2011. **11**(2): p. 135-146.
72. Freestone, I., et al., *The Lycurgus cup—a roman nanotechnology*. *Gold Bulletin*, 2007. **40**(4): p. 270-277.
73. Molina, G., et al., *Color and dichroism of silver-stained glasses*. *Journal of nanoparticle research*, 2013. **15**(9): p. 1-13.
74. Reibold, M., et al., *Materials: Carbon nanotubes in an ancient Damascus sabre*. *Nature*, 2006. **444**(7117): p. 286-286.
75. Wickson, F., K. Grieger, and A. Baun, *Nature and nanotechnology: science, ideology and policy*. *International Journal of Emerging Technologies and Society*, 2010. **8**(1): p. 5-23.
76. Sitti, M. and R.S. Fearing, *Synthetic gecko foot-hair micro/nano-structures as dry adhesives*. *Journal of Adhesion Science and Technology*, 2003. **17**(8): p. 1055-1073.
77. Autumn, K. and N. Gravish, *Gecko adhesion: evolutionary nanotechnology*. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, 2008. **366**(1870): p. 1575-1590.
78. Shah, G.J. and M. Sitti, *Modeling and design of biomimetic adhesives inspired by gecko foot-hairs*. in *Robotics and Biomimetics*, 2004. *ROBIO 2004. IEEE International Conference on*. 2004. IEEE.
79. Parker, A.R. and H.E. Townley, *Biomimetics of photonic nanostructures*. *Nature nanotechnology*, 2007. **2**(6): p. 347-353.
80. Toumey, C., *Plenty of room, plenty of history*. *Nature nanotechnology*, 2009. **4**(12): p. 783-784.
81. Toumey, C., *The man who understood the Feynman machine*. *Nature nanotechnology*, 2007. **2**(1): p. 9-10.
82. Roco, M.C., *The journal of nanoparticle research at 10 years*. *Journal of Nanoparticle Research*, 2008. **10**(1): p. 1-2.
83. Foster, L.E., *Nanotechnology: science, innovation, and opportunity*. 2005: Prentice Hall PTR.

84. Whitesides, G.M., *The right size in nanobiotechnology*. Nature biotechnology, 2003. **21**(10): p. 1161-1165.
85. Sanvicens, N. and M.P. Marco, *Multifunctional nanoparticles—properties and prospects for their use in human medicine*. Trends in biotechnology, 2008. **26**(8): p. 425-433.
86. El-Sayed, M.A., *Some interesting properties of metals confined in time and nanometer space of different shapes*. Accounts of chemical research, 2001. **34**(4): p. 257-264.
87. Shi, W., et al. *Size and shape-controlled synthesis of bifunctional gold/Fe₃O₄ nanoparticles*. in *AIChE Annual Meeting, Conference Proceedings*. 2005.
88. Velasco, V., et al., *Chemically synthesized Au-Fe₃O₄ nanostructures with controlled optical and magnetic properties*. Journal of Physics D: Applied Physics, 2015. **48**(3).
89. Ke, P.C. and M.H. Lamm, *A biophysical perspective of understanding nanoparticles at large*. Physical Chemistry Chemical Physics, 2011. **13**(16): p. 7273-7283.
90. Gillberg-LaForce, G.E. and G. Khanarian, *Organic-inorganic composites with enhanced nonlinear optical response*. 1989, Google Patents.
91. Takita, K., et al., *Observation of surface evaporation of Hg from HgTe crystals by means of energetic oxygen ion backscattering*. Applied Physics Letters, 1980. **37**(5): p. 460-462.
92. Rajeshwar, K., N.R. de Tacconi, and C. Chenthamarakshan, *Semiconductor-based composite materials: preparation, properties, and performance*. Chemistry of Materials, 2001. **13**(9): p. 2765-2782.
93. Arfsten, N.J., et al., *Core-shell nanoparticles*. 2007, Google Patents.
94. Ghosh Chaudhuri, R. and S. Paria, *Core/shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications*. Chemical reviews, 2011. **112**(4): p. 2373-2433.
95. Kong, X.Y., Y. Ding, and Z.L. Wang, *Metal-semiconductor Zn-ZnO core-shell nanobelts and nanotubes*. The Journal of Physical Chemistry B, 2004. **108**(2): p. 570-574.
96. Alayoglu, S., et al., *Ru-Pt core-shell nanoparticles for preferential oxidation of carbon monoxide in hydrogen*. Nature materials, 2008. **7**(4): p. 333-338.
97. Tak, Y., et al., *Fabrication of ZnO/CdS core/shell nanowire arrays for efficient solar energy conversion*. Journal of Materials Chemistry, 2009. **19**(33): p. 5945-5951.
98. Verma, N.K., et al., *Magnetic core-shell nanoparticles for drug delivery by nebulization*. J Nanobiotechnol, 2013. **11**(1).
99. Zhou, S., et al., *Pt-Cu core-shell and alloy nanoparticles for heterogeneous NO_x reduction: anomalous stability and reactivity of a core-shell nanostructure*. Angewandte Chemie, 2005. **117**(29): p. 4615-4619.
100. Viswanath, R., et al., *Luminescence properties of blue-red emitting multilayer coated single structure ZnS/MnS/ZnS nanocomposites*. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2014. **125**: p. 222-227.
101. Maye, M.M., et al. *Gold based core-shell nanoparticles as fuel cell catalysts*. in *ACS Division of Fuel Chemistry, Preprints*. 2003.
102. Aslan, K., et al., *Fluorescent core-shell Ag@ SiO₂ nanocomposites for metal-enhanced fluorescence and single nanoparticle sensing platforms*. Journal of the American Chemical Society, 2007. **129**(6): p. 1524-1525.
103. Fan, X.M., et al., *Preparation of Au@SiO₂ core-shell nanoparticles and their surface-enhanced Raman spectra*. Gaodeng Xuexiao Huaxue Xuebao/Chemical Journal of Chinese Universities, 2008. **29**(1): p. 130-134.
104. Sun, S., *Recent advances in chemical synthesis, self-assembly, and applications of FePt nanoparticles*. Advanced Materials, 2006. **18**(4): p. 393-404.
105. Li, J., et al., *Coating urchinlike gold nanoparticles with polypyrrole thin shells to produce photothermal agents with high stability and photothermal transduction efficiency*. Langmuir, 2013. **29**(23): p. 7102-7110.
106. Ahmed, O.S., et al., *Synthesis of the newly developed core-shell Au/Fe₃O₄ magneto-plasmonic nanocomposite in cancer cells*. Life Science Journal, 2014. **11**(10): p. 182-187.
107. Hodak, J.H., et al., *Laser-Induced Inter-Diffusion in AuAg Core-Shell Nanoparticles*. Journal of Physical Chemistry B, 2000. **104**(49): p. 11708-11718.
108. Mukhopadhyay, K., et al., *Gold nanoparticles assembled on amine-functionalized Na - Y zeolite: A biocompatible surface for enzyme immobilization*. Langmuir, 2003. **19**(9): p. 3858-3863.
109. Du, Y., et al., *The preparation and characterization of Au-core/Pt-shell nanoparticles*. Colloid and Polymer Science, 2007. **285**(5): p. 553-556.
110. Bao, F., et al., *Synthesis and characterization of Au@Co and Au@Ni core - Shell nanoparticles and their applications in surface-enhanced Raman spectroscopy*. Journal of Physical Chemistry C, 2008. **112**(2): p. 345-350.
111. Contreras-Cáceres, R., et al., *Au@pNIPAM thermosensitive nanostructures: Control over shell cross-linking, overall dimensions, and core growth*. Advanced Functional Materials, 2009. **19**(19): p. 3070-3076.
112. AbouZeid, K.M., M.B. Mohamed, and M.S. El-Shall, *Hybrid Au-CdSe and Ag-CdSe Nanoflowers and Core-Shell Nanocrystals via One-Pot Heterogeneous Nucleation and Growth*. Small, 2011. **7**(23): p. 3299-3307.
113. Chen, T., et al., *Core-shell nanocarriers with ZnO quantum dots-conjugated Au nanoparticle for tumor-targeted drug delivery*. Carbohydrate Polymers, 2013. **92**(2): p. 1124-1132.
114. Chung, R.J. and H.T. Shih, *Preparation of multifunctional Fe@Au core-shell nanoparticles with surface grafting as a potential treatment for magnetic hyperthermia*. Materials, 2014. **7**(2): p. 653-661.
115. Cai, K., et al., *Facile Synthesis of Quasi-One-Dimensional Au/PtAu Heterojunction Nanotubes and Their Application as Catalysts in an Oxygen-Reduction Reaction*. Chemistry-A European Journal, 2015. **21**(20): p. 7556-7561.
116. El-Sayed, I.H., X. Huang, and M.A. El-Sayed, *Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer*. Nano letters, 2005. **5**(5): p. 829-834.
117. Levy, R., et al., *Gold nanoparticles delivery in mammalian live cells: a critical review*. Nano reviews, 2010. **1**.
118. Khlebtsov, N. and L. Dykman, *Biodistribution and toxicity of engineered gold nanoparticles: a review of in vitro and in vivo studies*. Chemical Society Reviews, 2011. **40**(3): p. 1647-1671.
119. Daniel, M.-C. and D. Astruc, *Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology*. Chemical reviews, 2004. **104**(1): p. 293-346.
120. Jain, P.K., I.H. El-Sayed, and M.A. El-Sayed, *Au nanoparticles target cancer*. Nano Today, 2007. **2**(1): p. 18-29.

121. Ghosh, P., et al., *Gold nanoparticles in delivery applications*. Advanced drug delivery reviews, 2008. **60**(11): p. 1307-1315.
122. Huang, X., et al., *Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods*. Journal of the American Chemical Society, 2006. **128**(6): p. 2115-2120.
123. Chaudhuri, R.G. and S. Paria, *Gold-Based Core/Shell and Hollow Nanoparticles*. Kirk-Othmer Encyclopedia of Chemical Technology, 2013.
124. Wang, Y. and Y. Xia, *Bottom-up and top-down approaches to the synthesis of monodispersed spherical colloids of low melting-point metals*. Nano Letters, 2004. **4**(10): p. 2047-2050.
125. Thakkar, K.N., S.S. Mhatre, and R.Y. Parikh, *Biological synthesis of metallic nanoparticles*. Nanomedicine: Nanotechnology, Biology and Medicine, 2010. **6**(2): p. 257-262.
126. Tiwari, D.K., J. Behari, and P. Sen, *Time and dose-dependent antimicrobial potential of Ag nanoparticles synthesized by top-down approach*. Curr Sci, 2008. **95**: p. 647-655.
127. Watt, F., et al., *Ion beam lithography and nanofabrication: a review*. International Journal of Nanoscience, 2005. **4**(03): p. 269-286.
128. Ko, S.H., et al., *All-inkjet-printed flexible electronics fabrication on a polymer substrate by low-temperature high-resolution selective laser sintering of metal nanoparticles*. Nanotechnology, 2007. **18**(34): p. 345202.
129. Nashner, M.S., et al., *Core shell inversion during nucleation and growth of bimetallic Pt/Ru nanoparticles*. Journal of the American Chemical Society, 1998. **120**(32): p. 8093-8101.
130. Mandal, S., et al., *Keggin ions as UV-switchable reducing agents in the synthesis of Au core-Ag shell nanoparticles*. Journal of the American Chemical Society, 2003. **125**(28): p. 8440-8441.
131. Tom, R.T., et al., *Freely dispersible Au@ TiO₂, Au@ ZrO₂, Ag@ TiO₂, and Ag@ ZrO₂ core-shell nanoparticles: One-step synthesis, characterization, spectroscopy, and optical limiting properties*. Langmuir, 2003. **19**(8): p. 3439-3445.
132. Jones, C.D. and L.A. Lyon, *Dependence of shell thickness on core compression in acrylic acid modified poly (N-isopropylacrylamide) core/shell microgels*. Langmuir, 2003. **19**(11): p. 4544-4547.
133. Li, J.J., et al., *Large-scale synthesis of nearly monodisperse CdSe/CdS core/shell nanocrystals using air-stable reagents via successive ion layer adsorption and reaction*. Journal of the American Chemical Society, 2003. **125**(41): p. 12567-12575.
134. Reiss, P., M. Protiere, and L. Li, *Core/shell semiconductor nanocrystals*. small, 2009. **5**(2): p. 154-168.
135. Zhao, M., et al., *Core-shell palladium nanoparticle@ metal-organic frameworks as multifunctional catalysts for cascade reactions*. Journal of the American Chemical Society, 2014. **136**(5): p. 1738-1741.
136. Yang, H., J. Shao, and B.Q. Li. *Self-Assembly of Ordered SiO₂@ Au Core-Shell Nanoparticle Arrays*. in ASME 2014 International Mechanical Engineering Congress and Exposition. 2014. American Society of Mechanical Engineers.
137. Tanaka, A., et al., *Functionalization of Au/TiO₂ Plasmonic Photocatalysts with Pd by Formation of a Core-Shell Structure for Effective Dechlorination of Chlorobenzene under Irradiation of Visible Light*. The Journal of Physical Chemistry C, 2013. **117**(33): p. 16983-16989.
138. Kennedy, L.C., et al., *A New Era for Cancer Treatment: Gold-Nanoparticle-Mediated Thermal Therapies*. Small, 2011. **7**(2): p. 169-183.
139. Samal, A.K., et al., *Size Tunable Au@ Ag core-shell nanoparticles: synthesis and surface-enhanced raman scattering properties*. Langmuir, 2013. **29**(48): p. 15076-15082.
140. Kalska-Szostko, B., et al., *Chemical preparation of core-shell nanoparticles*. Nukleonika, 2013. **58**: p. 35-38.
141. Khlebtsov, N., et al., *Analytical and theranostic applications of gold nanoparticles and multifunctional nanocomposites*. Theranostics, 2013. **3**(3): p. 167.
142. Maye, M.M. and C.-J. Zhong, *manipulating core-shell reactivities for processing nanoparticle sizes and shapes*. Journal of Materials Chemistry, 2000. **10**(8): p. 1895-1901.
143. Shankar, S.S., et al., *Rapid synthesis of Au, Ag, and bimetallic Au core-Ag shell nanoparticles using Neem (Azadirachta indica) leaf broth*. Journal of colloid and interface science, 2004. **275**(2): p. 496-502.
144. Lee, Y.W., et al., *One-step synthesis of Au@Pd core-shell nanooctahedron*. Journal of the American Chemical Society, 2009. **131**(47): p. 17036-17037.
145. Jiang, H.-L., et al., *Synergistic catalysis of Au@ Ag core-shell nanoparticles stabilized on metal-organic framework*. Journal of the American Chemical Society, 2011. **133**(5): p. 1304-1306.
146. Mallik, K., et al., *Seed Mediated Formation of Bimetallic Nanoparticles by UV Irradiation: A Photochemical Approach for the Preparation of "Core-Shell" Type Structures*. Nano Letters, 2001. **1**(6): p. 319-322.
147. Qian, L. and X. Yang, *Preparation and characterization of Ag(Au) bimetallic core-shell nanoparticles with new seed growth method*. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2005. **260**(1-3): p. 79-85.
148. Lu, Y., et al., *Synthesis and Self-Assembly of Au@SiO₂ Core-Shell Colloids*. Nano Letters, 2002. **2**(7): p. 785-788.
149. Chu, Z., et al., *Surface plasmon enhanced drug efficacy using core-shell Au@SiO₂ nanoparticle carrier*. Nanoscale, 2013. **5**(8): p. 3406-3411.
150. Wu, X.-F., et al., *Synthesis of core-shell Au@ TiO₂ nanoparticles with truncated wedge-shaped morphology and their photocatalytic properties*. Langmuir, 2009. **25**(11): p. 6438-6447.
151. Liu, W.L., et al. *Influence of morphology on the plasmonic enhancement effect of Au@TiO₂ core-shell nanoparticles in dye-sensitized solar cells*. in CLEO: QELS Fundamental Science, CLEO:QELS FS 2013. 2013.
152. Liu, L., et al., *Au-ZnO hybrid nanoparticles exhibiting strong charge-transfer-induced SERS for recyclable SERS-active substrates*. Nanoscale, 2015. **7**(12): p. 5147-5151.
153. Prabhakaran, S., *Dual-functional core-shell Au@Fe₃O₄ nanoparticles for cellular imaging and magnetic hyperthermia*. Magnetohydrodynamics, 2013. **49**(3-4): p. 609-614.
154. Zhang, L., D.A. Blom, and H. Wang, *Au-Cu₂O core-shell nanoparticles: a hybrid metal-semiconductor heteronanostructure with geometrically tunable optical properties*. Chemistry of Materials, 2011. **23**(20): p. 4587-4598.
155. Zhu, J., et al., *Tunable surface plasmon resonance of Au@Ag₂S core-shell nanostructures containing voids*. Journal of Materials Chemistry, 2009. **19**(46): p. 8871-8875.
156. Peng, Z.F., et al., *Synthesis and characterization of Au/CdS-core/shell nanocomposite by electrostatic interaction*. Rengong Jingti Xuebao/Journal of Synthetic Crystals, 2008. **37**(4): p. 931-935.
157. Cai, H. and P. Yao, *In situ preparation of gold nanoparticle-loaded lysozyme-dextran nanogels and*

- applications for cell imaging and drug delivery. *Nanoscale*, 2013. **5**(7): p. 2892-2900.
158. Hu, Y., et al., *Formation of multifunctional Fe₃O₄/Au composite nanoparticles for dual-mode MR/CT imaging applications*. *Chinese Physics B*, 2014. **23**(7).
 159. Kim, Y.K., et al., *One-pot synthesis of multifunctional Au@Graphene oxide nanocolloid core@shell nanoparticles for raman bioimaging, photothermal, and photodynamic therapy*. *Small*, 2015. **11**(21): p. 2527-2535.
 160. Prabakaran, M., et al., *Gold nanoparticles with a monolayer of doxorubicin-conjugated amphiphilic block copolymer for tumor-targeted drug delivery*. *Biomaterials*, 2009. **30**(30): p. 6065-6075.
 161. Li, D., et al., *Assembled core-shell nanostructures of gold nanoparticles with biocompatible polymers toward biology*. *Current Topics in Medicinal Chemistry*, 2014. **14**(5): p. 595-616.
 162. Kim, J.-Y., S.J. Kim, and D.-J. Jang, *Laser-Induced Fabrication of Au@CdS Core-Shell Nanowires*. *The Journal of Physical Chemistry C*, 2010. **115**(3): p. 672-675.
 163. Rajasekhar Pullabhotla, V.S.R. and N. Revaprasadu, *A novel route to cysteine capped Au-CdSe hybrid nanoparticles*. *Materials Letters*, 2009. **63**(24-25): p. 2097-2099.
 164. Zhang, S., et al., *Thiol-PEG-carboxyl-stabilized Fe₂O₃/Au nanoparticles targeted to CD105: Synthesis, characterization and application in MR imaging of tumor angiogenesis*. *European Journal of Radiology*, 2014. **83**(7): p. 1190-1198.
 165. Garcia-Gutierrez, D.I., et al., *Temperature effect on the synthesis of Au-Pt bimetallic nanoparticles*. *Journal of Physical Chemistry B*, 2005. **109**(9): p. 3813-3821.
 166. Yu, K., et al., *High-temperature-stable Au@SnO₂ core/shell supported catalyst for CO oxidation*. *Journal of Physical Chemistry C*, 2008. **112**(7): p. 2244-2247.
 167. Alvarez-Paneque, A.F., et al., *Shape-templated growth of Au@Cu nanoparticles*. *Journal of Physical Chemistry C*, 2013. **117**(6): p. 2474-2479.
 168. Encina, E.R., M.A. Pérez, and E.A. Coronado, *Au@ZnO hybrid nanostructures: correlation between morphology and optical response*. *RSC Advances*, 2015. **5**(69): p. 56210-56218.
 169. Ko, F.-H., et al., *Au-Ag core-shell nanoparticles with controllable shell thicknesses for the detection of adenosine by surface enhanced Raman scattering*. *Sensors and Actuators B: Chemical*, 2015. **211**: p. 283-289.
 170. Monga, A. and B. Pal, *Improved catalytic activity and surface electro-kinetics of bimetallic Au-Ag core-shell nanocomposites*. *New Journal of Chemistry*, 2015. **39**(1): p. 304-313.
 171. Wang, H., et al., *Optimized core-shell Au@Ag nanoparticles for label-free Raman determination of trace Rhodamine B with cancer risk in food product*. *Food Chemistry*, 2015. **188**: p. 137-142.
 172. Zhang, Y., et al., *Label-free immunosensor based on Au@Ag₂S nanoparticles/magnetic chitosan matrix for sensitive determination of ractopamine*. *Journal of Electroanalytical Chemistry*, 2015. **741**: p. 14-19.
 173. Zhang, G., et al., *Green synthesis and characterization of Au@Pt core-shell bimetallic nanoparticles using gallic acid*. *Journal of Physics and Chemistry of Solids*, 2015. **81**: p. 79-87.
 174. Thongthai, K., et al., *Morphology and catalytic activity of gold core-platinum shell nanoparticles*. *Chiang Mai Journal of Science*, 2015. **42**(2): p. 481-489.
 175. Yan, J.-M., et al., *One-step seeding growth of magnetically recyclable Au@Co core-shell nanoparticles: highly efficient catalyst for hydrolytic dehydrogenation of ammonia borane*. *Journal of the American Chemical Society*, 2010. **132**(15): p. 5326-5327.
 176. Zhang, T.T., et al., *Electrochemiluminescence immunosensor for highly sensitive detection of 8-hydroxy-2'-deoxyguanosine based on carbon quantum dot coated Au/SiO₂ core-shell nanoparticles*. *Talanta*, 2015. **131**: p. 379-385.
 177. Huo, X., et al., *Synthesis of Au yolk/LDH shell nanoparticles as anticancer vehicles*. *RSC Advances*, 2015. **5**(12): p. 8689-8692.
 178. Oldfield, G., T. Ung, and P. Mulvaney, *Au@SnO₂ core-shell nanocapacitors*. *Advanced Materials*, 2000. **12**(20): p. 1519-1522.
 179. Wu, X.F., et al., *Synthesis of core-shell Au@TiO₂ nanoparticles with truncated wedge-shaped morphology and their photocatalytic properties*. *Langmuir*, 2009. **25**(11): p. 6438-6447.
 180. Li, M., et al., *Synthesis of Au-CdS Core-Shell Hetero-Nanorods with Efficient Exciton-Plasmon Interactions*. *Advanced Functional Materials*, 2011. **21**(10): p. 1788-1794.
 181. Kim, Y.K., et al., *One-Pot Synthesis of Multifunctional Au@Graphene Oxide Nanocolloid Core@Shell Nanoparticles for Raman Bioimaging, Photothermal, and Photodynamic Therapy*. *Small*, 2015.
 182. Turcheniuk, K., et al., *Plasmonic photothermal destruction of uropathogenic E. coli with reduced graphene oxide and core/shell nanocomposites of gold nanorods/reduced graphene oxide*. *Journal of Materials Chemistry B*, 2015. **3**(3): p. 375-386.
 183. Misra, M., et al., *Influence of gold core concentration on visible photocatalytic activity of gold-zinc sulfide core-shell nanoparticle*. *Journal of Power Sources*, 2015. **294**: p. 580-587.
 184. Li, X., et al., *Au@In₂O₃ core-shell composites: A metal-semiconductor heterostructure for gas sensing applications*. *RSC Advances*, 2015. **5**(1): p. 545-551.
 185. Yin, H., et al., *Heterostructured catalysts prepared by dispersing Au@Fe₂O₃ core-shell structures on supports and their performance in CO oxidation*. *Catalysis Today*, 2011. **160**(1): p. 87-95.
 186. Venditti, I., et al., *Bioconjugation of gold-polymer core-shell nanoparticles with bovine serum amine oxidase for biomedical applications*. *Colloids and Surfaces B: Biointerfaces*, 2015. **134**: p. 314-321.
 187. Cao, Y., R. Jin, and C.A. Mirkin, *DNA-modified core-shell Ag/Au nanoparticles*. *Journal of the American Chemical Society*, 2001. **123**(32): p. 7961-7962.
 188. Huang, H., et al., *Preparation of Au-decorated Ag@C nanoparticle and its biosensing application*. *Chemistry Letters*, 2014. **43**(6): p. 781-783.
 189. Vinod, M. and K. Gopchandran, *Ag@Au core-shell nanoparticles synthesized by pulsed laser ablation in water: Effect of plasmon coupling and their SERS performance*. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2015. **149**: p. 913-919.
 190. Gong, M., et al., *Superparamagnetic core/shell GoldMag nanoparticles: size-, concentration- and time-dependent cellular nanotoxicity on human umbilical vein endothelial cells and the suitable conditions for magnetic resonance imaging*. *Journal of nanobiotechnology*, 2015. **13**(1): p. 24.
 191. Sodipo, B.K., A.A. Aziz, and M. Mustapa, *Facile synthesis and characteristics of gold coated superparamagnetic iron oxide nanoparticles via sonication*. *Int. J. Nanoelectronics and Materials*, 2015. **8**: p. 1-6.
 192. Karamipour, S., M. Sadjadi, and N. Farhadyar, *Fabrication and spectroscopic studies of folic acid-conjugated Fe₃O₄@Au core-shell for targeted drug delivery application*. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2015. **148**: p. 146-155.

193. Ke, F., L. Wang, and J. Zhu, *Multifunctional Au-Fe₃O₄@MOF core-shell nanocomposite catalysts with controllable reactivity and magnetic recyclability*. *Nanoscale*, 2015. **7**(3): p. 1201-1208.
194. Langlois, C., et al., *Fully Crystalline Faceted Fe-Au Core-Shell Nanoparticles*. *Nano letters*, 2015. **15**(8): p. 5075-5080.
195. Park, J.-I. and J. Cheon, *Synthesis of "solid solution" and "core-shell" type cobalt-platinum magnetic nanoparticles via transmetalation reactions*. *Journal of the American Chemical Society*, 2001. **123**(24): p. 5743-5746.
196. Pal, T., et al., *Au@Pd Core-shell Nanoparticles Decorated Reduced Graphene Oxide: A Highly Sensitive and Selective Platform for Electrochemical Detection of Hydrazine*. *RSC Advances*, 2015.
197. Zeng, J., et al., *A colorimetric assay for measuring iodide using Au@Ag core-shell nanoparticles coupled with Cu²⁺*. *Analytica chimica acta*, 2015.
198. Peng, H.P., et al., *Facile preparation of novel core-shell enzyme-Au-polydopamine-Fe₃O₄ magnetic bionanoparticles for glucose sensor*. *Biosensors and Bioelectronics*, 2013. **42**(1): p. 293-299.
199. Eksi, H., et al., *Fabrication of an Electrochemical E. coli Biosensor in Biowells Using Bimetallic Nanoparticle-Labelled Antibodies*. *Electroanalysis*, 2015. **27**(2): p. 343-352.
200. Nagaonkar, D. and M. Rai, *Sequentially reduced biogenic silver-gold nanoparticles with enhanced antimicrobial potential over silver and gold monometallic nanoparticles*. *Advanced Materials Letters*, 2015. **6**(4): p. 334-341.
201. Kristian, N. and X. Wang, *Pt shell-Au core/C electrocatalyst with a controlled shell thickness and improved Pt utilization for fuel cell reactions*. *Electrochemistry Communications*, 2008. **10**(1): p. 12-15.
202. Rajoua, K., L. Baklouti, and F. Favier, *Electronic and Mechanical Antagonist Effects in Resistive Hydrogen Sensors Based on Pd@Au Core-Shell Nanoparticle Assemblies Prepared by Langmuir-Blodgett*. *The Journal of Physical Chemistry C*, 2015. **119**(18): p. 10130-10139.
203. Nik Roselina, N.R., et al., *Synthesis and characterization of Ni-Au bimetallic nanoparticles*. *International Journal of Modern Physics B*, 2015. **29**(10-11).
204. Mayoral, A., D. Llamasa, and Y. Huttel, *A novel Co@Au structure formed in bimetallic core@shell nanoparticles*. *Chemical Communications*, 2015. **51**(40): p. 8442-8445.
205. Kandpal, D., S. Kalele, and S. Kulkarni, *Synthesis and characterization of silica-gold core-shell (SiO₂@Au) nanoparticles*. *Pramana*, 2007. **69**(2): p. 277-283.
206. Subramanian, V., E.E. Wolf, and P.V. Kamat, *Influence of metal/metal ion concentration on the photocatalytic activity of TiO₂-Au composite nanoparticles*. *Langmuir*, 2003. **19**(2): p. 469-474.
207. Wei, Y., et al., *ZnO nanorods/Au hybrid nanocomposites for glucose biosensor*. *Biosensors and Bioelectronics*, 2010. **26**(1): p. 275-278.
208. Shen, X., Z. Ge, and Y. Pang, *Conjugating folate on superparamagnetic Fe₃O₄@Au nanoparticles using click chemistry*. *Journal of Solid State Chemistry*, 2015. **222**: p. 37-43.
209. Karamipour, S., M.S. Sadjadi, and N. Farhadyar, *Fabrication and spectroscopic studies of folic acid-conjugated Fe₃O₄@Au core-shell for targeted drug delivery application*. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, 2015. **148**: p. 146-155.
210. He, X., et al., *Lectin-conjugated Fe₂O₃@Au core@shell nanoparticles as dual mode contrast agents for in vivo detection of tumor*. *Molecular Pharmaceutics*, 2014. **11**(3): p. 738-745.
211. Salata, O.V., *Applications of nanoparticles in biology and medicine*. *Journal of nanobiotechnology*, 2004. **2**(1): p. 3.
212. De, M., P.S. Ghosh, and V.M. Rotello, *Applications of nanoparticles in biology*. *Advanced Materials*, 2008. **20**(22): p. 4225-4241.
213. Ahmed, N., H. Fessi, and A. Elaissari, *Theranostic applications of nanoparticles in cancer*. *Drug Discovery Today*, 2012. **17**(17): p. 928-934.
214. Zeng, H., et al., *Tailoring magnetic properties of core/shell nanoparticles*. *Applied physics letters*, 2004. **85**: p. 792-794.
215. Zhu, Q., et al., *Facile fabrication of Au-F127 nanocolloids with different morphologies and their potential bioapplications*. *Australian Journal of Chemistry*, 2013. **66**(3): p. 381-390.
216. Huang, D.-M., et al., *Highly efficient cellular labeling of mesoporous nanoparticles in human mesenchymal stem cells: implication for stem cell tracking*. *The FASEB journal*, 2005. **19**(14): p. 2014-2016.
217. Toso, C., et al., *Clinical magnetic resonance imaging of pancreatic islet grafts after iron nanoparticle labeling*. *American Journal of Transplantation*, 2008. **8**(3): p. 701-706.
218. Kanwar, J.R., et al., *Nanoparticles in the treatment and diagnosis of neurological disorders: untamed dragon with fire power to heal*. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2012. **8**(4): p. 399-414.
219. Abdulla-Al-Mamun, M., et al., *Au-ultrathin functionalized core-shell (Fe₃O₄@Au) monodispersed nanocubes for a combination of magnetic/plasmonic photothermal cancer cell killing*. *RSC Advances*, 2013. **3**(21): p. 7816-7827.
220. Addae, E., et al., *Investigation of antimicrobial activity of photothermal therapeutic gold/copper sulfide core/shell nanoparticles to bacterial spores and cells*. *Journal of Biological Engineering*, 2014. **8**(1).
221. Wang, X., et al., *Multifunctional Fe₃O₄@P(Si/MAA)@Chitosan@Au core/shell nanoparticles for dual imaging and photothermal therapy*. *ACS Applied Materials and Interfaces*, 2013. **5**(11): p. 4966-4971.
222. Xu, B., et al., *TLyP-1-conjugated Au-nanorod@SiO₂ core-shell nanoparticles for tumor-targeted drug delivery and photothermal therapy*. *Langmuir*, 2014. **30**(26): p. 7789-7797.
223. Zhou, Z., et al., *Iron/iron oxide core/shell nanoparticles for magnetic targeting MRI and near-infrared photothermal therapy*. *Biomaterials*, 2014. **35**(26): p. 7470-7478.
224. Chen, Y.-S., et al., *Enhanced thermal stability of silica-coated gold nanorods for photoacoustic imaging and image-guided therapy*. *Optics express*, 2010. **18**(9): p. 8867-8878.
225. Wong, J.K., S.P. Yip, and T.M. Lee, *Ultra-Stable oligonucleotide-gold and-silver nanoparticle conjugates prepared by a facile silica reinforcement method*. *Nano Research*, 2012. **5**(9): p. 585-594.
226. Biju, V., *Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy*. *Chemical Society Reviews*, 2014. **43**(3): p. 744-764.
227. Liu, R., et al., *Core-shell Fe₃O₄ polydopamine nanoparticles serve multipurpose as drug carrier, catalyst support and carbon adsorbent*. *ACS Applied Materials and Interfaces*, 2013. **5**(18): p. 9167-9171.
228. Melancon, M.P., M. Zhou, and C. Li, *Cancer theranostics with near-infrared light-activatable multimodal nanoparticles*. *Accounts of chemical research*, 2011. **44**(10): p. 947-956.

229. Melancon, M.P., et al., *In vitro and in vivo targeting of hollow gold nanoshells directed at epidermal growth factor receptor for photothermal ablation therapy*. Molecular cancer therapeutics, 2008. **7**(6): p. 1730-1739.
230. Melancon, M.P., et al., *Theranostics with multifunctional magnetic gold nanoshells: photothermal therapy and t2* magnetic resonance imaging*. Investigative radiology, 2011. **46**(2): p. 132.
231. Li, J., S. Gupta, and C. Li, *Research perspectives: gold nanoparticles in cancer theranostics*. Quantitative imaging in medicine and surgery, 2013. **3**(6): p. 284.
232. Ke, H., et al., *Gold nanoshelled liquid perfluorocarbon magnetic nanocapsules: a nanotheranostic platform for bimodal ultrasound/magnetic resonance imaging guided photothermal tumor ablation*. Theranostics, 2014. **4**(1): p. 12.
233. Wang, H., et al., *Tailor-made Au@Ag core-shell nanoparticle 2D arrays on protein-coated graphene oxide with assembly enhanced antibacterial activity*. Nanotechnology, 2013. **24**(20).
234. Huo, D., et al., *Fabrication of AuAg core-shell NPs as enhanced CT contrast agents with broad antibacterial properties*. Colloids and Surfaces B: Biointerfaces, 2014. **117**: p. 29-35.
235. Lee, I.H., J.M. Lee, and Y. Jung, *Controlled protein embedment onto Au/Ag core-shell nanoparticles for immuno-labeling of nanosilver surface*. ACS Applied Materials and Interfaces, 2014. **6**(10): p. 7659-7664.
236. Gangopadhyay, P., et al., *Novel superparamagnetic core (shell) nanoparticles for magnetic targeted drug delivery and hyperthermia treatment*. Magnetics, IEEE Transactions on, 2005. **41**(10): p. 4194-4196.
237. Kayal, S. and R.V. Ramanujan, *Anti-cancer drug loaded iron-gold core-shell nanoparticles (Fe@ Au) for magnetic drug targeting*. Journal of nanoscience and nanotechnology, 2010. **10**(9): p. 5527-5539.
238. Sinha, R., et al., *Nanotechnology in cancer therapeutics: bioconjugated nanoparticles for drug delivery*. Molecular cancer therapeutics, 2006. **5**(8): p. 1909-1917.
239. Cho, K., et al., *Therapeutic nanoparticles for drug delivery in cancer*. Clinical cancer research, 2008. **14**(5): p. 1310-1316.
240. Chow, E.K.-H. and D. Ho, *Cancer nanomedicine: from drug delivery to imaging*. Science translational medicine, 2013. **5**(216): p. 216rv4-216rv4.
241. Zhang, Z., et al., *Near infrared laser-induced targeted cancer therapy using thermoresponsive polymer encapsulated gold nanorods*. Journal of the American Chemical Society, 2014. **136**(20): p. 7317-7326.
242. Holsapple, M.P., et al., *Research strategies for safety evaluation of nanomaterials, part II: toxicological and safety evaluation of nanomaterials, current challenges and data needs*. Toxicological Sciences, 2005. **88**(1): p. 12-17.
243. Hodgson, E. and J. Goldstein, *Introduction to biochemical toxicology*. Toxicology, 2001. **2**: p. 2.
244. Cohen, M.H., et al., *United States Food and Drug Administration drug approval summary gefitinib (ZD1839; Iressa) tablets*. Clinical Cancer Research, 2004. **10**(4): p. 1212-1218.
245. Dhawan, A., V. Sharma, and D. Parmar, *Nanomaterials: a challenge for toxicologists*. Nanotoxicology, 2009. **3**(1): p. 1-9.
246. Oberdörster, G., *Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology*. Journal of internal medicine, 2010. **267**(1): p. 89-105.
247. Wittmaack, K., *Dose and Response Metrics in Nanotoxicology: Wittmaack Responds to Oberdörster et al. and Stoeger et al.* Environmental health perspectives, 2007. **115**(6): p. A291.
248. Kong, B., et al., *Experimental considerations on the cytotoxicity of nanoparticles*. Nanomedicine, 2011. **6**(5): p. 929-941.
249. Lewinski, N., V. Colvin, and R. Drezek, *Cytotoxicity of nanoparticles*. small, 2008. **4**(1): p. 26-49.
250. Demaurex, N., *pH Homeostasis of cellular organelles*. Physiology, 2002. **17**(1): p. 1-5.
251. Verma, A. and F. Stellacci, *Effect of surface properties on nanoparticle-cell interactions*. Small, 2010. **6**(1): p. 12-21.
252. Lassen, N.A., D.H. Ingvar, and E. Skinhøj, *Brain function and blood flow*. Sci. Am.,(United States), 1978. **239**(4).
253. Almeida, J.P.M., et al., *In vivo biodistribution of nanoparticles*. Nanomedicine, 2011. **6**(5): p. 815-835.
254. Hillegass, J.M., et al., *Assessing nanotoxicity in cells in vitro*. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2010. **2**(3): p. 219-231.
255. Fischer, H.C. and W.C. Chan, *Nanotoxicity: the growing need for in vivo study*. Current opinion in biotechnology, 2007. **18**(6): p. 565-571.
256. Wu, T., et al., *Effects of external magnetic field on biodistribution of nanoparticles: A histological study*. Journal of Magnetism and Magnetic Materials, 2007. **311**(1): p. 372-375.
257. Dadmehr, M., et al., *DNA methylation detection by a novel fluorimetric nanobiosensor for early cancer diagnosis*. Biosensors and Bioelectronics, 2014. **60**: p. 35-44.
258. Li, L., et al., *A photosensitizer-conjugated magnetic iron oxide/gold hybrid nanoparticle as an activatable platform for photodynamic cancer therapy*. Journal of Materials Chemistry B, 2014. **2**(19): p. 2929-2937.
259. Liu, T.M., et al., *One-step shell polymerization of inorganic nanoparticles and their applications in SERS/nonlinear optical imaging, drug delivery, and catalysis*. Scientific Reports, 2014. **4**.
260. Bandyopadhyay, S., et al., *Synthesis and in vitro cellular interactions of superparamagnetic iron nanoparticles with a crystalline gold shell*. Applied Surface Science, 2014. **316**(1): p. 171-178.
261. Perera, V.S., et al., *Cell-permeable Au@ZnMoS₄ core-shell nanoparticles: Toward a novel cellular copper detoxifying drug for wilson's disease*. Chemistry of Materials, 2013. **25**(23): p. 4703-4709.
262. Quyen, T.T.B., et al., *Au@SiO₂ core/shell nanoparticle assemblage used for highly sensitive SERS-based determination of glucose and uric acid*. Journal of Raman Spectroscopy, 2013. **44**(12): p. 1671-1677.
263. Yu, C., et al., *Bovine serum albumin nanospheres synchronously encapsulating gold selenium/gold nanoparticles and photosensitizer for high-efficiency cancer phototherapy*. Applied Biochemistry and Biotechnology, 2013. **169**(5): p. 1566-1578.
264. Cheng, X., et al., *Revealing silver cytotoxicity using Au nanorods/Ag shell nanostructures: Disrupting cell membrane and causing apoptosis through oxidative damage*. RSC Advances, 2013. **3**(7): p. 2296-2305.
265. Zhang, W., et al., *Designed self-assembled hybrid Au@CdS core-shell nanoparticles with negative charge and their application as highly selective biosensors*. Journal of Materials Chemistry B, 2015. **3**(2): p. 217-224.

CHAPTER TWO: The synthesis and characterisation of biocompatible ZnTe nanoparticles

2.1 Introduction

Semiconductor nanoparticles are assembled through the combination of cations from elements in group 12 and anions from elements in group 16 of the periodic table. Semiconductor nanoparticles display a unique array of optical and electrical properties that can be exploited for multiple applications in pigments, dyes, night vision aids, high quality light-emitting diodes (LEDs'), and various bio-applications such as bio-imaging, biosensors and disease diagnosis tools (**Figure 2.1**). Semiconductor nanoparticles display size dependent changes in optical, electrical and band gap properties that are different to their bulk materials. The optical properties of semiconducting nanoparticles can be tuned by controlling the size of the particle. The size of the nanomaterial corresponds to specific wavelengths which determines the colour fluorescence property of the quantum dot or semiconductor nanoparticle.

The electrons within a semiconductor nanoparticle has pre-defined energies allocated to each orbital known as orbital energy. Electrons occupy each orbital in a specific manner from the lowest energy level and the energy within each level is controlled according to Pauli's Exclusion Principle and the solution of Schrödinger's equations. The band gap in bulk semiconducting materials are stable however the band gap of semiconductor nanoparticles can vary relative to size. It is well demonstrated that as the nanoparticle decreases in size the band gap increases (**Figure 2.2**). The band gap is the region between the valence and conduction bands with no energy levels. The valence band is the most occupied in terms of energy because the electrons occupy each orbital from the lowest energy level. The conduction band comprises of electrons from the upper region of the bandgap.

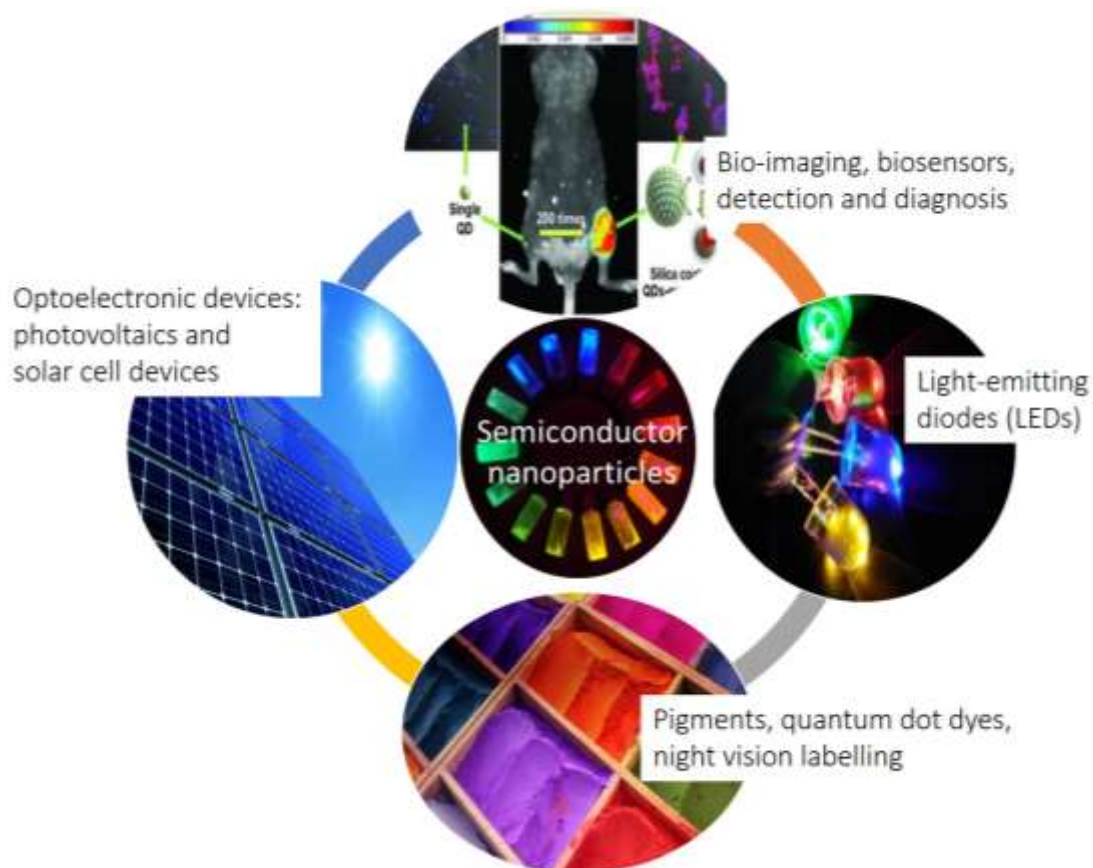


Figure 2.1 Applications of semiconductor nanoparticles. Their extraordinary optical and electrical properties are utilized for applications in pigment and dye technology, LEDs and bio-imaging diagnostic tools. (Figure modified from <https://dborck.files.wordpress.com/2015/03/1000017182-1.jpg> http://onlinelibrary.wiley.com/store/10.1002/adfm.201102930/asset/image_m/mcontent.jpg?v=1&s=2df65f806428e599778f38c3531c2aa6086375d2 <http://media.mehrnews.com/d/2015/09/21/3/1840466.jpg>, 2015).

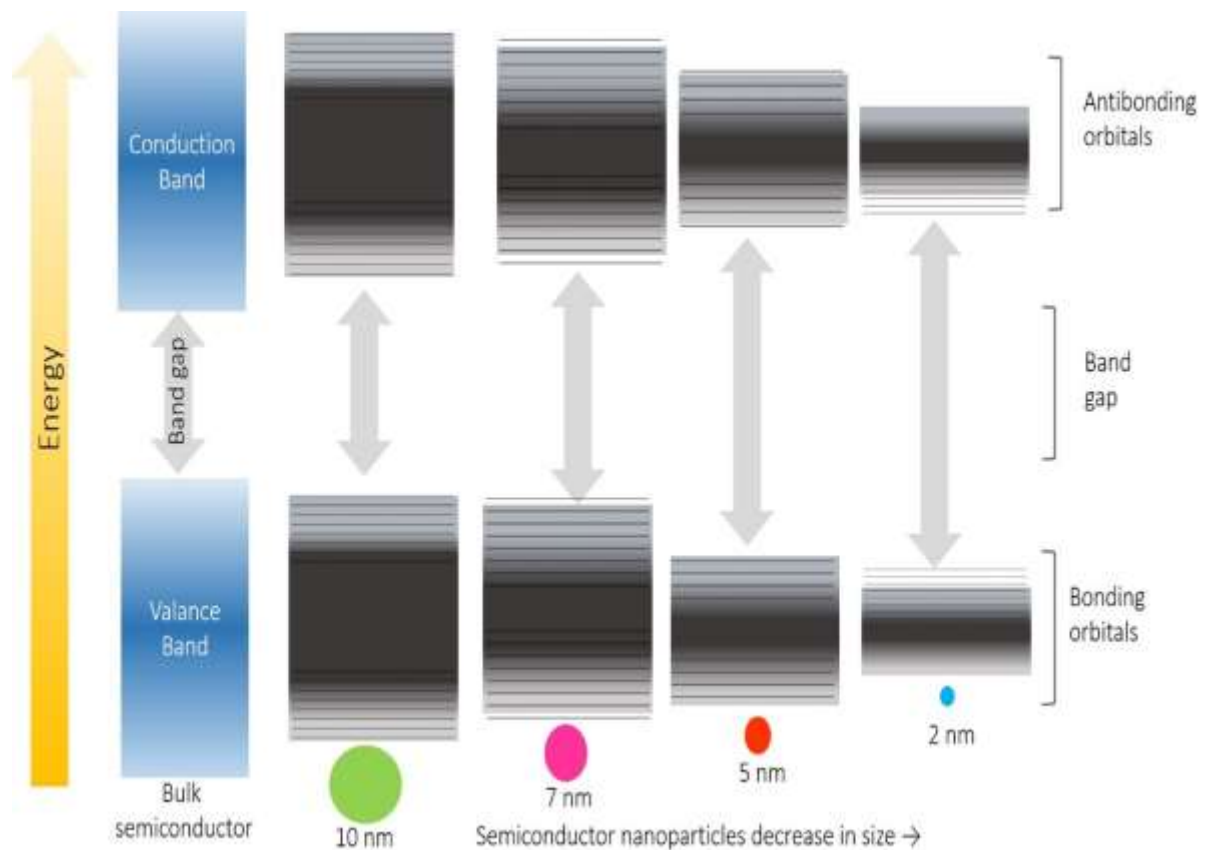


Figure 2.2 Band gap properties relative to nanoparticle size. The band gap and energy of nanoparticles increases as the particle size decreases.

The electrons in the valence band are required to have enough energy to move across the band gap region into the conduction band region. After the electrons are excited, they subsequently revert back to the valence band position, also termed returning to rest state followed by a subsequent emission of radiation at specific wavelengths. During excitation and movement of electrons from the valence to conduction bands, an absence of electron or energy is formed within the valence band, known as a hole. Together the electron pair (electron in the conduction band and hole in the valence band) is termed an exciton. Excitons are characterised by a specific separation distances between the corresponding hole and electron, this distance is known as the exciton Bohr radius. The electronic structure is responsible for most of the applications of

semiconductor nanoparticles (**Figure 2.1** and **Figure 2.2**). Other factors that can be manipulated to deliver specific applications of semiconductor nanoparticles include the use of surface ligands, size, surface charge and solubility.

The synthesis of functionalized semiconductor nanoparticles has attracted interest in the fields of nanoscience and medicine [1-3]. Scientists are constantly searching for alternative materials that may be utilized in bio-imaging and labelling applications. A key component in designing such nanomaterials incorporates water solubility, functionalization, relatively low toxicity and materials that are not susceptible to photobleaching. Literature describes many advances in applications of biocompatible semiconductor nanoparticles, these include live cell labelling, nanosensors for glucose and immunochromatographic assays.

Semiconducting materials may be engineered at sizes that are smaller than or similar to those of a cell, peptides, enzymes and other subcellular organelles [4, 5]. In addition their surface features may be altered to facilitate its interaction and entry into the biological entity of choice resulting in a variety of applications across plant biology and the biomedical sciences [6-9]. A challenge in synthesizing semiconducting nanomaterials for bio-applications is the accumulation of component ions that result in toxicity. A well-known example is cadmium based semiconductor nanoparticles, studies show that cadmium toxicity is due to bio-accumulation [10, 11]. Mechanisms to improve the biosafety of cadmium based nanoparticles include the use of coating layers that are non-toxic and biocompatible [12-14]. ZnTe is an interesting II-VI semiconducting nanomaterial that is less toxic when compared to cadmium

based chalcogenides[9]. ZnTe has been synthesized via physical routes such as mechanical alloying [15, 16] and pulsed laser ablation [17]. It is a challenge to prepare ZnTe nanostructures with controllable size and shape because of the lack of suitable precursors for controlling the thermodynamics and kinetics of the nanocrystal nucleation stage. ZnTe in the form of nanodots [18-21], nanoflowers [21] nanowires and nanorods [21-24] have been reported using various colloidal and high temperature routes. In many cases the growth of the anisotropic structures is attributed to the oriented attachment growth mechanism [25-29]. An aqueous route for the synthesis of fluorescent ZnTe/dendrimer nanocomposites was reported by Ghosh and co-workers, their study investigated antimicrobial properties of the ZnTe nanocomposites and revealed their potential in therapeutic applications [30]. Water-dispersible 1D Te@ZnTe core-shell nanoparticles have also been reported whereby the ZnTe nanoparticles were used as precursors. The 1D nanoparticles have a crystalline Te core and an amorphous ZnTe shell [31]. The design of biocompatible ZnTe nanoparticles is accomplished through the optimal use of pH, temperature and appropriate coating materials. Thioglycerol capped ZnTe nanoparticles was prepared under the pH range 7.1-10.0, this study showed that the choice of ligands influences the stability of the ZnTe nanoparticles [32]. In another study the synthesis of ZnTe nanowires using the vapor-liquid-solid method was reported. This study showed a direct correlation between temperature effects and changes in morphology. The temperature dependent growth patterns produced highly ordered structures of ZnTe nanorods, wires and ribbons. The effect of pH, capping materials and temperature has been shown to produce ZnTe nanomaterials with varying morphologies [15, 22, 25].

The morphology of ZnTe nanomaterials is associated to its fluorescence properties [33]. The use of long-alkyl-chain fatty acid as the capping ligand in the preparation of ZnTe nanorods, exhibited stable blue fluorescence with high quality quantum yield that may be used in blue light-emitting diodes [34]. This study reports a solution based, low thermal route for the synthesis of biocompatible ZnTe nanoparticles. The aim was to synthesize and characterise biocompatible ZnTe nanoparticles that could be subsequently utilised for its fluorescence properties. The synthesis method used in this work was adapted from the route previously reported for the synthesis of cysteine capped CdSe nanoparticles [12]. The as-prepared cysteine capped ZnTe nanoparticles was evaluated to establish optical, crystalline and morphology features. Additionally the fluorescence properties and cytotoxicity of cysteine capped ZnTe nanoparticles are demonstrated.

2.2 Experimental details

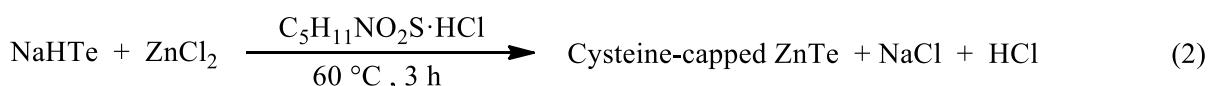
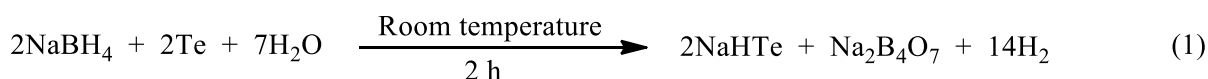
2.2.1 Materials

Zinc chloride, L-cysteine ethyl ester hydrochloride, tellurium powder, sodium borohydride, deionised water (HPLC grade) and acetone were obtained from Sigma Aldrich. All the chemicals were of analytical grade and used as purchased.

2.2.2 Synthesis of cysteine capped ZnTe nanoparticles

Tellurium powder (0.32 mmol) was mixed with 20 ml of deionized water in a three necked round bottom flask at room temperature. Sodium borohydride (0.81 mmol) was added to the reaction mixture under inert conditions (Equation 1). After 4 hours 20 ml of 3.2×10^{-4} M ZnCl_2 (zinc salt) and L-cysteine ethyl ester hydrochloride (capping agent) was added to the reaction mixture in molar

ratios of 1:10 (Equation 2). The reaction mixture was then heated at 60 °C for 3 hours under nitrogen gas. The pH of the reaction was adjusted to 4, 7 and 11 within the first 30 minutes of adding the zinc salt. An aliquot of the reaction mixture was removed and purified for characterisation at 1, 2 and 3 hours to determine the effect of reaction time on nanoparticle growth and morphology. The ZnTe nanoparticles were precipitated using acetone. This product was centrifuged to give the pellet that was then dried under vacuum and weighed out to give a dark brown material that was readily dispersed in water.



2.2.3 Characterisation of nanoparticles

2.2.3.1 Ultra-violet-visible spectroscopy analysis

A Perkin-Elmer Lambda 20 UV-vis spectrophotometer was used to carry out optical measurements in the 200-1100 nm wavelength range at room temperature. Samples of cysteine capped ZnTe nanoparticles were placed in quartz cuvettes (1 cm path length) and the absorbance was recorded.

2.2.3.2 Photoluminescence spectroscopy analysis

Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 luminescence spectrometer with xenon lamp over range of 200-800 nm. The samples were placed in quartz cuvettes (1 cm path length) and the excitation peaks were analysed and recorded.

2.2.3.3 Morphological characterisation (TEM, HRTEM and SEM)

TEM imaging of ZnTe nanoparticles was performed using a Philips CM120 BIOTWIN TEM operated at 120kV. A JOEL JEM 3010 URP high resolution transmission electron microscope (HRTEM) operated at 300 kV was used for high resolution analysis of a small aliquot of the ZnTe samples. Images were recorded using a 1024 x 1024 CCD camera. SEM measurements were done using a Zeiss Ultra Plus FEG SEM at 10 kV and EDX at 20 kV.

2.2.3.4 X-ray diffraction analysis

XRD measurements of the ZnTe nanoparticles were performed using a Bruker aXS D8 advanced diffractometer with Cu-K α radiation (λ = 1.5406 Å) operated at 40 kV and 40 mA. TEM imaging of the cellular uptake of nanoparticles was performed using a Philips CM120 BIOTWIN TEM operated at an accelerating voltage of 120 kV with the sample cooled to 80K.

2.2.3.5 Surface charge analysis

The Zeta potential of cysteine capped ZnTe was determined by a Zetasizer, Nanoseries, Malvern Instrument. Samples were filtered several times through a 0.22 mm Millipore membrane filter prior to recording surface charge measurements.

2.2.3.6 Fluorescence of ZnTe nanoparticles in the presence of DNA plasmid pGEMT easy

An aliquot of 2 μ l of DNA plasmid was treated with 50 μ g/ml of cysteine capped ZnTe nanoparticles and placed on a heating block at 37°C. Fluorescence images were recorded using a Nikon Eclipse 80i fluorescence and phase-contrast microscope. The aqueous sample of ZnTe nanoparticles was placed on a slide and viewed under a 365-395 nm filter set.

2.2.3.7 Cytotoxicity of ZnTe nanoparticles using MTT assay

All the cell culture reagents were supplied by Invitrogen. Adhesion cell line, *Human mammary adenocarcinoma* (MCF7) cells were grown using EMEM culture media containing 10% fetal bovine serum and 1-2% penstrep fungizone. Cells that grew as monolayers were passaged, trypsinized and harvested for experimentation before reaching 100 % confluency. Cells were seeded at cell density of 5000 cells per well, into a 96-well plate. Following adherence to the plate, the growth medium was replaced with fresh medium (100 μ L per well) and ZnTe nanoparticles (20, 40, 60, 80 and 100 μ g/ml). After 48 hours the control wells were treated with culture medium only. Wells containing culture medium without cells were used as blanks. All experiments were performed with six replicates. After the 48 hour incubation, the culture medium and compounds were removed and replaced with fresh medium (100 μ L) and 100 μ L of MTT solution (5 mg/mL in PBS) in each well. After 4 hour incubation, the media and MTT solution was removed and 100 μ L of DMSO was added to each well to solubilize the MTT formazan. The optical density (OD) of each well was measured on a microplate spectrophotometer (Mindray MR-96A) at a wavelength of 540 nm [35].

2.3 Results

2.3.1 Optical properties of ZnTe Nanoparticles

Cysteine capped ZnTe nanoparticles were synthesized using a one pot route method under inert experimental conditions. During the reduction step a dark purple colour was observed after 4 hours. This solution turned dark brownish to black in colour after the addition of zinc chloride and L-cysteine ethyl ester hydrochloride (**Figure 2.3 A**). This method was carried out at acidic, neutral and alkaline pH. The growth of the nanoparticles was investigated at 1, 2 and 3 hour time intervals. The material from this reaction was purified and quantified for characterisation analysis. The absorption properties of the cysteine capped ZnTe nanoparticles was measured for each sample. The cysteine capped ZnTe nanoparticles synthesized at pH 4 showed an absorption peak at 269 nm (**Figure 2.3 B**). The optical band gap of the ZnTe nanoparticles can be estimated from the UV-spectra by extrapolating the linear portion of the curve into X-axis. The blue shift observed from both the absorption edges and peaks can be related to the quantum confinement effect (QCE). For the ZnTe samples synthesized at acidic reaction conditions, there is evidence of quantum confinement. The absorption band edge of the ZnTe synthesized at pH 4, 2hrs is observed at 295 nm (4.2 eV), which is higher than the bulk value of 548 nm (2.26 eV) ^[36] as shown in (**Figure 2.3 B and D**). The absorption properties of ZnTe nanoparticles under varying pH conditions over different time intervals are shown in (**Figure 2.4 A-C**). The optical properties of ZnTe nanoparticles showed stability at six months after synthesis (**Figure 2.4 D**). The corresponding photoluminescence spectrum shows narrow emission in the 365-420 nm range (**Figure 2.3 C**).

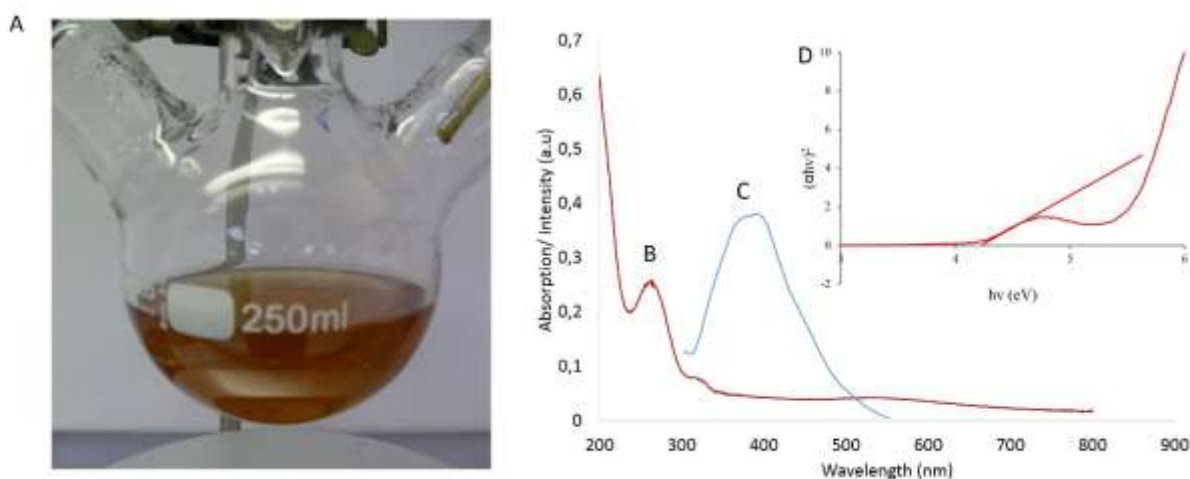


Figure 2.3 Optical properties of cysteine passivated ZnTe nanoparticles ZnTe nanoparticles at pH 4, 2 hours. **A.** Solution of ZnTe nanoparticles. **B.** shows an absorption peak at 269 nm. **C.** displays the emission spectrum in the 365-415nm range. **D.** displays the extrapolation of the band edge for the sample at pH 4, 2hours.

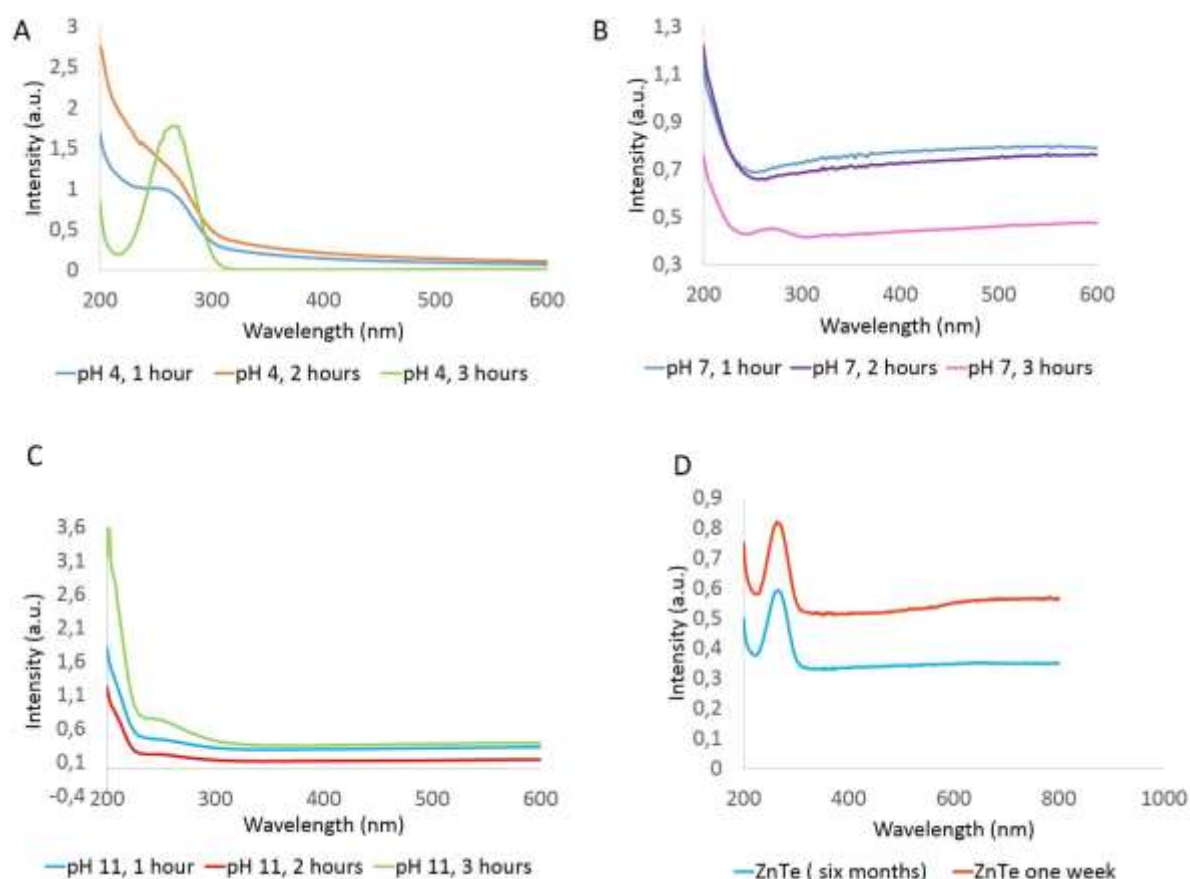


Figure 2.4 Optical properties of ZnTe at different pH conditions. **A.** Optical properties at pH 4. **B.** Optical properties at pH 7. **C.** Optical properties at pH 11. **D.** Stability of ZnTe after six months, optical analysis.

2.3.2 Morphological characterisation of ZnTe nanoparticles

The ZnTe nanoparticles were characterized using TEM, HRTEM and SEM techniques. **Figure 2.5** shows TEM micrographs of ZnTe nanoparticles at different pH and time intervals. The ZnTe nanoparticles synthesized at pH 7, 1-3 hour intervals appeared aggregated and undefined in shaped and size (**Figure 2.5, D-F**). The ZnTe nanoparticles synthesized at pH 11, 1-3 hour intervals appeared close to spherical in shape and of irregular size (**Figure 2.5, G-I**). The ZnTe nanoparticles synthesized at pH 4, 1-3 hour intervals showed interesting features in their growth patterns, size and shape (**Figure 2.5, A-C**) and therefore was subjected to in-depth analysis in this study. The size and growth of ZnTe nanoparticles was controlled by time intervals, we restricted the growth of ZnTe nanomaterials to 3 hours because we observed relatively larger nanorods with an average size of 250 nm in length at 24 hours (**Figure 2.6**).

ZnTe nanoparticles from pH 4, 1 hour interval showed spherical nanoparticles that appeared closely agglomerated. ZnTe nanoparticles from pH 4, 2 hour interval showed a combination of nanospheres and nanorods. The ZnTe nanoparticles at pH 4, 1 hour time interval consisted of large spheres in the 40-50 nm size range. As the reaction proceeded to the 2 hour time interval the sample began to display a transition phase in which nanospheres develop into nanorods that are between 60-80 nm in length and 20-25 nm in diameter (**Figure 2.5 B** and **Figure 2.7 A-C**). After 3 hours the transformation of the spheres into rods is completed as highlighted within the red ring (**Figure 2.5 C** and **Figure 2.8 A-C**).

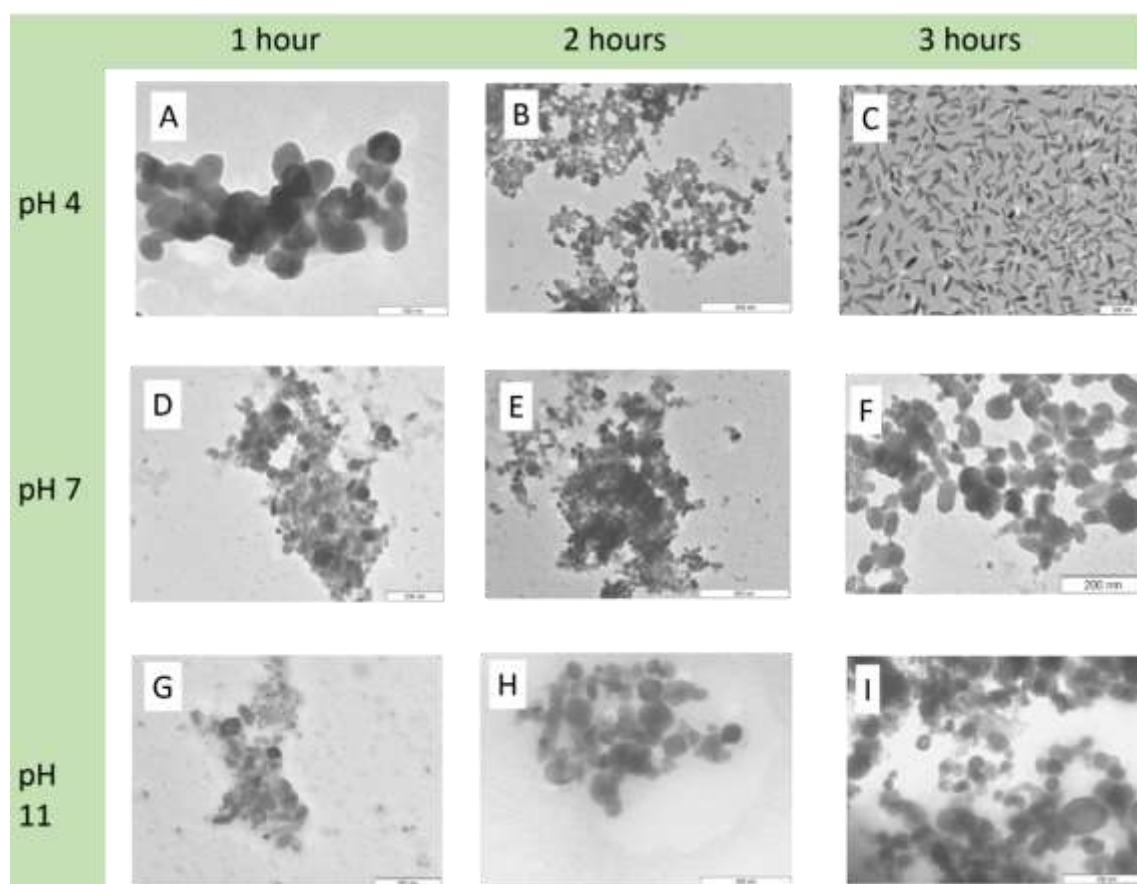


Figure 2.5 TEM characterisation of ZnTe nanoparticles synthesized at varying pH and time intervals. **A.** pH 4, 1 hour interval. **B.** pH 4, 2 hour interval. **C.** pH 4, 3 hour interval. **D.** pH 7, 1 hour interval. **E.** pH 7, 2 hour interval. **F.** pH 7, 3 hour interval. **G.** pH 11, 1 hour interval. **H.** pH 11, 2 hour interval. **I.** pH 11, 3 hour interval.

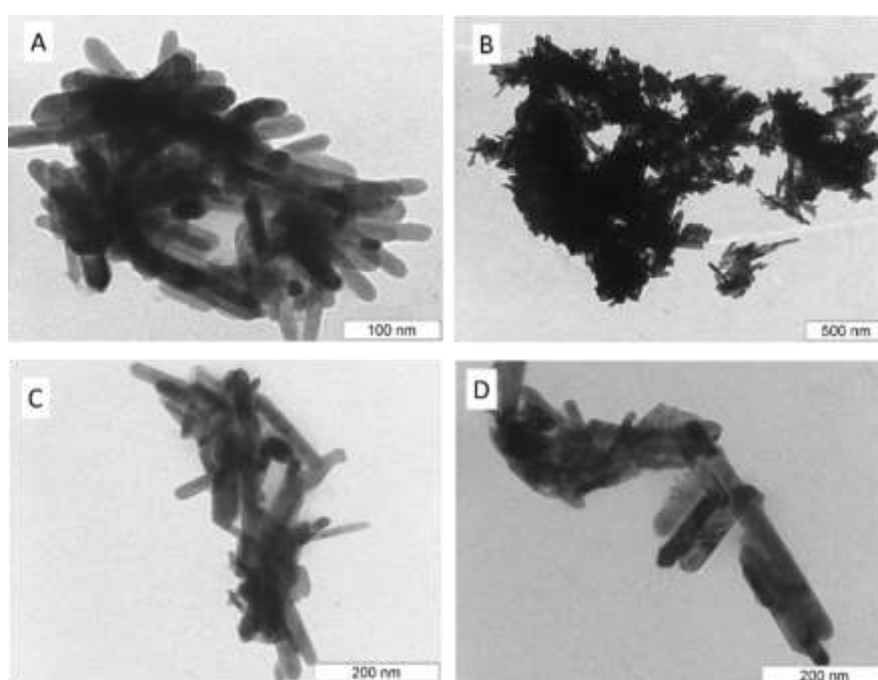


Figure 2.6. TEM micrographs of cysteine capped ZnTe nanoparticles, show nanoparticle growth at pH 4, 24 hour interval.

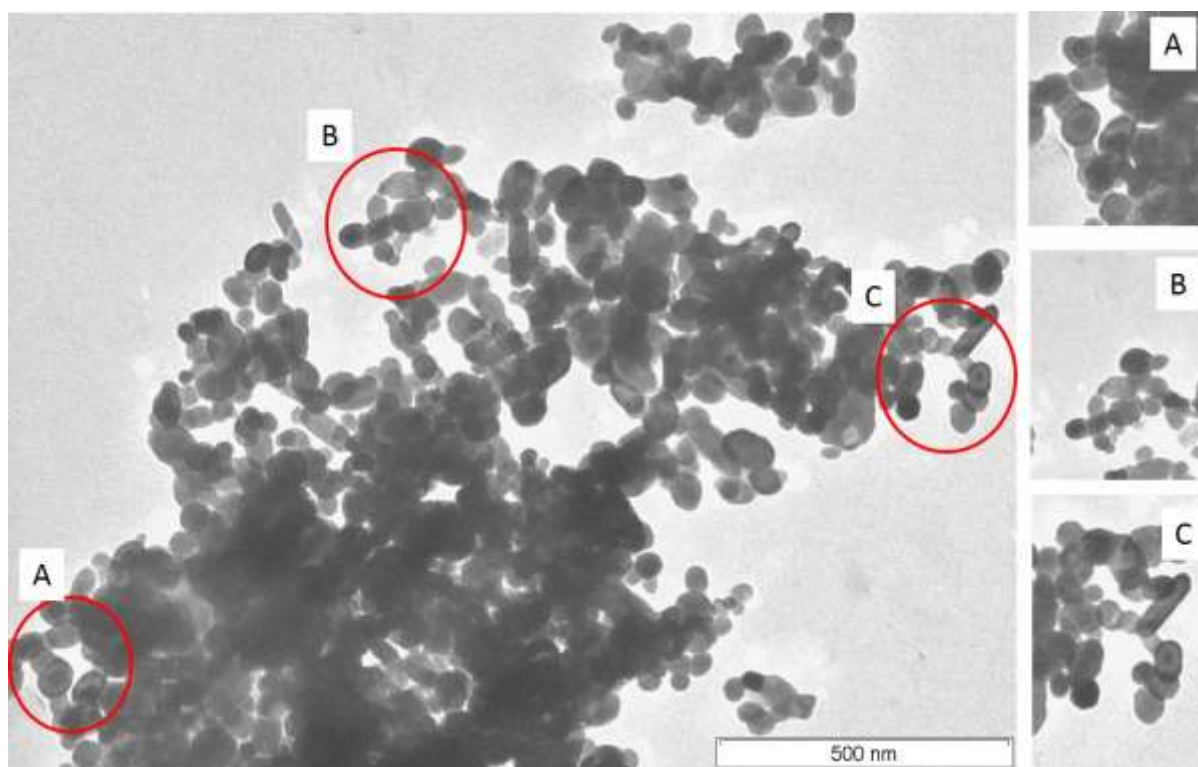


Figure 2.7 ZnTe nanoparticles using TEM analysis for pH 4, 2 hour interval (A-C).

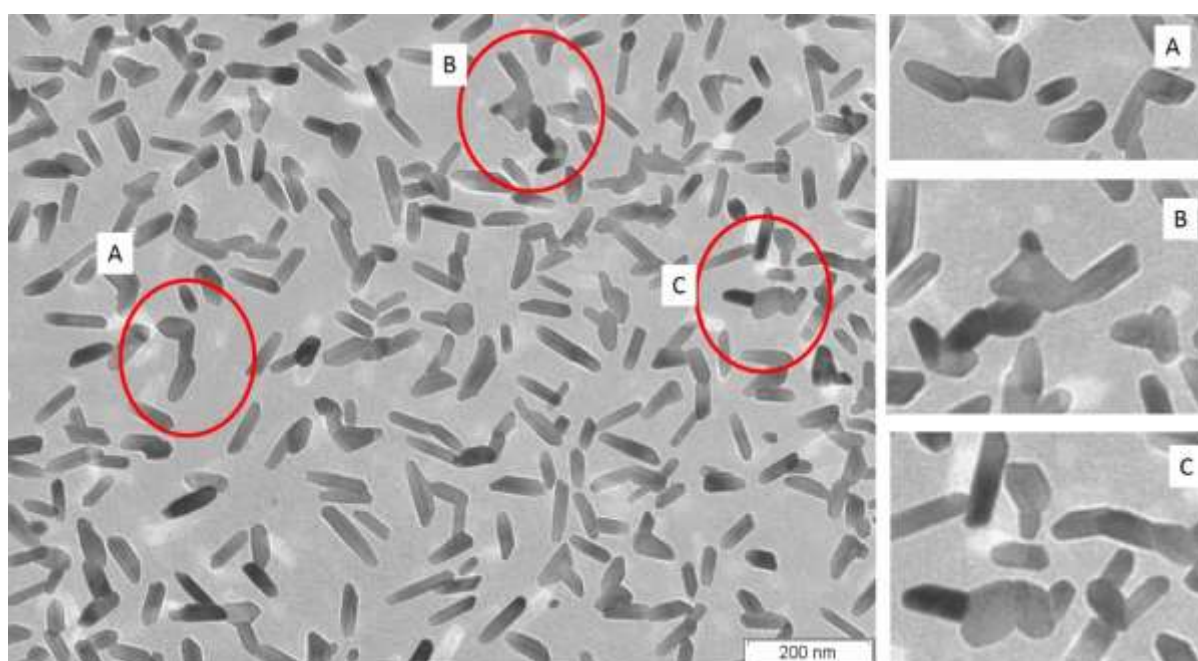


Figure 2.8 TEM characterisation of ZnTe nanoparticles, **A-C** demonstrates the oriented attachment mechanisms of growth resulting in the formation of uniform nanorods, pH 4, 2 hour.

The growth patterns observed in the TEM images in (**Figure 2.7, A-C** and **Figure 2.8, A-C**) clearly demonstrate oriented attachment of nanospheres in the transition phase leading to the formation of well defined, uniform nanorods. **Figure 2.9 A**, shows spherical ZnTe nanoparticles (pH 4, 2 hours), that are in the process of alignment and coalescence. In **Figure 2.9 .B-D** the fusion of the particles is more evident. The clearly defined lattice fringes in varying directions are observed with the spacing of 0.36 and 0.39 nm in (**Figure 2.9, B-C**) is assigned to the (111) plane of cubic ZnTe, respectively. The SAED pattern observed for the ZnTe nanorods (**Figure 2.9, F**) corresponds to the cubic crystal phase.

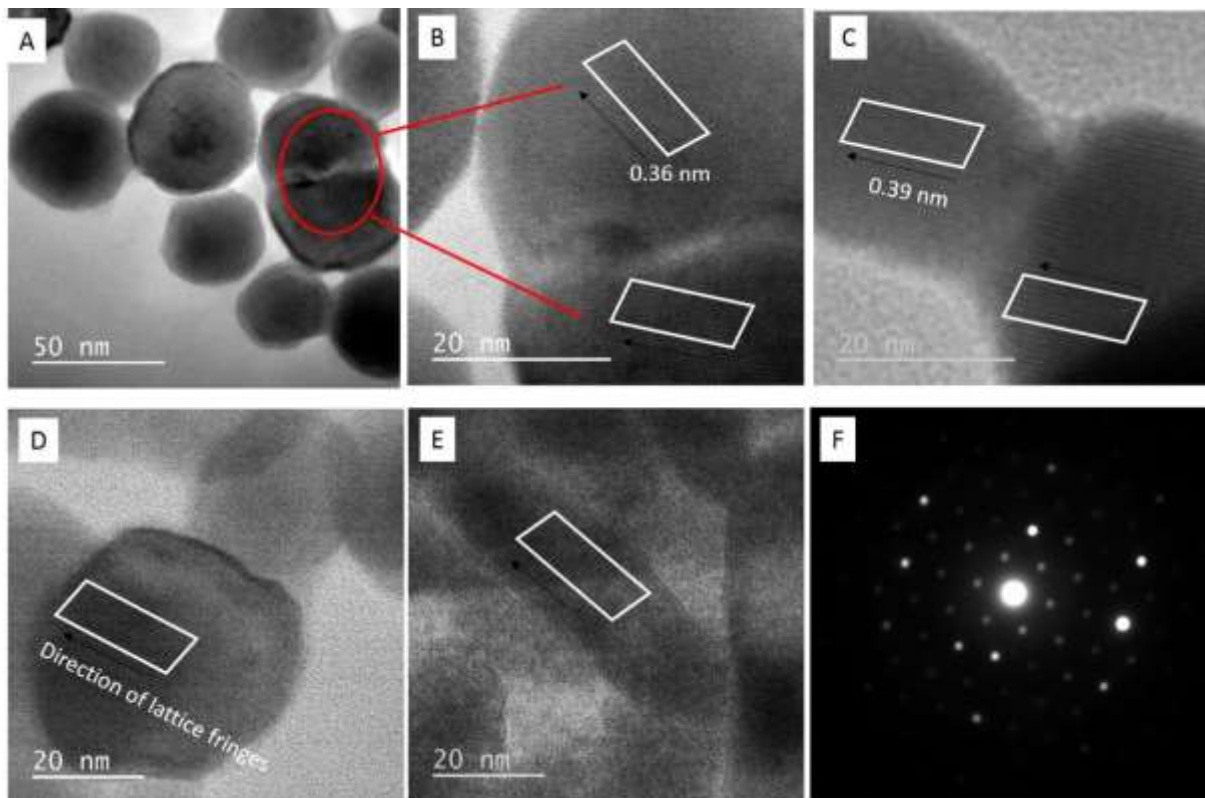


Figure 2.9 A, B, C and D. demonstrates the oriented attachment mechanisms of growth. **B** and **C** indicates clear lattice fringes. **E.** Displays the orientation of lattice fringes observed in the nanorods. **F.** Shows the SAED pattern for the nanorods observed in **E**.

The SEM EDX and elemental mapping was utilized to provide more information on the surface morphology and elemental ratios of ZnTe nanoparticles. The SEM micrograph shown in **Figure 2.10, A** confirmed the transition phase for the ZnTe nanoparticles synthesized at pH 4, 2hr, showing the presence of spherical and rod shaped particles. The EDX pattern shown in **Figure 2.10, B**, confirmed the presence of Te and Zn components. **Figure 2.10, C**, shows the elemental mapping of Zn and Te displaying their respective ratios.

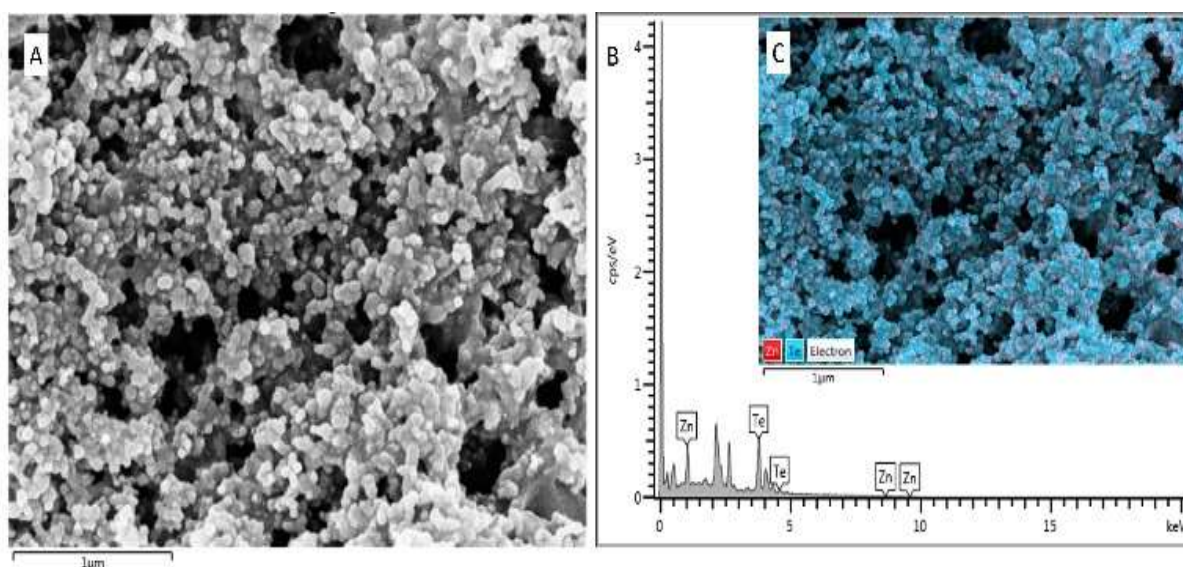


Figure 2.10 **A.** SEM micrograph displaying the surface morphology of ZnTe nanospheres and nanorods. **B.** EDX spectrum showing the presence of Te and Zn. **C.** Elemental mapping of Te and Zn in the cysteine capped ZnTe nanomaterial.

ZnTe nanoparticles can exist in both cubic zinc blende and wurtzite phases.¹¹⁻¹⁷ The miller indices attributed to peaks (200), (220), (311) and (400) identified in **Figure 2.11**, correspond to the cubic phase of ZnTe (ICDD number: 015:0746, see appendix B for supplementary data sheet). XRD diffraction peaks from unreacted Te, Zn, or other ZnTe phases are observed in the pattern.

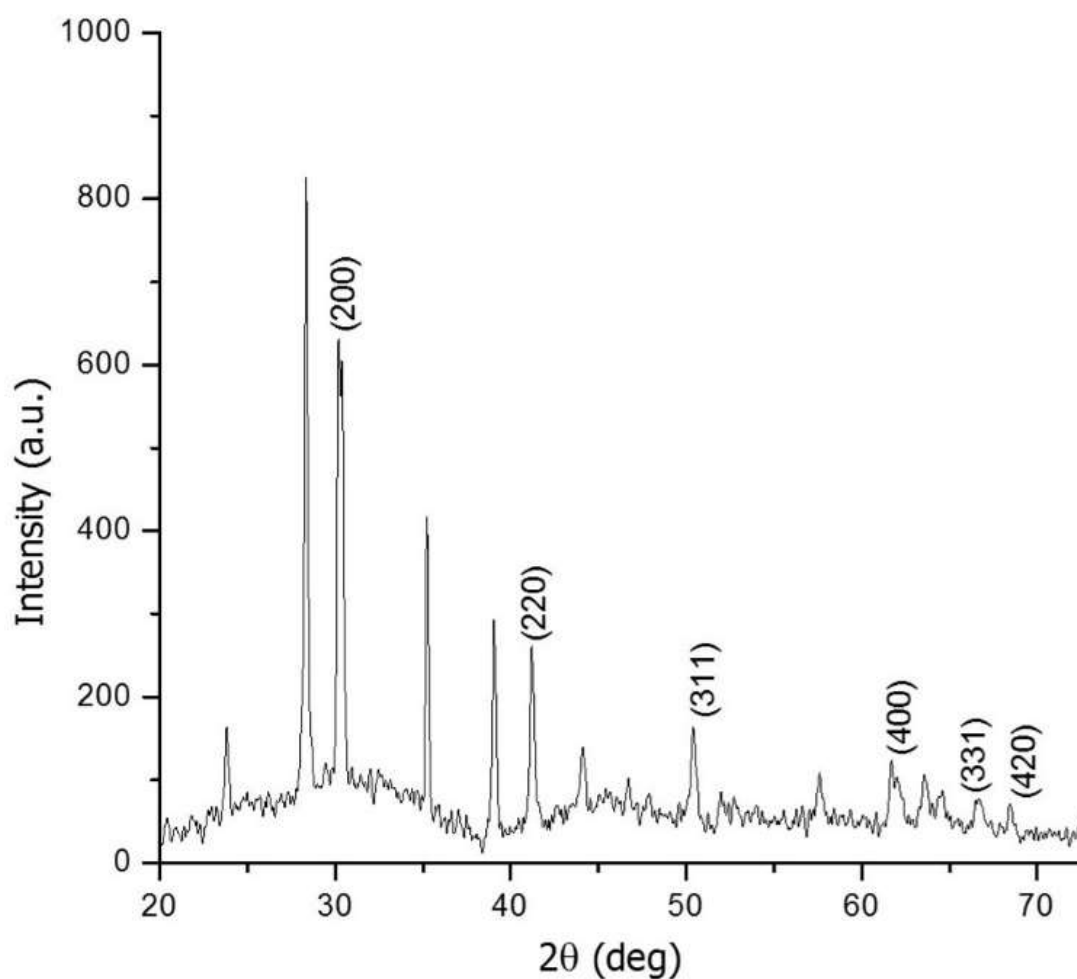


Figure 2.11 XRD pattern for cysteine capped ZnTe nanoparticles, showing a cubic phase.

2.3.3 Surface charge of ZnTe nanoparticles

The Zeta potential is used to measure the surface charge of nanoparticles. In addition it may be used to determine how efficiently the capping agent has passivated the nanoparticle surface and confirm the size of the nanoparticles. The surface charge may be altered by the attachment of bioconjugates. Monitoring the surface charge allows control in electrostatic and hydrophobic interactions within biological systems resulting in intracellular targeting and drug carrier applications. The surface charge properties of cysteine capped ZnTe was evaluated and displayed a negative charge of -11.7 mV.

2.3.4 Fluorescence properties of ZnTe nanoparticles

The fluorescence properties and optical stability were studied using fluorescence and phase-contrast microscopes. **Figure 2.12, A.** shows the ZnTe nanoparticle emitting in the blue region under the influence of UV 2A 330-380 nm filter. The blue light corresponds to the absorption wavelength displayed in the UV spectroscopy results. **Figure 2.12, B.** shows the ZnTe nanoparticles fluorescing between the 365-395 filter.

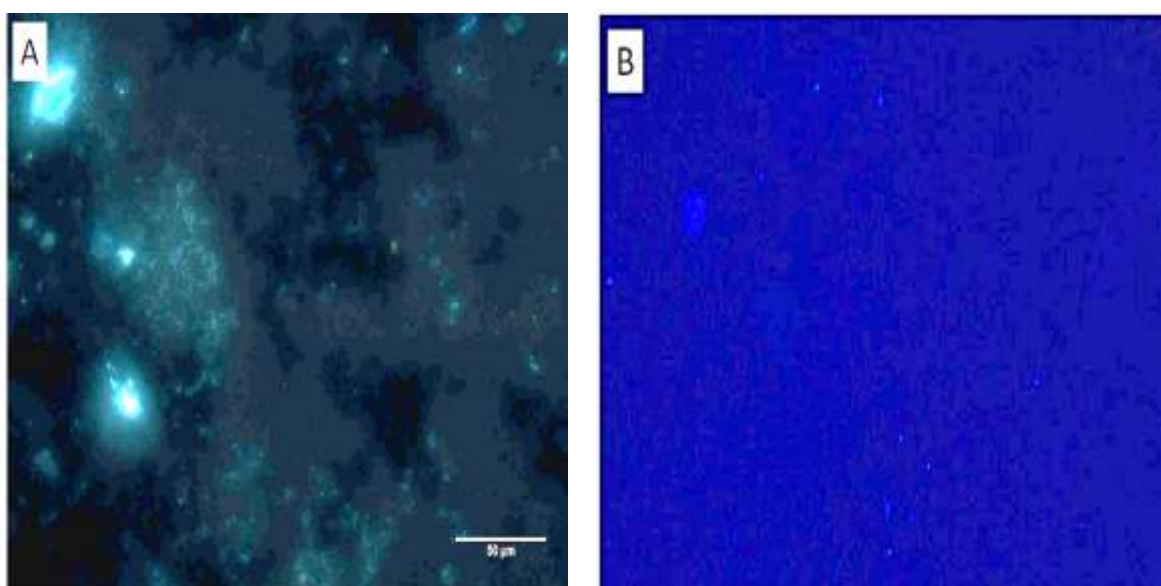


Figure 2.12 Fluorescence of cysteine capped ZnTe nanoparticles. Fig. 8. **A.** UV 2A filter in the presence of DNA plasmid pGEMT Easy. **B.** 365-395 filter set in the presence of DNA plasmid pGEMT Easy.

2.3.5 Cytotoxicity evaluation of ZnTe nanoparticles

Following the successful synthesis and characterisation of ZnTe nanoparticles an *in vitro* cell culture system was used to determine its biological safety. Cytotoxicity evaluations were performed on MCF7 cells (**Figure 2.13, B**) using the MTT cell viability assay. The MTT assay is based on the biochemical reduction of MTT dye by viable cells by testing their enzymatic dehydrogenase activity. The results showed that cysteine capped ZnTe nanoparticles induced

no toxic effects on the growth or viability of MCF7 cells. The percentage cell viability was between 85 to 95 % (**Figure 2.13, A**) for concentrations ranging from 20 -100 $\mu\text{g/ml}$. A one-way ANOVA, with a non-parametric Kruskal-Wallis test showed no significant differences ($p > 0.05$) between the ZnTe treated and control cells.

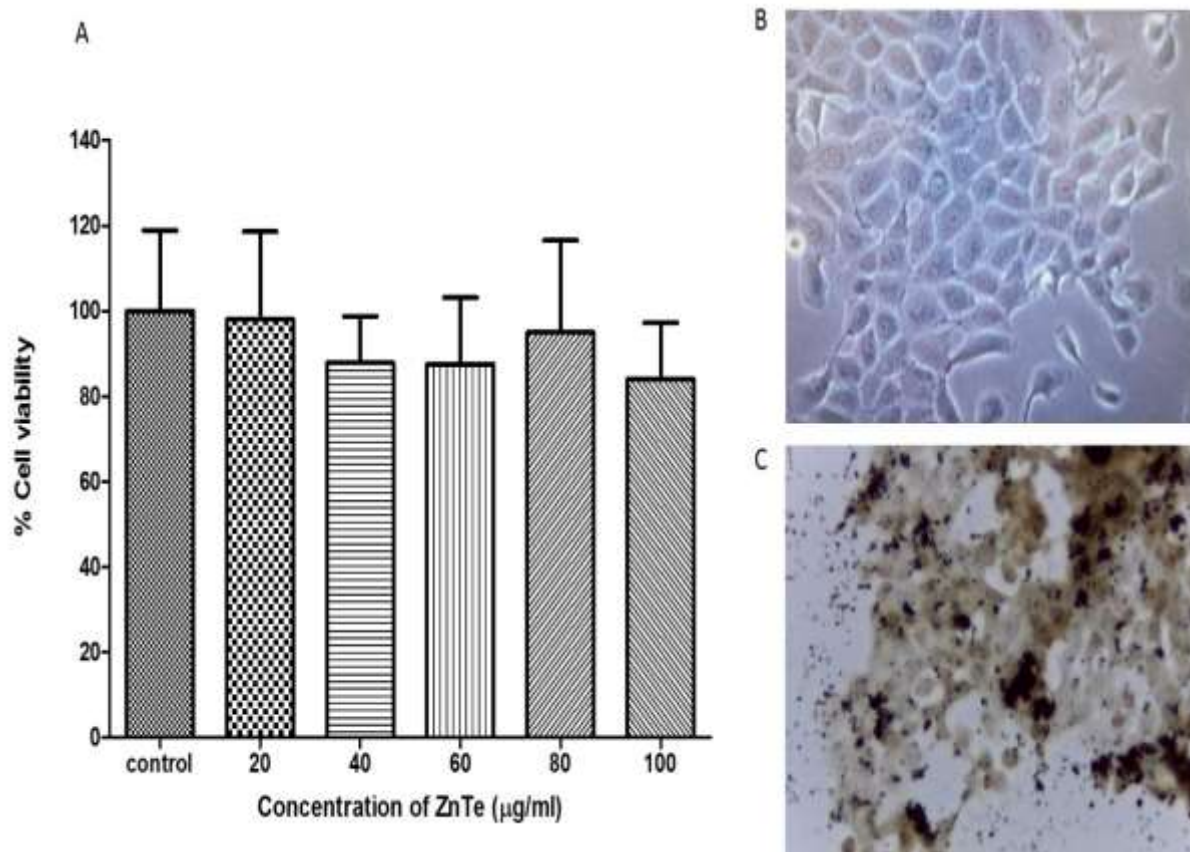


Figure 2.13 Cytotoxicity of ZnTe nanoparticles using MTT assay. **A.** The percentage cell viability relative to exposure to ZnTe nanoparticles at different concentrations is graphically represented. **B.** Untreated control, MCF7 cancer cells. **C.** Resin embedded MCF7 cells treated with ZnTe nanoparticles.

2.4 Discussion

The synthesis and functionalisation of ZnTe nanoparticles for bio-applications such as bio-imaging can be a useful tool in nanomedicine. The optical properties can be tuned by varying the size and shape of the ZnTe nanoparticles. The absorption peak observed in **Figure 2.3, B** is attributed to the Zn-ligand complex as reported previously for cysteine capped CdTe [20, 37]. The photoluminescence emission in **Figure 2.3, C** are in accordance with previous reports which show emission in the 400-600 nm range [38, 39].

The size of a nanoparticle is an important parameter for biological applications, generally smaller nanoparticles display advantages such as higher biocompatibility or efficacy within *in vivo* systems. Smaller nanoparticles are easily functionalised and are able to evade immune scavenging cells such as monocytes and neutrophils [40, 41]. Larger sized nanoparticles are considered unfavourable for biological applications [2, 3]. The growth patterns observed for ZnTe nanoparticles displayed a transition phase from spherical into rod shaped nanoparticles (**Figure 2.7**). This transition phase is similar to the morphology reported by Zhang et al, who described the growth of nanolumps or dots into nanowires[26]. The formation of the ZnTe nanoparticles relative to reaction temperature, pH and time is typical of the oriented attachment (OA) growth mechanism which involves the controlled morphosynthesis of nanoparticles under the influence of crystal growth factors [27]. OA allows the nanocrystals to develop by the alignment and coalescence of neighbouring nanospheres by eliminating the capping agent boundaries [28]. It is common for OA to lead to the formation and growth of anisotropic nanostructures such as nanorods [29]. A higher surface energy of the zinc-blende can lead to the termination of the

(111) faces resulting in strong dipolar interactions between the particles facilitating OA growth [27-30]. The use of surface passivating agents such as amines or thiols are commonly used to control and stabilize the particle growth at the order of nanometers to achieve quantum confinement effects [42].

In addition to the functionalization and stability that cysteine provides to ZnTe nanoparticles during the growth phase, it also functions as an agent for solubilisation in water, and finally for the possible conjugation with biomolecules to the free amine groups [12]. The correlation between the growth kinetics and properties of the nanomaterials is related to the type of passivating agent employed during synthesis. In this report, functionalizing ZnTe with cysteine will allow for the attachment of various drugs or biological agents. The capping agent not only stabilises the nanoparticle and influences its shape and size but also determines the surface charge of the ZnTe nanoparticles. Work reported by Ghosh *et al.* showed that ZnTe nanoparticles capped with carboxyl, hydroxyl and succinamic acid displayed negative surface charges [30]. The negative charge displayed in cysteine capped ZnTe nanoparticles will accommodate the functionalization of this nanomaterial for further applications in cell targeting and bio-imaging. Preliminary fluorescence and phase-contrast microscope evaluations indicate the bio-imaging potential of ZnTe nanoparticles (**Figure 2.12**). These findings provide a rationale for establishing more robust studies that can clearly demonstrate the use of ZnTe nanomaterials in bio-imaging applications across cell lines and whole animal studies. To date the literature has successfully demonstrated the use of various other quantum dots in bio-imaging of bacterial, plant and human cells.[6-8]. The cytotoxicity studies display the biocompatibility of ZnTe nanoparticles (**Figure 2.13**). These initial cellular

responses therefore suggest the biosafety of cysteine capped ZnTe nanoparticles.

2.5 Conclusions

Cysteine capped ZnTe nanoparticles were synthesized at different pH using a simple solution based route. The ZnTe nanoparticles obtained at pH 4 showed interesting morphological features whereby a transition phase was identified in which nanospheres of 40-50 nm in size range developed into nanorods that were between 60-80 nm in length and 20-25 nm in diameter. This transformation was attributed to the oriented growth attachment mechanism. The particles synthesized at these optimum reaction conditions also showed quantum confinement and narrow band-edge emission. The cysteine capped ZnTe nanoparticles displayed a negative surface charge suggesting that the particles would be conducive to functionalization for further applications in cell targeting and bio-imaging. Initial fluorescence studies of the ZnTe nanoparticles within DNA plasmid shows emission in the blue region. Preliminary *in vitro* cytotoxicity studies showed that ZnTe nanoparticles do not induce adverse or harmful effects to human MCF7 cells. In addition to the synthesis and characterisation of water soluble ZnTe nanoparticles, its biosafety and bio-imaging potential has been demonstrated. ZnTe nanoparticles has the potential to be utilised independently in various *in vitro* and *in vivo* drug delivery and imaging applications. Furthermore ZnTe nanoparticles can be used in the assembly of multicomponent heterostructured or core-shell nanoparticles for enhanced biological applications.

References

- Qi, L. and X. Gao, *Emerging application of quantum dots for drug delivery and therapy*. 2008.
- Li, Y., et al., *Nitrogen-doped graphene quantum dots with oxygen-rich functional groups*. Journal of the American Chemical Society, 2011. **134**(1): p. 15-18.
- Bera, D., et al., *Quantum dots and their multimodal applications: a review*. Materials, 2010. **3**(4): p. 2260-2345.
- Li, H., W.Y. Shih, and W.-H. Shih, *Synthesis and characterization of aqueous carboxyl-capped CdS quantum dots for bioapplications*. Industrial & Engineering Chemistry Research, 2007. **46**(7): p. 2013-2019.
- Byrappa, K., S. Ohara, and T. Adschiri, *Nanoparticles synthesis using supercritical fluid technology-towards biomedical applications*. Advanced Drug Delivery Reviews, 2008. **60**(3): p. 299-327.
- Valizadeh, A., et al., *Quantum dots: synthesis, bioapplications, and toxicity*. Nanoscale research letters, 2012. **7**(1): p. 1-14.
- Shao, L., Y. Gao, and F. Yan, *Semiconductor quantum dots for biomedical applications*. Sensors, 2011. **11**(12): p. 11736-11751.
- Bentolila, L., et al., *Quantum dots for molecular imaging and cancer medicine*. Discovery medicine, 2005. **5**(26): p. 213.
- Smith, A.M., et al., *Multicolor quantum dots for molecular diagnostics of cancer*. 2006.
- Spehar, R.L., R.L. Anderson, and J.T. Fiantdt, *Toxicity and bioaccumulation of cadmium and lead in aquatic invertebrates*. Environmental Pollution (1970), 1978. **15**(3): p. 195-208.
- Godt, J., et al., *The toxicity of cadmium and resulting hazards for human health*. J Occup Med Toxicol, 2006. **1**(22): p. 1-6.
- Dunpall, R., et al., *An in vitro assessment of the interaction of cadmium selenide quantum dots with DNA, iron, and blood platelets*. IUBMB life, 2012. **64**(12): p. 995-1002.
- Oluwafemi, S., N. Revaprasadu, and A. Ramirez, *A novel one-pot route for the synthesis of water-soluble cadmium selenide nanoparticles*. Journal of Crystal Growth, 2008. **310**(13): p. 3230-3234.
- Oluwafemi, O.S., *A novel "green" synthesis of starch-capped CdSe nanostructures*. Colloids and Surfaces B: Biointerfaces, 2009. **73**(2): p. 382-386.
- Patra, S. and S.K. Pradhan, *Microstructural, optical and quantum confinement effect study of mechanically synthesized ZnTe quantum dots*. Acta Materialia, 2012. **60**(1): p. 131-138.
- Patra, S. and S. Pradhan, *Quickest single-step one pot mechanosynthesis and characterization of ZnTe quantum dots*. Journal of Alloys and Compounds, 2011. **509**(18): p. 5567-5570.
- Liu, Y., et al., *Fabrication and photocatalytic activity of high-efficiency visible-light-responsive photocatalyst ZnTe/TiO₂ nanotube arrays*. Journal of Solid State Chemistry, 2011. **184**(3): p. 684-689.
- Zhang, J., et al., *Shape-control of ZnTe nanocrystal growth in organic solution*. The Journal of Physical Chemistry C, 2008. **112**(14): p. 5454-5458.
- Zhang, B., F. Guo, and W. Wang, *Synthesis and characterization of ZnTe hierarchical nanostructures*. Journal of Nanomaterials, 2012. **2012**: p. 8.
- Mntungwa, N., V.S.R. Pullabhotla, and N. Revaprasadu, *A facile hybrid route to luminescent ZnTe nanoparticles*. Materials Letters, 2012. **81**: p. 108-111.
- Lee, S.H., Y.J. Kim, and J. Park, *Shape evolution of ZnTe nanocrystals: nanoflowers, nanodots, and nanorods*. Chemistry of Materials, 2007. **19**(19): p. 4670-4675.
- Zhang, J., et al., *Synthesis and characterization of wurtzite ZnTe nanorods with controllable aspect ratios*. Journal of the American Chemical Society, 2011. **133**(39): p. 15324-15327.
- Kim, D.-J., et al., *Formation of 1-D ZnTe nanocrystals by aerosol-assisted spray pyrolysis*. Korean Journal of Chemical Engineering, 2011. **28**(4): p. 1120-1125.
- Yong, K.-T., et al., *Formation of ZnTe nanowires by oriented attachment*. Chemistry of materials, 2007. **19**(17): p. 4108-4110.
- Jiang, F., et al., *Ligand-tuned shape control, oriented assembly, and electrochemical characterization of colloidal ZnTe nanocrystals*. Chemistry of Materials, 2010. **22**(16): p. 4632-4641.
- Zhang, Q., S.-J. Liu, and S.-H. Yu, *Recent advances in oriented attachment growth and synthesis of functional materials: concept, evidence, mechanism, and future*. Journal of Materials Chemistry, 2009. **19**(2): p. 191-207.
- Lee, E.J., et al., *Oriented attachment: An effective mechanism in the formation of anisotropic nanocrystals*. The Journal of Physical Chemistry B, 2005. **109**(44): p. 20842-20846.
- Zhang, J., F. Huang, and Z. Lin, *Progress of nanocrystalline growth kinetics based on oriented attachment*. Nanoscale, 2010. **2**(1): p. 18-34.
- Gong, M., A. Kirkemide, and S. Ren, *Symmetry-Defying Iron Pyrite (FeS₂) Nanocrystals through Oriented Attachment*. Scientific reports, 2013. **3**.
- Ghosh, S., et al., *Aqueous synthesis of ZnTe/dendrimer nanocomposites and their antimicrobial activity: implications in therapeutics*. Nanoscale, 2011. **3**(3): p. 1139-1148.
- Xu, S., et al., *Synthesis of water-dispersible one-dimensional Te@ZnTe core-shell nanoparticles*. Journal of Materials Chemistry, 2011. **21**(41): p. 16427-16429.
- Xu, S., et al., *Key roles of solution pH and ligands in the synthesis of aqueous ZnTe nanoparticles*. Chemistry of Materials, 2010. **22**(21): p. 5838-5844.
- Shkir, M., et al., *Synthesis of ZnTe Nanoparticles by Microwave Irradiation Technique, and Their Characterization*. Nanoscience and Nanotechnology Letters, 2012. **4**(4): p. 405-408.
- Hou, L., et al., *Interfacial fabrication of single-crystalline ZnTe nanorods with high blue fluorescence*. Journal of the American Chemical Society, 2013. **135**(29): p. 10618-10621.
- Rambharose, S., et al., *Novel dendritic derivatives of unsaturated fatty acids as promising transdermal permeation enhancers for tenofovir*. Journal of Materials Chemistry B, 2015. **3**(32): p. 6662-6675.
- Thompson, D.P. and P. Boudjouk, *A convenient synthesis of alkali metal selenides and diselenides in tetrahydrofuran and the reactivity differences exhibited by these salts toward organic bromides. Effect of ultrasound*. The Journal of Organic Chemistry, 1988. **53**(9): p. 2109-2112.
- Mntungwa, N., V.S.R. Pullabhotla, and N. Revaprasadu, *Facile synthesis of cysteine and triethanolamine capped CdTe nanoparticles*. Colloids and Surfaces B: Biointerfaces, 2013. **101**: p. 450-456.
- Jun, Y.-w., C.-S. Choi, and J. Cheon, *Size and shape controlled ZnTe nanocrystals with quantum confinement effect*. Chemical Communications, 2001(1): p. 101-102.

39. Dwivedi, D., D. Ashankar, and M. Dubey, *Synthesis, structural and optical characterization of CdS nanoparticles*. J. Ovon. Resea, 2010. **6**(1): p. 57.
40. Dobrovolskaia, M.A., et al., *Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution*. Molecular pharmaceutics, 2008. **5**(4): p. 487-495.
41. Walkey, C.D., et al., *Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake*. Journal of the American Chemical Society, 2012. **134**(4): p. 2139-2147.
42. Mlowe, S., et al., *Lead chalcogenides stabilized by anacardic acid*. Materials Science in Semiconductor Processing, 2013. **16**(2): p. 263-268.

CHAPTER THREE: The Synthesis and characterisation of biocompatible Au-ZnTe core/shell nanoparticles

3.1 Introduction

The National Cancer Registry (NCR) and international cancer research institutes provide statistics that show an increase in the prevalence of cancer patients worldwide. The Fight against cancer has evolved into a multidisciplinary approach involving clinicians, chemists, biomedical engineers and materials scientists [1]. Chemotherapy is a commonly used treatment for cancer. It involves the use of various cytotoxic drugs that frequently affect both cancer and healthy cells. This treatment regime often results in adverse side effects such as hair and weight loss, nausea, diarrhoea, cancer cachexia, opportunistic infections and sometimes even death [2]. The challenge that researchers are faced with is the design of chemotherapeutic drugs that are not only cancer specific but also exhibit higher therapeutic efficacy. Advanced nanosystems such as core/shell nanoparticles have the potential to support this application if designed appropriately. The synthesis of heterostructured nanoparticles has received much attention recently due to their potential in catalysis, electronics and biomedical sciences [3-5].

Additionally the design and application of gold based core-shell nanoparticles remain at the forefront of cancer therapeutics and diagnostic applications such as photothermal ablation treatment of tumours [6, 7], localized bio-imaging of tumour or cancer tissues [8] and targeted drug delivery to specific cancer cells [9, 10]. The combination of two different types of material that function as a single entity can confer particular advantages such as enhanced optical, electrical or structural properties. A challenge in designing such materials arises from determining the structure of a two-component system and in identifying compatible core/shell component elements. The controlled synthesis of

bimetallic heterosystems such as Pd/Pt or Ag/Au core/shell systems is possible because of the relatively close match between the crystal lattices of their component parts. In contrast, core/shell metal-semiconductor systems are more difficult to synthesize due to their differing crystal structures and to the large crystal lattice mismatches [11, 12]. There have been few reports of metal–semiconductor core/shell nanostructures [13-16]. Wang and co-workers described a general method for the preparation of Au nanocrystals of different shapes and sizes as starting material for the synthesis of water-dispersible Au–metal sulfide core/shell structures [17]. Salant *et al.* conducted elaborate work on the growth of gold tips on anisotropic CdSe particles which was used to establish a relationship between the growth pattern and morphology that develops from a stabilized core [18-20]. The synthesis of gold shelled heterostructures with the core composed of a magnetic nanomaterial such as iron oxide is common [21-23]. The gold shell reduces the toxicity of the iron oxide and also enhances the biocompatibility properties.[24] When gold is used as the core component it exhibits certain advantages; playing a direct role in the size tunability and morphology of the core/shell or heterostructured nanoparticle. The advantages of using gold as a core or shell component depends also on its chemical interaction with other materials [25-28].

Recent studies show improvements in targeted drug delivery using gold based core/shell nanoparticles as the delivery vehicle. These studies illustrate the versatility of gold nanoparticles as either a core or shell component [29-35]. The surface plasmon resonance features displayed by gold nanomaterials results in enhanced light scattering and absorption properties. These unique optical properties enable the rapid conversion of absorbed light into heat, resulting in

photothermal energy which may be applied in the treatment and destruction of cancer or tumour tissues. In addition gold nanoparticles are highly stable, non-toxic and easily synthesized in a variety of sizes and surface modifications [36]. In general metal/semiconductor hybrid nanostructures are very interesting as they possess strong interactions between the metal plasmon and the semiconductor exciton i.e. electron/hole pair [37-40]. In this study we have chosen ZnTe which has a direct band gap of 2.26 eV and attractive optical properties as the secondary component of our heterostructured material [41-44]. The presence of ZnTe, is an attractive semiconducting nanomaterial that will facilitate bio-imaging of cells, tissues and even track the location of the core/shell within biological systems without having to use additional dyes and fluorescence tagging molecules. We have recently shown that ZnTe nanoparticles displayed fluorescence properties that are useful in bioapplications [45]. The presence of cysteine has the potential to support conjugation of biomolecules and chemotherapeutic drugs which may enable this system to be applied in targeted drug delivery and bio-imaging applications within the domain of oncology therapeutics and diagnostics. We report the synthesis of cysteine-capped Au-ZnTe core/shell nanoparticles using a simple solution-based route. **Figure 3.1** shows the core/shell nanoparticle design and illustrates its potential applications. The amino functional groups on cysteine have the potential to support conjugation of cellular ligands and a combination of chemotherapeutic drugs to produce a bio-modified version of Au-ZnTe

core/shell nanoparticles. To the best of our knowledge, this is the first report of a Au-ZnTe core/shell nanoparticle system.

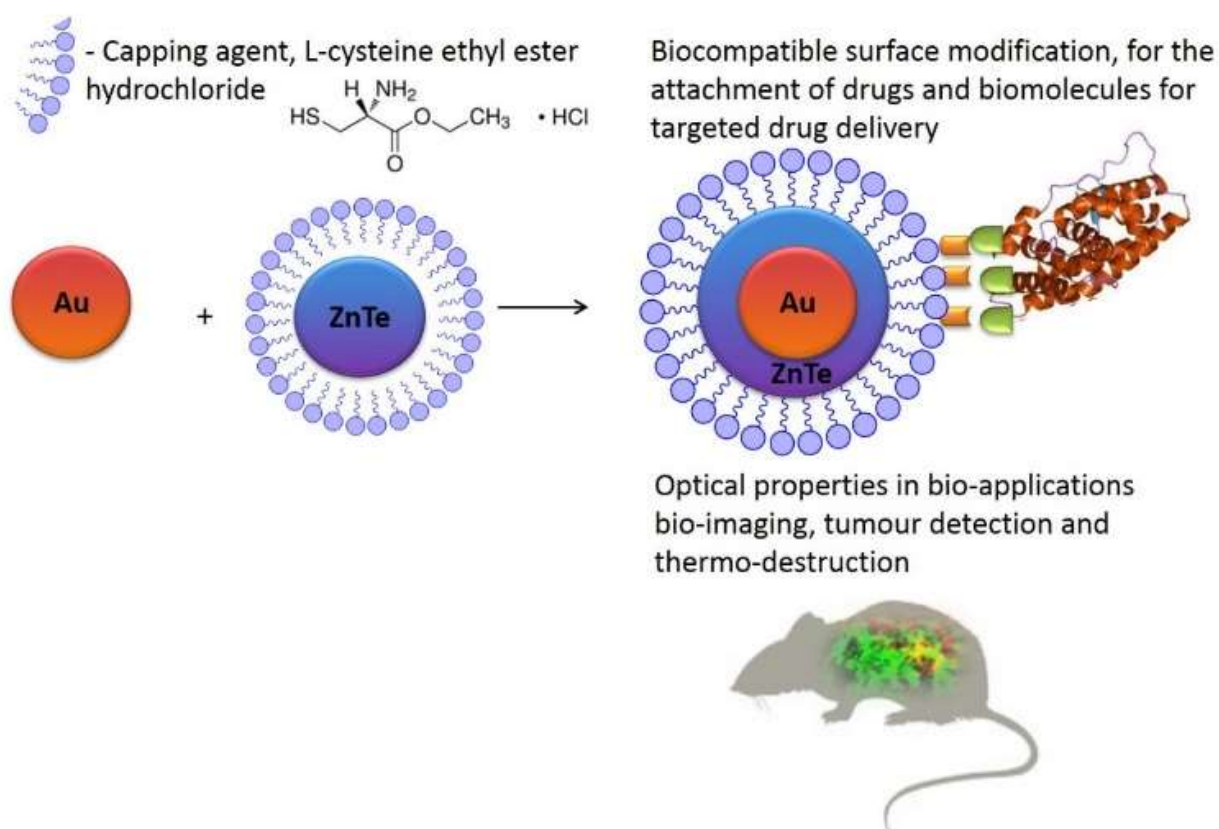


Figure 3.1 Synthesis concept for Au-ZnTe core/shell nanoparticles. The shell component is surface modified with L-cysteine ethyl ester hydrochloride for water solubility and biocompatibility features. Amino groups from the capping agent may be used to attach cell specific ligands and chemotherapeutic drugs for application in targeted drug delivery and bio-imaging.

3.2 Experimental

3.2.1 Materials

Gold (III) chloride was obtained from Sigma Aldrich. All the chemicals were of analytical grade and used as purchased.

3.2.2 Synthesis of gold nanoparticles

The gold nanoparticles were prepared via the Turkevich route.[46] A solution of 5.0×10^{-3} M gold chloride (HAuCl_4) and a solution of 5.69×10^{-2} M sodium citrate was prepared. Aliquots of 2 ml of gold chloride and 10 ml of sodium citrate was added to 500 ml of boiling distilled water and the reaction proceeded for 10 minutes until a dark red solution was observed.

3.2.3 Synthesis of Au-ZnTe core/shell nanoparticles

In a typical room temperature reaction, tellurium powder (0.041 g, 0.32 mmol) was mixed with sterile distilled water (15.0 ml) in a three-necked round bottom flask. A 15.0 ml aqueous solution of sodium borohydride (0.031 g, 0.79 mmol) was carefully added to the tellurium solution under inert conditions. After 2 h of reduction, 20.0 ml aqueous solution of ZnCl_2 (0.0436 g, 0.32 mmol) and 40.0 ml aqueous solution of L-cysteine ethyl ester hydrochloride (0.1188 g, 0.32 mmol) was simultaneously added to the dark purple tellurium ion solution. The solution was stirred for 30 minutes followed by pH adjustment. The reaction was allowed to proceed at pH 7, for 3 hours at 60°C . An aliquot of 40 ml of gold nanoparticle solution was added to the cysteine capped ZnTe solution and the reaction was allowed to proceed for 4 hours. The reaction mixture was filtered and centrifuged to give a dark greenish black material that was readily dispersed in sterile distilled water.

3.2.4 Characterisation of nanoparticles

3.2.4.1 Ultra-violet-visible spectroscopy

A Perkin-Elmer Lambda 20 UV-vis spectrophotometer was used to carry out optical measurements in the 200-1100 nm wavelength range at room temperature. Samples were placed in quartz cuvettes (1 cm path length) and the absorbance was recorded.

3.2.4.2 Photoluminescence spectroscopy

Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 luminescence spectrometer with xenon lamp over range of 200-800 nm. The samples were placed in quartz cuvettes (1 cm path length) and the excitation peaks were analysed and recorded.

3.2.4.3 Morphology and elemental mapping

Samples for S/TEM imaging and spectroscopic analysis were prepared by drop casting an aliquot solution of Au-ZnTe nanoparticle material onto a TEM support grid (consisting of a holey carbon amorphous film supported on a copper mesh) and then allowing the solvent to evaporate at room temperature. Preliminary TEM imaging of the Au, ZnTe and the Au-ZnTe nanoparticles was performed using a Philips CM120 BIOTWIN TEM operated at 120kV. A JEOL JEM 3010 URP high resolution transmission electron microscope (HRTEM) operated at 300 kV was used for high resolution imaging of the Au-ZnTe samples. High angle annular dark field (HAADF) imaging was performed in Titan G2 80-200 ChemiSTEM™ scanning transmission electron microscope (STEM) operated at 200 kV with a convergence angle of 18mrad and a HAADF inner angle of 54 mrad. X-ray energy dispersive spectroscopy (XEDS) was performed using the

Titan's Super-X X-ray detector system which has a solid collection angle of 0.7 srad.

3.2.4.4 X-ray diffraction

XRD measurements of the Au-ZnTe samples were performed using a Bruker aXS D8 advanced diffractometer with Cu-K α radiation ($\lambda = 1.5406 \text{ \AA}$) operated at 40 kV and 40 mA. TEM imaging of the cellular uptake of nanoparticles was performed using a Philips CM120 BIOTWIN TEM operated at an accelerating voltage of 120 kV with the sample cooled to 80K.

3.2.4.5 Tissue culture media and cell line

Dulbecco's modified eagle medium (DMEM), Dulbecco's Phosphate buffered saline (PBS), Trypsin, 100x pen-strep Fungizone (PSF) and fetal calf serum (FCS). All the cell tissue culture reagents were supplied by Invitrogen. Human pancreas adenocarcinoma cell line PL45, was purchase from American Type Culture Collection (ATCC) and used in the experiments. This adhesion cell line was grown in DMEM medium containing 10% FBS and 1-2% PSF at a cell density of 5000. Cells that grew as monolayers were passaged, trypsinized and harvested for experimentation before reaching 100 % confluency.

3.2.4.6 WST-1 cell viability assay

Cell viability was evaluated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Nanoparticle treated and control cells were seeded in a 96 well plate at a cell density of 10^5 per well in 100 μ l of appropriate culture media. Cells were cultured in a CO₂ at 37°C for 48 hours. Reconstituted WST-1 reagent was added to each treatment well and incubated for 2 hours at 37°C. The plate was shaken for 30

seconds and the absorbance was measured at 450 nm using a Multiskan FC microplate reader. All experiments were repeated in triplicates and the results were represented as mean $\pm$ SD.

3.2.4.7 Cellular uptake of Au-ZnTe core/shell nanoparticles

PL45 cell lines were treated with Au-ZnTe core/shell nanoparticles for 48 hours at the following concentrations, 3.125, 6.25, 12.5, 25 and 50 μ g/ml. The treated cells were then fixed with 2.5 % glutaraldehyde for 24 hours, followed by a phosphate buffer wash. A postfix of 0.5 % Osmium tetroxide was added to the cells and incubated for 1 hour at room temperature followed by a phosphate buffer wash. The cells were then dehydrated with 30, 50, 75 and 100 % acetone. Resin and acetone were added to the sample in equal volumes followed by 4 hour incubation at room temperature. Cells were placed in moulds and whole resin, followed by an overnight incubation at 70 °C. Resin sample blocks were sectioned into ultrathin cross sections using a microtome, stained and viewed with a transmission electron microscope.

3.2.4.8 Bio-imaging analysis of Au-ZnTe nanoparticles

Fluorescence images were recorded using a Carl Zeiss Axio Imager 2 fluorescence and phase-contrast microscope. The cross-section of PL45 cell sample was placed on a slide and viewed under a Dapi and Alexa filter set.

3.3 Results

3.3.1 Optical properties of Au-ZnTe Nanoparticles

Au-ZnTe core/shell nanoparticles were synthesized using a novel one pot method under an inert atmospheric conditions. A dark purple colour was observed after 2 hours, indicating the reduction of tellurium powder. The solution turned dark brownish to black in colour after the addition of zinc chloride and L-cysteine ethyl ester hydrochloride. The material from this reaction was purified for characterisation analysis.

The absorption properties of the parent and the core/shell material were measured. The gold nanoparticles exhibited their characteristic surface plasmon resonance (SPR) absorbance at approximately 515 nm, whilst ZnTe displayed an absorption peak at 269 nm (**Figure 3.2 A, B**) respectively. The absorption spectra of the Au-ZnTe nanoparticles showed an absorption peak at 538 nm (**Figure 3.2 C**), a small red shift in relation to the absorption of the gold. The absorbance of Au-ZnTe is dominated by the absorption properties of gold. The photoluminescence spectra of the Au-ZnTe core/shell nanoparticles showed sharp, Gaussian shaped emission peaks (**Figure 3.3**). Excitation at different wavelengths (300, 310 and 320 nm) produced a constant peak at 402 nm in the PL spectra (**Figure 3.3 A, B and C**).

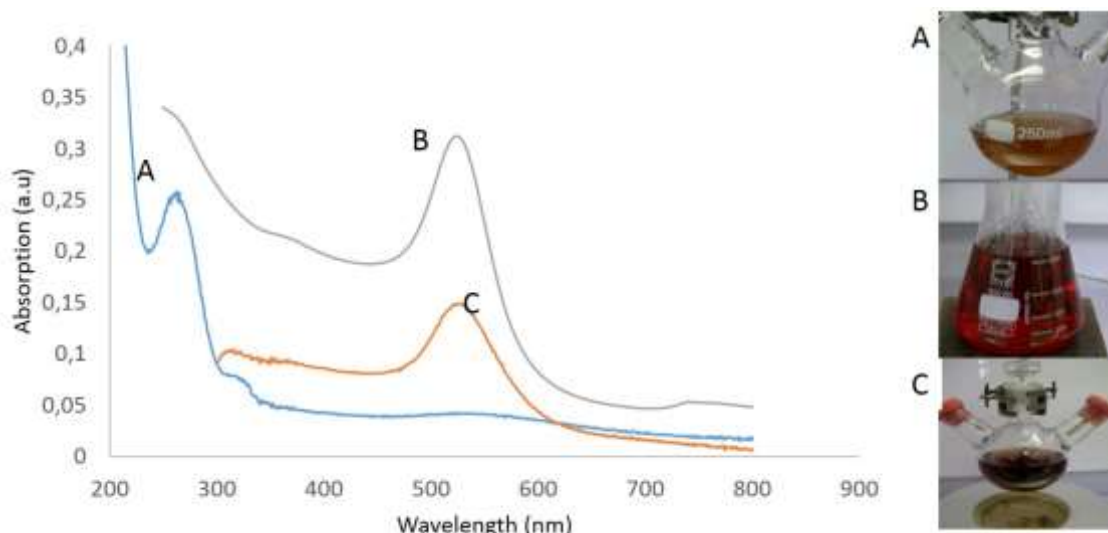


Figure 3.2 **A.** Cysteine capped ZnTe nanoparticles exhibit an absorbance peak at 269 nm, a brownish material is observed. **B.** Gold nanoparticles show an absorbance peak at 519 nm, a red colloidal solution is observed. **C.** Au-ZnTe core/shell nanoparticles exhibit an absorbance peak at 538 nm, a dark purple material is observed.

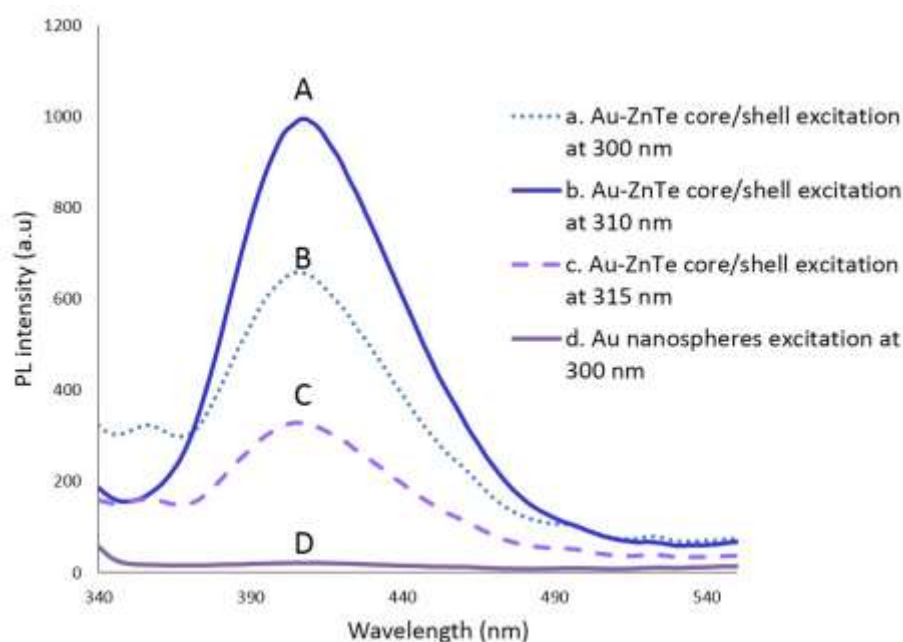


Figure 3.3. Photoluminescence spectra of Au-ZnTe core/shell nanoparticles at various excitations are represented in **A**, **B** and **C**, the peak observed displays constant emission maxima at 402 nm, **D.** Au nanoparticles displays no emission peak for excitation at 300 nm

3.3.2 Morphological characterisation of Au-ZnTe nanoparticles

TEM images of Au-ZnTe showed spherical particles with diameters in the 2-10 nm range (**Figure 3.4 A**). The average size of the particles are 7 nm, SD $\pm$ 3.74 nm (**Figure 3.4 B**). High resolution transmission electron microscope (HRTEM) imaging of the agglomerated clusters revealed the particles to be roughly spherical with dark cores and lower contrast outer shells consistent with an Au-ZnTe core-shell morphology. This proposed structure is further supported by the presence of 0.34nm lattice spacings in the outer shell region (**Figure 3.4 C**), which is close to the **111** planes of cubic ZnTe (0.34 nm). AFM analysis of Au-ZnTe core/shells showed uniform morphology of the particle surface (**Figure 3.4 D**). The shell component is relatively inhomogeneous. The spatial distributions of the elements, seen in the spectrum images and lines scans, are consistent with that expected for a core-shell structure. The spectrum image is a 2D projection of a 3-dimensional structure so some Zn and Te counts are expected to coincide with Au rich areas, corresponding to regions of shell above and below the core.

The presence of some alloying at the interfaces cannot be ruled out but we are confident that the EDX supports segregation of the two phases. Gold was found to be localised in the core with the gold distribution shown in red in (**Figure 3.5 C and D**) whilst Zn and Te were observed to be principally associated with the particle surface. Line scan data extracted from the EDX spectrum images (**Figure 3.5 B**) suggests a shell thickness of ~1-4 nm. Semi quantitative analysis of the atomic percentage of Au, Zn and Te in the nanoparticles yielded a value of 45.4, 19.1 and 35.5 respectively.

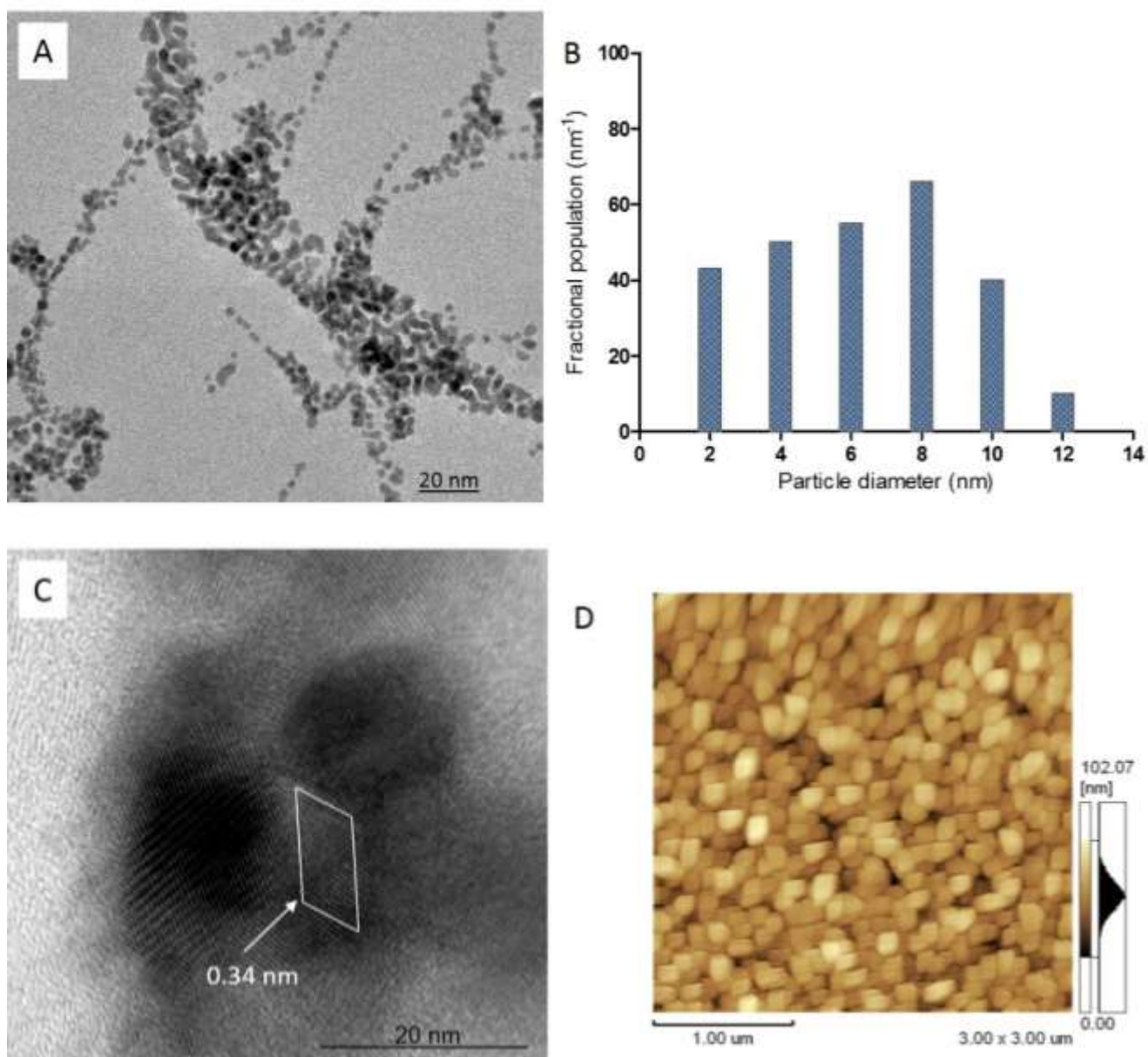


Figure 3.4. Morphological characterisation of Au-ZnTe. **A-B.** Particle morphology and size distribution using TEM **C.** Crystalline properties using HRTEM, **D.** Particle surface morphology using AFM.

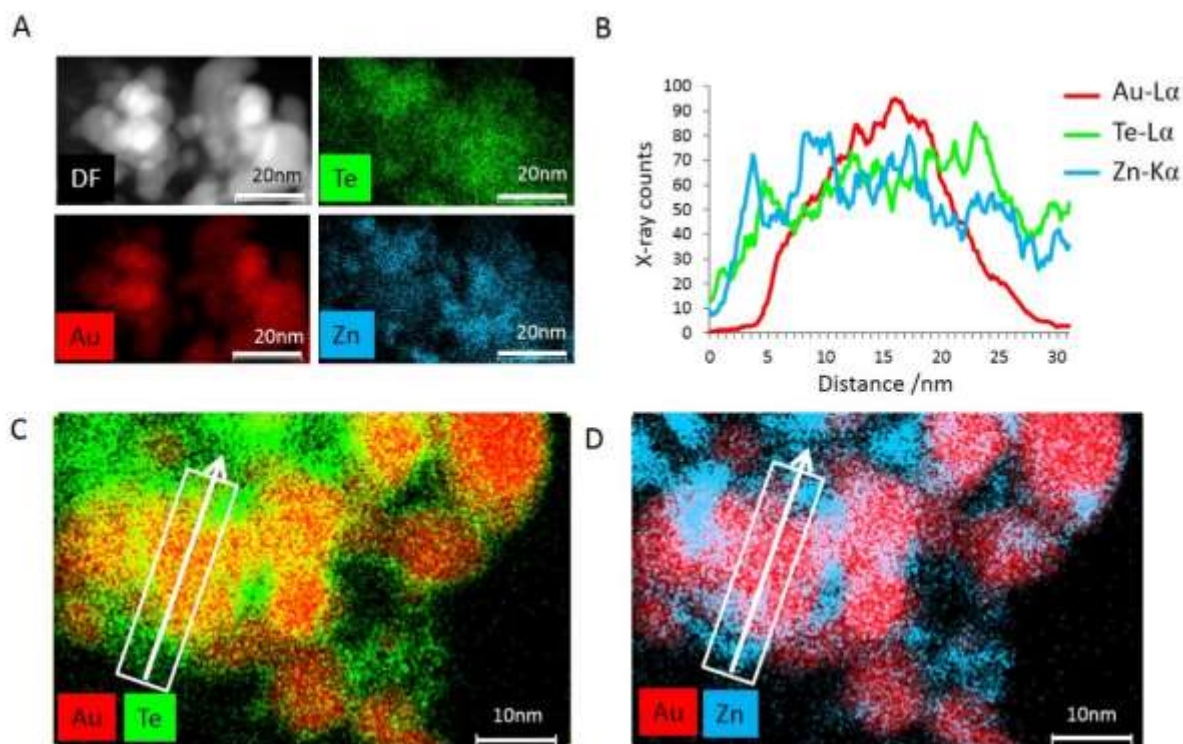


Figure 3.5. Elemental analysis of core-shell structure using HAADF-STEM and EDS spectrum imaging. **A-D.** Displays evidence for core-shell structure **B-** Line scans taken at positions indicated in **C** and **D** are shown.

3.3.3 Crystalline analysis of Au-ZnTe nanoparticles

The powder X-ray diffraction pattern of Au, ZnTe and Au-ZnTe nanoparticles is shown in (**Figure 3.6**). The pattern reveals some of the expected characteristic peaks for Au, ZnTe and Au-ZnTe. The (111), (200), and (311) planes of cubic gold (ICDD: 00-004-0784) are visible in the Au-ZnTe diffraction pattern. Characteristic (200), (311) and (511) planes of cubic zinc telluride phase (ICDD: 01-079-0004) are also present. The diffraction pattern showed additional peaks attributed to ZnO, and elemental tellurium.

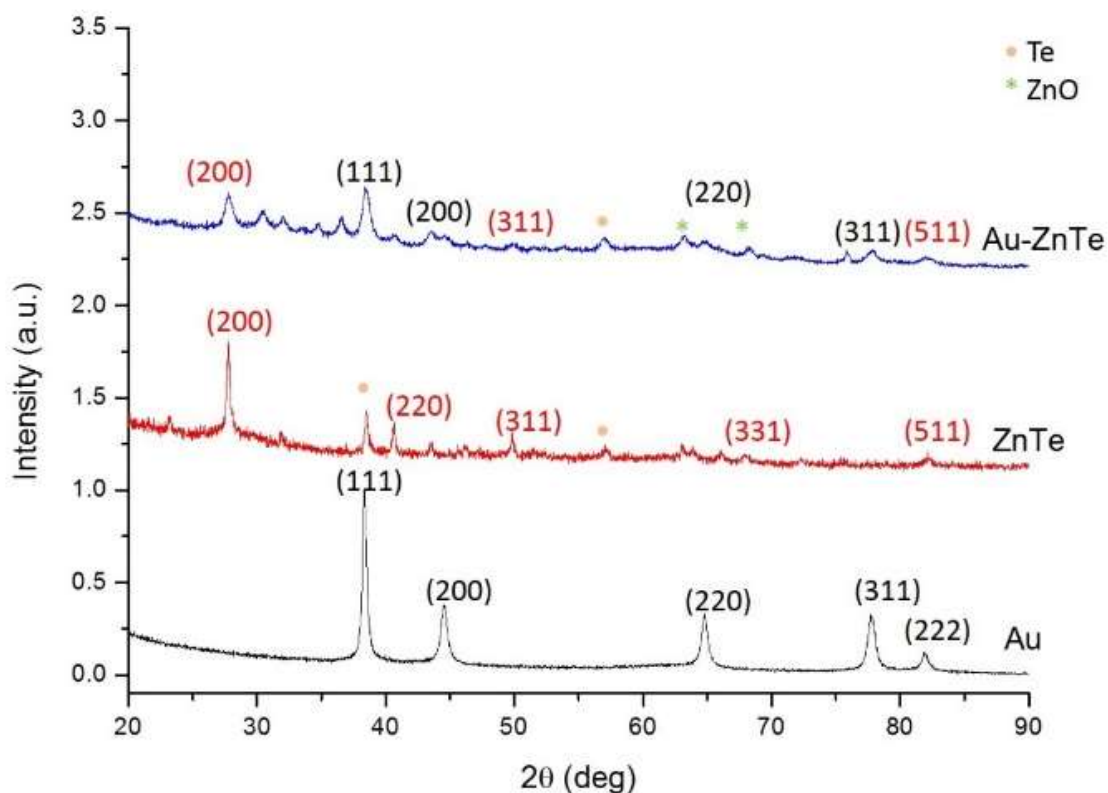


Figure 3.6. XRD pattern of Au-ZnTe, ZnTe and Au nanoparticles

Furthermore, the chemical composition of the Au-ZnTe nanocrystals was confirmed by the XPS spectra of the Zn-3d, Te-4d, and Au-4f electrons (**Figure 3.7**). The binding energies of Zn-3d, Te-4d, and Au-4f electrons in the Au-ZnTe nanocrystals are observed at Au4f₅ (86.6 eV); Au4f₇ (83.7 eV); Zn2p₁ (1045 eV); Zn2p₃ (1018 eV); Te3d₃ (576 eV) and Te3d₅ (573 eV). The binding energy of Te can be described by the two peaks, which, respectively, are assigned to the uncharged Te (573.6 eV) from the shell bound material and positively charged Te (576.5 eV) that is presence in excess.

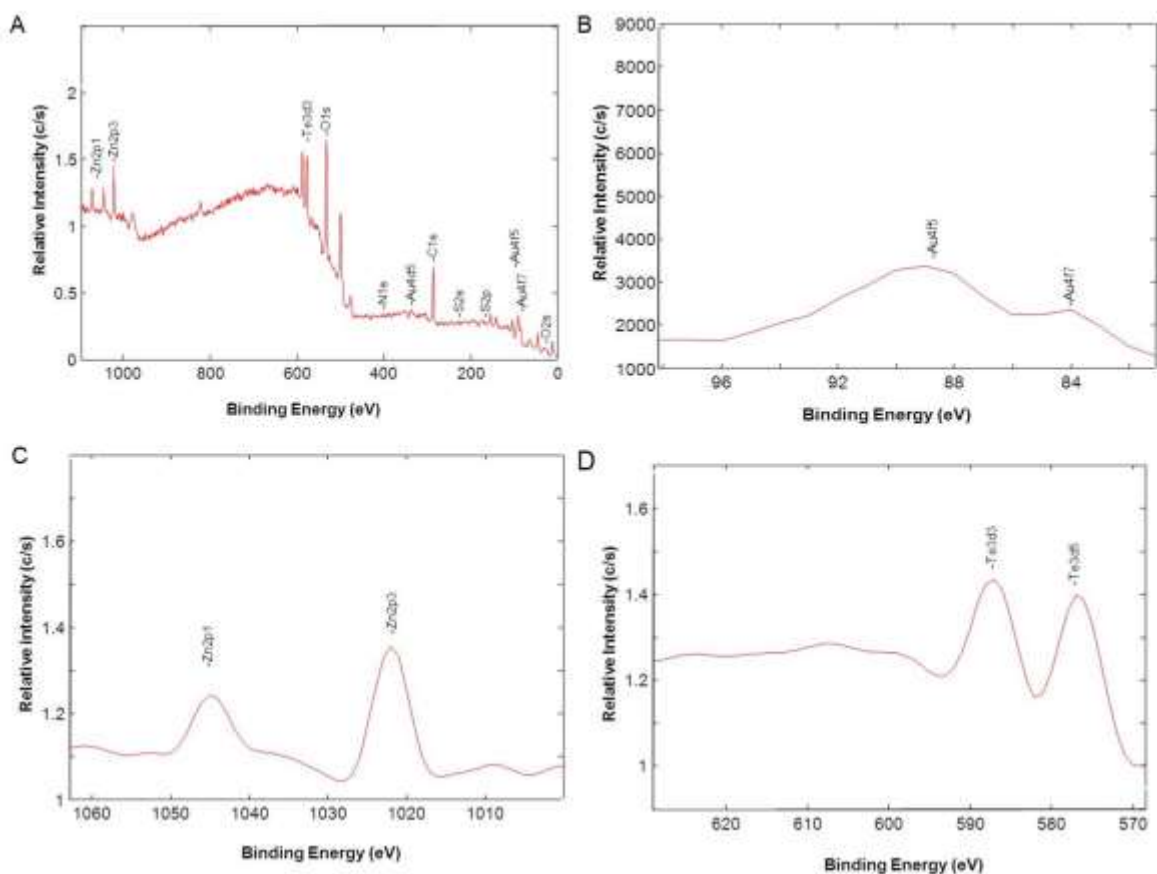


Figure 3.7 XPS absorbance of Au-ZnTe nanoparticles. **A.** XPS spectrum of Au-ZnTe core shell nanoparticles, general elemental scan. **B.** Binding energy spectrum of Au. **C.** Binding energy spectrum of Zn. **D.** Binding energy spectrum of Te.

3.3.4 Preliminary cytotoxicity evaluation of Au-ZnTe nanoparticles

The biocompatibility and cytotoxicity of the core/shell system was investigated using human pancreas adenocarcinoma PL45 cell line that was co-cultured with Au-ZnTe core/shell nanoparticles. PL45 cells showed no dose-dependent inhibition on cell proliferation after 72 hours of exposure to Au-ZnTe core/shell nanoparticles at the following concentrations 3.125, 6.25, 12.5, 25 and 50 $\mu\text{g/ml}$ (**Figure 3.8 A**). A comparison of cellular morphology after exposure to Au-ZnTe

core/shell nanoparticles is displayed in (**Figure 3.8 B, C and D**). No significant morphology variations were observed in the pancreas cancer cell line.

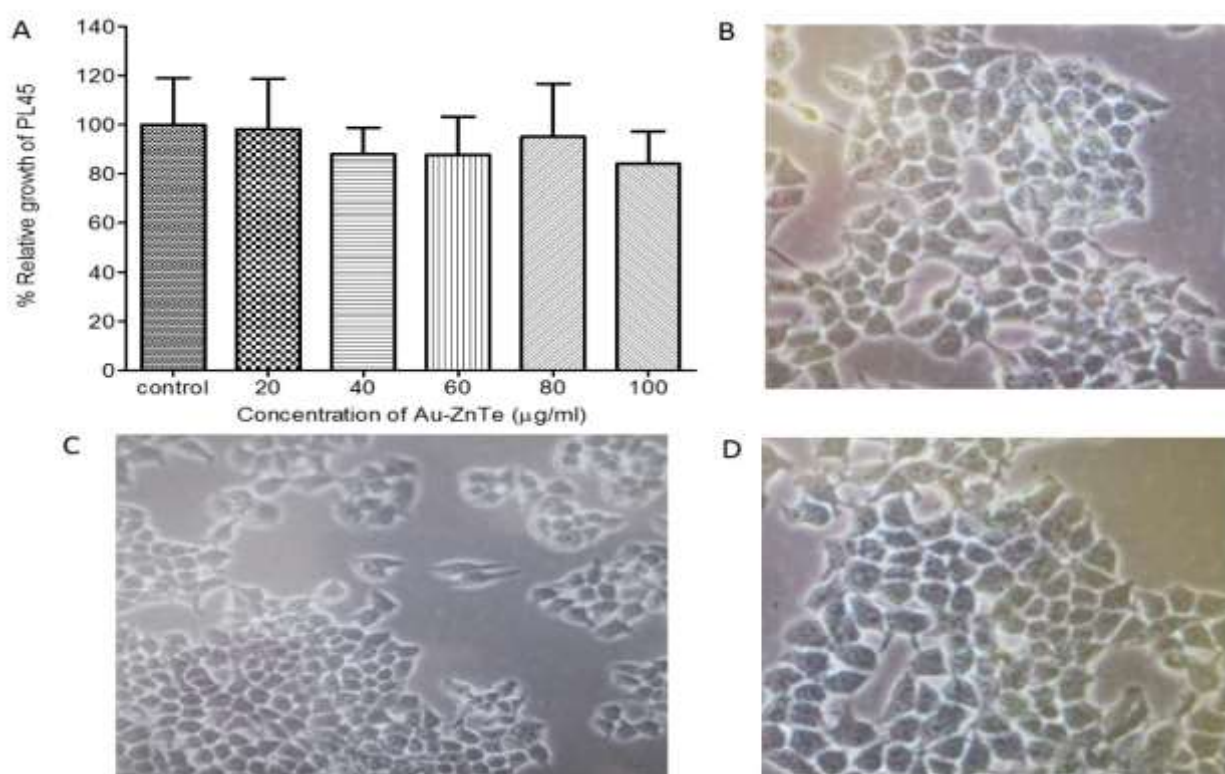


Figure 3.8 A. Displays the relative growth of PL45 cells after treatment with Au-ZnTe core/shell nanoparticles. **B.** Displays the untreated control PL45 cells. **C.** displays PL45 cells growing at 60 % confluency under the influence of 25 μg/ml. **D.** displays PL45 growing at 80 % confluency under the influence of 50 μg/ml.

3.3.5 Cellular uptake and bio-interaction of Au-ZnTe nanoparticles

The interaction of Au-ZnTe core/shell nanoparticles and PL45 was investigated using embedding, ultra-thin microtome sectioning and TEM imaging. Cellular uptake and isolation of the core/shell nanoparticles through membrane interactions was confirmed. The cysteine surface layer may have been responsible for the membrane interactions with the core/shell nanoparticles (**Figure 3.9 A-F**).

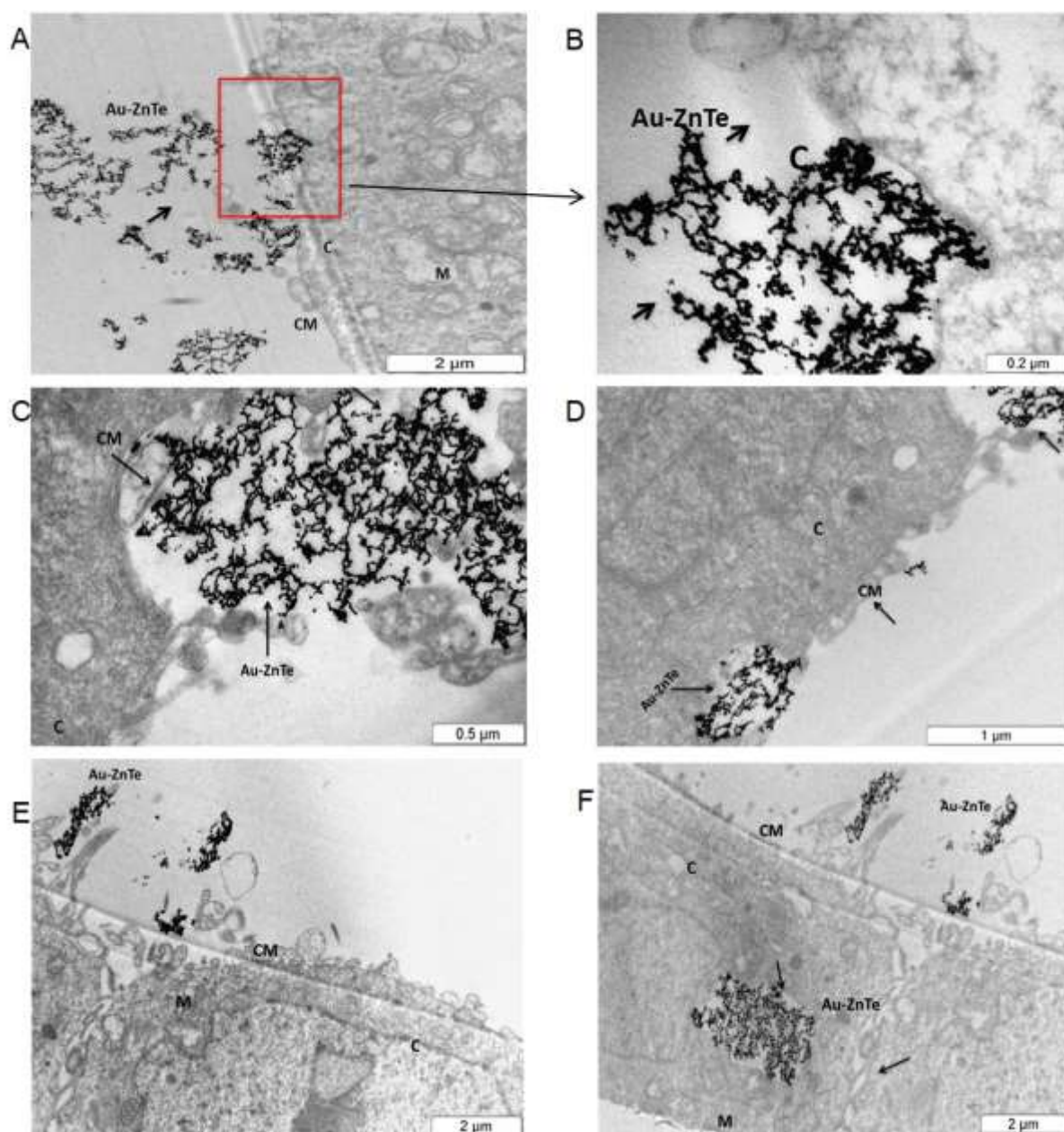


Figure 3.9 A-B, Cross-section through resin embedded PL45 cells, showing cellular membrane interaction with Au-ZnTe core/ shell nanoparticles. **C, D, E** and **F**, displays two adherent PL45 cells showing both membrane interactions with core/shell nanoparticles and Isolation of Au-ZnTe core/shell nanoparticles within a vacuole in the cells cytoplasm. C-Cytoplasm, CM-cytoplasmic membrane and M- mitochondria.

3.3.6 Bio-imaging potential of Au-ZnTe core-shell nanoparticles

To test the bio-imaging functionality of Au-ZnTe nanoparticles the fluorescence properties of Au-ZnTe core/shell nanoparticles within the PL45 cell lines were studied using fluorescence and phase-contrast light microscopes. The results shown in (**Figure 3.10**) illustrate imaging by Au-ZnTe core/shell nanoparticles. (**Figure 3.10 A**) shows the cross section of resin embedded PL45 cells treated with Au-ZnTe core/shell nanoparticles under a bright field imaging mode. (**Figure 3.10 B**), shows the same slide under a Dapi fluorescent filter set. The blue light emissions correspond to the absorption wavelength between 365- 415 nm and is due to emission from ZnTe. **Figure 3.10 C** and **D**, display various sections on the sample slide that fluoresce due to the presence of the semiconductor under the Dapi and Alexa 388 filter sets. In addition the measurement of the Zeta potential was used to determine the surface charge of the parent precursors, Au-ZnTe core/shell and cysteine. In addition it was used to determine how efficiently the capping agent has passivated the outermost surface of the Au-ZnTe core/shell nanoparticles. The Au and ZnTe parent precursors displayed negative charges of -65.1 mV and -11.7 mV respectively. Interestingly the Au-ZnTe core/shell nanoparticles displayed a positive close to neutral charge of 0.0519 mV. Cysteine displayed a positive charge of 3.95 mV. The results confirmed that the capping agent influenced the surface charge of Au-ZnTe and that the core/shell nanoparticles are efficiently passivated by an outer capping layer of cysteine. The surface charge of the core/shell nanoparticles will accommodate the functionalization of this nanomaterial for further applications in cell targeting and therapeutics.

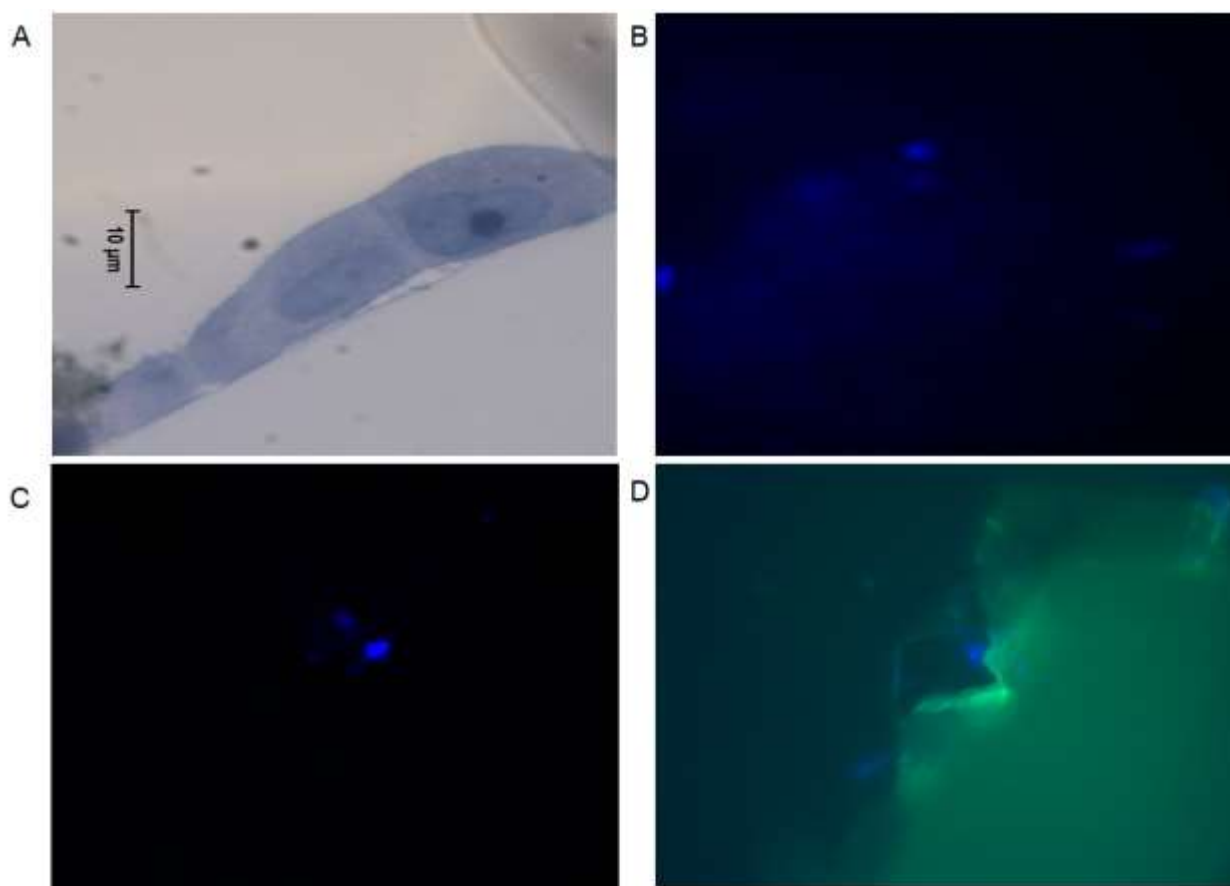


Figure 3.10 **A.** Displays a cross-section through pancreas cancer cell PL45 under bright field microscopy. **B.** shows a cross-section through pancreas cancer cell PL45 under fluorescence microscopy Dapi filter set. **C.** showing the fluorescence properties of Au-ZnTe core/ shell nanoparticles on different areas of the sample slides. **D.** Displays multi-fluorescence overlays of PL45 cells treated with Au-ZnTe core/shell nanoparticles under fluorescence Zeiss filter sets Dapi and Alexa 388.

3.4 Discussion

Au-ZnTe core/shell nanoparticles were successfully prepared using a novel one pot route. The average yield of Au-ZnTe nanoparticles filtered per reaction is between 800-1000 μg . Optical characterisation of Au-ZnTe core-shell nanoparticles confirmed that the core-shell nanoparticles displayed an optical absorbance peak characteristic of gold nanoparticles. The cysteine capped ZnTe nanoparticles showed an absorption peak at 269 nm (**Figure 3.2 A**) which is attributed to the Zn-ligand complex as observed previously for cysteine capped CdTe [36, 41]. Previous reports on ZnTe nanoparticles show emission in the 400-600 nm range depending on its size, shape and surface modifications.[41-45, 47-49]. In the case of materials where the gold core is surrounded by a semiconductor shell the emission from the shell material is quenched due to electron transfer to the metal core [50]. It is therefore conceivable that the emission in (**Figure 3.3**) is due to bare ZnTe nanoparticles or alloyed Au-ZnTe present in the sample.

The morphology of the Au-ZnTe nanoparticles were uniform and spherically shaped. In addition the use of cysteine as a capping material contributed to a self-assembly pattern of arrangement that is largely due to the formation of disulfide bonds between cysteine molecules. The self-assembled arrangement of the Au-ZnTe nanoparticles on the carbon grid is similar to that reported for Au-CdSe core-shell nanoparticles [34]. HAADF-STEM and EDX spectrum imaging can be used to confirm the structure and ratio of the heterostructured core/shell nanomaterials [11]. As shown in **Figure 3.5**, the elemental maps extracted from the EDX spectrum images data support the formation of Au-ZnTe nanoparticles within core/shell structure. The shell component is relatively

inhomogeneous. The spatial distributions of the elements, seen in the spectrum images and lines scans, are consistent with that expected for a core-shell structure. The spectrum image is a 2D projection of a 3-dimensional structure so some Zn and Te counts are expected to coincide with Au rich areas, corresponding to regions of shell above and below the core. The presence of some alloying at the interfaces can't be ruled out but we are confident that the EDX supports segregation of the two phases.

Gold was found to be localised in the core with the gold distribution shown in red in (Figure 3.5) whilst Zn and Te were observed to be principally associated with the particle surface. Line scan data extracted from the EDX spectrum images (Figure 3.5 B) suggests a shell thickness of ~1-4 nm. Semi quantitative analysis of the atomic percentage of Au, Zn and Te in the nanoparticles yielded a value of 45.4, 19.1 and 35.5 respectively. The mapping also confirms that elemental Te and bare ZnTe are present. This would explain the emission spectra in **Figure 3.3** which is attributed to the ZnTe nanoparticles.

The crystalline properties of Au-ZnTe core-shell nanoparticles displayed characteristic crystalline features from both Au and ZnTe crystals. Additionally the formation of ZnS as a secondary nanomaterial was considered as reported previously for cysteine capped CdTe whereby the sulfur from the cysteine is hydrolysed and reacts with cadmium to form an additional CdS shell [34, 47]. We did not find evidence from the XRD data for any ZnS formation. There are a number of methods that have been reported for the fabrication and design of various heterostructures and core/shell nanomaterials. The displacement method, redox-transmetalation, seeding- reduction, direct metal-salt reduction and the vapour deposition processes have been highlighted by Song and co-

workers [51] The formation of heterostructured materials by the fusion of the two components through the direct reaction or mixing is also a distinct possibility [52].

In this work, the formation of the Au-ZnTe is largely attributed to the use of citrate capped Au nanoparticles as the seeding material. The formation of shape and size controlled gold is determined by the concentration of citrate. Higher concentrations of citrate yield smaller particles whereas lower concentrations of citrate yield larger agglomerated particles. The citrate anions absorb onto the gold nanoparticles at specific facets or planes via carboxylate groups. Work reported by Park and Shumaker-Perry demonstrates the interaction of citrate anions on the Au nanoparticle surface [53]. The Au (111) plane is the most likely facet for citrate binding activity, due to the low surface energy. The absorbed citrate interacts with an adjacent absorbed species and a dangling free species of citrate through hydrogen bonds between the terminal carboxylic acid groups [53]. During the first phase of the reaction stabilised citrate capped gold nanoparticles was synthesized using an optimum citrate concentration. When the gold solution is added to the ZnTe solution, some Au planes are available to bind to the ZnTe thereby inducing a fusion-mediated growth of Au-ZnTe core-shell and alloy heterostructures. However more knowledge on the growth mechanism is required since there is limited literature on solution based growth of heterostructures. Carbone and Cozzoli reported that careful regulation of thermodynamics parameters and growth dynamics in solution under varying solvents, ligands and capping groups play a direct role in the growth mechanisms of heterostructured nanocrystals [54]. The control of

thermodynamic and kinetic parameters is important to obtain a heterostructure with the desired composition and structure.

The biocompatibility, bio-interaction and preliminary cytotoxicity was investigated using human pancreas adenocarcinoma PL45 cell line and co-cultured with Au-ZnTe core/shell nanoparticles. The cellular toxicity was studied using the WST-1 assay which is based on the cleavage of water soluble tetrazolium salt to a formazan dye by succinate-tetrazolium reductase, this reaction occurs in the mitochondrial respiratory chain and is active only in viable cells [55-58]. The results confirmed that Au-ZnTe nanoparticles do interact with the cells and are not cytotoxic under the experiment concentrations. In addition the Au-ZnTe nanoparticles displayed cell membrane interaction and cellular uptake. Similar observations were reported in other nanoparticle toxicology studies, however the mechanism by which the nanomaterial enters the cellular membranes is still under investigation [59-64]. The surface charge of nanomaterials is an important factor that may be altered by the attachment of bioconjugates. Monitoring the surface charge allows control in electrostatic and hydrophobic interactions within biological systems resulting in intracellular targeting and drug carrier applications [59, 60]. The surface charge and surface chemistry properties of the nanoparticle enables its cellular entry, interaction and applications. Many reports have demonstrated the use of various quantum dots, nanoparticles with core/shell structures and other nanomaterials in bio-imaging of bacterial, plant and human cells [65]. The preliminary bio-imaging potential of Au-ZnTe nanoparticles have been confirmed using fluorescent microscopy techniques.

The focus of this study was optimising the synthetic procedures and to understand the properties of Au-ZnTe core/shell nanoparticles. Modification of the core shell surface with cysteine allowed the particles to be water soluble as well as biocompatible. The amino functional groups of cysteine can be utilized for the attachment of biological receptors, tags or ligands. The chain-like arrangement observed for Au-ZnTe core/shell nanoparticles is characteristic of cysteine capped gold nanoparticles. In their study, Horovitz and co-workers investigated the assembly of gold nanoparticles which were induced by cysteine, an amino acid possessing an additional thiol functional group besides the alpha-amine [62]. The cysteine assembly effect was explained by considering the zwitterion-type electrostatic interactions between the charged amine and acid groups of cysteine molecules, bound to the gold nanoparticles by their –SH groups. They also did not rule out the interaction between cysteine and citrate bound to gold nanoparticles. In this work the cysteine is bound to the ZnTe shell with electrostatic interactions as well as with the citrate covered gold forming a network or assembly of particles. Previous reports on Au particles covered with metal shells showed that this surface plasmon absorption peak can be tuned by changing the thickness of the shell. Lu *et al*, demonstrated that the plasmon absorption of gold gradually shifts until a peak characteristic of the shell material emerges [66]. The optical properties displayed by Au-ZnTe core shell nanoparticles provide evidence that the ZnTe shell is fluorescent. The emission from the Au-ZnTe nanoparticles could be useful in biomedical applications such as tumour imaging. The efficient emission properties can be harnessed in order to track the movement of the Au-ZnTe drug coated particles within cells or for monitoring tumour size after drug treatments. These preliminary cytotoxicity and biocompatibility results indicate that these Au-ZnTe core/shell nanoparticles are biocompatible. These results provide a platform for further

studies to investigate the mechanistic interactions between the cellular membranes and core/shell nanoparticles and also to venture into the research of surface attachment of complimentary drugs and cellular ligands. The findings from this work demand a critical need to further understand the bio-interaction and biosafety of Au-ZnTe nanoparticles before they may be exploited in bio-applications such as targeted drug delivery and bio-imaging of cancer or tumour tissues.

3.5 Conclusions

The work presented in this chapter demonstrates an optimized synthesis route to produce water soluble Au-ZnTe core/shell nanoparticles. The research concept was to design a novel biocompatible heterostructure by combining beneficial properties from both parental nanomaterials to function as a single entity. The synthesized material displayed enhanced optical and stability features. The morphological analysis displayed uniform distribution of particle size and shape. In addition the elemental mapping data supports the core-shell structure composed of gold cores surrounded by a shell material comprised of ZnTe. The presence of some alloys and bare ZnTe nanomaterials were also present in the sample. These findings indicate the need for developing methods that refine and separate the prepared material on the nanoscale.

Preliminary studies using PL45 cancer cells showed the biocompatibility, biosafety and bio-interaction of Au-ZnTe nanoparticles. More especially the use of cysteine as the surface layer enabled cellular uptake of Au-ZnTe nanoparticles. The mechanism by which the membrane interacts with the cysteine functionalised Au-ZnTe nanoparticles can be further evaluated in future studies. Although the initial results show that Au-ZnTe are biocompatible and not toxic a

more in-depth account of nanotoxicology needs to be established using comparative assessments from various cell types and *in vivo* animal models. A thorough toxicity and biosafety evaluation will support the future use of Au-ZnTe in targeted drug delivery and bio-imaging applications.

References

- Danhier, F., O. Feron, and V. Préat, *To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery*. Journal of Controlled Release, 2010. **148**(2): p. 135-146.
- Vigderman, L. and E.R. Zubarev, *Therapeutic platforms based on gold nanoparticles and their covalent conjugates with drug molecules*. Advanced drug delivery reviews, 2013. **65**(5): p. 663-676.
- Ghosh Chaudhuri, R. and S. Paria, *Core/shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications*. Chemical reviews, 2011. **112**(4): p. 2373-2433.
- Huang, X., et al., *Gold nanoparticles: interesting optical properties and recent applications in cancer diagnostics and therapy*. Nanomedicine, 2007.
- Hsu, C., et al., *Au/Pd core-shell nanoparticles with varied hollow Au cores for enhanced formic acid oxidation*. Nanoscale research letters, 2013. **8**(1): p. 1-7.
- Lal, S., S.E. Clare, and N.J. Halas, *Nanoshell-enabled photothermal cancer therapy: impending clinical impact*. Accounts of chemical research, 2008. **41**(12): p. 1842-1851.
- Hou, H., et al., *Fine-Tuning LSPR Response of Gold Nanorod/Polyaniline Core-Shell Nanoparticles with High Photothermal Efficiency for Cancer Cell Ablation*. Journal of Materials Chemistry B, 2015.
- Topete, A., et al., *Fluorescent drug-loaded, polymeric-based, branched gold nanoshells for localized multimodal therapy and imaging of tumoral cells*. ACS nano, 2014. **8**(3): p. 2725-2738.
- Lee, D., et al., *Ca²⁺-Dependent Intracellular Drug Delivery System Developed with "Raspberry-Type" Particles-on-a-Particle Comprising Mesoporous Silica Core and α -Synuclein-Coated Gold Nanoparticles*. ACS nano, 2014. **8**(9): p. 8887-8895.
- Xu, B., et al., *tLyP-1-Conjugated Au-Nanorod@SiO₂ Core-Shell Nanoparticles for Tumor-Targeted Drug Delivery and Photothermal Therapy*. Langmuir, 2014. **30**(26): p. 7789-7797.
- Slater, T.J., et al., *Correlating catalytic activity of Ag-Au nanoparticles with 3D compositional variations*. Nano letters, 2014. **14**(4): p. 1921-1926.
- Lee, J.-S., E.V. Shevchenko, and D.V. Talapin, *Au-PbS core-shell nanocrystals: Plasmonic absorption enhancement and electrical doping via intra-particle charge transfer*. Journal of the American Chemical Society, 2008. **130**(30): p. 9673-9675.
- Li, J. and H.C. Zeng, *Size tuning, functionalization, and reactivation of Au in TiO₂ nanoreactors*. Angewandte Chemie, 2005. **117**(28): p. 4416-4419.
- Liu, M. and P. Guyot-Sionnest, *Preparation and optical properties of silver chalcogenide coated gold nanorods*. Journal of Materials Chemistry, 2006. **16**(40): p. 3942-3945.
- Shevchenko, E.V., et al., *Gold/iron oxide core/hollow-shell nanoparticles*. Adv. Mater., 2008. **20**(22): p. 4323-4329.
- Qian, L., Y. Sha, and X. Yang, *Simple and convenient preparation of Au-Pt core-shell nanoparticles on surface via a seed growth method*. Thin Solid Films, 2006. **515**(4): p. 1349-1353.
- Sun, Z., et al., *A general approach to the synthesis of gold-metal sulfide core-shell and heterostructures*. Angewandte Chemie International Edition, 2009. **48**(16): p. 2881-2885.
- Salant, A., E. Amitay-Sadovsky, and U. Banin, *Directed self-assembly of gold-tipped CdSe nanorods*. Journal of the American Chemical Society, 2006. **128**(31): p. 10006-10007.
- Mokari, T., et al., *Formation of asymmetric one-sided metal-tipped semiconductor nanocrystal dots and rods*. Nature Materials, 2005. **4**(11): p. 855-863.
- Mokari, T., et al., *Selective growth of metal tips onto semiconductor quantum rods and tetrapods*. Science, 2004. **304**(5678): p. 1787-1790.
- Goon, I.Y., et al., *Fabrication and dispersion of gold-shell-protected magnetite nanoparticles: systematic control using polyethyleneimine*. Chemistry of Materials, 2009. **21**(4): p. 673-681.
- Zhang, J., et al., *Laser-assisted synthesis of superparamagnetic Fe@Au core-shell nanoparticles*. The Journal of Physical Chemistry B, 2006. **110**(14): p. 7122-7128.
- Bao, Y., H. Calderon, and K.M. Krishnan, *Synthesis and characterization of magnetic-optical Co-Au core-shell nanoparticles*. The Journal of Physical Chemistry C, 2007. **111**(5): p. 1941-1944.
- Pal, S., et al., *Synthesis and magnetic properties of gold coated iron oxide nanoparticles*. Journal of Applied Physics, 2009. **105**(7): p. 7B504.
- Sardar, D., et al., *A facile method for synthesis of Co-core Au-shell nanohybrid*. New J. Chem., 2014. **38**: p. 4107-4114.
- Wang, L., et al., *Monodispersed core-shell Fe₃O₄@Au nanoparticles*. The Journal of Physical Chemistry B, 2005. **109**(46): p. 21593-21601.
- Li, S., et al., *Controlled hybridization of Sn-SnO₂ nanoparticles via simple-programmed microfluidic processes for tunable ultraviolet and blue emissions*. Journal of Materials Chemistry C, 2014. **2**(36): p. 7687-7694.
- Yoshida, Y., et al., *Synthesis and growth mechanism of Au@Cu core-shell nanorods having excellent antioxidative properties*. CrystEngComm, 2014. **16**(25): p. 5672-5680.
- Chiang, I.C. and D.H. Chen, *Synthesis of monodisperse FeAu nanoparticles with tunable magnetic and optical properties*. Advanced Functional Materials, 2007. **17**(8): p. 1311-1316.
- Wei, Y., et al., *Synthesis, shape control, and optical properties of hybrid Au/Fe₃O₄ "nanoflowers"*. Small, 2008. **4**(10): p. 1635-1639.
- Melancon, M.P., W. Lu, and C. Li, *Gold-based magneto/optical nanostructures: challenges for in vivo applications in cancer diagnostics and therapy*. MRS bulletin, 2009. **34**(06): p. 415-421.
- Kayal, S. and R.V. Ramanujan, *Anti-cancer drug loaded iron-gold core-shell nanoparticles (Fe@Au) for magnetic drug targeting*. Journal of nanoscience and nanotechnology, 2010. **10**(9): p. 5527-5539.
- Pullabhotla, V.R. and N. Revaprasadu, *A novel route to cysteine capped Au-CdSe hybrid nanoparticles*. Materials Letters, 2009. **63**(24): p. 2097-2099.
- Duncan, B., C. Kim, and V.M. Rotello, *Gold nanoparticle platforms as drug and biomacromolecule delivery systems*. Journal of Controlled Release, 2010. **148**(1): p. 122-127.
- Jain, P.K., I.H. El-Sayed, and M.A. El-Sayed, *Au nanoparticles target cancer*. Nano Today, 2007. **2**(1): p. 18-29.
- Crowder, B., F. Morehead, and P. Wagner, *Efficient injection electroluminescence in ZnTe by avalanche breakdown*. Applied Physics Letters, 1966. **8**(6): p. 148-149.
- Nag, A., J. Kundu, and A. Hazarika, *Seeded-growth, nanocrystal-fusion, ion-exchange and inorganic-ligand mediated formation of semiconductor-based colloidal heterostructured nanocrystals*. CrystEngComm, 2014. **16**(40): p. 9391-9407.
- Bigall, N.C., W.J. Parak, and D. Dorfs, *Fluorescent, magnetic and plasmonic—Hybrid multifunctional*

- colloidal nano objects. *Nano Today*, 2012. **7**(4): p. 282-296.
39. Feng, X., G. Hu, and J. Hu, *Solution-phase synthesis of metal and/or semiconductor homojunction/heterojunction nanomaterials*. *Nanoscale*, 2011. **3**(5): p. 2099-2117.
40. Subramanian, V., E.E. Wolf, and P.V. Kamat, *Influence of metal/metal ion concentration on the photocatalytic activity of TiO₂-Au composite nanoparticles*. *Langmuir*, 2003. **19**(2): p. 469-474.
41. Sou, I., et al., *Highly efficient light emission from ZnS_{1-x}Te_x alloys*. *Applied physics letters*, 1995. **66**(15): p. 1915-1917.
42. Wu, Q., M. Litz, and X.C. Zhang, *Broadband detection capability of ZnTe electro-optic field detectors*. *Applied Physics Letters*, 1996. **68**(21): p. 2924-2926.
43. Bhunia, S. and D. Bose, *Microwave synthesis, single crystal growth and characterization of ZnTe*. *Journal of crystal growth*, 1998. **186**(4): p. 535-542.
44. Mntungwa, N., V.S.R. Pullabhotla, and N. Revaprasadu, *Facile synthesis of cysteine and triethanolamine capped CdTe nanoparticles*. *Colloids and Surfaces B: Biointerfaces*, 2013. **101**: p. 450-456.
45. Dunpall, R., S. Mlowe, and N. Revaprasadu, *Evidence of oriented attachment in the growth of functionalized ZnTe nanoparticles for potential applications in bio-imaging*. *New Journal of Chemistry*, 2014. **38**(12): p. 6002-6007.
46. Turkevich, J., P.C. Stevenson, and J. Hillier, *A study of the nucleation and growth processes in the synthesis of colloidal gold*. *Discussions of the Faraday Society*, 1951. **11**: p. 55-75.
47. Jiang, F., et al., *Ligand-tuned shape control, oriented assembly, and electrochemical characterization of colloidal ZnTe nanocrystals*. *Chemistry of Materials*, 2010. **22**(16): p. 4632-4641.
48. Gadalla, A.M., M. and Hamad, D., *Synthesis and Characterization of Au/CdSe & Ag/CdSe Core/Shell Nanoparticles*. *International Journal of Metallurgical & Materials Science and Engineering (IJMMSE)*, 2013. **3**(2): p. 59-68.
49. Kang, S.W., et al., *One-pot synthesis of trimetallic Au@PdPt core-shell nanoparticles with high catalytic performance*. *ACS nano*, 2013. **7**(9): p. 7945-7955.
50. L'Azou, B., et al., *In vitro effects of nanoparticles on renal cells*. *Part Fibre Toxicol*, 2008. **5**(22): p. 651-63.
51. Song, Y., et al., *Shell-driven Fine Structure Transition of Core Materials in Co@Au Core-shell Nanoparticles*. *Nano-Micro Letters*, 2012. **4**(4): p. 235-242.
52. Ray, M., et al., *Highly lattice-mismatched semiconductor-metal hybrid nanostructures: gold nanoparticle encapsulated luminescent silicon quantum dots*. *Nanoscale*, 2014. **6**(4): p. 2201-2210.
53. Park, J.-W. and J.S. Shumaker-Parry, *Structural study of citrate layers on gold nanoparticles: role of intermolecular interactions in stabilizing nanoparticles*. *Journal of the American Chemical Society*, 2014. **136**(5): p. 1907-1921.
54. Carbone, L. and P.D. Cozzoli, *Colloidal heterostructured nanocrystals: Synthesis and growth mechanisms*. *Nano Today*, 2010. **5**(5): p. 449-493.
55. Gibson, J.D., B.P. Khanal, and E.R. Zubarev, *Paclitaxel-functionalized gold nanoparticles*. *Journal of the American Chemical Society*, 2007. **129**(37): p. 11653-11661.
56. Patra, C.R., et al., *Fabrication of gold nanoparticles for targeted therapy in pancreatic cancer*. *Advanced drug delivery reviews*, 2010. **62**(3): p. 346-361.
57. Pujalté, I., et al., *Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells*. *Part Fibre Toxicol*, 2011. **8**(10): p. 1-16.
58. Parveen, S., R. Misra, and S.K. Sahoo, *Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging*. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2012. **8**(2): p. 147-166.
59. Liang, H.-F., et al., *Paclitaxel-loaded poly (γ-glutamic acid)-poly (lactide) nanoparticles as a targeted drug delivery system for the treatment of liver cancer*. *Biomaterials*, 2006. **27**(9): p. 2051-2059.
60. Iversen, T.-G., T. Skotland, and K. Sandvig, *Endocytosis and intracellular transport of nanoparticles: present knowledge and need for future studies*. *Nano Today*, 2011. **6**(2): p. 176-185.
61. Labhasetwar, V., *Nanotechnology for drug and gene therapy: the importance of understanding molecular mechanisms of delivery*. *Current opinion in biotechnology*, 2005. **16**(6): p. 674-680.
62. Mocanu, A., et al., *Self-assembly characteristics of gold nanoparticles in the presence of cysteine*. *Colloids and Surfaces A: Physicochemical And Engineering Aspects*, 2009. **338**(1): p. 93-101.
63. Zhang, Y., et al., *Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells*. *Biomedical microdevices*, 2008. **10**(2): p. 321-328.
64. Valizadeh, A., et al., *Quantum dots: synthesis, bioapplications, and toxicity*. *Nanoscale research letters*, 2012. **7**(1): p. 1-14.
65. Shao, L., Y. Gao, and F. Yan, *Semiconductor quantum dots for biomedical applications*. *Sensors*, 2011. **11**(12): p. 11736-11751.
66. Lu, L., et al., *Core-shell gold/silver nanoparticles: Synthesis and optical properties*. *Journal of colloid and interface science*, 2013. **392**: p. 90-95.

**CHAPTER FOUR: The bio-interaction, biosafety and nanotoxicology of Au-ZnTe
core-shell nanoparticles**

4.1 Introduction

An important factor in understanding the properties of nanomaterials is knowledge of its potential toxicity, biosafety and biocompatible properties. It is estimated that nanomaterials on the global scale are produced in large quantities. Due to toxicity, safety, risk assessments and hazard regulations only a small percentage of these nanomaterials end up in downstream healthcare applications such as clinical trials, pharmaceuticals and nanomedicine [1-6]. It therefore is critical to establish the biosafety data of core-shell nanomaterials across all platforms of research [7].

The parameters used to synthesize core-shell nanoparticles for biological applications involve precise optimisation of materials compatibility, temperature, pH, biocompatible surfactants and water solubility [8]. Work reported by Sun *et al.*, [9] describes a general high temperature method for the synthesis of water-dispersible gold metallic core surrounded by different transitional metal sulfide semiconductors (ZnS, CdS, AgS, NiS, and CuS). The gold-metal sulfide core-shell nanoparticles displayed enhanced optical and morphology properties that could be utilized in plasmonics, and optic applications [9]. In an independent report, Lim and co-workers described the embedding of DNA within Au/Ag core-shell nanoparticles designed specifically for applications in biorecognition and optical stability studies [10]. Other research trends describe gold coated iron oxide nanoparticles that are used as enhanced contrast agents in magnetic resonance imaging [11]. This study displayed the effect of combining two compatible types of nanomaterials that function as a single unit to enhance features such as size tunability, magnetism, optical and surface binding properties. In another study Fe/Au core-shell nanoparticles were surface modified with anti-cancer drugs and used to enhance drug release in cancer

cells [12]. These findings demonstrate the versatility of gold based core-shell nanomaterials in various bioapplications [8-12]. Scientists are currently focused on understanding the properties and surface chemistry features of the core-shell nanomaterials as much as demonstrating their potential applications [13]. Of particular interest to our research area are gold based metallic/semiconductor core-shell nanomaterials [14, 15]. Work done by Cui *et al.*, illustrated the importance of the surface chemistry of nanomaterials and the role it plays in bio-interactions with cells and other biological components [16]. In addition literature reports that cytotoxicity of nanomaterials are attributed to a combination of varying factors such as morphology, composition and cell type [17-20]. A report by Liu *et al.*, investigated the *in vivo* toxicity of PEGylated FePt@Fe₂O₃ core-shell magnetic nanoparticles using various human cell lines and *in vivo* mouse models [21]. Their results displayed the importance of correlating various *in vitro* and *in vivo* techniques and experimental approaches to establish biosafety and toxicity on a broader platform. Biocompatibility studies of Au-Ag bimetallic alloy and core-shell nanoparticles were demonstrated using the survival rates of microorganism *Daphnia magna* [22]. This work demonstrated the various *in vitro* approaches that may be used to determine the biosafety limits of heterostructured nanomaterials. Their study further highlighted the importance of toxicity studies across biological and environmental systems.

A biosafety study aims to evaluate the effects of nanoparticles on biological units and their functions, in addition it is used to establish safe concentration/ dose ranges that may be used in bioapplications such as drug delivery, bio-imaging, diagnostics and therapeutics [23]. The toxic effects and bio-interactions of nanoparticles can be studied

across the molecular, cellular, organ and integrated organs or whole animal systems [24]. Although initial reports on Au-ZnTe core-shell nanoparticles established its cytotoxicity and cellular uptake within human pancreas cancer cells, a more in-depth understanding of the potential interaction of these core-shells within several *in vitro* cellular and *in vivo* animal models is required, in which homeostasis, thousands of cell cycle events, and organ responses are simultaneously ongoing. In a thorough biosafety evaluation *in vitro* experiments are limited to cell cycle events and isolated cell culture experiments which lead to comparative studies across various cell and tissue types. *In vitro* studies provide vital links in understanding how nanomaterials interact with cellular organelles and biomolecules providing important information on mechanisms of cell death and pathway interactions [24]. Nanoparticles may enter biological systems through inhalation, dermal and ingestion routes. These exposure routes inevitably lead to bio-interactions with DNA, cells, tissues, organs and the blood circulatory system. The main objective of this study was to establish the biosafety profile of the core-shell nanoparticles. In this chapter we evaluate the biocompatibility and biosafety effects of Au-ZnTe core-shell nanoparticles on human carcinoma and normal epithelial cell lines from different organs, its interaction with human peripheral blood mononuclear immune cells and identify the clinical effects of its acute exposure within Sprague-Dawley rats.

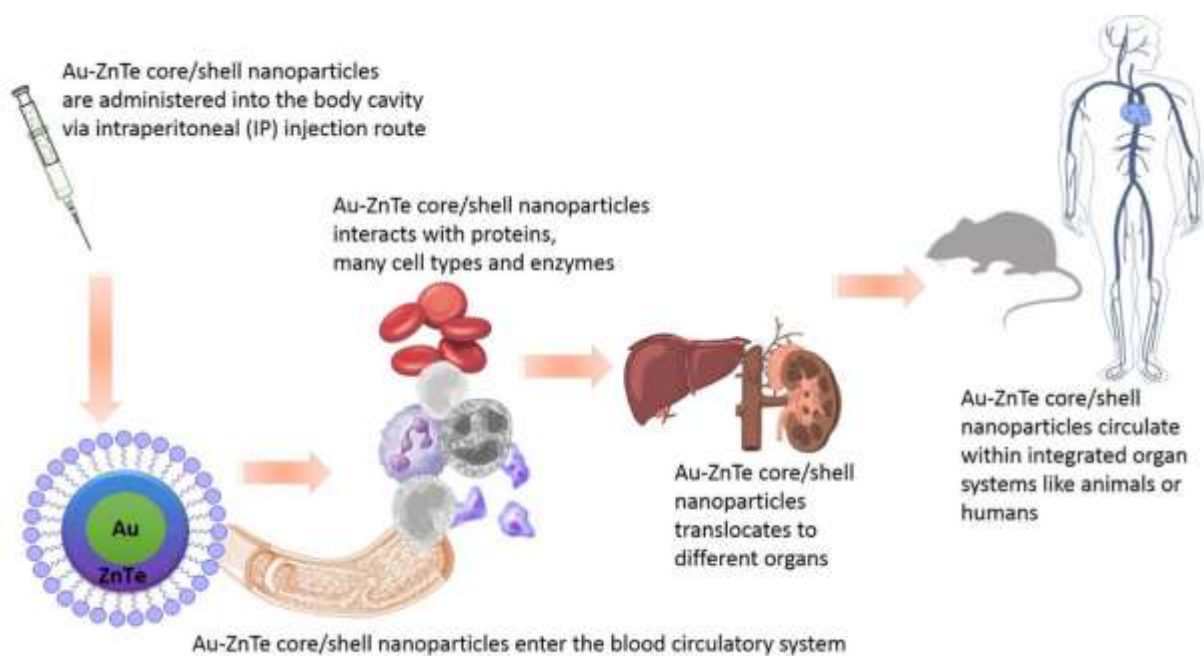


Figure 4.1 Exposure routes of Au-ZnTe nanoparticles. Illustration of Au-ZnTe core-shell nanoparticles that enter biological systems through the IP injection route. A pathway of biochemical interactions across different cell types, organs and integrated animal system are inevitable.

4.2 Materials and Methods

4.2.1 Ethics Statement

- This research was approved by the University of KwaZulu Natal (UKZN) Animal Ethics Committee. All animal research was conducted at the Biomedical Resource Unit, UKZN. Animal Ethics number: 085/14/Animal. The Animal ethics and animal welfare, laboratory animal science and 3R's:
- **Replacement:** Involves the selection of alternate non-animal methods to accomplish the same scientific aims of the study.
- **Reduction:** Minimum number of animals that can provide information that is statistically and scientifically valid.
- **Refinement:** Focuses on ensuring the animal welfare through well designed experimental protocols that reduce discomfort, pain and suffering.

Guidelines for reporting *in vivo* animal experiments was strictly adhered to. In accordance with the guidelines for animal ethics approval, it was compulsory to design and implement humane endpoint forms that are used to closely monitor the pain, activity and distress that the animals experience on a daily basis. Access to veterinary care was ensured and available constantly by resident staff of the Biomedical Resource Unit (BRU) of UKZN. Veterinary surgeon- Dr. S D Singh (BVSc, MS, CVE, LAS), Veterinary technologist- Dr. L A Bester (M. Vet Tech, PhD), Laboratory animal technician-Mr. D Mompe (LAT). Additionally ethics approval was obtained from the University of Zululand for this project. See appendix B for ethics approval documents and humane endpoint form.

4.2.2 Synthesis and characterisation of Au-ZnTe core-shell nanoparticles

The synthesis and characterisation of Au-ZnTe was performed according to the prescribed methods described in **chapter 3, section 3.2**.

4.2.3 Cell culture

Modified eagle medium (MEM), Ham's F-12K (Kaighn's) Medium, McCoy's 5a medium Dulbecco's Phosphate buffered saline (PBS), Trypsin, 100x pen-strep Fungizone (PSF) and fetal calf serum (FCS). All the cell tissue culture reagents were supplied by Invitrogen. Human normal mammary breast epithelial (MCF12A), normal colon (CCD 841 CoN) and breast (MCT-7), prostate (PC3), colon colorectal (HT29) adenocarcinoma cell lines were purchased from American Type Culture Collection (ATCC) and used in the experiments. These adhesion cell line was grown in appropriate culture media (MCF7, MCF12A, CCD 841 CoN-MEM, PC3-F12K, HT29-McCoy's 5a) containing 10% FBS and 1-2% PSF at a cell density of 5000. Cells that grew as monolayers were passaged, trypsinized and harvested for experimentation before reaching 100 % confluency.

4.2.4 WST-1 cell viability assay

Cell viability was evaluated by WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Roche diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Nanoparticle treated and control cells were seeded in a 96 well plate at a cell density of 5000 per well in 100 μ l of appropriate culture media. Cells were cultured in 5% CO₂ at 37 °C for 48 hours. Reconstituted WST-1 reagent was added to each treatment well and incubated for 2 hours at 37°C. The plate was shaken for 30 seconds and the absorbance was measured at 450 nm using a Multiskan FC microplate reader. All experiments were repeated in triplicates and the results were

represented as mean $\pm$ SD.

4.2.5 Cellular interaction with Au-ZnTe core/shell nanoparticles

MCF7 cell lines were treated with Au-ZnTe core/shell nanoparticles for 48 hours at the following concentrations, 3.125, 6.25, 12.5, 25 and 50 $\mu\text{g/ml}$. The treated cells were then fixed with 2.5 % glutaraldehyde for 24 hours, followed by a phosphate buffer wash. A postfix of 0.5 % Osmium tetroxide was added to the cells and incubated for 1 hour at room temperature followed by a phosphate buffer wash. The cells were then dehydrated with 30, 50, 75 and 100 % acetone. Resin and acetone were added to the sample in equal volumes followed by 4 hour incubation at room temperature. Cells were placed in molds and whole resin, followed by an overnight incubation at 70 °C. Resin sample blocks were sectioned into ultrathin cross sections using a microtome, stained and viewed with a transmission electron microscope.

4.2.6 Human peripheral blood mononuclear cell culture

Suspension cell line, Human peripheral blood mononuclear cells (PBMC) were isolated from donor blood samples obtained from the South African Blood bank. Density gradient centrifugation using Ficoll-Paque Plus method was used to isolate PBMC's from whole blood. The PBMC's were resuspended in RPMI-1640 cell culture media

4.2.7 Alamar blue assay

Alamar blue assay was used to evaluate the viability of PBMC's. PBMC's at a cell density of 5000 per well was seeded in a 96-well plate. The cells were stimulated with Phytohemagglutinin (PHA) whilst cells in another test group remained non-stimulated. After 4 hours of incubation with PHA, all cells were treated with Au-ZnTe nanoparticles in the following concentrations (0, 0.09, 0.19, 0.39, 0.78, 1.5, 3.125, 6.25, 12.5, 25, 50

µg/ml). After 72 hours of exposure 10 µl of Alamar blue dye was added to each well and the absorbance was monitored at 570nm using a Multiskan FC microplate reader. All experiments were repeated in triplicates and the results were represented as mean SEM.

4.2.8 Cytokine analysis

The concentrations of proinflammatory cytokines, Tumor necrosis factor (TNFα), Interferon (IFNγ) and Interleukin (IL-1β) from Phytohemagglutinin (PHA) stimulated and non-stimulated PBMCs were measured using commercially available BD Bioscience ELISA kits. Each well was coated with 100 µl of capture antibody and incubated overnight at 4 °C. PBMC fluid serum was added to each well after washing and blocking with assay diluent. Cytokine containing culture media and cytokine standards were added to each well and incubated at room temperature for 2 hours. After washing the detection antibody was added to each well and incubated at room temperature for 1 hour. Plates washed again and incubated with Avidin-Horseradish peroxidase for 30 min followed by detection with Tetramethylbenzidine solution. The reaction was stopped by the addition of H₂PO₄ and the absorbance was measured at 450 nm using a Multiskan FC microplate reader. All experiments were carried out in quadruplicates and represented as mean ± SD. The cytokine concentrations were calculated from standard curve (range: 10-1000 pg/ml) derived from cytokine standards available in the BD Bioscience ELISA kits.

4.2.9 Animals and conditions

Four-week-old female, specific-pathogen free (SPF) Sprague Dawley rats were purchased from Biomedical Resource Unit, University of KwaZulu Natal and acclimated for 7 days before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (maximum of 3 rats per cage) in a

room with controlled temperature ($22.2 \pm 1.7^{\circ}\text{C}$) and humidity ($48.4 \pm 6.0\%$), and a 12-h light/dark cycle. The rats were fed a rodent pellet food and water ad libitum. Au-ZnTe core-shell nanoparticles was filtered, weighed, resuspended in phosphate buffered saline and prepared for administration to the rats using intraperitoneal (IP) injection techniques. The rats were divided into 4 groups ($n = 3$): untreated control (Phosphate buffered saline), low-dose group ($50 \mu\text{g/ml}$), intermediated-dose group ($500 \mu\text{g/ml}$) and high-dose group ($1500 \mu\text{g/ml}$). After the acclimatization period, a volume of 0.5 ml Au-ZnTe core-shell nanoparticles were administered to the rats via IP injection routes. Animals were then placed individually in metabolic cages for 24 hours for observation and urine analysis using commercial urine dipsticks. The animals were observed for a further 7 days. The animal studies and protocols were approved by the Animal Ethics Committee of University of KwaZulu Natal.

4.2.10 Biochemical evaluation of liver and kidney function

Food was withheld for 12 hours before necropsy at the conclusion of the 14-day acute toxicity study. On day 14 of the study the animals were anaesthetised by an overdose of halothane followed by immediate bleeding and exsanguination by cardiac puncture. The blood was collected in heparinized vacutainers, and analysed for alkaline phosphatase (ALK Phos), Alanine Aminotransferase (ALT), Gamma-glutamyl transpeptidase (Gamma-GT), Lactate dehydrogenase (LDH), Creatinine (CRE) and Urea using a Beckman Coulter DXC600/800. The white blood cell (WBC) counts, red blood cell (RBC) counts, platelet (PLT) counts were analysed using a Roche Sysmex XT 1800.

4.2.11 Histopathology analysis of liver and kidney tissue

The kidney and liver organs were carefully harvested, weighed and fixed in a 10% formalin solution containing neutral phosphate-buffered saline. For light microscopy (LM) evaluations, the tissue samples were removed from the fixative and dehydrated using a 50 to 96% ethanol gradient with the ethanol being subsequently cleared from the tissue using xylene. The tissue was then embedded in paraffin wax using standard procedures. The wax embedded tissue were sliced into 3 μm sections using a microtome, collected on glass slides, dried and stained with hematoxylin and eosin (H&E) using standard protocols. The stained sections were examined using a light microscope (LM; Nikon 80i, Kanagawa, Japan) and images were digitally captured using NIS Elements D software and a camera (Nikon U2, Kanagawa, Japan). All experiment/evaluations were performed using a minimum of three replicates.

4.2.12 Statistical analyses

The statistical analyses were performed using GraphPad Prism® Version 5 software. The statistical evaluation included a two-tailed Student's t-test or analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

4.3 Results

4.3.1 Cytotoxicity of Au-ZnTe core-shell nanoparticles using WST-1 assay

The cytotoxicity of Au-ZnTe core-shell nanoparticles were established using the WST-1 assay which is based on the cleavage of water soluble tetrazolium salt to a formazan dye by succinate-tetrazolium reductase, this reaction occurs in the mitochondrial respiratory chain and is active only in viable cells. HT29, MCF7 and PC3 cells (**Figure 4.2**), CCD 841 CoN and MCF12A (**Figure 4.2**) showed no adverse effects on cellular growth during exposure to core-shell nanoparticles, the treated cells displayed relative growth that was statistically insignificant ($p > 0.05$) when compared to the control. Additionally, no significant morphology changes, depicting apoptosis or necrosis were observed across the different cell types during the co-culture exposure to Au-ZnTe core-shell nanoparticles.

4.3.2 Cellular interaction and biocompatibility studies using MCF7 cells

Pre-exposed MCF7 cancer cells were resin embedded and sectioned for transmission electron microscopy analysis (**Figure 4.3 A**). The treated cell sections displayed bio-interactions of the core-shell along the cellular membrane. The cysteine capped core-shell interacts with the cell membrane (**Figure 4.3 B-D**) and is shown to enter the cells internal environment through cellular mediated endocytosis. The findings in **figure 4.3 C-D**, shows evidence of cellular uptake of the core-shells. This analysis not only confirms the bio-interaction of the core-shells but also confirms the cellular uptake and isolation of these nanomaterials within the cells [25]. The use of cysteine as a capping agent plays a key role in stabilising the nanomaterial and facilitating its biocompatibility.

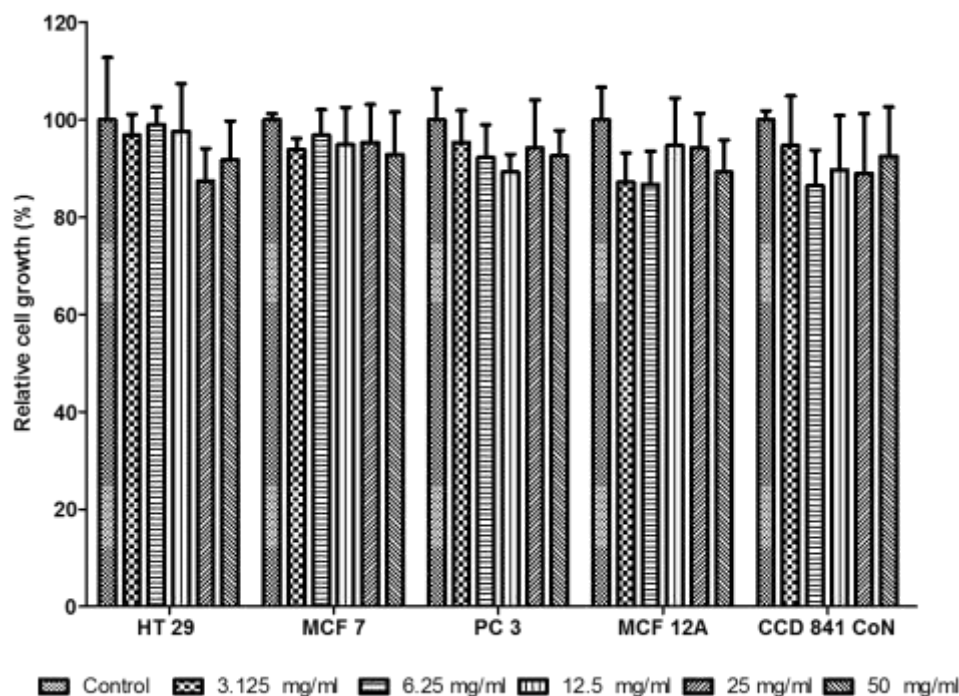


Figure 4.2 Cytotoxicity of Au-ZnTe core-shell nanoparticles. Displays relative growth of HT29, MCF7 and PC3 cancer cell lines; MCF12A and CCD 841 CoN normal cell lines.

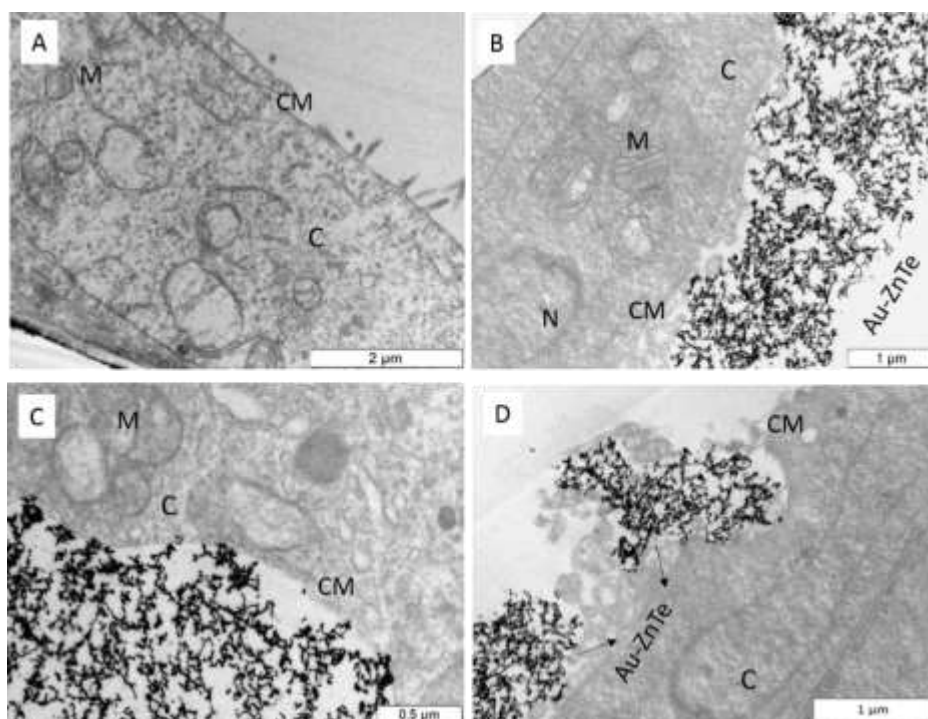


Figure 4.3. A. Cross section of control MCF7 cells. B-C Cross-section of MCF7 cells showing Au-ZnTe core-shell nanoparticles aligning along and interacting with the cellular membrane. D. Cellular mediated endocytosis of Au-ZnTe core-shell nanoparticles and cellular uptake of cysteine capped Au-ZnTe core-shell nanoparticles.

4.3.3 Immunotoxicity effects of Au-ZnTe core-shell nanoparticles on human PBMC's

PHA-stimulated and non-stimulated human PBMCs were exposed to the various concentrations of Au-ZnTe core-shell nanoparticles (50, 25, 12.5, 6.25, 3.125, 1.5, 0.78, 0.39, 0.19 and 0.09 $\mu\text{g/ml}$) for 72 hours. The cell viability was then evaluated using Alamar blue cell proliferation assay. Au-ZnTe core-shell nanoparticles exposure at concentration below 3.125 $\mu\text{g/ml}$ did not induce a dose-dependent reduction in the cell viability of the PHA-treated and non-stimulated PBMCs. The viability of the cells were moderately lower (80-95% viability) as compared to their respective untreated control (**Figure 4.4 A**). There was a clear dose-dependent reduction in the cell viability of the PHA-stimulated and non-stimulated PBMC exposed to 6.25 $\mu\text{g/ml}$ or higher concentrations of Au-ZnTe core/shell nanoparticles. The Au-ZnTe core-shell nanoparticles at 50 $\mu\text{g/ml}$, induced 20 % reduction in cell viability in both PHA-stimulated and non-stimulated PBMC's, which was statistically significant ($p < 0.05$). Culture media of PHA-stimulated and non-stimulated PBMC's exposed to non-cytotoxic concentrations of Au-ZnTe core-shell nanoparticles were evaluated for expression of IFN- γ , IL-1 β and TNF- α using commercial ELISA kits. In the non-stimulated PBMC, the Au-ZnTe core-shell nanoparticles induced a dose-dependent increase in IL-1 β expression. The highest concentration tested, 3.125 $\mu\text{g/ml}$ of Au-ZnTe core shells induced 643 pg/ml of IL-1 β (**Figure 4.4 B**). In the PHA-stimulated PBMC, the Au-ZnTe core-shell nanoparticles induced a dose-dependent increase in both TNF- α and IL-1 β secretion (**Figure 4.4 C**). Au-ZnTe core-shell nanoparticles did not induce detectable levels of IFN- γ in both PHA-stimulated and non-stimulated PBMC's.

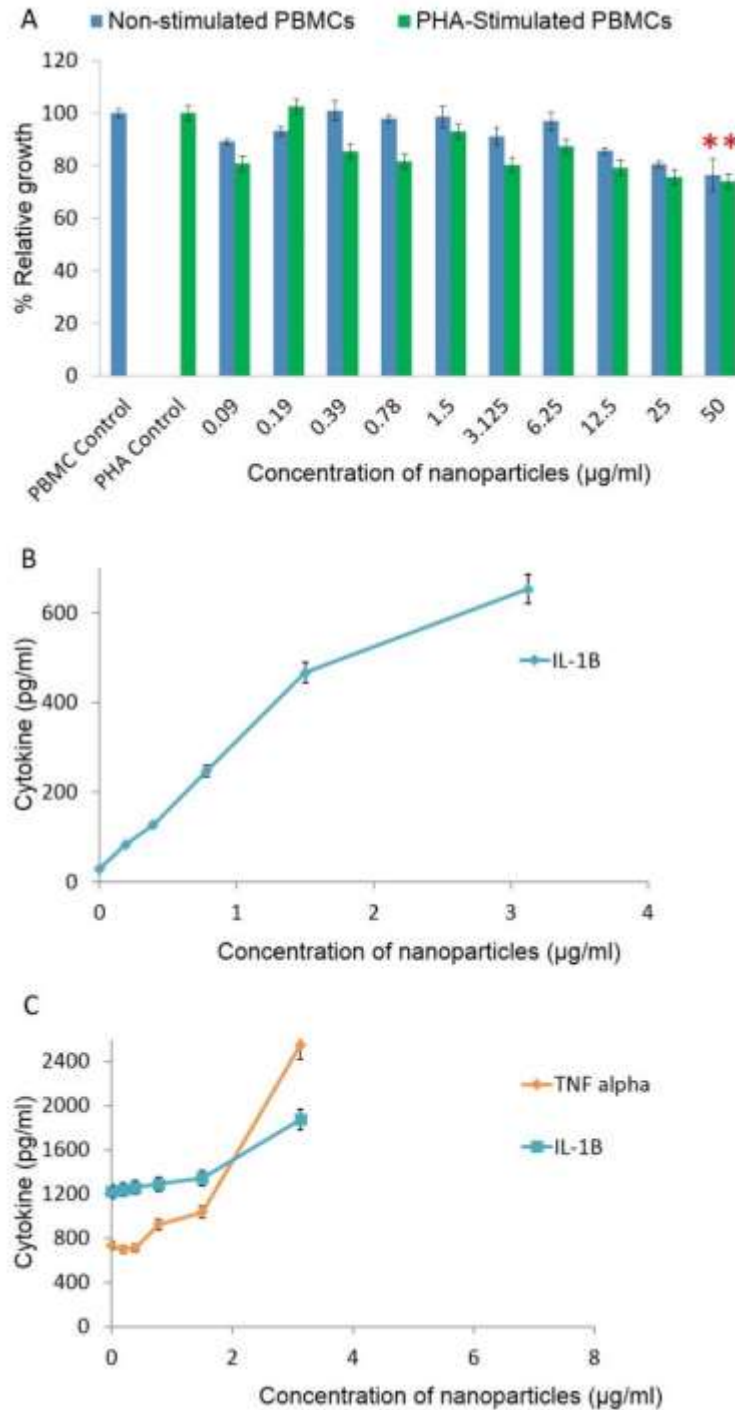


Figure 4.4 Immunotoxicity effects of Au-ZnTe core-shell nanoparticles on human peripheral blood mononuclear cells and cytokine expression. (A) Relative growth of PHA-stimulated and non-stimulated PBMC's during co-culture with Au-ZnTe core-shell nanoparticles. (B) Cytokine expression of non-stimulated PBMC's. (C) Cytokine expression of PHA stimulated PBMC's. All values compared to their respective untreated control, n=3. (* = $p < 0.05$).

4.3.4 Effects of Au-ZnTe core-shell nanoparticles on full blood counts in rat models

During the experiments, each animal maintained the regular alert, active, grooming and eating patterns. The animals were handled with care and all procedures were performed under the care of the institutional veterinarian. Blood collected from the rats after acute exposure to Au-ZnTe core-shell nanoparticles was analysed for full blood counts to detect abnormalities or changes in the blood composition. The accepted rat hematological reference range for red blood cells (RBC's) and white blood cells (WBC's) is $6.76\text{--}9.75 \times 10^6/\text{mm}^3$ and $6.6\text{--}12.6 \times 10^3/\text{mm}^3$ respectively. Blood platelets were analysed using a reference range of $150\text{--}460 \times 10^3/\text{ml}$. No statistically significant changes ($p > 0.05$) were observed in the hematological parameters measured across the untreated control and nanoparticle treated samples (**Figure 4.5**).

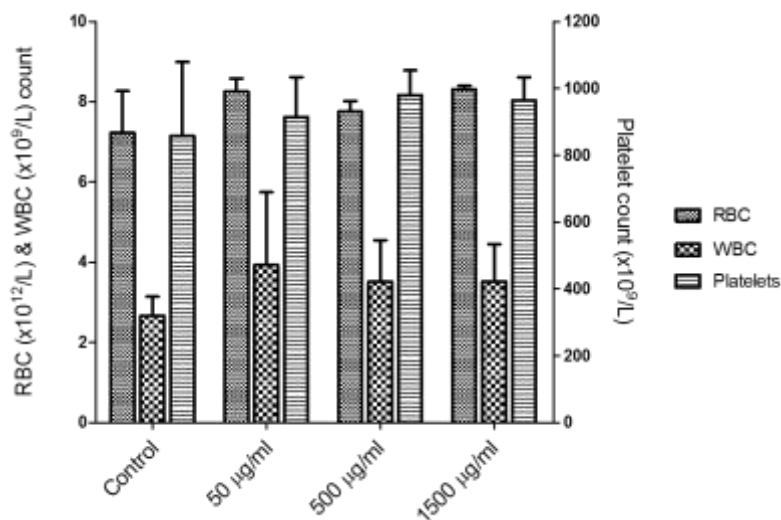


Figure 4.5 Hematological analysis of animals treated with Au-ZnTe core-shell nanoparticles. Red, white and platelet blood cells were evaluated. All values compared to the untreated control, $n=3$

4.3.5 Effects of Au-ZnTe core-shell nanoparticles on liver and kidney function in rat models

The biochemical parameters included the liver and kidney function due to its central role in detoxification and excretion Figure 4.6. Serum levels of liver enzymes referenced within the following ranges were measured ALT: 17.5-30.2 IU/L, ALP: 56.8-128 IU/L, Gamma-GT: > 60 IU/L and LDH: 100-250 IU/L. The results showed no statistically significant ($p > 0.05$) changes across the untreated control and nanoparticles treated animals (**Figure 4.6 A**). Creatine and urea serum levels indicated no statistically significant ($p > 0.05$) changes in the kidney function from animals in the untreated control group compared to the nanoparticle treated groups (**Figure 4.6 B**). The 24 hour urine analysis of all 12 animals displayed no significant differences across the untreated control and nanoparticle treated animals. The urine pH was observed at 6.8. The presence of glucose, ketones, bilirubin, blood or haemoglobin appeared negative on the urine dipstick test. Preliminary metal analysis using inductively coupled plasma tests (ICP) indicated the presence of Au-ZnTe materials in small amounts.

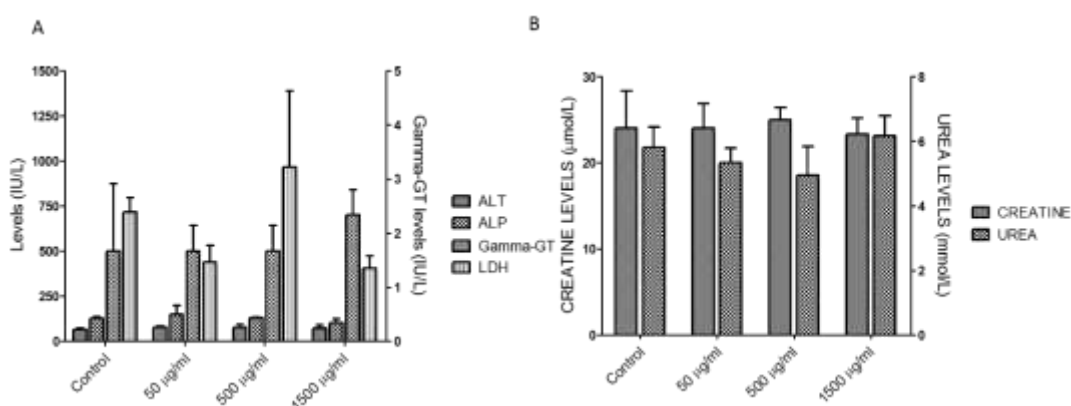


Figure 4.6 Effects of Au-ZnTe core-shell nanoparticles on liver and kidney function in Sprague-Dawley rats. (A) Liver function tests, showing concentrations of ALT, ALP, Gamma-GT and LDH. (B) Kidney function tests, showing concentrations of creatine and urea. All values compared to their respective untreated control, $n=3$.

4.3.6 Histological effects of Au-ZnTe core-shell nanoparticles on liver and kidney organs in Sprague-Dawley rats

Histology studies were performed on freshly harvested kidney and liver tissue from the experimental rats. The H&E images obtained from the control samples (**Figure 4.7 A**) closely resemble the expected morphology of normal healthy renal tissue. The H&E micrographs of the treated samples are all similar to that of the controls, displaying normal tissue morphology and cellular arrangement.

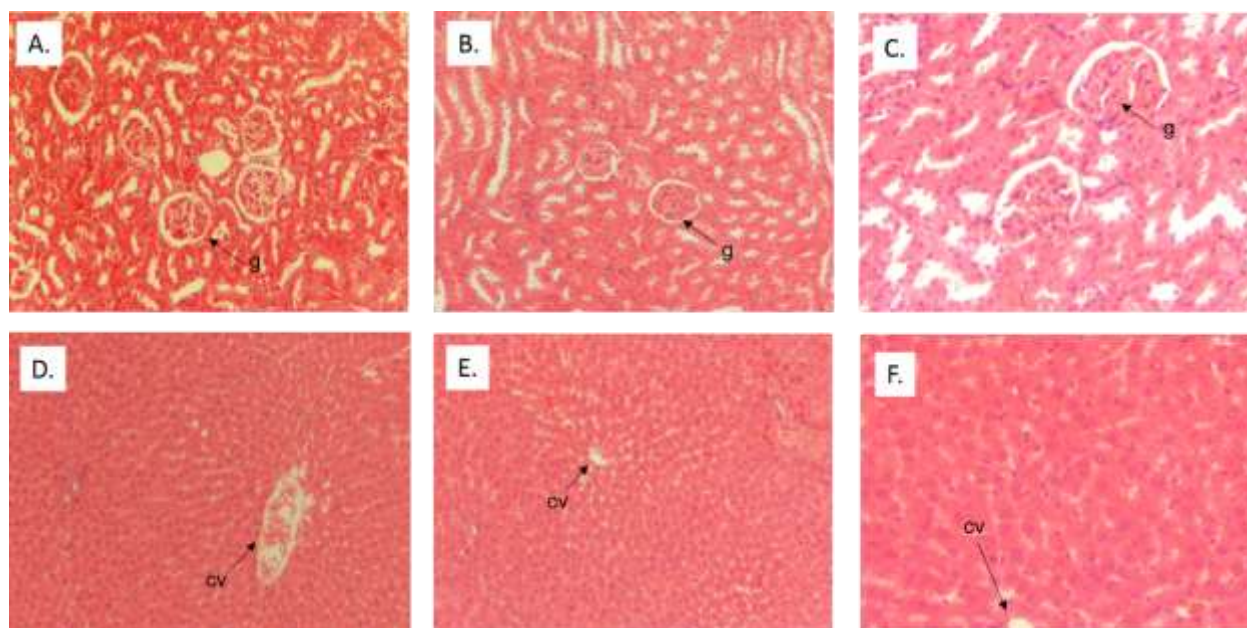


Figure 4.7 Histological effects of Au-ZnTe core-shell nanoparticles on liver and kidney organs in Sprague-Dawley rats. (A) Tissue section from the untreated control kidney 10x. (B) Low-magnification micrograph of kidney tissue from the high dose (1500 $\mu\text{g/ml}$) treated animal 10x. (C) high-magnification micrograph of kidney tissue from the high dose (1500 $\mu\text{g/ml}$) treated animal 20x. (D) Tissue section from the untreated control liver 10x. (E) Low-magnification micrograph of liver tissue from the high dose (1500 $\mu\text{g/ml}$) treated animal 10x. (F) high-magnification micrograph of liver tissue from the high dose (1500 $\mu\text{g/ml}$) treated animal 20x. (g- glomerulus, cv-central vein).

Micrographs of the high-dose samples show no sign of tissue damage or any lesions being present in the entirety of tissue at low magnification (**Figure 4.7 B**). At a higher magnification (**Figure 4.7 C**) which allows for a closer inspection of the functional unit of the kidney, there appears to be no damage to the Bowman's capsule or the glomerulus in the treated samples. H&E images of the control sample (**Figure 4.7 D**) closely resembles that of normal liver morphology. The images obtained from all the treated samples are similar to that of the controls. At low magnification of the treated samples (**Figure 4.7 E**) there are no observable tissue lesions, and at higher magnification (**Figure 4.7 F**) the morphology of the hepatocytes appear to be normal, and similar to that of the control. The assessment of these LM images show no loss of cellular integrity within the samples studied.

4.4 Discussion

The objective of this study was to synthesis, characterise and evaluate the biosafety of Au-ZnTe core-shell nanoparticles under *in vitro* and *in vivo* experimental conditions. The surface chemistry of this core-shell nanoparticle can be further developed for potential applications as a drug delivery template that can support surface modifications to target many different types of cancer. In order to develop the bioapplications of these core-shell nanoparticles, an understanding of the bio-interaction across many cell lines must be demonstrated. In this study we used three cancer cell lines from different organs to evaluate any changes in cytotoxicity responses across cell types. The results showed that Au-ZnTe nanoparticles displayed no adverse effect on cellular proliferation of MCF-7, HT29 and PC3 at the concentrations tested. In addition normal breast epithelial and colon cells grown under co-culture exposure to Au-ZnTe core-shell nanoparticles showed no cytotoxic effects from bio-interactions with core-shell nanoparticles.

In general cytotoxicity and biosafety evaluations of novel materials can be assessed through the use of any eukaryotic cell line as the basal cell physiological functions remain similar across any cell type. In an independent study, dose-dependent toxicity of nanoparticles have been observed on renal cell lines, this study demonstrated the effect of size and concentration of nanomaterials on renal cell function. Literature highlights the importance of standardizing *in vitro* cellular systems for nanotoxicity and biosafety screening and in establishing a safe dose/ concentration range that could be used for bioapplications [19, 20, 23-27]. An important aspect of biosafety of nanomaterials is related to their size and composition of the nanomaterials. Some studies demonstrate that exposure to larger sized nanoparticles are known to trigger macrophage responses

that activate the immune system [28]. In the current study, human peripheral blood mononuclear cells were used to simulate an *in vitro* immune system. Phytohemagglutinin (PHA) is a protein molecule that is used as a mitogen to trigger T-lymphocyte cell division and to activate growth of human peripheral lymphocytes [29]. The function of PHA stimulation is to mimic an active immune system that is stimulated to produce more active mononuclear cells, while the PBMC's that grow at their normal rate or are unstimulated represent an inactive immune system. The effects of Au-ZnTe core-shell nanoparticles on both an active and inactive immune system shows a 20 % decrease in cell viability at 50 µg/ml. Work reported by Paino *et al.*, [30] showed cytotoxic effects of sodium citrate and polyamidoamine capped gold nanoparticles on human PBMC's in a dose-dependent manner. The results also confirmed that immune cells are less sensitive to DNA damage when compared to cancer HepG2 cells.

Cellular responses during exposure or co-culture with nanomaterials are interpreted based on what is observed, however the thousands of cell cycle events that take place regardless of our observation may well be the missing link to enable scientists to monitor cellular immune functions [29, 30]. The effects of Au-ZnTe core-shell nanoparticles were further evaluated by investigating the expression of cell signaling proteins called cytokines. The serum from both PHA stimulated and non-stimulated PBMC's were evaluated for expression of IFN- γ , IL-1 β and TNF- α . The results showed expression of IL-1 β and TNF- α in very small amounts relative to concentration of core-shell nanoparticles, indicating the immunomodulatory effects of Au-ZnTe nanoparticles and the presence of a systematic functional immune system at the *in vitro* level. These results clearly indicate that accumulation of high concentrations of Au-ZnTe nanoparticles within

biological systems may trigger immune responses through the dose-dependent increased expression of these cytokines. The use of animals in laboratory research allows for biosafety evaluations across integrated organ systems and whole animal studies, providing more information on short and long term immune responses, enzyme function, cellular responses, cyto and genotoxicity. The acute toxicity effects and bio-interaction studies of Au-ZnTe core-shell nanoparticles were tested on Sprague-Dawley rats over an exposure of 7 days. The animals used in this study were handled in accordance with animal ethics committee and laboratory animal science protocols. Literature often highlights the importance of maintaining vital cellular and enzyme functions so that homeostasis is not interrupted through the interaction of nanoparticles [25-33]. In the blood circulatory system, red blood cells function in gaseous exchange, white blood cells provide immune protection whilst platelets initiate coagulation of blood [24, 25].

In addition the enzymes produced in the liver and kidney organs provide vital roles in detoxification and excretion physiology. ALT catalyses the transfer of an amino group from L-alanine to α -ketoglutarate, leading to the production of pyruvate and L-glutamate. ALP is responsible for dephosphorylating biomolecules. Gamma-GT plays a regulatory role at various levels in cellular signal transduction and cellular pathophysiology. Lactate dehydrogenase is found extensively in body tissues and cells. During tissue damage, LDH is released from destructed membranes, this enzyme is used as a marker of common injuries and disease. In the kidney urea serves an important role in the metabolism of nitrogen-containing compounds and the nitrogenous organic acid, known as creatine helps to supply energy to all cells in the body, primarily the muscle through

the increased formation of adenosine triphosphate [28-31]. These enzymes and protein molecules function systemically to maintain optimal liver and kidney functions. Evaluations of these parameters provide valuable information on the status of the kidney and liver functions during acute exposure to nanoparticles. Histomorphological evaluations are vital tools that can be used to assess tissue damage and/or cellular abnormalities after exposure to a treatment regimen. Although limited, LM evaluations can provide insight to the potential disruptions at the tissue level, thereby inferring change in functionality of the organ system. The functional unit of the kidney is the nephron, which comprises the Bowman's capsule and the glomerulus. LM evaluations show no significant changes in the treated samples which therefore suggests that there is no loss of kidney function in the treated groups as compared to the control. This notion is further confirmed by the data obtained for the renal functionality tests (**Figure 4.6 B**) that displayed no significant differences between the measurements for creatine and urea levels between the control and nanoparticle treated experimental animals.

The liver is comprised of many lobules which make up its functional unit. Each lobule contains millions of hepatocytes which are the basic metabolic cells of the liver. Assessments of the treated samples showed no change in the morphology of the hepatocytes which is therefore indicative of similar functionality of the liver between the treated and control animals. Interestingly, the data obtained for the ALT, ALT Phos, Gamma GT and LDH levels (**Figure 4.6 A**) from the liver functionality tests displayed no significant differences between control and treated groups, which directly correlates with inferences made from these histological evaluations. It can therefore be deduced that there are no acute toxicity of these organ systems after exposure to Au-ZnTe core-shell

nanoparticles for seven days. Throughout the experimental period, no behaviour changes or unusual responses were observed in the animals treated with low, intermediate and high doses of Au-ZnTe core-shell nanoparticles. The full blood count measurements indicated no significant changes across the control and nanoparticle treated rats. In addition the liver and kidney enzymes from the nanoparticle treated rats showed no significant differences when compared to the untreated control animals. Work reported by Lee *et al.*, showed the biopersistence effects of silver nanoparticle exposure to Sprague-Dawley rats [33].

The results confirmed that the size of the silver nanoparticles did not affect tissue distribution and other hematological parameters, however the exposure to silver nanoparticles did indicate a certain level of liver abnormalities. In another study silver nanoparticles and silver acetate was administered to rats over 28 days. This study showed that 63 % silver nanoparticles were excreted in the excrement [34]. In the present study a small percentage of Au-ZnTe core-shell nanoparticles were excreted in the urine. The liver and kidney tissues showed no signs of tissue damage or visible lesions. Work reported by Magaye *et al.*, used Sprague-Dawley rats to establish acute toxicity of nickel nanoparticles, the results showed no significant changes in red and white blood cell counts. In addition there were no significant histopathological changes in tissue sections from the rat lung, liver and spleen [35]. These findings suggest that increased exposure times and concentration of nanoparticles during acute animal studies play an important role in assessing *in vivo* biosafety of nanomaterials.

4.5 Conclusions

This chapter illustrates the *in vitro* and *in vivo* biosafety of Au-ZnTe core-shell nanoparticles through the use of various human cells and Sprague-Dawley rats. The use of human cell lines with different cellular environments indicated a comparative biosafety and bio-interaction potential of Au-ZnTe nanoparticles. The *in vitro* simulation of an immune response was clearly demonstrated. In addition, the results support the principle that demonstrates any material can be toxic if used in excessive quantities/ doses. The cell membranous interaction and cellular uptake observed in this work provides a key link in understanding the role of capping materials and surface chemistry of biocompatible nanoparticles. The study also provided important correlations between cellular, organ interactions and systemic circulation effects of Au-ZnTe nanoparticles. The full blood counts, liver and kidney functions appeared normal. The histological analysis of the liver and kidney tissue showed that the organs were not adversely affected by exposure to Au-ZnTe nanoparticles. These findings may be correlated to the serum levels of Gamma-glutamyl transpeptidase, alkaline phosphatase (ALK Phos), Alanine Aminotransferase (ALT), Lactate dehydrogenase (LDH), Urea and Creatinine (CRE). Future research will aim to understand and evaluate the degradation and elimination effects of bio-accumulated Au-ZnTe core-shell nanoparticles. Bio-accumulation of nanomaterials is an important contributing factor in nanotoxicity studies. Overall, the current study successfully demonstrated the biosafety and biocompatibility of Au-ZnTe nanoparticles through multiple experimental environments and sets the platform for future applications in drug delivery and bio-imaging through the use of surface modifications.

Reference

- Chatterjee, K., et al., *Core/shell nanoparticles in biomedical applications*. Advances in colloid and interface science, 2014. **209**: p. 8-39.
- Li, J., et al., *Toxicity of inorganic nanomaterials in biomedical imaging*. Biotechnology advances, 2014. **32**(4): p. 727-743.
- Ghosh, P., et al., *Gold nanoparticles in delivery applications*. Advanced drug delivery reviews, 2008. **60**(11): p. 1307-1315.
- Kondath, S., et al., *Synthesis and characterisation of morin reduced gold nanoparticles and its cytotoxicity in MCF-7 cells*. Chemico-biological interactions, 2014. **224**: p. 78-88.
- Kirchner, C., et al., *Cytotoxicity of nanoparticle-loaded polymer capsules*. Talanta, 2005. **67**(3): p. 486-491.
- Borm, P. and V. Castranova, *Toxicology of Nanomaterials: Permanent interactive learning*. Part Fibre Toxicol, 2009. **6**(3).
- Kumar KS, K.V., Paik, Pradip, *Recent advancement in functional core-shell nanoparticles of polymers: synthesis, physical properties, and applications in medical biotechnology*. Journal of Nanoparticles, 2013. **2013**.
- Thanh, N.T. and L.A. Green, *Functionalisation of nanoparticles for biomedical applications*. Nano Today, 2010. **5**(3): p. 213-230.
- Sun, Z., et al., *A general approach to the synthesis of gold-metal sulfide core-shell and heterostructures*. Angewandte Chemie International Edition, 2009. **48**(16): p. 2881-2885.
- Lim, D.-K., I.-J. Kim, and J.-M. Nam, *DNA-embedded Au/Ag core-shell nanoparticles*. Chemical Communications, 2008(42): p. 5312-5314.
- Park, H.-Y., et al., *Fabrication of magnetic core@ shell Fe oxide@ Au nanoparticles for interfacial bioactivity and bio-separation*. Langmuir, 2007. **23**(17): p. 9050-9056.
- Kayal, S. and R.V. Ramanujan, *Anti-cancer drug loaded iron-gold core-shell nanoparticles (Fe@ Au) for magnetic drug targeting*. Journal of nanoscience and nanotechnology, 2010. **10**(9): p. 5527-5539.
- Stark, W.J., *Nanoparticles in biological systems*. Angewandte Chemie International Edition, 2011. **50**(6): p. 1242-1258.
- Ghosh Chaudhuri, R. and S. Paria, *Core/shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications*. Chemical reviews, 2011. **112**(4): p. 2373-2433.
- Bigall, N.C., W.J. Parak, and D. Dorfs, *Fluorescent, magnetic and plasmonic—Hybrid multifunctional colloidal nano objects*. Nano Today, 2012. **7**(4): p. 282-296.
- Cui, W., et al., *Effects of aggregation and the surface properties of gold nanoparticles on cytotoxicity and cell growth*. Nanomedicine: Nanotechnology, Biology and Medicine, 2012. **8**(1): p. 46-53.
- Sohaebuddin, S.K., et al., *Nanomaterial cytotoxicity is composition, size, and cell type dependent*. Particle and fibre toxicology, 2010. **7**(1): p. 22.
- Sha, B., et al., *Cytotoxicity of titanium dioxide nanoparticles differs in four liver cells from human and rat*. Composites Part B: Engineering, 2011. **42**(8): p. 2136-2144.
- Freese, C., et al., *Uptake and cytotoxicity of citrate-coated gold nanospheres: Comparative studies on human endothelial and epithelial cells*. Part Fibre Toxicol, 2012. **9**(1): p. 23.
- Gliga, A.R., et al., *Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release*. Part Fibre Toxicol, 2014. **11**(11).
- Liu, Y., et al., *PEGylated FePt@ Fe₂O₃ core-shell magnetic nanoparticles: Potential theranostic applications and in vivo toxicity studies*. Nanomedicine: Nanotechnology, Biology and Medicine, 2013. **9**(7): p. 1077-1088.
- Li, T., et al., *Comparative toxicity study of Ag, Au, and Ag-Au bimetallic nanoparticles on Daphnia magna*. Analytical and bioanalytical chemistry, 2010. **398**(2): p. 689-700.
- Borm, P.J., et al., *The potential risks of nanomaterials: a review carried out for ECETOC*. Particle and fibre toxicology, 2006. **3**(1): p. 11.
- Oberdörster, G., et al., *Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy*. Particle and Fibre toxicology, 2005. **2**(1): p. 8.
- Dunpall, R., et al., *Synthesis of biocompatible Au-ZnTe core-shell nanoparticles*. Journal of Materials Chemistry B, 2015. **3**(14): p. 2826-2833.
- L'Azou, B., et al., *In vitro effects of nanoparticles on renal cells*. Part Fibre Toxicol, 2008. **5**(22): p. 651-63.
- Pujalté, I., et al., *Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells*. Part Fibre Toxicol, 2011. **8**(10): p. 1-16.
- Shann, S.Y., et al., *Size-and charge-dependent non-specific uptake of PEGylated nanoparticles by macrophages*. International journal of nanomedicine, 2012. **7**: p. 799.
- Vlata, Z., et al., *A study of zearalenone cytotoxicity on human peripheral blood mononuclear cells*. Toxicology letters, 2006. **165**(3): p. 274-281.
- Paino, I.M.M., et al., *Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells*. Toxicology letters, 2012. **215**(2): p. 119-125.
- Eidi, H., et al., *Cytotoxicity assessment of heparin nanoparticles in NR8383 macrophages*. International journal of pharmaceutics, 2010. **396**(1): p. 156-165.
- Reddy, M., et al., *Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function*. Journal of immunological methods, 2004. **293**(1): p. 127-142.
- Lee, J.H., et al., *Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats*. Part Fibre Toxicol, 2013. **10**(36): p. 16968-16973.
- Loeschner, K., et al., *Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate*. Part Fibre Toxicol, 2011. **8**(1): p. 18.
- Magaye, R.R., et al., *Acute toxicity of nickel nanoparticles in rats after intravenous injection*. International journal of nanomedicine, 2014. **9**: p. 1393.

**CHAPTER FIVE: The preparation, evaluation and *in vitro* application of Surface
Conjugated Drug-Antibody Au-ZnTe Core-Shell Nanoparticles**

5.1. Introduction

The medical application of chemotherapy drugs to treat cancer dates back to the early 20th century, during this period transplantable tumours in rodents was one of the first animal models that was used to screen anti-cancer compounds and establish their various molecular mechanisms of killing genetically damaged cells [1]. Chemotherapy is a well-established practice however due to the lack of targeting cancer cells specifically, this form of therapy presents several side-effects such as excessive nausea, diarrhoea, blood coagulation, hair and weight loss, compromised immune responses, organ failure and opportunistic infections [1, 2]. In addition to these side effects patients have to endure higher therapeutic dosing of the drugs to ensure adequate systemic circulation, constant serum level and bioavailability that is sufficient to kill the cancer cells or reduce tumour growth [3]. These challenges cause a decrease in patient compliance and clinical outcome, as a result there is a need to develop strategies that can improve the use of conventional chemotherapy drugs. Modern technologies within the discipline of pharmaceuticals and nanobiotechnology aim to address these challenges by improving the delivery of the chemotherapeutics to the target site, thereby decreasing the required unit dose and enhancing the efficacy of the drug [4-6]. In addition to their attractive size features nanoparticles offer multiple advantages that can be exploited for the development of novel, efficient and cost effective approaches to improving already existing cytotoxic drugs. Currently there are several nano-chemotherapeutic drug formulations that are in pre-clinical, clinical or FDA approved status for cancer therapeutics [7-9]. These formulations involve the combination of paclitaxel, doxorubicin, 6-mercaptopurine, cisplatin, 5-fluorouracil and various other anti-cancer drugs that are either internally loaded or externally surface conjugated to nanoparticles [10-12]. These nano-drug delivery vehicles can be

designed in the form of nano lipids, dendrimers, liposomes, magnetic, semiconductors or core-shell heterostructured nanoparticles [3]. A common characteristic that these nanoparticles share is a size range that does not exceed 100 nm. Doxil® is a FDA approved nano-drug that supports improved anti-cancer drug activity of doxorubicin by increasing the circulation time of the drug, decreasing the standard dose units and directing the drug to the target cell. A combination of these factors ensures more of the drug is delivered to the cancer cell and minimal systemic side effects are observed [7]. The nanoparticle therefore functions as a drug carrier and does not change the mechanism of action of the drug itself. Antimetabolite drug 5-fluorouracil or 5-FU, is a common cytotoxic agent that is used to treat head, neck, colon, pancreas, prostate, ovarian and breast cancers [11]. This drug displays a broad spectrum of activity because it acts by competitively inhibiting the enzyme thymidylate synthetase which is responsible for the synthesis of thymine nucleotides [13] (**Figure 1.5** Mechanistic pathway 5-fluorouracil). Thymine is one of four compulsory nucleotides that assembly to form DNA during the synthesis phase of cell division. 5-FU is able to act as a pyrimidine analogue substituting thymine in the nucleotide sequence which stops DNA synthesis and causes cell death. The cytotoxic mechanism of 5-FU is remarkable in destroying undesired cancer cells however this mechanism is not limited to cancer cells alone, during systemic circulation 5-FU is able to destroy normal cells and cause unwanted side-effects for the patient. The drug is metabolised rapidly within the body and therefore higher therapeutic doses are required to maintain serum levels in order to achieve the desired cytotoxic response. Another disadvantage is that aggressive tumour cells can develop resistance to the drug and evade the entry of 5-FU into the cell. The efficacy of 5-FU can be improved by localising the treatment to the tumour site, this would increase the bioavailability, cellular uptake and therapeutic activity of

5-FU. Nanotechnology provides a platform for developing unique strategies to improve and enhance the delivery and efficacy of 5-FU to cancer cells. These nano formulations are designed to provide chemical and physical stability, uniform capillary biodistribution and easy release of the drug once it has reached the target site. The nanoparticle itself should be non-toxic or biodegradable. In a study reported by Wang and co-workers, increased cell targeting and 5-FU efficacy comparative to 5-FU alone was demonstrated on HT29 cancer cells through the application of PLGA-1,3-diaminopropane folic acid nanoparticles. [14] Literature reports the use of numerous types of dendrimers, chitosan, lipid polymer, polysaccharide, and metal nanoparticles that was developed to improve the delivery and efficacy of 5-FU. **Table 5.1** illustrates the variations in nano-5-FU formulations and its applications.

Table 5.1 Nano-pharmaceutical formulations for improved efficacy of 5-FU

Nano-drug formulation	Nanomaterial properties	Method of toxicity/ efficacy evaluation	Application	Reference
1,4,7,10-tetraazacyclododecane dendritic polymers	Conjugated 5-FU	Hydrolysis of conjugated dendrimers for drug release	Controlled release of anti-tumour drugs	[15]
Poly(amidoamine) dendrimer	¹⁴ C labelled 5FU	Skin permeation of 5-FU was performed using Franz diffusion cell	Transdermal drug delivery of 5-FU	[16]
Ferritin conjugated solid lipid nanoparticles	5-FU loaded liposomes	<i>In vitro</i> drug release and cytotoxicity using MDA-MB-468 breast cancer cells	<i>In vitro</i> and <i>in vivo</i> tumour targeting	[11]
poly D, L (lactide-co-glycolide)(PLGA) nanoparticles	5-FU loaded	<i>In vitro</i> drug release, pharmacokinetics of 5-FU conjugates	Anti-microbial activity	[17]
N,O-carboxymethyl chitosan nanoparticles	5-FU loaded	Cellular uptake and drug release	Cancer drug delivery	[18]
Manganese doped zinc sulphide quantum dot nanoparticles	Folate acid 5-FU encapsulation	Cytotoxicity and cellular uptake of 5-FU loaded chitosan nanoparticles	Targeted therapeutics	[19]
Chitosan/gold nanocomposite	5-FU loaded	Cytotoxicity using MCF7 cells	Enhanced therapeutic efficacy of 5-FU	[20]
Layered double hydroxide nanoparticles	5-FU intercalated	<i>In vivo</i> tissue distribution	Drug delivery and biodistribution	[21]
Magnetite-polymeric core-shell nanoparticles	5-FU loaded	Release of 5-FU loaded nanoparticles	Active drug targeting	[3, 22]
Fe ₃ O ₄ @PSMA core-shell nanoparticles	5-FU conjugated	Tumour hyperthermia using magnetic nanoparticles	Receptor-ligand mediated drug targeting	[23]
Hollow mesoporous silica nanoparticles	5-FU grafted	Colorectal anti-cancer therapy using SW480 cells	Targeted-antibody EGF drug delivery	[12]

The understanding of cancer on the molecular and cellular level provides further innovative strategies to develop novel targeted nano-drug systems [3, 24-26]. Due to the excessive metabolic demands of the rapidly dividing cancer cells, the surface chemistry of a cancer cell is quite different from that of normal cell. Cancer cells require an additional supply of glucose, nutrients, oxygen, growth factors, proteins and hormones in order to sustain its uncontrolled growth [27]. Cancer cells evolve with each successive division to present enhanced cell membrane features such as over-expression of cell-surface proteins known as receptor or antigen molecules [28]. These proteins are able to specifically bind to their corresponding antibody or ligand molecules, attracting more growth factors and proteins into the cell which offset a series of cell cycles and support continuous cellular proliferation of damaged cells [25].

The selectivity of chemotherapeutic agents can be achieved through passive or active targeting using biocompatible nanoparticles [29]. Tumour tissues display disrupted vasculature that develops through the assembly of fractional or asymmetrical endothelial cell junctions which create pore like entry sites within the tumour mass [30]. The abnormal tumour vasculature displays enhanced permeability and retention effects which allow the nano-chemotherapeutics to passively enter and accumulate within the tumour site. This feature allows more nano-chemotherapeutics to selectively enter the tumour site especially since normal tissue displays regular vasculature that is less permeable to nano-chemotherapeutics. Alternatively active targeting of nano-chemotherapeutics involves the application of modified biocompatible nanoparticles that are covalently linked to suitable antibody molecules [31]. The use of antibody conjugates to improve delivery and uptake of various drugs is a well-known tool in pharmaceutical applications for cellular targeting. Some of these antibodies include the transforming growth factor alpha protein, vascular endothelial growth factor

antibody, anti-transferrin antibody and epidermal growth factors (EGF) [30]. These antibodies display unique binding mechanisms to their corresponding surface receptor sites, upon binding signal transduction, proliferation, tumourigenesis and angiogenesis is activated. The use of suitable drug directing or antibody molecules has been shown to enhance targeted drug delivery and efficacy of 5-FU [11]. The application of hollow mesoporous silica nanoparticles conjugated with epidermal growth factor and 5-FU was used to treat drug resistant colorectal cancer cells. The results displayed superior selectivity, internalisation and cytotoxicity of nano-chemotherapeutics that were conjugated to the EGF [12].

The selectivity and increased affinity of antibody EGF was illustrated using gold nanorods-EGF conjugates and a combination of malignant and non-malignant epithelial cells [32]. The study provided evidence of active targeting of nanoparticles even in the absence of a chemotherapeutic drugs. In another report, folic acid conjugated nanoparticles was used to demonstrate enhanced anti-cancer activity in cancer cells that display over-expression of folate receptors [33]. The exploitation of over-expressed cellular receptors have shown promising results in the development and application of nano-chemotherapeutics for breast, colon, prostate, ovarian, pancreas, lung and various other solid tumours [34]. The use of lipid, polymer, metallic and heterostructured nanomaterials can be assembled to support any combination of chemotherapeutic drug and antibody molecule [35]. A key concept in successful nano-chemotherapeutics would involve minimal drug accumulation in normal tissues, direct administration to the tumour mass, resulting in enhanced anti-cancer therapy and decreased associated harmful side effects. The diversity of such drug-antibody conjugates lays the foundation to revolutionise existing chemotherapy regimens allowing more nano-drug formulations to enter clinical trials [26].

The work presented in this chapter, reports the first synthesis and characterisation of drug-antibody surface conjugated Au-ZnTe nanoparticles using 5-fluorouracil and epidermal growth factor for enhanced *in vitro* anti-cancer therapy.

5.2 Experimental

5.2.1 Materials

Zinc chloride, L-cysteine ethyl ester hydrochloride, gold salt, tellurium powder, sodium borohydride, sodium citrate, 5 Fluorouracil and deionised water (HPLC grade) and acetone were obtained from Sigma Aldrich. Human epidermal growth factor antibody, fetal calf serum, pen-strep fungizone, trypsin and phosphate buffered saline was purchased from Whitehead Scientific. All the chemicals were of analytical grade and used as purchased.

5.2.2 Synthesis of surface conjugated 5FU-EGF-Au-ZnTe nanoparticles

In a 250 mL three-necked round bottom flask, tellurium powder (0.041 g, 0.32 mmol) was mixed with sterile distilled water (15.0 mL) at room temperature. A 15.0 mL aqueous solution of sodium borohydride (0.031 g, 0.79 mmol) was carefully added to the tellurium solution under inert conditions. After 2 h of reduction, 20.0 mL aqueous solution of ZnCl_2 (0.0436 g, 0.32 mmol) and 40.0 mL aqueous solution of L-cysteine ethyl ester hydrochloride (0.1188 g, 0.32 mmol) was simultaneously added to the dark purple tellurium ion solution. The solution was stirred for 30 minutes followed by pH adjustment. The reaction was allowed to proceed at pH 7, for 3 hours at 60°C. An aliquot of 40 mL of gold nanoparticle seed solution was added to the cysteine capped ZnTe solution and the reaction was allowed to proceed for 4 hours. The reaction mixture was filtered and centrifuged to give a dark greenish black material that was weighed and readily dispersed in sterile phosphate buffered saline at a concentration

of 1 mg/ mL. A solution of 1 mg/ mL of 5-FU was added to the solution of Au-ZnTe under sterile conditions and placed in a sonicator at 37 °C for 30 minutes. Thereafter a solution of human epidermal growth factor at 200 ng/ ML was added to the 5-FU-Au-ZnTe solution and sonicated for a further 30 minutes. The drug-antibody surface conjugated Au-ZnTe was then characterised and evaluated for anti-cancer efficacy studies.

5.2.3 Characterisation of surface conjugated 5FU-EGF-Au-ZnTe nanoparticles

5.2.3.1 Transmission electron microscopy

The drug-antibody Au-ZnTe nanoparticle solution was carefully placed on a formvar coated TEM grid. TEM imaging was performed using a Philips CM120 BIOTWIN TEM operated at 120kV.

5.2.3.2 Ultra-violet-visible spectroscopy

A Perkin-Elmer Lambda 20 UV-vis spectrophotometer was used to carry out optical measurements in the 200-1100 nm wavelength range at room temperature. Samples of Au-ZnTe and drug-antibody Au-ZnTe nanoparticles were placed in quartz cuvettes (1 cm path length) and the absorbance was recorded.

5.2.3.3 Photoluminescence spectroscopy

Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 luminescence spectrometer with xenon lamp over range of 200-800 nm. The samples were placed in quartz cuvettes (1 cm path length) and the excitation peaks were analysed and recorded.

5.2.3.4 Fourier Transform Infrared spectroscopy (FTIR)

Infrared spectra were recorded using a Bruker FT-IR tensor 27 spectro-photometer directly on small dry samples of cysteine, Au-ZnTe nanoparticles, 5-FU and drug-antibody conjugated Au-ZnTe nanoparticles in the range 450-4000 cm^{-1} .

5.2.3.5 Zeta potential

The Zeta potential of cysteine capped Au-ZnTe, 5-FU, cysteine and drug-antibody Au-ZnTe nanoparticles was determined by dynamic light scattering using a Zetasizer, (Malvern Nanoseries). Samples were filtered several times through a 0.22 μm Millipore membrane filter prior to recording surface charge measurements.

5.2.3.6 Cell culture and cytotoxicity

Cell culture reagents were supplied by Invitrogen and Whitehead Scientific. Adhesion cell lines, Human mammary adenocarcinoma (MCF7) cells were grown using EMEM culture media containing 10% fetal bovine serum and 1-2% penstrep fungizone. Cells that grew as monolayers were passaged, trypsinized and harvested for experimentation before reaching 100 % confluency. Cells were seeded at cell density of 4000 cells per well, into a 96-well plate. Following a 24 hour incubation, the growth medium was replaced with fresh medium (100 μL per well) and varying concentrations of Au-ZnTe, 5-FU-Au-ZnTe, 5-FU-EGF-Au-ZnTe nanoparticles and 5-FU (20, 40, 60, 80 and 100 $\mu\text{g/ml}$). The plate was further incubated for 48 hours. The control wells were treated with culture medium only. Wells containing culture medium without cells were used as blanks. All experiments were performed with five replicates. After the 48 hour incubation, the culture medium and nanoparticles were removed and replaced with fresh medium (100 μL) and 100 μL of MTT solution (5 mg/mL in PBS) in each well. After 4 hour incubation, the media and MTT solution was removed and 100 μL of

DMSO was added to each well to solubilize the MTT formazan. The optical density (OD) of each well was measured on a microplate spectrophotometer (Mindray MR-96A) at a wavelength of 540 nm.

5.2.3.7 Infra-red light assisted cytotoxicity

MCF7 cells were seeded at cell density of 4000 cells per well, into a 96-well plate. The cells were allowed to adhere to the plate for 24 hours, thereafter the growth medium was replaced with fresh medium (100 μ L per well) and 100 μ g/ml of Au-ZnTe, 5-FU and 5-FU-EGF-Au-ZnTe nanoparticles. A medical grade Infrared lamp was used to exposure the pre-treated MCF7 cells. The light exposure cycles were done for 5, 10 and 15 minute intervals at least 4 times per day. This procedure was repeated for 3 days before the cytotoxicity was evaluated using the MTT assay.

5.3 Results

5.3.1 Surface conjugation of drug-antibody Au-ZnTe nanoparticles

Cysteine capped Au-ZnTe nanoparticles were first prepared using the experimental protocol as described in chapter three. The reaction solution was maintained at physiological pH. The nanomaterial from this reaction was purified, quantified and resuspended in sterile phosphate buffered saline. During the first sonication step, 5-FU was added to Au-ZnTe nanoparticles in a 1:1 ratio. After 5-FU reacted with Au-ZnTe nanoparticles the solution appeared grey in colour. The addition of human EGF was followed by a second sonication step, after 30 min no colour changes was observed within the reaction solution. The material from the drug-antibody conjugation reaction was analysed for morphology, surface charge and optical properties to confirm the surface binding of 5-FU and EGF to Au-ZnTe nanoparticles.

5.3.2 Characterisation 5-FU-EGF-Au-ZnTe nanoparticles

FTIR spectra of cysteine, Au-ZnTe (**Figure 5.1**), 5-FU and 5-FU-EGF-Au-ZnTe materials (**Figure 5.2**) were compared to establish possible cysteine and drug-antibody binding interactions with the nanoparticles. The intensity of the characteristic $2550\text{--}2620\text{ cm}^{-1}$ S-H peak observed in the cysteine spectra is greatly diminished in the cysteine capped Au-ZnTe spectra, which could be due to the thiol linkage on the surface of Au-ZnTe nanoparticles (**Figure 5.1**). The FTIR spectra for 5-FU displays distinctive peaks in the regions $1448\text{--}1645\text{ cm}^{-1}$ and $3000\text{--}2995\text{ cm}^{-1}$ which represent C=N or C=C and stretches after C-H respectively. The peak at 1179 cm^{-1} was assigned to C-O stretch (**Figure 5.2**). Following the attachment of 5-FU to Au-ZnTe, there is a broad signal around $3350\text{ to }3500\text{ cm}^{-1}$ and new peak around $1000\text{ to }1200\text{ cm}^{-1}$, indicating one of the carbonyl group may be converted to O-H, as O-H displays a broad

signal around 3500 to 3200 cm^{-1} . In addition, N-H signal should appear between 3400 to 3500 cm^{-1} , but a shift to lower value around 3200 to 3300 cm^{-1} could also mean linkage to the nanoparticle. It is presumed that the amine group of cysteine reduces the carbonyl group of 5-FU forming a cyano group that stabilises 5-Fu to cysteine capped Au-ZnTe, this condensation reaction releases H_2O .

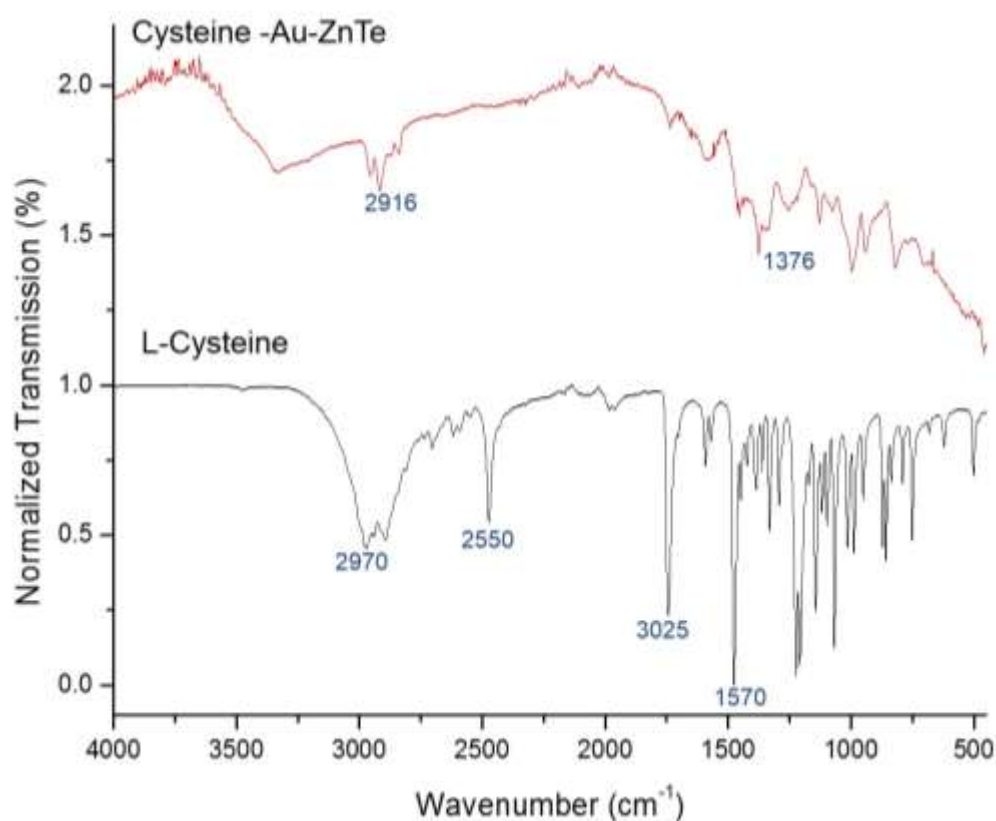


Figure 5.1 Comparative FTIR scans of cysteine capped Au-ZnTe nanoparticles and cysteine.

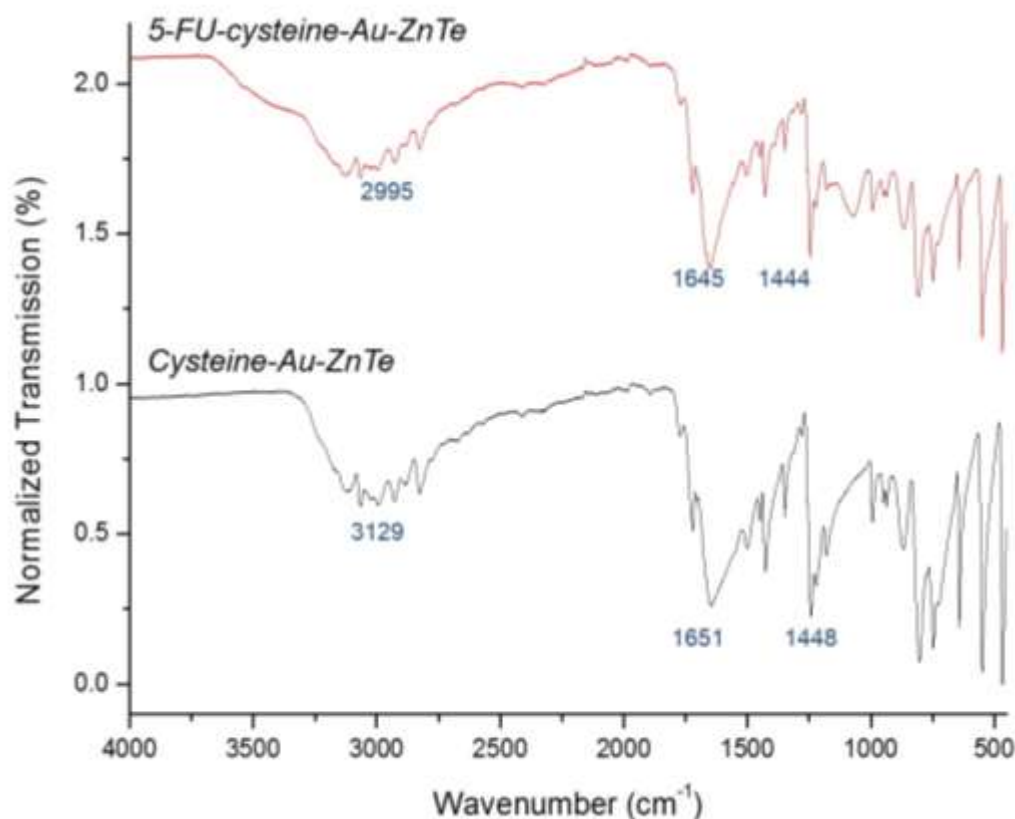


Figure 5.2 Comparative FTIR scans of 5-FU and antibody-drug conjugated Au-ZnTe nanoparticles.

The optical properties of 5-FU-EGF-Au-ZnTe nanoparticles was studied to confirm the surface conjugation of the drug and antibody. The photoluminescence spectra of the ZnTe and Au-ZnTe nanoparticles displayed a well-defined, gaussian shaped emission peak when excited at 300 nm which produced a constant peak at 415 nm in the PL spectra (**Figure 5.3 A**). The PL spectra in (**Figure 5.3 B**) displays the emission for EGF and 5-FU at 360 nm and 385 nm respectively. Additionally an emission peak at 385 nm with the highest intensity was observed when 5-FU-EGF-Au-ZnTe nanoparticles was excited at 315 nm (**Figure 5.3 B**). The drug-antibody surface conjugated Au-ZnTe nanoparticles displayed blue shifted emission properties from that of Au-ZnTe nanoparticles. The UV spectra of EGF and 5-FU displayed an absorption peak at 282 nm and 278 nm respectively (**Figure 5.3 C**). The absorption

properties of Au-ZnTe nanoparticles is dominated by the optical properties of Au showing an absorption maxima of 534 nm. The absorption of 5-FU-EGF-Au-ZnTe nanoparticles was observed at 272 nm. This absorption peak confirms a surface interaction between the drug and antibody (**Figure 5.3 D**). The changes in optical properties of EGF, 5-FU, Au-ZnTe and 5-FU-EGF-Au-ZnTe suggest that 5-FU and EGF was successfully attached to the surface of Au-ZnTe nanoparticles.

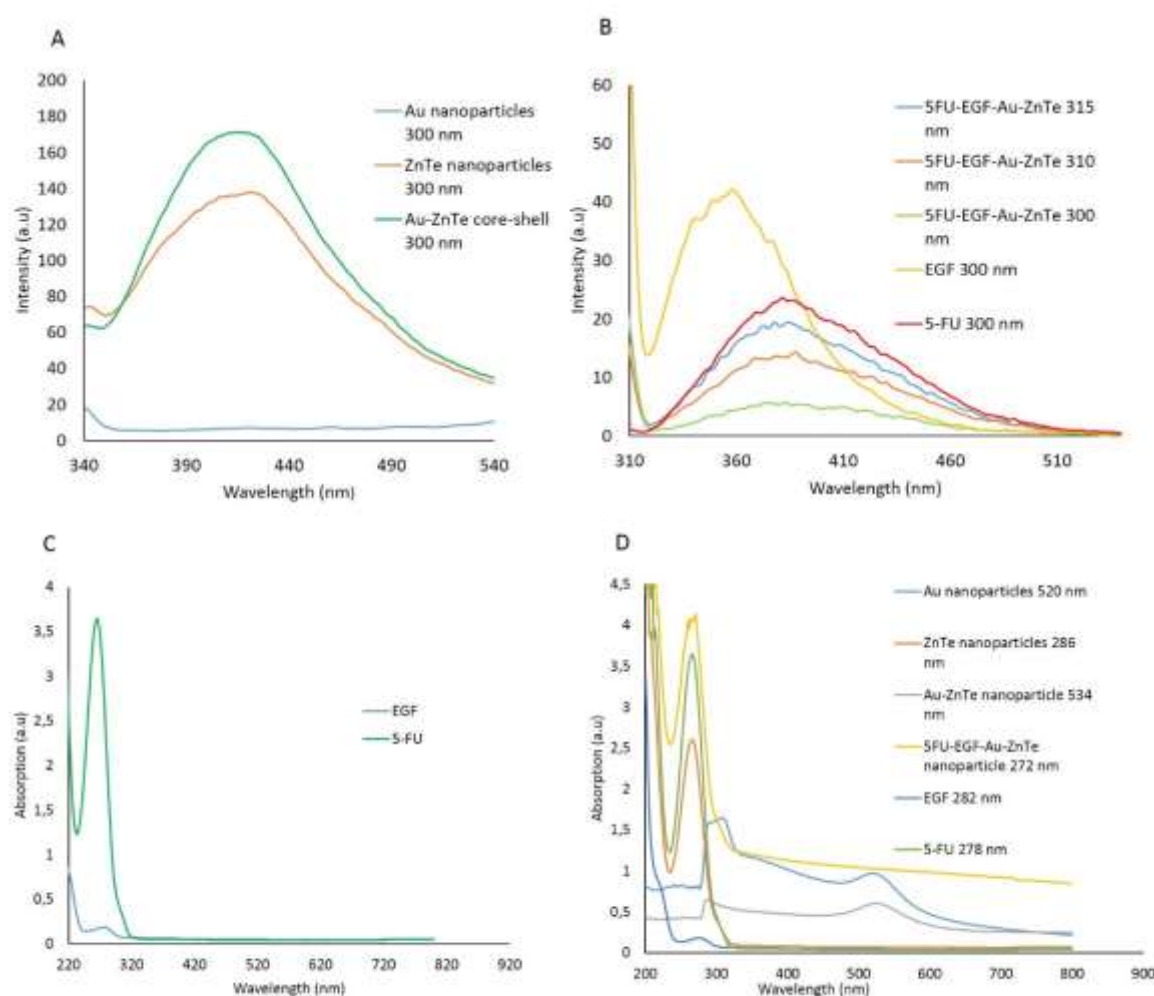


Figure 5.3 Optical properties of 5-FU-EGF-Au-ZnTe nanoparticles. **A-B.** PL spectra of Au, ZnTe and Au-ZnTe nanoparticles and corresponding absorption spectra of 5-FU-EGF and 5-FU-EGF-Au-ZnTe nanoparticle. **C.** Absorption spectra of EGF and 5-FU. **D.** Absorption spectra of the surface modified Au-ZnTe nanoparticle.

The morphological features of 5-FU-EGF-Au-ZnTe nanoparticles was studied to confirm any changes in size, shape or orientation that may be observed after surface conjugation. TEM micrographs of the original Au-ZnTe nanoparticles showed

spherically shaped particles with diameters in the 2-10 nm range (**Figure 5.4 A**). The average size of the particles are 7 ± 3.74 nm. TEM micrographs of 5-FU-EGF-Au-ZnTe nanoparticles displayed similar morphology to that of the original non-conjugated nanoparticles with diameters in the 7-12 nm range. The drug-antibody conjugated particles displayed a thin matrix layer that surrounded the assembly of nanomaterial (**Figure 5.4 B-E**). In addition new morphology patterns was observed as thin-stalk like threads or networks attached to bundles of uniform spherical particles. The drug-antibody nanoparticle sample also displayed some areas of aggregated particles. However the polydispersity index (PDI) using dynamic light scattering displayed a value of 0.44 ± 0.009 , indicating that although there was some degree of dispersity within the sample, the size distribution was not broad. The PDI value could be attributed to the interaction of 5-FU and EGF with the cysteine capped nanoparticle (**Figure 5.4 B-F**)

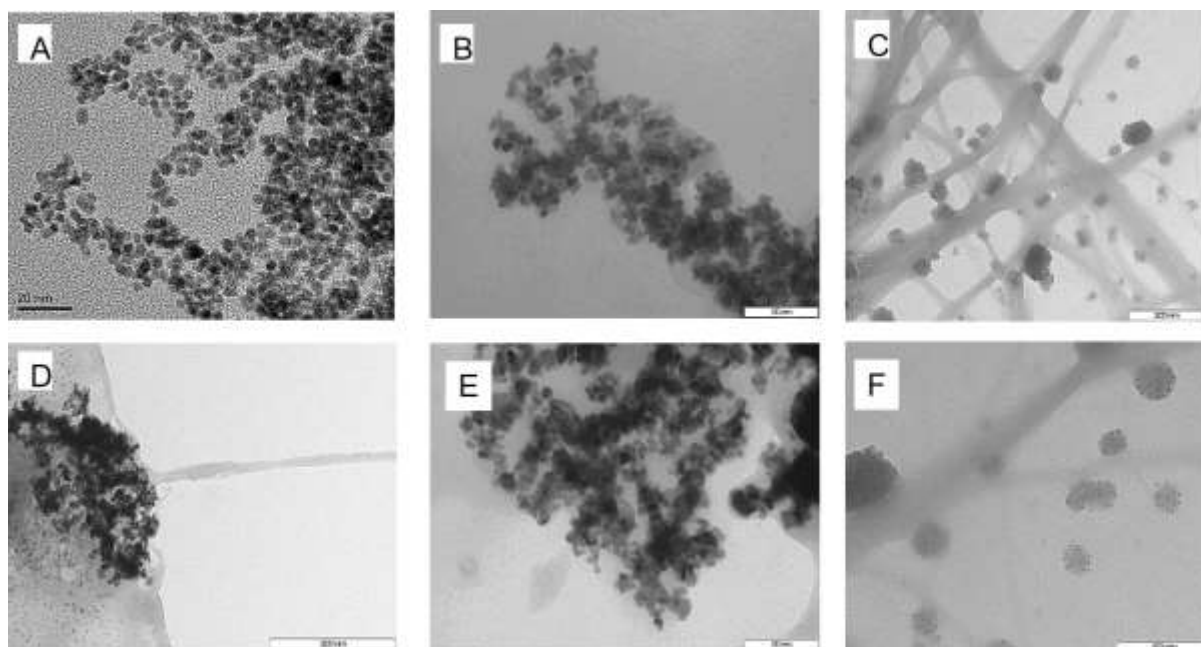


Figure 5.4 Morphology of 5-FU-EGF-Au-ZnTe (**A**) and antibody-drug conjugated Au-ZnTe nanoparticles (**B-F**)

The zeta potential properties of 5-FU-EGF-Au-ZnTe nanoparticles was analysed and compared to Au-ZnTe nanoparticles to confirm the changes in surface chemistry due to conjugation of drug-antibody molecules. The zeta potential values displayed in **Table 5.2** shows the respective surface charges of each material. The data indicates that cysteine capped the surface of Au-ZnTe nanoparticles influencing the core-shell particle charge to a lower value of -23.80 ± 1.90 mV. 5-FU displays a negative charge. Human EGF is 6045 dalton protein characterised as an assembly of 53 amino acids in the following sequence Asn-Ser-Asp-Ser-Glu-Cys-Pro-Leu-Ser-His-Asp-Gly-Tyr-Cys-Leu-His-Asp-Gly-Val-Cys-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-Asn-Cys-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys-Gln-Tyr-Arg-Asp-Leu-Lys-Trp-Trp-Glu-Leu-Arg. The combination of these 53 amino acids consists of non-polar aliphatic, aromatic, polar uncharged, negative and positively charged side groups however the surface charge of EGF is influenced largely by the total number of negatively charged side groups. This characteristic contributes to the negative surface charge -35.00 ± 0.60 mV observed for the 5-FU-EGF-Au-ZnTe sample.

Table 5.2 Zeta potential analysis

Material	Zeta Potential (mV)
Gold nanoparticles	-50.50 ± 1.05
Zinc telluride nanoparticles	-34.63 ± 1.00
Cysteine capping agent	-23.46 ± 2.65
Au-ZnTe core-shell nanoparticle	-23.80 ± 1.90
5-Fluorouracil	-17.73 ± 3.88
Drug-Antibody Au-ZnTe nanoparticles	-35.00 ± 0.60

Results represented as mean $\pm$ SD, n = 3

5.3.3 *In vitro* application of 5-FU-EGF-Au-ZnTe nanoparticles

An *in vitro* cytotoxicity study was used to establish the comparative therapeutic efficacy of 5-FU-EGF-Au-ZnTe nanoparticles. The cytotoxicity of 5-FU was established using MCF7 cancer cells. 5-FU displayed an IC_{50} value between 80-100 $\mu\text{g/ml}$, killing between 54-43 % of the MCF7 cell population respectively (**Figure 5.5 A**). For the purpose of this comparative study 100 $\mu\text{g/ml}$ of 5-FU was used as a negative control.

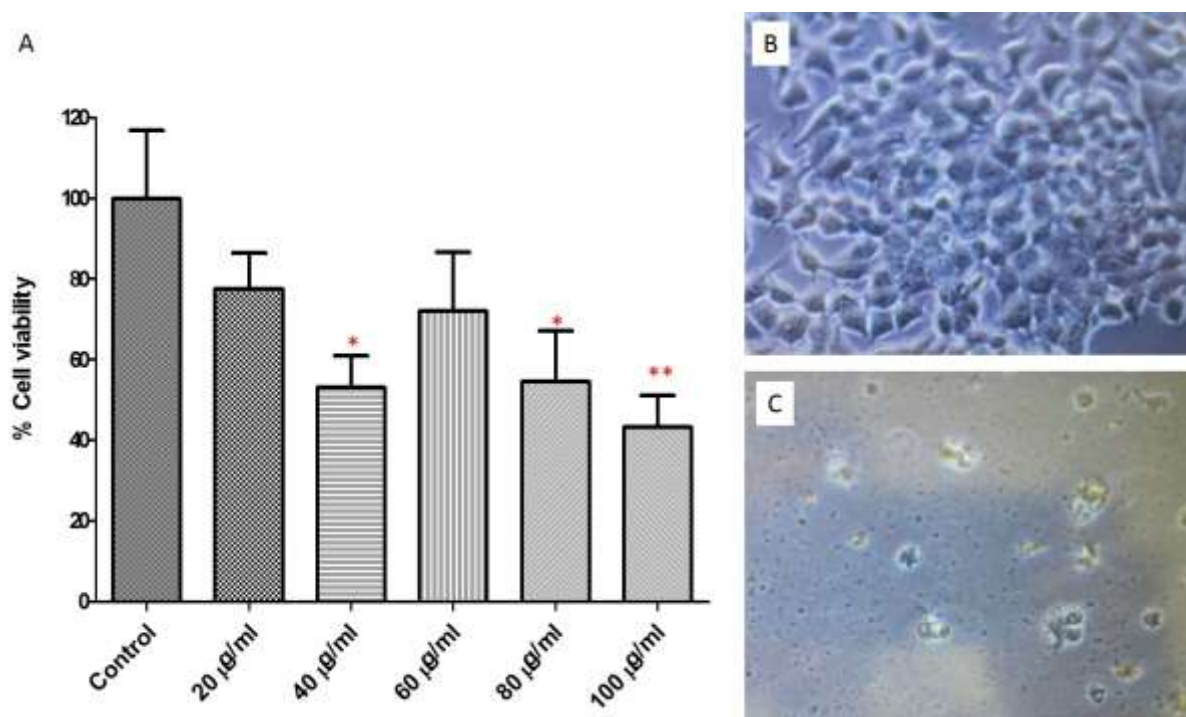


Figure 5.5. Cytotoxicity of 5-FU. **A.** Displays determination of the IC_{50} value for 5-FU activity against MCF7 cells. **B.** Untreated control MCF7 cells **C.** Displays the cytotoxicity effects of 100 $\mu\text{g/ml}$ of 5-FU on MCF7 cancer cells when compared to untreated MCF7 control cells.

The *in vitro* anti-cancer therapeutic efficacy study was performed using the MTT cytotoxicity assay. The study involved a comparative assessment of MCF7 breast cancer cells treated with Au-ZnTe, 5-FU and drug-antibody conjugated Au-ZnTe nanoparticles (**Figure 5.6 A**). The untreated MCF7 cells was used as a positive control, additionally cysteine capped Au-ZnTe nanoparticles was used as a second positive control to confirm their biosafety and non-toxic interaction with MCF7 cells

(**Figure 5.6 B, C**). As expected MCF7 cells treated with Au-ZnTe nanoparticles between 20-100 $\mu\text{g/ml}$ displayed no adverse effects on the relative growth of MCF7 cells, showing cell viabilities in the between 95-97% (**Figure 5.6 A**). Anti-cancer drug 5-FU at 100 $\mu\text{g/ml}$ induced 56.33 % cell viability (**Figure 5.6 D**). MCF7 cells treated with drug-antibody or 5-FU-EGF conjugated Au-ZnTe nanoparticles displayed enhanced anti-cancer effects over 5-FU alone (**Figure 5.6 A**).

The results show that 5-FU-EGF-Au-ZnTe nanoparticles are 24.74 % more effective than 5-FU at equivalent concentrations displaying a cell viability of 31.5%. In addition this data was supported by statistical analysis that showed extremely significant *p* value of 0.0079 of 5-FU-EGF-Au-ZnTe nanoparticles at 100 $\mu\text{g/ml}$ when compared to the untreated control and 5-FU treated samples. Furthermore the enhanced therapeutic efficacy of 5-FU-EGF-Au-ZnTe nanoparticles display 11.07, 9.08, 7.98 and 7.17 % increased cytotoxic effects at 80, 60, 40 and 20 $\mu\text{g/ml}$ of 5-FU-EGF-Au-ZnTe nanoparticles. MCF7 cells treated with 100 $\mu\text{g/ml}$ of 5-FU-EGF-Au-ZnTe nanoparticles displayed a huge population of dead cells, showing compromised cellular integrity, great loss in cell adhesion properties and low density of live cells (**Figure 5.6 E**). The anti-cancer effect of 5-FU conjugated to Au-ZnTe nanoparticles in the absence of EGF was studied (**Figure 5.7**). The results indicated cell viability of 80-88 and 54% for 5-FU conjugated Au-ZnTe and 5-FU respectively.

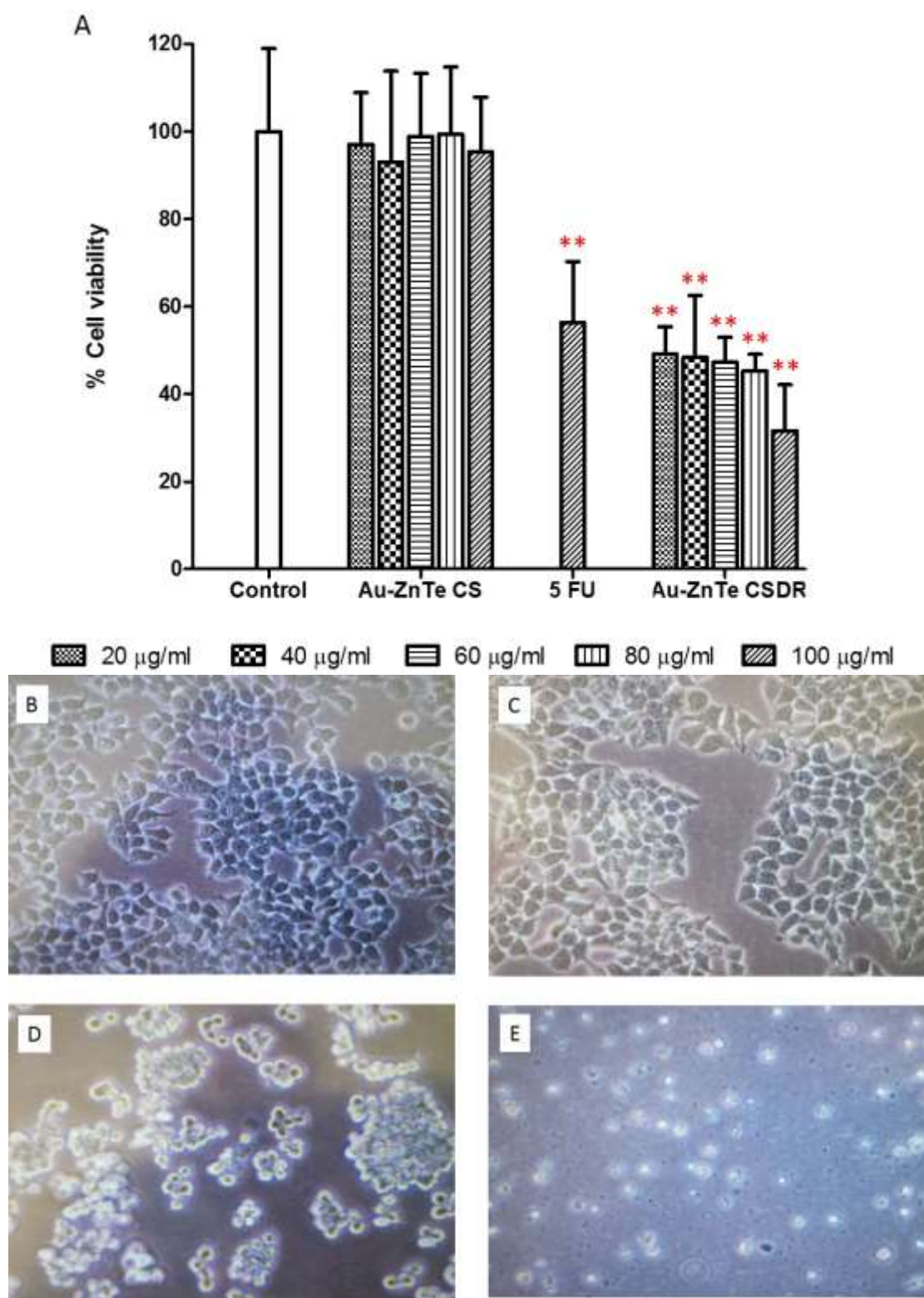


Figure 5.6 *In vitro* therapeutic efficacy. **A.** Displays comparative cytotoxicity of 5-FU and drug-antibody conjugated nanoparticles. **B.** Shows the morphology of untreated MCF7 control cells. **C.** Shows the morphology of Au-ZnTe treated cells. **D.** Shows the morphology of dead cells treated with 100 µg/ml of 5-FU. **E.** Shows the morphology of cells treated with drug-antibody conjugated Au-ZnTe nanoparticles.

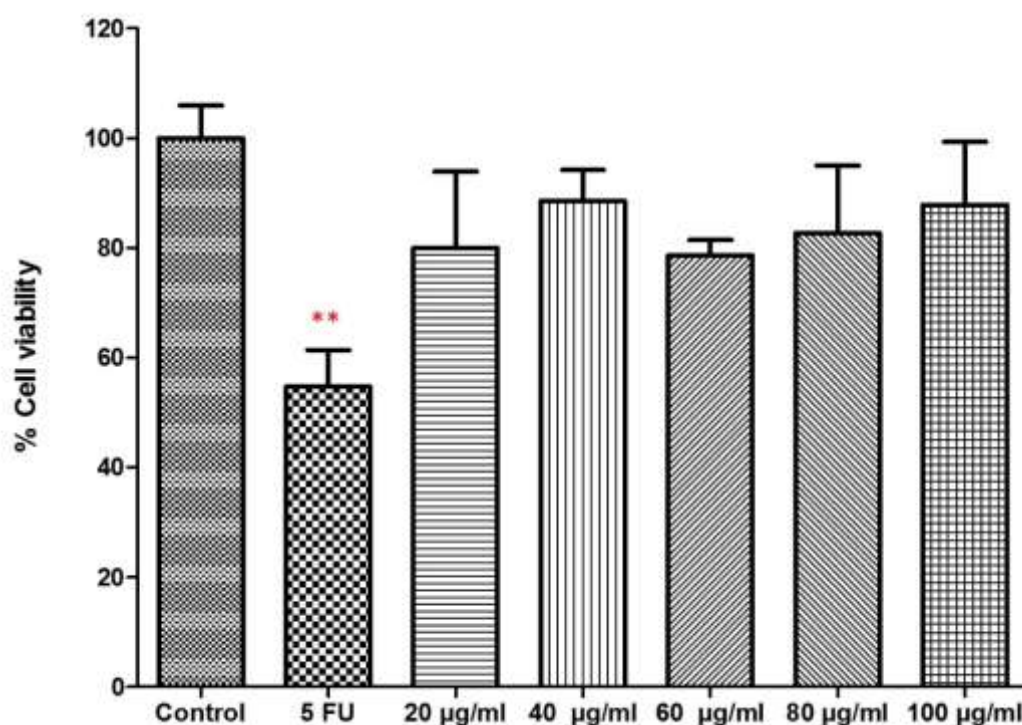


Figure 5.7 *In vitro* therapeutic efficacy of 5-FU-Au-ZnTe nanoparticles in the absence of human EGF.

In addition the *in vitro* anti-Cancer efficacy under infra-red light exposure of conjugated and non-conjugated Au-ZnTe nanoparticles was studied. Untreated control, 5-FU, Au-ZnTe and 5FU-EGF-Au-ZnTe nanoparticles at 100 µg/ml was used to study the effects of IR-light exposure (**Figure 5.8**). The infra-red light assisted cytotoxicity study was carried out 4 times a day for a duration of 5, 10 and 15 minutes (**Figure 5.8 A-D**). The MCF7 cells were treated with the respective controls and a medical grade IR-light, the results displayed no significant enhanced cytotoxicity when compared to the data from the cytotoxicity study presented in **Figure 5.6 A**. However the data did show statistically significant cytotoxicity when 5-FU was compared to the untreated MCF7 cells. Au-ZnTe nanoparticles at 100 µg/ml displayed no toxic effects to MCF7 cells. Additionally 5-FU-EGF-Au-ZnTe nanoparticles at 100 µg/ml displayed enhanced cytotoxicity when compared to 5-FU alone, however the results were not statistically

significant with relative cell viability between 41, 46 and 49% for 5, 10 and 15 minute IR-light exposed samples (**Figure 5.8 B-D**).

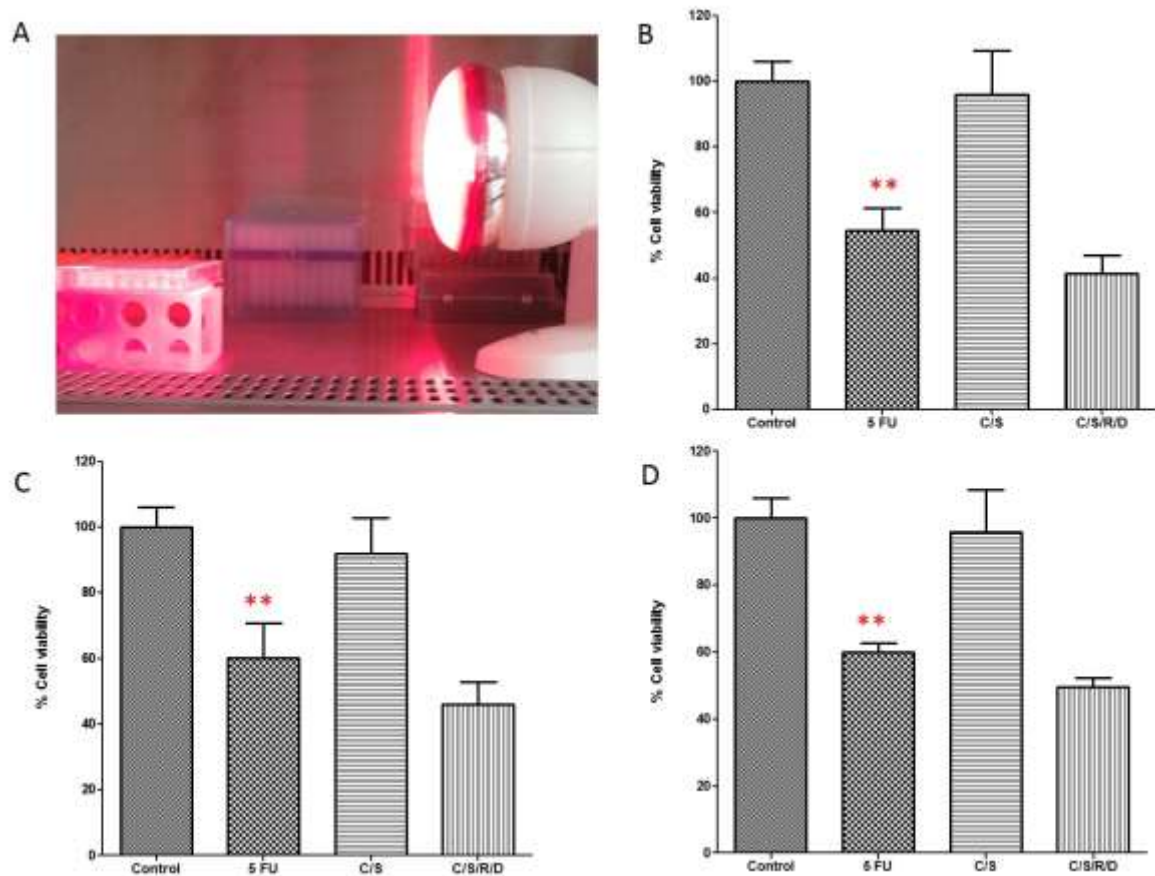


Figure 5.8 Infrared light assisted anti-cancer therapeutic efficacy. **A.** Medical graded Infrared light exposure to MCF7 treated cells. **B.** 5 minutes exposure to IR. **C.** 10 minutes exposure to IR. **D.** 15 minutes exposure to IR.

5.4 Discussion

5-Fluorouracil is one of the most successful anti-metabolite agents used to destroy rapidly dividing cells whilst in the **S** phase of the growth cycle. The application of 5-FU is however limited due to the lack of cell selectivity between normal and cancer cells. The formulation of 5-FU and nanoparticle complexes have been able to provide innovative strategies in targeting the cytotoxic drug directly to the desired mutated cells. Nanomaterials containing gold have received much recognition as colloidal drug carriers mainly due to their inherent optical and chemical properties which allow them to be both biocompatible and non-toxic. Gold-based nanomaterials can be designed to facilitate targeted drug delivery, tissue-imaging and photothermal ablation cancer therapy applications. There are two strategies used to formulate 5-FU+nanoarticle complexes, these involve either entrapment or loading of 5-FU inside the nanoparticle or conjugation of 5-FU to the surface of the nanoparticle.

In this study, 5-FU and human EGF was surface attached to Au-ZnTe nanoparticles using a simple, cost effective sonication method. The surface modified Au-ZnTe nanoparticles was evaluated to confirm this conjugation. FTIR analysis confirmed that cysteine binds to the surface of Au-ZnTe through the thiol group. The thiol functional group displays high binding affinity to metal nanoparticles. In an independent report L-cysteine displayed a thiol signal present at 2550 cm^{-1} however the thiol signal was not present in the L-cysteine capped gold nanoparticle spectra indicating that S-H had interacted with the surface of the gold nanoparticles forming Au-S during the growth process [36]. Similar observations were reported for cysteine interactions with CdS, CdSe, CdTe nanoparticles [37, 38]. The FTIR data shown in **Figure 5.2** supports the binding of 5-FU through interaction with the amine group from cysteine, followed by the subsequent cyano group signal in the FTIR spectra. This observation is common

for cysteine and 5-FU nanoparticle formulations. Work reported for the conjugation of 5-FU and Fe₃O₄ magnetic nanoparticles demonstrates that the N-H signal in 5-FU alone is present at 3100-3500 cm⁻¹ and in 5-FU- Fe₃O₄ nanoparticles the signal for the ring vibration at 1668 cm⁻¹ and the C=O stretch at 1726 cm⁻¹ appeared more intense indicating the presence of 5-FU [39]. The accumulation and conjugation of human EGF at the nanoparticle surface is possible through one of the following mechanisms such as conjugation via the carboxylic group of cysteine, hydrophobic and hydrophilic interactions from the 53 amino acid side group interactions, ion pairing or hydrogen bonding [40].

The photoluminescence emission for EGF and 5-FU was observed at 360 and 385 nm, similarly the emission peak for 5-FU-EGF-Au-ZnTe nanoparticles was observed at 385 nm, displaying a small blue shift from the emission wavelength recorded for Au-ZnTe nanoparticles (**Figure 5.3 A-B**). Additionally the UV spectra demonstrated a blue-shifted absorption peak for 5-FU-EGF-Au-ZnTe nanoparticles when compared to Au-ZnTe nanoparticles. The results indicate that both the drug and EGF dominates the emission and absorption properties of surface conjugated Au-ZnTe nanoparticles (**Figure 5.3 C-D**). The optical characterisation supports the interaction between the drug, antibody and the nanoparticle. The stability of the optical properties of nanoparticles is an important criteria especially for bio-imaging applications [41]. The optical properties of nanomaterials can be altered or enhanced through both surface conjugation and encapsulation of drugs [42, 43]. In addition size and shape plays key roles in the bio-applications of chemo-nano drug formulations. The morphology data shown in (**Figure 5.4**) displays similar size and shape features in both bare and drug-antibody conjugated Au-ZnTe nanoparticles. 5-FU-EGF-Au-ZnTe nanoparticles appeared as a self-assembly pattern of nanoparticles within a surrounding matrix. The

polydispersity value of 0.4 could be due to the surrounding matrix that forms after conjugation with 5-FU and EGF. The self-assembly and close interaction of nanoparticles is due to the biocompatible surface features which support conjugation of 5-FU [39].

The zeta potential analysis of 5-FU-EGF-Au-ZnTe nanoparticles displays a stronger negative charge when compared to Au-ZnTe nanoparticles and is therefore more stable for bio-applications [21]. The use of IR-light assisted therapy was used to determine how the optical properties of Au-ZnTe and conjugated Au-ZnTe nanoparticles could be used to facilitate enhanced or improved efficacy and drug knock off within the cancer cells. It is well known that gold-based nanoparticles display surface plasmon resonance properties which results in enhanced light scattering and absorption properties which enable the conversion of absorbed light into heat thereby destroying the cancer cell [44]. The data shown in **(Figure 5.8)** does not exhibit additional toxicity after IR-light exposure this is due to the size and shape of the gold based nanoparticles. In order for gold-based nanoparticles to cause photothermal destruction the nanoparticles need to be in a specific shapes, sizes, aspect length/width ratios that provides optical properties in the near-infrared region (650-900 nm) [32]. The size shape and surface chemistry of the nano-chemotherapeutics display key features in understanding both the improved anti-cancer efficacy and mechanism of destroying cancer cells. Chemo-nano drug formulations do not change the mechanism of the specific anti-cancer drug however the nanoformulations provide distinct advantages that are not present in conventional chemotherapeutics. Biocompatible nanoparticles act by increasing the surface area, bioavailability, maintaining serum concentrations and half-life properties of anti-cancer drugs. Conjugation with cancer cell markers enable increased efficacy and cell selectivity of

chemo-nano drug formulations. Cytotoxicity results highlighted in (**Figure 5.6**) demonstrates the favourable features that arise from chemo-nano drug formulations, showing an increased efficacy of 24.74% when compared to 5-FU alone. All concentrations of 5-FU-EGF-Au-ZnTe nanoparticles displayed higher cytotoxicity than of 5-FU alone. Additionally the results confirmed the biosafety of Au-ZnTe nanoparticles. The cytotoxicity results presented in (**Figure 5.6 and Figure 5.8**) confirm the biosafety and non-toxic effects of cysteine capped Au-ZnTe nanoparticles. In another report, Cysteine was used as a surfactant material to enable the stability, biodistribution and increase the uptake of 5-FU loaded poly-L-lactide nanoparticles. The *in vitro* cytotoxicity of 5-FU and 5-FU loaded poly-L-lactide nanoparticles was studied using normal human lung fibroblast cells, the data illustrated the biosafety of cysteine capped nanoparticles [45].

The use of nanoparticles to enhance therapeutic efficacy of various chemotherapeutics is well documented. Work reported for nanoformulations of 5-FU and Fe₃O₄ magnetic nanoparticles showed a similar trend in which HepG2 cells showed significant cell death after treatment with 5-FU conjugated nanoparticles in comparison to 5-FU alone [39]. In another comparative study 5-FU-folic acid conjugated PLGA nanoparticles was used to illustrate enhanced anti-cancer therapy when compared to 5-FU alone. A concentration of 50 µg/ml of 5-FU-FA-PLGA nanoparticles induced 28.02% cytotoxicity and free 5-FU induced 37.67% cell viability at the equivalent concentration. The data also showed increased cytotoxic effects of HT29 cancer cells especially for nanoparticles conjugated to folic acid. [14]. The use of targeting ligands incorporated into chemo-nano drug formulations often show drastic improvements in cytotoxicity of cancer cells [46]. A similar observation was illustrated for 5-FU-EGF-Au-ZnTe nanoparticles (**Figure 5.6 and Figure 5.7**). 5-FU-

Au-ZnTe nanoparticles was unable to induce enhanced cytotoxicity in the absence of human EGF (**Figure 5.7**). Anti-cancer therapeutics for breast cancer is strongly influenced by HER2 receptors which specifically bind to EGF [40]. Mechanisms by which chemo-nano drug formulations enter and exit cells are important aspects in developing future nanotherapeutics. Most gold based nanoparticles are taken up by non-specific mechanisms such as endocytosis or interactions with serum proteins [47].

Once the epidermal growth factor binds to the epidermal growth factor receptor present on the cancer cell surface, the ligand-receptor complex is activated offsetting a cascade of reactions causing the drug-antibody nanoformulation to undergo internalisation. The internalised drug-antibody nanoformulation is either isolated within vacuoles or after its breakdown the components such as the EGF are reprocessed to the cellular membrane to retain signal transduction function [48, 49]. The compromised cellular integrity displayed in (**Figure 5.6 E**) creates a challenge in identifying the cellular uptake of 5-FU-EGF-Au-ZnTe nanoparticles. In the illustration (**Figure 5.9**) we are able to demonstrate how 5-FU-EGF-Au-ZnTe nanoparticles can possibly enter the cell based on the molecular mechanisms of EGF.

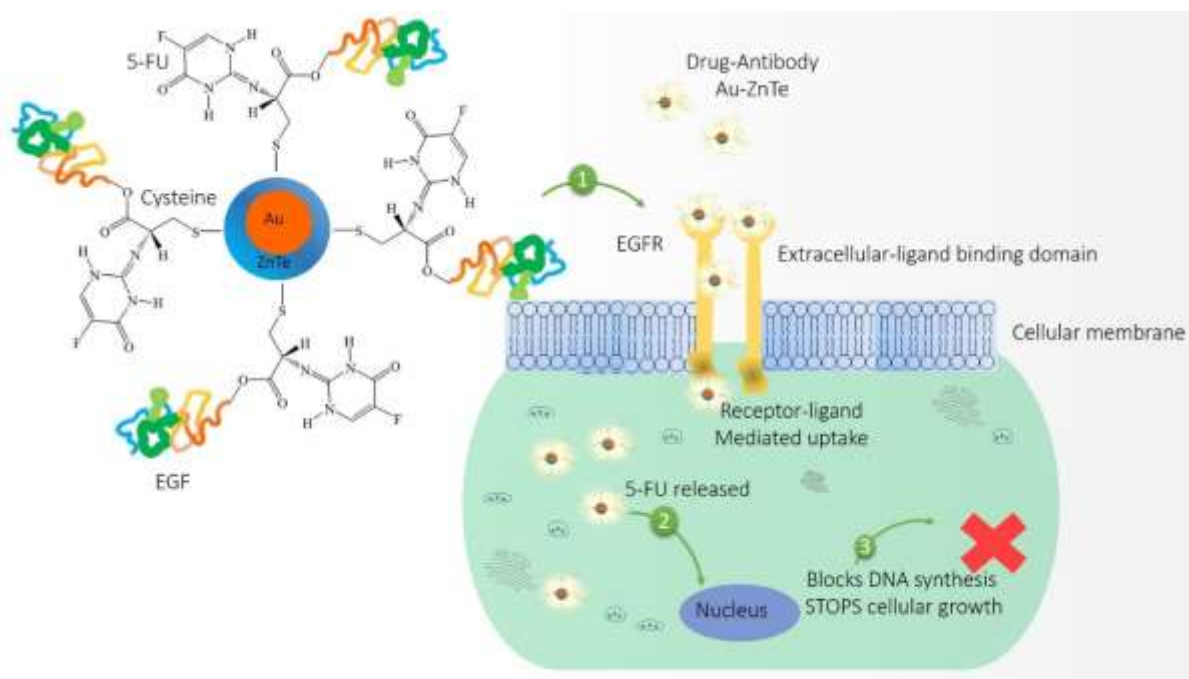


Figure 5.9 Receptor-ligand EGF-EGFR mediated cellular uptake and release of 5-FU-EGF-Au-ZnTe nanoparticles

The cellular uptake and isolation of Au-ZnTe nanoparticles has been previously presented [50] (**Chapter 3, Figure 3.9**). The Au-ZnTe nanoparticles displayed close interaction with the cell membrane and endocytosis. 5-FU has been shown to enter the cells through voltage gated channels presented on the cell surface [13]. In the present study the antibody plays a key role in directing the 5-FU-EGF-Au-ZnTe nanoparticle into the cell. The antibody EGF directs the chemo-nano drug formulation selectively to the cancer cells by binding to the highly expressed receptor sites located within the cell membrane (**Figure 5.9**). This process of internalising the drug-antibody nanoparticle is commonly known as receptor-mediated cellular endocytosis, which triggers a number of reactions that cause the drug-antibody nanoparticle formulation to disassociate once the EGF is signalled to return to the membrane. 5-FU and Au-ZnTe nanoparticles also separates once the EGF disassociates from 5-FU-EGF-Au-ZnTe nanoparticle formulation. 5-FU is then free to induce cytotoxic destruction of the cancer cell. This mechanism can be used to explain the poor cytotoxic response from 5-FU-Au-ZnTe nanoparticles in the absence of EGF (**Figure 5.7**), mainly because 5-

FU and Au-ZnTe nanoparticles form a complex that is unable to enter the cancer cell or it may enter the cell but the 5-FU remains conjugated to Au-ZnTe nanoparticles, without the energy released during the disassociation of EGF the drug and nanoparticle is not stimulated to separate within the cells cytoplasm. The results displayed in **Figure 5.7** shows 5-FU-Au-ZnTe nanoparticles induce 87% cell viability on MCF7 cells at 100 µg/ml. This implies that the overall cytotoxicity efficacy of 5-FU-Au-ZnTe nanoparticles is only 13% while at equivalent concentrations of 5-FU-EGF-Au-ZnTe nanoparticles the cell viability is 31.5% indicating cell death of 68.5%.

5.5. Conclusion

This study has successfully demonstrated the synthesis of surface conjugated 5-FU-EGF-Au-ZnTe nanoparticles. In addition the application of these nanoparticles displayed *in vitro* enhanced therapeutic efficacy in MCF7 cancer cells. The cytotoxicity studies have shown that all components in the 5-FU-EGF-Au-ZnTe nanoparticle formulation plays specific roles in improving anti-cancer therapy, in addition the drug, antibody and nanoparticle function synergistically to attack cancer cells. The pharmacokinetics of the 5-FU-EGF-Au-ZnTe nanoparticles will need to be investigated to provide clear understanding of its cytotoxic properties. Additionally the selectivity or targeted drug delivery needs to be demonstrated using co-cultures of normal and cancer cells in a single experiment or the use of *in vivo* models to illustrate the cell selectivity or targeting of 5-FU-EGF-Au-ZnTe nanoparticles. The use of further analytical and molecular modelling techniques must be investigated to establish further mechanistic insights between the drug, antibody and nanoparticle.

Reference

- DeVita, V.T. and E. Chu, *A history of cancer chemotherapy*. Cancer research, 2008. **68**(21): p. 8643-8653.
- Coates, A., et al., *On the receiving end—patient perception of the side-effects of cancer chemotherapy*. European Journal of Cancer and Clinical Oncology, 1983. **19**(2): p. 203-208.
- Arias, J.L., *Novel strategies to improve the anticancer action of 5-fluorouracil by using drug delivery systems*. Molecules, 2008. **13**(10): p. 2340-2369.
- Ahmed, N., H. Fessi, and A. Elaissari, *Theranostic applications of nanoparticles in cancer*. Drug Discovery Today, 2012. **17**(17): p. 928-934.
- Ali, I., et al., *Advances in nano drugs for cancer chemotherapy*. Current cancer drug targets, 2011. **11**(2): p. 135-146.
- Brannon-Peppas, L. and J.O. Blanchette, *Nanoparticle and targeted systems for cancer therapy*. Advanced drug delivery reviews, 2012. **64**: p. 206-212.
- Barenholz, Y.C., *Doxil®—the first FDA-approved nano-drug: lessons learned*. Journal of Controlled Release, 2012. **160**(2): p. 117-134.
- Li, S.Y. and M. Wang, *Novel core-shell structured Paclitaxel-loaded PLGA@Ag-Au nanoparticles*. Materials Letters, 2013. **92**: p. 350-353.
- Le, V.-M., et al., *An investigation of antitumor efficiency of novel sustained and targeted 5-fluorouracil nanoparticles*. European journal of medicinal chemistry, 2015. **92**: p. 882-889.
- Pullarkat, S., et al., *Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy*. The pharmacogenomics journal, 2001. **1**(1): p. 65-70.
- Jain, S.K., et al., *Development and characterization of 5-FU bearing ferritin appended solid lipid nanoparticles for tumour targeting*. Journal of microencapsulation, 2008. **25**(5): p. 289-297.
- Chen, L., et al., *Overcoming acquired drug resistance in colorectal cancer cells by targeted delivery of 5-FU with EGF grafted hollow mesoporous silica nanoparticles*. Nanoscale, 2015. **7**(33): p. 14080-14092.
- Longley, D.B., D.P. Harkin, and P.G. Johnston, *5-fluorouracil: mechanisms of action and clinical strategies*. Nature Reviews Cancer, 2003. **3**(5): p. 330-338.
- Wang, Y., et al., *Targeted delivery of 5-fluorouracil to HT-29 cells using high efficient folic acid-conjugated nanoparticles*. Drug delivery, 2015. **22**(2): p. 191-198.
- Zhuo, R.X., B. Du, and Z.R. Lu, *In vitro release of 5-fluorouracil with cyclic core dendritic polymer*. Journal of controlled release, 1999. **57**(3): p. 249-257.
- Venuganti, V.V.K. and O.P. Perumal, *Effect of poly (amidoamine)(PAMAM) dendrimer on skin permeation of 5-fluorouracil*. International journal of pharmaceutics, 2008. **361**(1): p. 230-238.
- Bozkir, A. and O.M. Saka, *Formulation and investigation of 5-FU nanoparticles with factorial design-based studies*. Il Farmaco, 2005. **60**(10): p. 840-846.
- Anitha, A., et al., *5-fluorouracil loaded N, O-carboxymethyl chitosan nanoparticles as an anticancer nanomedicine for breast cancer*. Journal of biomedical nanotechnology, 2012. **8**(1): p. 29-42.
- Mathew, M.E., et al., *Folate conjugated carboxymethyl chitosan-manganese doped zinc sulphide nanoparticles for targeted drug delivery and imaging of cancer cells*. Carbohydrate Polymers, 2010. **80**(2): p. 442-448.
- Nivethaa, E., et al., *An in vitro cytotoxicity study of 5-fluorouracil encapsulated chitosan/gold nanocomposites towards MCF-7 cells*. RSC Advances, 2015. **5**(2): p. 1024-1032.
- Choi, S.-J., J.-M. Oh, and J.-H. Choy, *Biocompatible nanoparticles intercalated with anticancer drug for target delivery: pharmacokinetic and biodistribution study*. Journal of nanoscience and nanotechnology, 2010. **10**(4): p. 2913-2916.
- Arias, J.L., et al., *Magnetite/poly (alkylcyanoacrylate)(core/shell) nanoparticles as 5-Fluorouracil delivery systems for active targeting*. European journal of pharmaceutics and biopharmaceutics, 2008. **69**(1): p. 54-63.
- Li, T.-J., et al., *In vivo anti-cancer efficacy of magnetite nanocrystal-based system using locoregional hyperthermia combined with 5-fluorouracil chemotherapy*. Biomaterials, 2013. **34**(32): p. 7873-7883.
- Shay, J.W., W.E. Wright, and H. Werbin, *Toward a molecular understanding of human breast cancer: a hypothesis*. Breast cancer research and treatment, 1993. **25**(1): p. 83-94.
- Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. cell, 2011. **144**(5): p. 646-674.
- Carter, P.J. and P.D. Senter, *Antibody-drug conjugates for cancer therapy*. The Cancer Journal, 2008. **14**(3): p. 154-169.
- Hsu, P.P. and D.M. Sabatini, *Cancer cell metabolism: Warburg and beyond*. Cell, 2008. **134**(5): p. 703-707.
- Weber, G., *Enzymology of cancer cells*. New England Journal of Medicine, 1977. **296**(10): p. 541-551.
- Mitra, S., et al., *Tumour targeted delivery of encapsulated dextran-doxorubicin conjugate using chitosan nanoparticles as carrier*. Journal of Controlled Release, 2001. **74**(1): p. 317-323.
- Acharya, S. and S.K. Sahoo, *PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect*. Advanced drug delivery reviews, 2011. **63**(3): p. 170-183.
- Haley, B. and E. Frenkel, *Nanoparticles for drug delivery in cancer treatment*. in *Urologic Oncology: Seminars and original investigations*. 2008. Elsevier.
- Huang, X., et al., *Cancer cell imaging and photothermal therapy in the near-infrared region by using gold nanorods*. Journal of the American Chemical Society, 2006. **128**(6): p. 2115-2120.
- Garcia-Bennett, A., M. Nees, and B. Fadeel, *In search of the holy grail: folate-targeted nanoparticles for cancer therapy*. Biochemical pharmacology, 2011. **81**(8): p. 976-984.
- Danhier, F., O. Feron, and V. Préat, *To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery*. Journal of Controlled Release, 2010. **148**(2): p. 135-146.
- Sega, E.I. and P.S. Low, *Tumor detection using folate receptor-targeted imaging agents*. Cancer and Metastasis Reviews, 2008. **27**(4): p. 655-664.
- Sarangi, S., A. Hussain, and S. Sahu, *Strong UV absorption and emission from L-cysteine capped monodispersed gold nanoparticles*. Applied Physics Letters, 2009. **95**(7): p. 073109.
- Priyam, A., et al., *Synthesis and spectral studies of cysteine-capped CdS nanoparticles*. Research on chemical intermediates, 2005. **31**(7): p. 691-702.
- Zhao, W., et al., *L-cysteine-capped CdTe quantum dots as a fluorescence probe for determination of*

- cardiolipin. *Analytical Sciences*, 2010. **26**(8): p. 879-884.
39. Banerji, B., et al., *Synthesis, characterization and cytotoxicity study of magnetic (Fe₃O₄) nanoparticles and their drug conjugate*. RSC Advances, 2012. **2**(6): p. 2493-2497.
40. MA Sadat, S., et al., *Nano-pharmaceutical Formulations for Targeted Drug Delivery against HER2 in Breast Cancer*. *Current cancer drug targets*, 2015. **15**(1): p. 71-86.
41. Qian, J., et al., *Bio-molecule-conjugated fluorescent organically modified silica nanoparticles as optical probes for cancer cell imaging*. *Optics express*, 2008. **16**(24): p. 19568-19578.
42. Chandran, P.R. and N. Sandhyarani, *An electric field responsive drug delivery system based on chitosan-gold nanocomposites for site specific and controlled delivery of 5-fluorouracil*. RSC Advances, 2014. **4**(85): p. 44922-44929.
43. Zhou, J.D., et al., *Study of the biological effectiveness of a nanosilver-epidermal growth factor sustained-release carrier*. *Experimental and therapeutic medicine*, 2013. **5**(4): p. 1231-1235.
44. Jain, P.K., I.H. El-Sayed, and M.A. El-Sayed, *Au nanoparticles target cancer*. *Nano Today*, 2007. **2**(1): p. 18-29.
45. Mishra, B.J., A. Kaul, and P. Trivedi, *L-Cysteine conjugated poly L-lactide nanoparticles containing 5-fluorouracil: formulation, characterization, release and uptake by tissues in vivo*. *Drug delivery*, 2015. **22**(2): p. 214-222.
46. Trail, P.A., *Antibody drug conjugates as cancer therapeutics*. *Antibodies*, 2013. **2**(1): p. 113-129.
47. Tomuleasa, C., et al., *Gold nanoparticles conjugated with cisplatin/doxorubicin/capecitabine lower the chemoresistance of hepatocellular carcinoma-derived cancer cells*. *J Gastrointest Liver Dis*, 2012. **21**(2): p. 187-196.
48. Sokolov, K., et al., *Real-time vital optical imaging of precancer using anti-epidermal growth factor receptor antibodies conjugated to gold nanoparticles*. *Cancer research*, 2003. **63**(9): p. 1999-2004.
49. Bouras, A., M. Kaluzova, and C.G. Hadjipanayis, *Radiosensitivity enhancement of radioresistant glioblastoma by epidermal growth factor receptor antibody-conjugated iron-oxide nanoparticles*. *Journal of neuro-oncology*, 2015: p. 1-10.
50. Dunpall, R., et al., *Synthesis of biocompatible Au-ZnTe core-shell nanoparticles*. *Journal of Materials Chemistry B*, 2015. **3**(14): p. 2826-2833.

**CHAPTER SIX: Development and characterisation of MCF7 mammary carcinoma
xenografts in a non-immunocompromised animal models**

6.1 Introduction

Over more than a century, the contribution of animals in scientific research has pioneered remarkable advances in medicine which has improved the quality of life for billions of humans and animals worldwide [1]. The discovery and application of prescribed drugs, antibiotics, vaccines, blood transfusions and organ transplants would not have been possible without the use of animal models. Animal research has provided key links in transforming medical technology, diagnosis, therapeutics and management of countless health disorders such as the human immunodeficiency virus, diabetes, high blood pressure, bacterial and viral infections, organ failure, neurodegenerative disorders and cancer. The study of cancer however is currently one of the most extensive and dynamic fields in the forefront of animal research [1, 2]. Chemotherapy is currently a commonly used treatment regime in breast cancer, which involves the use of cytotoxic drugs such as alkylating agents, antimetabolites, anti-tumour antibiotics, topoisomerase and mitotic inhibitors. The various classes of cytotoxic drugs are categorized according to their chemical structure and the mechanism of action in destroying viable cells [3-5].

Herceptin and aromatase inhibitors were amongst the first therapeutics investigated to treat breast cancer through its application in animal research [1]. Aromatase inhibitors are a class of drugs used to block the production of oestrogen hormone by inhibiting the enzyme aromatase which catalyses the conversion of androgens into oestrogens. Preliminary studies displayed positive therapeutic response of aromatase inhibitors in mice with oestrogen-receptor-positive cancers. These findings allowed aromatase inhibitors to undergo successful clinical trials and there after directed its FDA approval for use in patients suffering with hormone driven breast cancers which has since improved

the quality of life for millions of females worldwide [6]. *In vitro* research studies within the domain of breast cancer is also an important factor in establishing the efficacy of new chemotherapy drugs, tumour morphology, disease diagnosis and in understanding apoptotic pathways [7]. The human breast adenocarcinoma adherent cell line (MCF7) was established in 1973 by the Michigan Cancer Foundation, currently the MCF7 is one of the most predominant cell lines used to study breast cancer in both *in vitro* and *in vivo* experiments [8]. The MCF7 was derived from metastatic sites and display remarkable hormone sensitivity due to the overexpression of oestrogen receptors (ER) on the cell surface. In addition the cell line is well characterised and therefore a suitable choice for breast cancer research involving tumour growth, hormone and other targeted drug related therapies. *In vitro* techniques such as 3D and 2D cell culture, subcellular fractions, purified enzymes, proteins and nucleic acids have laid the foundation in cancer research [7, 9, 10]. These techniques are rapid and inexpensive. However *in vitro* techniques are often isolated and lack the ability to simulate homeostatic mechanisms presented within integrated organs and whole animal systems. The most common *in vivo* technique within animal models involves the study of tumours [11]. The growth of tumours are induced by chemical carcinogens, human tumour xenografts, transgenic or knockout models followed by the administration of chemotherapeutics or anti-tumour drugs [12].

Work reported by various researchers demonstrated the induction of mammary tumours in rats through the use of chemical carcinogens such as N-methylnitrosurea, dimethylhydrazine and 7, 12-dimethylbenz (a) anthracene [13-15]. These findings showed the importance of using tumours in understanding the pathogenesis of cancer and tumour development. Other chemical inducers of tumours include the administration

of high doses of growth hormones, oestrogens or aminofluorene compounds that stimulate the development of mammary tumours [16]. These methods succeeded in producing mammary tumours and providing a platform of ideal animal models that have generated innumerable links of knowledge in the area of oncology diagnosis and therapeutics. However, there are limitations to these techniques of inducing tumours, the chemical inducers are carcinogenic and toxic, harmful to the animal upon administration and offers low tumourigenicity rates that take up to 3-4 months before the appearance of an external tumour growth [17]. The development of rapid and efficient tumourigenicity or growth rate in xenograft techniques was achieved through the application of basement membrane extract (BME) during the early 1990s [18-20]. The basement membrane is the thin extracellular matrices that lie beneath epithelial, endothelial tissues and functions in cell adherence [20]. This membrane extract was first isolated from the Engelbreth-Holm-Swarm murine tumour and is composed of collagen IV, heparin sulphate proteoglycan, laminin and various other growth factors that work synergistically to promote cell growth and tumour formation [21]. Numerous research has been conducted to demonstrate the ability of BME to increase uniform tumourigenicity in xenograft applications. Most of these studies were performed on nude mice to induce mammary, pancreatic, prostate, lung and colorectal cancer tumours [18-22]. A drawback of this animal model is the lack of a well-functioning immune system. These animals are immunocompromised and require specialized breeding, handling and application protocols [19, 21].

The administration of hormones such as estrogen, growth hormone or cortisone can be used to assist and promote mammary tumour growth in rat and mice models [23, 24]. Estrogen is a vital female hormone that both controls and regulates the reproductive

organs and is responsible for various female characteristics such as body shape, menstruation, ovulation, physical appearance and lactation. 17β -estradiol is a common synthetic steroid derivative of estrogen and functions in the same manner as the naturally secreted hormone [25]. Estradiol is able to bind to the receptor molecules present on the cells membrane surface initiating a cascade of reactions that stimulate expression of specific genes [23, 26]. Those genes encode for proteins that can perform specific functions in promoting rapid mammary tumour development [27]. Cortisone is a hormone naturally produced by the adrenal cortex. Synthetic versions of cortisone are used in medical applications that require anti-inflammatory and anti-allergic responses [26]. The combination of cortisone treatment in xenograft techniques is useful in suppressing the animal's immune response to the introduction of external cells of human origin, allowing the injected cancer cells time to adhere and proliferate at the tumour injection site within the animal [28].

These tumour models provide a platform for understanding the growth, development, metabolism and therapeutic responses during drug development. Several biocompatible nanomaterials are specifically designed to deliver cancer drugs directly to the tumour site thereby increasing therapeutic responses and lowering unwanted harmful side effects that occur once normal cells are exposed to cytotoxic anti-cancer drugs [29]. There are various reports, investigating the use of biocompatible nanomaterials and animal tumour models for the development and understanding of targeted drug delivery, pharmacokinetics responses, photothermal destruction and bio-imaging of cancer tumours in mice and rats [30-35]. In the previous chapter, the *in vitro* enhanced efficacy of 5-FU-EGF-Au-ZnTe nano formulation when compared to 5-FU alone was confirmed

using MCF7 cancer cells. The work presented in this chapter aimed to induce and develop viable tumours on rat or mice animal models, for the *in vivo* application of 5-FU-EGF-Au-ZnTe nanoparticles. This study aimed to demonstrate the tumour inducing ability of BME using Sprague-dawley rats that have normal functioning immune systems over a period of 70 days. The findings from the 70 day study led to a more complex investigation involving the use of estradiol and cortisone assisted xenografts of MCF7 for mammary tumour development in BALB/c mouse models. **Figure 6.1** illustrates the complex morphology of the mammary glandular systems present in female Sprague Dawley rats and BALB/c mice. The target of these experiments was to induce mammary tumours directly on the thoracic glandular region of the animal using MCF7 cells in combination with BME, estradiol and cortisone. Once the tumour development was measurable in size (1-2 cm) the main objective was to evaluate the *in vivo* efficacy of novel 5-FU-EGF-Au-ZnTe nanoparticles.

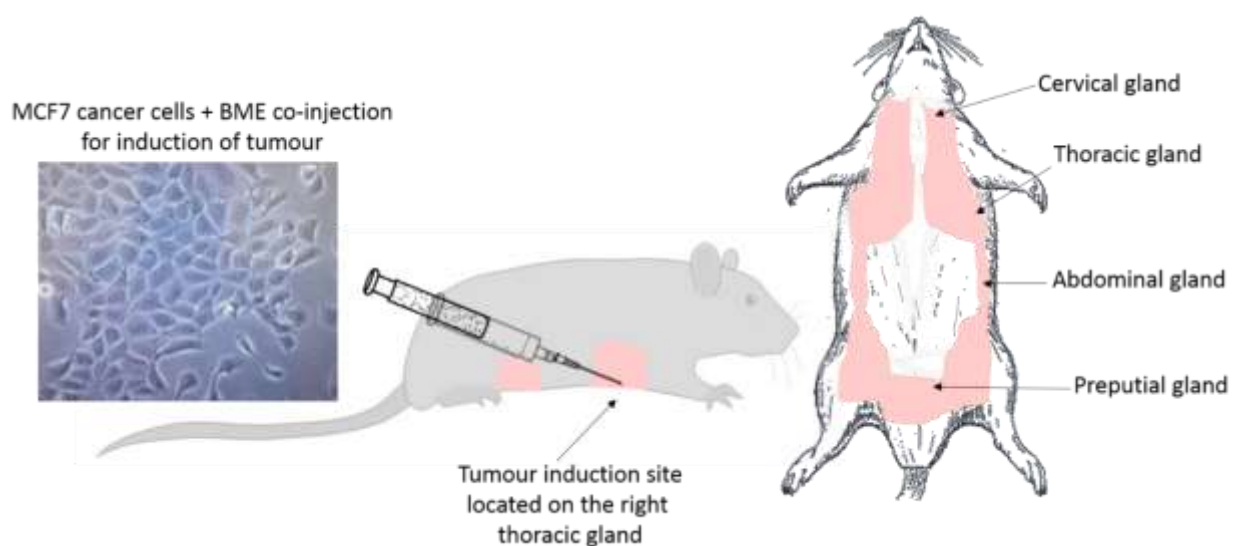


Figure. 6.1 Tumour induction site located on the right thoracic gland. An illustration of the mammary glands present in the Sprague Dawley rat and BALB/c animal model

6.2 Experimental

6.2.1 Ethics Statement

This research was approved by the University of KwaZulu Natal (UKZN) Animal Ethics Committee. All animal research was conducted at the Biomedical Resource Unit, UKZN. Animal Ethics number: 085/14/Animal and 002/15/Animal. The care and use of experimental animals in this study strictly complied with the approved University of KwaZulu-Natal animal welfare laws, guidelines and policies.

6.2.2 Cell culture

Modified eagle medium (MEM), Phosphate buffered saline (PBS), Trypsin, 100x pen-strep Fungizone (PSF) and fetal calf serum (FCS) were supplied by Whitehead Scientific. Estradiol and cortisone (Soli-Cortef) was purchased from Merck and Vet Care Animal Clinic respectively. Human breast (MCT-7) adenocarcinoma cell lines was purchased from American Type Culture Collection (ATCC) and used in the experiments. This adhesion cell line was grown in MEM culture media containing 10% FCS and 1-2% PSF.

6.2.3 Tumour induction using Sprague Dawley rats

The vial containing 15.79 mg/5ml of BME was removed from -80°C storage, placed on ice and allowed to thaw 24 hours prior to the tumour induction. Cells that grew as monolayers were trypsinized at passage two and calculated at a total cell density of 7.2×10^6 . Injections were prepared containing equal volumes of MCF7 cell and BME. A 22 gauge needle was used to carefully inject 1 ml of the preparations (MCF7 cells only; MCF7+BME) subcutaneously into the right thoracic gland of the female rats.

6.2.4 Tumour induction using BALB/c mice

The same cell culture and xenograft techniques were followed to induce tumours in BALB/c mice (Section 6.2.3). Estradiol powder containing 60 µg/kg body weight was dissolved in sesame seed oil and orally administered on alternate days using equal amounts of chocolate paste as described previously [36]. Cortisone at 4 mg/ml was administered daily for 3 consecutive days prior to the tumour induction through subcutaneous injections, each mouse received a total volume of 0.25 ml of Solu-Cortef as previously described [37-39]. Thereafter the same concentration of Solu-Cortef was administered in the drinking water every alternating day. The animals were monitored over a period of 91 Days.

6.2.5 Animals and conditions for tumour induction studies in Sprague Dawley rats

Four-week-old female, specific-pathogen free, Sprague Dawley rats were purchased from Biomedical Resource Unit, University of KwaZulu Natal. The 9 rats were housed and acclimatised for 7 days before starting the experiments. During the acclimatisation and experimental periods, the rats were housed in polycarbonate cages (maximum of 3 rats per cage) in a room with controlled temperature ($22.2 \pm 1.7^{\circ}\text{C}$), humidity ($48.4 \pm 6.0\%$), and a 12-h light/dark cycle. The rats were fed a rodent pellet food and water ad libitum. The rats were divided into three groups. Group A was the untreated control animals that received no injection. The animals in group B received a subcutaneous injection of human breast adenocarcinoma MCF7 cancer cells at a total cell density of 7.2×10^6 , the animals from group C, received a co-injection of human breast adenocarcinoma MCF7 cancer cells at a total cell density of $7.2 \times 10^6/5\text{ml}$ and 15.79 mg/5ml BME.

6.2.6 Animals and conditions for tumour induction studies in BALB/C mice

Four-week-old female, specific-pathogen free (SPF) BALB/c mice were purchased from Biomedical Resource Unit, University of KwaZulu Natal. The 25 mice were housed and acclimatised for 7 days before starting the experiments. During the acclimatisation and experimental periods, the mice were housed in polycarbonate cages (3, 5 and 6 per cage) in a room with controlled temperature ($22.2 \pm 1.7^{\circ}\text{C}$), humidity ($48.4 \pm 6.0\%$), and a 12-h light/dark cycle. The mice were fed a rodent pellet food and water *ad libitum*. The mice were divided into five groups (untreated control n=3, tumour induced untreated control n=5, tumour induced 5-FU control n=5 and two groups of tumour induced 5-FU-EGF-Au-ZnTe treated n=6). All animals in the study received subcutaneous injections of human breast adenocarcinoma MCF7 cancer cells at a total cell density of 12.2×10^6 mixed with 15.79 mg/5ml BME. The hormones were administered as described in section 6.2.4.

6.2.7 Hematological evaluation

On day 35 of the study, each animal was placed in a warming chamber for a few minutes, followed by blood collection through the lateral tail vein using a 22G needle. The blood (1 ml) was collected in heparinized vacutainers. White and red blood cell counts were analysed using a Beckman Coulter DXC600/800. Food was withheld for 12 hours before necropsy at the conclusion of the study. On day 70 of the study the animals were anaesthetised by an overdose of halothane followed by immediate bleeding and exsanguination by cardiac puncture. The blood was collected in heparinized vacutainers. Cell counts were analysed using a Beckman Coulter DXC600/800. The same procedure was followed on the last day of the hormone assisted tumour induction.

6.2.8 Histopathological evaluation

The injection site (mammary tissue) was carefully harvested, weighed and fixed in a 10% formalin solution containing neutral phosphate-buffered saline, embedded in paraffin, stained with hematoxylin and eosin, followed by microscopic histological evaluations using light microscopy techniques. For light microscopy (LM) evaluations, the tissue samples were removed from the fixative and dehydrated using a 50 to 96% ethanol gradient with the ethanol being subsequently cleared from the tissue using xylene. The tissue was then embedded in paraffin wax using standard procedures. The wax embedded tissue were sliced into 3 μm sections using a microtome, collected on glass slides, dried and stained with hematoxylin and eosin (H&E) using standard protocols. The stained sections were examined using a light microscope (LM; Nikon 80i, Kanagawa, Japan) and images were digitally captured using NIS Elements D software and a camera (Nikon U2, Kanagawa, Japan). All tissue evaluations were performed using a minimum of three replicates.

6.2.9 Statistical analyses

The statistical analyses were performed using GraphPad Prism® Version 5 software. The statistical evaluation included a two-tailed Student's t-test or analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

6.3 Results

6.3.1 Cell culture of MCF7 cells

The MCF7 cells were cultured under standard protocols until passage two, thereafter the cells were counted and prepared for injection into the tumour site of the rat according to the experimental design.

6.3.2 Animal observations during the 70 day tumour induction study

The animal's behaviour, activity, mobility, alertness, grooming, food and water consumption was monitored daily over the 70 day study. The animals from all groups appeared healthy, active and alert in addition their food and water consumption remained constant throughout the 70 days. The baseline weight of the animals at the start of the study was between 150-160 g (**Table 6.1**). The weight of the animals at the end of the study was between 217-227 g, which indicated a 70% weight increase across all groups over the 70 day period. Weekly examinations were performed to evaluate the presence of a tumour mass. No solid or external growth was observed in group B and C. The tissue from the injection site was removed and processed for histopathology analysis.

Table 6.1. Animal weight gain and tumour observations during the 70 day study.
The data is represented as mean $\pm$ SD (n=3)

	Group A Untreated control	Group B MCF7 cells	Group C MCF7 cells +BME
<i>Body weight at start (g)</i>	158 $\pm$ 5.57	161 $\pm$ 7.81	150.67 $\pm$ 6.66
<i>Body weight at end (g)</i>	225 $\pm$ 10.44	227 $\pm$ 5.51	217 $\pm$ 11.37
<i>% body weight increase</i>	70.40 $\pm$ 5.83	70.90 $\pm$ 5.21	69.44 $\pm$ 4.53
<i>Tissue removed from the tumour site weight (g)</i>	0.57 $\pm$ 0.16	0.81 $\pm$ 0.09	1.05 $\pm$ 0.14
<i>Clinical observations of tumor growth</i>	N/A	No signs of external tumour mass	No signs of external tumour mass

6.3.3 Hematological analysis of red and white blood cells

Blood vasculature is expected to increase as the tumour growth progresses. Hematological analyses were performed across all groups at day 35 and 70 to monitor any changes in the red and white blood cell concentrations. At day 35 the red blood cell (RBC) concentrations across all groups were $8.10 \times 10^6/\text{mm}^3$ (**Figure 6.2 A**), which is within the normal reference range of rat RBC ($6.76\text{-}9.75 \times 10^6/\text{mm}^3$). There was no statistically significant change ($p>0.05$) in the RBC values from day 35 to 70 as the values obtained on day 70 were between $7.64\text{-}7.87 \times 10^6/\text{mm}^3$ (**Figure 6.2 B**). The values obtained for the white blood cells (WBC) ranged between 3.5 and $4 \times 10^3/\text{mm}^3$ at day 35 (**Figure 6.2 A**). There was a decrease in the concentration ($1.5\text{-}2.07 \times 10^3/\text{mm}^3$) of WBC observed across all groups at day 70, however these decreases were not statistically significant when compared to day 35. The p values obtained were 0.1, 0.2 and 0.2 for group A, B and C respectively. Although there was a decrease in the WBC concentrations, group C, retained the highest WBC values at day 70 (**Figure 6.2 B**).

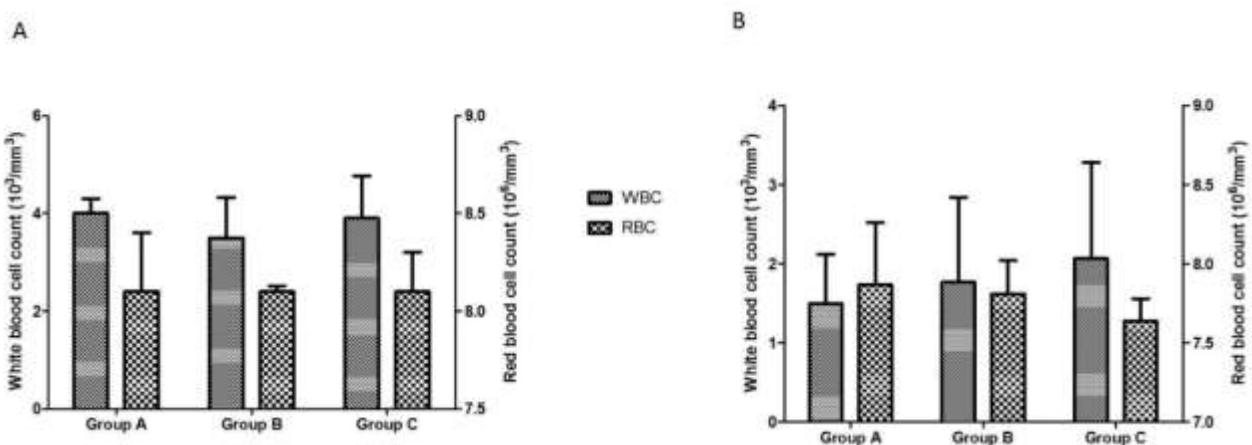


Figure 6.2 White and red blood cell counts (**A**) Shows the levels at day 35. (**B**) Shows levels at day 70. The data is represented as mean $\pm$ SD. All values compared to their respective control ($n=3$).

Differential WBC counts were performed to establish if there were any changes in the individual WBC populations from day 35 to day 70. The values obtained at day 35 were

all within reference range for these cell populations (**Figure 6.3 A**). With the exception of the monocytes and lymphocytes, the results showed a statistically non-significant decrease ($p>0.05$) in the other cell populations at day 70. The lymphocytes values increased from 65.23 to 77.67 % and 67.73 to 81.20 % in groups B and C respectively (**Figure 6.3 B**). Although there was an observable marked increase in these lymphocyte values, the increases were not statistically significant ($p>0.05$) when compared to animals from the control group.

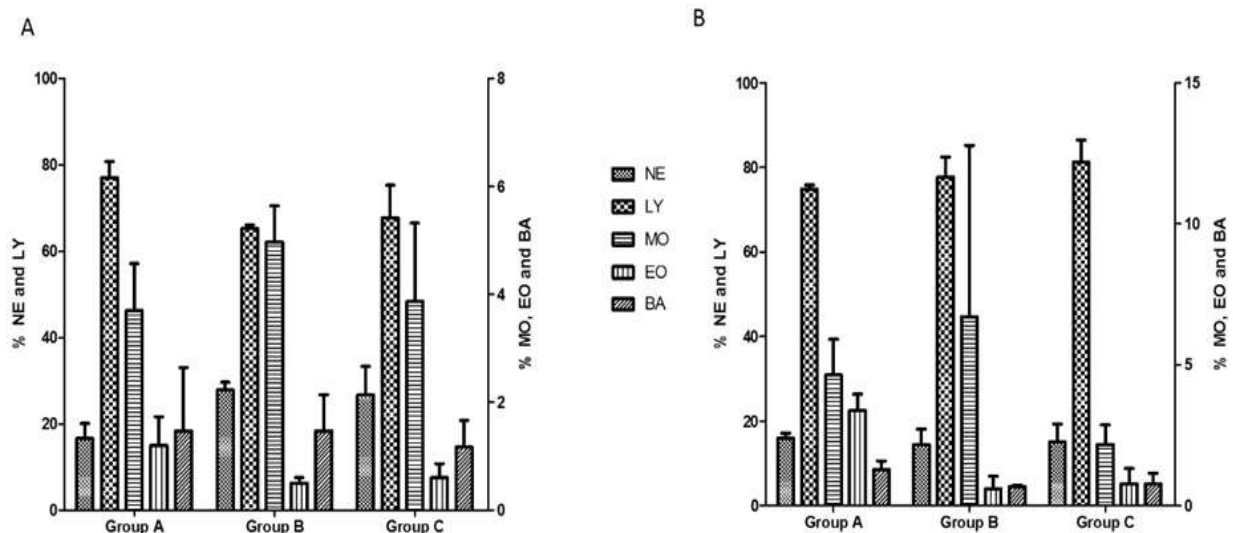


Figure 6.3 Differential white blood cell counts (**A**) Shows the percentage of cell populations at day 35. (**B**) Shows the percentage of cell populations at day 35. The data is represented as mean $\pm$ SD. All values compared to their respective control ($n=3$).

6.3.4 Histopathology evaluations of tumour site from rat models

The tissue of interest for histopathology evaluations was obtained from the injection site, situated on the right thoracic gland (**Figure 6.4 A**). The micrographs from the untreated control group, closely resembles that of normal white adipose tissue found in large closely packed masses between the lobules, with cells having regular morphology and peripherally located, darkly stained nuclei (**Figure 6.4 B**). Animals from group B (**Figure**

6.4 C and D) and group C (**Figure 6.4 E and F**), displayed the accumulation and growth of clusters of MCF7 cells between the adipose tissue cells. Although both the treated groups exhibited this growth pattern, the micrographs of the MCF7 cells + BME treated group showed larger clusters of MCF7 cells inter-dispersed between the adipose cells when compared to the MCF7 treatment (**Figure 6.4 E and F**). The larger clusters of cells observed in group B is attributed to the properties of BME in promoting and stimulating increased growth and adherence of higher cell numbers to the injection site. Quantitative analysis of the colony size was established through measuring the surface area occupied by the MCF7 cancer cells from multiple observations of the tissue sections. The MCF7 treated tissue sections displayed surface areas ranging from 504 to 788 μm^2 . The MCF7+BME treated tissue sections displayed surface areas ranging from 4693 to 7637 μm^2 (**Figure 6.4**).

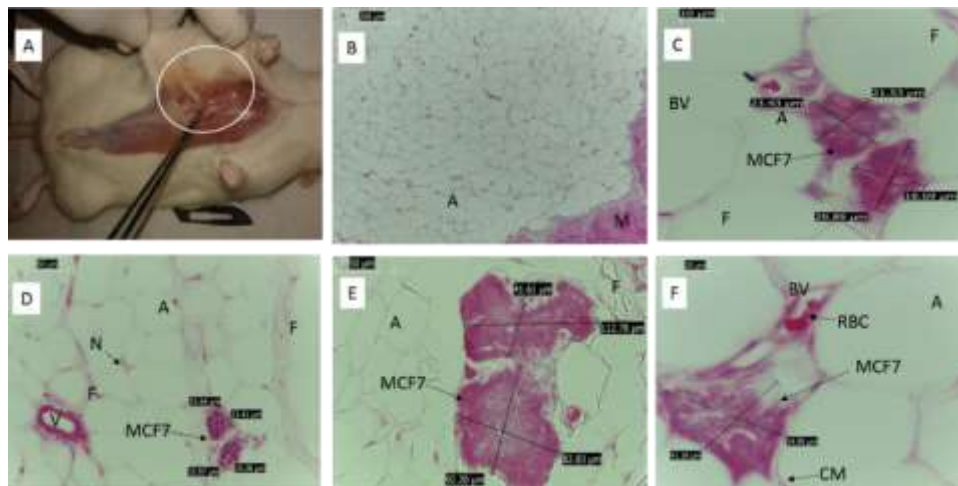


Figure 6.4 Histopathology evaluations of tissues for the tumour injection site using light microscopy. (A) Displays the location on the right thoracic gland that was used as the injection site on the Sprague-dawley rat. (B) Tissue morphology of mammary tissues from the untreated control group at 10x magnification. (C) Displays the localization of MCF7 cells within the adipose tissue at 40x magnification (D) shows the growth of MCF7 cells in close proximity of blood supply at 40x magnification. (E) Displays larger clusters of MCF7 cells within the adipose tissue at 40x magnification. (F) Displays MCF7 cells growing close to blood vessels within the adipose tissue at 100x magnification. (A=adipose cells, BV= blood vessel, F= fibrous interlobular tissue, N= Nucleus of adipose cells, CM= Cell membrane of adipose tissue, RBC= Red blood cel

6.3.5 Tumour development in BALB/c mice

The hormone assisted tumour induction in BALB/c mice (12-15 g in body weight) was investigated over a period of 91 days, during this time the animals appeared to display normal behavioral, eating and breathing patterns. The animals maintained normal weight gain throughout the study and weighed between 22-26 g at the end of the study. By day 30 of the study, 1 animal died from inflammatory uterine disorder, and 2 animals from an over-exposure of halothane. The histopathology of the tumour site was investigated on day 30. The effects of estradiol was evident in the enlarged uterus and bladder observed in **Figure 6.5 B** and **C**, upon closer histological examination the tumour injection site displayed regular white and brown adipose supporting normal mammary tissue morphology (**Figure 6.5 D-F**). No MCF-7 cells were observed.

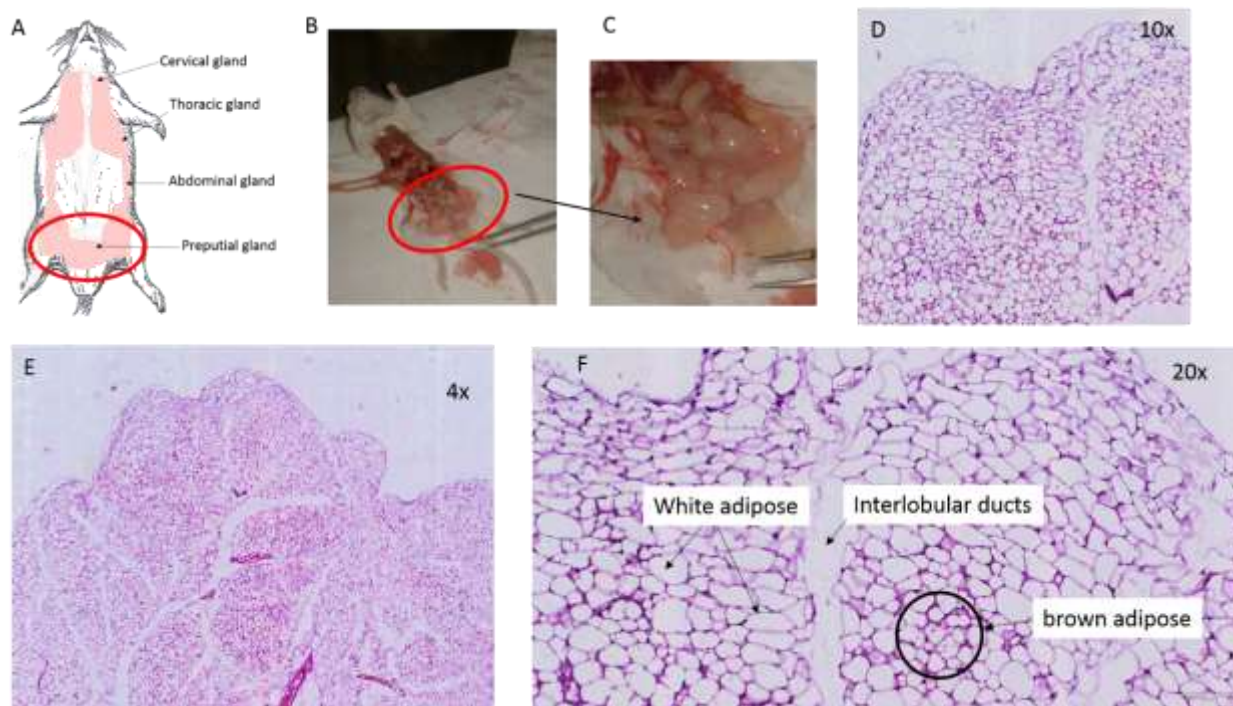


Figure 6.5 Histopathology of BALB/c mice at day 30. (A) Mammary gland systems, (B-C) Enlarged uterus and bladder, (D-F) Light microscope cross sections of tumour site tissues

6.3.6 Histopathology evaluations of tumour site from mouse models

After the observations from day 30 of the study, a second tumour induction was performed using cortisone in combination with Estradiol. During this study the animals displayed no signs of external tumour development at the site of injection. However following the administration of cortisone, it was observed that 1 animal died every 2 days. After terminating the study (day 91). The tumour site was studied carefully. Animals from the untreated control group showed normal breast tissue morphology at low magnification (**Figure 6.6 A**) with an abundance of white adipose tissue surrounding the lobules. At a higher magnification (**Figure 6.6 B**) the adipose tissue cells display regular morphology and darkly stained peripheral nuclei.

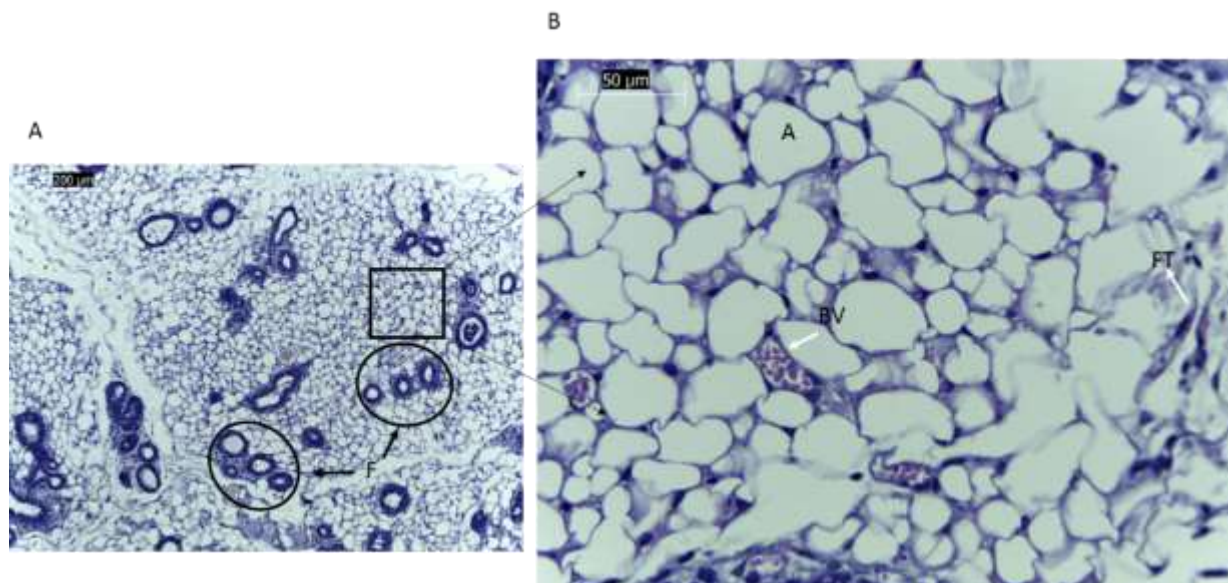


Figure 6. 6 Cross-section of mammary tissues from BALB/c mice. **(A)** Micrographs of control mammary tissue at 10x magnification. **(B)** Micrographs of control mammary tissue at 40x magnification. A=adipose cells, BV= blood vessels, FT= fibrous interlobular tissue, F= Acini alveoli)

The presence of glandular tissue in the breast is dependent on hormonal activity, stimulated by various hormones including estrogen. In the control slides there are a few lobules which are small in size and have small lumens. The lobules in these tissue

sections are typical of non-lactating breast tissue and are lined by two layers of epithelial cells. Low magnification micrographs (**Figure 6.7 A**) from the tumour induced treated group shows a significant increase in the number of lobules as compared to the untreated control group (**Figure 6.6 A**). At low (**Figure 6.7 A**) and high (**Figure 6.7 B**) magnification the tissue samples closely resemble that of lactating breast tissues. The lobules appear larger, with a huge lumen and contain secretions within them.

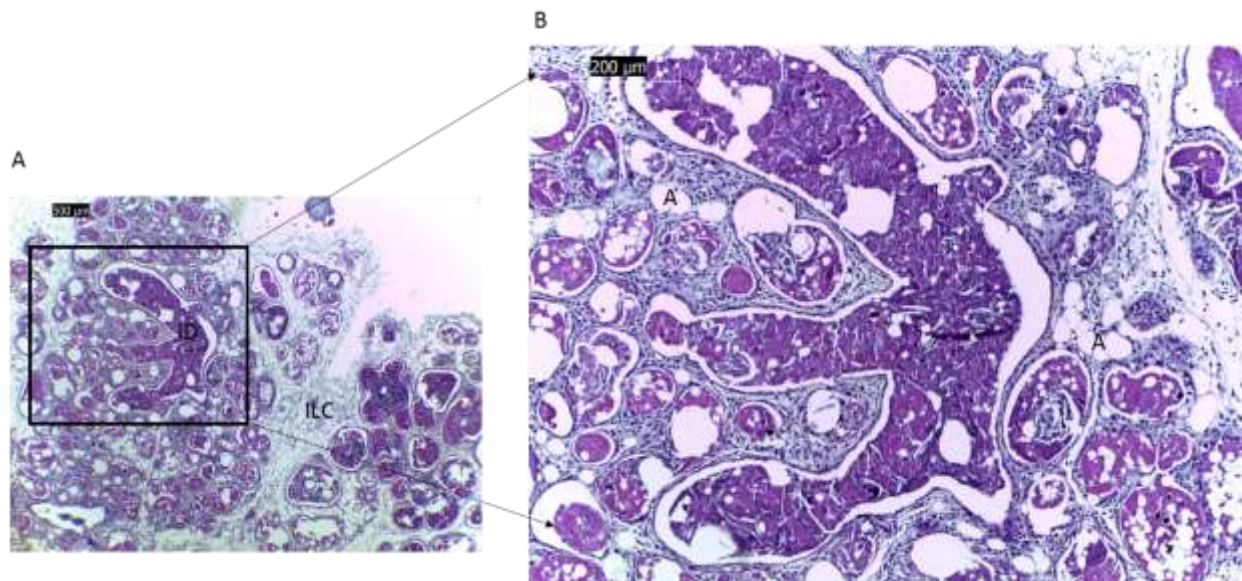


Figure 6.7 Cross-section of mammary tissues from tumour induced BALB/c mice. (A) Micrographs of mammary tissue at 10x magnification. (B) Micrographs of mammary tissue at 40x magnification. A=adipose cells, ID= interlobular ducts with milk secretion, ILC= interlobular connective tissue

The terminal duct lobular unit (TDLU) is significantly larger and is replete with milk secretions collected from the lobules. **Figure 6.8 A**, displays sections from the treated group that shows areas that contain the MCF7 cells. The cells are found surrounding the secretion filled lobules. Higher magnification images (**Figure 6.8 B and C**) show the clusters of MCF7 cells that are found amongst the lobules.

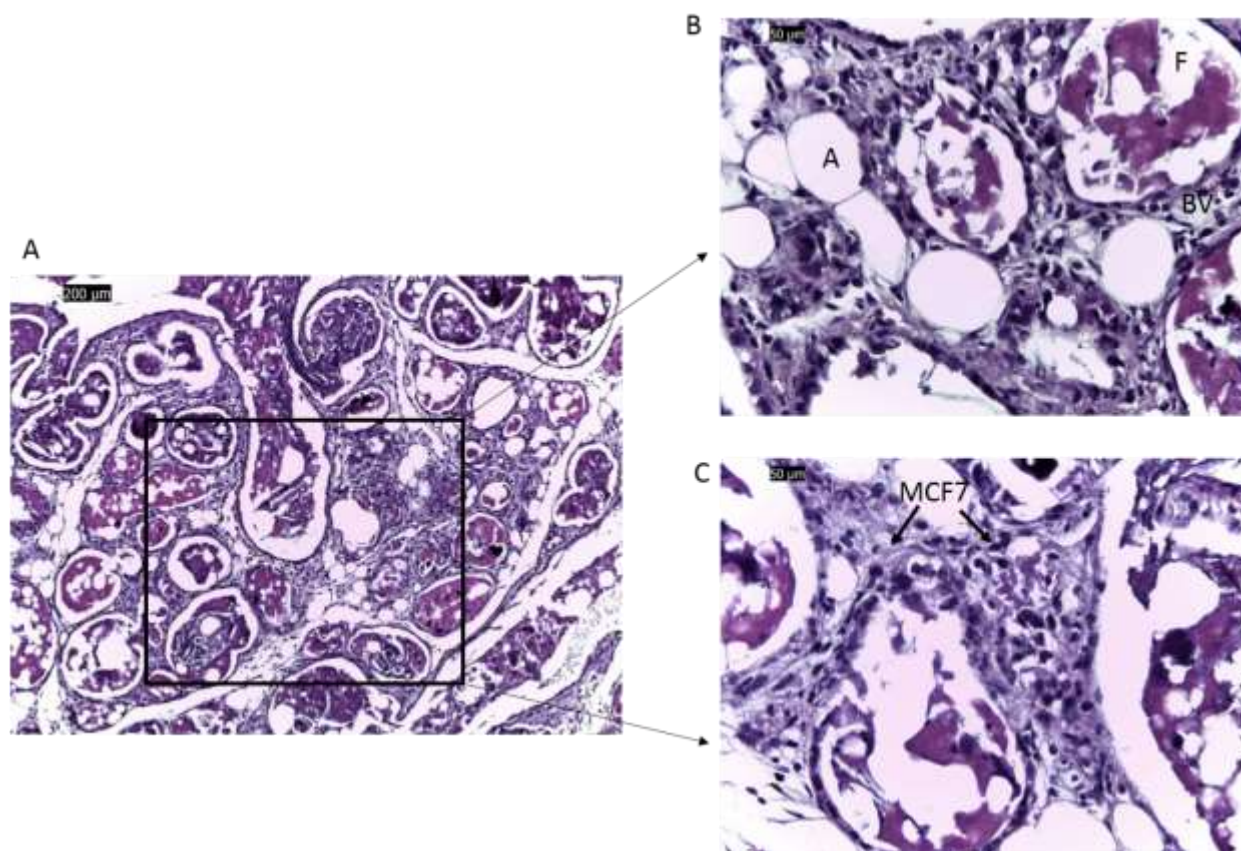


Figure 6.8 Cross-section of mammary tissues from tumour induced BALB/c mice. **(A)** Micrographs of mammary tissue at 10x magnification. **(B)** Micrographs of mammary tissue at 40x magnification. A=adipose cells, BV=blood vessels.

In the current study there was no observation of external tumour growth in BALB/c animal models, however during the harvesting of the breast tissue there were huge observable differences between the treated and the control group. The tissue from the treated group appeared larger and denser with a more granular appearance (**Figure 6.9**). Histological evaluations revealed that the control group displayed normal non- lactating breast tissue morphology with glandular units arranged in lobules, embedded in a mass of adipose tissue which is subdivided by collagenous and fibrous interlobular tissue (Figure **6.6**, **6.7** and **6.8**)

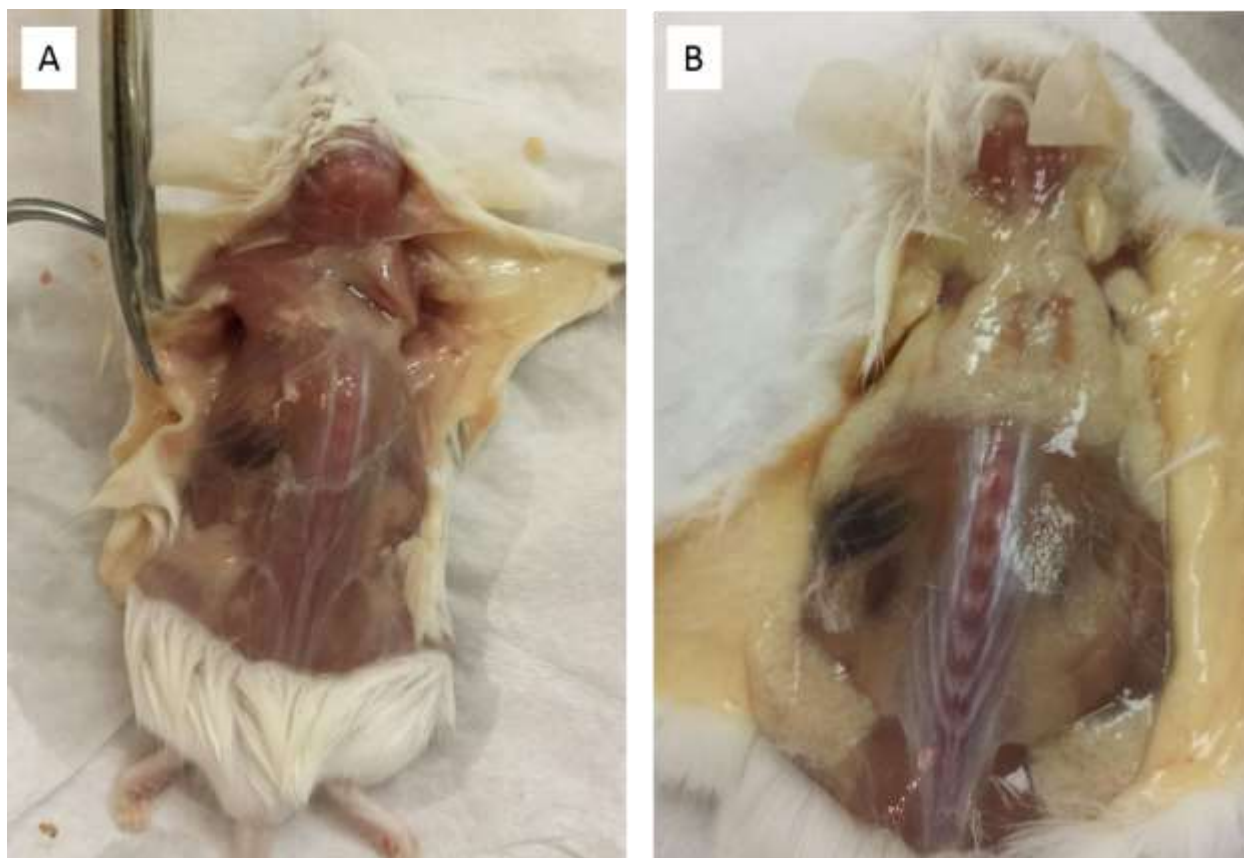


Figure 6.9 (A) Control and tumour induced (B) BALB/c mice on day 91 illustrating the lactation effects of estradiol within the mammary glands.

6.4 Discussion

The objective of this study was to induce mammary tumours in Sprague-dawley rats using MCF7 human cancer cells and xenograft techniques. The use of BME was employed to increase the MCF7 cell growth rate and promote the formation of a tumour. An additional aim was to demonstrate the tumour enhancing ability of BME in rat models. The main aim was to develop sizeable tumours that could be studied for targeted drug delivery and increased efficacy of 5-FU-EGF-Au-ZnTe nanoparticles. Similar methods of tumour induction has been well studied and highly reported using immunocompromised nude mice as the recipient animal model [40, 41].

In the current study we used nine Sprague-dawley rats. The rats were divided into three groups, group A was the untreated control, group B was the MCF7 induced animals and group C was the MCF7+BME induced animals. Throughout the 70 day study the animal's maintained normal behaviour, mobility, alertness and feeding patterns. Groups B and C showed no evidence of external tumour growth within the 70 day study. In a comparable study the use of rat derived mammary adenocarcinoma LA7 cells was used to induce tumours in Sprague Dawley rats [42]. This technique involved a subcutaneous injection of LA7 cells into the mammary fat pad of the rat. The presence of an external tumour was observed at day 30 of the study. Work reported by Barros *et al.*, showed the presence of mammary tumours in Sprague-dawley rats after 91 days of induction with 7,12-dimethylbenz(a)anthracene [43]. In another report tumour growth induced by the chemical carcinogen N-methyl-N-nitroso urea developed only after 140-175 days [15]. Generally a developing tumour requires increased blood supply to support the nutrient and oxygen demands of the rapidly growing cancer cells. This process is commonly termed

angiogenesis which results in consequent increases in blood cell density. During tumour growth the red blood cell counts are expected to increase. In this study the red blood cell counts remained within the normal range as the presence of a tumour mass was not observed in animals from the tumour induced groups B and C. During tumourigenicity, white blood cells play key roles in identifying and destroying cancer cells. The white blood cells are differentiated into many cell types that have specific functionalities within immune responses. Lymphocytes specifically have been reported to induce tumour cell destruction [44]. Abnormally low levels of lymphocytes have been implicated in the negative outcomes associated with cancer patients [45]. Studies show that increased levels of lymphocytes inhibit tumour growth and are also linked to reduced tumour recurrence [46]. The increased percentage of lymphocytes reported in this study could be in response to the introduction of the MCF7 cells in group B and C. The increase in the lymphocyte cell numbers has the potential to inhibit the growth of the injected MCF 7 cells and could therefore be implicated as one of the possible reasons for the restricted growth of these cells observed in the tumour induced groups. Breast tissue has unique morphology, composed of glandular units arranged in lobules, embedded in a mass of adipose tissue which is subdivided by collagenous and fibrous interlobular tissue [47]. In this study the presence of an external tumour was not observed in animals from group B and C, however light microscopy evaluations confirmed that the MCF7 cancer cells localized and grew within the adipose cells that surrounds the lobules of the mammary tissue. The presence of internalized MCF7 cell colonies confirms that the xenograft technique was successful. The tissue morphology from groups B and C (Figure 6.4 C-F) closely resembles the population density and orientation of MCF7 cells around the

adipose tissue as reported in a study by Chang et al., using xenografts in nude mice [48]. Additional reports on breast cancer tissue morphology also support the findings of the current study [49]. Interestingly in the present study, the injected MCF7 cells confined their aggregation and growth very closely to either the cell membranes of the adipose cells or in very close proximity to blood vessels. This can be attributed to the MCF7 cells adhering to areas that have a rich nutrient supply that would enable their successful survival and allow them the ideal conditions to promote tumour growth. In addition the histopathology studies confirmed that the xenograft technique using MCF7 cells and BME was correctly performed. The surface area or colony size that the cancer cells occupied within the MCF7+BME treated (group C) were extensively larger compared to the MCF7 treated (group B). This finding is attributed to the tumour enhancing properties of BME, which promoted higher colony numbers and colony size. The technique demonstrated the growth of MCF7 cells in the thoracic region of the mammary gland however the progression of external tumours in the presence of BME was unsuccessful.

This study demonstrated that the method of using cancer cells in combination with BME has the potential to induce tumours in rat models however the method required further optimization. The second step of this study involved the induction of mammary tumours in BALB/c mice using some of the following strategies; increasing the cell density of the cancer cells in the xenograft, addition of hormone supplements such as estrogen and immuno suppressing cortisone hormone. Changing the recipient animal from rats to mice was investigate because both rodent animals display similar morphological and physiological characteristics however BALB/c mice are approximately 10 times smaller than rats, display higher metabolic rates and are preferred for tumour induction studies

[50]. Additionally increasing the tumour growth time and increasing the concentration of tumour promoting BME was expected to further promote the formation of mammary tumours within the BALB/c model. Interestingly, the histological evaluations of the denser and granular appearing tissue from the tumour induced BALB/c mice displayed tissue morphology that is representative of a lactating breast. The increase in number and size of the lobules are dependent on hormonal activity and the supplementation of estrogen to the treated group could be responsible for the morphological changes observed within this group. The tissue sections confirmed that the MCF7 cancer cells were present within the tissue of the treated groups. The MCF7 cells were present around the secretion filled lobules of the mammary tissue. The presence of internalized MCF7 cell colonies confirms that the xenograft technique was successful. The injected MCF7 cells confined their aggregation and growth very closely to either the cell membranes of the lobular epithelial cells or in very close proximity to blood vessels. This can be attributed to the MCF7 cells adhering to areas that have a rich nutrient supply that would enable their successful survival and allow them the ideal conditions to promote tumour growth. In addition the histopathology studies confirmed that the xenograft technique using MCF7 cells and BME was correctly performed. The technique demonstrated the growth of MCF7 cells in the thoracic region of the mammary gland however the progression of external tumours in the presence of BME, estradiol and cortisone still remained unsuccessful. The induction of tumours using animal models that are not immunocompromised is a pivotal aspect of cancer research which has further downstream implications in clinical studies. The challenges in understanding cancer cell interactions and tumour physiology is important in developing effective treatment strategies for novel nano-drug systems.

6.5 Conclusions

In the current work, the findings show that the MCF7 mammary cancer cells survived in both rat and mice mammary tissues. The growth of MCF7 cancer cells within the mammary tissues confirmed the tumour enhancing properties of BME, estradiol and cortisone, however the presence of a measureable external tumour mass was not observed. Due to the lack of external tumour growth under the described experimental conditions, the *in vivo* application of 5-FU-EGF-Au-ZnTe nanoparticles could not be investigated. It is possible that the MCF7 cancer cells used to induce tumours in this study was not aggressive enough to proliferate into an external tumour mass. In order to demonstrate the *in vivo* efficacy and drug delivery application of 5-FU-EGF-Au-ZnTe nanoparticles a robust tumour model will need to be optimized through the use of more aggressive cancer cells or immunocompromised nude mice models.

References

1. RDS. *The contribution of animal science to the medical revolution: some case histories*. Medical Advances and Animal Research. 2007. Research Defense Society: Understanding Animal Research in Medicine and Coalition for Medical Progress © 2007
2. Van Der Worp, H.B., et al., *Can animal models of disease reliably inform human studies?* PLoS Medicine, 2010. **7**(3): p. e1000245.
3. DeVita, V.T. and E. Chu, *A history of cancer chemotherapy*. Cancer Research, 2008. **68**(21): p. 8643-8653.
4. Kaye, S.B., *New antimetabolites in cancer chemotherapy and their clinical impact*. British journal of cancer, 1998. **78**(Suppl 3): p. 1.
5. Boulikas, T. and M. Vougiouka, *Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs (review)*. Oncology reports, 2004. **11**(3): p. 559-595.
6. Santen, R., et al., *History of aromatase: saga of an important biological mediator and therapeutic target*. Endocrine Reviews, 2009. **30**(4): p. 343-375.
7. Bana, E. and D. Bagrel, *In Vitro Breast Cancer Models as Useful Tools in Therapeutics?* 2011: INTECH Open Access Publisher.
8. Holliday, D.L. and V. Speirs, *Choosing the right cell line for breast cancer research*. Breast Cancer Res, 2011. **13**(4): p. 215.
9. Ferreira, D., F. Adegá, and R. Chaves, *The Importance of Cancer Cell Lines as in vitro Models in Cancer Methylome Analysis and Anticancer Drugs Testing*. 2013: INTECH Open Access Publisher.
10. Lovitt, C.J., T.B. Shelper, and V.M. Avery, *Advanced cell culture techniques for cancer drug discovery*. Biology, 2014. **3**(2): p. 345-367.
11. Russo, J. and I.H. Russo, *Experimentally induced mammary tumors in rats*. Breast Cancer Research and Treatment, 1996. **39**(1): p. 7-20.
12. Fantozzi, A. and G. Christofori, *Mouse models of breast cancer metastasis*. Breast Cancer Research 2006. **8**(4): p. 212.
13. Roomi, M.W., et al., *Modulation of N-methyl-N-nitrosourea induced mammary tumors in Sprague-Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract*. Breast Cancer Research, 2005. **7**(3): p. 291-5.
14. Perše, M. and A. Cerar, *The dimethylhydrazine induced colorectal tumours in rat-experimental colorectal carcinogenesis*. Radiology and Oncology, 2005. **39**(1): p. 61-70.
15. Rajmani, R., et al., *Induction of mammary gland tumour in rats using N-methyl-N-nitroso urea and their histopathology*. Indian Journal of Veterinary Pathology 2011.
16. Howell, J., *The chemical induction of breast tumours in the rat: hormonal factors in tumour production*. British Journal of Cancer, 1960. **14**(4): p. 657.
17. Klaunig, J.E., *Chemical carcinogenesis*. Principles of Toxicology: Environmental and Industrial Applications, 2014: p. 259.
18. Fridman, R., et al., *Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines*. Proceedings of the National Academy of Sciences, 1990. **87**(17): p. 6698-6702.
19. Pretlow, T.G., et al., *Transplantation of human prostatic carcinoma into nude mice in Matrigel*. Cancer Research, 1991. **51**(14): p. 3814-3817.
20. Topley, P., et al., *Effect of reconstituted basement membrane components on the growth of a panel of human tumour cell lines in nude mice*. British Journal of Cancer, 1993. **67**(5): p. 953.
21. Noël, A., et al., *Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts*. British Journal of Cancer, 1993. **68**(5): p. 909.
22. Bao, L., et al., *Effects of inoculation site and Matrigel on growth and metastasis of human breast cancer cells*. British Journal of Cancer, 1994. **70**(2): p. 228-232.
23. Hayward, J., *Hormones and human breast cancer: an account of 15 years study*. 2013: Butterworth-Heinemann.
24. Grubbs, C.J., et al., *Chemoprevention of N-nitroso-N-methylurea-induced mammary cancers by pretreatment with 17 β -estradiol and progesterone*. Journal of the National Cancer Institute, 1985. **74**(4): p. 927-931.
25. Bradlow, H.L., et al., *Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice*. Carcinogenesis, 1991. **12**(9): p. 1571-1574.
26. Topper, Y., *Multiple hormone interactions in the development of mammary gland in vitro*. Recent Prog. Horm. Res, 2013. **26**: p. 287-3.
27. Dennison, K.L., et al., *Development and characterization of a novel rat model of estrogen-induced mammary cancer*. Endocrine-related cancer, 2015. **22**(2): p. 239-248.
28. Baserga, R., P. Shubik, and J. Pass, *The action of cortisone on transplanted and induced tumors in mice*. Cancer research, 1954. **14**(1): p. 12-16.
29. Wang, A.Z., R. Langer, and O.C. Farokhzad, *Nanoparticle delivery of cancer drugs*. Annual review of medicine, 2012. **63**: p. 185-198.
30. O'Neal, D.P., et al., *Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles*. Cancer letters, 2004. **209**(2): p. 171-176.
31. Huang, X., et al., *Plasmonic photothermal therapy (PPTT) using gold nanoparticles*. Lasers in medical science, 2008. **23**(3): p. 217-228.
32. Hainfeld, J.F., et al., *Infrared-transparent gold nanoparticles converted by tumors to infrared absorbers cure tumors in mice by photothermal therapy*. PloS one, 2014. **9**(2): p. e88414.
33. Cheng, L., et al., *Organic stealth nanoparticles for highly effective in vivo near-infrared photothermal therapy of cancer*. ACS nano, 2012. **6**(6): p. 5605-5613.
34. Zheng, M., et al., *Single-step assembly of DOX/ICG loaded lipid-polymer nanoparticles for highly effective chemo-photothermal combination therapy*. Acs Nano, 2013. **7**(3): p. 2056-2067.
35. Tian, G., et al., *Red-Emitting Upconverting Nanoparticles for Photodynamic Therapy in Cancer Cells Under Near-Infrared Excitation*. Small, 2013. **9**(11): p. 1929-1938.
36. Ingberg, E., et al., *Methods for long-term 17 β -estradiol administration to mice*. General and comparative endocrinology, 2012. **175**(1): p. 188-193.
37. Chaves, G.M., et al., *Model of experimental infection in healthy and immunosuppressed swiss albino mice (Mus musculus) using Candida albicans strains with different patterns of enzymatic activity*. Brazilian

- Journal of Microbiology, 2004. **35**(4): p. 311-315.
38. Stephens, R.S., W.-J. Chen, and C.-C. Kuo, *Effects of corticosteroids and cyclophosphamide on a mouse model of Chlamydia trachomatis pneumonitis*. Infection and immunity, 1982. **35**(2): p. 680-684.
39. Shtrassburg, S., et al., *Suppression of amyloidogenesis in a mouse model by corticosteroid intervention*. Translational Research, 2007. **150**(1): p. 66-72.
40. Fridman, R., et al., *Increased initiation and growth of tumor cell lines, cancer stem cells and biopsy material in mice using basement membrane matrix protein (Cultrex or Matrigel) co-injection*. Nature Protocols, 2012. **7**(6): p. 1138-1144.
41. Benton, G., et al., *Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells*. International Journal of Cancer, 2011. **128**(8): p. 1751-1757.
42. Abbasalipourkabir, R., et al., *Induction of mammary gland tumor in female Sprague-Dawley rats with LA7 cells*. African Journal of Biotechnology, 2010. **9**(28): p. 4491-4498.
43. Barros, A.C.S., et al., *Induction of experimental mammary carcinogenesis in rats with 7, 12-dimethylbenz (a) anthracene*. Revista do Hospital das Clínicas, 2004. **59**(5): p. 257-261.
44. Niederhuber, J.E., *Cancer vaccines: the molecular basis for T cell killing of tumor cells*. The Oncologist, 1997. **2**(5): p. 280-283.
45. Fogar, P., et al., *Decreased total lymphocyte counts in pancreatic cancer: an index of adverse outcome*. Pancreas, 2006. **32**(1): p. 22-28.
46. Azab, B., et al., *Usefulness of the neutrophil-to-lymphocyte ratio in predicting short-and long-term mortality in breast cancer patients*. Annals of Surgical Oncology, 2012. **19**(1): p. 217-224.
47. Young, B., P. Woodford, and G. O'Dowd, *Wheater's functional histology: a text and colour atlas*. 2013: Elsevier Health Sciences.
48. Chang, Y., et al., *Secretion of pleiotrophin stimulates breast cancer progression through remodeling of the tumor microenvironment*. Proceedings of the National Academy of Sciences, 2007. **104**(26): p. 10888-10893.
49. Uhlén, M., et al., *A human protein atlas for normal and cancer tissues based on antibody proteomics*. Molecular & Cellular Proteomics, 2005. **4**(12): p. 1920-1932.
50. Nandi, S., R.C. Guzman, and J. Yang, *Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis*. Proceedings of the National Academy of Sciences, 1995. **92**(9): p. 3650-3657.

CHAPTER SEVEN: Discussion and conclusion

7.1 Discussion

The need for novel and innovative biocompatible nanomaterials are always in demand to improve current therapeutic and diagnostic medical applications. Literature provides evidence that support the importance of gold based core-shell nanostructures synthesized specifically for drug delivery and bio-imaging applications within the field of oncology. The aim of this research project was to synthesize, characterise and demonstrate the various bio-applications of novel Au-ZnTe core-shell nanoparticles.

The work presented in chapter two and three, highlights the synthesis and characterisation of both the parent materials Au and ZnTe and Au-ZnTe core-shell nanoparticles. This study enables a clear understanding of the interaction between the parent materials and the mechanism of assembly into core-shell nanostructures. Additionally the preliminary *in vitro* biocompatibility findings supported the biosafety of ZnTe and Au-ZnTe nanoparticles. The bio-safety and functionality of these materials is largely attributed to the use of L-cysteine ethyl ester hydrochloride as a surface passivating agent.

Detailed investigations on the biocompatibility, bio-interaction and biosafety of Au-ZnTe nanoparticles were evaluated using suitable *in vitro* and *in vivo* experiments and are presented within chapter four. These findings established a toxicity profile for the Au-ZnTe nanoparticles and established that these nanoparticles are non-toxic between a concentration range of 20-100 µg/ml for *in vitro* cell culture experiments and 50-1500 µg/ml for *in vivo* animal experiments. The subsequent aim after demonstrating the biosafety of Au-ZnTe nanoparticles was to investigate the bio-functionalisation of Au-ZnTe nanoparticles. This was demonstrated using surface conjugation of 5-FU and human EGF. As described in chapter five, after physicochemical characterisation, the *in vitro* therapeutic efficacy application of the surface modified Au-ZnTe nanoparticle was investigated. The UV, PL, FTIR and Zeta potential analysis provided evidence that 5-FU and EGF dominated the optical properties of the surface modified Au-ZnTe nanoparticles. Additionally the comparative *in vitro* therapeutic efficacy studies showed that 5-FU-EGF-Au-ZnTe nanoparticles displayed enhanced cytotoxic effects on MCF7 cells when compared to 5-FU alone. Modern technologies within the discipline of pharmaceuticals, drug delivery, materials chemistry and nanobiotechnology aim to address the harmful side-effects

and challenges that result from conventional chemotherapy by improving the delivery of the chemotherapeutics directly to the target site, thereby decreasing the required unit dose, enhancing the efficacy of the drug and improving both drug development and patient compliance. The data displayed in chapter five shows statistically significant *in vitro* therapeutic activity confirming that the nano-drug formulation of 5-FU would be able to support drug specificity and improved therapeutic activity at a lower dose as compared to the plain drug. In order to investigate the *in vivo* application of 5-FU-EGF-Au-ZnTe nanoparticles, a well-optimised animal model that presents external mammary tumours had to be first developed and then treated with the nano-drug formulation of 5-FU.

In vivo tumour induction studies using xenograft techniques was performed on female Sprague Dawley rats and BALB/c mice and reported in chapter six. The xenograft consisted of human MCF7 cancer cells in combination with basement membrane matrix growth enhancers. In addition to this technique the animals received supplementation with estradiol and cortisone hormones. Estradiol functioned in stimulating the growth of the cancer cells and the cortisone functioned in suppressing the immune response allowing the introduction and growth of foreign cells into the animal's body. The results indicated tumour growth and MCF7 cellular invasion of surrounding tissues. However the presence of an external and sizeable tumour mass was not observed. Side effects of the cortisone administration caused two mice to die on a weekly basis. The study was terminated at 91 days due to ethical, animal welfare and humane endpoint protocols. The study was able to generate scientific knowledge and contribute to the optimisation and development of xenograft techniques using animals. The absence of a visible external tumour mass limited the project from demonstrating the *in vivo* application of 5-FU-EGF-Au-ZnTe nanoparticles.

7.2 Challenges

- I. This project involved various disciplines and therefore required access to specialised laboratory and instrument facilities. The lack of vital in-house research facilities such as cell culture laboratories, electron microscopy facilities, animal availability (numbers and strain), animal handling, ethics and welfare training at the Department of Biochemistry and Microbiology, University of Zululand was a huge challenge that led to a 6 months delay in the animal and application studies during 2013. In 2013, a collaboration was arranged with

the University of KwaZulu-Natal, Biomedical Resource Unit (BRU), followed by a series of animal ethics and lab animal science training and workshop. The animal ethics was approved through the UKZN animal ethics committee. Thereafter the animal studies were carried out under the close supervision of Dr S. Singh and Dr L. Bester (Vet and technical support).

- I. The absence of cell culture facilities at the University of Zululand led to a search for a collaborator, in close vicinity to the BRU (UKZN) where the animals were housed. In 2014, we entered into a collaboration with Dr J. Bodenstein, Pharmacology Department, UKZN. We were unable to simply purchase the cancer cells due to license restrictions, therefore an extensive search for the availability of human breast cancer cells was a key challenge in various aspects of the project. After months of searching the MCF7 cells were donated to us through Dr S. Naidu and Mr S Rambharose from UKZN, originally purchased from the ATCC.
- II. The animal studies were time consuming and the tumour induction studies took longer than anticipated. Additionally the requirements of the animal studies limited the opportunities to attend conferences and undertake international training opportunities such as collaborations or research visits abroad due to daily clinical observations and constant animal care that needed to be maintained specifically by the Ph.D student throughout the animal studies.
- III. Although the *in vitro* efficacy and drug delivery effects of anti-cancer 5-FU-EGF-Au-ZnTe nanoparticles using MCF7 cancer cells was demonstrated, the main challenge of the project was the inability to induce consistent external tumour growth in both rat and mice models. This phase of the project was exhausted under several experimental conditions (BME, Estradiol and cortisone) and limits the progress of *in vivo* application studies of 5-Fu-EGF-Au-ZnTe nanoparticles. The absence of cancer cell lines that are aggressive or tumourigenic remained a serious challenge throughout the project.

7.3 Conclusion

The synthesis of the parental materials Au and ZnTe nanoparticles was optimised using various experimental conditions prior to the synthesis of the core-shell structure. The ZnTe nanoparticles obtained at pH 4 showed interesting morphological features attributed to oriented growth attachment mechanisms. ZnTe nanoparticles showed

quantum confinement and narrow band-edge emission. The cysteine capped ZnTe nanoparticles displayed a negative surface charge suggesting that the particles would be conducive to functionalization for additional applications in cell targeting, transfection and bio-imaging. Initial fluorescence studies of the ZnTe nanoparticles within DNA plasmid shows emission in the blue region. *In vitro* cytotoxicity studies showed that ZnTe nanoparticles do not induce adverse or harmful effects to human MCF7 cells. ZnTe nanoparticles displayed the potential to be utilised independently in various *in vitro* and *in vivo* drug delivery and imaging applications. Furthermore ZnTe nanoparticles may be used as a shell component in the assembly of other core-shell nanostructures. The aim was to design a novel biocompatible heterostructure by combining Au and ZnTe nanomaterials to function as a single entity that delivered favourable physicochemical properties from both parental materials. The synthesized material displayed enhanced optical and stability features. The morphological analysis displayed uniform distribution of particle size and shape. In addition the elemental mapping data provided evidence of the core-shell structure composed of gold cores surrounded by a ZnTe shell. The presence of some alloys and bare ZnTe nanomaterials were also observed in the sample. These findings indicate the need for developing methods that refine and separate the prepared material on the nanoscale. Preliminary studies displayed the biosafety and biocompatibility features of Au-ZnTe nanoparticles using various *in vitro* cell culture techniques. A thorough toxicity and biosafety evaluation supported the use of Au-ZnTe in targeted drug delivery and bio-imaging applications in concentrations between 20-1500 µg/ml. Systemic circulation of Au-ZnTe in Sprague Dawley rat models confirmed the biosafety effects of Au-ZnTe nanoparticles. Future research should aim to understand and evaluate the degradation and elimination effects of bio-accumulated Au-ZnTe core-shell nanoparticles. Bio-accumulation of nanomaterials is an important contributing factor in nanotoxicity studies. The synthesis of surface conjugated 5-FU-EGF-Au-ZnTe nanoparticles displayed *in vitro* enhanced therapeutic efficacy in MCF7 cancer cells. The cytotoxicity data suggests that all the components of the 5-FU-EGF-Au-ZnTe nanoparticle formulation played specific roles in synergistically improving anti-cancer therapeutic efficacy. The growth of MCF7 cancer cells within the mammary tissues confirmed the tumour enhancing properties of BME, estradiol and cortisone, however the presence of a measureable external tumour mass was not observed and therefore limited the next phase of evaluating the *in vivo* application of the nano-drug formulation of 5-FU.

7.4 Recommendations

Recommendations for future work have the potential to support research projects and collaborations with perspective institutions on the MSc and PhD level under the following suggested research themes;

- The pharmacokinetics and stability of 5-FU-EGF-Au-ZnTe nanoparticles needs to be evaluated
- Co-culture combination techniques using normal and cancer to investigate the drug targeting effects of Au-ZnTe nanoparticles
- Molecular modelling to establish the theoretical interaction of 5-FU and EGF with Au-ZnTe nanoparticles
- The use of nude mice and aggressive cancer cells to develop external tumour masses that can be used to investigate the in vivo applications of the nano-drug formulation of 5-FU
- Surface conjugation of alternative chemotherapeutic drugs and other antibody ligands such as growth factors, folate acids or glucose molecules.
- Synthesis and design of Au-ZnTe nanorods core-shell structures for applications in bio-imaging and photothermal tumour destruction
- Detailed synthesis characterisation and bio-applications of ZnTe under the influence of additional capping agents
- Synthesis, characterisation and bio-applications of ZnTe-Au core-shell nanostructures

NJC



PAPER

View Article Online
View Journal | View IssueCite this: *New J. Chem.*, 2014,
38, 6002

Evidence of oriented attachment in the growth of functionalized ZnTe nanoparticles for potential applications in bio-imaging†

Rekha Dunpall, Sixberth Mlowe and Neerish Revaprasadu*

Received (in Victoria, Australia)
16th July 2014;
Accepted 12th September 2014

DOI: 10.1039/c4nj01183j

www.rsc.org/njc

This study reports a solution based, low temperature route towards the synthesis of water soluble cysteine capped zinc telluride (ZnTe) nanoparticles under the influence of variations in pH and reaction time. The optical properties of the ZnTe nanoparticles display broad emission peaks ranging between 365–415 nm, which make them useful for imaging and biological labelling applications. Transmission electron microscopy (TEM) and high resolution TEM studies indicated that the particles are uniform in size and shape, however at the 2 hour reaction time interval the nanoparticle growth enters a transition phase where the morphology changes from nanospheres to nanorods. The formation of the ZnTe nanoparticles relative to reaction temperature, pH and time is typical of the oriented attachment growth mechanism. X-ray diffraction patterns confirmed the crystalline cubic phase. The surface charge of the functionalised ZnTe was also determined. The fluorescence properties and optical stability of the ZnTe nanoparticles in DNA plasmid pGEMT Easy were studied using fluorescence (365–395 nm filter) and phase-contrast (UV 2A 330–380 nm filter) microscopy techniques.

Introduction

The synthesis of functionalized semiconductor nanoparticles has attracted interest in the fields of nanoscience and medicine.^{1–3} Scientists are constantly searching for alternative materials that may be utilized in bio-imaging and labelling applications. A key component in designing such nanomaterials incorporates water solubility, functionalization, relatively low toxicity and materials that are not susceptible to photobleaching. Semiconducting materials may be engineered at sizes that are smaller than or comparable to those of a cell, protein or gene. In addition their surface features may be altered to facilitate their interaction and entry into the biological entity of choice resulting in a variety of applications across plant biology and the biomedical sciences.^{4–7} A challenge in synthesizing semiconducting nanomaterials for bio-applications is the toxicity of the component ions. ZnTe is an interesting II–VI semiconducting nanomaterial that is less toxic when compared to cadmium based chalcogenides.⁷

ZnTe has been synthesized via physical routes such as mechanical alloying^{8,9} and pulsed laser ablation.¹⁰ It is a challenge to prepare ZnTe nanostructures with controllable size and

shape because of the lack of suitable precursors for controlling the thermodynamics and kinetics of the nanocrystal nucleation stage. ZnTe in the form of nanodots,^{11–14} nanoflowers,¹⁴ nanowires and nanorods^{14–17} has been reported using various colloidal and high temperature routes. In many cases the growth of the anisotropic structures is attributed to the oriented attachment growth mechanism.^{18–22}

An aqueous route for the synthesis of fluorescent ZnTe-dendrimer nanocomposites was reported by Ghosh *et al.*, their study investigated antimicrobial properties of the ZnTe nanocomposites and revealed their potential in therapeutic applications.²³ Water-dispersible 1D Te@ZnTe core-shell nanoparticles have also been reported whereby the ZnTe nanoparticles were used as precursors. The 1D nanoparticles have a crystalline Te core and an amorphous ZnTe shell.²⁴

This study reports a solution based, significantly lower thermal route for the synthesis of functionalized ZnTe nanoparticles. The synthesis method used in this work was adapted from the route previously reported for the synthesis of cysteine capped CdSe nanoparticles.²⁵

Experimental details

Materials

Zinc chloride, L-cysteine ethyl ester hydrochloride, tellurium powder, sodium borohydride, deionised water (HPLC grade) and acetone

Department of Chemistry, University of Zululand, Private Bag X1001, Kwa-Dlangezwa, 3886, South Africa. E-mail: RevaprasaduN@unizulu.ac.za
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4nj01183j

Cite this: *J. Mater. Chem. B*, 2015, 3,
2826Synthesis of biocompatible Au–ZnTe core–shell
nanoparticles†Rekha Dunpall,^a Edward A. Lewis,^{bc} Sarah J. Haigh,^{bc} Paul O'Brien^{bc}
and Neerish Revaprasadu^{a*}

A novel, solution-based route to biocompatible, cysteine-capped gold–zinc telluride (Au–ZnTe) core–shell nanoparticles with potential in biomedical applications is described. The optical properties of the core–shell nanoparticles show combined beneficial features of the individual parent components. The tunable emission properties of the semiconductor shell render the system useful for imaging and biological labeling applications. Powder X-ray diffraction analysis reveals the particles contain crystalline Au and ZnTe. Transmission electron microscope (TEM) imaging of the particles indicates they are largely spherical with sizes in the order of 2–10 nm. Elemental mapping using X-ray energy dispersive spectroscopy (XEDS) in the scanning transmission electron microscope (STEM) mode supports a core–shell morphology. The biocompatibility and cytotoxicity of the core–shells was investigated on a human pancreas adenocarcinoma (PL45) cell line using the WST-1 cell viability assay. The results showed that the core–shells had no adverse effects on the PL45 cellular proliferation or morphology. TEM imaging of PL45 cell cross sections confirmed the cellular uptake and isolation of the core–shell nanoparticles within the cytoplasm via membrane interactions. The fluorescence properties of the Au–ZnTe core–shell structures within the PL45 cell lines results confirmed their bio-imaging potential. The importance and novelty of this research lies in the combination of gold and zinc telluride used to produce a water soluble, biocompatible nanomaterial which may be exploited for drug delivery applications within the domain of oncology.

Received 27th October 2014
Accepted 13th February 2015

DOI: 10.1039/c4tb01779j

www.rsc.org/MaterialsB

Introduction

The National Cancer Registry (NCR) and international cancer research institutes provide statistics that show an increase in the prevalence of cancer patients worldwide. The fight against cancer has evolved into a multidisciplinary approach involving clinicians, chemists, biomedical engineers and materials scientists.¹ Chemotherapy is a commonly used treatment for cancer. It involves the use of various cytotoxic drugs that often affect both cancer and healthy cells. This treatment often results in adverse side effects such as hair and weight loss, nausea, diarrhoea, cancer cachexia, opportunistic infections and sometimes even death.² The challenge that researchers are faced with is the design of chemotherapeutic drugs that are not only cancer specific but also exhibit higher therapeutic efficacy. Advanced nanosystems such as core–shell

nanoparticles have the potential to support this application if designed appropriately. The synthesis of heterostructured nanoparticles has received much attention recently due to their potential in catalysis, electronics and biomedical sciences.^{3–7} The combination of two different types of material that function as a single entity can confer particular advantages such as enhanced optical, electrical or structural properties. A challenge in designing such materials arises from determining the structure of a two-component system and in identifying compatible core–shell component elements. The controlled synthesis of bimetallic heterosystems such as Pd/Pt or Ag/Au core–shell systems is possible because of the relatively close match between the crystal lattices of their component parts. In contrast, core–shell metal–semiconductor systems are more difficult to synthesize due to their differing crystal structures and to the large crystal lattice mismatches.^{8,9} There have been few reports of metal–semiconductor core–shell nanostructures.^{10–11} Wang and co-workers described a general method for the preparation of Au nanocrystals of different shapes and sizes as starting material for the synthesis of water-dispersible Au–metal sulfide core–shell structures.¹² Salant *et al.* conducted elaborate work on the growth of gold tips on anisotropic CdSe particles which was used to establish a relationship between the growth

^aDepartment of Chemistry, University of Zululand, Private Bag X1001, Kwa-Dlangezwa, 3886, South Africa. E-mail: RevaprasaduN@unizulu.ac.za^bSchool of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL, UK^cSchool of Materials Science, University of Manchester, Oxford Road, Manchester, M13 9PL, UK

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4tb01779j

1. (WO2015019297) THE SYNTHESIS OF CORE-SHELL METAL-SEMICONDUCTOR NANOMATERIALS

PCT Biblio. Data	Description	Claims	National Phase	Notices	Drawings	Documents
----------------------------------	-----------------------------	------------------------	--------------------------------	-------------------------	--------------------------	---------------------------

Latest bibliographic data on file with the International Bureau

Pub. No.: WO/2015/019297 International Application No.: PCT/IB2014/063729

Publication Date: 12.02.2015 International Filing Date: 06.08.2014

IPC: B82Y 5/00 (2011.01), A61K 38/48 (2006.01), B82Y 15/00 (2011.01), C08G 85/00 (2006.01), G01N 33/50 (2006.01)

Applicants: UNIVERSITY OF ZULULAND [ZA/ZA]; 24 Main Road 3886 KwaDlangezwa (ZA)

Inventors: REVAPRASADU, Neerish; (ZA)
DUNPALL, Rekha; (ZA)

Agent: SPOOR & FISHER; P O Box 454 0001 Pretoria (ZA)

Priority Data: 2013/05925 07.08.2013 ZA

Title: (EN) THE SYNTHESIS OF CORE-SHELL METAL-SEMICONDUCTOR NANOMATERIALS

PermaLink

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:

SPOOR & FISHER
P O BOX 454
0001 Pretoria
South Africa

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference PA160940/PCT	Date of mailing (day/month/year) 21 January 2015
International application No. PCT/IB2014/063729	FOR FURTHER ACTION See paragraphs 1 and 4 below
Applicant UNIVERSITY OF ZULULAND	International filing date 06 August 2014

- ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.
Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When?	The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.
How?	Directly to the International Bureau of WIPO preferably through cPCT or on paper to: 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see PCT Applicant's Guide, International Phase, paragraphs 9.004 – 9.011.
- ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
- ☐ **With regard to any protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:**

☐	the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
☐	no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.
- Reminders**
The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90bis.1 and 90bis.3).
Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices. In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the PCT Applicant's Guide, National Chapters.
Within 19 months from the priority date, the applicant may request that a supplementary international search be carried out by a different International Searching Authority that offers this service (Rule 45bis. 1). The procedure for requesting supplementary international search is described in the PCT Applicant's Guide, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
Email address: pct@ipaustralia.gov.au

Authorised officer

ISAAC TAN
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. 0262832814

Form PCT/ISA/220 (July 2014)

(See notes on accompanying sheet)

Appendix B 1 University of Zululand: Ethics approval

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-30- RA Level 01)



RESEARCH & INNOVATION

Website: <http://www.unizulu.ac.za>
Private Bag X1001
KwaDlangezwa 3886
Tel: 035 902 6887
Fax: 035 902 6222
Email: ManqeleS@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030-RA Level 01 PGD 2014/60					
Project Title	The synthesis, characterization and bio-applications of gold-zinc telluride core-shell nanoparticles					
Principal Researcher/ Investigator	R Dunpall					
Supervisor and Co- supervisor	Prof N Ravaprasadu			Prof AR Opoku		
Department	Biochemistry					
Nature of Project	Honours/4 th Year		Master's		Doctoral	x Departmental

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions:

- (1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
- (2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
X	X	X			
Low Risk		Medium Risk		High Risk	
				X	

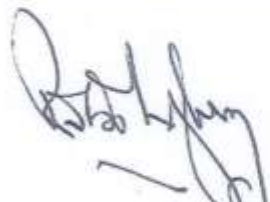
The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation			X
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants	X		
Informed consent from parent/guardian			X
Permission for access to sites/information/participants	X		
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire	X		
Data collection instrument in appropriate language			X
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
 - Any unethical principles or practices are revealed or suspected
 - Relevant information has been withheld or misrepresented
 - Regulatory changes of whatsoever nature so require
 - The conditions contained in this Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.



Professor Rob Midgley
 Deputy Vice-Chancellor, Research and Innovation
 Chairperson: University Research Ethics Committee
 20 August 2014

CHAIRPERSON
 UNIVERSITY OF ZULULAND RESEARCH
 ETHICS COMMITTEE (UZREC)
 REG NO: UZREC 171110-30

20-08-2014

RESEARCH & INNOVATION OFFICE

Appendix B 2 UKZN: Ethics approval 2014



21 April 2014

Reference: 085/14/Animal

Miss R Dunpall
Dept of Biochemistry
University of Zululand
C/o Biomedical Resource Unit
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Dunpall

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for 2014 on the following project:

"The synthesis, characterisation and bio-applications of gold-zinc telluride core/shell nanoparticles."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Supervisor – Prof. N Revaprasadu (Univ. of Zululand)
Co-Supervisor – Prof. A Opoku (Univ. of Zululand)
BRU – Dr S Singh

Animal Ethics Committee Professor Theresa HT Coetzer (Chair)

Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 Facsimile: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Founding Campuses:  Edgewood  Howard College  Medical School  Pietermaritzburg  Westville

INSPIRING GREATNESS



CERTIFICATE OF ATTENDANCE

INTEGRATIVE AND ORGANS SYSTEMS PHARMACOLOGY WORKSHOP A Satellite Meeting of WCP 2014

REKHA DUNPALL

8-11 July 2014

University of KwaZulu-Natal, Westville, South Africa



Convenor



Course Coordinator



Appendix B 4 Animal Ethics approval Renewal 2015 (UKZN)



15 December 2014

Reference: 002/15/Animal

Miss R Dunpall
Dept of Biochemistry
University of Zululand
C/o Biomedical Resource Unit
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Dunpall

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2015** on the following project:

"The synthesis, characterisation and bio-applications of gold-zinc telluride core/shell nanoparticles."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar
Research Office – Dr N Singh
Supervisor – Prof. N Revaprasadu (Univ. of Zululand)
Co-Supervisor – Prof. A Opoku (Univ. of Zululand)
BRU – Dr S Singh

Animal Ethics Committee Professor Theresa HT Coetzer (Chair)

Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 Facsimile: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Founding Campuses:  Edgewood  Howard College  Medical School  Pietermaritzburg  Westville

INSPIRING GREATNESS



Appendix B 5. Ethics amendment to animal strain (UKZN)



05 May 2015

Ms R Dunpall
Department of Biochemistry
University of Zululand

c/o Biomedical Resource Unit
University of KwaZulu-Natal
Westville Campus

Dear Ms Dunpall,

Protocol reference number: 085/14/Animal

Project title: The synthesis, characterization and bio-applications of gold-zinc telluride core/shell nanoparticles

Approval Notification – Amendment Application

This letter serves to notify that your request for an amendment received on 20 April 2015 has now been approved as follows:

- Change from Sprague-Dawley rats to Balb/c mice

Any alterations to the approved research protocol i.e. Title of the Project, Location of the Study, Research Methodology must be reviewed and approved through an amendment /modification prior to its implementation. In case you have further queries, please quote the above reference number.

PLEASE NOTE: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 15 December 2016.

Best wishes for the successful completion of your research protocol.

Yours faithfully

Dr Shahidul Islam
Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Prof N Revaprasadu (UNIZULU)
cc Co-Supervisor: Prof A Opoku (UNIZULU)
cc Registrar
cc BRU- Dr Sanil Singh

Animal Research Ethics Committee (AREC)

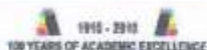
Ms Mariette Szymian (Administrator)

Westville Campus, Govan Mbeki Building

Postal Address: Private Bag X34001, Durban 4000

Telephone: +27 (0) 31 260 8350 Facsimile: +27 (0) 31 260 4608 Email: animalethics@ukzn.ac.za

Website: <http://research.ukzn.ac.za/Ethics/Animal-Ethics.aspx>



Funding Colleges: Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix B 6. Cancer research humane endpoint evaluations sheet for study

Name of study				Animal number:			
Animal group/cage							
Weight range (g)							
Date							
Day							
Time							
Observation analysis without handling							
Behavior							
Inactive							
Mobility							
Hunched posture							
Grooming							
Alertness							
Fatigue							
Ruffled coat							
Observation analysis on handling							
Alert and active							
Eating*							
Drinking*							
Vocalization on gentle palpation							
Body weight (g) (twice a week)							
% of baseline weight							
Type of breathing **							
Condition scoring 4 to 1 ***							
Specific clinical signs of tumour progression							
Size of tumour							
Shape of tumour							
Necrosis of tumour							
Bleeding of tumour							
Ulceration							
Nothing Abnormal Detected (NAD)							
Other observations							
Signature							

Special Husbandry Requirements:

After the tumour becomes visible, the frequency of observation and sizing of the tumour should be increased. Particular attention should be paid to the growth of the tumour. Photographs and calliper measurements will be documents

Scoring Details

*Eating/drinking: Y=Yes; N= No

** Breathing: R = rapid; S = Shallow; L = laboured; N = normal.

***Condition: 4 = Normal, 1 = emaciated.

Humane Endpoints and Actions:

- Any animal weighing less than 10% of the starting weight at any point of the study will be euthanized.
- Tumour size limit of 40cm³
- Poor condition, ill health, distressed behaviour patterns. Signs of compromised immune systems
- Necrosis and bleeding of the tumour.

Scientific Measures: Should the animal lose more than 10% of its starting weight, is showing clinical signs of distress or appears hunched back, ruffled hair, dark eyes and unhealthy a decision will be taken to kill the animal using a humanely acceptable approach to prevent the animal from suffering further pain and ill health even before the phase period. The animals will be euthanized using an overdose of Halothane or isoflurane 4% inhalation.