



**NANOTOXICOLOGY ASSESSMENT OF GOLD AND SILVER
NANOPARTICLES IN HUMAN LUNG CELLS**

By

BASHIRU IBRAHIM

**A thesis submitted to the University of Birmingham for the degree
of DOCTOR OF PHILOSOPHY**

School of Geography, Earth, and Environmental Sciences

College of Life and Environmental Sciences

University of Birmingham

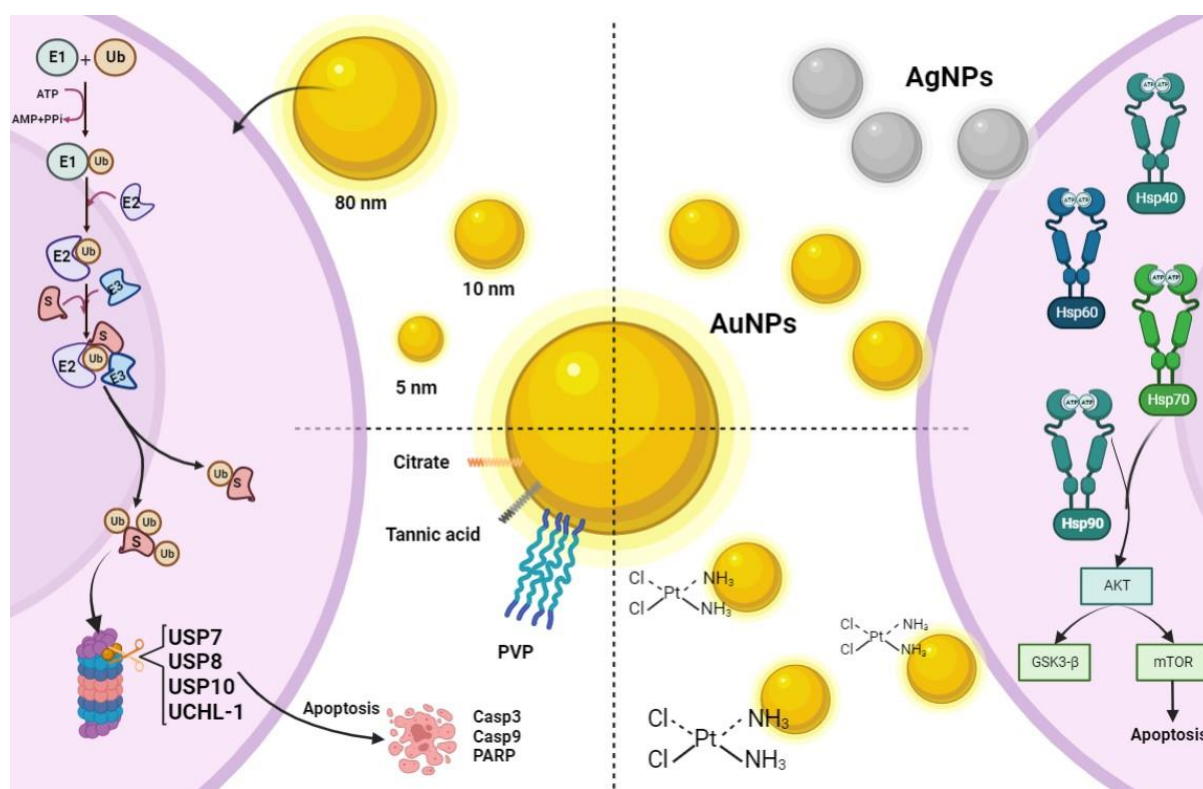
February 2023

Abstract

Engineered nanomaterials such as gold (AuNPs) and silver nanoparticles (AgNPs) have shown a great potential in biomedical applications such as cancer therapy, diagnostics, and drug delivery. However, it is crucial to understand their potential cytotoxicity *in vitro*. In this work, it was hypothesised that cellular exposure to different nanoparticles (NPs) can elicit various biological responses in human alveolar adenocarcinoma (A549) cells depending on their size and chemical functionalisation. Initially the effects of different sizes of AuNPs on the ubiquitin proteasome system (UPS) was investigated with a particular focus on deubiquitinating enzymes (DUBs) such as ubiquitin specific proteases (USP) and ubiquitin carboxyl-terminal hydrolases (UCHL-1). Secondly, the influences of different functionalisation on their rate of cytotoxicity to UPS was studied. Thirdly, the effects of different NPs composition on the molecular chaperone heat shock proteins (HSPs) were evaluated and finally the combination of AuNPs with cisplatin (CDDP) and its effects on the efficacy of the anticancer CDDP was evaluated. It was found that the exposure of NPs to complex biological media significantly affected their physicochemical properties inducing agglomeration and dissolution, with complete culture media (CCM) having a stabilising impact because of protein corona formation. In addition, the cytotoxicity of NPs in A549 cells was found to be size-, type, concentration-, and time-dependent as assessed by several toxicity assays. The internalisation of AuNPs in A549 increased to a greater extent with the increase in particle size (80 > 10 > 5 nm) and was mediated by different endocytic mechanisms such as clathrin- and caveolae-mediated endocytosis. The cellular internalisation of the particles triggered the production of reactive oxygen species (ROS), depletion of glutathione, a decrease in mitochondrial membrane potential (MMP), and the activation of mitochondrial-related apoptosis. Moreover, the expression of DUBs (USP7, USP8, USP10, and UCHL-1) and HSPs

(HSP40, HSP60, HSP70, and HSP90) was downregulated in a dose-dependent manner upon treatment with NPs. Combination of AuNP with CDDP had also shown pronounced effects on the expression of molecular chaperone HSPs. Additionally, it was found that a decrease in the expression of DUBs and HSPs proportionally affected the protein kinase B/mammalian target of rapamycin (P13K/AKT/mTOR) and wingless-related integration site (Wnt) pathways leading to the upregulation of poly-ADP ribose polymerase (PARP), caspase-3, and caspase-9. Overall, the findings of this work demonstrated a new effective strategy for reducing the proliferation of A549 cells through the inhibition of DUBs and HSPs. This thesis is presented in the “Alternative thesis format”

Graphical abstract



List of Publications and Conference Presentations

Published paper

Ibrahim, B., Akere, T. H., Chakraborty, S., Valsami-Jones, E., & Ali-Boucetta, H. (2023). Gold Nanoparticles Induced Size Dependent Cytotoxicity on Human Alveolar Adenocarcinoma Cells by Inhibiting the Ubiquitin Proteasome System. *Pharmaceutics*, 15(2). doi:10.3390/pharmaceutics15020432

Manuscripts Prepared for publication

Ibrahim, B., Valsami-Jones, E., & Ali-Boucetta, H. (2023). Functionalisation of Gold Nanoparticles (AuNPs) Plays a Pivotal Role in the Suppression of A549 Proliferation

Ibrahim, B., Valsami-Jones, E., & Ali-Boucetta, H (2023). Inhibiting the Molecular Chaperone Heat Shock Proteins (HSPs) with AgNPs and AuNPs Attenuate the Proliferation of A549 Cells.

Ibrahim, B., Valsami-Jones, E., & Ali-Boucetta, H (2023). Effect of Cisplatin and AuNPs Combination on Heat Shock Proteins in Human Lung Epithelial Cancer Cells.

Paper published that is not directly related to this work but completed during this PhD

Akere, T. H., de Medeiros, A. M. Z., Martinez, D. S. T., **Ibrahim, B.**, Ali-Boucetta, H., & Valsami-Jones, E. (2022). Synthesis and Characterisation of a Graphene Oxide-Gold Nanohybrid for Use as Test Material. *Nanomaterials (Basel)*, 13(1). doi:10.3390/nano13010033

Conference Presentations

- Postgraduate research conference, University of Birmingham, United Kingdom, 6th May 2019.
- 10th International conference on nanotoxicology (NanoTox 2021), 20th to 22nd April 2021 (Virtual conference). Organised by Biomaterials Risk management (BIORIMA), Patrols and Gracious.
- Total Nano-Safe International conference, 11th to 12th November 2021 (Virtual conference). Organised by NanoSAFE and NanoSafety Cluster.

Acknowledgments

First of all, I would like to thank the Petroleum Technology Development Fund (PTDF) for allowing me to benefit from the scholarship. My sincere gratitude goes out to my amazing supervisors Prof. Eugenia Valsami-Jones and Dr Hanene Ali-Boucetta for their leadership, mentoring, financial assistance, and patience in carefully overseeing my project from start to end. I also want to express my deep gratitude to Prof. Eugenia for giving me the chance to work on the NanoSolveIT project and for giving me all the necessary supplies for the project.

Finally, I want to express my gratitude to all my wonderful lab friends and family for everything they have done for me. Without your love, support, and believe in me, I would not have been able to do this.

Table of Contents

Abstract.....	ii
List of Publications and Conference Presentations	iv
Acknowledgments.....	vi
List of Schemes.....	xv
List of Figures	xviii
List of Tables	xxii
List of Abbreviations	xxiii
Chapter 1: Introduction.....	1
1.1 Background	1
1.2 Different Approaches for the Synthesis of AuNPs and AgNPs.....	2
1.3 Applications of AuNPs and AgNPs in Biomedical Research	4
1.4 Cellular Uptake of NPs (AuNPs and AgNPs) and their Behaviour in Cancer	6
1.4.1 Phagocytosis.....	7
1.4.2 Caveolae Mediated Endocytosis (CME)	8
1.4.3 Clathrin Mediated Endocytosis (CME)	9
1.5 Effects of Physicochemical Properties on Uptake and Cytotoxicity of NPs.....	10
1.5.1 Effects of Size on the Cellular Uptake of NPs.....	10
1.5.2 Effects of Surface Coating or Charge on the Cellular Uptake of NPs.....	14
1.5.3 Effects of Shape on the Cellular Uptake of NPs	18
1.5.4 Effects of Protein Corona on the Cellular Uptake of NPs.....	20

1.6 Assessment of AuNPs and AgNPs Mechanisms of Toxicity in Different Cell Types.....	21
1.6.1 Oxidative Stress Induction by AuNPs and AgNPs in Cells	21
1.6.2 Effects of AgNPs and AuNPs on Mitochondrial Function.....	22
1.6.3 Effects of AgNPs and AuNPs on Deoxyribonucleic Acid (DNA) (Genotoxicity)	23
1.6.4 Programmed Cell death (Apoptosis) Induced by AgNPs and AuNPs	25
1.6.5 Effects of AgNPs and AuNPs on Intracellular Signalling Pathways	30
1.6.5.1 PI3K/AKT/mTOR.....	30
1.6.5.2 Effects of AgNPs and AuNPs on the Wnt/ β -Catenin Pathway	34
1.7 Ubiquitin Proteasome System (UPS).	36
1.7.1 Dysregulation of UPS Proteins and their Role in Cancer Development	37
1.7.2 Inhibitors of Ubiquitin Proteasome System (UPS)	39
1.8 Molecular Chaperone Heat Shock Proteins (HSPs).....	45
1.8.1 Roles of HSPs Expression in Cancer Development.....	47
1.8.2 Targeting HSPs as Novel Cancer Modality	51
1.9 Hypothesis and Objectives of the Study	53
1.10 Objective	53
1.10.1 Specific objectives	53
Chapter 2: Materials and Methods.....	55
2.1 Materials	55
2.1.1 Nanoparticles (NPs).....	56

2.1.2 Cell Culture	56
2.1.3 Cell Counting with Hemocytometer.....	57
2.2 Methodologies.....	58
2.2.1 Characterisation of AuNPs and AgNPs	58
2.2.1.1 Morphology and Size Distribution	58
2.2.1.2 Absorption Spectra	58
2.2.1.3 Hydrodynamic Diameter	58
2.2.1.4 Zeta Potential.....	59
2.2.1.5 Dissolution of AuNPs and AgNPs in UPW and CCM	59
2.2.2 Cell Viability Assays	60
2.2.2.1 MTT Assay.....	60
2.2.2.2 Modified Lactate Dehydrogenase (mLDH)	61
2.2.2.3 Lactate Dehydrogenase (LDH) Release Assay	62
2.2.2.4 CellTiter-Glo® Luminescent Cell Viability Assay	63
2.2.2.5 Cell Recovery	64
2.2.2.6 A549 3D Spheroids Formation.....	64
2.2.2.7 Cell Viability of A549 3D Spheroids	65
2.2.3 Cellular Uptake of Gold Nanoparticles (AuNPs) by A549 Cell.....	65
2.2.3.1 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)	65
2.2.3.2 Flow Cytometry Analysis of AuNPs Uptake	66

2.2.3.3 Transmission Electron Microscopy Analysis AuNPs Uptake.....	67
2.2.4 Mechanisms of Cellular Uptake Using Pharmacological Inhibitors	67
2.2.4.1 Inductively Coupled Plasma Mass Spectrometry	67
2.2.4.2 Flow Cytometry	68
2.2.5 Mechanisms of AuNPs and AgNPs Toxicity	68
2.2.5.1 GSH/GSSG-Glo™ Assay	68
2.2.5.2 Dichloro-Dihydro-Fluorescein Diacetate (H2DCF-DA) Assay.....	70
2.2.5.3 CellRox Deep Red Flow Cytometry Assay	71
2.2.5.4 Mitochondrial Membrane Potential (MMP)	72
2.2.5.5 MitoProbe™ TMRM Flow Cytometry Assay	72
2.2.5.6 Caspase-Glo 3/7 Activity Measurement.....	73
2.2.5.7 Apoptosis and Necrosis Assay by Flow Cytometry.....	74
2.2.6 Effects of AuNPs and AgNPs on Different Pathways.....	75
2.2.6.1 Extraction of Protein.....	75
2.2.6.2 Bradford Assay.....	76
2.2.6.3 Western Blotting.....	76
2.2.7 Statistical Analysis	77
Chapter 3: Gold Nanoparticles Induced Size Dependent Cytotoxicity on Human Alveolar Adenocarcinoma Cells by Inhibiting the Ubiquitin Proteasome System	78
Abstract.....	78
3.1 Introduction	79

3.2. Results.....	81
3.2.1. Physicochemical Characterisation of Different Sizes of AuNPs	81
3.2.2 AuNPs Decreased Cell Viability and Increased LDH Release in A549 Cells	83
3.2.3 AuNPs Uptake in A549 cells by ICP-MS, Flow Cytometry, and Transmission Electron Microscopy (TEM)	86
3.2.4 Assessment of the Uptake Mechanism(s) of Different Sizes of AuNPs in A549 Cells.....	88
3.2.5 Upregulation of ROS Production Caused by Different Sizes of AuNPs.....	90
3.2.6 Mechanism of Cell Death of Different Sizes of AuNPs Assessed by Annexin-V/PI	92
3.2.7 Effects of Different Sizes of AuNPs on 19S Proteasome DUBs Enzymes.....	93
3.2.8 Effects of Different Sizes of AuNPs on the Expression Level of Apoptosis-Related Proteins	95
3.3 Discussion.....	98
3.4 Conclusion.....	101
Chapter 4: Functionalisation of Gold Nanoparticles (AuNPs) Plays a Pivotal Role in the Suppression of A549 Proliferation.....	103
Abstract.....	103
4.1 Introduction	103
4.2 Results.....	104
4.2.1 Physicochemical Characterisation of Different Functionalised AuNPs.....	104

4.2.2 Cytotoxicity of A549 Cells Exposed to Different Functionalised AuNPs	107
4.2.3 Cell Recovery Upon Exposure to Different Functionalised AuNPs	110
4.2.4 Effects of Different Functionalised 5 nm AuNPs on Oxidative Stress	112
4.2.5 The Effect of Functionalised AuNPs on Mitochondrial Membrane Potential (MMP)	114
4.2.6 Apoptosis Induction by Different Functionalised AuNPs	115
4.2.7 Determining the Expression Levels of DUBS, P13K/AKT/mTOR and Wnt Signalling Related Proteins in Normal and Cancerous Lung Cell Lines Without AuNPs.....	117
4.2.8 Effects of Different Functionalised AuNPs on DUBs Expression in A549 Cell Lines..	119
4.2.9 Effects of Surface Functionalised AuNPs on Proteins in the P13K/AKT/mTOR and Wnt Signaling Pathways in A549 Cell Lines.....	121
4.2.10 The Effect of Surface Functionalised AuNPs on the Expression of PARP, Caspase-3, and Caspase-9 in A549 Cell Lines	123
4.3 Discussion.....	124
4.4 Conclusion.....	129
Chapter 5: Inhibiting the Molecular Chaperone Heat Shock Proteins (HSPs) with AgNPs and AuNPs Attenuate the Proliferation of A549 Cells	131
Abstract.....	131
5.1 Introduction	131
5.2 Results.....	133
5.2.1 Physicochemical Characterisation of AgNPs and AuNPs	133

5.2.2 Dissolution of AgNPs and AuNPs in UPW and CCM	136
5.2.3 Cytotoxicity Assessment of AgNPs and AuNPs in A549 Cells.....	137
5.2.4 Uptake of AgNPs and AuNPs by Flow cytometry	139
5.2.5 Formation of 3D A549 Spheroid Model	140
5.2.6 Live/Dead Cell Viability Assay	142
5.2.7 Intracellular ROS Generation Upon Treated with AgNPs and AuNPs	143
5.2.8 Effect of AgNPs and AuNPs on Mitochondrial Membrane Potential (MMP)	144
5.2.9 Apoptosis Induced by AgNPs and AuNPs	145
5.2.10 Effects of AgNPs and AuNPs on Molecular Chaperone HSPs.....	146
3.2.11 Does Inhibition of Molecular Heat Shock Proteins Downregulate AKT Signaling Pathway Proteins	148
5.2.12 Apoptosis Induction by Downregulation of HSP-AKT Binding	151
5.3 Discussion.....	153
5.4 Conclusion.....	157
Chapter 6: Effect of Cisplatin and AuNPs Combination on Heat Shock Proteins in Human Lung Epithelial Cancer Cells	158
Abstract.....	158
6.1 Introduction	158
6.2 Results.....	159
6.2.1 Cell viability Assessment of CDDP, AuNPs alone	159
6.2.2 The Effect of CDDP and AuNPs Combination on Cell Viability.....	162

6.2.3 Changes in Cell's Morphological Appearance After Exposure to Combination of AuNPs & CDDP	163
6.2.4 Does AuNPs:CDDP Combination CDDP Cause Oxidative Stress?	165
6.2.5 Does AuNPs:CDDP Combination Affect Mitochondria Membrane Potential (MMP)?	166
6.2.6 AuNPs:CDDP Combination Enhances Apoptosis	167
6.2.7 Effects of AuNPs:CDDP Combination on Molecular Chaperone Heat-Shock Proteins (HSPs)	169
6.2.8 Effects of AuNPs:CDDP Combination on P13K/AKT/mTOR Pathway.....	170
6.2.9 Effects of AuNPs: CDDP Combination on Apoptosis Related Proteins	172
6.3 Discussion.....	173
6.4 Conclusion.....	176
Chapter 7: General Discussion, Conclusion, Future Work and Recommendations	178
7.1 General Discussion.....	178
7.2 Conclusion	186
7.3 Future work or recommendations.....	187
References	189
Supplementary Materials Chapter 3.....	247
Supplementary Materials Chapter 4.....	254
Supplementary Materials Chapter 5.....	257

List of Schemes

Scheme 1.1. Nanoscale size of ENMs compared to molecular material and the objects found in nature [3].	2
Figure 1.2. TEM images of AuNPs and AgNPs synthesised through different approaches.....	4
Scheme 1.3. Illustration of biomedical applications of AuNPs and AuNPs	6
Scheme 1.4. Schematic diagram showing different nanoparticle uptake mechanisms	10
Scheme 1.5. Schematic illustration of DNA damage response upon exposure to NPs.....	24
Scheme 1.6. Extrinsic and intrinsic apoptosis pathways are shown schematically when cells are exposed to NPs	26
Scheme 1.7. Schematic illustration of cytotoxic effects of NPs on A549 cells through PI3K/AKT/mTOR signalling pathway.	31
Scheme 1.8. Schematic illustration of canonical and noncanonical Wnt pathways activated by upon exposure to NPs. Illustration was created with BioRender.com.....	35
Scheme 1.9. Ubiquitin proteasome system pathway: A ubiquitin molecule first attaches to the carboxyl group of c-terminal glycine residue. The activated ubiquitin is then transferred to the active site of a cystine residue of E2 conjugating enzymes via a thioester bond linkage. The linkage between ubiquitin and lysine residue of substrate molecules is catalysed by E3 ligases.	37
Scheme 1.10. Response of tumour cells to stress inducing factors. In the presence of stress factors, HSFs are phosphorylated in the cytoplasm and translocate into the nucleus of the cell where they bind to HSE and trigger the transcription of HSPs	46
Scheme 1.11. Compounds that have been previously found to inhibit cancer proliferation through the inhibition of HSPs.....	52

Scheme 2.1. A diagram depicting steps involved in splitting cells into more T ₇₅ flask to increase yield for experiments. The scheme was created with BioRender.com.	57
Scheme 2.2. Enzymatic reduction of yellow MTT to purple formazan by mitochondrial reductase enzyme in viable cells. The scheme was created with BioRender.com.	61
Scheme 2.3. Lactate dehydrogenase (LDH) release from the cell exposed to AuNPs or AgNPs convert lactate to pyruvate and iodonitrotetrazolium violet (INT) in the presence of coenzyme (NAD ⁺) which further convert to formazan by diaphorase enzyme. Scheme was created with BioRender.com.	63
Scheme 2.4. Schematic representation of a luminescence-based assay for measuring the amount of GSH/GSSG ratio in culture cells. The amount of both reduced and oxidised glutathione (GSH and GSSG) measurements relied based on the reaction steps where GSH-dependent conversion of a GSH probe in the first step, Luciferin-NT to luciferin by an enzyme glutathione S-transferase (GST) which then coupled to a firefly luciferase reaction in the second step. The Scheme was created with BioRender.com.	69
Scheme 2.5. Reactive Oxygen Species (ROS) measurement in A549 cells using H ₂ DCFDA: The non-fluorescent 2',7'-dichlorofluorescein diacetate (H ₂ DCFDA) is deacetylated by the action of cellular esterase in the cell membrane to a non-fluorescent 2',7'-dichlorofluorescein (H ₂ DCF) which further oxidised to a fluorescent 2',7'-dichlorofluorescein (DCF). The scheme was created with BioRender.com.	70
Scheme 2.6. Diagrammatic illustration of apoptosis and necrosis assay using fluorescently labelled Annexin V and propidium iodide (PI) measured by BD LSRFortessa X-20 flow cytometry. The scheme was created with BioRender.com.	75
Scheme 3.1. UPS diagram. Ubiquitin-activating enzymes (E1) activate the first step of the process which forms a thioester bond in the presence of a higher energy compound (ATP).	

The activated ubiquitin is then transferred to the ubiquitin conjugation enzyme (E2) via a transthiolation reaction. Ubiquitin ligases (E3) provide substrate specificity for the reaction and catalyse the covalent binding of ubiquitin to the target substrate through an isopeptide bond. The polyubiquitinated substrate moves to the proteasome for degradation. Finally, DUBs associated with the 19S proteasome remove conjugated ubiquitin from the substrate prior to the degradation 102

Schematic 6.1: Schematic illustration summarising the findings of this chapter and the effects of CDDP and AuNPs on HSPs, P13K/AKT/mTOR and apoptosis in A549 cells. The green arrow indicates the proteins that were downregulated while the red arrow indicates the proteins that were downregulated upon exposure to AuNPs:CDDP 177

List of Figures

Figure 3.1. Characterisation of different sizes of AuNPs by DLS in UPW and CCM..	82
Figure 3.2. Cell viability and lactate dehydrogenase (LDH) release of A549 cells treated with 5–40 µg/mL of different sizes of AuNPs measured using MTT and LDH assays.....	85
Figure 3.3. Cellular uptake of different sizes of AuNPs in A549 cells after 24 h incubation...	87
Figure 3.4. Effects of pharmacological inhibitors on the internalisation of different sizes of AuNPs (30 µg/mL) in A549 cells.....	889
Figure 3.5. ROS production in A549 cells following exposure to different sizes of AuNPs analysed using flow cytometry with the CellRox deep red kit.	911
Figure 3.6. Bar graph analyses of apoptosis and necrosis assessed by flow cytometry after the incubation of A549 cells with.....	933
Figure 3.7. AuNPs downregulate the expression of DUBs in A549 cells..	955
Figure 3.8. AuNPs induced apoptosis in A549 cells through a mitochondrial signalling pathway.	977
Figure 4.1. Physicochemical characterisation of different surface functionalised AuNPs..	1066
Figure 4.2. Percentage cell viability and metabolic activity of A549 cells treated with 1.25-35 µg/mL of 5 nm AuNPs coated with tannic acid, citrate, and PVP with 20% DMSO used as a positive control measured using mLDH and MTT assay.	10909
Figure 4.3. A549 cells recovery following exposure to functionalised 5 nm AuNPs	1111
Figure 4.4. Oxidative stress induction in A549 cells after exposure to 5 nm functionalised AuNPs at a concentration range of 1.25-10 µg/mL.	1133
Figure 4.5. A549 mitochondrial membrane potential (MMP) after exposure to 5 nm tannic acid, citrate, and PVP-functionalised AuNPs.	1144

Figure 4.6. Flow cytometric analysis of A549 cells exposed to 1.25-10 µg/mL of different functionalised 5 nm AuNPs for 24 h stained with Annexin V and PI	1166
Figure 4.7. Baseline Proteins expression in normal MRC-5 and cancerous A549 cells detected by western blot and semi-quantified using ImageJ.	11818
Figure 4.8. Surface functionalised AuNPs inhibit Deubiquitinating enzymes (DUBS) in A549 cells treated with a concentration of 1.25-10 µg/mL for 24 and 48 h as detected by western blotting and semi-quantified using ImageJ.....	1200
Figure 4.9. Surface functionalised AuNPs inhibit P13K/AKT/mTOR and Wnt related proteins in A549 cells treated with a concentration range of 1.25-10 µg/mL for 24 and 48 h and analysed by western blotting and semi-quantified using ImageJ.....	1222
Figure 4.10. Surface functionalised AuNPs induced mitochondrial-related apoptosis in A549 cells treated with a concentrations of 1.25-10 µg/mL for 24 and 48 hs assessed by western blotting and semi-quantified using ImageJ.....	1244
Figure 5.1. Physicochemical characterisation of AgNPs and AuNPs. Morphology and size distribution.....	1355
Figure 5.2. Dissolution of Ag and Au, shown in the form of ionic release in UPW and CCM over a period of 0.25-24 h measured by ICP-MS	1377
Figure 5.3. Percentage cell viability of A549 cells treated with 1.25-35 µg/mL of AgNPs and AuNPs with 20% DMSO used as a positive control measured using mLDH and CellTiter Glo assays.	13939
Figure 5.4. Dot plots of side scatter area (SSC-A) versus forward scatter area (FSC-A) intensity of A549 cells exposed to different concentration (1.25-10 µg/mL) of NPs for 24 h.	1400
Figure 5.5. Formation of A549 3D spheroid over time using liquid overlay method.....	1421

Figure 5.6. Live and dead cell staining of A549 3D spheroid cultured for 4 days using hanging drop method..	1422
Figure 5.7. Intracellular ROS fluorescence intensity in A549 cell detected by flow cytometry using Cellroxx deep red kit.	1433
Figure 5.8. Mitochondrial membrane potential (MMP) of A549 cells exposed to 1.25-10 µg/mL of AgNPs and AuNPs for 24 h measured by using mitoProbe™ TMRM Flow cytometry assay kit..	1444
Figure 5.9. Apoptotic cell death and necrotic analysis of A549 cells measured by flow cytometry with double staining of annexin V/PI..	1466
Figure 5.10. AgNPs and AuNPs downregulate the expression of HSPs in A549 cells.....	14848
Figure 5.11. Downregulation of P13K/AKT/mTOR pathway and β-catenin protein.	1500
Figure 5.12. Apoptosis induction by inhibiting HSP-AKT binding complex.	1524
Figure 6.1. Percentage cell viability of A549 cells following treatment with different concentrations of AuNPs (0-45 µg/mL) or CDDP (0-10 µg/mL) for 24h measured by MTT and mLDH assays.	1611
Figure 6.2. Percentage cell viability of A549 cells following treatment with a combination of AuNPs (25 µg/mL) and CDDP (2.5 µg/mL) for 24 h measured by mLDH and MTT assays..	1622
Figure 6.3. Optical images of A549 cells following incubation with.....	163
Figure 6.4: Effects of AuNPs, CDDP, and AuNPs:CDDP combination on oxidative stress biomarkers in A549 cells after 24 h.	1655
Figure 6.5. Mitochondrial Membrane Potential of A549 cells following treatment for 24 h	16767
Figure 6.6: AuNPs:CDDP enhanced apoptosis in A549 cells following 24 h treatment	16868

Figure 6.7. Effects of AuNPs, CDDP and AuNPs:CDDP combination on the expression of HSPs.	17069
Figure 6.8. Effects of AuNPs, CDDP and the AuNPs:CDDP combination on the expression of P13K/AKT/mTOR pathway proteins.	1711
Figure 6.9. Effects of AuNPs, CDDP and AuNPs:CDDP combination on the expression of mitochondrial related-proteins	1733
Figure S3.1. Characterisation of different sizes AuNPs dispersed in Ultrapure water by Transmission of electron microscopy (TEM)	2478
Figure S3.2. Dot plots of side scatted intensity (SSC) versus forward scattered intensity (FSC) of A549 cells treated with different concentration of AuNPs for 24 h.	2501
Figure S3.3. Percentage cell viability of A549 cells determined using MTT assay for pre-treated with pharmacological inhibitors	2512
Figure S3.4: Dot plots effects of pharmacological inhibitors on the internalisation of different size AuNPs in A549 cells.	2523
Figure S3.5. Apoptosis and necrosis analysis of A549 cells following the exposure to AuNPs of different sizes flow cytometry at of 5-30 µg/mL for 24 h.	2534
Figure S4.1: Absorption spectra of 5 nm AuNPs dispersed in UPW measured by UV-Vis.	2545
Figure S4.2: Cell viability of A549 cells exposed to stabilising agents at 0.125-1 mM for 24 h.	2556
Figure S4.3: Mitochondrial membrane potential of A549 cells treated with different functionalised AuNPs at concentration of 1.25 -10 µg/mL stained with mitohealth staining kit.	2567
Figure S5.1: Absorption spectra of 10 nm of AgNPs and AuNPs dispersed in UPW measured by DLS.	2578

List of Tables

Table 1.1: Effects of size on the cellular uptake of AuNPs and AgNPs.....	12
Table 1.2: Effects of surface coating or charge on the cellular uptake of AuNPs and AgNPs.	16
Table 1.3: Effects of shape on the cellular uptake of AuNPs and AgNPs.	19
Table 1.4: Apoptosis induced by AgNPs and AuNPs in different cell types	28
Table 1.5: Effects of AgNPs and AuNPs on the PI3K/AKT/mTOR signalling pathway and its downstream target.	32
Table 1.6: Ubiquitin proteasome system inhibitors in different types of cells.....	41
Table 1.7: Role of molecular HSPs in cancer development.	48
Table 4.1: Calculated IC ₅₀ of 5 nm surface functionalised AuNPs in A549 cells.....	110
Table S3.1: Surface area (m ² /g) of different 5, 10 and 80 nm AuNPs at different mass concentrations used in the cell viability assays.	248
Table S3.2: The table show the number of particles per volume (NPs/ML) for the highest concentration used in the cell viability assay and the number of viable cells remained after exposed to different size AuNPs for 24 h.	249

List of Abbreviations

β -TrCP - Beta transducin repeat containing E3

2D - Two-dimensional

3D - Three-dimensional

16HBE14o - Human bronchial epithelial cell

3T3 - Fibroblast cell

A431 - Epidermoid carcinoma

A2780 - Human ovarian cancer

A549 - Human lung epithelial carcinoma cell

Ag⁺ - Silver ion

Au⁺ - Gold ion

Ag - Silver

Ag10 - 10 nm silver nanoparticles

AgNPs - Silver nanoparticles

AgNO₃ - Silver nitrate

AKT - Protein kinase B

ANOVA – Analysis of variance

APC - Adenomatous polyposis coli

ATP - Adenosine triphosphate

Au10 - 10 nm gold nanoparticles

Au5 - 5 nm gold nanoparticles

AuNPs - Gold nanoparticles

B16 - Melanoma

Bax - Bcl-2 associated X protein

Bcl-2 - B-cell lymphoma-2

Bid - Bh3 interacting domain

Bak - Bcl-2 antagonist killer

BSA - Bovine serum albumin

Caco-2 - Colorectal adenocarcinoma cell

CCCP - Carbonyl cyanide 3-chlorophenyldrazone

CK1 - Casein kinase 1

CNTs - Carbon nanotubes

CME – Clathrin/Caveolae mediated endocytosis

COVID-19 - Corona virus 19

c-Myc - Cellular myelocytomatosis oncogene

CDDP - Cisplatin

CTAB - Cetyl trimethyl ammonium bromide

CUO - Copper oxide

DLS - Dynamic light scattering

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DPBS - Dulbecco phosphate buffered saline

DSBs - Double strand breaks

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethylene glycol tetra acetic acid

EMEM - Eagle minimum essential medium

ENMs - Engineered nanomaterials

ER - Endoplasmic reticulum

ERK - Extracellular signal-regulated kinase

FBS - Fetal bovine serum

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GO - Graphene oxide

GS - Glutathione (Reduced)

GSK-3 β - Glycogen synthase kinase 3 beta protein

GSSG - Glutathione (Oxidised)

GQDs - Graphene quantum dots

HecaT - Human keratinocytes

HEK - Human embryonic kidney

HeLa - Lacks cervical cancer cells

HepG2 - Hepatocellular carcinoma

hMSC - Human mesenchymal stem cells

HSF-1 - Heat shock factor-1

HS-PEG-CO - Thiol carboxyl polyethyl glycol

HSPs - Heat shock proteins

Ht29 - Human colorectal cancer

Huh7 - Human liver cell

ICP-MS - Inductively coupled plasma-mass spectroscopy

ICP-OES - Inductively coupled plasma optical emission spectroscopy

J774A-1 - Mouse macrophage

LA-ICP-MS - Laser ablation inductively coupled plasma-mass spectrometry

LO2 - Human fetal hepatocyte

M β CD - Methyl- β -cyclodextrin

MDA-MB231 - Human breast cancer

MDM2 - Mouse double minute 2 homolog

MIC - Maximum inhibitory concentration

MGC-803 - Gastric carcinoma

mLDH - Modified lactate dehydrogenase

MMP - Mitochondrial membrane potential

MRC-5 - Lung fibroblast

mTOR - Mammalian target of rapamycin

NPs - Nanoparticles

NMs - Nanomaterials

NM - Nanometre

NSCLC - Non-small cell lung cancer

OVCAR-5 - Ovarian cancer

P-70S6K – Protein 70-S6 kinase

PARP - Poly (ADP-ribose) polymerase protein

PC-3 - Prostatic small cell carcinoma

PDDC - Polydiallyldimethyl ammonium chloride

PDI - Polydispersity index

PDK1 - Pyruvate dehydrogenase 1

pDRP1 - Phosphate dikinase regulatory protein 1

PeG - *Pelargonium graveolens*

PEG - Polyethylene glycol

PI3K - Phosphoinositide 3-kinase

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PIP3 - Phosphatidylinositol 3,4,5-trisphosphate

PVP - Polyvinyl pyrrolidone

PSS - Polystyrene sulfonate (PSS)

RAW264.7 - Macrophage

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RPMI-1640 - Roswell Park memorial institute medium

SARS-COV-2 - severe acute respiratory syndrome coronavirus-2

SaOs2 - Human osteosarcoma cell

SKBR-3 - Human breast cancer

SH - Thiol group

SGC-7901 - Human gastric cancer

SnO₂ - Stannic oxide

THP-1 - Monocytic cell line

TEM - Transmission electron microscope

TiO₂ - Titanium oxide

U937 - Malignant cells

Ub - Ubiquitin

UCHL-1 - Ubiquitin C-terminal hydrolase-L1

UPS - Ubiquitin proteasome system

USP - Ubiquitin specific proteases

Wnt - Wingless

ZIF-8 - Zeolitic imidazole framework 8

ZnO NPs - Zinc oxide nanoparticles

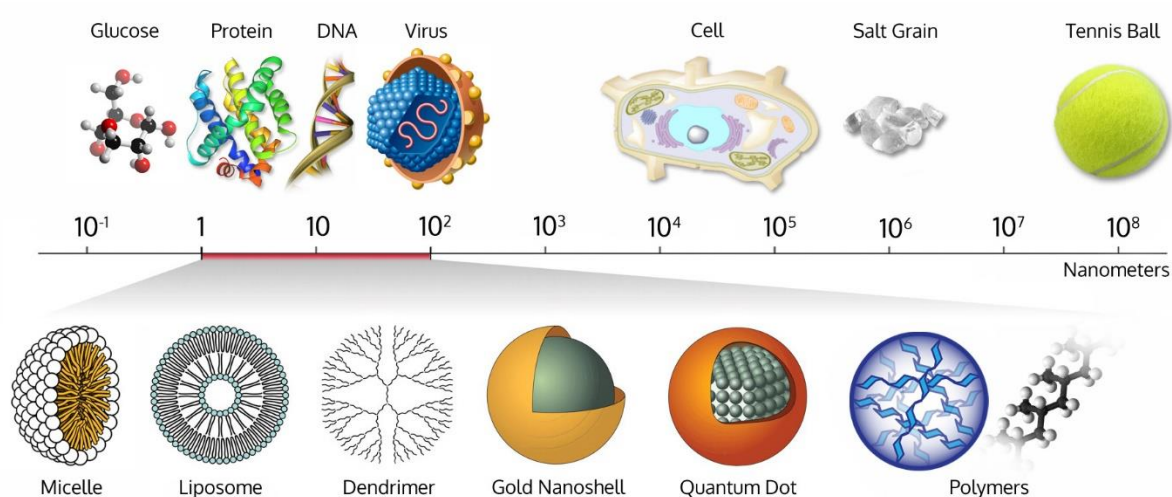
1.1 Background

Nanomaterials (NMs) are tiny nanoscale objects with at least one dimension in the size range 1-100 nm. They are incorporated into many products including food, cosmetics, agriculture, personal care, coating of medical devices, paints, pharmaceuticals, and electronics [1, 2]. Their nanoscale size ranges between molecular materials and the objects found in nature allowing them to maintain unique physicochemical characteristics (**Scheme 1.1**)[3]. They can be classified into naturally occurring, incidental and engineered nanomaterials. Naturally occurring NMs are found in the environment in volcanic ash, ocean spray, dust, and soil [4, 5]. The incidental NMs fall between the naturally, and engineered NMs which are as a result of human activities such as large scale mining, erosions, and industrialisation [6]. In contrast, engineered nanomaterials (ENMs) are the most important group of NMs and they are intentionally manufactured in the laboratory for specific applications with controlled diameter, morphology, surface charge, and chemical composition [7, 8]. These can be further categorised into:

- Carbon-based NMs, composed primarily of carbon such as carbon nanotubes (CNTs), fullerenes (C_{60} , C_{70} and C_{84}), graphene oxide (GO), graphene quantum dots (GQDs), and have been conventionally explored in biosensing, drug delivery and cancer therapeutics as a result of their robust electrostatic characteristics [9, 10].
- Metal-based NMs including nanogold, nanosilver, and metal oxides such as SnO_2 , TiO_2 , ZnO which offer unique electronic and optical behaviour compared to bulk ENMs [11, 12].

Other types of ENMs include spherical structures composed of core branched units classified as “dendimers” and material that incorporate nanosized particles into a matrix of standard material categorised as “Nanocomposite” such as nanofibers, nanowires, nanotubes [1, 13].

Gold nanoparticles (AuNPs) are widely used NMs due to their inert nature, stability, reduced cytotoxicity, biocompatibility and controlled method of synthesis [14]. Owing to their increased usage, they enter the body via ingestion, inhalation, blood circulation, skin absorption and may have an impact on human health [15]. These concerns have led to a rise in nanotoxicological research to investigate their toxicity and safety [16], but scientists are yet to draw conclusions regarding this.



Scheme 1.1. Nanoscale size of ENMs compared to molecular material and the objects found in nature [3].

1.2 Different Approaches for the Synthesis of AuNPs and AgNPs

NMs, such as AuNPs and AgNPs, are synthesised through an array of different approaches including chemical, green synthesis, biological, and physical methods. The chemical approach is the most common approach used for the synthesis of AuNPs, and AgNPs that involves reduction of tetrachloroauric (III) acid and silver Nitrate (AgNO_3) which occur in two different

stages [17]. In the first stage, organic molecules such as sodium borohydride (NaBH_4), sugars, formaldehyde, and hydrogen are used for the reduction of gold ion (Au^+) or silver ion (Ag^+) which are further reduced to an Au^0 or Ag^0 metallic ion. In the second stage, the capping or stabilising agents including citrate, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), and polymers are attached to their outer surface to prevent aggregation, surface charge loss and produce monodispersed AuNPs or AgNPs when dispersed in aqueous solution [18, 19]. Using such methods, researchers have reported the synthesis of AuNPs and AgNPs in a range of particle sizes. For instance, Piella et al., [20] pointed out that the chemical reduction process is used to produce sub-10 nm citrate capped AuNPs. This approach yields small monodispersed AuNPs with an average hydrodynamic distribution range of 3-10 nm. In addressing the issues of environmentally friendly, cost effective, and non-cytotoxic, a green alternative is considered for the synthesis of NMs using a renewable sources, such as plant extracts [21]. The use of plant extracts to produce AuNPs or AgNPs is considered to have economical and medicinal advantages due to the phytochemicals present in the plants; they also yield highly reproducible AuNPs or AgNPs [22, 23]. In this approach specific segments of plant extracts are used to produce the NMs. For example, leaves of *Pelargonium graveolens* (PeG) were used to synthesise decahedral and icosahedral AuNPs in a size range between 20-40 nm [24]. Likewise, spherical monodispersed AgNPs with a size range of 5-85 nm were synthesised from Aloe Vera leaf extracts [25]. In another study, extracts from *Morinda Citrifolia* roots were applied to synthesised 8-17 nm AuNPs [26]. In the biological approach, microorganisms like *Klebsiella pneumonia*, *Pseudomonas stutzeri* AG259, *Escherichia coli*, and *Candida utilis* were used to synthesise AuNPs or AgNPs due to their ability to reduce the Au^+ or Ag^+ to form metallic particles [27, 28]. One example of the physical approach, is a laser ablation technique used to synthesise AuNPs which involves the use of a beam to ablate

nanosized fragments from a metal surface immersed in an aqueous solution to produce NMs [29]. This process is regarded to be an alternative approach for AuNPs, and AgNPs synthesis which may involve lower cost, less contamination, temperature control, and the used of advanced equipment [30].

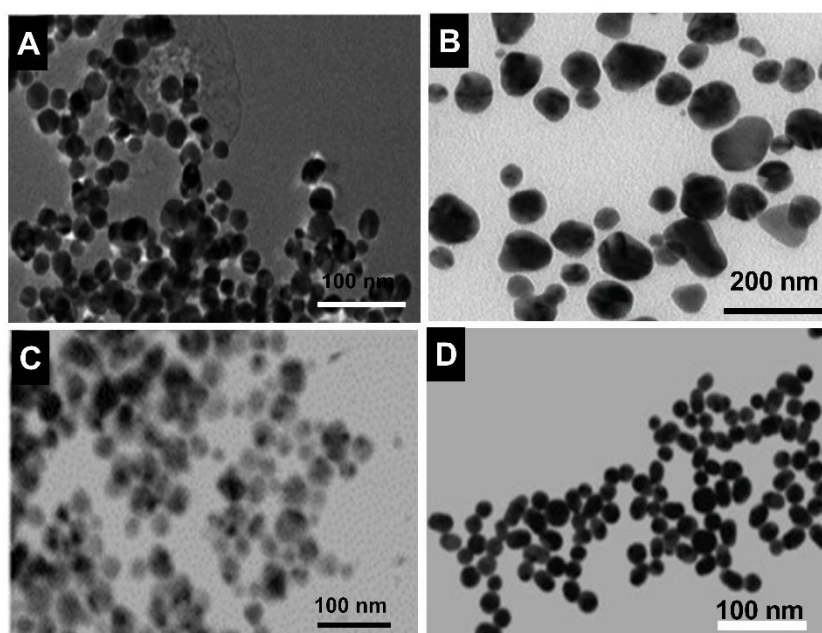


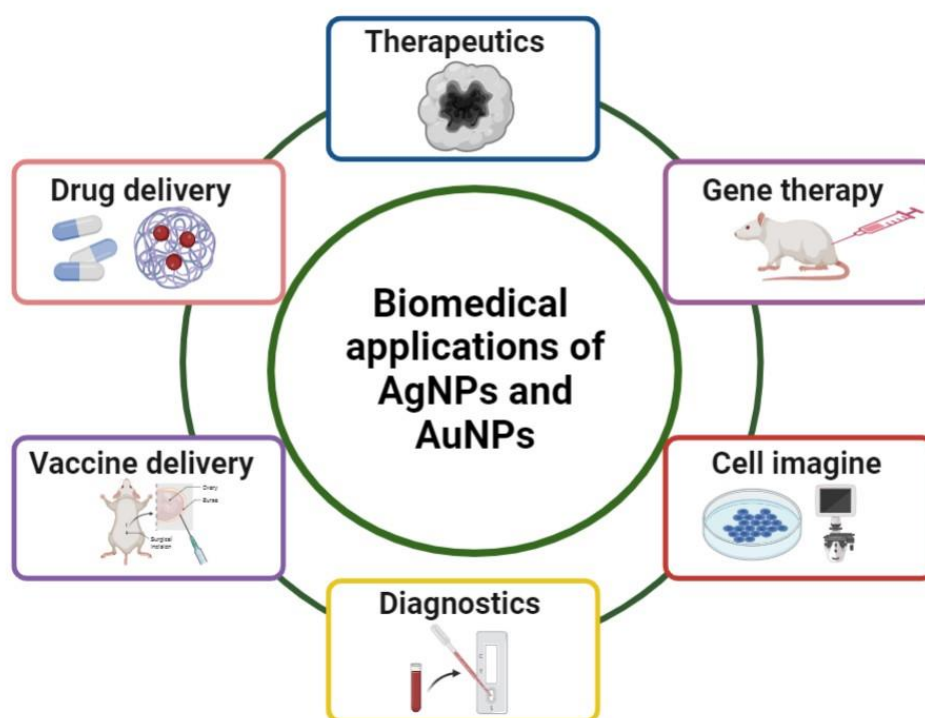
Figure 1.2. TEM images of AuNPs and AgNPs synthesised through different approaches. (A) AgNPs synthesised from chemical reduction (B) AuNPs synthesised from plant extracts (C) AuNPs synthesised from bacteria (D) AuNPs synthesised with laser ablation. (Scale bar: 100 nm was used for figure A, C and D, and 200 nm was used for figure B).

1.3 Applications of AuNPs and AgNPs in Biomedical Research

AuNPs and AgNPs have shown an impressive potential in virtually every branch of nanoscience ranging from cancer therapy, drug delivery, vaccine delivery, microbial, viral diagnostics, and gene therapy (**Scheme 1.3**). Because of their nanoscale (1-100 nm) related properties, which increase the surface area to volume ratio, they are better candidates for cancer therapy compared with their bulk counterpart [31, 32]. Researchers have shown that

their ability to generate reactive oxygen species (ROS) through intracellular oxidative stress, decrease MMP, and consequently cell death, promotes their use in cancer therapeutics [33, 34]. While the majority of drugs have been associated with poor solubility, reduced bioavailability, uncontrolled release, and high toxicity in biological systems [35]. AuNPs and AgNPs have gained significant interest as delivery systems for drugs to enhance their pharmacological properties and reduced adverse effects [36, 37]. The small size of AuNPs, biocompatibility, easy method of synthesis, and low rate of toxicity make them exceptional for drug delivery. For instance, tumour cells can become resistant because of repeated cisplatin administration by increasing the solid stress level [38]. However, AuNPs were able to reduce stress levels and thus enhance cisplatin delivery and ultimately improve chemotherapy [38]. Additionally, AuNPs and AgNPs are used as antibacterial agents due to their ability to limit the growth of bacteria, as they have the capacity to pass through the bacterial cell wall and change the conformational membrane structure, thereby creating damage to bacterial cells [39, 40]. Furthermore, they can bind to the thiol group (-SH) of many respiratory enzymes and deactivate them [41]. For example, the antibacterial and wound healing activity of AuNPs synthesised extracts of *Abelmoschus esculantus* opposed the strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis* using minimum inhibitory concentration (MIC) has shown that microorganisms lose their ability to replicate and enzymes of a metabolic pathway are deactivated [42]. Likewise, 20-80 nm AgNPs were discovered to be extremely effective against the strains of *Staphylococcus aureus* and *Escherichia coli* [43]. Recently, AuNPs and AgNPs were incorporated into COVID-19 rapid diagnostic kits which help isolating the target viral RNA from infected patients. This could be due to their powerful surface plasmon resonance that can be brought into play to detect this biological interaction [44, 45]. They also have shown the capacity to block viral binding to

host cells, inhibiting RNA replication and preventing it from binding to biological membrane cell receptors [46]. For instance, the anti-spike antibody attached to the outer surface of AuNPs was reported to block the replication of COVID-19 and prevent it from infecting other cells [47]. Mouth wash with AgNPs has been reported to reduce the morbidity of SAR-COV-2 infections in care workers who are exposed to patients diagnosed with the virus [48]



Scheme 1.3. Illustration of biomedical applications of AuNPs and AuNPs. Illustration was created with BioRender.com.

1.4 Cellular Uptake of NPs (AuNPs and AgNPs) and their Behaviour in Cancer

Understanding the interactions between engineered NMs and biological membranes may significantly explain the potential uptake, biodistribution, biomedical applications, and cytotoxicity of AuNPs and AgNPs in A549 cell [49]. The biological membrane is composed of a phospholipid bilayer, proteins (integral and peripheral), cholesterol and a small proportion

of lipopolysaccharides [50]. It plays an essential function in protecting the cell against the external environment, maintaining homeostasis, transporting materials such as glucose and ions from inside and outside of the organisms, and mediate cell to cell communications [51]. Upon inhalation, ingestion, and penetration through the surface of the skin. AuNPs or AgNPs can bind to membrane surface receptors resulting in systematic uptake via different mechanisms like phagocytosis, clathrin-, caveolae-mediated endocytosis and macropinocytosis [52].

1.4.1 Phagocytosis

Phagocytosis (also known as cell eating) is a form of an active mechanism by which the cells biological membrane engulfs debris, bacteria, large AuNPs or AgNPs into the cytoplasm through the formation of membrane vesicles and is used by neutrophils, macrophages, and monocyte [53]. Phagocytosis is initiated when the phagocytic receptors capture ligands such as immunoglobins (i.e antibodies), NPs, complement proteins or other blood proteins (i.e Laminin) to be ingested into the cells [54]. During phagocytosis, NMs such as AuNPs or AgNPs bind to a specific receptor on the surface of biological membranes, which stimulate the formation of a membrane-bound vesicle called a phagosome [55]. The phagosome invaginates and then pinches off from the biological membrane receptor, fuses with the lysosome to form phagolysosome and the NPs are degraded by the hydrolytic enzymes containing in the lysosome at a later stage (**Scheme 1.4**) [56]. Notably, Kuhn et al., [57] studied the uptake mechanism of 40 nm and 1 μ m NPs using a mouse macrophage (J774A.1) and human epithelial type II cell line (A549). The uptake of 1 μ m particles was only observed in J774A.1, but not in epithelial cells when incubated for 1 h [57]. Similarly, the uptake mechanism of murine macrophage was investigated using 20-45 nm AuNPs by TEM and optical microscopy. The latter showed that the AuNPs were efficiently internalised via this

uptake mechanism [58]. Likewise, the influence of NPs size (20 and 200 nm), functionalisation (amine and carboxyl), media content on cellular uptake of polystyrene NPs in phagocytotic (DMBM-2) and non-phagocytotic cells (A549) was investigated using confocal microscope and the image analysis showed that 200 nm, carboxy functionalised and non-functionalised were preferentially phagocytosed by macrophages more efficiently than the epithelial cells [59]. This suggests that a small size of AuNPs or AgNPs localise into the cells by other mechanisms other than phagocytosis [53, 60].

1.4.2 Caveolae Mediated Endocytosis (CME)

CME is another uptake mechanism for NPs that implicate flask-shaped invaginations of the plasma membrane called caveolae (approximately 50-80 nm), rich in lipid rafts present in endothelial cells, fibroblast, type I epithelial cells, and muscle cells [61]. In this process, AuNPs or AgNPs could be trapped in the caveolae binding to the plasma membrane surface. After internalisation, caveolae- or lipid raft-derived vesicles pinch up from the cell membrane and fuse with caveosomes, which mainly differ from endosomes in pH and contents. In caveosomes, internalised NPs or membrane constituents could reside and be sorted to the golgi complex, or to the endoplasmic reticulum (ER) by avoiding the lysosomal breakdown process (**Scheme 1.4**)[62]. Caveolae mediated endocytosis plays critical functions in the cells such as cell signalling, lipid regulation, fatty acids and vesicular transport [54]. Several cross-sectional studies suggest CME is the dominant cellular uptake mechanism of spherical AuNPs or AgNPs within epithelial and endothelial cells [60, 63]

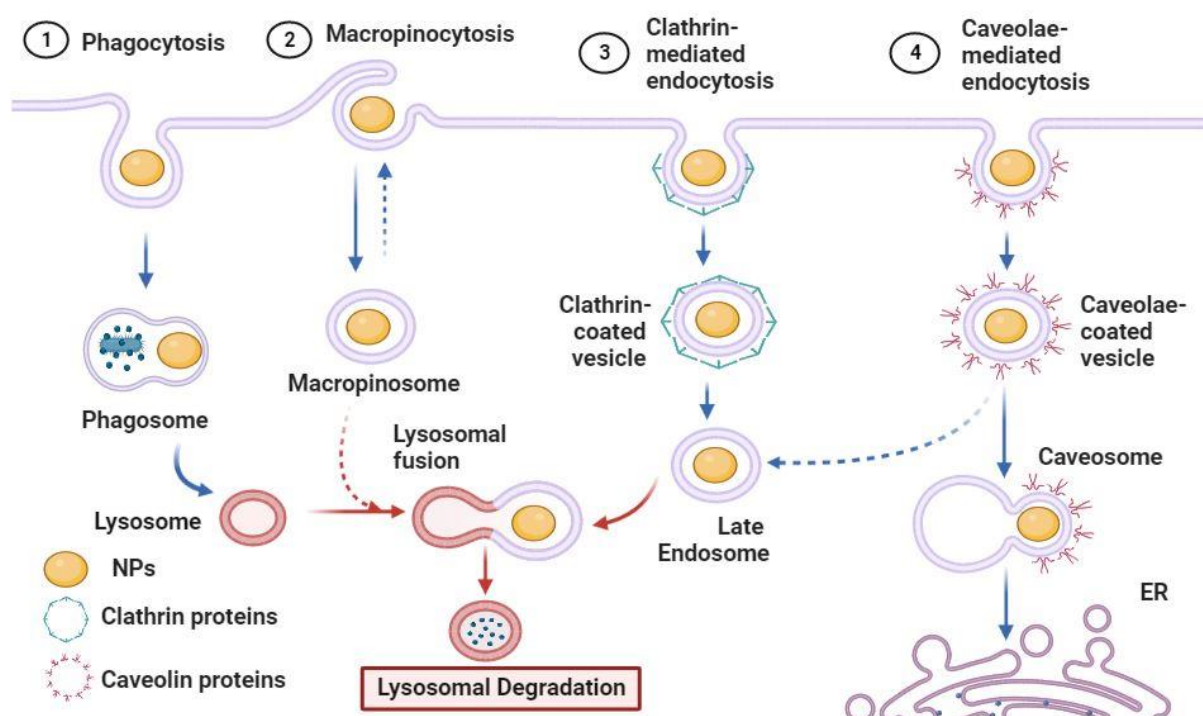
In addition, Hao and his co-workers studied the possible uptake mechanism of small size AuNPs with a diameter of 4.5 nm in HeLa cells. They reported that the small AuNPs were internalised through the CME pathway after incubation with methyl - β - cyclodextrin (M β CD) at 4 °C for 2 h [64]. In another study, flow cytometry and confocal microscopy were used to

investigate the quantitative and qualitative internalisation pathway of spherical and cylindrical functionalised glycol-nanoparticles in RAW264.7 after 4 h incubation with endocytic inhibitors namely: rottlerin, chlorpromazine, and genistein [65]. They found that spherical NPs were internalised in greater amounts via caveolae mediated endocytosis while cylindrical NPs were only internalised through clathrin mediated endocytosis [65]. Endocytic mechanisms of negatively charged polystyrene NPs functionalised with a carboxylic group (40 and 200 nm) were reported in HeLa, A549, and 1321N1 cells by Santos and his colleagues after incubation with different pharmacological inhibitors at 4 °C for 2:30 h [66].

1.4.3 Clathrin Mediated Endocytosis (CME)

CME is another type of receptor mediated endocytosis for the internalisation of external nutrients, integral membrane proteins, membrane phospholipid, NPs, cholesterol through low-density proteins, and iron via transferrin proteins as a carrier molecule into the cytoplasm [67]. During this process, the extracellular molecules or ligands attach to a specific receptor on the surface of the biological membrane to form a receptor-ligand complex [49]. The receptor-ligand complex moves to a region of the biological membrane with clathrin coated pits, where they are generally internalised via the formation of clathrin coated vesicles [49]. Upon entry into the cytoplasm, internalised materials are trapped in the endosomes which bind with the lysosome where the materials are degraded by hydrolytic enzymes (**Scheme 1.4**) [54, 67]. Clathrin-mediated endocytosis as an uptake mechanism for AuNPs and AgNPs has been studied recently by many investigators using different cells and pharmacological inhibitors. For instance, in one study the uptake mechanism of AuNPs with three different shapes (stars, rods and triangles) was evaluated in RAW264.7 cells using different transport inhibitors. All the three shapes of AuNPs were internalised into the RAW264.7 cells through the CME pathway efficiently after pre-treatment with sucrose [68]. Similarly, the route of

entry of transferrin coated spherical and nanorods in HeLa cells has been illustrated to be CME after pre-treatment with sodium azide, potassium, and sucrose which inhibit the formation of clathrin coated vesicles [69]. Moreover, the uptake mechanism of lung fibroblast (MRC-5) and Chang liver cells were observed using 20 nm AuNPs. The study reported that the 20 nm AuNPs were internalised via clathrin-mediated endocytosis after treatment with chlorpromazine A and concanavalin, as measured using ICP-MS [70].



Scheme 1.4. Schematic diagram showing different nanoparticle uptake mechanisms. Scheme was created with BioRender.com.

1.5 Effects of Physicochemical Properties on Uptake and Cytotoxicity of NPs

The efficient uptake of AuNPs and AgNPs relies on their physiochemical characteristics such as hydrodynamic size, surface coating or charge, and shape [70-74].

1.5.1 Effects of Size on the Cellular Uptake of NPs

Efficient cellular uptake and permeability of AuNPs or AgNPs across tumour cells are controlled by the size of NPs [75]. The hydrodynamic size of NPs primarily determines their

cellular uptake mechanism, internalisation, and cellular response [76]. If the particle size is less than 10 nm, they may be able to penetrate cells without requiring any active energy. On the other hand, increasing particle diameter requires active energy for the engulfment of the particles by the cells [77]. The impact of size on cellular uptake and cytotoxicity of AuNPs and AgNPs has been reported. For instance, Gliga et al., [78] investigated the effect of size on the cellular uptake and cytotoxicity of 10, 40, and 75 nm of AgNPs in human non-tumour epithelial cells derived from human lung tissue (BEAS-2B) using TEM. They described a no significant differences between the different size NPs in terms of uptake and intracellular localisation for all the concentrations used (5, 10, and 20 µg/mL) at 24 h [78]. Different ovarian cancer cells (OVCAR5, OVCAR8, and SKOV3) were used to measure the cellular uptake of 18, 40, 60, and 80 nm AuNPs. The findings indicated higher uptake of 18 nm AuNPs in all three cell types and 80 nm in OVCAR5 and SKOV3 followed by 60 nm. On the other hand, it was found that ovarian cancer cells do not favour the uptake of 40 nm AuNPs [79]. Similarly, Liu et al., [80] evaluated the cellular uptake of 5, 20, and 50 nm AgNPs using four different cell lines (A549, HepG2, SGC7901, and MCF-5) to elucidate the possible mechanisms of cytotoxicity. They reported size dependent cellular uptake of AgNPs (5 > 20 > 50 nm) respectively and concluded the 50-100 nm is the optimum range for cellular uptake of AgNPs in cells [80]. Moreover, **Table 1.1** presents the major outcome of several studies that investigated the effects of different sizes of AgNPs and AuNPs on cellular uptake.

Table 1.1: Effects of size on the cellular uptake of AuNPs and AgNPs

NPS	Size (nm)	Cell lines	Major outcomes	Reference
AuNPs	14, 30, 50, 74, 100	HeLa	50 nm is the ideal size for the uptake of nanomaterials	[73]
AuNPs	15, 50, 74	MCF-7, HeLa	Uptake of 50 nm after prolonged exposure to hypoxia condition was observed.	[81]
AuNPs	18, 40, 60, 80	Ovarian cancer	The ovarian cancer cells showed higher uptake of 18 nm particles.	[79]
AuNPs	2, 4, 6	HeLa	2 nm was internalised to a greater extent compared to its counterpart.	[82]
AuNPs	5, 20, 50	HepG2, L02	50 nm demonstrate a greater extent of localisation	[83]

AgNPs	10, 40, 75	BEAS-2B	No significance difference was observed between the different sizes in terms of uptake and intracellular localisation.	[78]
AgNPs	5, 20, 50	HepG2, A549, MCF-7, SGC7901	5 nm localised into the cells much easier and induced greater cytotoxicity when compared to other counterparts.	[80]
AgNPs	20, 40	THP-1	Both NPs are localised into the cells.	[84]
AgNPs	20, 50, 75	16HBE14	Findings demonstrated higher uptake of 50 than 75 and 20 nm of AgNPs on a mass basis in FCS free media.	[85]
AgNPs	5, 20, 50, 100	B16	50 and 100 nm showed higher uptake	[86]

1.5.2 Effects of Surface Coating or Charge on the Cellular Uptake of NPs

NPs are stabilised with different capping agents including citrate, polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), tannic acid, carboxylic acid, carbonated and polystyrene to prevent aggregation, inhibit overgrowth, improve biocompatibility, and stability of the NPs [69, 87]. The variability of the functionalising agents or ligands attached to AuNPs or AgNPs surface determines their cellular uptake [88]. The positively charged particles are readily attracted to biological membranes because of the negative electrical charges on the membrane generated by the phosphate group attached to its surface and internalised rapidly compared to neutral and negatively charged particles [89]. AuNPs or AgNPs modified with anionic or cationic functionalised agents have been shown to affect cellular uptake as investigated by different researchers. For instance, AuNPs functionalised with chitosan (positively charged) were internalised in THP-1 to a greater extent compared to AuNPs functionalised with citrate [90]. Research showed that when Cetyl Trimethylammonium Bromide (CTAB), Polydiallyldimethyl Ammonium Chloride (PDAC), Polystyrene Sulfonate (PSS), and Thiol Carboxy Polyethyl Glycol (HS-PEG-COOH) coated AuNPs uptake were evaluated in A549 cells, AuNPs coated with CTAB molecules induced more cell death and membrane defects compared to AuNPs with other surface coatings which could be due to interaction and greater internalisation in cells [91]. However, there is still a controversy regarding how surface coating influences the uptake rate of NPs. Regardless of the charge, when the NPs are dispersed in growth media, the zeta-potential is slightly modified due to protein adsorption which can affect their uptake [92]. For instance, it has been reported that dextran-coated NPs with zeta-potential between -50 and +5 mV can be internalised in Caco-2 cells despite having a higher negative zeta-potential [93]. In addition, **Table 1.2** summarised conflicting outcomes

regarding the influence of surface charge or coating on the cellular uptake of AgNPs and AuNPs.

Table 1.2: Effects of surface coating or charge on the cellular uptake of AuNPs and AgNPs.

NPs	Coating	Cell lines	Major outcomes	Reference.
AuNPs	CTAB, PDDC, PSS, HS-PEG-COOH	A549	CTAB AuNPs induced more defects because of their greater interactions with the biological membrane.	[91]
AuNPS	Citrate, Mercaptoundecanoic acid	HepG2	Both surface coatings internalise into the cells with no significant difference.	[94]
AuNPs	Positively, negatively charge	3T3 HUVECs, C17.2	Negatively charged AuNPs internalised into the cell to a lesser extent when compared to positively charged AuNPs.	[95]
AuNPs	Positively, negatively	U937	Positively charged NPs internalised faster into the cell via Macropinocytosis and induced more toxicity than the negatively charged NPs.	[96]

AgNPs	Chitosan, PVP, BSA	RAW 264.7	The finding illustrated rapid uptake of AgNPs coated with BSA.	[97]
-------	--------------------	-----------	---	------

1.5.3 Effects of Shape on the Cellular Uptake of NPs

AuNPs and AgNPs are synthesised in different geometries including nanospheres, nanorods, nanoshells, nanocages, nanoprisms, and nanostars. It is now becoming apparent that the particle's morphology plays a critical role in their cellular uptake, bioavailability, delivery, and toxicity in the cells [98, 99]. NPs shape is one of the major properties that influence the wrapping of the cell membrane during endocytosis and phagocytosis [99]. Chithrani and co-workers prepared spherical and rod AuNPs with a diameter of 14, 30, 50, 74, and 100 nm to measure the effects of shape on cellular uptake in HeLa cells. Their experimental results show greater uptake of spherical NPs than the rod-shape counterparts of the same diameter [73]. In a different study, Lee and co-workers compared the uptake of three different chitosan capped nanospheres, nanorods, and nanostars synthesised from green tea extract in human hepatocellular carcinoma (HepG2) using ICP-OES and LA-ICP-MS techniques. The results also demonstrated high uptake of nanosphere (58.0%), and nanorods (52.7%) compared to nanostars (41.5%) [100]. It could therefore be concluded that the time needed to wrap the NPs determines the degree of localisation with spherical NPs having less wrap time, and greater uptake than their counterparts. However, other researchers suggest that AuNPs with a greater aspect ratio demonstrate higher affinity to penetrate through biological membranes [101, 102]. AuNPs with triangular geometry and greater aspect ratio showed more effective cellular uptake than spherical AuNPs with less aspect ratio in HeLa cells [103]. Similarly, it has been demonstrated that nanotriangle AuNPs (3.33%) have greater internalisation into RAW264.7 than their nanorods (2.04%) and nanostars counterparts (0.38%) after 24 h incubation [68]. **Table 1.3** presents the major outcome of the effects of AgNPs and AuNPs shape on cellular uptake investigated by various researchers in different types of cell lines.

Table 1.3: Effects of shape on the cellular uptake of AuNPs and AgNPs.

NPs	Shape	Cell lines	Major outcomes	Reference
AuNPs	Nanosphere, nanorods, nanostars	HepG2	Illustrated higher uptake of nanospheres.	[100]
AuNPs	Spherical, Rod	HeLa	Higher efficient uptake of spherical AuNPs compared to rod.	[73]
AuNPs	Nanorods, Stars, Triangle	RAW264.7	The finding illustrated efficient uptake of triangle AuNPs.	[68]
AuNPs	Nanosphere, nanocages	SK-BR-3	Showed that shape did not influence cellular uptake.	[104]
AgNPs	Nanoprisms, spherical	hMSC's, HaCaT	Findings show greater uptake of nanoprism in hMSC's and showed no preference in HeCaT.	[105]

1.5.4 Effects of Protein Corona on the Cellular Uptake of NPs

Another factor that influences the cellular uptake of AuNPs or AgNPs is the formation of the protein corona. When NPs are dispersed in a growth media, the serum proteins and ions present are capable of being adsorbed onto the surface of NPs to form a NPs-protein complex known as the protein corona [106]. This complex plays a significant role in nanoparticle interactions with the cell membrane as it changes the morphology and surface chemistry of the particles [107]. For instance, Maiorano et al., [108] chose DMEM and RPMI-1640 cell culture media supplemented with 10% FBS to investigate how the composition of the media influences the formation of the protein corona and how the corona formed might affect the cellular uptake of different sizes AuNPs in HeLa and U937 cell lines. They found that AuNPs were internalised more rapidly when dispersed in RPMI-1640 than DMEM despite RPMI forming less time-dependent protein corona [108]. In another study of similar nature, the cellular uptake of AuNPs was reported to be less dependent on the proteins in the growth media [109]. In contrast to their study, Monteiro-Riviere et al., [110] demonstrated that the formation of protein corona enhances the interaction of AgNPs with the cell membrane and modulates their cellular uptake in HEK293 cells [110]. In this respect, there is still a lack of understanding of how the protein corona influence cellular uptake efficiency with others research suggests that, when NPs are dispersed in media containing serum proteins and ions, they tend to be aggregated which also affects their uptake efficiency [111-113].

1.6 Assessment of AuNPs and AgNPs Mechanisms of Toxicity in Different Cell Types

NPs such as AuNPs and AgNPs are commonly used in industrial products such as cosmetics, nanofluids, catalysts, painting, and medicines which made the discovery of their nanoscale properties possible [114, 115]. Their antimicrobial activity, together with their unique physicochemical properties, put them in high demand in industrial sectors [116]. A scientific report at the Woodrow Wilson International Centre for scholars showed that around 400 consumer products incorporated AgNPs [117, 118]. One negative impact of AuNPs or AgNPs usage is the increase in the potential human body exposure, thus, raising concern as to the effects they may present to the environment and humans [119-121]. After exposure to AgNPs or AuNPs through inhalation, digestion, or physical contact with the skin, they may penetrate the cells and interact with cellular organelles like mitochondria, nucleus, and lysosomes [122]. These interactions can lead to toxicological consequences such as a decrease in the metabolic activity, oxidative stress, genotoxicity, apoptosis, necrosis, autophagy, and modulation of signaling pathways [33, 121]. Several research studies demonstrated the cytotoxicity of AuNPs and AgNPs and the properties that potentiate their toxicity have been associated with their size, surface coating, concentrations, shape, and incubation period [77, 78, 97, 123-125].

1.6.1 Oxidative Stress Induction by AuNPs and AgNPs in Cells

During normal metabolic processes, cells produce free radicals because of normal cellular metabolic processes or by exposure to toxins in the environment such as radiation, cigarette smoking, industrial pollutants, UV light, and ozone [126, 127]. Excessive production of free radicals is expected to cause progressive adverse changes that bioaccumulate with time in the cells [128]. To reverse the changes, the body produces endogenous antioxidants such as Glutathione, Melatonin, Bilirubin, and Polyamines that interact with and neutralise the free radicals, thus, preventing them from cellular damage [129, 130]. An imbalance between

endogenous antioxidant defence and free radical generation in the cells is known as “Oxidative stress” and is associated with the initiation and progression of many diseases like cancer by increasing oxidation of DNA, proteins, and lipid peroxidation [131, 132]. Long-term oxidative stress contributes to the production of excessive ROS by damaging the electron transport chain (ETC) of oxidative phosphorylation and the release of free radical ions [127]. When generated, ROS mediates the activation of cellular metabolic mechanisms such as DNA damage and repair, mitochondrial dysfunction, apoptosis, necrosis, autophagy, and intracellular signalling pathways [133-135]. The role AuNPs and AgNPs in mediating ROS generation as one of the key mechanisms of their cytotoxicity has been postulated in different cell types, exposure conditions, and concentrations [21, 71, 74, 136, 137]. Despite these significant contributions to literatures, there is still an unclear conclusion regarding the actual concentrations, and exposure conditions of NPs that can generate ROS.

1.6.2 Effects of AgNPs and AuNPs on Mitochondrial Function

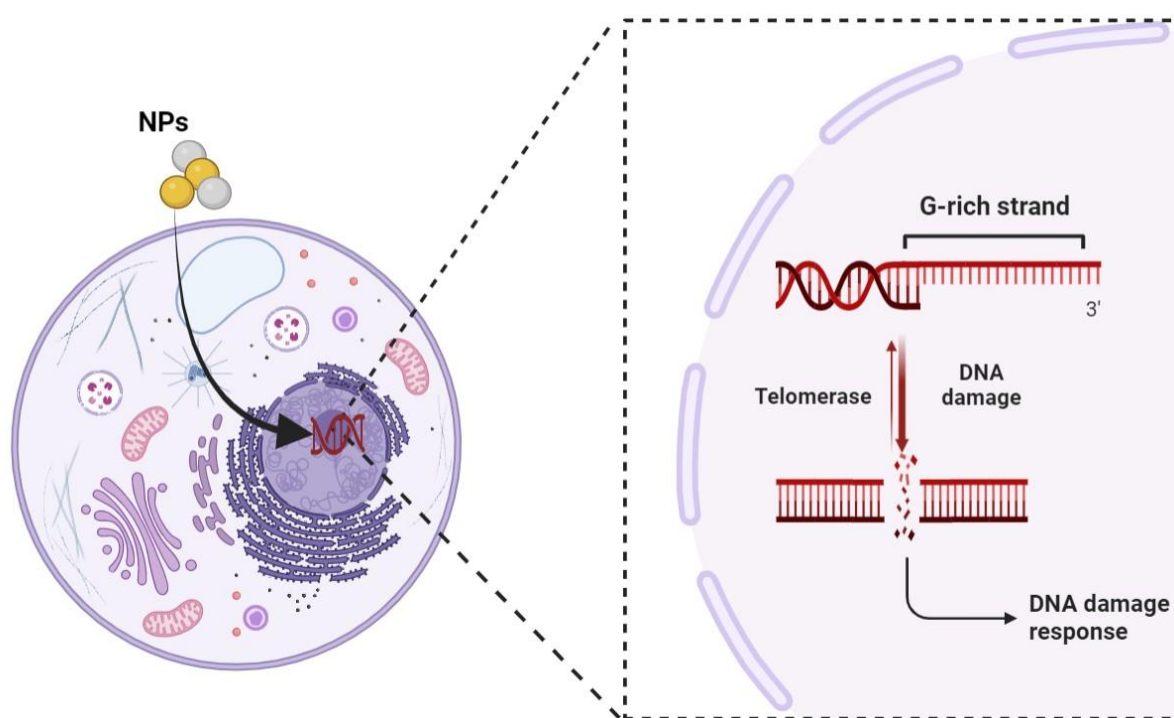
Mitochondria is a double-membrane organelle that is also regarded as the powerhouse of the cell where many cellular functions such as oxidative phosphorylation, calcium ion storage, energy production, regulation of metabolism, cell viability, and programmed cell death occur in eukaryotic organisms [138, 139]. When AuNPs or AgNPs are being internalised by the cell, NPs may interact with the mitochondria which may hinder the cellular function of the cell by affecting the ATP production and cellular function of cells [140, 141]. It has been demonstrated that exposure to AuNPs and AgNPs can cause negative alterations in the mitochondria in various types of cells [142-144]. For instance, it was reported that exposure to AuNPs coated with AAB (alkyl-ammonium bromide), ASC (alkyl-sodium carboxylate), and PEG triggers pronounced downregulations of genes encoding subunits of mitochondrial electron transport chain (ETC) leading to mitochondrial dysfunction, cell death and necrosis

in THP-1 cells [145]. In other research of similar nature, AuNPs coated with different surface functionalisation were reported to reduce tubular mitochondria, decrease ATP synthesis, and downregulate the expression of mitochondria respiratory chain complex V in Raw 264.7 [146]. Likewise, the activity of mitochondrial respiratory chain I-IV complexes was downregulated upon exposure to AgNPs leading to disruption of mitochondrial membrane potential (MMP) [147]. In human hepatocellular carcinoma (HepG2) cells, PVP capped AgNPs were found to cause loss in the mitochondrial ridge structure leading to upregulation in the expression of p-Drp1 associated with mitochondrial fission [143]. The loss of MMP further regulates cell growth, apoptosis, electron transport chain, and signalling pathways [148]. For example, a decrease in MMP regulates the expression of c-Jun U-terminal kinases (JNK) mediating mitochondrial apoptosis [149]. Likewise, the upregulation of apoptotic proteins like BAX and cytochrome C release into the cytoplasm can initiate caspase dependent mitochondrial cell death that is associated with the loss of MMP and integrity.

1.6.3 Effects of AgNPs and AuNPs on Deoxyribonucleic Acid (DNA) (Genotoxicity)

DNA is a complex molecule that is responsible for carrying and transmitting hereditary materials and genetic information from parent to offspring [150]. DNA damage can result in changes to genes involved in cell proliferation such as c-Myc, which can contribute to the emergence of diseases like cancer [151, 152]. DNA in living organisms can be subjected to many structural modifications that result from either cellular metabolic process (endogenous) such as alkylation, hydrolysis, or environmental substances (exogenous) like NMs and ionising radiation [153, 154]. DNA damage (genotoxicity) could therefore be taken as the first step of assessing the toxicological effect of NMs on cells [155]. Studies have shown that AuNPs and AgNPs can bind to DNA molecules and cause DNA double strand breaks, chromosomal damage, and DNA deletions (**Scheme 1.5**) [155, 156]. For example, exposure to 20 nm AgNPs

coated with starch induces the formation of single strand breaks in IMR-90 cells (normal human lung fibroblasts) in a dose-dependent manner [157]. Similarly, 20 nm AuNPs were also found to damage DNA, downregulate genes linked to genomic instability, and impair DNA damage repair in human embryonic lung fibroblasts (MRC-5) [158]. In other studies, inverse correlations were observed between the size of AuNPs and AgNPs and their cytotoxicity, ROS generation, and induction of genotoxicity in different cell types [159-164]. Additionally, the effect of surface coating (citrate and mercaptoundecanoic acid) on genotoxicity was examined using 20 nm AuNPs in human liver HepG2 cells. This study demonstrated that neither citrate nor mercaptoundecanoic acid cause cytotoxicity, even at concentrations of 100 μm , and only induces minimal genotoxicity in the cells [94].



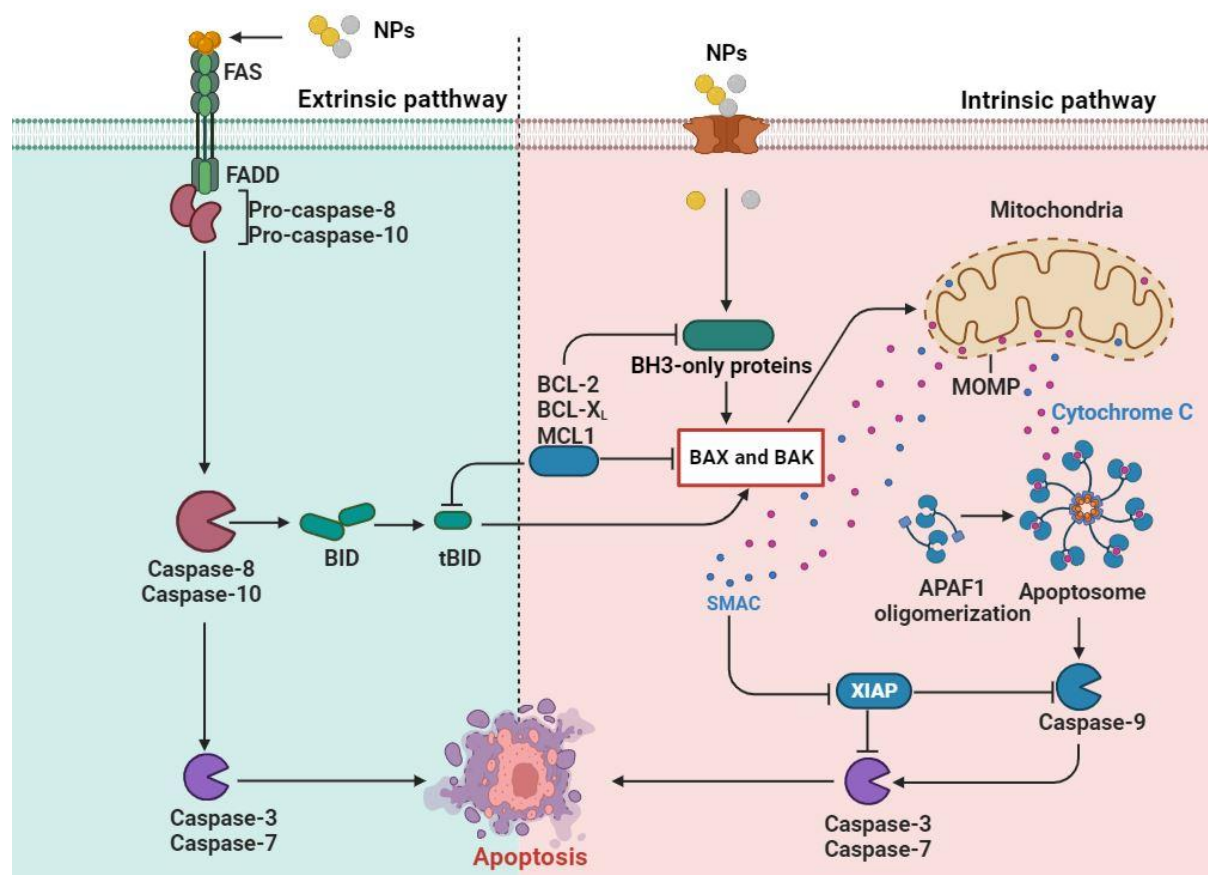
Scheme 1.5. Schematic illustration of DNA damage response upon exposure to NPs. Scheme was created with BioRender.com.

Abbreviations: Nanoparticles (NPs), Deoxyribonucleic acid (DNA)

1.6.4 Programmed Cell death (Apoptosis) Induced by AgNPs and AuNPs

Currie and colleagues described apoptosis (programmed cell death) in 1972 as a critical step in the progression of multicellular organisms and the continuity of tissue homeostasis [96]. Defects in apoptosis promote the emergence of different diseases such as neurodegenerative diseases, autoimmune diseases, cancer and make them resistant to treatment [165, 166]. Cell shrinkage, DNA fragmentation, chromatin condensation, extensive plasma membrane blebbing, and activation of cysteine aspartyl proteases such as caspase-3 and 9 are typical features of the natural mechanism of programmed cell death [167]. The apoptotic cell death machinery operates through either the mitochondrial (intrinsic) or death receptor (extrinsic) signalling pathways [165]. Numerous apoptotic stimulation signals, including oxidative stress, UV radiation, and exposure to NMs, activate the mitochondrial mediated pathway by activating Bax/Bak in the mitochondria and releasing cytochrome C into the cytoplasm [168]. A multi-protein complex called an "apoptosome" is created when cytochrome C binds to apoptosis protease activating factors 1 (Apaf-1) protein and inactive procaspase-9 [169]. The apoptosome hydrolyses adenosine triphosphate to cleave and activate caspase-9 initiator, which is then followed by activation of executioner caspase-3, 6, and 7, resulting in cell apoptosis [169, 170]. Contrarily, extracellular stimuli such as tumour necrosis factor related apoptosis inducing ligand (TRAIL), tumour necrosis factor (TNF), and fas ligand (Fas-L) activate transmembrane death receptors 4 (DR4) and death receptors 5 (DR5) to initiate extrinsic apoptotic pathway [171]. This sequence of events recruits other death domains that activate caspase-8 and caspase-10 which eventually activates and cleaves apoptosis executioner caspase-3 (**Scheme 1.6**) [168, 171]. According to several studies, exposure to AuNPs and AgNPs can induce apoptosis in several cell types, as indicated in **Table 1.4**. Combinations of NPs with certain drugs or flavonoids were also found to influence mitochondrial apoptosis in

a size- and -dose dependent manner. For instance, in human ovarian cancer cell lines (A2780), the combination of salinomycin and AgNPs was reported to induce apoptosis by upregulating Caspase-3, P53, Bax, and Bak and downregulating Bcl-2 [172]. Another comparable study found that combining AgNPs with gemcitabine selectively reduced the growth of A2780 cells by upregulating apoptotic protein expression and downregulating antiapoptotic protein expression [173].



Scheme 1.6. Extrinsic and intrinsic apoptosis pathways are shown schematically when cells are exposed to NPs. Scheme was created with BioRender.com.

Abbreviations: Fas cell surface death receptor (Fas), Fas-associated death domain (FADD), B-cell lymphoma 2 and X_L (Bcl-2 and X_L), Myeloid cell leukemia-1 (MCL-1), BH3-interacting domain (BID), BH3-antagonist/killer (BAK), BH3-antagonist X (BAX), X-linked inhibitor of apoptosis (XIAP), Apoptosis protease activating factor 1 (APAF-1), Mitochondria outer membrane permeabilisation (MOMP), nanoparticles (NPs), second mitochondria derived activator of caspase (SMAC).

Table 1.4: Apoptosis induced by AgNPs and AuNPs in different cell types

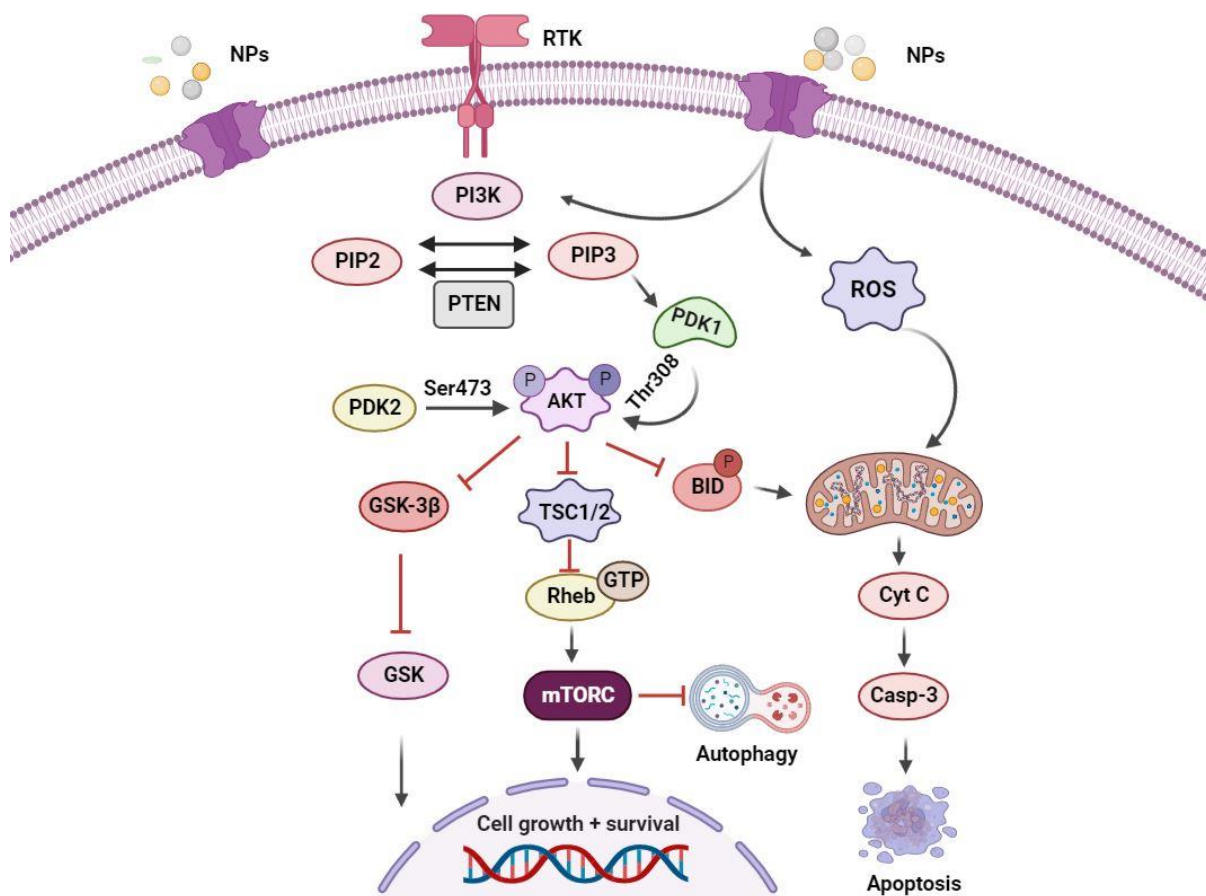
NPs	Size (nm)	Concentration ($\mu\text{g/mL}$)	Cell types	Apoptotic anti apoptotic proteins and their expression	Reference
AgNPs	30.71	0-100	HepG2	$\uparrow$ Bax, $\uparrow$ Caspase-3	[174]
AgNPS	52.4 ± 3.6	0-100	CHANG, HuH7	$\uparrow$ Bax, $\downarrow$ Bcl-2	[175]
AgNPs	25	1-37.5	SUM159	$\uparrow$ Caspase-3, $\uparrow$ Caspase-7	[176]
AgNPs	2	5-20	HepG2	$\uparrow$ Caspase-3, $\uparrow$ PARP, $\uparrow$ p53	[177]
AgNPS	86.6, 148.4, 315.1	1-500	HT-29	$\uparrow$ Caspase-3, $\uparrow$ Caspase-8, $\uparrow$ Caspase-9, $\uparrow$ Caspase-12	[178]
AgNPs	20	1	HeLa	$\uparrow$ P53, $\uparrow$ Cytochrome C, $\uparrow$ Bak, $\uparrow$ Bax, $\uparrow$ Bid, $\downarrow$ Bcl-2, $\downarrow$ Bcl-xL	[179]
AgNPs	480	0-100	A549	$\uparrow$ Caspase-3, $\uparrow$ Caspase-8, $\uparrow$ PARP, $\uparrow$ Bid, $\uparrow$ Bax $\downarrow$ Bcl-2, $\downarrow$ Bcl-w	[180]

AuNPs	-	0-200	MRC-7	↑Caspase-3, ↑Caspase-9, ↑Bax, ↑p53, ↓ Bcl-2	[181]
AuNPs	2, 40, 100	0-100	U937	↑Caspase-3, ↑Bax, ↓Bcl-xl, ↓XIAP, ↓Bid	[182]
AuNPs	13, 60	0-60	HK-2	↑Caspase-3, ↑Caspae-8, ↑Caspase-9	[183]
AuNPs	-	0-1000	MGC-803, GES-1, MCF-7	↑P53, ↑GSTP1	[184]
AuNPs	15, 50, 80	52.3	Calu-1, A549, H460	↑Capase-3, ↑DRP1, ↑TRAIL, ↑cleaved PARP	[185]
AuNPs	90-150	0-35	HEK293, Caco-2, HeLa, MCF-7	↑Caspase-3/7	[186]

1.6.5 Effects of AgNPs and AuNPs on Intracellular Signalling Pathways

1.6.5.1 PI3K/AKT/mTOR

PI3K/AKT/mTOR is formed through a threonine/serine kinase signalling pathway that mainly works to promote the proliferation of cells, movement, invasion, and prevention of apoptosis through activation of AKT, mTOR, and Bcl-2 proteins [187]. Growth factors, cytokines, neurotransmitters, and hormones are examples of molecular signals that activate the pathway by binding to surface receptors on the biological cell membrane, which then activate phosphoinositide-3- kinase (PI3K) by phosphorylating phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 proteins trigger the activation of phosphoinositide dependent kinase-1 (PDK1) which consists of the PH domain and promotes the phosphorylation of Thr308 in the cell, leading to protein kinase (AKT) activation. Activated AKT causes the phosphorylation of many downstream proteins including a mammalian target of rapamycin complex-1 (mTORC1), glycogen synthase kinase- β (GSK-3 β), ribosomal protein S6 kinase (p70S6K), Bcl-2, and caspases that promote cell proliferation, protein synthesis, apoptosis and cell cycle progression (**Scheme 1.7**) [188, 189]. The PI3K/AKT/mTOR signalling pathway has been found to be overexpressed in approximately 70% of all cancers and 30% of solid tumours, making it one of the most frequently mutated signalling pathways [190, 191]. Overexpression of PI3K/AKT/mTOR may be linked to genes mutation, growth factors, and upstream gene mutations [192, 193]. The overactivation of the pathway in cancer makes it a novel target for cancer treatment and many NPs have been identified to suppress the expression of this pathway through the induction of cytotoxicity [121, 194]. The effects of AgNPs and AuNPs on P13K/AKT/mTOR signalling pathway and it downstream targets studied by different researchers is illustrated in the **Table 1.5**.



Scheme 1.7. Schematic illustration of cytotoxic effects of NPs on A549 cells through PI3K/AKT/mTOR signalling pathway. Scheme was created with BioRender.com.

Abbreviations: Receptor tyrosine kinase (RTK), Nanoparticles (NPs), Phosphoinositide-3-kinase (PI3K), Phosphatidylinositol-4,5-phosphate (PIP2), Phosphatidylinositol-3,4,5-phosphate (PIP3), Phosphoinositide dependent kinase-1 and 3 (PDK1&2), Phosphatase and tension homolog (PTEN), Serine 473 (Ser473), Threonine 308 (Thr 308), Protein kinase B (AKT), Glycogen synthase kinase-3β (GSK-3β), Glycogen synthase kinase (GSK), Tuberous sclerosis 1/2 (TSC1/2), Ras homolog (Rheb), Guanosine-5-triphosphate (GTP), cytochrome c (Cyt c), Mammalian target rapamycin complex (mTOR), Caspase 3 (casp-3), BH3-interacting domain (BID).

Table 1.5: Effects of AgNPs and AuNPs on the PI3K/AKT/mTOR signalling pathway and its downstream target.

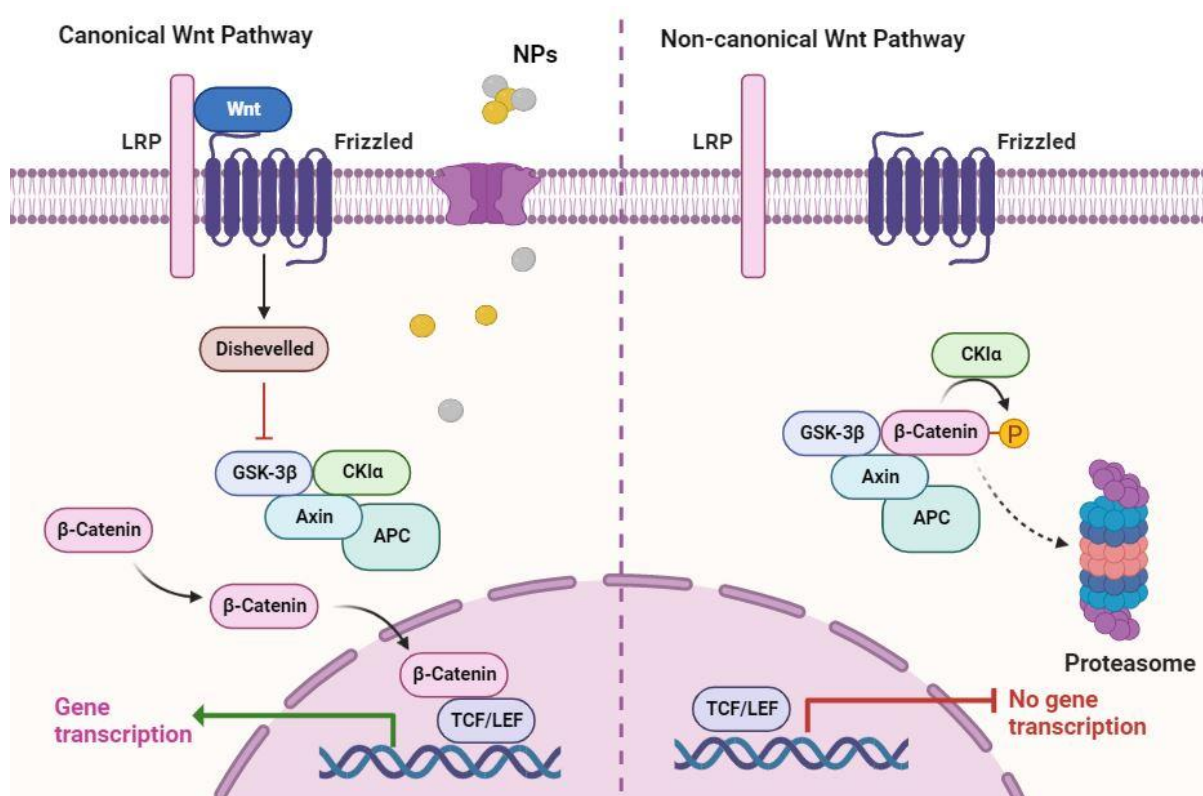
NPs	Size (nm)	Coating	Cells	Concentration ($\mu\text{g/mL}$)	Proteins	Reference
AgNPs	2	Citrate	HepG2	5, 10, 20	$\downarrow$ Akt, $\downarrow$ ERK, $\downarrow$ JNK, $\uparrow$ P38, $\uparrow$ P53, $\uparrow$ H2X	[177]
AgNPs	20, 30	-	MCF-7	2-14	$\downarrow$ PI3K, $\downarrow$ AKT, $\downarrow$ RAS, $\downarrow$ RAF, $\downarrow$ Bcl-2, $\uparrow$ VEGF, $\uparrow$ Cyto-C, $\uparrow$ Caspase-3	[195]
AgNPs	10, 100	-	HePG2	2, 4	$\downarrow$ ERK, $\uparrow$ P38, $\downarrow$ JNK	[196]
AgNPs	6, 20		A278	25-200	$\uparrow$ P53, $\uparrow$ P21, $\uparrow$ Bax, $\uparrow$ Bak, $\downarrow$ Bcl-2, $\uparrow$ Cyto-C, $\uparrow$ Caspase-3, $\uparrow$ Caspase-9	[197]
AgNPs	16, 20	-	A278	2-20	$\uparrow$ P53, $\uparrow$ p21, $\uparrow$ Bax, $\uparrow$ Bak, $\uparrow$ caspase-3, $\uparrow$ caspase-9	[172]
AgNPs	1-100		NIH3T3	50	$\uparrow$ P53, $\downarrow$ Bcl-2, $\uparrow$ PARP, $\downarrow$ JNK	[198]
AgNPs	5, 35	Citrate	U2OS, Saos-2	5-80	$\uparrow$ P53, $\uparrow$ Cyto-c	[199]
AgNPs	15		HepG2, LO2	20, 40, 80, 160	$\uparrow$ Cyto-c, $\downarrow$ Bcl-2, $\uparrow$ Bax	[200]

AgNPs	78	-	HEK293T, A498	10, 20, and 40	↓mTOR, ↓S6K	[201]
AuNPs	16-25	PEG	-	40	↓p-PI3K, ↓p-AKT, ↓p-AMPK	[33]
AuNPs	-	PEG	MCF-7	6	↓PI3K	[202]
AuNPs	3, 4.5		MCF-7, MDA- MB 231	50, 100	↓PI3K, ↓AKT, ↓PTEN, ↓mTOR, ↑GSK- 3β, ↓Bcl-2, ↑Bax, ↑caspase-3	[203]
AuNPs	-		HepG2	6.25	↓AKT, ↓mTOR, ↓Bcl-2, ↑Caspase-3, ↑FAAD, ↓ERK	[204]
AuNPs	10-20		A549	121	↑P53, ↑Bax, ↓Bcl-2, ↑caspase-3, ↑PARP	[205]
AuNPs	10	Citrate	A-431	5-25	↓AKT, ↓P-AKT, ↓P-ERK, ↓P-IKK	[206]
AuNPs	10, 70		HL-60	1-100	↑Caspase-3,7, 8	[207]
AuNPs	13	Citrate	A549	10	↓Bcl-2, ↑Bax, ↑P53, ↓Puma	[208]

1.6.5.2 Effects of AgNPs and AuNPs on the Wnt/ β -Catenin Pathway

The Wnt signalling pathway directly communicates information received from extracellular glycoproteins through the plasma membrane receptor to the target gene in the nucleus to control gene transcription [209]. The signalling pathway has been categorised as noncanonical and canonical. In the noncanonical state (in the absence of Wnt), cytoplasmic β -catenin is bound and degraded by the action of the destruction complex proteins including Axin, adenomatous polyposis coli (APC), GSK-3 β , and casein kinase 1 (CK1) [210]. The complex phosphorylates β -Catenin at the amino acid terminal region leading to its ubiquitination and proteasomal degradation by the action of E3 ubiquitin ligase enzyme β -TrCP [211]. The degradation of β -catenin prevents the protein from reaching the target gene in the nucleus, thereby reducing gene transcription (**Scheme 1.8**) [212]. In the Canonical Pathway (in the presence of Wnt) the Wnt gene is transported in an exosome from somewhere in the cell and activates the receptor Frizzled (Fz or Fzd) [213]. The activation of Fzd leads to the phosphorylation of a low-density lipoprotein receptor related protein (LRP) to form the Wnt-Fzd-LRP complex [212, 214]. This induces translocation of the destruction complex to a region of the membrane near the Wnt-Fzd-LRP complex resulting in Dishevelled (Dvl) activation [215]. Activated Dvl inhibits the destruction complex and therefore preventing β -catenin phosphorylation in the cytoplasm [216]. As a result, dephosphorylated β -catenin travels into the nucleus, where it binds with T-cell factor/ lymphoid enhance binding factor (TCF/LEF) transcription repressor and activates Wnt target gene expression [216] (**Scheme 1.8**). Dysregulation of the Wnt pathway occurs in various forms of chronic lymphocytic leukaemia, multiple myeloma, non-small cell lung cancer and is crucial for their pathogenesis [217, 218]. Overexpression of β -catenin transcription factor, CK1, Axin, APC, GSK-3 β , Dvl, and TCF/LEF has been reported to lead to cell proliferation, differentiation, apoptosis, and transcription

regulations [219-221]. Thus, controlling their overexpression might be vital for cancer treatment. Exposure to AgNPs (1 $\mu\text{g}/\text{mL}$) for 24 h lowers the level of cytoplasmic β -catenin in B35 neuroblastoma cells [222]. In another *in vitro* study, positively (cysteamine coated) and negatively (citrate coated) charged AuNPs induced apoptosis by inhibiting β -catenin overexpression in human breast cancer (MDA-MB-231). The study further demonstrated that exposure to both AuNPs induced toxicity to MDA-MB-468 cells by reducing the expression of GSK3- β and β -catenin levels [223].



Scheme 1.8. Schematic illustration of canonical and noncanonical Wnt pathways activated by upon exposure to NPs. Illustration was created with BioRender.com

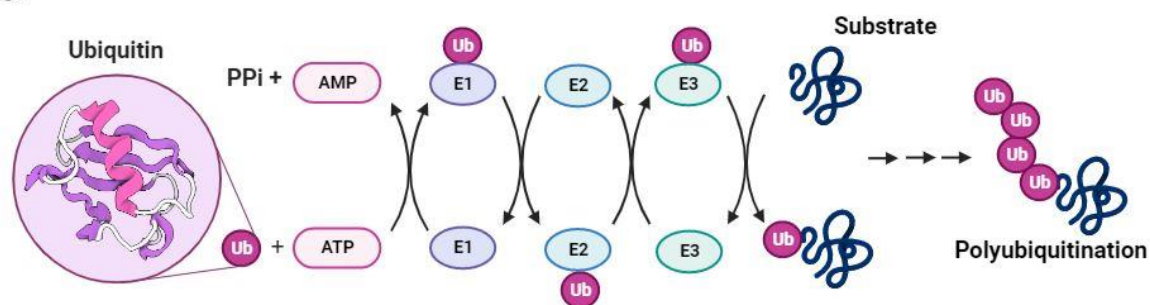
Abbreviations: Low density/lipoprotein receptor related protein (LRP), Glycogen synthase kinase-3 β (GSK-3 β), Adenomatous polyposis coli (APC), Casein kinase-1 (CK1), T-cell

factor/lymphoid enhance binding factor (TCF/LEF), Dishevelled (DVL), Frizzled (FZ or FZD), Wntless signaling (wnt), Nanoparticles (NPs), Beta-catenin (β -catenin).

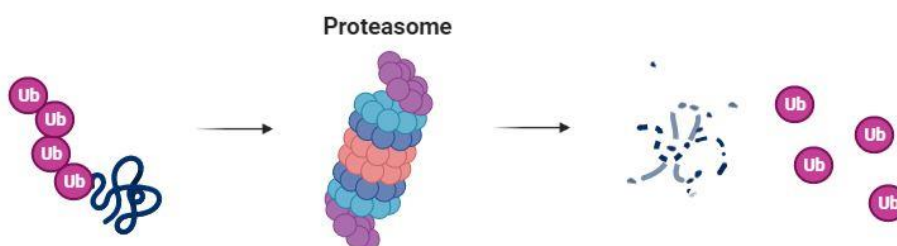
1.7 Ubiquitin Proteasome System (UPS).

Cell cycle progression, cell growth, apoptosis, intracellular signalling, eliminating mutants, and degradation of misfolded proteins are the key functions performed by UPS in eukaryotic cells. UPS involves covalent attachment of ubiquitin (76 amino acids) molecules to proteins to be degraded in 26S proteasome complex [224]. The process requires the action of three major successive enzymes: (a) the ubiquitin activating enzymes (E1), (b) ubiquitin conjugating enzymes (E2), and (c) ubiquitin ligases (E3) [225]. The process of ubiquitination begins with an activation step where Adenosine triphosphate molecules and ubiquitin are covalently attached to the alpha carboxyl group of the C-terminal of glycine residue forming a thioester linkage between the activating enzyme and ubiquitin. The activated ubiquitin molecule is then transferred from ubiquitin activating enzymes to an active site of a cysteine residue of the E2 conjugating enzymes and joined through thioester bond linkage [226]. Finally, the E3 ligases help with substrate recognition between the ubiquitin and lysine residue of substrate molecules, thereby catalysing the linkage between them [225]. Attachment of a single ubiquitin molecule to a substrate is generally termed as monoubiquitination while attachment of more than one molecule of ubiquitin is known as polyubiquitination and the reaction is formed on the K48 lysine residue of the substrate molecules (**Scheme 1.9**) [227].

1 Ubiquitination



2 Protein degradation



Scheme 1.9. Ubiquitin proteasome system pathway: A ubiquitin molecule first attaches to the carboxyl group of c-terminal glycine residue. The activated ubiquitin is then transferred to the active site of a cystine residue of E2 conjugating enzymes via a thioester bond linkage. The linkage between ubiquitin and lysine residue of substrate molecules is catalysed by E3 ligases. Illustration was created with BioRender.com.

Abbreviations: Ubiquitin (Ub), Adenosine monophosphate (AMP), Adenosine triphosphate (ATP), Inorganic pyrophosphate (PPi), Ubiquitin activating enzymes (E1), Ubiquitin conjugating enzymes (E2), Ubiquitin ligases (E3).

1.7.1 Dysregulation of UPS Proteins and their Role in Cancer Development

UPS play an important role in controlling the intracellular protein homeostasis network through discriminatory elimination of damage and unfolded proteins in cells [228]. The elevated level of UPS can directly or indirectly aid tumour progression, resistance to treatment, cell cycle progression, and activation of oncogenic signalling pathways [225].

Recent studies have demonstrated the relationship between UPS and the development of different tumours. For instance, ubiquitin activating enzyme UBA1 (E1) has been demonstrated to be overexpressed in many human cancer types including leukaemia, multiple myeloma, liver, and lung cancers and is associated with a poor prognosis regarding patient survival [229]. In another study, the aberrant expression of UBA1 has been detected in cutaneous squamous cell carcinoma and was also associated with its pathogenesis [230]. E2 conjugating enzymes are responsible for the attachment of ubiquitin to proteins or substrates during ubiquitination and the human proteome contains over 40 putative E2s in mammalian cells that are involved in the attachment of ubiquitin to cellular proteins [231]. Several studies have shown a significant correlation between E2 expression and an increase in cancer poor prognosis. For example, Luo et al., [232] showed that higher expression of ubiquitin E2 (UBE2) is associated with the early occurrence and poor outcomes in patients with non-small cell lung cancer NSCLC. Ubiquitin conjugating enzymes H5C (UBCH5C) overexpression has also been detected in pancreatic cancer and is associated with cell proliferation, metastasis, and resistance to treatment [233]. Another important group of enzymes that plays a significant role in cancer pathogenesis and development are deubiquitinating enzymes (DUBs). DUBs can remove attached ubiquitin from the substrate allowing the recycling of free ubiquitin for further use in the ubiquitin proteasome system [234]. These are classified into: Ubiquitin C-terminal hydrolases (UCHs), Ubiquitin specific proteases (USPs), Ovarian tumour proteases (OTUs), Machado Joseph domain proteases (MJDs), and monocyte chemotactic protein induced proteins (MCPs) which are members of the cysteine protease family and JAB1/MPN/Mov34 metalloenzyme (JAMM) which is part of the zinc-dependent metalloprotease family [235]. Among these DUBs, USPs and UCHs was the centre of research as they are highly mutated in human cancers, suggestive of their roles

in tumour progression [236]. For example, overexpressed USP2 was reported to play a critical role in human prostate cancer, glioma, and fibroblast progression through deubiquitinating antiapoptotic proteins such as MDM2, MDMx, and fatty acid synthase [237, 238]. Similarly, USP2 overexpression is crucial in promoting triple-negative breast cancer development, metastasis, and invasiveness by modulating the activity or expression of matrix metalloproteinase-2 (MMP2) protein [239]. It has been shown in liver, breast, colorectal cancer that the cell survival was promoted by an elevated level of USP4 by simultaneously inhibiting apoptotic proteins and promoting tumour metastasis [240]. USP5 plays a critical function in pancreatic cancer progression and tumourigenesis by stabilising Forkhead box M1 (FoxM1) proteins [241]. UCHL-1 overexpression has also been positively correlated with development, invasiveness, and chemotherapy resistance in some cancers including pancreatic, myeloma, neuroblastoma, NSCLC, prostate, and lymphoma [242].

1.7.2 Inhibitors of Ubiquitin Proteasome System (UPS)

UPS is frequently altered in all cancer and many solid tumours [243, 244]. The alterations can be attributed to the mutations in enzymes such as F-box proteins, E3s ligases, DUBs, or mutations in UPS target proteins [245-247]. The mutation may result in signalling abnormalities leading to tumour progression, genomic instability, metastasis, invasiveness, and resistance to chemotherapy [248, 249]. This is clearly illustrated by the recent study of Fhu & Ali (2021) who demonstrated that dysregulation of UPS contributes to the development of various malignancies, and conducted in human breast cancer (MDA-MB-231) with [250]. Therefore, inhibiting or modulating the expression of UPS could be a novel approach to fighting cancer progression and tumourigenesis. As shown in **Table 1.6**, several generations of UPS inhibitors have been developed including MDM2 antagonists such as RG7388 and tripeptide aldehydes such as MG-132. However, most of these inhibitors have a short half-

life, poor aqueous solubility, adverse side effects, uncompetitive binding to UPS, and display genomic instability in the cells by inhibiting non-proteasomal proteases [251, 252]. Therefore, novel, and safe inhibitors are urgently needed for cancer therapy. The effects of AuNPs on ubiquitin proteasome system have not yet been studied *in vitro*. Herein, the effects of different sizes (5, 10, and 80 nm) and functionalised (tannic acid, citrate, and PVP) AuNPs on UPS with a particular focus on DUBs such as USP7, USP8, USP10, and UCHL-1 was for the first-time studied in the first two experimental chapters of this thesis.

Table 1.6: Ubiquitin proteasome system inhibitors in different types of cells

USP inhibitors	Target	Role in cells	Binding proteins	Expression	Cancer types	Outcomes	Reference
MI-219 NVP- CGM097 RG7388	MDM2/X	Avoiding cell death	p53	Highly expressed	A549, H460, SKLU-1, MCF-7, T-47D	Suppression of tumour growth and p53 activation by disrupting MDM2-p53 binding	[253, 254]
Paclitaxel	SKP2	Cell cycle progression, Metastasis	p27, p21	Overexpressed	A549, NCI-H1299	Inhibition of SKP2 regulates the expression of MDM2 gene through P27-CDKs-E2F1	[255]
Doxycycline	β -TrCP	Substrate recognition	β -Catenin, I κ B, wee1	Overexpressed in some cells	LNCaP, 293T, AT2.1	Lower cell proliferation by inhibiting β -TrCP binding to the substrate	[256]

Apcin	Cdc20	Oncogenic role	P21, Cyclin a/b	Highly expressed in human tumours	MG63, U2OS	Inhibits cell growth by blocking the interaction of APC/C-Cdc20 complex	[257]
LDN-57444 Au (III)	UCHL1	Progression, Metastasis	β -Catenin, Smad2/3	Overexpressed	HSC3-4, NP69, NP69LMP1, MCF-7, HeLa	Inhibition of UCHL1 reduced the levels of pro-metastatic makers, induced apoptosis, cell cycle arrest	[258, 259]
b-AP15	UCHL5	Cell development	β -Catenin, WnT	Expressed	BFTC905	Increased apoptosis in cancer cell by decreasing the antiapoptotic protein Bcl2	[260]

MG132 ML364	USP2	Regulate the expression of oncoproteins	β -TrCP, FBXL14, Bmi1, cyclin D1	Highly elevated	MDA-MB-157, BT549	Induced cell cycle arrest by increasing cyclin D1 degradation	[261, 262]
MG132	USP4	Metastasis, recurrence	Twist1, ZEB1, Snail/Slug, I κ B	Expressed	A549, HEK293T, MCF-7, BT549	Stabilised Twist1 protein, promote stemness, inhibits cell growth	[263]
P2207 P5091 HBX19818	USP7	Cell growth, genomic instability	P53, MDM2	Overexpressed	IMR-32, HCT116, SH-SY5Y, PC3,	Suppressed cell growth, induction of apoptosis	[234, 264, 265]
WP1130 BXI-01294	USP9X	Promotes cell proliferation, invasion, Metastasis	P53, Mcl-1, C-Myc, MMP2	Frequently elevated	A549, Z138, HepG2, 293T	Reduced cell proliferation by reducing the expression of transcription factors	[266, 267]

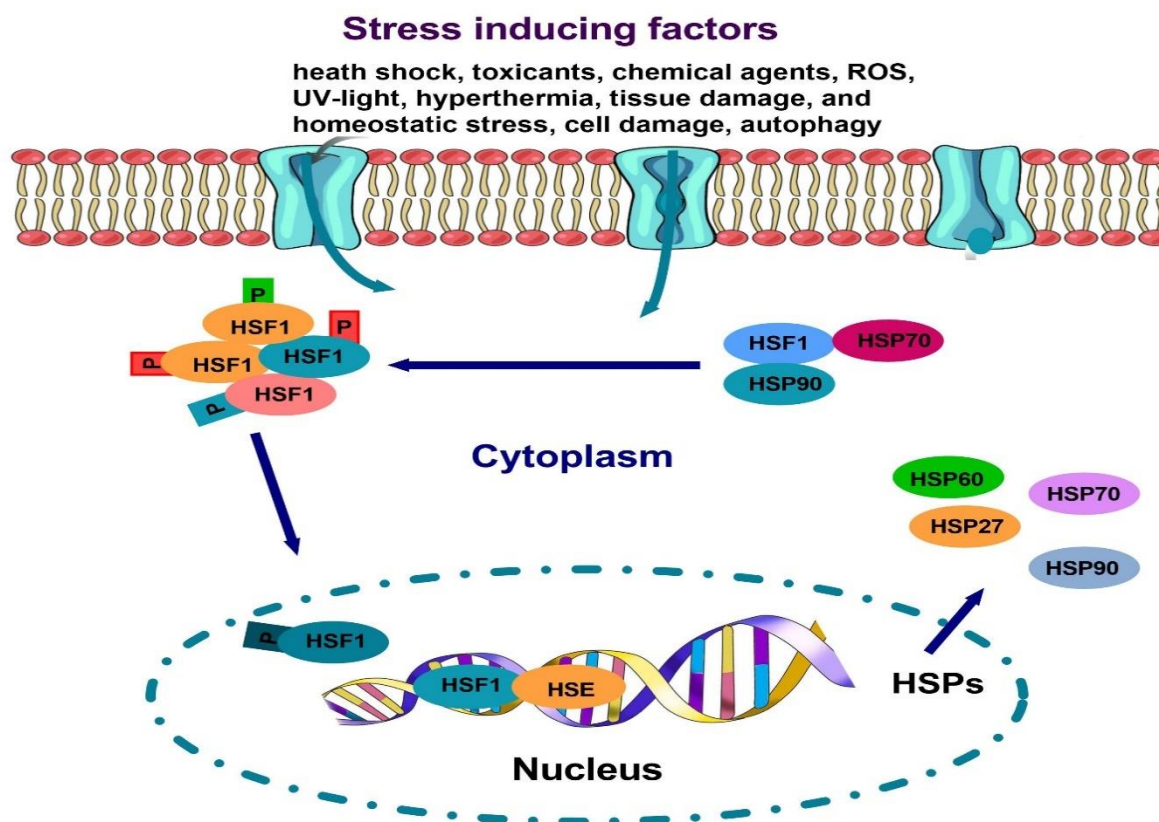
P2207 Spautin-1 HBX19818	USP10	Maintain intracellular protein homeostasis	PTEN, AKT, p53, c-Myc, mTOR	Overexpressed	NSCLC	Inhibits cell proliferation and invasion by upregulating PTEN, p53, down regulating AKT and c-Myc	[268, 269]
b-A15 IU1-47	USP14	Regulate tumourigenesis	β -Catenin, mTOR	Highly overexpressed	NSCLC, A549, MCF-7, T47D	Inhibition of USP14 mitochondrial apoptosis through caspase 3 activation, induced G2/M phase cell cycle arrest	[230, 270]

1.8 Molecular Chaperone Heat Shock Proteins (HSPs)

Heat shock proteins belong to the family of evolutionary molecular chaperones and are responsible for signal transduction, regulation of the cell cycle, mitochondrial cell death, development of cell, and maintaining cell survival [271, 272]. The heat shock proteins are classified into 3 main categories, small HSPs (HSP27), HSP40, the chaperone family of HSP60, HSP70, HSP90 and the large HSP (HSP110, glucose regulated proteins 170 GRP170) based on their molecular weight [273, 274]. Notably, when the cells are exposed to environmental conditions such as biochemical agents, ultra-violet light, hyperthermia, low level of oxygen, toxicants, and other homeostatic stress, the proteins are promptly elevated to augment the stress factor referred to as the heat shock response (HSR) which is controlled predominantly by the heat shock factor (HSF) [275, 276]. When HSF is phosphorylated, it rapidly binds to the heat shock element (HSE) in the nucleus and accelerates the overexpression of HSP (**Scheme 1.10**).

Under “normal” conditions increased in HSP in response to stress provides cytoprotective functions to normal cells. However, dysfunction of these proteins contributes to the development of several disorders and diseases such as cancer [277]. In cancer, the HSPs proteins are highly overexpressed in response to metabolic needs such as hypoxia, acidosis, and nutrient deprived tumour conditions [278]. The proliferation of cancer occurs through the activation of oncogene proteins, amplification and elevation of intracellular signalling proteins that require overexpression of HSPs for their stabilisation, ubiquitination, protein folding, aggregation, and proteolytic degradation [279, 280]. Thus, the inhibition of molecular HSPs may offer a novel way to prevent cancer proliferation and targeting multiple HSPs may

also bring a new direction in cancer treatment and the development of new therapeutics molecules.



Scheme 1.10. Response of tumour cells to stress inducing factors. In the presence of stress factors, HSFs are phosphorylated in the cytoplasm and translocate into the nucleus of the cell where they bind to HSE and trigger the transcription of HSPs. Scheme was created with GraphPad prism layout.

Abbreviations: Heat shock factor-1 (HSF-1), Heat shock element (HSE), Heat shock proteins (HSPs), Heat shock protein 27 (HSP27), Heat shock protein 60 (HSP60), Heat shock protein 70 (HSP70), Heat shock protein (HSP90).

1.8.1 Roles of HSPs Expression in Cancer Development

Current studies suggest that molecular chaperone HSPs play a significant role in cancer development [276, 281-283]. During cellular processes, they serve as a molecular chaperone that assists in folding proteins correctly or helps proteins to refold when threatened by environmental stress that may lead to their denaturation [284, 285]. Due to these, molecular chaperone proteins are highly involved in controlling cancer growth, development, signal transduction, apoptosis, and cell cycle regulation leading to cell survival [286]. Numerous studies indicate the role of HSPs in cancer development (**Table 1.7**).

Table 1.7: Role of molecular HSPs in cancer development.

HSPs	Cell lines	Outcome	Reference
HSP27	Prostate cancer (PCa)	Overexpressed HSP27 elevates PCa growth, motility, metastasis, and survival	[287]
HSP27	PC3, PC3-M	Elevated HSP27 mediates the invasion of human prostate cancer	[288]
HSP27	U87, DBTRG.05MG	Frequently expressed HSP27 plays critical role in gliomas progression	[289]
HSP27	K562, HL-60, THP-1	Elevated expression of HSP27 correlates with poor prognosis of leukaemia	[290]
HSP27	A549	High HSP27 expression has a promotional role in lung cancer proliferation	[291]
HSP27	NSCLC	HSP27 overexpression enhances the chemoresistance of lung cancer	[292]

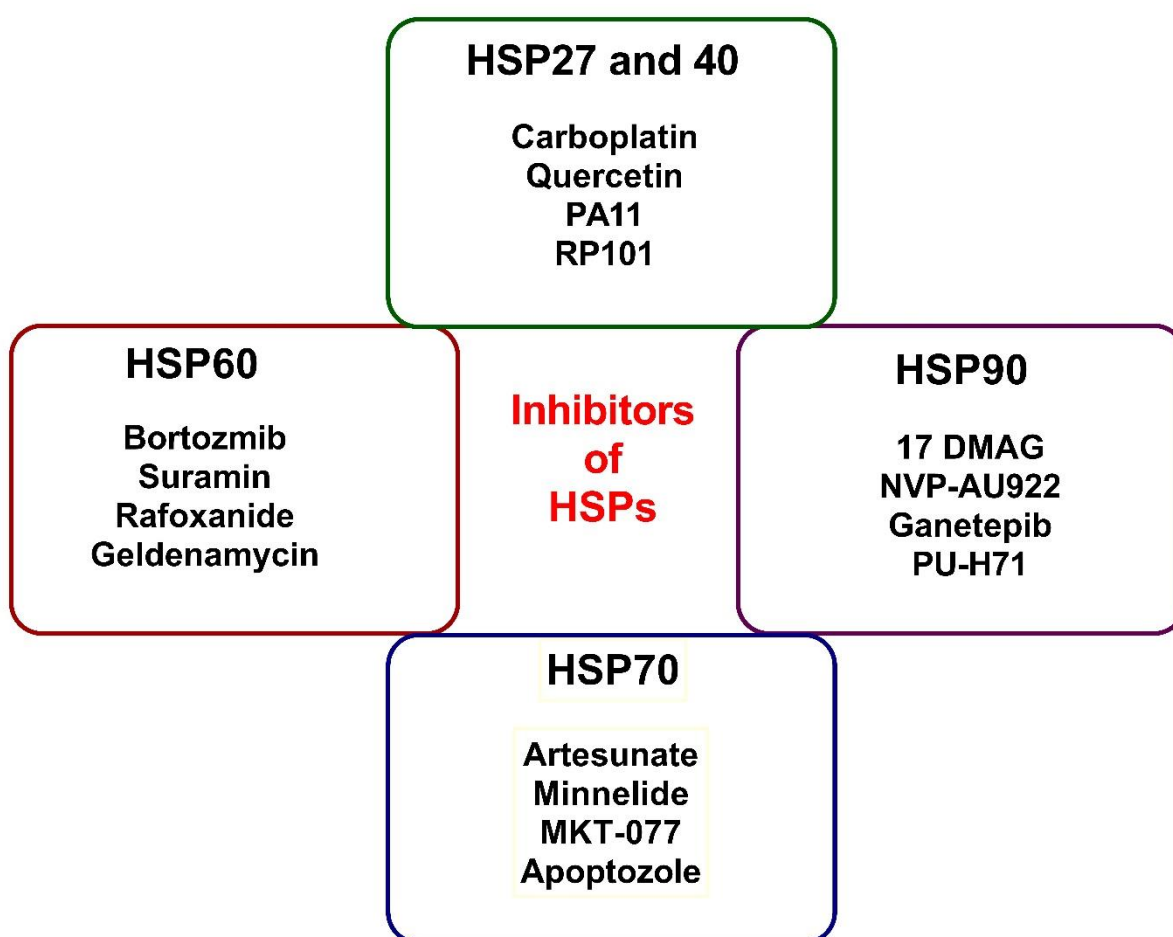
HSP40	A549	Elevated level of HSP40 is associated with propagation of A549 cells	[293]
HSP40	A549, NCI-H1975	HSP expression promotes lung cancer development and survival	[294]
HSP40	A549, HEK293T, HeLa, HepG2	High expression of HSP40 regulates proliferation of tumour cells	[295]
HSP40	NCI-H460	Expression of HS40 was associated with growth of lung cells	[296]
HSP60	NSCLC	HSP expression was found to be involved in NSCLC morphological alterations, proliferation, and migration.	[297]
HSP60	Breast cancer	HSP60 overexpression correlates to tumour progression	[298]
HSP60	Prostate cancer	Elevated level of HSP60 predicts survival of prostate cancer	[299]
HSP70	A549	Progression of A549 cells was regulated by HSP70 expression	[300]

HSP70	OV2008, A2780	Elevated level of HSP70 reverses cisplatin resistance in ovarian cancer	[301]
HSP70	A549	Heightened expression of HSP70 protects lung cells against hypoxia	[302]
HSP70	HepG2	There is significant correlation between HepG2 lipogenesis and HSP70	[303]
HSP70	HepG2	HSP70 and HSF-1 enhanced the growth of liver cancer by preventing programmed cell death	[304]
HSP90	HepG2	The proliferation of hepG2 is associated with HSP90 state	[305]
HSP90	MDA-MB-231	HSP90 overexpression promotes the metastasis and invasion of breast carcinoma	[306]
HSP90	HCC827, A549, H1437	The proliferation state of the lung cells was altered through the inhibition of HSP90	[307]
HSP90	Brain cancer	HSP90 overexpression significantly correlates with growth of pediatric brain tumours	[308]

1.8.2 Targeting HSPs as Novel Cancer Modality

Inhibition of HSPs is increasingly considered as a potential treatment method for cancer due to their association with cancer pathogenesis, development, and progression [309]. Two therapeutic approaches have been developed to target specific HSPs that are overexpressed in different cancer types: one is to control the activity and the expression of HSPs (e.g. Cisplatin and siRNA) and the other is to develop a distinct molecule that can bind to HSPs and inhibit their molecular activity (e.g. MKT-077, PES and JG-98) [310, 311]. At the time of this research, no drug has been clinically approved to control the activity and the expression of HSPs. Nevertheless, several known inhibitors have been developed, which are under various clinical evaluations (**Scheme 1.11**). For instance, the chemotherapeutic agent quercetin (bioflavonoid compound) has been shown to downregulate the expression of HSP27 in A549 cell line leading to a reduced percentage of cell viability and induction of apoptosis [292]. Likewise, a combination of quercetin and RP101 [(E)-5-(bromovinyl)-2'-deoxyuridine (BVDU)] led to apoptosis and prevented the acquisition of chemoresistance in pancreatic cancer, leukaemia, and glioblastoma [312, 313]. HSP40 is involved in cancer proliferation and migration by positively regulating the Wnt pathway and p53 mediated mitochondrial apoptosis when highly overexpressed [277]. Additionally, it has also been reported to enhance the proliferation of tumour cells by promoting ubiquitination and proteolytic degradation of tumour suppressors [314]. Multiple agents have shown effective anticancer properties through the inhibition of the overexpressed HSP40 in lung, liver, and pancreatic cancers [294, 315, 316]. HSP90 has been known to mediate cell proliferation and drug resistance by forming a complex with a survivin protein that inhibits mitochondrial apoptosis through downregulation of caspases and thus, proven to be an ideal candidate for cancer therapeutics [317, 318]. Geldanamycin has been shown to stabilise the overexpressed HSP90 in A549 cells

through binding to ATP at the ATP binding site and lower metabolic activity, leading to a decrease in cell survival [319, 320]. NVP-AU922 has also shown a promising impact in reducing cell growth of breast cancer and NSCLC through the inhibition of elevated HSP90 [321, 322]. Despite the development, it has been reported that Phase I clinical trials of the developed drugs have been suspended due to their strong cytotoxic impact, poor solubility, and lack of biocompatibility [323-325]. Therefore, alternative inhibitors are urgently needed for cancer therapy.



Scheme 1.11. Compounds that have been previously found to inhibit cancer proliferation through the inhibition of HSPs, although some (e.g. rafoxanide) are indirect inhibitors. Scheme was created with GraphPad prism layout.

1.9 Hypothesis and Objectives of the Study

Exposure to different NMs sizes (5, 10, and 80 nm), different functionalised AuNPs (tannic acid, citrate and PVP) and AgNPs may trigger cytotoxicity to lung adenocarcinoma cells (A549) by inhibiting the UPS, P13K/AKT/mTOR and Wnt signalling pathways and molecular chaperones HSPs leading to the induction of mitochondrial related apoptosis.

1.10 Objective

The main objective of the project was to explore the cytotoxicity effects of AuNPs and AgNPs on USP with a particular focus on the deubiquitinating enzymes (USP7, USP8, USP10, and UCHL-1) and molecular chaperone HSPs (HSP40, HSP60, HSP70, and HSP90) in lung adenocarcinoma (A549) which has never been investigated. To achieve this goal, the main objective was break down into specific objectives.

1.10.1 Specific objectives

1. Investigate the size-dependent effects of AuNPs (5, 10. and 80 nm) on ubiquitin proteasome system in A549 cells with a particular focus on deubiquitinating enzymes (USP7, USP8, USP10, and UCHL-1).
2. Evaluate the effects of different functionalised (tannic acid, citrate, and PVP) AuNPs on ubiquitin proteasome system in A549 cells with a particular focus on deubiquitinating enzymes (USP7, USP8, USP10, and UCHL-1).
3. Study the impact of AgNPs and AuNPs on molecular heat shock proteins in A549 cells with a particular focus on HSP40, HSP60, HSP70, and HSP90.

4. Assess the biological effects of cisplatin (CDDP) combination with 10 nm citrate functionalised AuNPs on molecular heat shock proteins in A549 cells with a particular focus on HSP40, HSP60, HSP70, and HSP90.

Chapter 2: Materials and Methods

2.1 Materials

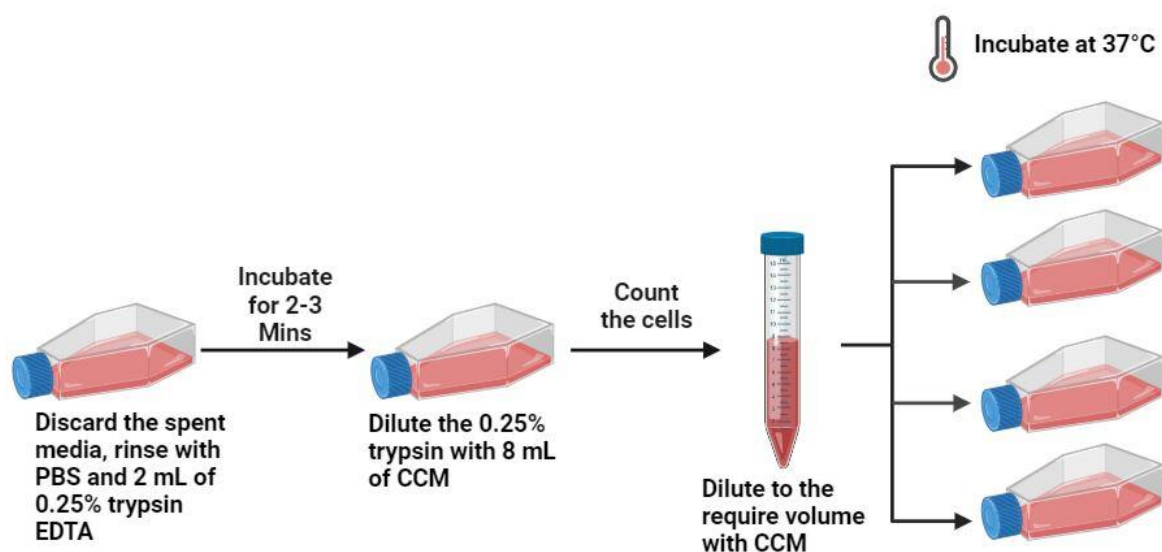
All the chemicals, reagents, and assay kits used for this thesis were procured from companies in the United Kingdom (UK) unless stated otherwise. Phosphate-buffered saline (PBS), F12 nutrimix media, Penicillin/Streptomycin, 0.25% trypsin-EDTA, fetal bovine serum (FBS), RPMI 1640, Hoechst 33342, HCS mitochondrial health were purchased from Thermo Fisher Scientific (Paisley, UK). CytoTox 96[®] non-radioactive cytotoxicity LDH, cellTiter-Glo[®] luminescent cell viability, caspase-Glo[®] 3/7, GSH/GSSG-Glo[™] luminescence assay kits were from Promega (Southampton, UK). Chlorpromazine hydrochloride, Wortmannin, Nystatin, Simvastatin, Methyl- β -cyclodextrin (M β CD), Menadione, Staurosporine, Dimethyl sulfoxide (DMSO), Cisplatin (CDDP), Tris-Base, Glycine, Sodium chloride (NaCl) were purchased from Sigma Aldrich (Dorset, UK). Bovine serum albumin (BSA), Acrylamide/bis-acrylamide, 4% paraformaldehyde, and ponceau were bought from Alfa Aesar (Lancashire, UK). Alexa fluor 488[®] Annexin V/PI cell death and CellRox deep read reagent kits were purchased Thermo Fisher Scientific (Paisley, UK). PVDF membrane and ECL[™] were bought from GE health care (Buckinghamshire, UK). USP7, USP8, USP10, UCHL-1, HSP40, HSP60, HSP70, HSP90, AKT, mTOR, PTEN, MDM2, GSK-3 β , β -Catenin, p- β -Catenin, Caspase-3, Caspase-9, PARP, and GAPDH primary antibodies and Anti-Rabbit IgG horseradish peroxidase HRP-linked antibody were purchased from cell signalling technology (CST) (London, UK).

2.1.1 Nanoparticles (NPs)

Nanospheres AuNPs of sizes 5 nm, 10 nm, and 80 nm capped with citrate provided at 0.05 mg/mL in 2 mM sodium solution, 5 nm AuNPs capped with tannic acid and PVP provided at mass concentration 0.05 mg/mL in water and 10 nm AgNPs citrate capped provided at mass concentration 0.02 mg/mL in 2 mM sodium citrate solution were obtained from NanoComposix (San Diego, CA, USA). The particle number concentrations were $4.2\text{E}+13$, $3.3\text{E}+12$, $1.1\text{E}+10$, $3.7\text{E}+12$ particles/mL, and the surface areas 60.6, 26.4, 3.9, and $53.0\text{ m}^2/\text{g}$ for 5, 10, 80 nm AuNPs, and 10 nm AgNPs coated with citrate.

2.1.2 Cell Culture

A549 cells cryopreserved at a very low passage number of five in our laboratory were thawed and cultured in F-12 nutrimix media. The media was supplemented with 10% FBS, 1% 100 $\mu\text{g}/\text{mL}$ penicillin, and 100 mg/mL streptomycin at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 . The cells were passage into more culturing vessels to increase cell yield for experiments once they had reached 70-80% confluency using 0.25% trypsin-EDTA phenol red (Thermo Fisher Scientific, Paisley, UK). A549 cells were used in this study because lungs represent the first portal to the body once NMs are inhaled and therefore can be used for studying AuNPs' cytotoxicity on one type of lung cells. The passage numbers of both cell lines were taken into account throughout the studies, where the cells were maintained at passage numbers ranging from 5 to 20.



Scheme 2.1. A diagram depicting steps involved in splitting cells into more T₇₅ flask to increase yield for experiments. The scheme was created with BioRender.com.

2.1.3 Cell Counting with Hemocytometer

The A549 cells were counted manually using a hemocytometer which was cleaned with 70% ethanol and the cover slide was gently placed on the top of the counting chambers. After thawing and resuspension in CCM, a 20 μ L of cell suspension was diluted with trypan blue at the ratio of 1:1 in 1.5 mL tube and visualised by optical microscope (Primovert, Carl Zeiss AG, Suzhou, China) at 10X magnification. The number of cells in the top left, bottom left, top right, and bottom squares (0.06 mm²) were counted and the average result represented the number of cells in 1 mL of cell suspension.

2.2 Methodologies

2.2.1 Characterisation of AuNPs and AgNPs

2.2.1.1 Morphology and Size Distribution

A freshly prepared 5 μ L of AuNPs or AgNPs (10 μ g/mL) dispersed in UPW was deposited onto a copper 300 Mesh TEM grids (EM Resolutions Ltd., Sheffield, UK) and allowed to dry for 2 h in a safety cabinet. The morphology and dispersibility of the particles were examined after air dried using TEM (JEOL Ltd, Hertfordshire, UK) operated at an accelerating voltage of KeV (JEOL 1400 TEM, University of Birmingham, Centre for Electron Microscope). The size distribution of AuNPs and AgNPs was measured by imaging software (ImageJ, version, 2.0.0, Public domain, BSD-2).

2.2.1.2 Absorption Spectra

Ultraviolet–visible spectroscopy (UV-2600 SHIMADZU model, Kyoto, Japan) was used to measure the absorption spectra of AuNPs and AgNPs. The particles were freshly prepared in 1.5 mL Eppendorf tubes at 10 μ g/mL and transferred to disposable quartz cuvettes (1 cm path length) (Thermo Fisher Scientific, Paisley, UK). The UV-Vis was set to collect the spectra from 200 to 800 nm using a spectra width of 1 nm.

2.2.1.3 Hydrodynamic Diameter

Dynamic light scattering (DLS) (Malvern Instruments Ltd., Worcestershire, UK) technique was used to measure the average hydrodynamic diameter and polydispersity index (PDI) of AuNPs (5, 10, and 80 nm), functionalised 5 nm AuNPs (Tannic acid, citrate, and PVP) and 10 nm AgNPs dispersed in ultrapure water (UPW), phosphate buffer saline (PBS), free culture media (FCM) and complete culture media (CCM) at 0 and 24 h at 25 °C. 1.4 mL of the dispersed AuNPs or AgNPs (10 μ g/mL) were pipetted into a disposable polystyrene cuvettes (Thermo Fisher scientific, Paisley, UK)

and carefully inserted into DLS for the measurement equipped with an avalanche photodiode detector (APD) and a green laser (532 nm, 4 mW). A total of 10 measurements were taken with a fixed equilibrated run time of 20 seconds for each of the AuNPs and AgNPs.

2.2.1.4 Zeta Potential

1.5 mL of AuNPs or AgNPs dispersed in UPW or CCM (10 µg/mL) was freshly prepared in 1.8 mL Eppendorf tubes and loaded into a disposable capillary cell cuvette (Malvern, DTS1070) using 1 mL BD Plastipak™ Syringe (Thermo Fisher Scientific, Paisley, UK). The zeta potential of the particles was measured using Zetasizer Nano ZS90 DLS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 173° detection angle and 4 mW red laser (633 nm).

2.2.1.5 Dissolution of AuNPs and AgNPs in UPW and CCM

Dissolution experiment was performed according to method of Queveda et al [326]. In brief, 20 mL of AuNPs and AgNPs were prepared in UPW at 5 µg/mL from the stock concentration and incubated at 37 °C with gentle agitation (Infors AG CH-4103, Bottmingen, Switzerland). 400 µL of the samples were taken at different time intervals (0.25, 0.50, 1, 2 and 6 h) and transferred into a microcentrifuge tube (Amicon® ultra 0.5 mL, 3KDa, UFC5003, Sigma Aldrich, Dorset, UK). The samples were centrifuged at 17,175 g for 10 min at 37 °C. 300 µL of the filtered supernatant samples were transferred to a 15 mL tube and diluted with 6 mL of 2% HNO₃ (Nitric Acid 67-69%, Trace Metal™, for Trace Metal Analysis, Fisher Chemical™). For dissolution in CCM, the filter was additionally washed three times with 400 µL of distilled water and centrifuged at 17,135 g for 30 min at 37 °C. At the end of the centrifugation, the recovered supernatant was added to the first centrifuged tube and diluted to 6 mL

with 2% HNO₃ (Nitric Acid 67-69%, Trace Metal™, for Trace Metal Analysis, Fisher Chemical™). The samples were analysed by ICP-MS (NexION 300X, Perkin Elmer, USA). A calibration curve of gold and silver standards was prepared (0, 0.25, 0.5, 1, 5, 10, 20, 50, and 100 ppb) in 2% HNO₃.

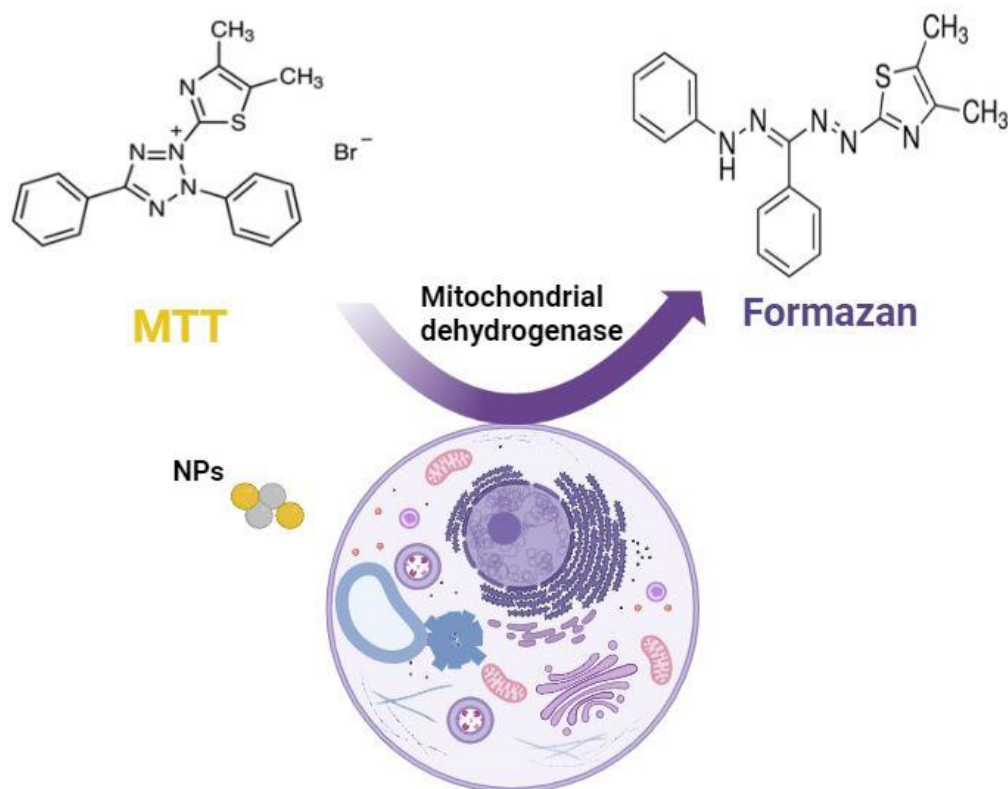
2.2.2 Cell Viability Assays

2.2.2.1 MTT Assay

The metabolic activity of A549 cell line exposed to NPs was measured using MTT assay which is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH) dependent oxidoreductase enzymes to metabolise the tetrazolium MTT dye to insoluble formazan crystals. MTT assay was used as previously described [72]. Briefly, A549 cells were seeded in flat bottom 96 well plates at a density of 7×10^3 cells/well diluted in 100 μ L of CCM and allowed to attach overnight at 37 °C in a 5% CO₂ humidified incubator. The cells were treated with different sizes AuNPs (0-40 μ g/mL), functionalised AuNPs (0-35 μ g/mL), AgNPs (0 - 45 μ g/mL), and CDDP (0-10 μ g/mL) for 24 and 48 h. Negative control cells were incubated with CCM while the positive control cells were treated with 20% DMSO diluted in CCM. The MTT solution was prepared as 5 mg/mL stock in sterile PBS and filtered with 0.22 μ m Acrodisc filter (Sigma Aldrich, Dorset, UK). After the treatment, the media was removed and 120 μ L of MTT solution was added to each well, diluted in CCM at a ratio of 1:6 for 4 h. The MTT solution was then replaced by adding 200 μ L of DMSO for 10-15 min to solubilize the formazan crystals in dark with gentle agitation on the shaker. The absorbance was recorded using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) at 570 nm emission wavelength to determine the percentage of viable cells of the A549 cells. The percentage of cell viability was calculated using the following equation:

% Cell Viability

$$= [\text{Absorbance of treated cells} / \text{Average absorbance of control cells}] \times 100$$



Scheme 2.2. Enzymatic reduction of yellow MTT to purple formazan by mitochondrial reductase enzyme in viable cells. The scheme was created with BioRender.com.

2.2.2.2 Modified Lactate Dehydrogenase (mLDH)

The percentage of cell viability was also assessed using mLDH assay by Promega CytoTox 96 non-radioactive cytotoxicity assay according to a protocol described [72]. Briefly, A549 cells were seeded in 96-well plates at a density of 7×10^3 cells/well and left to adhere overnight. The cells were then exposed to different concentrations of AuNPs (0-45 $\mu\text{g/mL}$), AgNPs (0-35 $\mu\text{g/mL}$), and CDDP (0-10 $\mu\text{g/mL}$). Untreated cells were used as negative control while 20% DMSO was used as a positive control for 24 and 48 h. After the incubation period, the cells were then lysed with 0.9% Triton X-100

and incubated for another 1 h at 37 °C. The lysates were collected in Eppendorf tubes and centrifuged for 5 min at 4 °C. 50 µL of the supernatants were transferred to a new 96-well plate and 50 µL of substrate mixture was added and incubated for 15 min at room temperature protected from light. After the incubation, 50 µL of stop solution was then added and the absorbance of each sample was measured using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) at 490 nm. The percentage of cell viability was calculated using the following equation:

% Cell Viability

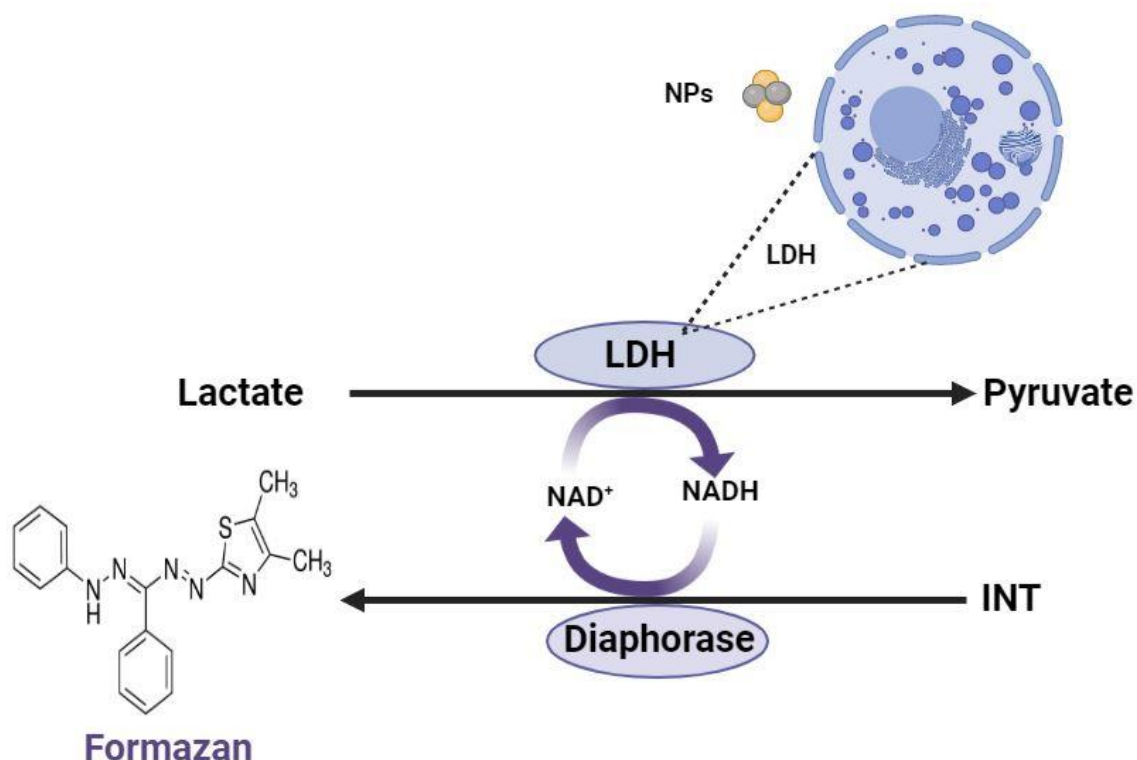
$$= [\text{Absorbance of treated cells} / \text{Average absorbance of control cells}] \times 100$$

2.2.2.3 Lactate Dehydrogenase (LDH) Release Assay

The LDH assay was used to measure the percentage of cell membrane damage induced by the NPs associated with the release of the LDH enzyme from the cytoplasm. The LDH assay was detected using Cytotox 96 non-radioactive cytotoxicity assay Kit (Promega, Southampton, UK) according to a protocol described previously [72]. After incubation with AuNPs (5, 10, and 80 nm) as described in section 2.2.2.2, 50 µL of media containing the NPs was transferred to a new flat-bottom 96-well plate and 50 µL of the reconstituted substrate was added to each well and incubated for 15 min in the dark. After the incubation period, the reaction was terminated by adding 50 µL of stop solution. The absorbance was measured using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) at 490 nm emission wavelength. The absorbance of supernatant corresponds to the LDH released in the media which indicates the number of damaged caused by the AuNPs exposure. The percentage of LDH released was calculated by the following equation:

% LDH released

$$= [\text{Absorbance of treated cells} / \text{Average absorbance of control cells}] \times 100$$



Scheme 2.3. Lactate dehydrogenase (LDH) release from the cell exposed to AuNPs or AgNPs convert lactate to pyruvate and idonitrotetrazolium violet (INT) in the presence of coenzyme (NAD⁺) which further convert to formazan by diaphorase enzyme. Scheme was created with BioRender.com.

2.2.2.4 CellTiter-Glo® Luminescent Cell Viability Assay

For the CellTiter-Glo (CTG) assay a protocol previously described [327] was used. In brief, A549 cells were seeded at a density of 7×10^3 cells/well in a 96-opaque walled multiwell plate (Thermo Fisher Scientific, Paisley, UK) and treated with 10 nm AuNPs and AgNPs coated with citrate at the concentration range of 0-35 $\mu\text{g/mL}$ for 24 and 48 h. After the desired incubation period, the 96-well plate was allowed to equilibrate at room temperature for 30 min and the CTG reagent was mixed at a 1:1 ratio with the volume of CCM containing the NPs in each well. The mixture was then incubated at room temperature for 10 min on an orbital shaker with

gentle agitation, followed by luminescence measurement using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK). The percentage of cell viability was calculated using the equation:

%Cell viability

= [Absorbance of treated cells/Average absorbance of control cells] X100

2.2.2.5 Cell Recovery

To evaluate the A549 cells' recovery capacity upon exposure to 5 nm functionalised AuNPs (Tannic acid, citrate. and PVP) a protocol previously described [328] was used. The A549 cells were seeded in a 96-well plate at a density of 7×10^3 cells/well overnight to adhere and then exposed to 0-10 $\mu\text{g/mL}$ of different functionalised AuNPs for 24 h. After the exposure period, the cells were then washed three times with PBS and 100 μL of fresh growth CCM was added every 24 h. The number of recovered cells was measured on days 1, 2, 3, and 4 using mLDH according to section 2.2.2.2.

2.2.2.6 A549 3D Spheroids Formation

3D spheroids were formed by the liquid overlay method using 1% agarose prepared in distilled water according to a protocol previously described [329]. A549 cells cultured in monolayers were maintained in Ham's F12 CCM. 100 μL of the cell suspension were seeded in 96-well plates coated with 1% agarose at a density of 8×10^3 cells/well and incubated for 8 days, with fresh CCM added every two days of the experiment duration. The morphology of the spheroids was observed each day using an EVOS™ FL imaging system (Washington, NJ, USA) with 20X magnification. The diameter and circumference of the spheroids were determined using Imaging software (ImageJ, Version 2.0.0, Public domain, BSD-2).

2.2.2.7 Cell Viability of A549 3D Spheroids

The viability of A549 3D spheroids was assessed using a live/dead cell staining kit which indicates green fluorescence for the live cells and red fluorescence for the dead cells as described in the protocol of [330]. After 4 days of seeding the spheroids, the spheroids were treated with different concentrations (0-10 $\mu\text{g/mL}$) of AgNPs and AuNPs for 24 h. A certain number of spheroids (5-10) were counted and gently washed three times with 50 μL of PBS. The spheroids were then stained with live/dead cell staining according to the manufacturer's instructions. The fluorescence was observed using EVOS™ FL imaging system (Washington, NJ, USA) with 20X magnification.

2.2.3 Cellular Uptake of Gold Nanoparticles (AuNPs) by A549 Cell

2.2.3.1 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

ICP-MS was used to measure the amount of Au uptake by A549 cells. The cellular uptake of AuNPs experiment was conducted as previously described [83]. In brief, A549 cells were seeded in six well plates at a density of 2×10^5 cells/well in 3 mL of CCM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C. At 24 h post-seeding, A549 cells were exposed to 5, 15, and 30 $\mu\text{g/mL}$ of different sizes of AuNPs dispersed in CCM for 24 h. After the exposure, the cells were washed three times with PBS, harvested, and transferred to a glass vial (Ampulla, Lancashire, UK). The cells were digested with 3 mL of aqua regia prepared with 37% hydrochloric acid (HCl) and 70% nitric acid (HNO_3), then 1 mL of hydrogen peroxide was added to the cell suspension. The glass vials were properly closed and heated at 120 °C for 30 min. The samples were diluted to a final acid concentration of 2% HNO_3 and filtered with a 0.22 μM syringe filter (Sigma Aldrich, UK) to ensure the removal of any debris. The total Au

concentration in the samples was measured by ICP-MS (NexION 300X, Perkin Elmer, Waltham, Massachusetts, USA) in the Helium KED mode (Helium Gas Flow at 5 mL/min). A calibration curve of gold standard series was also prepared (0, 0.25, 0.5, 1, 2, 5, 10, 20, 50, and 100 ppb) in 2% HNO₃ to set up the ICP-MS.

2.2.3.2 Flow Cytometry Analysis of AuNPs Uptake

For the measurement of AuNP uptake using flow cytometry it was conducted according to methods previously described [72]. Briefly, A549 cells were seeded in six well plates at a density of 2×10^5 cells/well in 3 mL of CCM and allowed to adhere overnight at 37 °C in 5% CO₂. Then, the cells were exposed to different sizes of AuNPs at concentrations of 5-30 µg/mL, 10 nm AgNPs, and AuNPs at concentrations range of 1.25-10µg/mL while the control cells were treated with CCM for 24 h. At the end of the exposure time, the cells were washed with PBS three times to remove any unattached NPs and were then harvested and centrifuged at 1,904 g for 5 min at 4 °C. The supernatant was discarded, and cells were resuspended in 500 µL of PBS. The samples were gently transferred to FACS round bottom polystyrene test tubes and kept on ice for analysis by BD LSRFortessa™ X-20 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The cells in suspension were injected into a thin tubing in a laminar flow of a sheath fluid with hydrodynamic focussing to allow single-cell analysis. Side scatter area (SSC-A) and forward scattered area (FSC-A) were recorded for each gated event as they pass through the laser while excluding the region of cell debris. The data obtained were then analysed using FlowJo software version 10.7.1 (Ashland, Oregon USA) by comparing the SSC intensity of control to that of incubated with NPs. The data were plotted as the mean SCC intensity of treated NPs samples compared to that of the control.

2.2.3.3 Transmission Electron Microscopy Analysis AuNPs Uptake

The uptake of different sizes of AuNPs by A549 cells was conducted as described previously with modifications [72]. In brief, A549 cells were seeded at a density of 2×10^5 cells/well onto a 13 mm diameter sterilised coverslip in six well plates and allowed to adhere overnight at 37 °C in 5% CO₂. The next day, the cells were incubated with different sizes of AuNPs at 30 µg/mL for 24 h. At the end of the exposure time, the cells were rinsed with PBS to remove unattached AuNPs. This was followed by fixation with 2.5% glutaraldehyde in 0.1M PO₄ buffer diluted in PBS for 24 h at 4 °C. The samples were then taken to the Electron microscopy centre (University of Birmingham, Birmingham, UK) for processing. The cells were dehydrated in ethanol and embedded in epoxy resin. An ultrathin section (70–90 nm) parallel to the cover glass was cut with a diamond knife and mounted onto 300 mesh copper grids on a carbon film. Images were visualised with TEM (JEOL 1400) operated at an acceleration voltage of 80 keV in imaging mode.

2.2.4 Mechanisms of Cellular Uptake Using Pharmacological Inhibitors

2.2.4.1 Inductively Coupled Plasma Mass Spectrometry

The amount of AuNPs internalised into the cells was determined according to the method previously described [83]. In brief, A549 cells were seeded in six well plates at a density of 2×10^5 cells/well in 3 mL of CCM, supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C. At 24 h post-seeding, the cells were pre-treated with either 10 µg/mL of chlorpromazine (clathrin-coated pit inhibitor), 5 µg/mL of nystatin (caveolae-mediated inhibitor), 2.5 µM of methyl-β-cyclodextrin (MβCD) (caveolae-mediated/cholesterol inhibitor), and 10 µg/mL of wortmannin (macropinocytosis inhibitor) for 1 h. This was followed by treatment with 30 µg/mL of different sizes of AuNPs dispersed in CCM for 3 h. After exposure, the cells were washed three times

with PBS, harvested, and transferred to a glass vial. The cells were digested with 3 mL of aqua regia prepared with 37% HCL and 70% HNO₃. Then, 1 mL of hydrogen peroxide was added to the cell suspension. The glass vials were properly closed and heated at 120 °C for 30 min. The samples were diluted to a final acid concentration of 2% HNO₃ and filtered with a 0.22 µM syringe filter (Sigma Aldrich, UK) to ensure the removal of any debris. The total Au concentration in the samples was measured using the same procedure as described in Section 2.2.3.1.

2.2.4.2 Flow Cytometry

Flow cytometry experiment was performed according to a protocol described [72]. A549 cells were seeded in six well plates at a density of 2×10^5 cells/well in 3 mL of CCM and allowed to adhere overnight at 37 °C in 5% CO₂. The next day, the cells were pre-treated with either chlorpromazine (10 µg/mL), nystatin (5 µg/mL), MβCD (2.5 µM), or wortmannin (10 µg/mL) for 1 h, followed by treatment with 30 µg/mL of different sizes of AuNPs dispersed in CCM for 3 h. At the end of the exposure time, the cells were rinsed with PBS to remove unattached AuNPs, followed by harvesting and centrifugation at 1,904 g for 5 min at 4°C. The supernatant was discarded and resuspended in 500 µL of PBS. The samples were then analysed using the same procedure as described in Section 2.2.3.2.

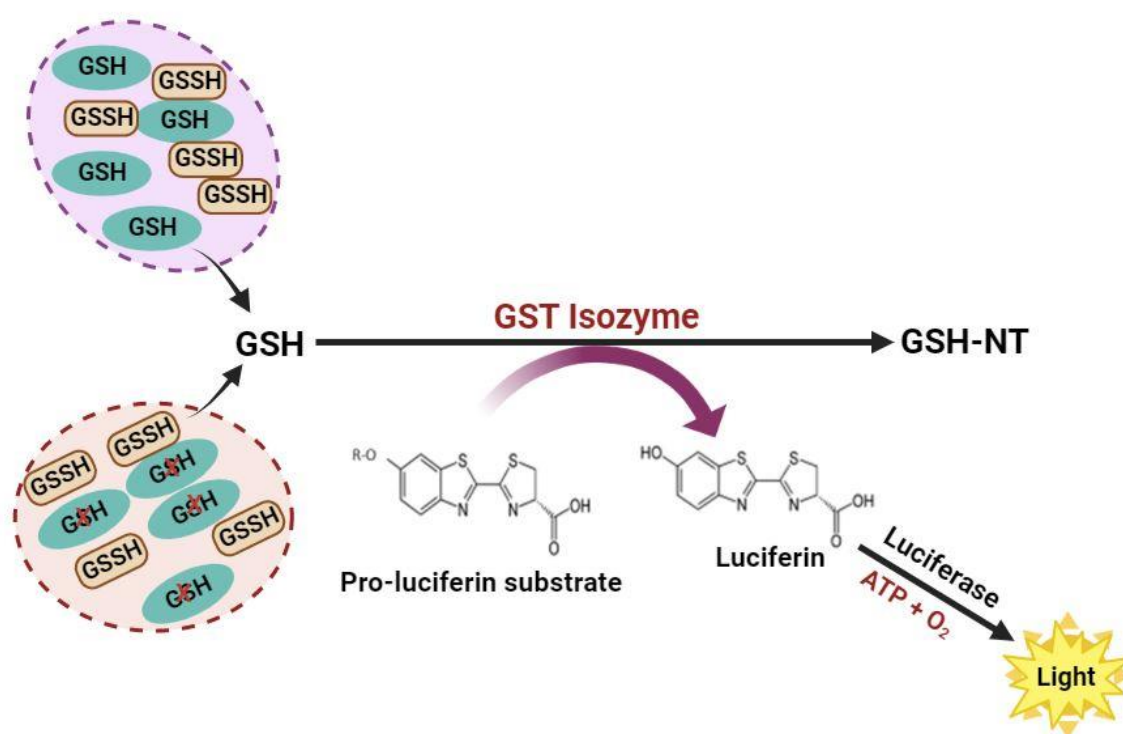
2.2.5 Mechanisms of AuNPs and AgNPs Toxicity

2.2.5.1 GSH/GSSG-Glo™ Assay

A luminescence-based Promega GSH/GSSG-Glo assay kit was used to quantify the intracellular glutathione in A549 cells exposed to 5 nm functionalised AuNPs (Tannic acid, citrate, and PVP) and a combination of CDDP (2.191 µg/mL) and 10 nm AuNPs (25 µg/mL) according to a protocol described [331]. After the treatment, the cells were washed three times with PBS

to remove any remaining unbound NPs. The cells were lysed with 50 μ L of either total glutathione or oxidized glutathione reagent and placed on a shaker for 5 min at room temperature. 50 μ L of luciferin generation reagent were added to all the wells and incubated for another 30 min at room temperature. Finally, 100 μ L of luciferin detection reagent was added and equilibrated for 15 min at room temperature followed by luminescence reading by a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK). The ratio of glutathione content was calculated using the relative light units (RLU) using the equation:

$$\text{GSH/GSSG ratio} = (\text{Net treated GSH RLU} - \text{Net treated GSSG RLU}) / (\text{Net treated GSSG RLU} / 2)$$

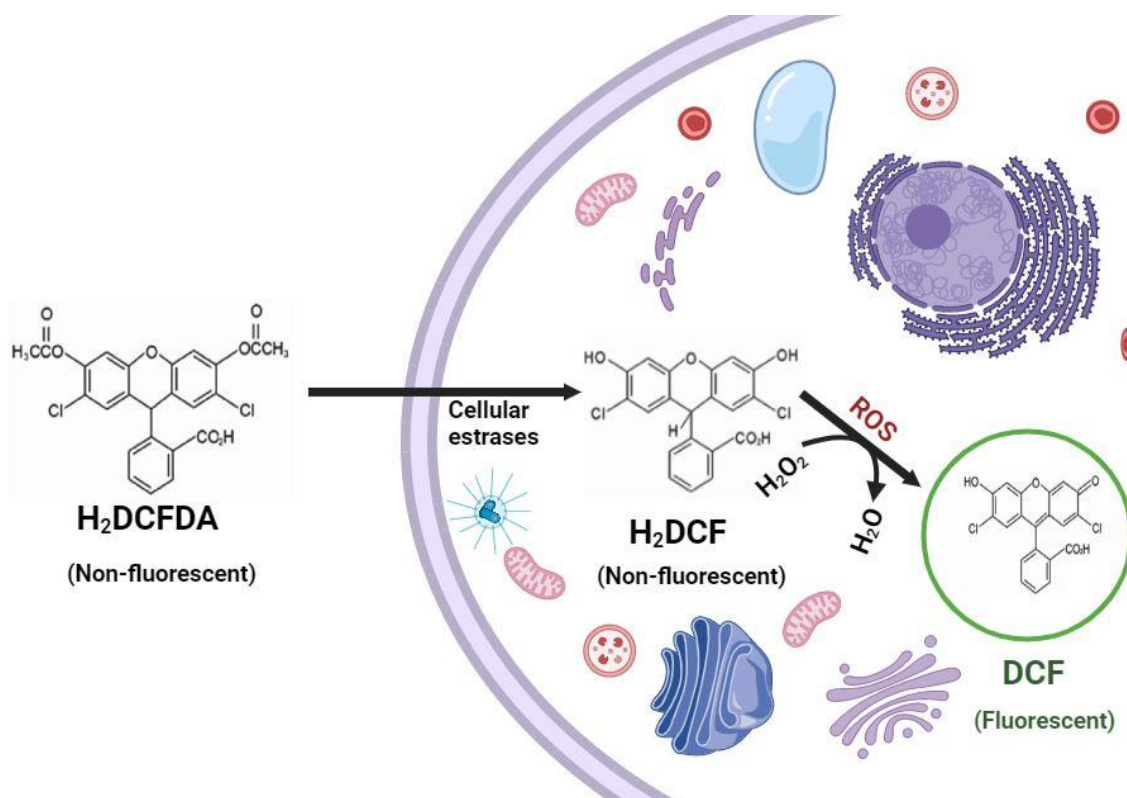


Scheme 2.4. Schematic representation of a luminescence-based assay for measuring the amount of GSH/GSSG ratio in culture cells. The amount of both reduced and oxidised glutathione (GSH and GSSG) measurements relied based on the reaction steps where GSH-dependent conversion of a GSH probe in the first step, Luciferin-NT to luciferin by an enzyme

glutathione S-transferase (GST) which then coupled to a firefly luciferase reaction in the second step. The Scheme was created with BioRender.com.

2.2.5.2 Dichloro-Dihydro-Fluorescein Diacetate (H₂DCF-DA) Assay

The Intracellular ROS generation of treated A549 cells was detected by staining the cells with H₂DCF-DA as previously reported [177]. A549 cells suspension were seeded at a density of 8×10^3 cells/well in the 96 well plates and treated 5 nm functionalised AuNPs (Tannic acid, citrate, and PVP) at the concentrations of (0-10 $\mu\text{g/mL}$) and a combination of CDDP (2.191 $\mu\text{g/mL}$) and AuNPs (25 $\mu\text{g/mL}$) for 24 and 48 h. Positive control was incubated with 50 μM of menadione one for 1 h before the end of each time point and the cells were washed three times with PBS. The cells were further incubated with 10 μM H₂DCF-DA (diluted in PBS) for 30-45 min at 4 °C. The fluorescence intensity was measured by FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) at excitation of 488 nm and emission of 525 nm.



Scheme 2.5. Reactive Oxygen Species (ROS) measurement in A549 cells using H₂DCFDA: The non-fluorescent 2',7'-dichlorofluorecin diacetate (H₂DCFDA) is deacetylated by the action of cellular esterase in the cell membrane to a non-fluorescent 2',7'-dichlorofluorescin (H₂DCF) which further oxidised to a fluorescent 2',7'-dichlorofluorescin (DCF). The scheme was created with BioRender.com.

2.2.5.3 CellRox Deep Red Flow Cytometry Assay

The CellRox™ deep red reagent kit was used for oxidative stress detection in A549 cells as per the manufacturer's instructions according to a protocol described [332]. A549 cells were seeded in twelve well plates at a density of 2×10^4 cells/well and incubated overnight to adhere. The cells were then treated with 5-30 µg/mL of AuNPs (5, 10, and 80 nm), 10 nm AuNPs, and AgNPs at the concentration range of 1.25-10 µg/mL for 24 h and the negative control was incubated with CCM while a 50 µM menadione treatment for 1 h was used as the positive control. Following the incubation, the cells were detached by trypsinisation and centrifuged at 1,904 g for 5 min. The cells were then stained with 5 µM CellRox deep red reagent diluted in phenol-free RPMI 1640 media for 1 h at 37 °C and protected from light. The cells were then centrifuged for 5 min at 1,904 g after the staining and the pellet was resuspended with 1 mL of RPMI 1640. The samples were gently transferred to FACS round bottom polystyrene test tubes and kept on ice for analysis by BD LSRFortessa™ X-20 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) equipped with 488 argon and UVB laser. Histograms of fluorescence (Alexa Fluor 700-A) intensity were acquired with a FACScalibur cytometer (BD Bioscience) equipped with a 488 argon and 635 red diode lasers with at least 10,000 events acquired for each sample. The data recorded were then analysed by

FlowJo software version 10.7.1 (Ashland, Oregon, USA) and statistically analysed by GraphPad prism software.

2.2.5.4 Mitochondrial Membrane Potential (MMP)

The changes in MMP induced by different coated 5nm AuNPs on A549 cells were evaluated according to a protocol reported [326]. Briefly, A549 cells were seeded at a density of 7×10^3 cells/well in 96 well plates and allowed to attach overnight. The CCM was then removed, and cells were exposed to 5 nm functionalised AuNPs (tannic acid, citrate, and PVP) at a concentration range of 1.25-10 $\mu\text{g/mL}$ and a combination of CDDP (2.191 $\mu\text{g/mL}$) and AuNPs (25 $\mu\text{g/mL}$) diluted in CCM for 24 h. After the incubation, 50 μL of mitochondrial staining solution was added to each well and incubated for 30 min under normal cell culture conditions. Afterward, the media was removed, and cells were counterstained with 100 μL of Hoechst 33342 staining solution and incubated for another 15 min at normal room temperature. The cells were then washed three times with 100 μL of PBS and finally 200 μL of PBS was added to each well. Finally, the MMP changes were measured using two different techniques. First, the fluorescence intensity was captured by FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) in time-resolved fluorescence manner. Second, the quality of fluorescence intensity was captured by EVOS™ FL imaging system (Washington, NJ, USA) equipped with an appropriate filter for DAPI and GFP at 20X magnification.

2.2.5.5 MitoProbe™ TMRM Flow Cytometry Assay

The MitoProbe™ TMRM assay kit was used to detect the MMP according to a protocol previously described [333]. In brief, the A549 cells were seeded at a density of 5×10^4 cells/well in 6 well plates in 1.5 mL of CCM overnight to adhere. The cells were then treated with AgNPs and AuNPs at the concentration of 0-10 $\mu\text{g/mL}$ for 24 h. 50 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control for 5 min incubated in 5% CO_2

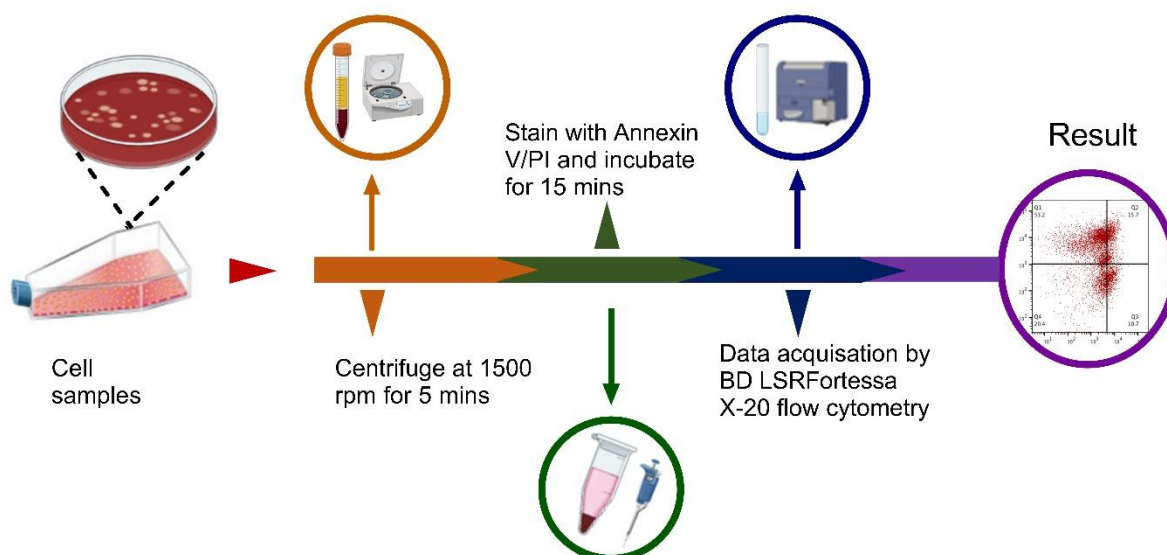
at 37 °C. After the incubation, the cells were detached and centrifuged at 1,904 g for 10 min at 4 °C and re-suspended in 1 mL of culture media with 1 µL TMRM reagent solution and incubated for 30 min at 37 °C in 5% CO₂ instructed by the manufacturer. The cells were then harvested by centrifugation for 5 min at 1,904 g and re-suspended in 500 µl PBS. The samples were gently transferred to FACS round bottom polystyrene test tubes and immediately analysed by BD LSRFortessa™ X-20 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). TMRM fluorescence intensity was measured at an approximate excitation (561 nm) and emission wavelength (585 nm) with at least 10,000 events acquired for each sample. The fluorescence intensity recorded was then analysed by FlowJo software version 10.7.1 (Ashland, Oregon, USA) and presented as a percentage of TMRM against concentration.

2.2.5.6 Caspase-Glo 3/7 Activity Measurement

The activity of caspase 3/7 was measured according to the manufacturer's instructions (Promega, Southampton, UK) according to a protocol described in published study [334]. Briefly, the A549 cells were seeded at a density of 7×10^3 cells/well in 96- opaque well plates and allowed to attach for 24 h. The cells were treated with CDDP (2.191 µg/mL), AuNPs (25 µg/mL) or a combination of both CDDP and AuNPs with Staurosporine (1 µM) treated for 6 h used as a positive control. After the treatment time point, the cells were additionally allowed to equilibrate for 1 h at room temperature. The media containing the treatments was gently removed and replaced with 100 µL of caspase-Glo 3/7 reagent. The contents were gently mixed for 60 seconds using a plate shaker at 445 g and then incubated at room temperature in a light protected area. The luminescence was finally read using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK).

2.2.5.7 Apoptosis and Necrosis Assay by Flow Cytometry

Apoptosis induced after exposure to different sizes of AuNPs was evaluated using the Annexin V-FITC/PI assay kit (Thermo Fisher Scientific, Paisley, UK) according to a protocol described [72]. A549 cells were seeded in six well plates overnight at a density of 2×10^5 cells/well and treated with different concentrations of NPs for 24 h. The positive controls were incubated with either 10% DMSO or 1 μ M Staurosporine for 6 h as inducers of necrosis and apoptosis, respectively. The cells were then collected by trypsinisation and centrifuged at 1,904 g for 5 min at 4 °C. The pellet was resuspended with 100 μ L of Annexin binding buffer and incubated with 5 μ L of Annexin V-FITC and 1 μ L of PI for 15 min in the dark at room temperature. After the incubation, the cell suspension was further diluted with 400 μ L of Annexin binding buffer. The samples were gently transferred to FACS round bottom polystyrene test tubes and immediately analysed by BD LSRFortessa™ X-20 flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) using FITC and Texas red dyes filters with 499/521 and 535/617 excitation/emission fluorescence wavelength. Electronic compensation was performed with unstained cells (Negative), cells labelled with single stain PI, and cells labelled with single stain annexin-V to eliminate spectral overlap between the fluorophores. The data recorded were then analysed by FlowJo software version 10.7.1 (Ashland, Oregon, USA) and expressed as a percentage cell population stained with PI or Annexin V staining.



Scheme 2.6. Diagrammatic illustration of apoptosis and necrosis assay using fluorescently labelled Annexin V and propidium iodide (PI) measured by BD LSRFortessa X-20 flow cytometry. The scheme was created with BioRender.com.

2.2.6 Effects of AuNPs and AgNPs on Different Pathways

2.2.6.1 Extraction of Protein

A549 cells were seeded at a density of 2.5×10^5 cells/well in six well plates to adhere before treatment with different concentrations of NPs, CDDP, and a combination of AuNPs with CDDP for either 24 or 48 h. After each period of treatment, the cells were washed three times with 2 mL of cold PBS and lysed in 100-200 μ L of NP40 lysis buffer containing protease inhibitors cocktail (Containing 0.1 mM PMSF, 1 mM Benzamidine, and 0.1% β -mearptoethanol). The lysed cells were carefully scraped from the plates and transferred to a 1.8 mL Eppendorf tube. The samples were mixed by pipetting up and down and later centrifuged at 17,078 g for 10 min at 4 °C. 2 μ L of the supernatant was used to determine the total protein concentration by Bradford assay as described previously [335].

2.2.6.2 Bradford Assay

5 µL of BSA protein standard prepared in the concentrations (0.125, 0.25, 0.5, 1, and 2 mg/mL) were pipetted into a non-sterile 96 well plate in triplicate. 2 µL of the supernatant (protein extracted) was added into the sample well and followed by adding Bradford reagent (280 µL) to all well containing the samples and the protein standards. The plate was gently mixed on a shaker to assure a homogenous solution and the absorbance was read using a FLUOstar Omega microplate reader (BMG LABTECH Ltd, Aylesbury, UK) at 595 nm wavelength. The protein concentration was calculated from the standard curve and diluted with sodium dodecyl sulfate (SDS), heated at 90 °C for 5 min, and stored in the freezer (-20) until needed for western blotting.

2.2.6.3 Western Blotting

20 µg of the lysates were loaded in 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prepared from resolving gel (1.5 M Tris (pH=8.8), 30% acrylamide, 20% SDS, 10% APS, water and TEMED) and stacking gel (0.5 M tris (pH=6.8), 30% acrylamide, 20% SDS, 10% APS, water and TEMED) according to the molecular weight of the target proteins and electrophoresed for 1 h at 160 V. Proteins separated were transferred to onto 0.45 µM PVDF membrane (GE Healthcare hybond) using 20X NuPAGE™ Transfer Buffer (Thermo Fisher Scientific) at 85 V for 1.45 h. The membranes were blocked in TBST (Tris Base, NaCl, pH 8.3) prepared with Tween-20 (0.1%) and skim milk (5%) for 1 h at room temperature with gentle agitation. The membranes were then washed three times with TBST for 8 min, followed by incubation with monoclonal primary antibodies purchased from cell signalling technology (CST) (London, UK) such as HSP40 (C64B4), HSP60 (D6F1), HSP70 (4872), HSP90 (C45G5), GAPDH (14C10) AKT (C67E7), P-AKT (D25E6), β-catenin(D10A8), P-β-Catenin (D8E11), GKS-3β (D5C5Z),

PTEN (138G6), MDM2 (D1V2Z), mTOR (7C10), USP7(D17C6), USP8 (D18F6), USP10 (D7A5), UCHL-1 (D3T2E), caspase-3 (D3R6Y), caspase-9 (C9), and PARP (9542) diluted in TBST containing 5% bovine serum albumin (1:1000) overnight at 4 °C with gentle agitation. After washing with TBST three times, membranes were then incubated with a secondary antibody (Horseradish peroxidase-conjugated anti-rabbit IgG (7074) or anti-mouse IgG (7076)) diluted in TBST with 5% BSA (1:2000) for 1 h at room temperature. The membranes were washed three with TBST and enhanced chemiluminescent reagents were added to detect the signal intensity of the proteins.

2.2.7 Statistical Analysis

Statistical analyses for the results obtained were performed using GraphPad Prism software version 9.0. BD LSRFortessa X20 data was analysed by FlowJo software for windows version 10.7.1 (Ashland, Oregon, USA). All the data were presented as mean $\pm$ standard deviation for at least three independent experiments. One way analysis of variance (ANOVA) was used to calculate the statistical significance between control, AuNPs, AgNPs or CDDP treated groups, followed by Bonferroni post hoc multiples comparison. The images from TEM and western blotting bands intensity were quantified by imaging software (ImageJ, Version 2.0.0, Public domain, BSD-2).

Chapter 3: Gold Nanoparticles Induced Size Dependent Cytotoxicity on Human Alveolar Adenocarcinoma Cells by Inhibiting the Ubiquitin Proteasome System

Abstract

Gold nanoparticles (AuNPs) are widely used in biomedicine due to their remarkable therapeutic applications. However, little is known about their cytotoxic effects on the ubiquitin proteasome system (UPS). Herein, the cytotoxicity of different sizes of AuNPs (5, 10, and 80 nm) on the UPS was investigated with a particular focus on deubiquitinating enzymes (DUBs) such as ubiquitin-specific proteases (USP) and ubiquitin carboxyl-terminal hydrolases (UCHL-1) in human alveolar epithelial adenocarcinoma (A549). It was found that all sizes of AuNPs reduced the percentage of viable A549 cells and increased lactate dehydrogenase (LDH) release, measured using the MTT and LDH assays, respectively. Furthermore, the 5 nm AuNPs were found to exhibit greater cytotoxicity than the 10 and 80 nm AuNPs. In addition, apoptosis and necrosis were activated through reactive oxygen species (ROS) generation due to AuNPs exposure. The internalisation of AuNPs in A549 cells increased with increasing particle size (80 > 10 > 5 nm). Interestingly, the expression of USP7, USP8, USP10, and UCHL-1 was significantly ($p < 0.001$) downregulated upon treatment with 5–30 $\mu\text{g/mL}$ of all of the AuNPs sizes compared to control cells. Moreover, the inhibition of these proteins triggered mitochondrial-related apoptosis through the upregulation of poly (ADP-ribose) polymerase (PARP), caspase-3, and caspase-9. Collectively, these results indicate that AuNPs suppress the proliferation of A549 cells and can potentially be used as novel inhibitors of the proteasome.

3.1 Introduction

In recent years there has been an increase in AuNP-related research due to their potential applications in cancer treatment [336, 337]. Their unique size-related (1–100 nm) physicochemical properties and high surface area to volume ratio enhance their reactivity with other molecules and enables them to cross biological barriers [34, 338]. Additionally, AuNPs can be used as drug carriers to improve drug delivery and reduce side effects through more effective targeting [339, 340]. Recent advances in AuNPs development have shown that they may also be ideal candidates for viral targeting and diagnosis [341-343] and may be used as stand-alone entities or carriers for other drugs or vaccine delivery [344-346].

Despite having various applications in cancer research, bioimaging, vaccines, and drug delivery, there is a growing concern about their short- and long-term health effects [16, 347]. Studies have revealed that the accumulation of AuNPs may impede the biological performance of cells through the induction of oxidative stress, reduction in metabolic activities, decrease in mitochondrial membrane potential, depletion of glutathione, and increase in lipid peroxidation, consequently leading to apoptosis and necrosis [348-350]. For instance, exposure to AuNPs induced the apoptosis of A549 cells and was characterised by cell shrinkage, reactive oxygen species (ROS) generation, and an increase in apoptotic protein expression [351, 352]. Apart from cytotoxicity, the induction of cell cycle arrest in different cell phases upon exposure to AuNPs has also been reported [353, 354]. Prasad et al., [141] found that AuNPs induced oxidative stress and G0/G1 cell cycle arrest by blocking the S phase, therefore inducing apoptotic cell death. Similar results were also reported using modified AuNPs [186]. The toxicity of AuNPs is considered to depend on their size, shape, surface charge, composition, concentrations, and cell type [115, 355, 356].

The UPS is the major intracellular pathway for the degradation of 80–90% of proteins in eukaryotic cells [357, 358]. During the process, ubiquitin (76 amino acids) covalently binds to the proteins to be degraded in the proteasome. This is accomplished by three enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (**Scheme 3.1**) [359, 360]. DUBs reverse the degradation process by removing the covalently attached ubiquitin from proteins, allowing the recycling of ubiquitin for further use by the proteasome [361]. The enzymes play an important role in controlling the process and are involved in many signalling activations such as DNA repair, apoptosis, and cell cycle progression [229, 362]. Due to their regulatory functions, tumour cells have been demonstrated to depend on DUBs for proliferation [363, 364]. Recently, many molecules have been tested for their toxicity against DUB activity and implicated in cancer treatment. For instance, cisplatin has been shown to suppress the proliferation of ovarian cancer through proteasome inhibition [231]. In breast cancer, the small molecule bis-benzylidene piperidone 15 (b-AP15) and platinum pyrithione (PtPT) were tested against elevated USP14, UCHL-5, and RPN11 proteases. These compounds were found to induce cell cycle arrest and cell death associated with caspase activation, endoplasmic reticulum stress, and downregulation of the endoplasmic reticulum by downregulating DUBs [365]. Although these compounds have shown a promising effect, there is still the issue of their limited efficacy, specificity, and biocompatibility [366]. Therefore, alternative DUB inhibitors are urgently needed in cancer treatment to improve efficacy and biocompatibility. Herein, the effects of different sizes of AuNPs (5, 10, and 80 nm) coated with citrate on UPS was investigated, with a particular focus on DUBs such as the ubiquitin-specific protease (USP) and ubiquitin carboxyl-terminal hydrolase L1 (UCHL-1) in A549 cells. Extensive insight into the underlying mechanisms of AuNPs (5, 10, and 80 nm) toxicity in A549 cells was further investigated by assessing their

metabolic activity, ROS, expression of apoptotic-related proteins, and their cellular uptake mechanisms. The sizes of AuNPs were selected due to lack of standardised characterisation methods and techniques for smaller AuNPs, definitive regulatory framework for nanoparticle-specific data on size dependent cytotoxicity and limited data on the toxicity of different sizes of AuNPs on the UPS.

3.2. Results

3.2.1. Physicochemical Characterisation of Different Sizes of AuNPs

The hydrodynamic size distribution, polydispersity index (PDI), and zeta potential of 5, 10, and 80 nm AuNPs used in this study was measured using DLS in UPW and CCM at 0 and 24 h. The DLS measurement revealed that the hydrodynamic diameter of 5, 10, and 80 nm were 12.92 ± 1.45 nm, 16.40 ± 0.39 nm, and 85.85 ± 0.76 nm, respectively, in UPW at 0 h (**Figure 3.1A**). Moreover, when the AuNPs were kept in UPW for 24 h, a slight increase in hydrodynamic diameter was observed compared to the hydrodynamic diameter at 0 h, as shown in **Figure 3.1A**. In addition, the PDI also showed an apparent change in AuNPs stability for 0 and 24 h in the UPW. For instance, the 5 nm AuNPs showed an Initial PDI value of 0.38 ± 0.08 for 0 h which changed slightly to 0.48 ± 0.09 after 24 h. Likewise, the PDI values of 10 nm AuNPs increased from 0.27 ± 0.01 at 0 h to 0.36 ± 0.02 after 24 h, whereas 80 nm showed a noticeable PDI decrease at 24 h. To further understand how AuNPs behave in the exposed CCM, the particles were dispersed in CCM containing 10% FBS and 1% penicillin/streptomycin at the concentration of 10 $\mu\text{g/mL}$. As seen in **Figure 3.1B**, there was an increase in the hydrodynamic diameter of all the AuNPs once exposed to CCM when compared to the UPW. This could be due to the presence of macromolecules in the CCM media such as

vitamins, amino acids, antibiotics, glucose, and protein supplements. The PDI values in the CCM show higher values compared to the UPW with all the NPs, and slightly fluctuating between 0 and 24 h. While the PDI values slightly increased for the 5 and 10 nm AuNPs after 24 h, there was a slight decrease in PDI value for the 80 nm AuNPs as shown in **Figure 3.1B**. As expected, the measured zeta potential showed that all the NPs were negatively charged in UPW and CCM (**Figure 3.1C–D**). Interestingly, the zeta potential values of the AuNPs showed an apparent decrease in UPW for all NPs as shown in **Figure 3.1C**. However, the zeta potential of the AuNPs noticeably increased when assessed in CCM at both time points (**Figure 3.1D**). TEM images of all three sized AuNPs showed that the particles are spherical with a uniform size distribution (**Figure S3.1 in Supplementary Materials**).

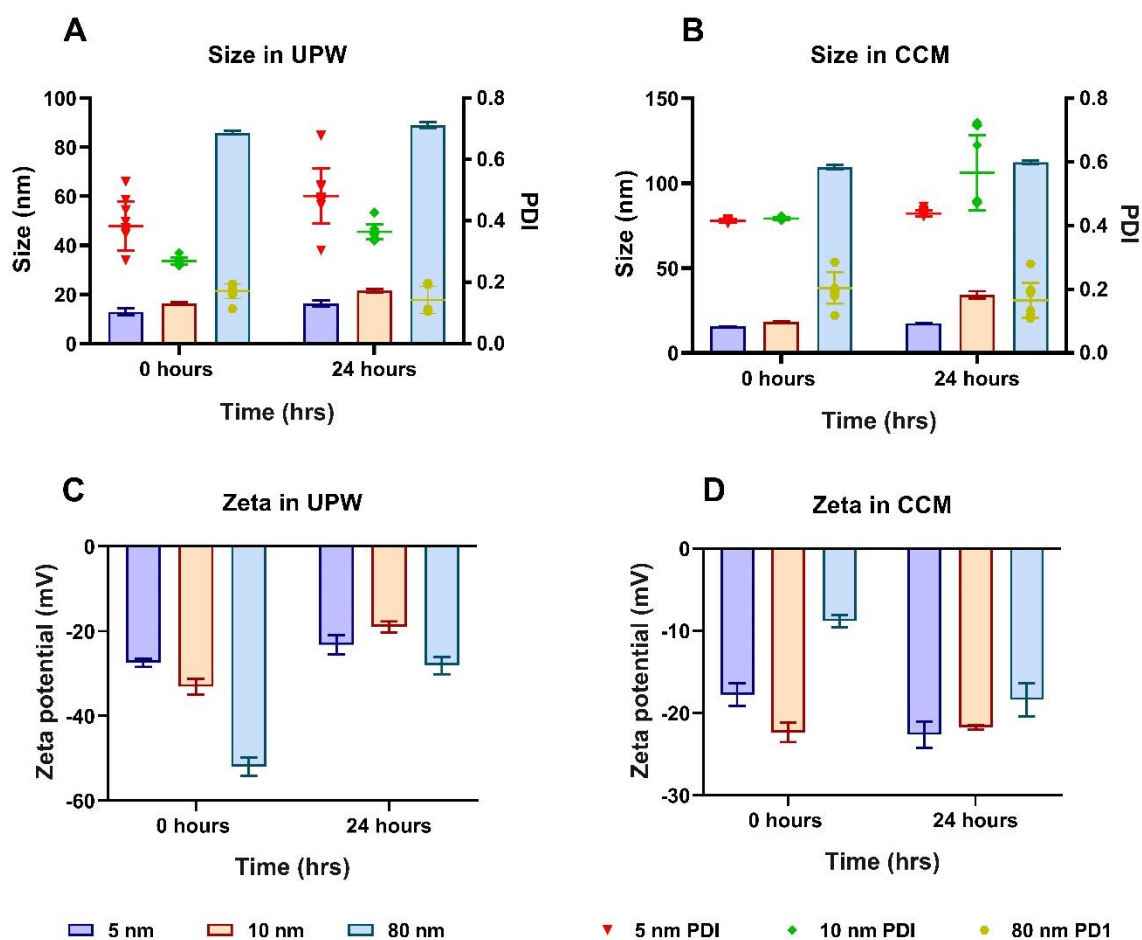


Figure 3.1. Characterisation of different sizes of AuNPs by DLS in UPW and CCM. Hydrodynamic size distribution and PDI of 5, 10, and 80 nm AuNPs in (A) UPW after 0 and 24 h and (B) CCM after 0 and 24 h. Zeta potential values of 5, 10, and 80 nm AuNPs in (C) UPW after 0 and 24 h and (D) CCM after 0 and 24 h. Plotted graphs represent the means $\pm$ standard deviation (SD) of ten different measurements.

3.2.2 AuNPs Decreased Cell Viability and Increased LDH Release in A549 Cells

In the current study, the cytotoxicity of 5, 10, and 80 nm AuNPs was measured based on the metabolic activity and LDH release from A549 cells using the MTT and LDH assays, respectively. As shown in **Figure 3.2**, the decrease in percentage cell viability and release of LDH is related to the increase in AuNP concentrations (5–40 $\mu\text{g/mL}$) and incubation period (24–48 h). In **Figure 3.2A**, the cell viability of A549 cells was maintained with up to 10 $\mu\text{g/mL}$ for 5, 10, and 80 nm AuNPs after 24 h incubation. As the concentrations increased above 10 $\mu\text{g/mL}$, the cell viability of A549 cells decreased for all three sizes of AuNPs. AuNPs at 40 $\mu\text{g/mL}$ showed the most significant decrease in cell viability (9.24%, 12.91%, and 24.08%) for the 5, 10, and 80 nm, respectively. Using the same concentrations, the cell viability measured after 48 h dramatically decreased at a greater rate (**Figure 3.2B**), with low concentration (5 $\mu\text{g/mL}$) having a minor impact (91%, 87.1%, and 85.4% for the 80, 10, and 5 nm, respectively). The membrane integrity effect induced by AuNPs on A549 cells was also assessed by LDH release in cell culture media. It was found that AuNPs showed a dose-dependent increase in LDH release at a concentration of 20 $\mu\text{g/mL}$ and higher (**Figure 3.2C**). In addition, an increase in LDH release was observed from a concentration of 10 $\mu\text{g/mL}$ (5.2% increase compared to control) for all the NPs (**Figure 3.2D**). A statistically

significant difference ($p < 0.01$) between the treated concentrations and control was observed at the exposure levels (25–40 $\mu\text{g/mL}$) and (20–40 $\mu\text{g/mL}$) for 24 and 48 h, respectively. In summary, the assays revealed that 5 nm was more cytotoxic than 10 and 80 nm at both 24 and 48 h. Although mass concentrations were used for the viability assays, the study explored if the cytotoxicity of the NPs is related to the surface area of the AuNPs and particle number concentration by normalising the surface area and particle number concentration from the corresponding concentrations used in the MTT assay. It was found that surface area varies with the variation of particle diameter; at the same concentration (40 $\mu\text{g/mL}$), 5 nm AuNPs ($4.13 \times 10^{-5} \text{ m}^2/\text{g}$) have a higher surface area compared to the 10 nm ($1.09 \times 10^{-5} \text{ m}^2/\text{g}$) and 80 nm ($1.02 \times 10^{-6} \text{ m}^2/\text{g}$) AuNPs which could explain why they are more cytotoxic (**Table S3.1 in Supplementary Materials**). Conversely, normalisation of mass concentration to particle number concentration revealed that 80 nm AuNPs were more cytotoxic than 5 and 10 nm AuNPs due to the fact that 80 nm AuNPs require a lower amount of particle number concentration ($8.46 \times 10^8 \text{ NPs/mL}$) to inhibit the percentage of cell viability at the same mass concentration (40 $\mu\text{g/mL}$) compared to the 5 nm and 10 nm AuNPs ($3.17 \times 10^{13} \text{ NPs/mL}$, $2.49 \times 10^{12} \text{ NPs/mL}$) (**Table S3.2 in Supplementary Materials**).

Based on these experiments, concentrations of 5, 15, and 30 $\mu\text{g/mL}$ were selected for subsequent experiments to evaluate other modes of toxicities.

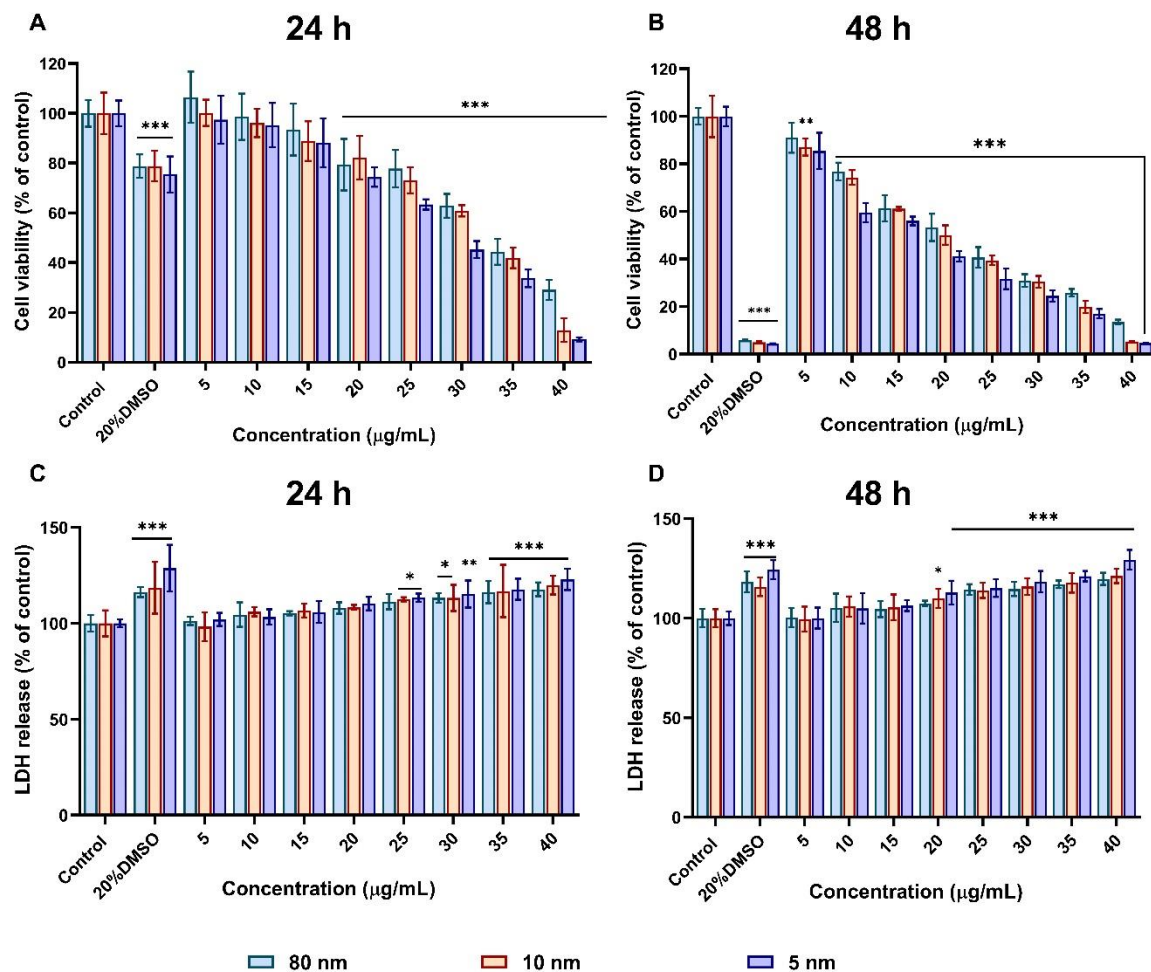


Figure 3.2. Cell viability and lactate dehydrogenase (LDH) release of A549 cells treated with 5–40 µg/mL of different sizes of AuNPs measured using MTT and LDH assays. Percentage of cell viability after incubation with AuNPs for **(A)** 24 h and **(B)** 48 h. Percentage of LDH release in media after incubation with AuNPs for **(C)** 24 h and **(D)** 48 h. 20% DMSO was used as a positive control. Plotted graphs represent the means $\pm$ SD of six independent experiments. Bars with an asterisk (*) show statistical differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) compared with the control.

3.2.3 AuNPs Uptake in A549 cells by ICP-MS, Flow Cytometry, and Transmission Electron Microscopy (TEM)

ICP-MS, flow cytometry, and TEM were used to measure the cellular uptake of different sizes of AuNPs after incubation for 24 h. When the mass concentrations of AuNPs in the cells were quantitatively measured using ICP-MS, it was observed that the uptake of AuNPs was highly dependent on the concentration and size of the NPs, as shown in **Figure 3.3A**. The uptake was lowest at 5 $\mu\text{g/mL}$ for the 5, 10, and 80 nm AuNPs (with 5.62, 22.90, and 38.97 Au pg/ 10^4 cells, respectively). As the concentration was tripled, the uptake slightly increased for the 10 nm (25.84 Au pg/ 10^4 cells) and 80 nm (59.08 Au pg/ 10^4 cells) but did not increase for the 5 nm AuNPs. Interestingly, when the concentration of AuNPs was further increased to 30 $\mu\text{g/mL}$, the uptake only increased with the 80 nm NPs (118.38 Au pg/ 10^4 cells). It was interesting to find that the order of AuNPs uptake (80 > 10 > 5 nm) did not correspond with the order of cell viability decrease and LDH release, thus, the 80 nm AuNPs proved to be less cytotoxic than the 5 and 10 nm.

Flow cytometry method was further used to assess the cellular uptake of AuNPs into A549 cells. Flow cytometry was used to measure the intensity signal of side scatter (SSC) which indicates the cells' internal granularity and complexity [367]. This technique was previously used to evaluate the uptake of functionalised carbon nanotubes in A549 cells [72], and AuNPs in MDA-MB-231 cells [368]. When the cells were incubated with the same concentrations used for ICP-MS at 24 h, the SSC intensity signals increased in a dose-dependent manner when compared with the control, which could be attributed to the uptake of AuNPs (**Figure 3.3B**). On the other hand, a maximal SSC intensity was observed with the 80 nm size, suggesting greater

uptake compared to the 5 and 10 nm (**Figure S3.2 in Supplementary Materials**). This is in complete agreement with that of ICP-MS which also showed greater uptake of the 80 nm. In order to accurately assess the localisation of AuNPs in the cells during the uptake process, A549 cells were incubated with 30 $\mu\text{g/mL}$ of different sizes of AuNPs for 24 h and sectioned using TEM. Ultra-thin sections of cells were visualised with TEM to assess the localisation of AuNPs in various organelles. All AuNPs were found to be localised within vesicles of cells as shown in **Figures 3.3C–E**. Moreover, the 5 nm AuNPs were also seen to be slightly bonded to the cell surface (**Figure 3.3C**).

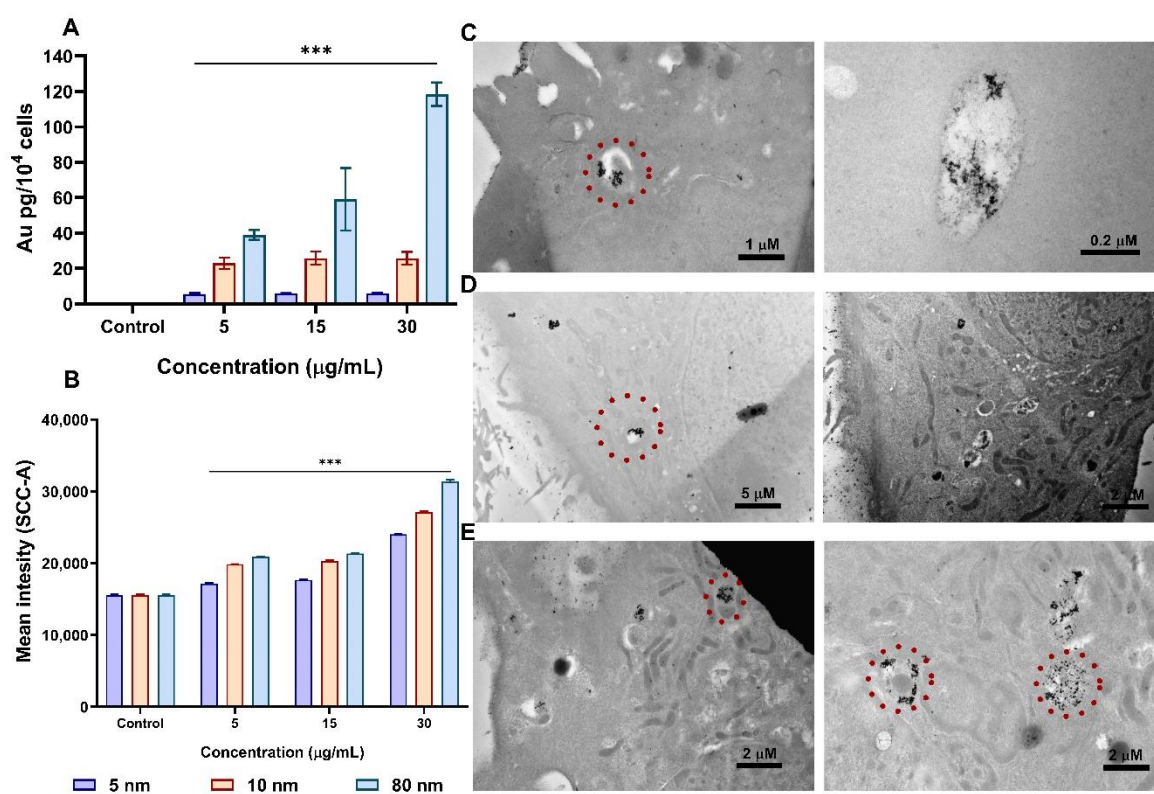


Figure 3.3. Cellular uptake of different sizes of AuNPs in A549 cells after 24 h incubation. (A) The Au content in A549 cells measured with inductively coupled plasma mass spectrometry (ICP-MS) (B) Side scatter (SSC) intensity changes due to the uptake of AuNPs analysed using flow cytometry. TEM Images of the internalisation of AuNPs into A549 cells after treatment with 30 $\mu\text{g/mL}$ for 24 h of (C) 5 nm, (D) 10 nm, (E) 80 nm AuNPs. AuNPs are indicated by

circles. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show a statistical difference (***) $p < 0.001$ when compared with the control

3.2.4 Assessment of the Uptake Mechanism(s) of Different Sizes of AuNPs in A549 Cells

Chlorpromazine, nystatin, M β CD, and wortmannin endocytic inhibitors were selected to investigate the uptake mechanism(s) of AuNPs in A549 cells. These pharmacological inhibitors were chosen because they have been previously used to inhibit specific endocytotic uptake mechanisms through the depletion of cholesterol in the biological membrane (M β CD and nystatin), serum protein transferrin inhibition blocking of transglutaminase 2 enzyme (chlorpromazine), and show fewer effects on the actin cytoskeleton (wortmannin) [57, 369-371]. First, the toxicity of these inhibitors in A549 cells was evaluated using the MTT assay after 1 h of incubation (**Figure S3.3 in Supplementary Materials**). The concentrations that is non-toxic to the cell membrane were selected to avoid interference with the actin filaments. As shown in **Figure 3.4A**, the A549 cells were pre-treated with chlorpromazine (10 μ g/mL) for 1 h. Chlorpromazine is used to inhibit clathrin-coated pit formation in order to assess whether it will disturb the internalisation of AuNPs. As assessed by ICP-MS, the pre-incubation with chlorpromazine demonstrated a stronger inhibition of the uptake for 5 nm (11.34 Au pg/10⁴ cells) and 10 nm (11.85 Au pg/10⁴ cells) compared to 80 nm (32 Au pg/10⁴ cells) in A549 cells. This indicates that all the AuNPs could be internalised via clathrin-mediated endocytosis. In the presence of nystatin, which specifically inhibits caveolae-mediated endocytosis, the internalisation of all the AuNPs remained relatively close (11.12, 11.71, and 11.17 Au pg/10⁴ cells) for 5, 10, and 80 nm. A similar

trend was also observed when the cells were incubated with another caveolae inhibitor or cholesterol inhibitor (M β CD). Likewise, when cells were pre-treated with a specific macropinocytosis inhibitor (wortmannin), the uptake of 5 and 10 nm AuNPs decreased (9.63 and 13.29 Au pg/10⁴ cells, respectively) compared to the control (100 Au pg/10⁴ cells). However, the uptake of 80 nm reduced to 23.41 Au pg/10⁴ cells but was still much higher than that of the 5 and 10 nm AuNPs. Similar outcomes were observed when A549 cells were pre-incubated with the same endocytic inhibitors and the change in SSC signal intensity was assessed using flow cytometry as shown in **Figure 3.4B**. A dot plot analysis of the SSC against FSC signal intensities can be found in **Figure S3.4 in the Supplementary Materials**. This show that the uptake of all AuNPs could be via either clathrin- or caveolae-mediated endocytosis.

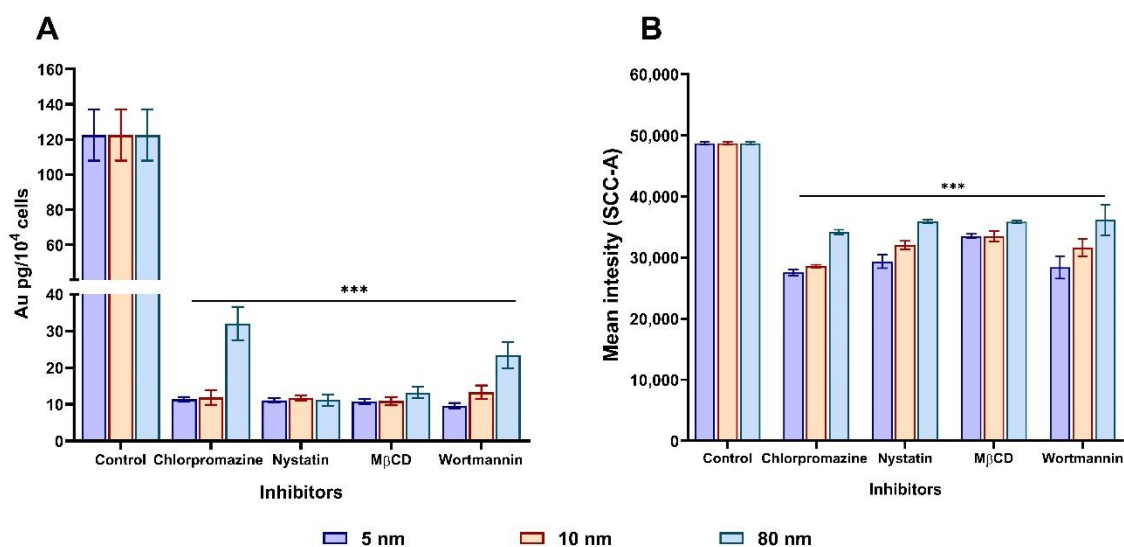


Figure 3.4. Effects of pharmacological inhibitors on the internalisation of different sizes of AuNPs (30 μ g/mL) in A549 cells. Cells were separately pre-incubated with chlorpromazine, nystatin, M β CD, and wortmannin for 1 h, followed by incubation with AuNPs for 3 h and analysed by (A) ICP-MS and (B) flow cytometry. Plotted graphs represent the means $\pm$ SD of

three independent experiments. Bars with an asterisk (*) show a statistical difference (***) ($p < 0.001$) when compared with the control.

3.2.5 Upregulation of ROS Production Caused by Different Sizes of AuNPs

Reactive oxygen species (ROS) are molecules that are formed as a by-product of the normal metabolism of oxygen [133]. They are regarded as activators of many signalling pathways in tumour cells such as AKT signalling, apoptotic and Wnt pathways [183]. The production of these molecules is triggered mainly by exposure to environmental stress and toxic materials, which may also include NPs. In the current study, CellRox deep red reagent kit was used to detect the production of ROS by different sizes of AuNPs after 24 h incubation at a concentration of 5, 15, and 30 $\mu\text{g/mL}$. In total, 50 μM of menadione was used as an inducer of oxidative stress (positive control) in A549 cells. **Figure 3.5A–C** shows that the fluorescence intensity gradually increased after exposure to different concentrations of different sizes of AuNPs which indicates an increase in intracellular ROS generation in A549 cells. For instance, 5 nm shows the highest increase in fluorescence intensity at 30 $\mu\text{g/mL}$ compared to 10 and 80 nm AuNPs. When the median fluorescence intensity (MFI) was quantified, 5 nm AuNPs showed a greater ROS generation in A549 cells when incubated with 5–30 $\mu\text{g/mL}$ compared to the control. Notably, a lower MFI (188 ± 2.65) was observed with 10 nm AuNPs when compared with 5 nm AuNPs (211 ± 1.78) at 30 $\mu\text{g/mL}$. However, at 5 $\mu\text{g/mL}$, 10 nm AuNPs showed a significant increase in MFI (140 ± 1.53) compared to 5 nm AuNPs (127.33 ± 1.52). In contrast, 80 nm AuNPs successfully showed a dose-dependent increase in MFI from 87.40 ± 10.67 at 5 $\mu\text{g/mL}$ to 178 ± 15.39 at 30 $\mu\text{g/mL}$ ($p < 0.001$ compared to the control). Overall, 30 $\mu\text{g/mL}$ was observed to induce a

greater ROS production in all the AuNPS. In addition, 5 nm AuNPS have significant effects compared to 10 and 80 nm AuNPs as shown in **Figure 3.5D**.

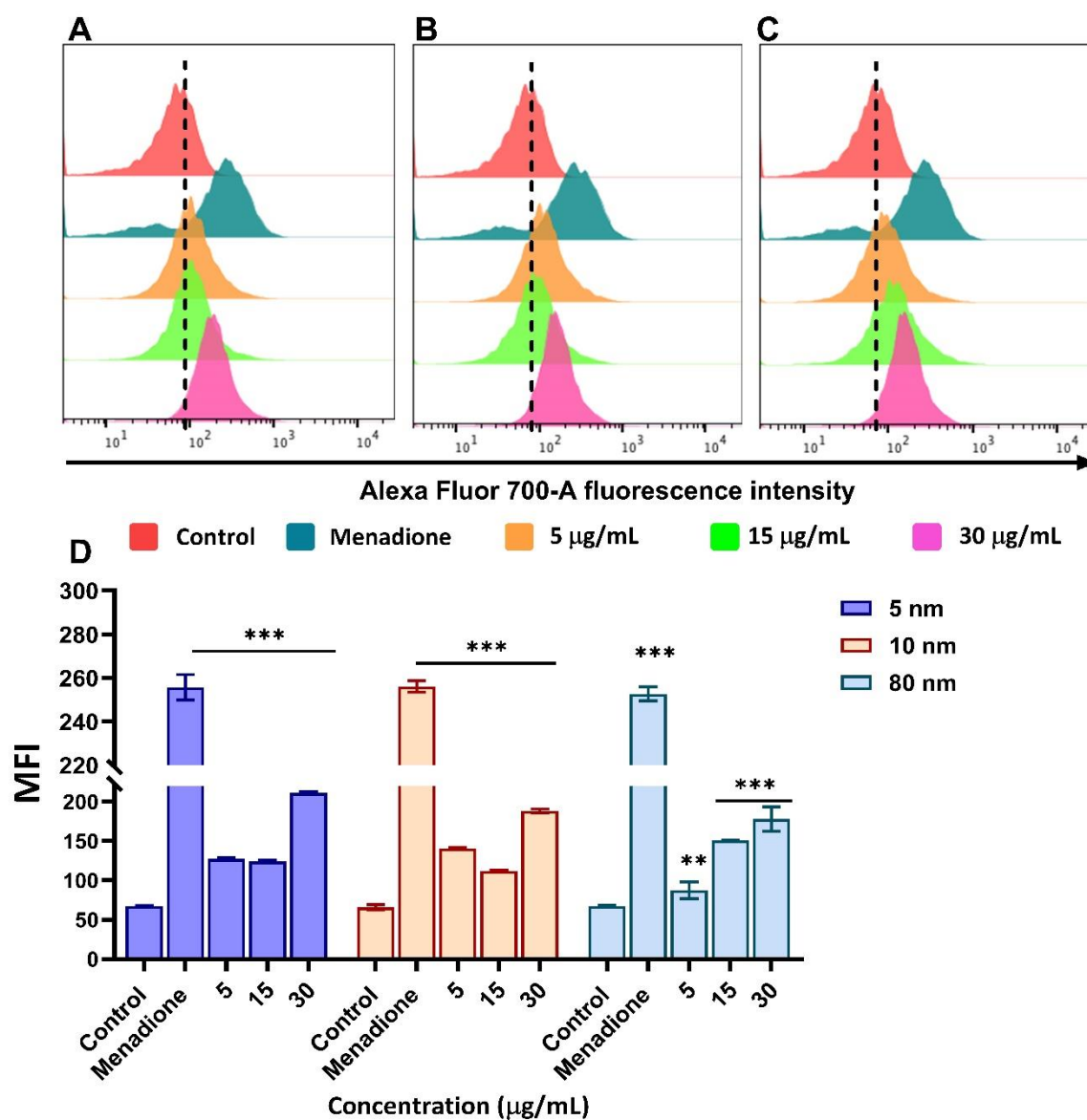


Figure 3.5. ROS production in A549 cells following exposure to different sizes of AuNPs analysed using flow cytometry with the CellRox deep red kit. Histograms of flow cytometry treated with (A) 5 nm (B) 10 nm and (C) 80 nm AuNPs. (D) Bar graphs represent the median fluorescence intensity (MFI) of A549 cells exposed to 5, 10, and 80 nm AuNPs. Menadione (50 µM) was used as a positive control. Plotted graphs represent the means $\pm$ SD of three

independent experiments. Bars with an asterisk (*) show a statistical difference (** $p < 0.01$, and *** $p < 0.001$) when compared with the control.

3.2.6 Mechanism of Cell Death of Different Sizes of AuNPs Assessed by Annexin-V/PI

The mechanisms by which A549 cell death could be induced upon exposure to different sizes of AuNPs was studied using flow cytometry. During early apoptosis, the cells undergo physiological changes which are characterised by the flip flop of the phosphatidyl-serine to the outer layer of the biological membrane to which fluorescently labelled Annexin V binds and emits green fluorescence signals, showing that apoptosis is occurring. At a late stage of the process, propidium iodide (PI) enters the cells, binds to the nucleus (DNA), and emits red fluorescence, indicating necrosis. As shown in **Figures 3.6A-B**, 5 and 10 nm AuNPs slightly induced both apoptosis and necrosis in a similar manner as the concentration of AuNPs increased. However, 80 nm AuNPs induced minimal apoptosis and necrosis compared to 5 and 10 nm (**Figure 3.6C**). FlowJo scatter dot plots can be found in the supporting information (**Figure S3.5 in Supplementary Materials**).

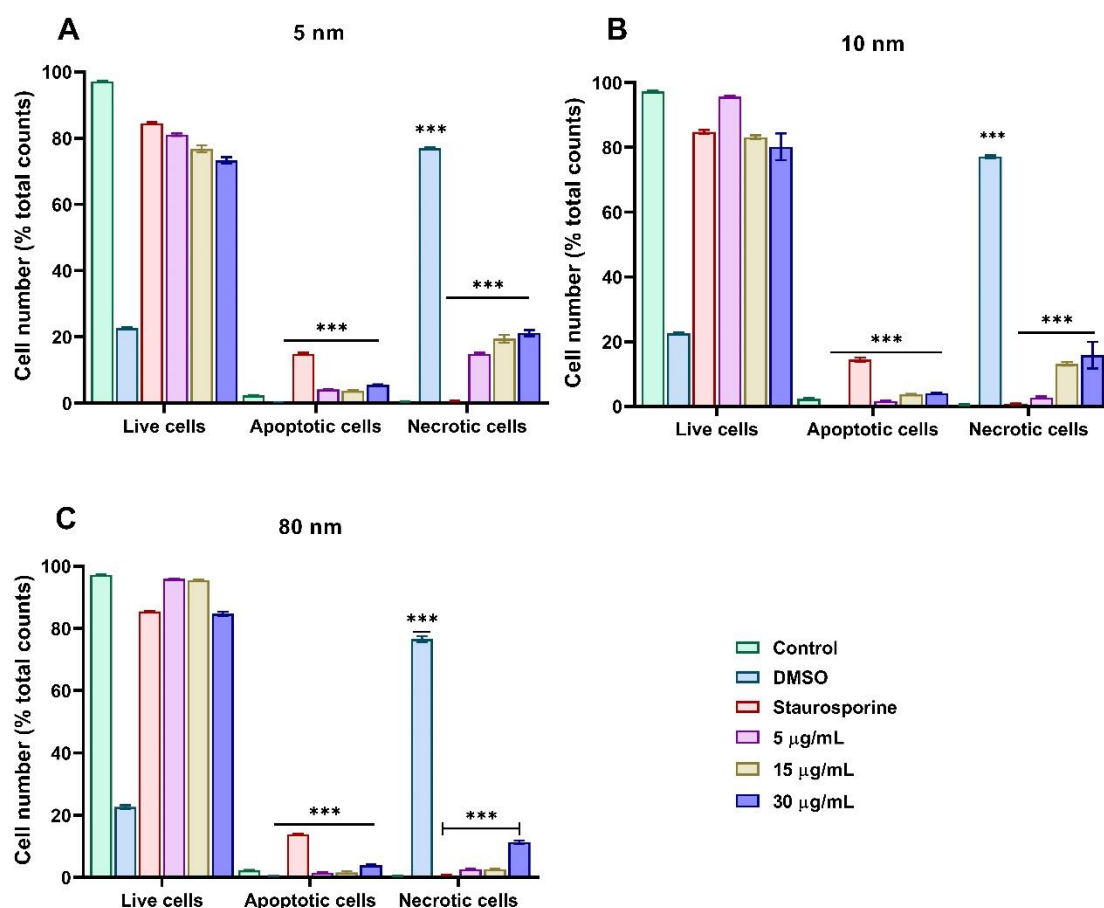


Figure 3.6. Bar graph analyses of apoptosis and necrosis assessed by flow cytometry after the incubation of A549 cells with (A) 5 nm, (B) 10 nm, and (C) 80 nm AuNPs at concentrations of 5–30 µg/mL. Treatment with 93taurosporineosporine and 10% DMSO for 6 h were used as positive controls for apoptosis and necrosis, respectively. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show a statistical difference (***) $p < 0.001$) when compared with the control.

3.2.7 Effects of Different Sizes of AuNPs on 19S Proteasome DUBs Enzymes

To establish whether AuNPs affect the UPS, the expression of USP7, USP8, USP10, and UCHL-1 which are involved in the removal of covalently attached ubiquitin from target proteins to be degraded was investigated using western blotting. The enzymes' activities regulate various signalling pathways, cell homeostasis, and protein stability

and are heavily linked to cancer proliferation [269]. In A549 cells, the enzymes are highly expressed [372]. Exposure to AuNPs resulted in the strong inhibition of these enzymes in a dose-dependent manner at 24 h. At the lowest concentration of AuNPs (5 µg/mL), it was found that all AuNPs did not inhibit the activity of USP7, USP8, USP10, and UCHL-1 (**Figure 3.7A**). Likewise, at 15 µg/mL AuNPs slightly inhibited the activities of these enzymes compared to control cells and the lower concentration. However, exposure of A549 cells to 30 µg/mL AuNPs resulted in a significant downregulation of these proteins. Interestingly, 30 µg/mL AuNPs inhibited the expression of proteins in a similar way to the positive control (MG132) which has been shown to inhibit the proteasome. The changes of the relative protein expression of DUBs exposed to 5 nm AuNPs decreased significantly ($p < 0.001$) in a dose-dependent manner with a greater decrease observed at 30 µg/mL for USP7, USP8, USP10, and UCHL-1 as shown in **Figure 3.7B**. Similarly, 10 nm AuNPs decreased the relative protein expressions of USP7, USP8, USP10, and UCHL-1 as shown in **Figure 3.7C** ($p \leq 0.001$). In addition, 80 nm AuNPs significantly decreased the relative protein expression of USP7, USP8, USP10, and UCHL-1 at 30 µg/mL (**Figure 3.7D**) ($p < 0.001$).

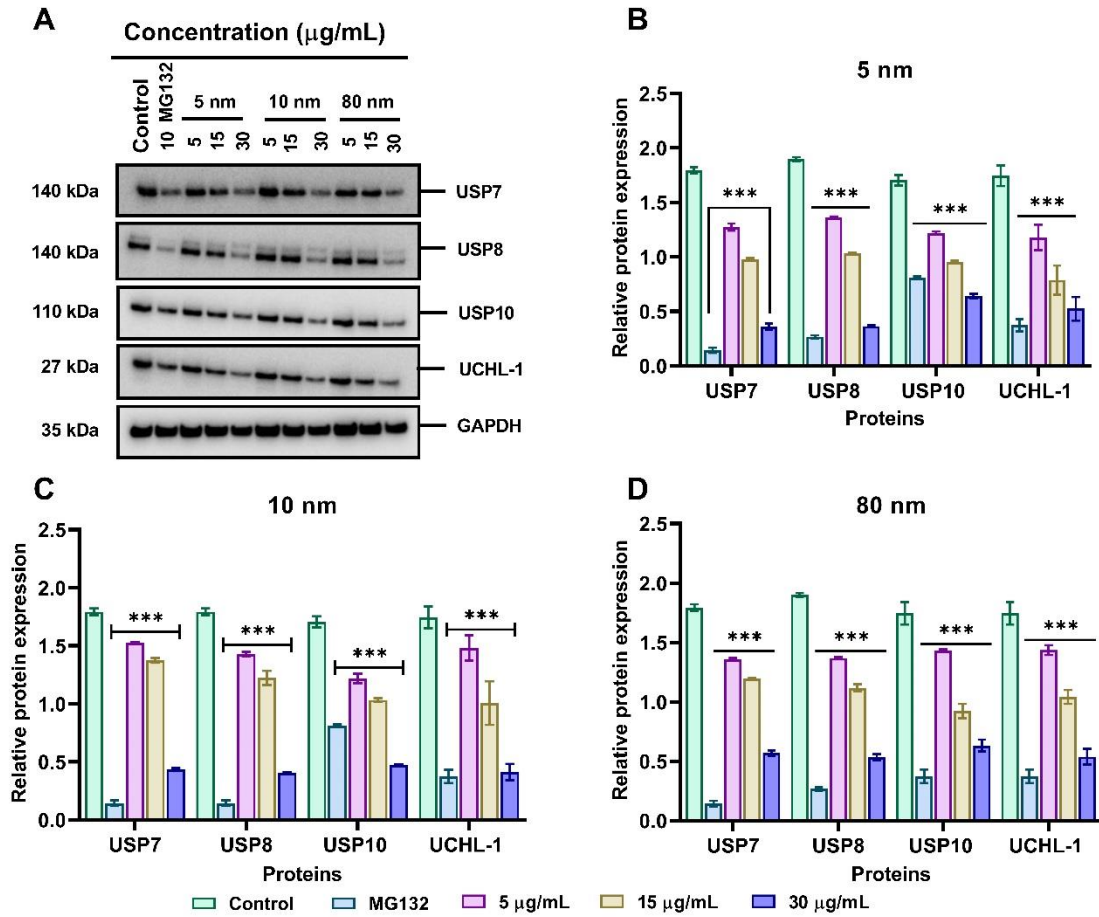


Figure 3.7. AuNPs downregulate the expression of DUBs in A549 cells. **(A)** Expression levels of DUBs detected by Western blot after A549 cells were incubated with 5, 15, and 30 µg/mL of different sizes of AuNPs for 24 h. MG132 (10 µg/mL) was used as a positive control. The relative protein expression signal intensity was semi-quantified by ImageJ software for **(B)** 5 nm, **(C)** 10 nm, and **(D)** 80 nm AuNPs. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show a statistical difference (***) $p < 0.001$) when compared with the control.

3.2.8 Effects of Different Sizes of AuNPs on the Expression Level of Apoptosis-Related Proteins

The induction of apoptosis of 5, 10, and 80 nm AuNPs was explored by checking the expression of Poly (ADP-ribose) polymerase (PARP), caspase-3, and caspase-9 when

incubated with 5–30 µg/mL AuNPs for 24 h. Following exposure to 5 µg/mL AuNPs, the expression of PARP and caspase-3 did not increase compared to the control. As cells were incubated with 15 µg/mL AuNPs, PARP, caspase-3, and caspase-9 were slightly upregulated as shown in **Figure 3.8A**. As the concentration increased to 30 µg/mL AuNPs, PARP, caspase-3, and caspase-9 were significantly upregulated, indicating the induction of apoptosis at higher concentrations. As shown in **Figure 3.8B**, the relative protein expression of pro-apoptotic proteins was upregulated in a dose-dependent manner when incubated with 5 nm AuNPs with the highest concentration showing a higher upregulation of PARP (1.54), caspase-3 (1.33), and caspase-9 (1.57) compared to the control (0.522) ($p < 0.001$). In addition, **Figure 3.8C** shows that the relative protein expression of PARP, caspase-3, and caspase-9 significantly increased when treated with 30 µg/mL of AuNPs. Likewise, following the treatment of A549 cells with 80 nm AuNPs, the relative protein expression of PARP, caspase-3, and caspase-9 increased but to a lesser extent compared to 5 and 10 nm AuNPs ($p < 0.001$) (**Figure 3.8D**).

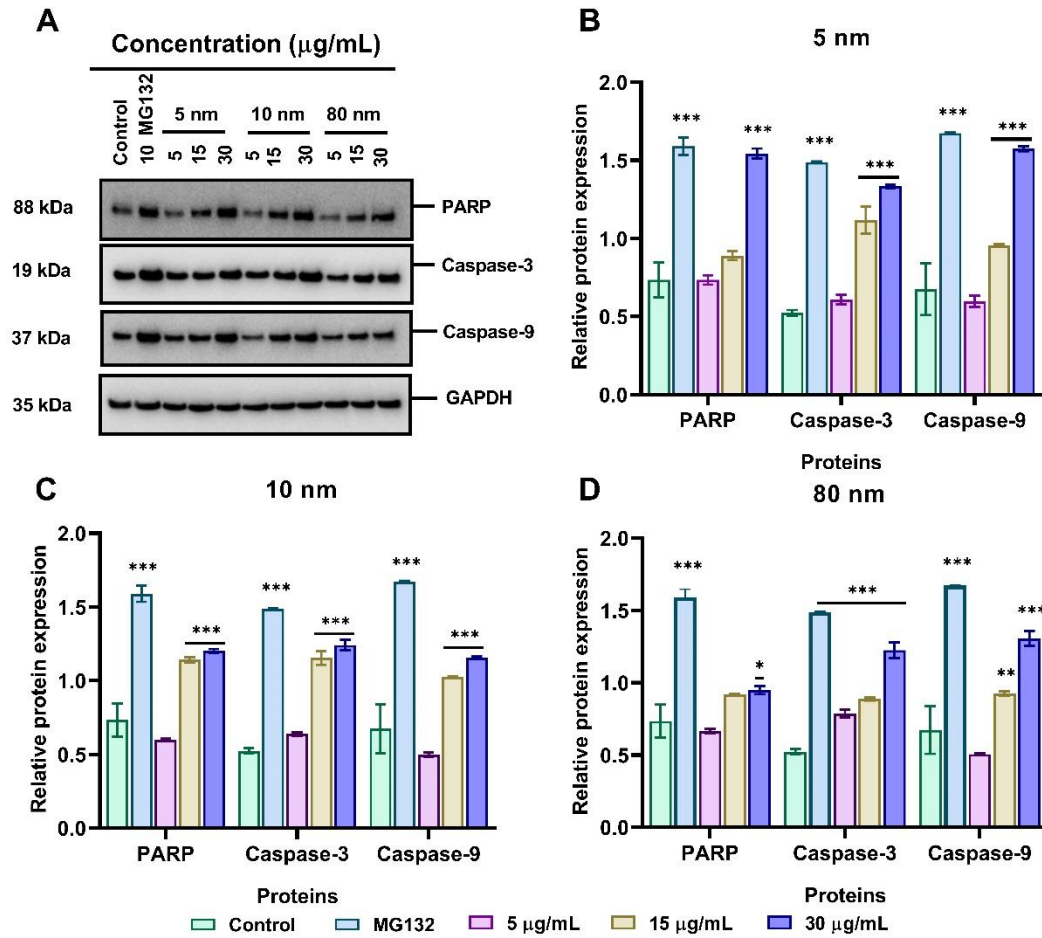


Figure 3.8. AuNPs induced apoptosis in A549 cells through a mitochondrial signalling pathway.

(A) The expression of apoptosis-related protein levels detected by Western blot after A549 cells were incubated with 5, 15, and 30 $\mu\text{g/mL}$ of different sizes of AuNPs for 24 h. MG132 (10 $\mu\text{g/mL}$) was used as a positive control. The relative expression signal intensity of apoptosis-related proteins was semi-quantified by ImageJ software for (B) 5 nm, (C) 10 nm, and (D) 80 nm AuNPs. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show a statistical difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) when compared with the control.

3.3 Discussion

AuNPs are extensively used in biomedicine because of the relative ease of controlling their physicochemical properties, such as size and shape, and their apparent biocompatibility compared with other metallic NPs [373, 374]. However, it has also been proposed that increasing exposure to AuNPs through inhalation and ingestion can potentially cause adverse effects on human health even at a low dose [375]. In this study, the cytotoxicity of different sizes of AuNPs and their effects on the UPS of A549 cells was investigated. Initially, the hydrodynamic size of all the AuNPs was measured and was found to be slightly lower at 0 h compared to 24 h in both UPW and CCM. In addition, the size distribution of the particles was greater in CCM compared to UPW. This is because the characteristics of NPs change in biological media in the presence of growth-stimulating factors such as vitamins, amino acids, and supplemented FBS. These growth stimulants could lead to the formation of a protein corona [376]. Moreover, all the AuNPs sizes exhibited negative surface charges in UPW and CCM, with a noticeable increase at 24 h, demonstrating that certain serum proteins in the media could mask the surface charge of the AuNPs [377].

The biological effects of AuNPs on A549 cells was the studied. It was found that AuNPs induced cytotoxicity to A549 cells in an increasing order of their sizes ($5 > 10 > 80$ nm), with the smallest AuNPs (5 nm) causing the most cytotoxicity. In addition, when cells were exposed to a high concentration of 40 $\mu\text{g/mL}$ for 24 and 48 h, the cell viability significantly decreased while the LDH release increased, indicating that cytotoxicity occurs at high AuNPs concentrations with all AuNPs sizes. In addition, these results demonstrate that the cytotoxicity of AuNPs *in vitro* depends not only on their sizes but also on their concentrations and incubation times, which is similar to what was

previously reported [79, 378]. Moreover, it was observed that AuNPs affected oxidative phosphorylation in the mitochondria and caused the generation of intracellular ROS in A549 cells. The generation of intracellular ROS upon exposure to AuNPs can result in oxidative damage to mitochondrial proteins, redox imbalance, and hindrance in the ability of the cells to carry out metabolic activities. This can consequently lead to pathological conditions [379, 380]. It was observed that ROS generation was dependent on the concentration and size of AuNPs. Surprisingly, 80 nm AuNPs were found to induce greater ROS generation than 10 nm, even though they showed the least cytotoxicity in the MTT and LDH assays. Using ICP-MS, flow cytometry, and TEM, the cellular uptake of AuNPs was studied after 24 h incubation. It was observed that A549 cells uptake 80 nm AuNPs to a greater extent (by mass), especially at high concentrations, compared to 10 and 5 nm, indicating that the uptake of NPs increased proportionally with the increase in their size. Interestingly, this shows that the cytotoxicity of AuNPs does not correlate with their cellular uptake. The degree of uptake of AuNPs likely depends on other factors such as the diffusion of the particle, the density of the particles, and interaction with the cellular membrane [100, 381, 382]. The cellular uptake results are in agreement with those of Chithrani et al., [73] who compared the uptake of different sizes of AuNPs in HeLa cells and found 50 nm to be the ideal AuNP size for their cellular internalisation. Furthermore, the TEM imaging of A549 cells showed all NPs were localised in vesicles, as evidenced by endosome formation. To gain further insight into the different mechanisms of AuNP internalisation, A549 cells were pre-treated with different pharmacological inhibitors for 1 h, followed by 30 µg/mL of different sizes of AuNPs (5, 10, and 80 nm) for 3 h. It was previously suggested that A549 cells can internalise AuNPs through pinocytosis

[49, 120]. Pinocytosis is an ideal internalisation mechanism for endothelial cells [350]. Accordingly, a particle size between 1–200 nm is expected to be internalised through either clathrin- or caveolae-mediated endocytosis [68]. In the current study, it was found that the internalisation of AuNPs gradually decreased when pre-incubated with clathrin- or caveolae-mediated inhibitors. This suggests that the uptake of the AuNPs is via clathrin- and caveolae-dependent pathways.

Several cytotoxicity studies have suggested that ROS generation could be a prominent mechanism of AuNP toxicity [383, 384]. The possible mechanisms of cell death, especially that the activation of apoptosis and necrosis was studied following treatment with AuNPs which can be related to their cellular uptake and ROS generation as reported by others [33, 385]. As shown in flow cytometry results using the double staining of Annexin V/propidium iodide, all the AuNPs sizes induced both apoptosis and necrosis in a dose-dependent manner. The study suggests that the cytotoxicity of AuNPs in A549 cells may involve different molecular mechanisms and aligns with the work of Xia et al., [83] who demonstrated that 5 nm AuNPs induced both apoptosis and necrosis through the production of ROS.

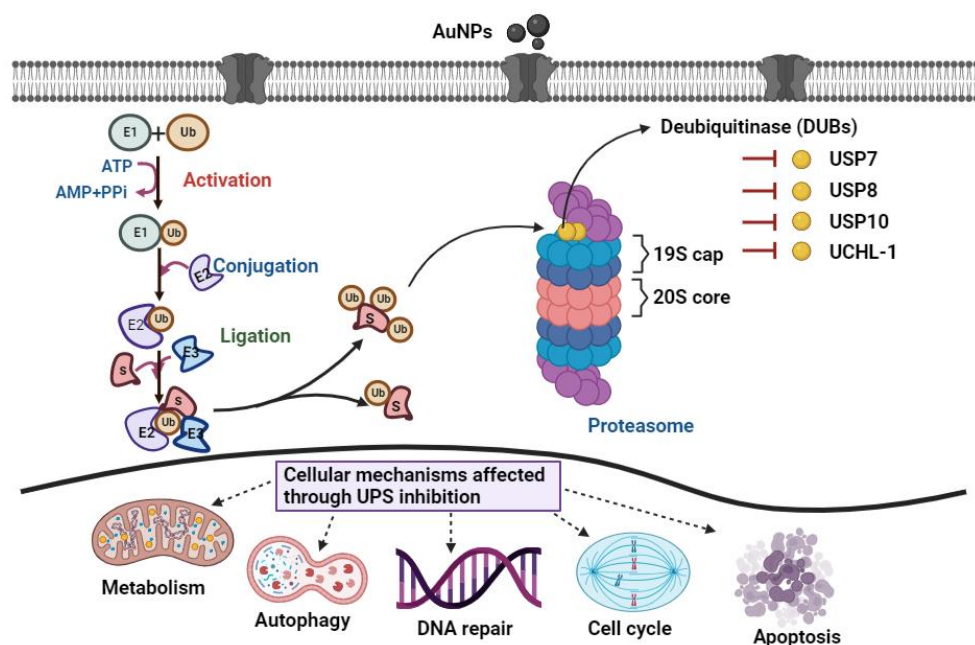
The UPS plays a critical role in protein degradation, including misfolded proteins, intracellular proteins involved in cell cycle arrest, overexpressed proteins, and apoptosis-related proteins [386]. Dysregulation of UPS has been reported to be associated with the pathogenesis and progression of various tumour cells, therefore inhibition of UPS activity might be an important target in cancer treatment [229, 365, 387]. Although the inhibitors of proteasome such as bortezomib, carfilzomib, and MG 132 have been identified and proven to suppress the proliferation of tumour cells via proteasome inhibition [251, 366, 388, 389], the lack of specificity and solubility of these

compounds prompts an urgent call for other effective proteasome inhibitors. The effectiveness of different sizes of AuNPs on proteasome inhibition was tested, with a specific focus on 19S proteasome DUB activity such as USP7, USP8, USP10, and UCHL-1. The western blotting analysis confirmed that 30 µg/mL of all sizes of AuNPs could downregulate the expression of these enzymes in the same way as the 10 µg/mL positive control (MG 132). This is the first study to show that AuNPs can affect the UPS of A549 cells. Others have shown that TiO₂ NPs downregulated the expression of UCHL-1 in rat PC12 cells [390]. The study further investigated if the inhibition of 19s proteasome DUBs could lead to the induction of apoptosis since tumour cells use these proteins in the degradation of tumour suppressors [391, 392]. Interestingly, it was found that AuNPs treatment in A549 cells upregulates the levels of PARP, caspase-3, and caspase-9 in a dose-dependent manner starting at 15 µg/mL of NPs. This agrees with previous findings which demonstrated that AuNPs inhibited the proliferation of cells through the induction of apoptosis [33, 159, 393]. For instance, the study agrees with Jawaid et al., [182] who confirmed the size-dependent apoptotic induction of different sizes of AuNPs (2, 40, and 80 nm) through caspase-3 upregulation.

3.4 Conclusion

In conclusion, the present study was undertaken to investigate the effects of different sizes of AuNPs on the UPS in A549 cells. The exposure of A549 cells to different sizes of AuNPs resulted in a decrease in percentage cell viability, an increase in LDH release, induction of apoptosis, and intracellular ROS generation that may be involved in cellular oxidative stress. A549 cells' toxicity following treatment with AuNPs was observed to be highly dependent on their size and concentration. Furthermore, exposure to AuNPs downregulates the expression

of USP enzymes such as USP7, USP8, USP10, and UCHL-1 and upregulates the expression of caspase-3, caspase-9, and PARP. Thus, this study sheds light on the mechanisms of AuNP cytotoxicity on lung carcinoma cell lines, as well as highlights their inhibitory effects on the UPS.



Scheme 3.1. UPS diagram. Ubiquitin-activating enzymes (E1) activate the first step of the process which forms a thioester bond in the presence of a higher energy compound (ATP). The activated ubiquitin is then transferred to the ubiquitin conjugation enzyme (E2) via a transthioesterification reaction. Ubiquitin ligases (E3) provide substrate specificity for the reaction and catalyse the covalent binding of ubiquitin to the target substrate through an isopeptide bond. The polyubiquitinated substrate moves to the proteasome for degradation. Finally, DUBs associated with the 19S proteasome remove conjugated ubiquitin from the substrate prior to the degradation. The scheme was created with BioRender.com

Chapter 4: Functionalisation of Gold Nanoparticles (AuNPs) Plays a Pivotal Role in the Suppression of A549 Proliferation

Abstract

This study investigates the cytotoxicity effects of 5 nm spherical AuNPs functionalised with tannic acid, citrate, and PVP in A549 cells. It was found that functionalised AuNPs reduce the percentage of cell viability in a concentration- and time-dependent manner as measured by the mLDH and MTT assays regardless of the functionalisation. An increased generation of intracellular ROS and depletion of GSH/GSSG ratio was observed with the highest AuNPs concentration of 10 µg/mL. The expression of deubiquitinating enzymes (DUBs) such as UPS7, USP8, and USP10 proteins were slightly inhibited when treated with concentrations above 2.5 µg/mL in a time-dependent fashion. Moreover, functionalised AuNPs showed an inhibitory effect on P13K/AKT/mTOR and Wnt signalling, and this could further trigger mitochondrial related-apoptosis by the upregulation of caspase-3, caspase-9, and PARP in A549 cells. The study warrants future work to investigate the effect of functionalised AuNPs on UPS in different cells type both *in vitro* and *in vivo*.

4.1 Introduction

It is known that functionalised AuNPs are widely used in cancer research, photothermal therapy [394] bioimaging, and drug delivery due to their low cytotoxicity compared to other NMs [375, 395]. However, prolonged exposure to functionalised AuNPs could result in adverse effects [396]. Oxidative stress imbalance, membrane damage, genotoxicity, apoptosis, necrosis, and activation of signalling pathways such as AKT, WNT, MAPK, and JNK were described as possible mechanisms of toxicity associated with the exposure to

functionalised AuNPs [203, 349, 397]. In addition, the importance of functionalisation in the toxicity of AuNPs have been demonstrated in several studies [384, 398]. For instance, Zhao et al., [399] found that AuNPs functionalised with PEG suppressed the proliferation of cells through ROS generation and reduction in mitochondrial activity [399]. In A549 cells, AuNPs functionalised with citrate showed higher toxicity in comparison to polyethylene imine functionalised AuNPs [400]. In another study, the role of functionalisation on the genotoxicity and cytotoxicity has been investigated using carboxylate, ammonium, and PEG in BEAS-2B cells and revealed that AuNPs functionalised with ammonium were more genotoxic and cytotoxic compared to AuNPs functionalised with carboxylate and PEG [401]. However, none of the previous studies investigated the effects of different functionalised AuNPs on DUBs. The recent literature is also currently biased towards larger functionalised AuNPs, which are easier to study and characterise and there is currently a lack of reliable data on the toxicity of smaller (5 nm) functionalised AuNPs on DUBs. In this study, the effects of tannic acid, citrate and PVP functionalised 5 nm AuNPs on DUBs particularly focussing on USPs and UCHL-1 in A549 cells were investigated. The study further explored if the inhibition of these DUBs could lead to the downregulation of AKT, and Wnt relative proteins and activation of apoptosis. The expression levels of DUBs, AKT and Wnt signalling proteins in normal (MRC-5) and cancerous cells (A549) without AuNPs were also evaluated.

4.2 Results

4.2.1 Physicochemical Characterisation of Different Functionalised AuNPs

It is crucial to study the physicochemical properties of the NPs in order to understand their interaction of different surface functionalised AuNPs with the biological milieu. Different surface functionalised AuNPs were first analysed using different characterisation techniques

such as transmission electron microscopy (TEM), dynamic light scattering (DLS), and ultra-violet spectroscopy (UV-Vis). **Figure 4.1** shows the size distribution, surface morphology, aggregation, and particle dispersion of each of the differently surface functionalised AuNPs dispersed in UPW. TEM images of all AuNPs NPs showed monodispersed spherical morphology with different average core size distributions closer to the size distribution mentioned by the manufacturer. Tannic acid, citrate, and PVP functionalised AuNPs showed around 6.3 ± 3.02 , 5.5 ± 0.34 , and 5.8 ± 1.02 nm respectively as calculated using ImageJ from the TEM images (**Figure 4.1A**). The hydrodynamic diameter and polydispersity index in ultrapure water (UPW), Phosphate buffer saline (PBS), free culture media (FCM), and complete culture media (CCM) supplemented with 10% fetal bovine serum were measured using DLS at a temperature of 25 °C. DLS measurements revealed that the hydrodynamic diameter of 5 nm tannic acid functionalised AuNPs were very close in UPW, PBS, and FCM but higher in CCM as shown in **Figure 4.1B**. Similar observations were seen with the 5 nm citrate functionalised AuNPs in which the hydrodynamic diameter was similar in PBS and FCM though a little smaller in UPW and higher in CCM as observed with the tannic acid functionalised AuNPs (**Figure 4.1B**). Interestingly, the 5 nm PVP functionalised AuNPs hydrodynamic diameter was larger in PBS, FCM, and CCM compared to UPW as highlighted in **Figure 4.1B**. Overall, all three surfaces functionalised AuNPs showed a slight increase in size in PBS, FCM, and CCM compared to UPW as shown in **Figure 4.1B**. Noticeably, DLS demonstrated that all the particles have PDI (polydispersity index) of less than 0.6 in UPW, PBS, FCM, and CCM as shown in **Figure 4.1B**.

Moreover, all AuNPs showed a negative surface charge (zeta potential) in the different media including 5nm tannic acid, citrated, and PVP functionalised AuNPs as shown in **Figure 4.1C**. In addition, the absorption spectra of the AuNPs were characterized by UV–Vis in the 300–800

nm range. At this wavelength range, AuNPs showed a distinct optical feature commonly referred to as localised surface plasmon resonance (LSPR) and the position and intensity of the LSPR band depend on the size and surface morphology of particles [402-404]. For the 5 nm citrate, PVP, or tannic acid functionalised AuNPs, the absorption peak was observed around 517-518 nm which indicates the formation of small-size AuNPs (**Figure 4.1 in Supplementary Materials**).

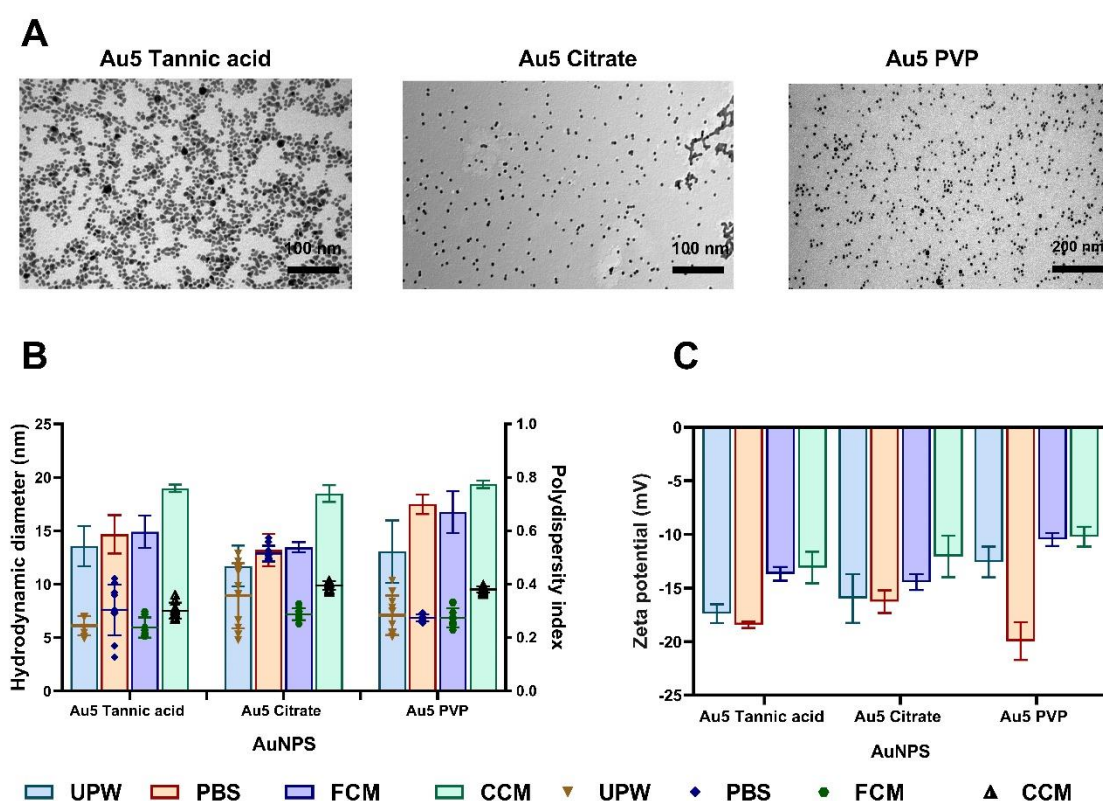


Figure 4.1. Physicochemical characterisation of different surface functionalised AuNPs. (**A**) Transmission electron images of 5 nm AuNPs dispersed in UPW. (**B**) Hydrodynamic diameter and polydispersity index of 5nm AuNPs dispersed in UPW, PBS, FCM and CCM with 10% FBS. (**C**) Zeta potentials of AuNPs dispersed in UPW, PBS, FCM, and CCM. Plotted graphs represent the means $\pm$ standard deviation (S.D) of ten different measurements. (Scale bar: 100 nm were

used for tannic acid and citrate functionalised AuNPs. 200 nm was used for PVP functionalised AuNPs).

4.2.2 Cytotoxicity of A549 Cells Exposed to Different Functionalised AuNPs

A549 cellular response to 5 nm tannic acid, citrate, and PVP functionalised AuNPs at a range of concentrations (1.25- 35 $\mu\text{g/mL}$) was evaluated using the modified lactate dehydrogenase (mLDH) and MTT assay for 24 and 48 h. As seen in **Figure 4.2A**, there is a clear decrease in cell viability of A549 cells in a concentration-dependent manner with all types of AuNPs at 24 h. While AuNPs functionalised with tannic acid at concentrations of 1.25-10 $\mu\text{g/mL}$ did not cause any significant reduction in A549 cell viability after 24 h, a concentration of 35 $\mu\text{g/mL}$ showed a higher reduction in cell viability (64.82%). On other hand, 5 nm citrate functionalised AuNPs started to show a reduction in percentage cell viability at 2.5 $\mu\text{g/mL}$ of AuNPs with the highest concentration showing 63% of cell viability. Likewise, treatment with 35 $\mu\text{g/mL}$ of PVP functionalised AuNPs for 24 h showed a reduction in cell viability similar to that seen with the same concentration of tannic acid functionalised AuNPs. Further incubation for 48 h revealed a similar dose-dependent effects on cell viability for all the three functionalised AuNPs. In comparison to 24 h, the result showed a decrease in percentage cell viability after treatment with 1.25 $\mu\text{g/mL}$ for all three AuNPs (**Figure 4.2B**). As expected, the higher concentration (35 $\mu\text{g/mL}$) of all AuNPs has shown more cytotoxicity to A549 cells after 48 h incubation.

The study then focussed on whether the three functionalised 5 nm AuNPs might affect metabolic activity in A549 cells. As shown in **Figure 4.2C-DA-B** A549 cells cytotoxicity was not only dependent on AuNPs concentrations but also on the incubation time. Moreover, the higher concentration of all AuNPs decreased A549 metabolic activity at all time-points. Overall, for the same concentration, 5nm AuNPs PVP functionalised has caused a significant

($p < 0.05$) cytotoxicity compared to citrate and tannic acid functionalised AuNPs. In order to understand if the effects seen with the functionalised AuNPs are not due to the functionalisation alone, 2mM tannic acid, citrate, and PVP stabiliser were prepared in UPW which was equivalent to the concentration used in AuNPs. A549 cells were further exposed to these stabilising agents (tannic acid, citrate, and PVP) at the concentrations of 0.125-1 mM diluted in CCM which is equivalent to 1.25-10 $\mu\text{g/mL}$ for 24 h. At the end of the exposure, all three stabilising agents exhibited a dose-dependent cytotoxicity to A549 cells (**Figure S4.2 in Supplementary Materials**). With 0.125 mM, tannic acid, and citrate show no sign of cytotoxicity compared to PVP which decreased the percentage cell viability to $84.6 \pm 1.89\%$. When the concentrations of both citrate and PVP agents is doubled to 0.25 mM, the percentage cell viability was $87.83 \pm 5.0\%$ and $73.1 \pm 0.73\%$ respectively compared to tannic acid $100.75 \pm 1.65\%$. On the other hand, with 0.5 and 1 mM all the stabilising agents significantly ($p < 0.001$) reduced A549 cell viability with PVP having the most significant ($p < 0.05$) effects (**Figure S4.3 in Supplementary Materials**). This shows that there was a positive correlation between the stabilising agents used in the NPs and toxicity. Hence, for the rest of the experiments a concentration range of 1.25-10 $\mu\text{g/mL}$ for all the functionalised AuNPs were used.

Based on the mLDH and MTT assays, the inhibitory concentrations (IC_{50}) of tannic acid, citrate, and PVP-functionalised AuNPs (5 nm) were calculated from the linear regression curve using GraphPad prism after 24 and 48 h as shown in **Table 4.1**. IC_{50} represents an inhibitory concentration of AuNPs that decreases the percentage of cell viability to 50% after 24 or 48 h incubation. The IC_{50} values obtained by mLDH are lower than the values obtained by the MTT assay at all incubation periods which could indicate that the decrease in cell viability observed by AuNPs is greater than that observed with the metabolic activity. Furthermore, as surface

functionalised AuNPs are compared, it can be seen that both tannic acid- and citrate-functionalised 5 nm AuNPs demonstrated low IC₅₀ values in A549 cells compared to PVP – functionalised AuNPs.

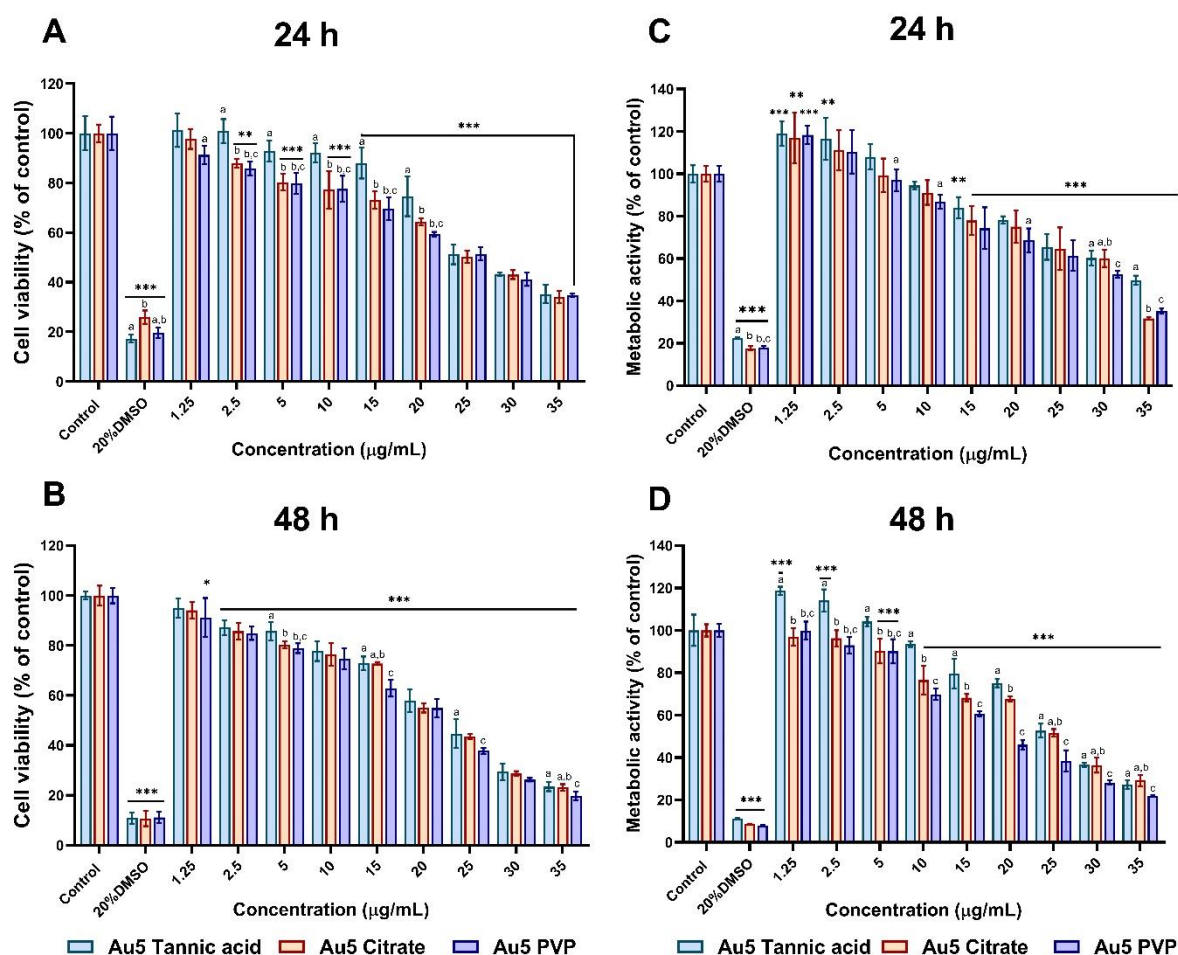


Figure 4.2. Percentage cell viability and metabolic activity of A549 cells treated with 1.25-35 µg/mL of 5 nm AuNPs coated with tannic acid, citrate, and PVP with 20% DMSO used as a positive control measured using mLDH and MTT assay. **(A)** Percentage of cell viability after 24 h **(B)** Percentage of cell viability after 48 h **(C)** Metabolic activity after 24 h **(D)** Metabolic activity after 48 h. Plotted graphs represent the means $\pm$ standard deviation (SD) of six independent experiments. Bars with an asterisk (*) show statistical differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) when compared with the control. Data with different letters above

the bars represent the statistically significant difference ($p < 0.05$) between the functionalised AuNPs.

Table 4.1: Calculated IC_{50} of 5 nm surface functionalised AuNPs in A549 cells

	IC_{50} Concentration ($\mu\text{g/mL}$)			
	mLDH		MTT	
	24 h	48 h	24 h	48 h
5 nm AuNPs				
Tannic acid	28.00 $\pm$ 0.83	23.33 $\pm$ 1.19	34.58 $\pm$ 1.45	25.84 $\pm$ 0.755
Citrate	27.23 $\pm$ 0.42	22.98 $\pm$ 0.61	30.43 $\pm$ 2.23	23.96 $\pm$ 0.436
PVP	26.27 $\pm$ 0.65	20.96 $\pm$ 1.50	29.00 $\pm$ 1.78	17.92 $\pm$ 0.787

Note: The inhibitory concentration of 5 nm AuNPs that decrease 50% of A549 cells proliferation after 24 and 48 h measured by mLDH and MTT assay.

4.2.3 Cell Recovery Upon Exposure to Different Functionalised AuNPs

The A549 cells' capacity to recover their proliferative state following AuNPs exposure was measured using mLDH assay at a concentrations range of 1.25-10 $\mu\text{g/mL}$ which were selected based on the previous cell viability assay results. In **Figure 4.3A**, the resumption of cells proliferation capacity was observed after cells were treated with 1.25 $\mu\text{g/mL}$ of 5 nm tannic acid functionalised AuNPs for 2 days. When incubated with 2.5 $\mu\text{g/mL}$, the cells recovered only at day four. In addition, treatment with 5 and 10 $\mu\text{g/mL}$ did not allow for cell recovery from tannic acid functionalised AuNPs toxicity. In the case of 5 nm citrate functionalised AuNPs, the cells also recovered at days 2 and 3. Surprisingly, the cells were able to recover when incubated with 2.5 $\mu\text{g/mL}$ at day 4 (**Figure 4.3B**). With the 5 nm PVP functionalised AuNPs, the cells were not able to recover to their proliferation state at any concentration on

days 2 and 3. However, with 1.25 and 2.5 $\mu\text{g/mL}$, the cells slightly recovered at day 4 compared with untreated cells (**Figure 4.3C**).

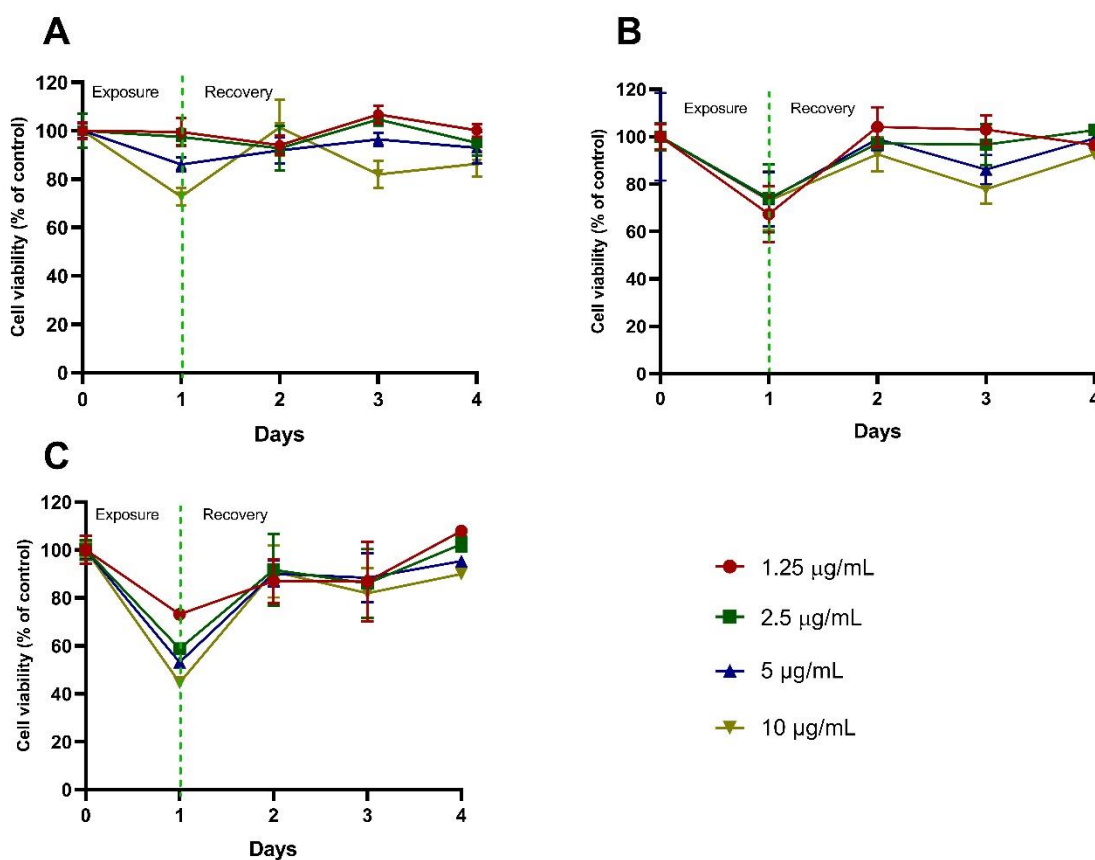


Figure 4.3. A549 cells recovery following exposure to functionalised 5 nm AuNPs. The cells were treated with AuNPs (1.25-10 $\mu\text{g/mL}$) for 1 day then they were allowed to recover by changing their media in days 2, 3, and 4. The percentage cells viability was then measured using mLDH. **(A)** Cell recovery after exposure to 5 nm tannic acid – functionalised AuNPs **(B)** Cell recovery following exposure to 5 nm citrate functionalised AuNPs **(C)** Cell recovery after exposure to 5 nm PVP functionalised AuNPs. Plotted graphs represent the means $\pm$ standard deviation of three independent experiments.

4.2.4 Effects of Different Functionalised 5 nm AuNPs on Oxidative Stress

ROS generation can play a significant function in oxidative damage and apoptosis induction [74, 202, 383]. In order to explore the effects of AuNPs in the induction of programmed cell death, the intracellular level of ROS was assessed in A549 cells after exposure to the three functionalised AuNPs for 24 and 48 h at a concentrations range of 1.25-10 $\mu\text{g/mL}$ with 50 μM used as a positive control for 1 h. As shown in **Figure 4.4A-B**, no difference was observed when A549 cells were treated at the lowest concentration (1.25 $\mu\text{g/mL}$) with all AuNPs after 24 and 48 h when compared to the control. As the AuNPs concentration increased, the intracellular level of ROS evidently increased in a time-dependent manner in comparison to the control. Additionally, 5 nm PVP functionalised AuNPs showed a significant ($p < 0.05$) increase in ROS compared to their citrate and tannic acid counterparts.

Furthermore, the effects of functionalised 5 nm AuNPs were examined on intracellular glutathione levels in A549 cells. Glutathione plays a crucial role in maintaining redox balance in cells, cell proliferation, apoptosis, and signal transduction [405, 406]. A dose-dependent depletion of the GSH/GSSG ratio was observed after 24 h as shown in **Figure 4.4C**. In addition, 10 $\mu\text{g/mL}$ of all the AuNPs showed a reduction in the GSH/GSSG ratio to 80% when incubated for 24 h (**Figure 4.4C**). Similarly, when cells were exposed to 2.5, 5, and 10 $\mu\text{g/mL}$ of tannic acid functionalised AuNPs for 48 h, the GSH/GSSG ratio was reduced to 82%, 75% 69% respectively (**Figure 4.4C**). A similar extent of reduction in the GSH/GSSG ratio was observed with citrate functionalised AuNPs after 48 h as shown in **Figure 4.4D**. However, exposure of A549 cells to 5 nm PVP functionalised AuNPs for 48 h have shown greater depletion of GSH/GSSG ratio to 78%, 70%, and 65% at a concentration of 2.5, 5, and 10 $\mu\text{g/mL}$ respectively as shown in **Figure 4.4D**.

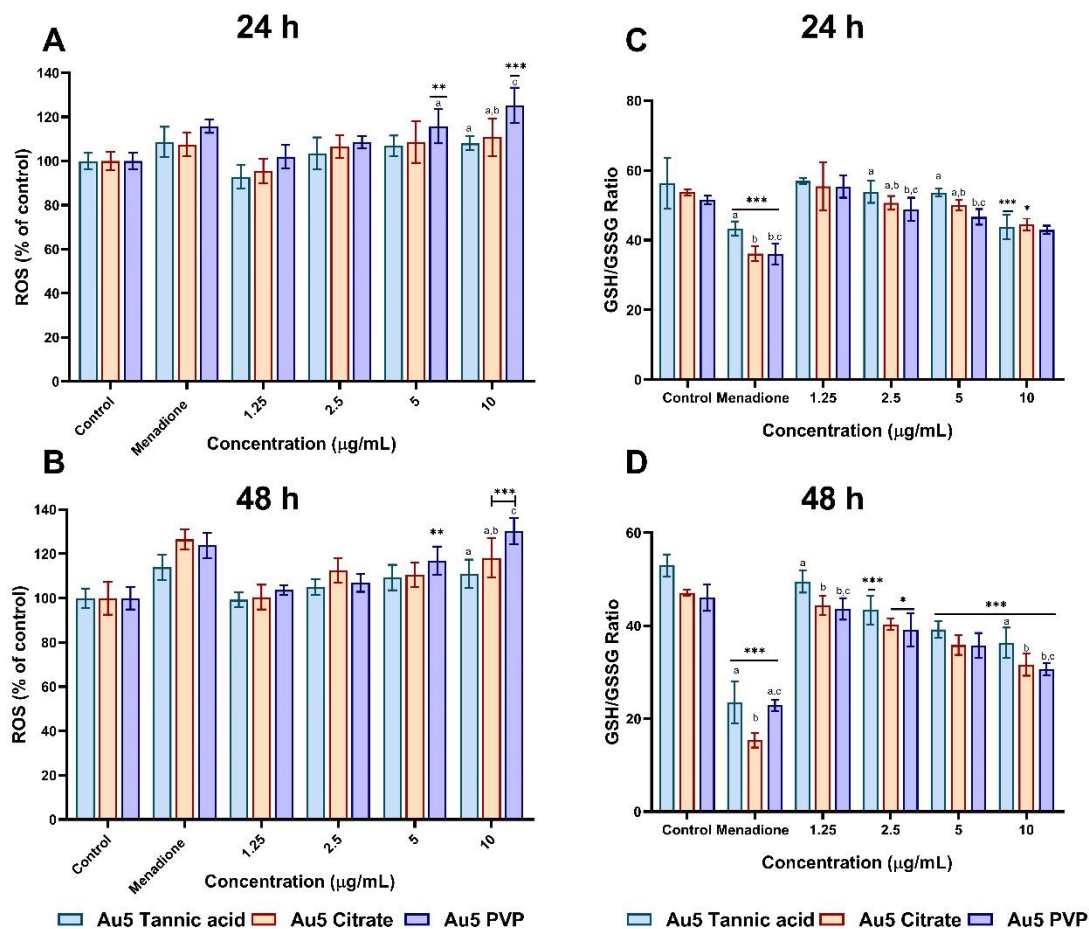


Figure 4.4. Oxidative stress induction in A549 cells after exposure to 5 nm functionalised AuNPs at a concentration range of 1.25-10 µg/mL. Intracellular ROS induced after (A) 24 h, (B) 48 h. GSH/GSSG ratio level after (C) 24 h (D) 48 h. 50 µM of menadione was used as a positive control for 1 h. Plotted graphs represent the means $\pm$ standard deviation of five independent experiments. Bars with an asterisk (*) show statistical difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) when compared with control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the functionalised AuNPs.

4.2.5 The Effect of Functionalised AuNPs on Mitochondrial Membrane Potential (MMP)

To assess the effect of surface functionalised AuNPs on A549 MMPI, the MMP depletion was measured using HCS Mitochondrial Health assay [326]. As shown in **Figure 4.5** a slight decrease in MMP was observed in A549 cells after 24 h incubation with all the AuNPs concentration range of 1.25-10 $\mu\text{g/mL}$. At the highest concentration (10 $\mu\text{g/mL}$) PVP functionalised AuNPs caused a slight decrease (87%) in MMP, followed by tannic acid-(92%) and citrate-functionalised AuNPs (93%). Incubation of A549 cells with the positive control (H_2O_2) in another hand showed much greater decrease in MMP compared to the control and AuNPs treated. Similar observations were confirmed with fluorescence intensity images at the highest concentration of PVP functionalised AuNPs compared to untreated (**Figure S4.4 in Supplementary Materials**).

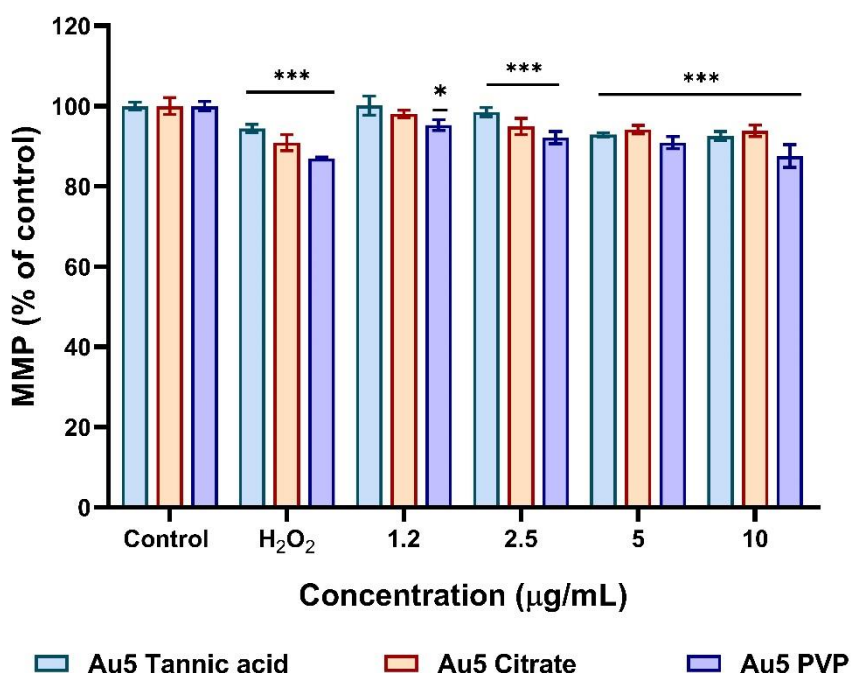


Figure 4.5. A549 mitochondrial membrane potential (MMP) after exposure to 5 nm tannic acid, citrate, and PVP-functionalised AuNPs at a concentration range of 1.25-10 $\mu\text{g/mL}$ for 24 h assayed using HCS Mitochondrial Health Kit. 100 μM of hydrogen peroxide (H_2O_2) was used

as a positive control for 1 h Plotted graphs represent the means $\pm$ SD of five independent experiments. Bars with an asterisk (*) show statistical difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) when compared with the control.

4.2.6 Apoptosis Induction by Different Functionalised AuNPs

Given that ROS generated by AuNPs can cause damage to membrane organelles which can consequently lead to the activation of apoptosis in A549 cells [136, 407], Herein, the correlation between the ROS generated, and apoptosis induction by AuNPs was investigated using Annexin V and PI assay. As shown in **Figure 4.6A**, 10 $\mu\text{g/mL}$ tannic acid functionalised AuNPs increase the percentage of apoptosis to $6.37 \pm 0.61\%$ which was significantly greater ($p < 0.001$) than control cell (2.34 ± 0.20). Citrate functionalised AuNPs were found to similarly induce apoptosis to $6.13 \pm 0.97\%$ with the highest concentration of 10 $\mu\text{g/mL}$ (**Figure 4.6B**). Interestingly at the same concentration, 5 nm PVP AuNPs showed the highest percentage of apoptotic cells ($13.39 \pm 1.71\%$) (**Figure 4.6C**). In addition, FlowJo scatters dot plots clearly highlight that the percentage of apoptotic cells in A549 cells incubated with 10 $\mu\text{g/mL}$ of PVP AuNPs is the highest compared to their tannic acid and citrate functionalised counterparts (**Figure 4.6D**). This could indicate that the PVP-AuNPs could suppress A549 cell proliferation through the induction of apoptosis.

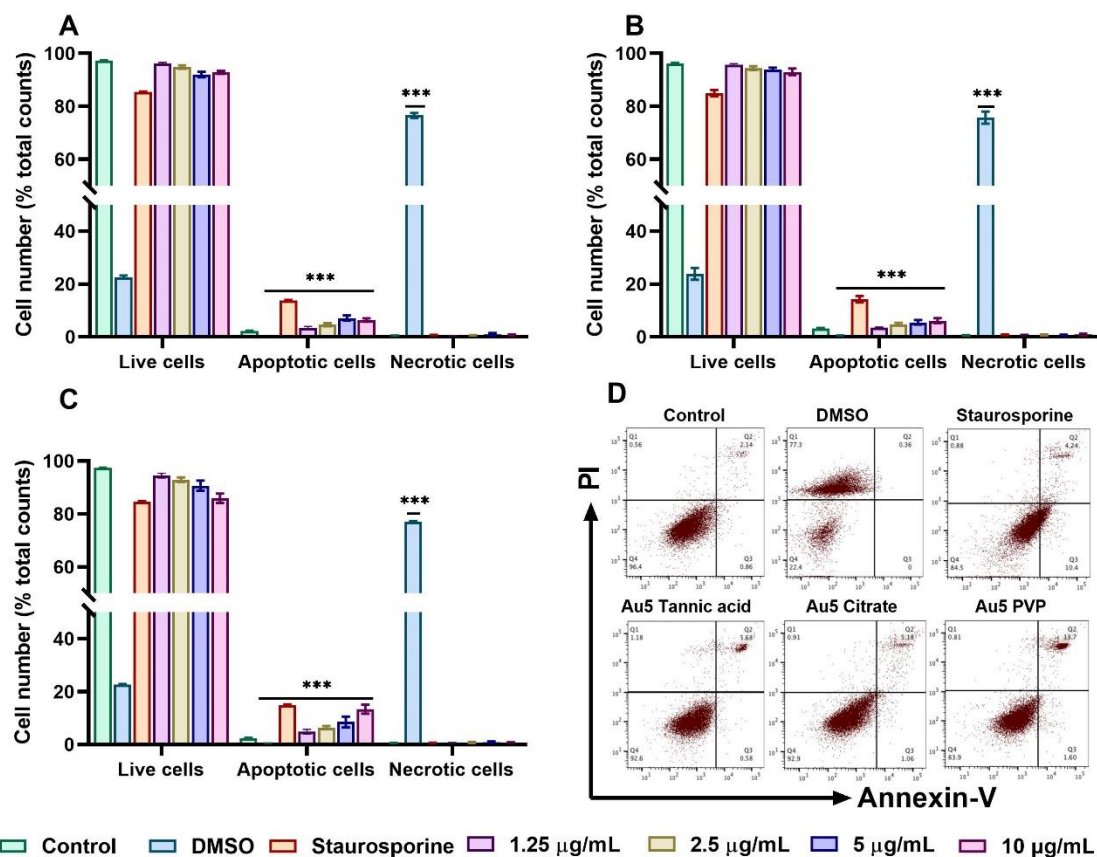


Figure 4.6. Flow cytometric analysis of A549 cells exposed to 1.25-10 $\mu\text{g/mL}$ of different functionalised 5 nm AuNPs for 24 h stained with Annexin V and PI. **(A)** A549 cells treated with tannic acid functionalised AuNPs **(B)** A549 cells treated with citrate functionalised AuNPs **(C)** A549 cells treated with PVP functionalised AuNPs **(D)** Dot plots analysis of apoptosis incubated with 10 $\mu\text{g/mL}$ of tannic acid, citrate, and PVP functionalised AuNPs. Treatment with 1 μM staurosporine and 10% DMSO for 6 h was used as a positive control for apoptosis and necrosis. Plotted graphs represent the means $\pm$ standard deviation of three independent experiments. Bars with an asterisk (*) show statistical difference (***) $p < 0.001$ when compared with the control.

4.2.7 Determining the Expression Levels of DUBs, P13K/AKT/mTOR and Wnt Signalling Related Proteins in Normal and Cancerous Lung Cell Lines Without AuNPs

In order to explore the potential effect(s) of the different functionalised AuNPs on different intracellular protein levels, the expression of DUBs, P13K/AKT/mTOR, and Wnt related proteins were initially assessed in A549 cells and normal lung fibroblast (MRC-5) to determine their baselines without AuNPs. Western blotting was used to determine those levels and images were semi-quantified using ImageJ software. Interestingly, all the investigated proteins were highly upregulated in the cancerous A549 cells compared to normal MRC-5 cells as shown in **Figures 4.7A-C**. In order to easily compare between the western blots bands, the levels of proteins were semi-quantified using ImageJ. The latter highlighted that some proteins (USP7, USP8, USP10, UCHL-1, AKT, p-AKT, mTOR, β -Catenin, p- β -Catenin) were upregulated in A549 cells after 24 h which was statistically significant compared to MRC-5 cells (**Figures 4.7B-D**). However, the expression levels of apoptotic proteins (PARP, caspase-3, and caspase-9) were downregulated in A549 cells compared to normal lung fibroblast (MRC-5) as shown in (**Figures 4.7E-F**).

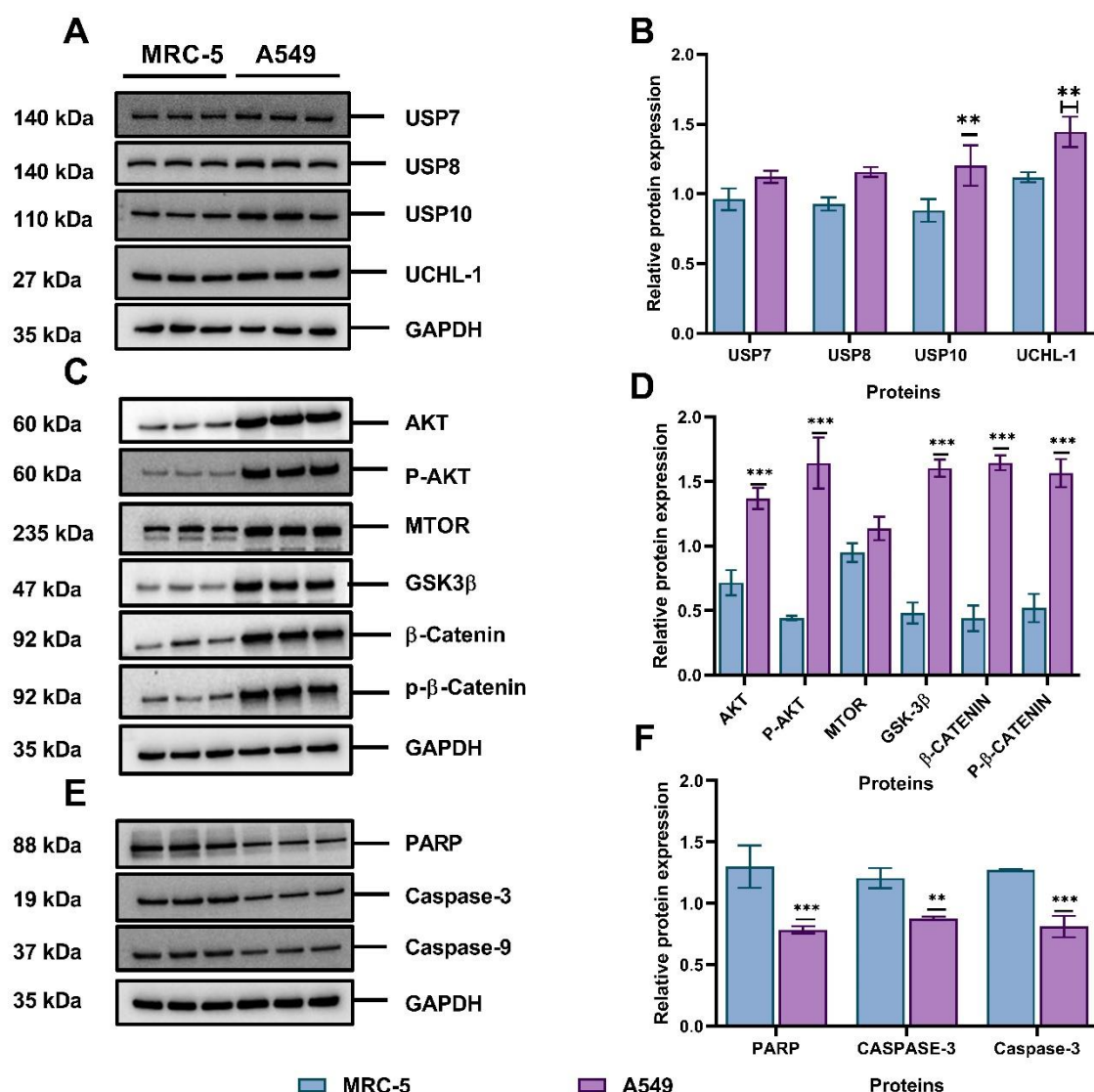


Figure 4.7. Baseline Proteins expression in normal MRC-5 and cancerous A549 cells detected by Western blot and semi-quantified using ImageJ. **(A)** and **(B)** DUBs proteins expression level, **(C)** and **(D)** P13K/AKT/mTOR and Wnt signalling pathway related proteins, **(E)** and **(F)** Apoptosis related proteins expression. Plotted graphs represent the mean $\pm$ SD (n=3). Bars with an asterisk (*) show statistical difference (**p < 0.01 and ***p < 0.001) when A549 cells compared to MRC-5.

4.2.8 Effects of Different Functionalised AuNPs on DUBs Expression in A549 Cell Lines

To investigate the effects of functionalised AuNPs on the ubiquitin-proteasome system (UPS), protein levels of deubiquitinating enzymes such as USP7, USP8, USP10, and UCHL-1 (involved in ubiquitin removal during the ubiquitination process) were investigated following treatment with the different surface functionalised AuNPs for 24 and 48 h by western blotting. As shown in **Figure 4.8A**, only 10 µg/mL of tannic acid functionalised AuNPs affected protein levels of USP7, USP8, and USP10 when incubated for 24 and 48 h. However, the level of UCHL-1 remained unchanged after 24 h but was followed by a slight decrease after incubation for 48 h. The semi-quantification of protein levels using ImageJ highlighted that the effects were more pronounced at 10 µg/mL as shown in **Figure 4.8B**. Similarly, citrate functionalised AuNPs slightly reduced the expression of USP7, USP8, and USP10 at 10 µg/mL but there was a significant change in the total protein level of UCHL-1 in comparison to the control ($p < 0.001$) (**Figure 4.8C-D**). Likewise, AuNPs functionalised with PVP slightly downregulated the levels of USP7, USP8, USP10, and UCHL1 in A549 cells at 24 and 48 h with 10 µg/mL (**Figure 4.8E-F**).

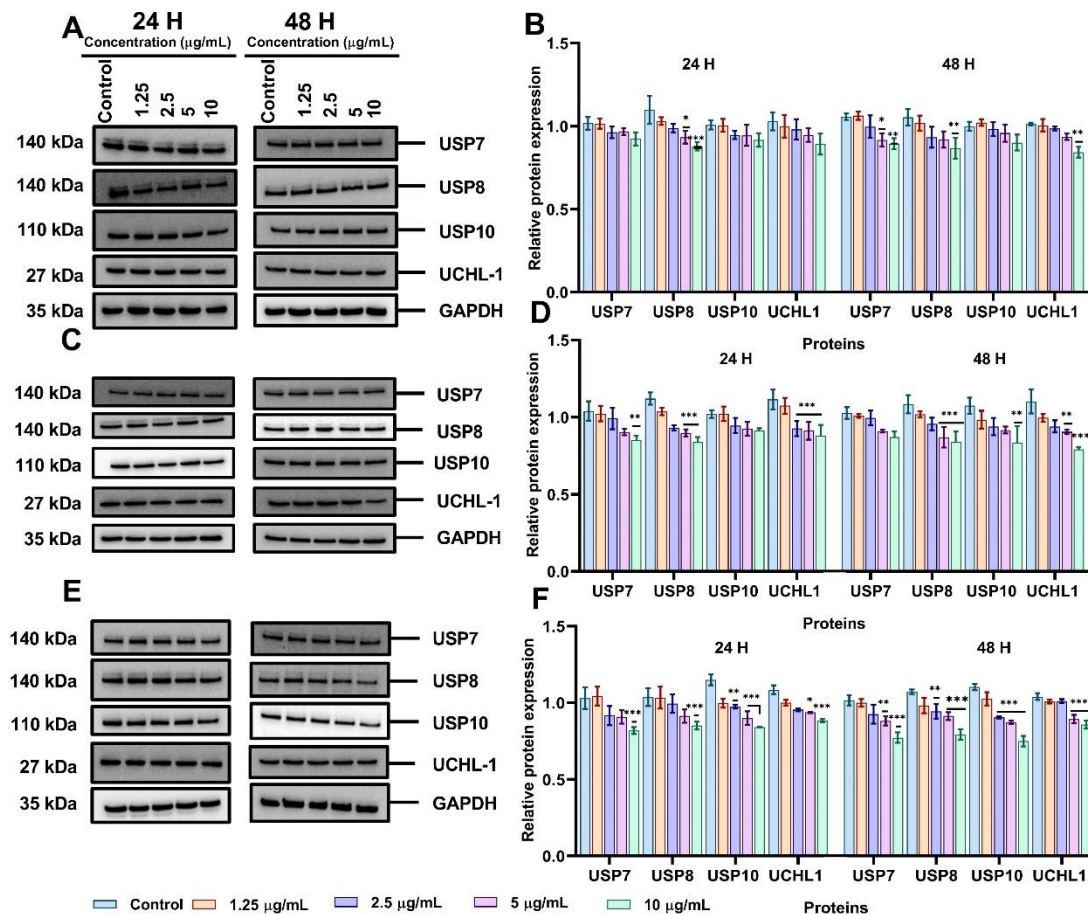


Figure 4.8. Surface functionalised AuNPs inhibit Deubiquitinating enzymes (DUBs) in A549 cells treated with a concentration of 1.25-10 µg/mL for 24 and 48 h as detected by Western blotting and semi-quantified using ImageJ. DUBs expression exposed to tannic acid functionalised AuNPs (**A**) and (**B**), citrate functionalised AuNPs (**C**) and (**D**), PVP functionalised AuNPs (**E**) and (**F**). Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) when compared with the control.

4.2.9 Effects of Surface Functionalised AuNPs on Proteins in the P13K/AKT/mTOR and Wnt Signaling Pathways in A549 Cell Lines

To further explore the effect of AuNPs on intracellular signaling pathways following the inhibition of DUBs, the expression of AKT, p-AKT, mTOR, GSK-3 β , β -Catenin and p- β -Catenin proteins were evaluated upon exposure to different functionalised AuNPs. As shown in **Figure 4.9A**, tannic acid functionalised AuNPs significantly affected the level of AKT, p-AKT, mTOR, β -Catenin, and p- β -Catenin in a dose- and time-dependent manner. However, GSK-3 β protein levels slightly increased at 24 h but decreased as the concentration increased following 48 h incubation as compared to the control. Relative protein expression as semi-quantified by ImageJ (**Figure 4.9B**) confirmed that protein levels of AKT, p-AKT, mTOR, β -catenin, and p- β -catenin in A549 cells were indeed significantly ($p < 0.05$, $p < 0.001$) downregulated with 10 $\mu\text{g/mL}$ at 48 h ($p < 0.001$). The decrease in the expression of p-AKT and p- β -catenin proteins might be due to downregulation of AKT and β -catenin proteins downregulation. Interestingly, similar downregulation of proteins was observed with both citrate (**Figure 4.9C- D**) and PVP functionalised AuNPs in A549 cells for 24 and 48 h (**Figure 4.9E- F**).

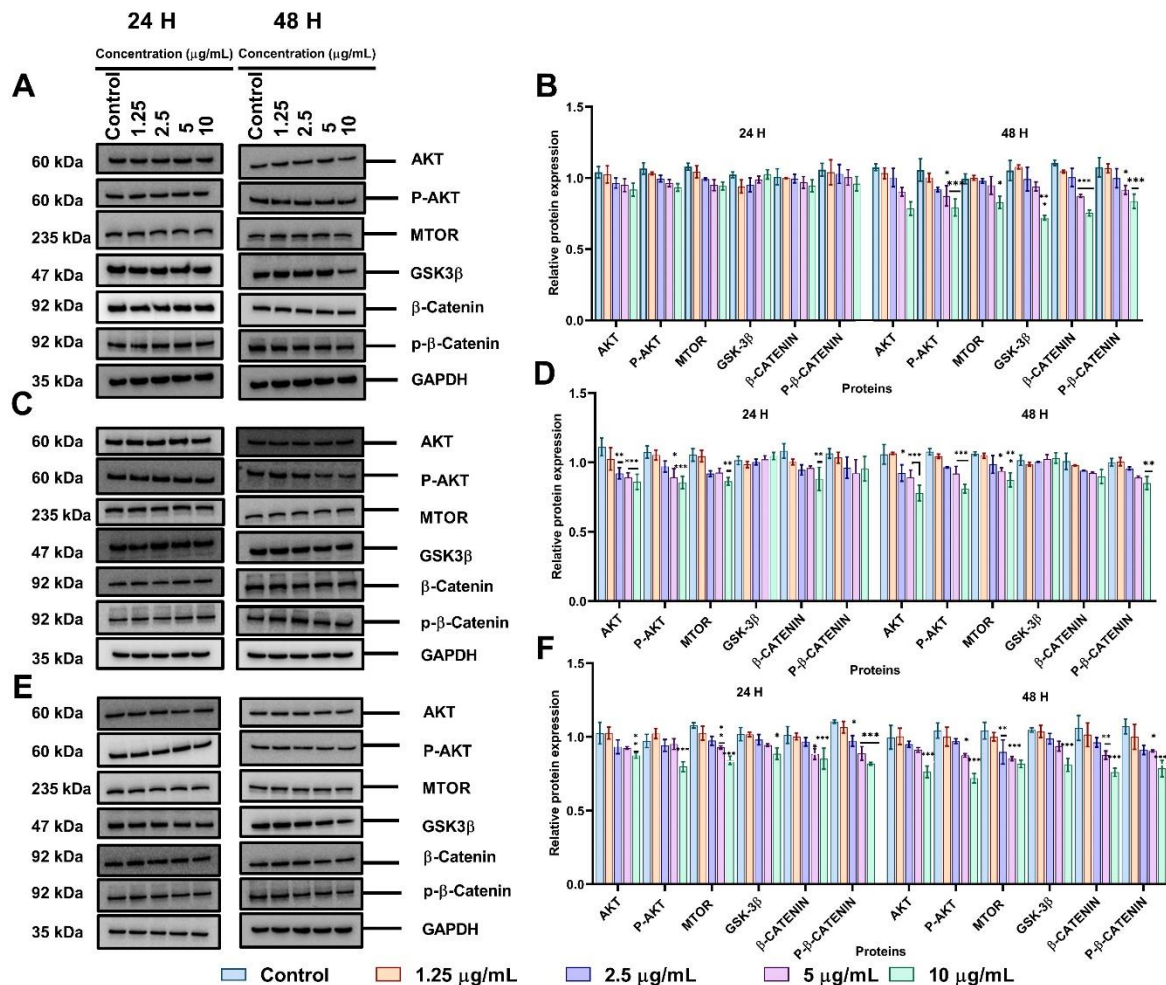


Figure 4.9. Surface functionalised AuNPs inhibit P13K/AKT/mTOR and Wnt related proteins in A549 cells treated with a concentration range of 1.25-10 $\mu\text{g/mL}$ for 24 and 48 h and analysed by Western blotting and semi-quantified using ImageJ. **(A)** and **(B)** P13K/AKT/mTOR and Wnt signalling proteins expression exposed to tannic acid functionalised AuNPs **(A-B)**, citrate functionalised AuNPs **(C-D)**, **(E-F)** PVP functionalised AuNPs. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) when compared with the control.

4.2.10 The Effect of Surface Functionalised AuNPs on the Expression of PARP, Caspase-3, and Caspase-9 in A549 Cell Lines

To determine whether the different functionalised AuNPs also affected some proteins which are crucial in apoptosis; the expression of PARP, caspase-3, and caspase-9 was assessed using western blotting. As shown in **Figure 4.10A-B**, treatment with tannic acid functionalised AuNPs upregulated the expression of PARP, caspase-3, and caspase-9 in a dose-dependent manner after 24 and 48 h. Likewise, the expression of PARP and Caspase-3 protein levels were also observed to be upregulated with citrate functionalised AuNPs after 24 and 48 h (**Figure 4.10D-C**). Interestingly, PVP functionalised AuNPs only affected caspase-9 protein expression at 10 µg/mL when compared to the untreated control while the expression levels of PARP, and caspase-3 showed a dose- and time-dependent increase after treatment (**Figure 4.10E-F**).

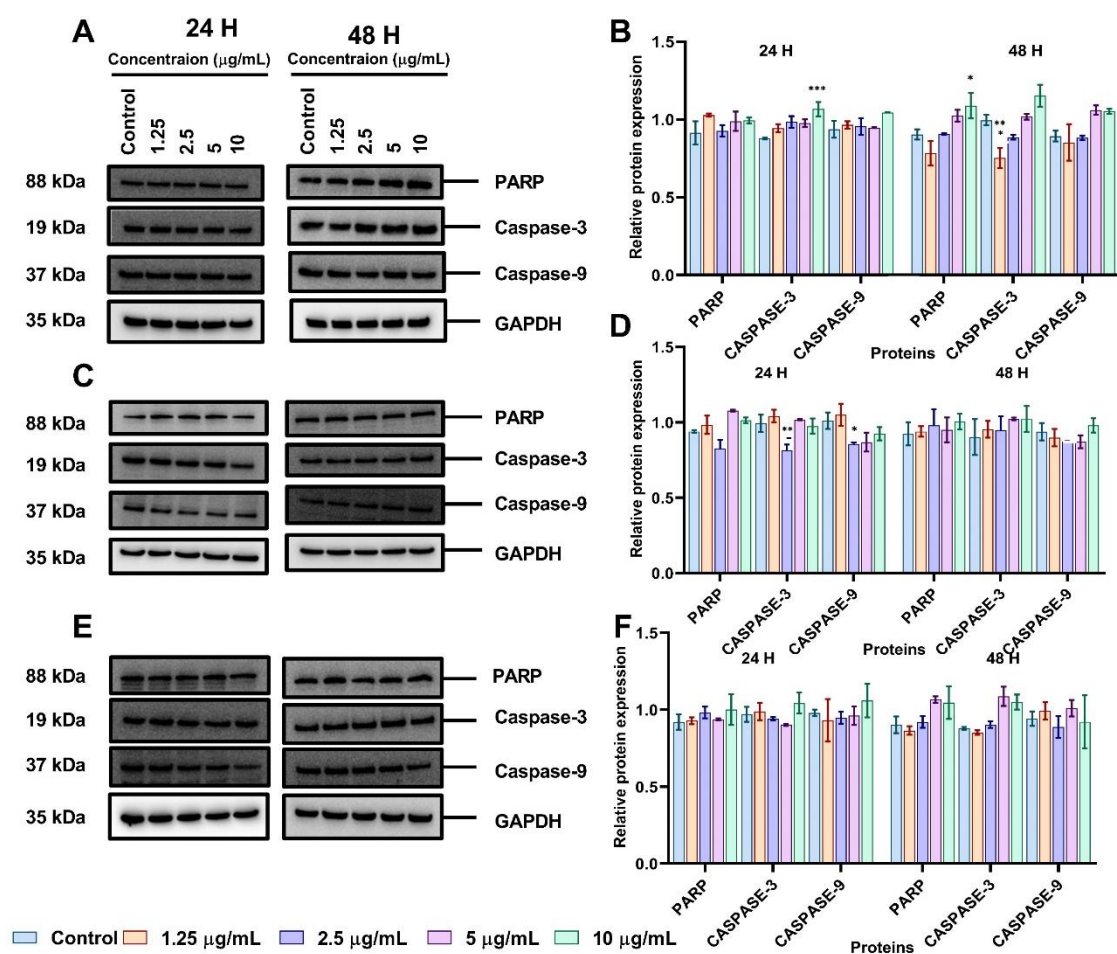


Figure 4.10. Surface functionalised AuNPs induced mitochondrial-related apoptosis in A549 cells treated with a concentrations of 1.25-10 μg/mL for 24 and 48 hs assessed by Western blotting and semi-quantified using ImageJ. PARP, caspase-3, caspase-9 Proteins expression after treatment with tannic acid functionalised AuNPs (**A-B**), citrate functionalised AuNPs (**C-D**), PVP functionalised AuNPs (**E-F**). Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) when compared with the control.

4.3 Discussion

AuNPs have received enormous attention in the field of nanotechnology due to their intrinsic properties which have shown great use in biomedical applications such as tumour imaging

and drug delivery [379, 408]. As their applications are becoming increasingly popular, toxicological effects and environmental hazards should also be considered. NPs can enter the body through different routes and become accumulated in the lungs, kidneys, and liver causing various toxic effects because of their different sizes and surface chemistry [409]. It is fundamental to determine the toxic response in lung cells because it is one of the major routes of NPs entry into human cells. A549 lung cells have been used as model cell to study the cytotoxicity of several NPs [370, 410, 411] and hence they have been used in this work as an *in vitro* model to investigate the cytotoxic effects induced by different surface functionalised AuNPs. Prior to toxicity assessment, various characterisation techniques were used to characterise the size, and zeta potential in different biological milieu and morphology in UPW. The size distribution obtained when the AuNPs were dispersed in the CCM was more than the size obtained in PBS, FCM, and UPW. The cause of the size discrepancy could be due to the components contained in the CCM that provides nutrient to the cells [412]. Morphological observations using TEM showed that all the functionalised AuNPs are spherical and evenly dispersed. Zeta potential measurement indicated that all the functionalised AuNPs were negatively charged analysed using Zetasizer nano and the negatively charged is attributed to the presence of functionalisation agents in the AuNPs [413]. Modified lactate dehydrogenase (mLDH) and MTT assays were then used to determine percentage cell viability as an indication of the cellular response of A549 cells to AuNPs [72]. The mLDH and MTT assays highlighted that citrate, PVP, and tannic acid- functionalised AuNPs significantly decreased cell viability and metabolic activity respectively in a dose- and time-dependent manner. Furthermore, PVP functionalised AuNPs showed a significant ($p < 0.05$) decrease in cytotoxicity in A549 cells compared to citrate and tannic acid functionalised AuNPs. This means that functionalisation plays an important role in determining the toxicity of AuNPs.

The variability of functionalising agents on AuNPs determines their uptake fate as well as their rate of toxicity [347, 414]. Similar to current findings, several studies have highlighted the influence of functionalisation on the toxicity of AuNPs [94, 223, 415]. They found that AuNPs functionalised with a positive charge have a stronger binding affinity for the cell membrane, which promotes their uptake and toxicity compared to AuNPs functionalised with a negative charge or neutral. Although negatively charge functionalised AuNPs were used in this study, it was somewhat observed that the toxicity of AuNPs does not only depend on the surface charge of the functionalised AuNPs, but also on the functionalising agents used during the synthesis. Based on the cell viability results, the capacity of A549 cells to recover from the detrimental effects caused by AuNPs was studied. Results on cells' recovery demonstrated that A549 cells have the capacity to return to normal proliferation at low AuNPs concentrations; however, cell viability continued to reduce at higher concentrations. The ability of the higher concentrations to interfere with cell proliferation is probably due to both AuNPs and the ions released by the dissolution may be retained inside the cell even after AuNPs removal from the CCM. In accordance with the current findings, other studies reported that cells can return to their normal state if not compromised by mechanical damage such as massive internalisation [328, 416, 417]. Given that ROS is directly related to AuNPs cytotoxicity, the level of ROS was measured after 24 and 48 h incubation with 1.25-10 µg/mL of the three different functionalised AuNPs in A549 cells. ROS can dynamically influence the tumour microenvironment survival, proliferation, and metastasis by enhancing intracellular oxidative stress [418, 419]. Consequently, a potent method of combating cancer may be realised by disrupting the equilibrium of intracellular ROS to cause their elevation [420]. In this study, an elevated level of intracellular ROS was observed when the cells were treated with different functionalised AuNPs compared to untreated cells. The elevated level of ROS is

initiated by the metal ions released during AuNPs exposure that impairs with mitochondrial respiration [421]. Activation of nicotinamide adenine dinucleotide phosphate oxidase that converts NADPH into NADP⁺ as well as cytochrome c oxidation in the mitochondria, can both contribute to an increase in ROS elevation by functionalised AuNPs [183]. Likewise, intracellular glutathione levels were significantly decreased in A549 cells in a dose- and time-dependent manner after exposure to the three functionalised AuNPs. The elevation of intracellular ROS along with the depletion of intracellular glutathione suggests that oxidative stress induction may be the primary mechanism for the toxicity of functionalised AuNPs in A549 cells. This was in agreement with others who demonstrated that functionalised AuNPs might induce cytotoxicity through ROS production and the depletion of intracellular glutathione [379, 422]. Since excessive production of ROS in the cells is associated with mitochondrial damage [423], the mitochondrial membrane potential (MMP) induced by different functionalised AuNPs was further investigated. Evidence from the results revealed that functionalised AuNPs altered mitochondrial functions through a decrease in MMP which was more pronounced with PVP functionalised AuNPs. In addition to the current studies, previous results showed that mitochondrial stress and a decrease in MMP occur in different cell types following treatment with functionalised AuNPs [145, 424]. Loss of MMP coupled with the increase in the production of ROS in the cells may trigger the initiation of mitochondrial apoptosis by cytochrome c release into the cytoplasm which could result in the activation of the caspase cascade [200, 425]. The results of the study revealed that all the functionalised AuNPs increased the number of apoptotic cells in a concentration-dependent manner with PVP-AuNPs showing slightly higher induction. This correlates with cell viability or cell membrane damage, MMP, and ROS analysis that shows that at the same concentration, PVP functionalised AuNPs have greater effects to A549 cells compared with

AuNPs functionalised with citrate and tannic acid. Several studies have suggested that the most predominant mechanism of cytotoxicity through which AuNPs suppressed the proliferation of cancer cells is apoptosis [83, 426]. For instance, the percentage of apoptotic cells was demonstrated to increase when A549 cells were treated with citrate functionalised 5 nm AuNPs compared to 5 nm polyethylene imine functionalised AuNP, confirming that there could be a functionalisation-dependent cell death [400]. Elsewhere, the number of apoptotic cells was reported to gradually increase when HepG2 liver cells were treated with polyethylene glycol (PEG) and lithocholic acid functionalised AuNPs [399]. Their observations revealed that when the functionalised AuNPs are absorbed by the cells, they release Au ions which led to mitochondrial dysfunction and oxidative stress through ROS accumulations, ultimately leading to apoptosis.

The effect of surface functionalisation on DUBs expression in A549 cells was next investigated using Western blotting. Several studies have shown that targeting DUBs might be an effective approach in cancer therapy due to their significant role in controlling cellular mechanisms such as apoptosis, cell proliferation, DNA transcription and repair, cell cycle progression, immune response, and protein modification [427-430]. In this study, the protein expression of DUBs in cancerous A549 and MRC-5 normal lung fibroblast cells without AuNPs treatment was first analysed. It was found that proteins were upregulated in A549 cells compared to MRC-5 which correlated with the proliferation of A549 cells being a cancerous cell. Interestingly, when A549 cells were treated with 1.25-10 $\mu\text{g/mL}$ of different surfaces functionalised AuNPs, the protein expression levels of DUBs in A549 cells significantly decreased. This is the first attempt to study the effects of functionalised AuNPs on the ubiquitin proteasome system of A549 cells. Study in the previous chapter highlight that AuNPs (5, 10 and 80 nm) were able to induced size dependent cytotoxicity to A549 cells by inhibiting

ubiquitin proteasome system enzymes such USP7, USP8, USP10 and UCHL-1 [431]. Other studies have also reported that small molecules like P5091, HBX19818, P22077, b-AP15 and WP1130 can attenuate the proliferation of different cell lines through the inhibition of ubiquitin-specific proteases such as USP7 [234, 241, 264, 432], USP8 [433-435], USP10 [267-269, 436], USP14 [230, 437] and Ubiquitin C-terminal hydrolases such as UCHL5 [230]. The study then further investigated if the downregulation of deubiquitylating enzymes can modulate the expression of AKT and Wnt signalling proteins after exposure to different functionalised AuNPs. A decrease in the expression of AKT and Wnt signalling related proteins were observed when compared with untreated cells. Studies showed that functionalised AuNPs inhibited the proliferation of cancer cells through modulation of the P13K/AKT/mTOR pathway [202, 203].

The most common mechanism through which NPs inhibit the proliferation of cancer cells is through the induction of apoptosis [71, 96, 203, 438-440]. The study further explored if the exposure of three functionalised AuNPs induced apoptosis by analysing the mitochondrial expression of PARP, caspase-3, and caspase-9 using western blotting. During apoptosis, these proteins are activated and play a significant role in the execution of mitochondrial-dependent apoptosis [438, 441]. The findings demonstrated that all the functionalised AuNPs induced mitochondrial-related apoptosis in a dose-dependent manner by increasing the expression of PARP, caspase-3, and caspase-9 proteins, which corroborate with apoptosis induction by AuNPs [83, 370].

4.4 Conclusion

This chapter showed that all three functionalised AuNPs induced A549 cells cytotoxicity through a decrease in mitochondrial activity, cell membrane integrity, glutathione,

mitochondrial membrane potential depletion, and an increase in ROS production. In depth analysis demonstrated that 5 nm functionalised AuNPs might affect the USP by slightly inhibiting the expression level of DUBS. Furthermore, the data demonstrated that the AuNPs may induce mitochondrial-related apoptosis in A549 cells through P13K/AKT/mTOR and β -Wnt signalling pathways by decreasing the expression of related proteins which could be associated with the production of ROS. The study warrants future work to investigate the effect of functionalised AuNPs on UPS in different cells type both *in vitro* and *in vivo*.

Chapter 5: Inhibiting the Molecular Chaperone Heat Shock Proteins (HSPs) with AgNPs and AuNPs Attenuate the Proliferation of A549 Cells

Abstract

Heat shock proteins are a large family of molecular chaperones that are overexpressed in tumour cells and confer cytoprotection against many varieties of external stimuli which consequently regulate cell development, differentiation, and death. Herein, the effects of 10 nm AgNPs and AuNPs citrate stabilised were investigated on molecular heat shock proteins in lung adenocarcinoma cells (A549). The study demonstrated that AgNPs exhibited greater cytotoxicity in both 2D and 3D spheroid models, reactive oxygen species (ROS) generation, and mitochondrial membrane damage compared to AuNPs of the same concentration and incubation period. The treatment of A549 cells with 10 µg/mL of AgNPs showed a decrease in the expression of molecular heat shock proteins (HSP40, HSP60, HSP70, and HSP90) using western blot. Interestingly, AgNPs decreased the proliferation of A549 cells through inhibition of AKT, mTOR, β -catenin and activated cell death by upregulating PARP, caspase-3, and caspase-9 but AuNPs did not show any effect on AKT, mTOR, β -catenin and pro-apoptotic proteins. It further confirmed that AgNPs upregulated GSK-3 β and downregulated β -catenin proteins. Altogether, the study found that HSP40, HSP60, HSP70, and HSP90 played a critical role in A549 cell proliferation and can be inhibited by AgNPs but not AuNPs.

5.1 Introduction

Heat shock proteins (HSPs) are a large family of molecular chaperone proteins that are rapidly overexpressed when cells are exposed to physiological and environmental stimuli such as hyperthermia, anoxia, UV-light, and change in osmolarity [442, 443]. These proteins are

broadly classified into families according to their relative molecular mass including small heat shock proteins (HSP27, HSP40, HSP60, HSP70, and HSP90) and the family of larger heat shock proteins (HSP110) [444, 445]. HSPs participate in protein folding, quality control of misfolded proteins, and controlling degradation after severe protein damage [446-448]. Targeting HSPs has received increasing attention in recent years, mostly due to their overexpression in tumour cells [277, 321]. The elevated levels of these proteins aid in cell proliferation, resistance to drug treatment, and suppression of apoptosis by regulating specific signaling pathways [283, 449, 450]. Considering the critical role of HSPs in tumour cells, many studies have shown that HSPs inhibition can suppress the growth of many tumour cells through the induction of cell death and mediated cell cycle arrest [291, 451, 452]. Both 5-fluorouracil and carboplatin exert potent anti-cancer activity by inhibition of HSP27 and HSP40 in hepatocarcinoma cells [315]. Ganetespib (STA-9090) has been described as a potent inhibitor of HSP90, functioning by blocking the ability of HSP to bind to biotinylated geldanamycin and disrupting the association of HSP90 with its cochaperone P23 [453]. Recent studies using C086 alone or in combination with gefitinib are shown to reduce the growth of A549 cells and increase the activation of proapoptotic proteins via the inhibition of HSP90 [454]. However, the developed inhibitors of HSPs have a weak effect on tumour cells due to a lack of sensitivity and specificity [276, 455]. Alternative HSPs inhibitors with different targets for HSPs activity sites are urgently needed to make more significant effects on the treatment of cancer cells.

Recently, AgNPs and AuNPs have shown a promising effect in reducing cell proliferation, inducing apoptosis, inhibiting cell cycle progression, and regulating the various cell mechanisms in different cell types [78, 379, 405, 453, 456]. It has been noted that AgNPs and AuNPs participate in the regulation of cell death and attenuation of cell growth in different intracellular signaling pathways mediated through reactive oxygen species (ROS) generation

[74, 84, 457]. Most of the studies have focused on intracellular signaling pathways without giving due attention to molecular chaperone heat shock proteins. The current literature is also biased towards comparative study of larger (>20 nm) NPs, which are easier to synthesise and there is currently a lack of reliable data on the comparative study of 10 nm NPs toxicity on HSPs in A549 cells. In this study, the effects of 10 nm citrate coated AgNPs and AuNPs on molecular chaperone HSPs were investigated in A549 cells. The study further investigates if the inhibition of HSPs could affect the expressions of P13K/AKT/mTOR signaling and mitochondrial-related apoptosis.

5.2 Results

5.2.1 Physicochemical Characterisation of AgNPs and AuNPs

The morphology, hydrodynamic size, and size distribution of AgNPs and AuNPs dispersed in ultrapure water were characterised by TEM. As shown in **Figure 5.1A**, AgNPs are spherical in shape and densely agglomerated with an average size distribution of 11 ± 1.51 nm. The image of AuNPs indicates spherical, monodispersed particles with an excellent dispersion quality and average size distribution of 8-14 nm (**Figure 5.1B**). The hydrodynamic size distribution, PDI, and zeta potential of AgNPs and AuNPs in UPW, FCM, and CCM were characterised by DLS in 0 and 24 h. At 0 h, the hydrodynamic size of AgNPs and AuNPs in UPW were 17.96 ± 1.68 nm and 15.09 ± 0.21 nm respectively. In FCM, the hydrodynamic diameter changes to 19.66 ± 2.42 nm and 19.99 ± 3.05 nm for AgNPs and AuNPs, while in CCM, the hydrodynamic size of both AgNPs and AuNPs increased to 35.57 ± 1.92 nm and 35.53 ± 0.83 nm respectively (**Figure 5.1C**). At 24 h, the hydrodynamic size of AgNPs increased slightly to 19.49 ± 0.70 nm but the AuNPs size remained statistically the same in water, showing the stability of AuNPs after 24 h. A slight change in hydrodynamic size for both AgNPs and AuNPs in FCM ($21.13 \pm$

4.133 nm, and 28.40 ± 5.25 nm) was seen after 24 h. In CCM, it was noted a larger change in hydrodynamic size for both AgNPs and AuNPs (40.68 ± 2.95 nm and 36.53 ± 2.31 nm) after 24 h (**Figure 5.1D**). The variation in hydrodynamic size of both AgNPs and AuNPs in CCM could be attributed to the interactions of NPs with the number of molecules in CCM. In UPW, AgNPs showed PDI values of 0.295 ± 0.104 in UPW and FCM which slightly change 0.707 ± 0.106 in CCM at 0 h. For the AuNPs, the PDI values in CCM (0.749 ± 0.03) and FCM (0.33 ± 0.04) were higher than UPW (0.213 ± 0.03) at 0 h. At 24 h timepoint, the PDI values of AgNPs in UPW and FCM slightly increased to (0.36 ± 0.04 and 0.53 ± 0.10) but decreased in CCM (0.45 ± 0.02) compared to 0 h. AuNPs PDI values slightly decrease in UPW (0.19 ± 0.02) and CCM (0.43 ± 0.01) but increase in FCM (0.52 ± 0.10). In both 0 and 24 h, the PDI values were higher in FCM and CCM than UPW, demonstrating higher stability in UPW. The zeta potential results demonstrated that all the particles were negatively charged in UPW, FCM, and CCM at both incubation times (**Figure 1E-F**).

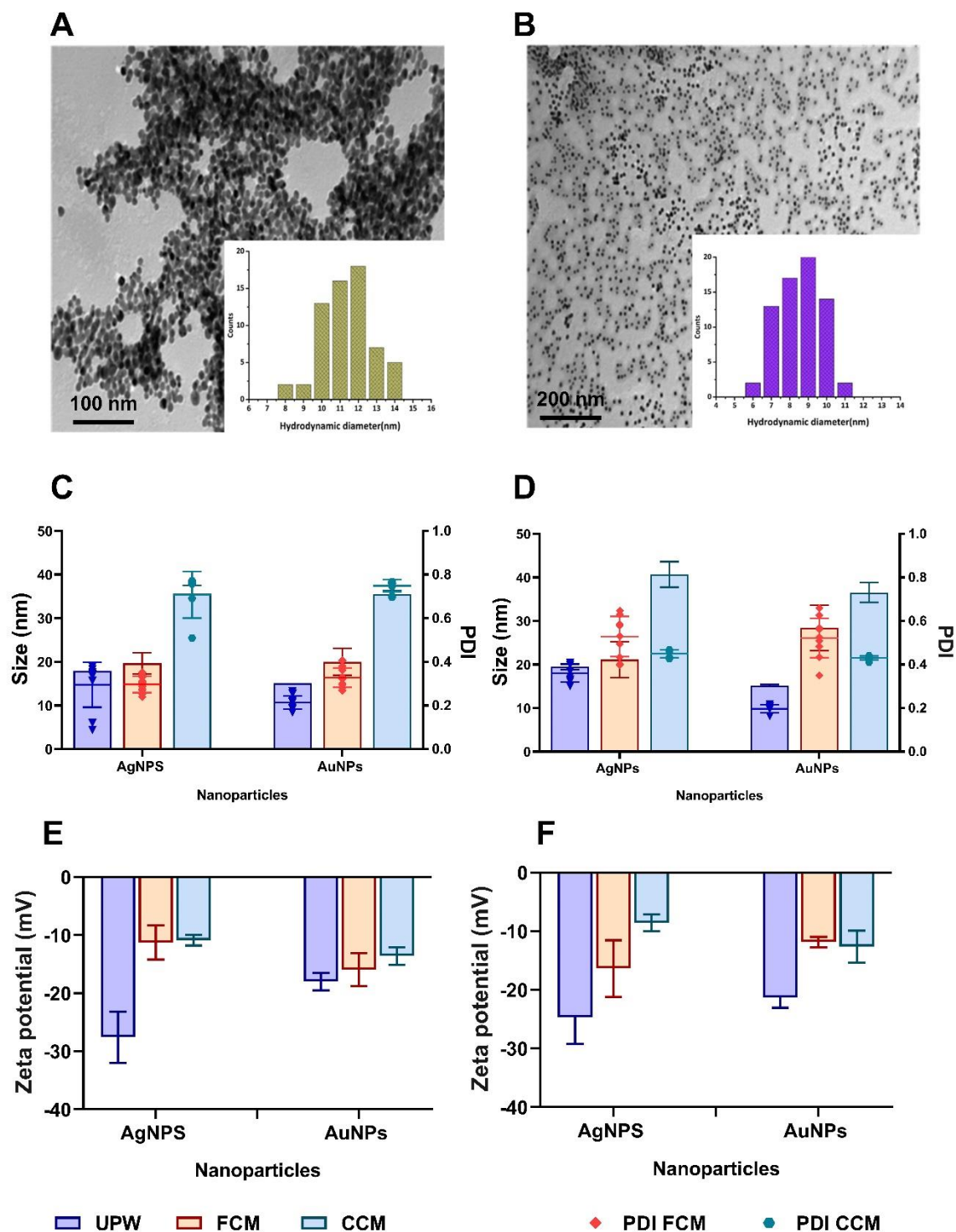


Figure 5.1. Physicochemical characterisation of AgNPs and AuNPs. Morphology and size distribution of (A) AgNPs (B) AuNPs. A total of 100 particles were measured by using ImageJ software (n=100) to calculate the average hydrodynamic size distribution. Hydrodynamic size distribution, and PDI of AgNPs and AuNPs in UPW, FCM, and CCM after (C) 0 h (D) 24 h. Zeta

potential of AgNPs, and AuNPs in UPW, FCM, and CCM (E) 0 h (F) 24 h. Plotted graphs represent the means $\pm$ standard deviation (SD) of ten different measurements. (Scale bar: 100 nm was used for AgNPs, and 200 nm was used AuNPs).

5.2.2 Dissolution of AgNPs and AuNPs in UPW and CCM

The degree of AgNPs and AuNPs dissolution was measured over time (0.25-24 h) in UPW and CCM using ICP-MS. As shown in **Figure 5.2A**, from 15-30 min the amount of dissolved Ag and Au ions detected remained below 1 $\mu\text{g/mL}$ in UPW. After 1 h, the dissolution slightly increased to 1.74 $\mu\text{g/mL}$ and 1.65 $\mu\text{g/mL}$ for AgNPs and AuNPs respectively and reached an apparent plateau from 6 to 24 h. In more complex media (CCM), the presence of glutamine, glucose, bicarbonate, and high ionic content may influence the degree of dissolution of AgNPs and AuNPs, due to competitive complexation, aggregation, or formation of protein corona on their surface as noticed in **Figure 5.2B**. The dissolution initiated rapidly with an initial Ag ions concentration of $0.082 \pm 0.02 \mu\text{g/mL}$ (8.23%) detected immediately after 15 min and the final concentration of $0.147 \pm 0.011 \mu\text{g/mL}$ (14.7%) after 24 h incubation for AgNPs. Unlike AgNPs, AuNPs started with a somewhat lower concentration of $0.050 \pm 0.014 \mu\text{g/mL}$ (5%) at 15 min and gradually increased with time for upto 1 h with a concentration of $0.064 \pm 0.004 \mu\text{g/mL}$ (6.43%) and remained at similar levels till 24 h $0.091 \pm 0.01 \mu\text{g/mL}$ (9.1%).

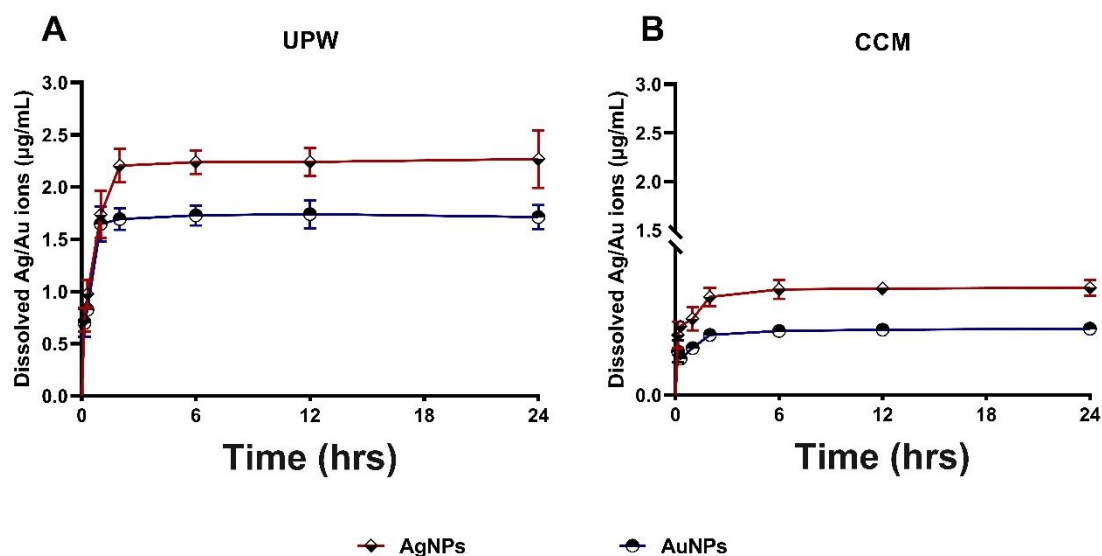


Figure 5.2. Dissolution of Ag and Au, shown in the form of ionic release in UPW and CCM over a period of 0.25-24 h measured by ICP-MS: **(A)** Ag and Au ions released in UPW **(B)** Ag and Au released in CCM. Plotted graphs represent the means $\pm$ SD of three independent experiments.

5.2.3 Cytotoxicity Assessment of AgNPs and AuNPs in A549 Cells

The percentage of cell membrane damage induced by AgNPs and AuNPs was measured using the modified lactate dehydrogenase (mLDH) assay. The assay is a modified version of the LDH assay for screening the cytotoxicity assay of NMs developed previously [72]. The original version of the LDH assay may interfere with extrinsic properties of NMs such as absorption spectra, fluorescence in optical signal detection, and interaction of NPs with the media components and enzymes in the assay [72, 144, 326]. The risk of interference was minimized in mLDH by centrifuging the A549 cell lysate after incubation with either AgNPs or AuNPs before incubation with a substrate mixture. As shown in **Figure 5.3A**, there is no significant reduction in the percentage of cell survival when A549 cells were exposed to 1.25-2.5 $\mu\text{g/mL}$ of AgNPs or AuNPs for 24 h. When the cells were incubated with 5 $\mu\text{g/mL}$ the percentage of cell survival significantly ($p < 0.05$) decreased to (73%) and (97%) for AgNPs and AuNPs. The

percentage of cell survival proportionally decreased with an increase in concentrations with a noticeable reduction at 35 $\mu\text{g/mL}$ (30%, 39%) for the AgNPs and AuNPs. Exposure to AgNPs at 48 h showed an apparent significant reduction in the percentage of cell survival in all the concentrations ($p < 0.001$) with a noticeable reduction at 35 $\mu\text{g/mL}$ (19%). No sign of a significant reduction in the percentage of cell survival was noted when incubated with a concentration of 1.25-10 $\mu\text{g/mL}$ of AuNPs. However, when the concentration was greater than 10 $\mu\text{g/mL}$ the corresponding percentages of cell survival were (80%, 63%, 45%, 34%, and 26%) for the 15, 20, 25, 30, and 35 $\mu\text{g/mL}$ (**Figure 5.3B**). Celltiter Glo assay was used to measure metabolic activity as a function of A549 cell viability after exposure to AgNPs and AuNPs which is based on the reduction of metabolic activity of the cells. At lower concentrations (1.25-5 $\mu\text{g/mL}$) no significant reduction in the cell viability was noted for both AgNPs and AuNPs. When the concentration increased, a significant of cell viability reduction was observed, with 35 $\mu\text{g/mL}$ having greater effects on A549 cells (19%, 26% cell survival) for AgNPs and AuNPs (**Figure 5.3C**). Likewise, when the A549 cells were incubated with the same concentrations for 48 h (**Figure 5.3D**), it was observed that the concentration of 1.25-2.5 $\mu\text{g/mL}$ of both AgNPs and AuNPs did not induce any decrease in the cell viability. When the concentration was increased to 5 $\mu\text{g/mL}$, a significant reduction of cell viability (78%, 87%) was observed for both AgNPs and AuNPs. At a concentration of 10 $\mu\text{g/mL}$, the cell viability further decreased to 55% for AgNPs and 68% for the AuNPs. Only 17% and 20% cell viability were noted when incubated with a concentration of 35 $\mu\text{g/mL}$ for AgNPs and AuNPs. AgNPs were shown to be significantly ($p < 0.05$) cytotoxic than AuNPs, suggesting that NPs toxicity depends on the nanoparticle chemical composition.

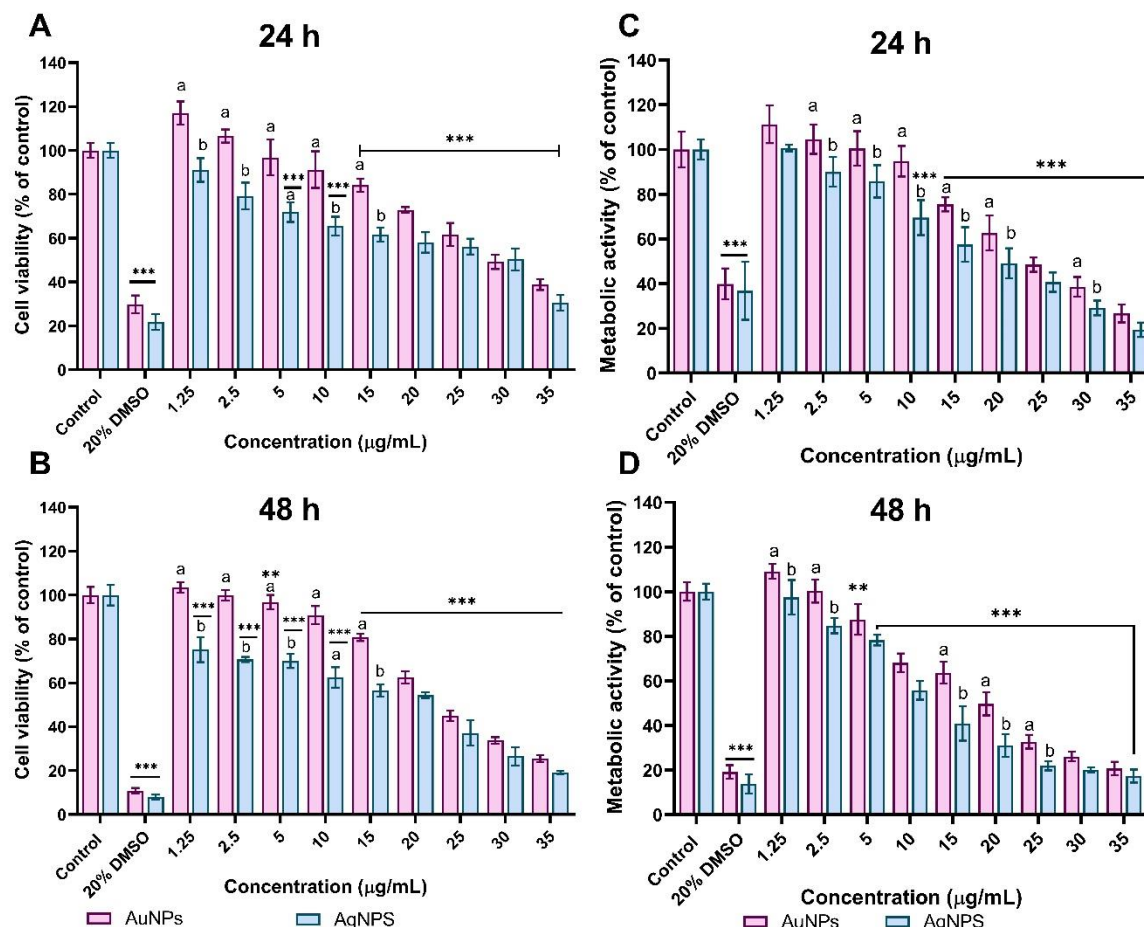


Figure 5.3. Percentage cell viability of A549 cells treated with 1.25–35 µg/mL of AgNPs and AuNPs with 20% DMSO used as a positive control measured using mLDH and CellTiter Glo assays. **(A)** Percentage of cell survival incubated for 24 h **(B)** Percentage of cell survival incubated for 48 h **(C)** Cell viability incubated for 24 h **(D)** Cell viability incubated for 48 h. Plotted graphs represent the means $\pm$ SD of six independent experiments. Bars with an asterisk (*) show statistical differences (** $p < 0.01$, *** $p < 0.001$) when compared with the control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between AgNPs and AuNPs.

5.2.4 Uptake of AgNPs and AuNPs by Flow cytometry

The flow cytometry measurement results showed an increase in the side scatter intensity signal which corresponds to the complexity of the cells or intracellular localisation of the

AgNPs or AuNPs. The results demonstrated dose-dependent increase in SSC-A intensity for AgNPs with maximal intensity observed at 10 $\mu\text{g/mL}$ (**Figure 5.4A**). Similarly, with AuNPs it was noted the same trend of SSC-A intensity signals (**Figure 5.4B**). However, the results demonstrated a greater uptake of AgNPs than AuNPs based on an increase in SCC-A.

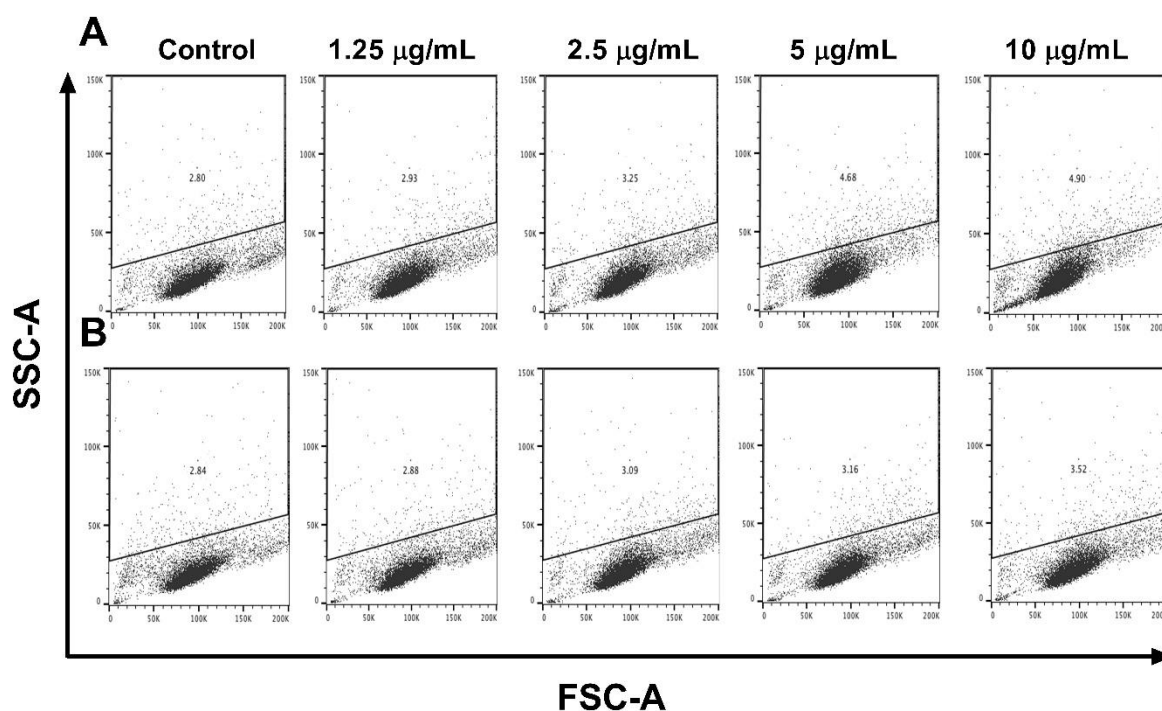


Figure 5.4. Dot plots of side scatter area (SSC-A) versus forward scatter area (FSC-A) intensity of A549 cells exposed to different concentration (1.25-10 $\mu\text{g/mL}$) of NPs for 24 h. **(A)** Dot plot of AgNPs **(B)** Dot plots of AuNPs.

5.2.5 Formation of 3D A549 Spheroid Model

An *in vitro* 3D model of A549 cells was developed to test the toxicity of AgNPs and AuNPs using a liquid overlay method which depends on the coating of 96 well plates with 1% agarose to prevent direct attachment of the cells to the surface of the well plate. As shown in **Figure 5.5A**, the morphology of the 3D spheroids formed at a density of 8000 cells/well between days 1-8. Based on the images it can be concluded that the A549 3D spheroid began from day

1 and smoothly aggregated through the 8 days. According to the quantitative results by ImageJ, the diameter of the 3D spheroid ranges from approximately 400-1000 μM between days 1 to 8 (**Figure 5.5B**). The spheroid was assumed to be spherical in shape, and hence the circumference of the 3D spheroids was calculated from the area measured from ImageJ. The circumference of the spheroid decreased over time as the spheroid compacted together with an average circumference of 135 μM (**Figure 5.5C**).

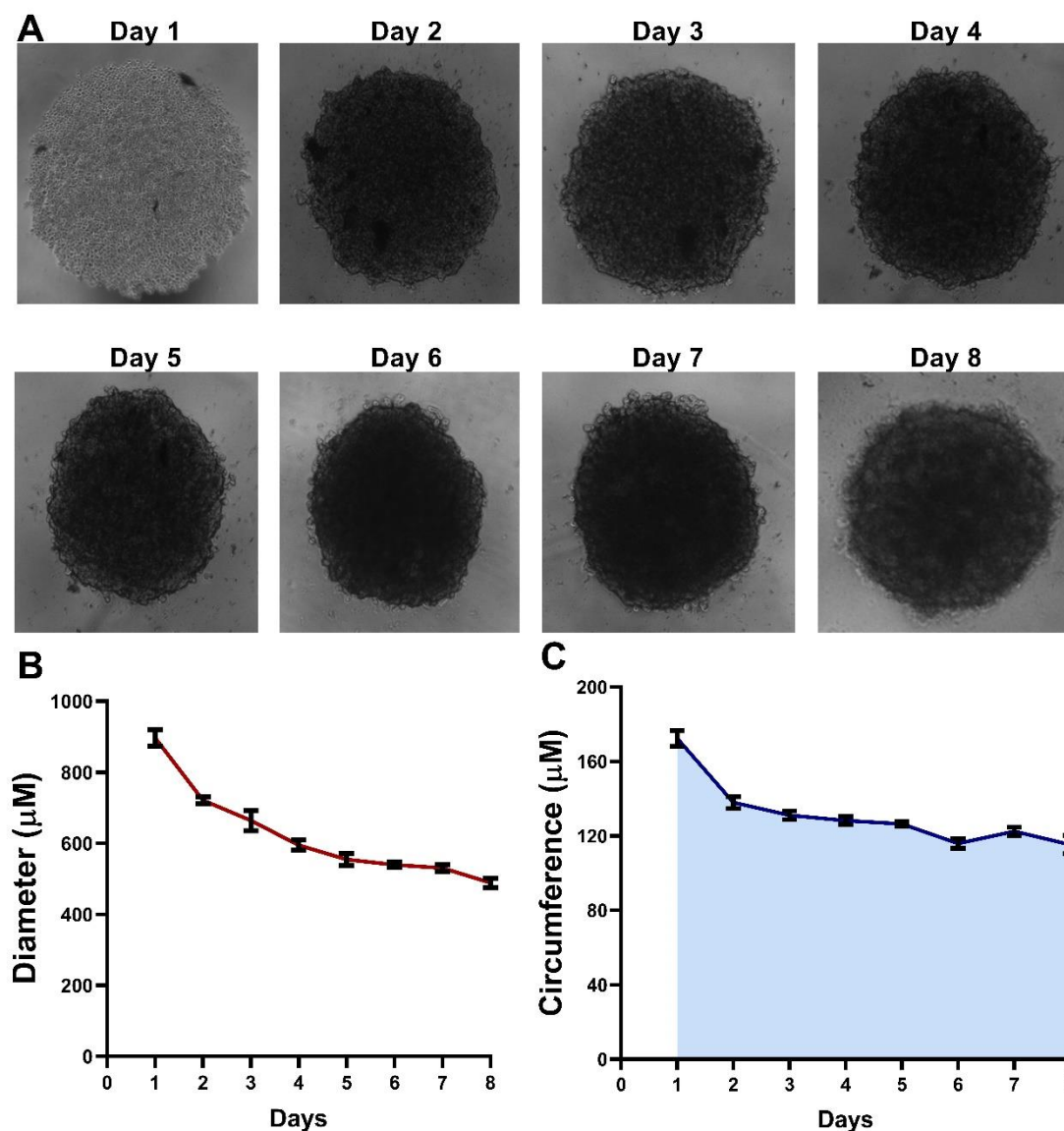


Figure 5.5. Formation of A549 3D spheroid over time using liquid overlay method. (A) Optical morphology of A549 3D spheroid (B) Diameter of A549 spheroid growth curve (C) Circumference of A549 spheroid growth curve calculated from area quantified by ImageJ software. Plotted graphs represent the means $\pm$ SD of three independent 3D spheroid.

5.2.6 Live/Dead Cell Viability Assay

The cytotoxicity of AgNPs and AuNPs in the 3D spheroid was investigated using live/dead cells assay kit at a concentration range of 1.25-10 $\mu\text{g/mL}$ for 24 h. The red signal indicates the number of dead cells, and the green signal indicates the number of live cells respectively. The live/dead cell staining showed that the 3D spheroid maintained its viability when it was exposed to a concentration of 1.25 $\mu\text{g/mL}$ and 2.5 $\mu\text{g/mL}$ of AgNPs. When the concentration further increased from 5-10 $\mu\text{g/mL}$, it showed that the 3D spheroid started losing its viability (Figure 5.6A). When the cells were treated with AuNPs, a slight red signal was noted with 10 $\mu\text{g/mL}$, showing a decrease in cell viability (Figure 5.6B).

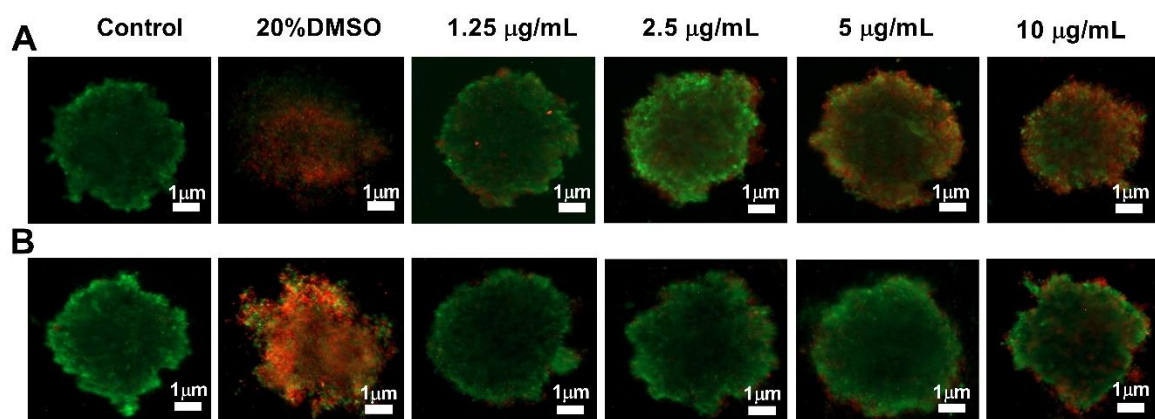


Figure 5.6. Live and dead cell staining of A549 3D spheroid cultured for 4 days using hanging drop method. (A) Fluorescence of live and dead cells of 3D spheroid treated with 1.25-10 $\mu\text{g/mL}$ of AgNPs for 24 h (B) Fluorescence of live and dead cells of 3D spheroid treated with 1.25-10 $\mu\text{g/mL}$ of AuNPs for 24 h.

5.2.7 Intracellular ROS Generation Upon Treated with AgNPs and AuNPs

The intensity of intracellular ROS generated after exposure to AgNPs and AuNPs was assessed using cell red deep reagent by flow cytometry after incubation with 1.25-10 $\mu\text{g/mL}$ for 24 h. Neither AgNPs nor AuNPs shifts the fluorescence intensity when treated with 1.25 $\mu\text{g/mL}$ and 2.5 $\mu\text{g/mL}$ for 24 h. At 5 $\mu\text{g/mL}$ the fluorescence intensity slightly shifts with AgNPs but not with AuNPs. When the concentration was increased to 10 $\mu\text{g/mL}$, the fluorescence intensity increased with both AgNPs and AuNPs indicating the generation of intracellular ROS.

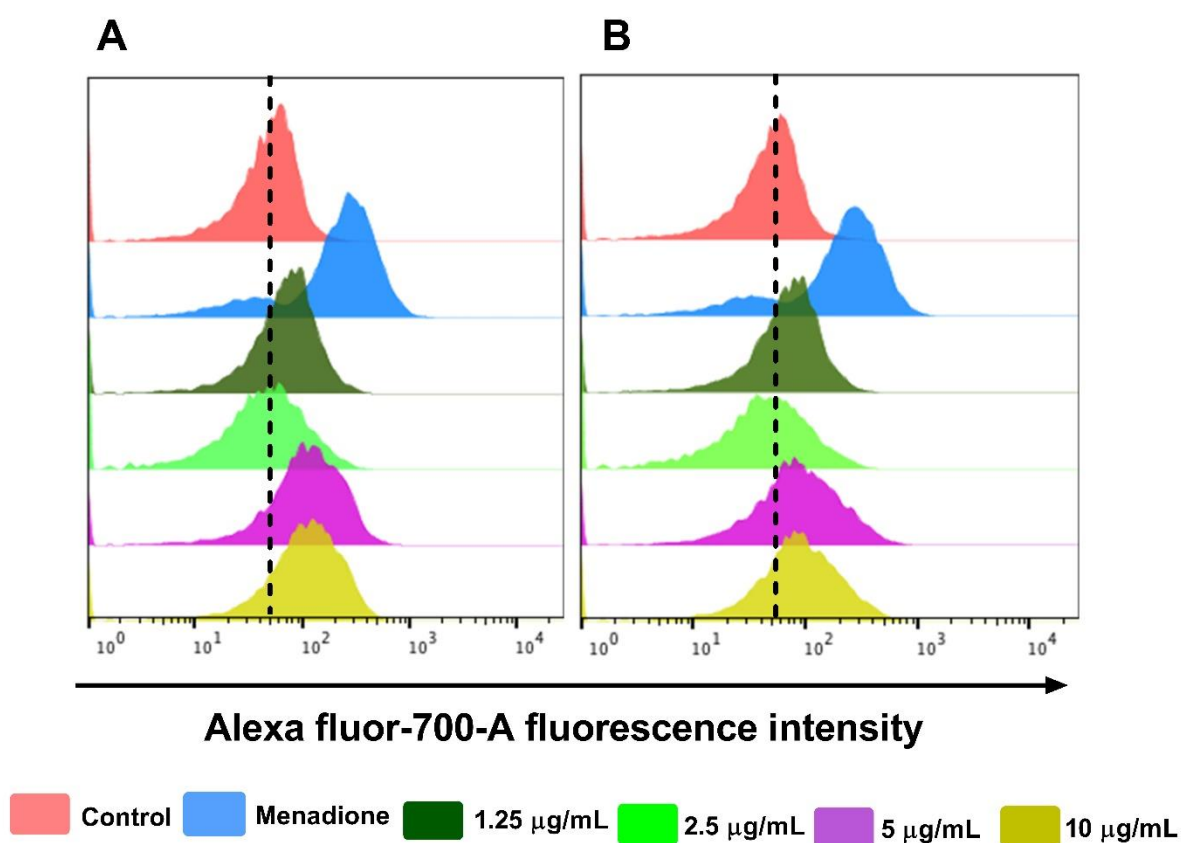


Figure 5.7. Intracellular ROS fluorescence intensity in A549 cell detected by flow cytometry using Cellroxx deep red kit. (A) Histogram of flow cytometry treated with 1.25-10 $\mu\text{g/mL}$ AgNPs for 24 h (B) Histogram of flow cytometry treated with 1.25-10 $\mu\text{g/mL}$ AuNPs for 24 h. 50 μM menadione was used as a positive control for 1 h.

5.2.8 Effect of AgNPs and AuNPs on Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential of A549 cells was measured by TMRM fluorescent probe. After 24 h, AgNPs incubation with 1.25 $\mu\text{g/mL}$ slightly reduce the MMP to 97% compared to control A549 cells (100%), while pre-treatment with 2.5, 5, and 10 $\mu\text{g/mL}$ show a significant reduction of MMP (81%, 75%, and 65%) ($p < 0.001$) respectively (**Figure 5.8A**). Exposure to AuNPs (up to 2.5 $\mu\text{g/mL}$) did not alter MMP in A549 cells. However, when the concentration was increased to 5 and 10 $\mu\text{g/mL}$, the MMP slightly decreased to 93% and 84% respectively (**Figure 5.8B**). The results support the observed reduction in the percentage metabolic activity measured by cellTiter glo assay and partly increased intracellular production of ROS.

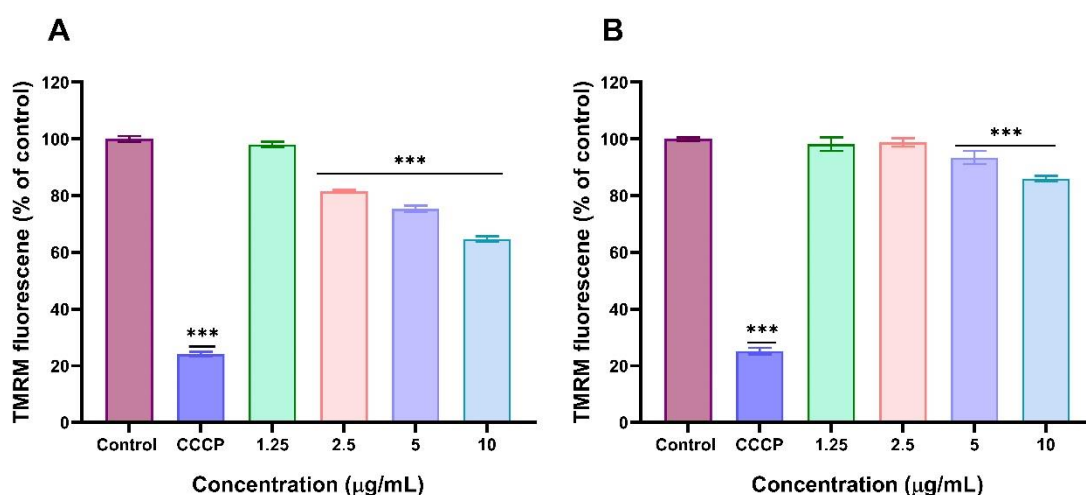


Figure 5.8. Mitochondrial membrane potential (MMP) of A549 cells exposed to 1.25-10 $\mu\text{g/mL}$ of AgNPs and AuNPs for 24 h measured by using mitoProbe™ TMRM Flow cytometry assay kit. **(A)** Bar graph of AgNPs **(B)** Bar graph of AuNPs. 50 μM of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control for 5 min. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference ($***p < 0.001$) when compared with the control.

5.2.9 Apoptosis Induced by AgNPs and AuNPs

The generation of intracellular ROS might draw forth biological responses such as apoptosis and intracellular signaling pathways activation. Apoptosis plays an essential role in cell growth and development, as well as the removal of faulty cells. The effect of exposure to AgNPs or AuNPs induced apoptosis in A549 cells was investigated with double staining of Annexin V/PI assay kit. At the time of apoptosis, the biological cell membrane is disrupted, and the inner phosphatidylserine becomes exposed to the outside of the cell membrane. The Annexin V strongly binds to phosphatidylserine and gives a fluorescence signal indication of apoptosis. As shown in figure below, there is a profound increase in the percentage of apoptosis after exposure to AgNPs for 24 h with the calculated percentage around 4.6%, 5.23%, 6.32%, and 15.94% for the 1.25, 2.5, 5, and 10 $\mu\text{g/mL}$ respectively (**Figure 5.9A**). The corresponding percentage when incubated with AuNPs were (3.1%, 3.24%, 3.35%, and 4.13%) for 1.25, 2.5, 5, 10 $\mu\text{g/mL}$ respectively (**Figure 5.9B**). Statistically significant differences between the exposure concentrations and control cells for the AgNPs and AuNPs were presented in **Figure 5.9C and D**.

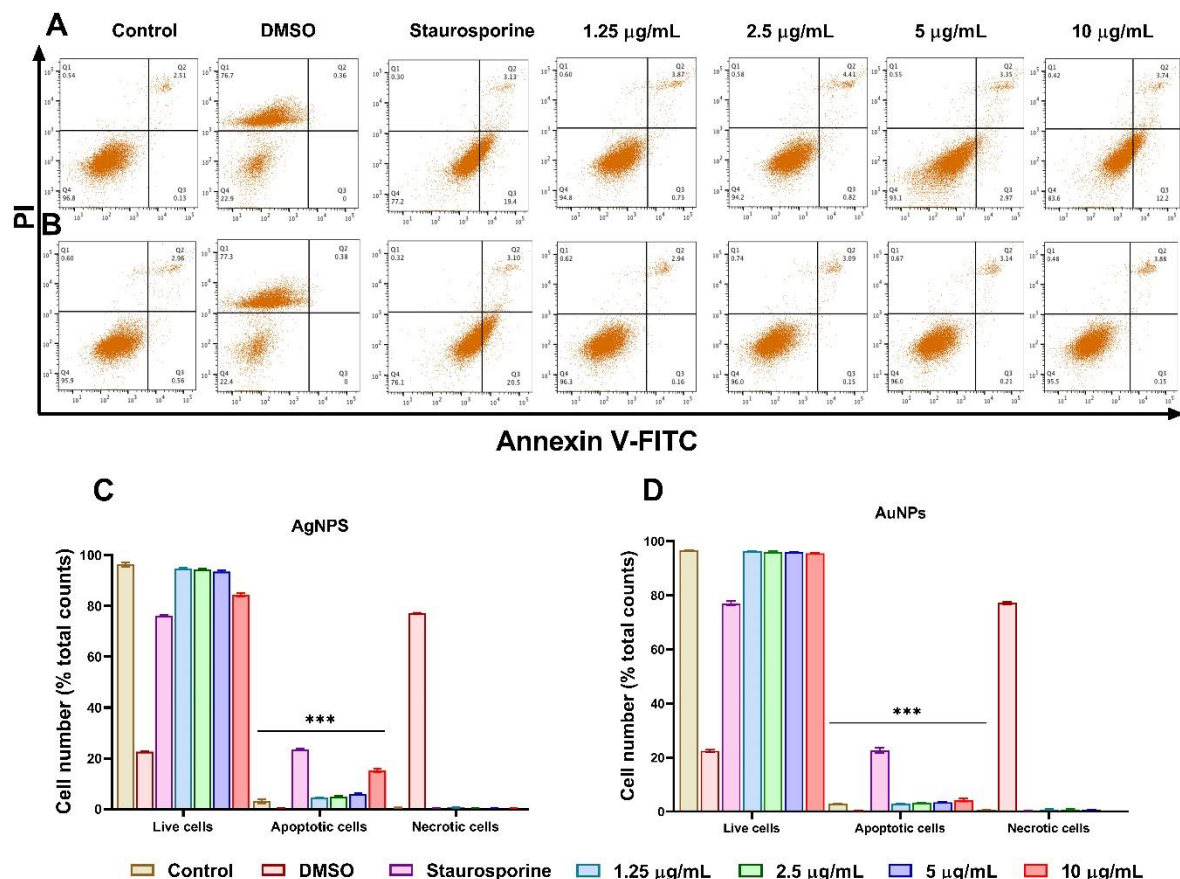


Figure 5.9. Apoptotic cell death and necrotic analysis of A549 cells measured by flow cytometry with double staining of annexin V/PI. Dot plots of A549 cells exposed to 1.25-10 µg/mL of (A) AgNPs for 24 h (B) AuNPs for 24 h. Percentage number of apoptotic and necrotic cells exposed to 1.25-10 µg/mL of (C) AgNPs for 24h (D) AuNPs for 24 h. 1 µM of Staurosporine and 10 % DMSO was used as a positive control for apoptosis and necrosis. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (***) $p < 0.001$ when compared with the control.

5.2.10 Effects of AgNPs and AuNPs on Molecular Chaperone HSPs

To explore the potential of attenuating A549 cells proliferation through molecular chaperone inhibition, the expression of HSP40, HSP60, HSP70, and HSP90 proteins after incubation with a concentration of 1.25-10 µg/mL of AgNPs or AuNPs for 24 h was studied by western blot.

As observed in **Figure 5.10A**, the expression of HSP40, HSP60, HSP70, and HSP90 was downregulated when treated with 10 $\mu\text{g/mL}$ of AgNPs. The relative protein expression changes showed that 1.25 and 2.5 $\mu\text{g/mL}$ have no effects on the protein expression of HSP40, HSP60, HSP70, and HSP90. At 5 $\mu\text{g/mL}$, the relative protein expressions of HSP40, HSP60, HSP70, and HSP90 decreased to 0.85, 0.87, 0.90, and 0.86 respectively. Likewise, when treated with 10 $\mu\text{g/mL}$, the relative protein expression of HSP40, HSP60, HSP70, and HSP90 significantly ($p < 0.001$) changed to 0.5, 0.66, 0.73, and 0.54 respectively (**Figure 5.10B**). The expression of these proteins exposed to AuNPs was examined, but it was found that none of the concentrations had an impact on the expression of HPS40, HSP60, HSP70, and HSP90 proteins (**Figure 5.10C**). No significant changes in the relative protein expression were further recognized when semi-quantified with ImageJ (**Figure 5.10D**).

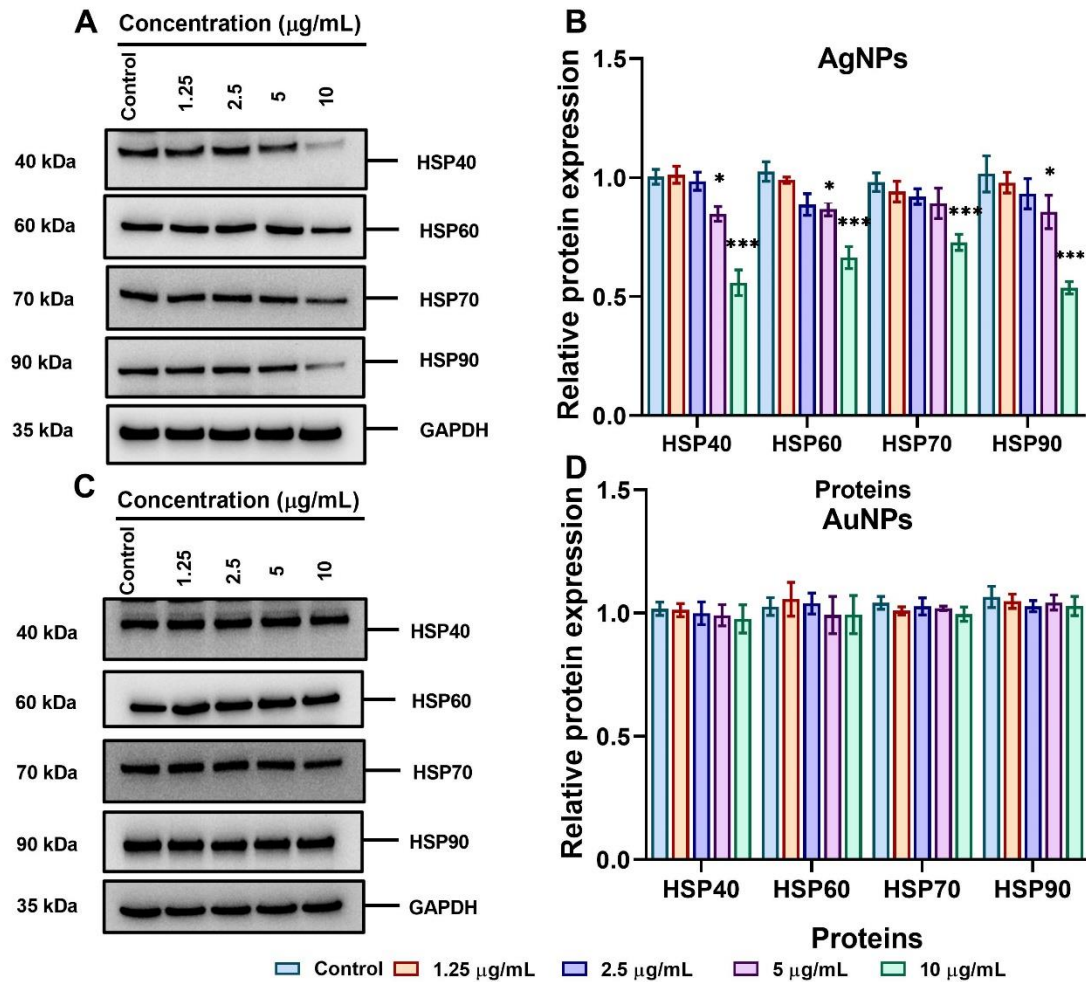


Figure 5.10. AgNPs and AuNPs downregulate the expression of HSPs in A549 cells. **(A)** A549 cells treated with AgNPs at a concentration of 1.25-10 $\mu\text{g/mL}$ for 24 h **(B)** Relative protein expression signal intensity of AgNPs **(C)** A549 cells treated with AuNPs at a concentration of 1.25-10 $\mu\text{g/mL}$ for 24 h **(D)** Relative protein expression signal intensity of AuNPs. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical differences (* $p < 0.05$, *** $p < 0.001$) when compared with the control.

3.2.11 Does Inhibition of Molecular Heat Shock Proteins Downregulate AKT Signaling Pathway Proteins

HSP was identified to form a molecular complex with AKT in tumour cell which is involved in protecting the cells from cell death [458, 459]. The balance between the expression of HSP

and AKT kinase determines the formation of this complex [460]. Therefore, inhibiting the HSP proteins might potentially decrease the activity of AKT signaling leading to the sensitivity of A549 cells to undergo cell death. The expression of AKT, MTOR, GSK-3 β , and β -catenin proteins upon exposure to AgNPs or AuNPs was analysed. Compare with the control cells, AgNPs treatment downregulates the expression of AKT, mTOR, and β -catenin and slightly upregulates GSK-3 β expression (**Figure 5.11A**). When the relative protein expression of the proteins was semi-quantified, it showed that the level of AKT, mTOR and β -catenin were significantly downregulated ($p < 0.001$) while the level of GSK3- β slightly increased dose-dependently with 10 $\mu\text{g/mL}$ having significant increased ($p < 0.01$) (**Figure 5.11B**). However, treatment of A549 cells with AuNPs did not affect the expression of AKT, mTOR, and β -catenin. Surprisingly, with 10 $\mu\text{g/mL}$ of AuNPs the expression of GSK β slightly decreased, showing that the concentration inhibits the expression of the protein (**Figure 5.11C**). When the relative expression of the proteins was quantified, no significant difference between the treated concentration and the control was noted (**Figure 5.11D**).

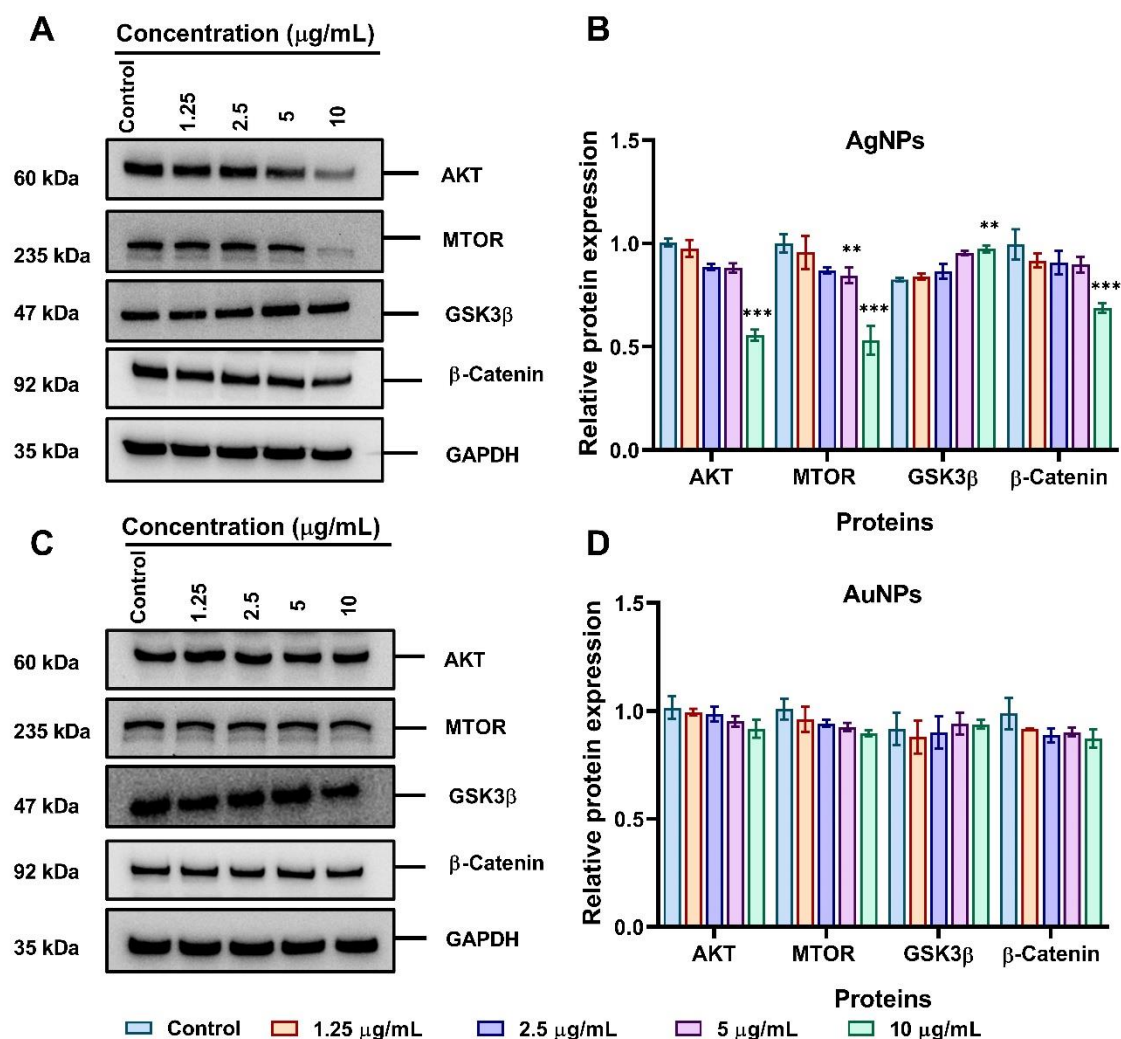


Figure 5.11. Downregulation of P13K/AKT/mTOR pathway and β-catenin protein. (A) Expression of AKT, mTOR, GSK-3β, and β-catenin proteins in A549 cells treated with AgNPs at a concentration of 1.25-10 μg/mL for 24 h (B) Relative protein expression of AKT, mTOR, GSK-3β, and β-catenin proteins signal intensity of AgNPs (C) Expression of AKT, mTOR, GSK-3β, and β-catenin proteins in A549 cells treated with AuNPs at a concentration of 1.25-10 μg/mL for 24 h (D) Relative protein expression of AKT, mTOR, GSK-3β, and β-catenin proteins signal intensity of AuNPs. Plotted graphs represent the means ± SD of three independent experiments. Bars with an asterisk (*) show statistical differences (**p < 0.01, ***p < 0.001) when compared with the control.

5.2.12 Apoptosis Induction by Downregulation of HSP-AKT Binding

The study next tested if the downregulation of molecular heat shock proteins and AKT signaling could lead to mitochondrial cell death in A549 cells by measuring the expression of PARP, caspase-3, and caspase-9 after exposure to different concentrations of AgNPs or AuNPs for 24 h. It was noticed that upon exposure to AgNPs in **Figure 5.12A**, A549 cells showed upregulation of PARP, caspase-3, and caspase-9 in a concentration-dependent manner designating mitochondrial-related apoptosis. Similarly, **Figure 5.12B** shows the relative expression of PARP, caspase-3, and caspase-9 following exposure 1.25 to 5 $\mu\text{g/mL}$ of AgNPs, the expression of PARP and caspase-3 were not upregulated, but the relative expression of caspase-9 increased. At 10 $\mu\text{g/mL}$, the relative expression of PARP, caspase-3, and caspase-9 were significantly upregulated ($p < 0.001$). When the expression of pro-apoptotic PARP, caspase-3, and caspase-9 was studied upon treatment with AuNPs, it was noticed that AuNPs did not upregulate PARP, but caspase-3 and caspase-9 were slightly upregulated at 10 $\mu\text{g/mL}$ (**Figure 5.12C**). Likewise, the relative protein expression of these proteins did not significantly change when semi-quantified with image software (**Figure 5.12D**). The result of western blotting correlate with the flow cytometry data showing that AuNPs only induced minimal apoptosis.

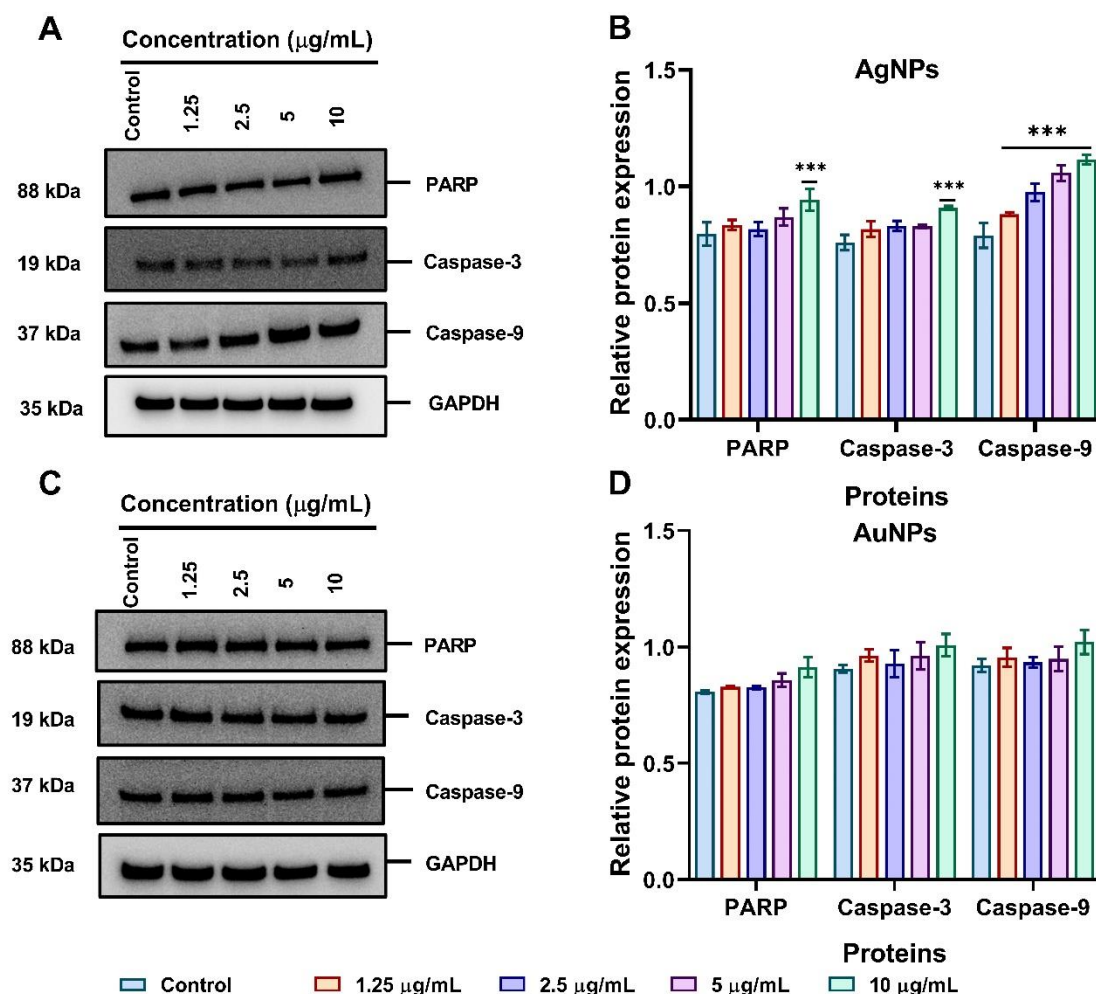


Figure 5.12. Apoptosis induction by inhibiting HSP-AKT binding complex. **(A)** Expression of apoptosis related proteins in A549 cells treated with AgNPs at a concentration of 1.25-10 $\mu\text{g/mL}$ for 24 h **(B)** Relative protein expression of apoptotic proteins signal intensity of AgNPs **(C)** Expression of apoptosis-related proteins in A549 cells treated with AuNPs at a concentration of 1.25-10 $\mu\text{g/mL}$ for 24 h **(D)** Relative protein expression of apoptotic proteins signal intensity of AuNPs. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (** $p < 0.001$) when compared with the control.

5.3 Discussion

AgNPs and AuNPs have attracted considerable attention in the field of nanomedicine due to their increased usage in cancer therapy, bioimaging, and drug delivery [373, 374, 461]. Given their increased use in biological systems, they become accumulated in the lungs, kidney, and liver, causing various toxicologic effects [74, 409]. The toxicological effects on biological systems should therefore be considered. Predicting the toxicity of AgNPs, and AuNPs is difficult because of their varying physicochemical properties such as size, shape, surface charge, and their interaction with biological entities (membrane, media components, and cell type) [51, 326, 462]. To clearly understand the behavior of our AgNPs and AuNPs in different biological fluids, the NPs dispersed in UPW, FCM, and CCM (containing 10% FBS, and 1% antibiotics) were characterised using DLS and TEM.

The hydrodynamic diameter of AgNPs, as observed by TEM, revealed that the AgNPs were spherical in shape with a dense aggregation while the AuNPs image indicated spherical, monodispersed particles with an excellent dispersion. The DLS results of both AgNPs and AuNPs showed an increase in hydrodynamic size distribution when dispersed in FCM and CCM in both 0 and 24 h in comparison to UPW. The growth factors and other biomolecules present in the 10% FBS added in CCM may bind to the surface of the particles. This may result in the formation of the protein corona, thereby increasing the particle size [376, 463]. The zeta potential is a crucial parameter that is related to the surface charge on the NPs. It explains a wide range characteristic of colloidal NPs such as their stability, behaviour in suspension and how they interact with biological phospholipids and cell membranes [464, 465]. The zeta potential measurements revealed that both AgNPs and AuNPs have a negative surface net charge with zeta values greater than -20 mV, indicating that the particles have enough electrostatic repulsion to remain stable in an aqueous solution. Dissolution experiment was

carried out to understand how AgNPs and AuNPs behave in UPW and the biological media with neutral pH. It was observed that particles in biological media show lower levels of dissolution compared to the particles in UPW. The lower dissolution of the particles in CCM might be related to the ions present in media that may bind to active surface sites on the particles, thus inhibiting dissolution. The results are consistent with previous studies that revealed lower dissolution of NPs in biological media compared to UPW [466-468].

The comparative effects of 10 nm AgNPs and AuNPs stabilised with citrate on A549 cells using 1.25-35 µg/mL concentration was studied for 24 and 48 h. The toxicity assays demonstrated that toxicity was strongly dependent on the concentration and incubation period with AgNPs having greater effects on A549 cells than AuNPs, suggesting that the type of NPs was the main property that trigger toxicity. In accordance with the results, earlier research assessed the effects of 10 nm AgNPs, AuNPs, and SPION on signal transduction in epithelial cell lines (A-431) and the authors found that AgNPs caused a larger response than its counterpart [206]. Huang et al., [440] found that the proliferation rates of human dermal fibroblasts were greater than 90% when exposed to 200 µM concentration of 20 nm AuNPs compared to AgNPs of the same size in which the proliferation rate decreased to 69.3% after 72 h incubation, indicating that the cytotoxicity of AgNPs is far greater than that of AuNPs. Parveen et al., [469] studied the effects of biologically synthesised 21 nm AuNPs and 20 nm AgNPs on human cell lines (A549, MDA-MB, and LNCap-FGC). The author demonstrated that IC_{50} of AgNPs is less than 10 µg/mL compared to AuNPs with IC_{50} greater than 30 µg/mL, further suggesting that AuNPs are less cytotoxic than AgNPs [469]. Cellular internalisation of AgNPs and AuNPs might lead to greater cytotoxic effects in the cells as reported by many researchers [49, 63, 470]. In the work presented here, there were no significant differences in SCC-A intensity which relates to the uptake of AgNPs or AuNPs by the cells. It was pointed out that

the uptake of NPs is well related to the size and surface chemistry of NPs instead of chemical composition [79, 94, 163, 471, 472].

3D spheroids A549 based model was designed to further investigate the toxicity of AgNPs and AuNPs using a concentration of 1.25 - 10 $\mu\text{g/mL}$ by a liquid overlay method. Spheroids are proficient in recapitulating some important properties of solid tumours such as cell-cell interactions, drug resistance, specific gene expression and reflect on the actual toxicity of NPs compared to the 2D cell culture model [473, 474]. Interestingly, the results revealed that AgNPs induced greater cytotoxicity even in 3D spheroids than AuNPs. AgNPs and AuNPs have shown cytotoxicity in tumour cells through ROS generation [71, 475]. The ROS level generated by the NPs are proposed to be used as therapeutic agents that suppressed the proliferation and selectively killed tumour cells through cell death induction [476]. The results demonstrated that AgNPs were more efficient in ROS generation than AuNPs treated A549 cells in a concentration-dependent manner. The release of Ag^+ cations by AgNPs which is far greater than the Au^+ radical release by AuNPs could be the major factor that contribute to it greater ROS generation. The Ag^+ cations bind to cellular organelles and cause potential toxic effects to the cells like ROS generation, activation of signalling pathways and ultimately cell death [477, 478]. This finding is consistent with other studies that have shown that AgNPs induced greater ROS generation compared to AuNPs in different tumour cells [206, 379]. Chairuangkitti et al., [479] have similarly reported that AgNPs exposure induced ROS accumulation in A549 cells and suppressed cell proliferation by activating apoptosis. The production of ROS by A549 cells is highly interlinked to a decrease in mitochondrial membrane potential [145, 480]. Notably, it was observed a dose-dependent decrease in MMP after AgNPs incubation to greater extent than AuNPs. In agreement with current findings, a previous study demonstrated that AgNPs caused a significant decrease in the percentage

MMP in A549 cells [479]. To gain mechanistic understanding of ROS generation involvement in apoptosis induction, A549 cells were double stained with annexin V/PI staining kit after exposure to AgNPs and AuNPs for 24 h. In contrast, only AgNPs synergistically enhanced apoptosis at higher concentrations compared to AuNPs in A549 cells. This suggests that lower concentrations of AgNPs and AuNPs may not activate A549 cell death, which agrees with various studies that report dose-dependent induction of apoptosis in tumour cells [137, 158, 457, 479, 481].

A growing number of studies have shown that molecular chaperone HSPs are highly expressed and play a crucial role in tumour cell progression [299, 482-484]. Some inhibitors of HSP27, HSP60, HSP70, and HSP90 have been developed by many research scientists which suppressed the proliferation of tumour cells [485, 486]. However, the lack of toxicity data, specificity, and sensitivity has limited their applications in clinical trials [487]. To find alternative inhibitors, the potential effects of AgNPs and AuNPs on A549 cells were examined which has not been previously tested. The current study observed that AgNPs might downregulate the high expression of molecular HSPs of A549 cells in a concentration-dependent manner. The study further tested the same concentrations of AuNPs at the same incubation time and it noticed no effects on the expression of molecular HSP even at the highest concentration (10 µg/mL). In contrast to our results, Xin et al., [488] demonstrated that AgNPs increased the expression of HSPA1A in A549 and HepG2 detected by western blot. However, no evidence of interactions of AgNPs or AuNPs with HSP40, HSP60, HSP70, and HSP90 was observed in their study. Another study showed that 10 nm AgNPs citrate upregulated the expression of HSP70 in *Drosophila melanogaster* [489]. Downregulation of HSPs may lead to downregulation of AKT pathway proteins since HSPs form a complex with AKT that regulates the proliferation, development, and apoptosis in tumour cells [458, 490,

491]. To confirm the effect on AKT pathway, the activity of AgNPs and AuNPs on AKT, mTOR, GSK-3 β , and β -catenin in A549 cells was studied. A significant decrease in the expression of AKT, mTOR, β -Catenin proteins ($p < 0.001$) and an increase in the expression of GSK-3 β ($p < 0.01$) with AgNPs was noted. Likewise, it was observed that AuNPs have only a slight effect on mTOR proteins. Consequently, this provides good evidence that downregulating the expression of HSPs might proportionally change the expression of Akt and its downstream target. Apoptosis is tightly regulated by AKT through its upregulation of Bcl-2, inhibition of PARP and caspases [177, 203, 437, 492]. To explore if inhibition AKT could result in the induction of cell death, A549 cells were treated with AgNPs and AuNPs for 24 h and the expression of PARP, caspase-3, and caspase-9 was detected by western blot. The results from the analysis suggest that only AgNPs induced cell death in A549 cells which is associated with PARP, caspase-3, and caspase-3 upregulation. In accordance with the current study, previous findings have demonstrated that several agents inhibited the proliferation of cells through the induction of apoptosis [321, 493-495].

5.4 Conclusion

In conclusion, the chapter reported that 10 nm AgNPs and AuNPs may inhibit molecular chaperone by downregulation of HSP40, HSP60, HSP60, HSP70, and HSP90 and disrupting the AKT-HSPs complex by downregulating the AKT protein and its downstream target. Furthermore, it showed that the tested concentrations of AgNPs could activate mitochondrial-related apoptosis through the upregulation of PARP, caspase-3, and caspase-9.

Chapter 6: Effect of Cisplatin and AuNPs Combination on Heat Shock Proteins in Human Lung Epithelial Cancer Cells

Abstract

The current study evaluated the effects of AuNPs combination with cisplatin (CDDP) on molecular chaperone heat shock proteins (HSPs) in A549 cells. It was found that AuNPs:CDDP combination decreased the percentage of cell viability measured using modified lactated dehydrogenase (mLDH) and MTT assays. AuNPs:CDDP exposure caused a significant ($p < 0.05$) increase in ROS generation, apoptosis induction and a decrease in the mitochondrial membrane potential (MMP) compared to AuNPs or CDDP alone. Similarly, exposure to AuNPs:CDDP combination had pronounced cytotoxic effects on the expression of HSPs and P13K/AKT/mTOR as well as mitochondrial apoptosis-related proteins. The results demonstrate that the combination of AuNPs with CDDP might enhance the anticancer efficacy of CDDP.

6.1 Introduction

Cisplatin (CDDP) is a well known chemotherapeutic drug that has been used to inhibit the growth of numerous human cancers including lung epithelial cells (A549) [496, 497]. It depletes the growth of tumour cells by interfering with the complementary bases of DNA leading to DNA damage, disabling repair mechanisms, and subsequently resulting in cell death [498, 499]. However, it is well known that CDDP can potentially induce severe side effects to cancer patients including kidney failure, immunity infections, irreversible hearing loss, renal and neural damage which has limited its further use in anticancer therapy [498]. In addition, tumour cells can promote resistance to CDDP by reducing its accumulation which would limit

its further use as an anticancer therapeutics in those patients [38]. Combination therapy with other drugs or NPs has been thoroughly considered to suppresses drug resistance, lower the rate of toxicity, decrease cancer cell viability, and enhance CDDP delivery to the targeted tissue [500, 501]. For instance, the combination of CDDP with AuNPs has been reported to selectively decrease the proliferation of A549 (lung), MG-63 (human osteosarcoma) , and Saos-2 (Sarcoma osteogenic) through reactive oxygen species (ROS) generation [502]. Similarly, the co-administration of CDDP conjugated PEG-AuNPs was found to induce the cytotoxicity of A549 cells by overcoming CDDP resistance [503]. However, the precise molecular effects of AuNPs and CDDP combination against HSPs in human alveolar adenocarcinoma cells (A549) have never been investigated.

Herein, it was hypothesised that combination of AuNPs with CDDP could enhance its anticancer activity by reducing the overexpression of HSPs, P13K/AKT/mTOR pathway, and upregulating the expression of mitochondria-mediated apoptosis related proteins. Firstly, the cytotoxicity of AuNPs and CDDP alone was evaluated in A549 cells. Secondly, the cell viability of A549 following the combination of AuNPs with CDDP was studied . Thirdly, the underlying mechanisms of cytotoxicity including oxidative stress, mitochondrial damage, and apoptosis caused by AuNPs and CDDP co-administration was investigated. Finally the effects of AuNPs and CDDP combination on molecular heat-shock proteins (HSPs), P13K/AKT/mTOR signaling, and apoptotic proteins in A549 cells was evaluated.

6.2 Results

6.2.1 Cell viability Assessment of CDDP, AuNPs alone

A549 cells response to CDDP, and AuNPs alone was assessed using the MTT and mLDH assays. A dose-dependent decrease in the percentage cell viability following treatment with

AuNPs and CDDP alone for 24 and 48 h was observed with both assays CDDP (**Figure 6.1**). As shown in **Figure 6.1A**, exposure to AuNPs caused minimal cell toxicity at the tested concentrations (0-45 $\mu\text{g/mL}$) with a maximum cell viability (43%) at the highest concentration (45 $\mu\text{g/mL}$) after 24 h. As expected, exposure to CDDP alone showed a significant reduction in cell viability up to 38.9% after 24 h at the highest concentration tested (10 $\mu\text{g/mL}$) (**Figure 6.1B**). In addition, A549 cell survival was measured using the mLDH assay which measures the number of viable cells that remain after exposure to AuNPs or CDDP. mLDH was used because is considered to be the most reliable and has less interference with NMs [72]. As shown in **Figure 6.1C**, AuNPs have less effect ($\text{IC}_{50}=43.01 \mu\text{g/mL}$) on A549 cells compared to CDDP ($\text{IC}_{50}=2.191 \mu\text{g/mL}$) in **Figure 6.1D**

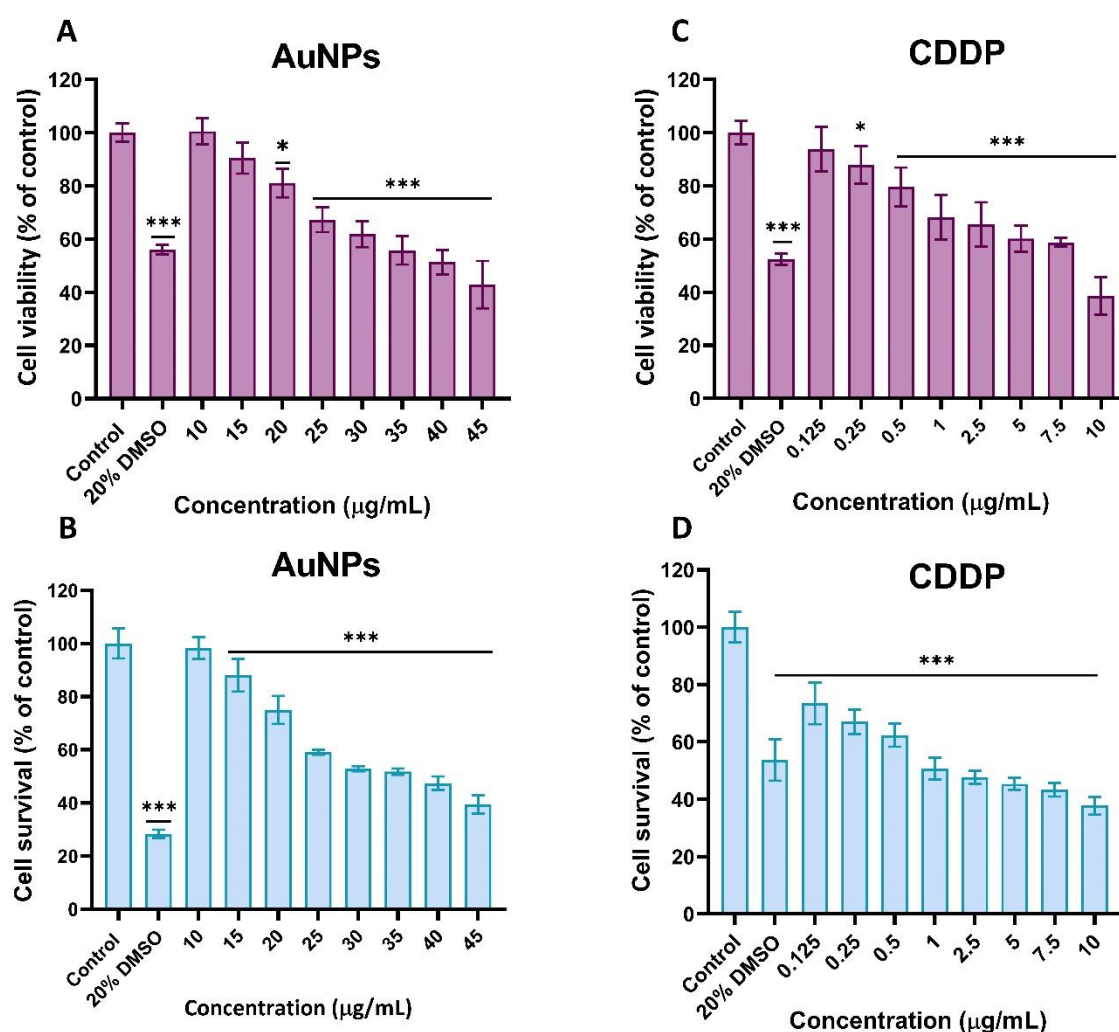


Figure 6.1. Percentage cell viability of A549 cells following treatment with different concentrations of AuNPs (0-45 μg/mL) or CDDP (0-10 μg/mL) for 24h measured by MTT and mLDH assays. (A) MTT assay of AuNPs, (B) mLDH assay of AuNPs, (C) MTT assay of CDDP, (D) mLDH assay of CDDP. 20% DMSO was used as a positive control. Plotted graphs represent the means ± standard deviation (SD) of six independent experiments. Bars with an asterisk (*) show statistical differences (*p < 0.05, **p < 0.01, and ***p < 0.001) compared with the control.

6.2.2 The Effect of CDDP and AuNPs Combination on Cell Viability

Based on viability data, the IC₅₀ of CDDP (2.191 µg/mL) and 25 µg/mL of AuNPs were used to investigate the effects of the combination of CDDP with AuNPs. As shown in **Figure 6.2A**, the combination of AuNPs and CDDP significantly ($p < 0.05$) reduced the percentage of cell survival to (38.5%) compared to AuNPs (87%) and CDDP alone (49.5%) as assessed by the mLDH assay. The cell viability of the cells following treatment with the combination of AuNPs:CDDP was further examined using the MTT assay. It was found that combination of AuNP and CDDP significantly ($p < 0.05$) decreased the percentage cell viability to (47%) compared to AuNPs (83%) and CDDP (57%) alone (**Figure 6.2B**). The results indicate that combinations of AuNPs and CDDP can enhance the reduction of A549 cell proliferation.

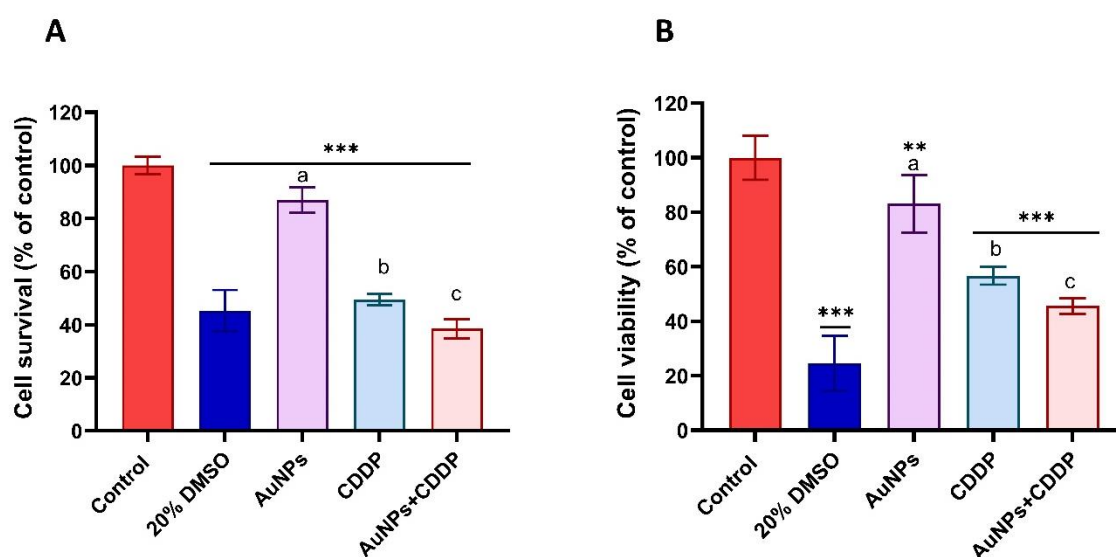


Figure 6.2. Percentage cell viability of A549 cells following treatment with a combination of AuNPs (25 µg/mL) and CDDP (2.191 µg/mL) for 24 h measured by mLDH and MTT assays. **(A)** mLDH assay of AuNPs, CDDP or combination, **(B)** MTT assay of AuNPs, CDDP or combination. 20% DMSO was used as a positive control. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical differences (** $p < 0.01$, *** $p < 0.001$) compared with the control. Data with different letters above the bars represent

the statistically significant difference ($p < 0.05$) between the combination of CDDP:AuNPs, CDDP, and AuNPs alone.

6.2.3 Changes in Cell's Morphological Appearance After Exposure to Combination of AuNPs & CDDP

Cells morphological changes following 24 h exposure to CDDP (2.191 $\mu\text{g/mL}$), AuNPs (25 $\mu\text{g/mL}$) and the combination were also evaluated using optical microscope. CDDP as seen in **Figure 6.3**, AuNPs:CDDP combination caused not only a decrease in cell numbers but also cell shrinkage and detachment from the plates compared to CDDP, and AuNPs alone.

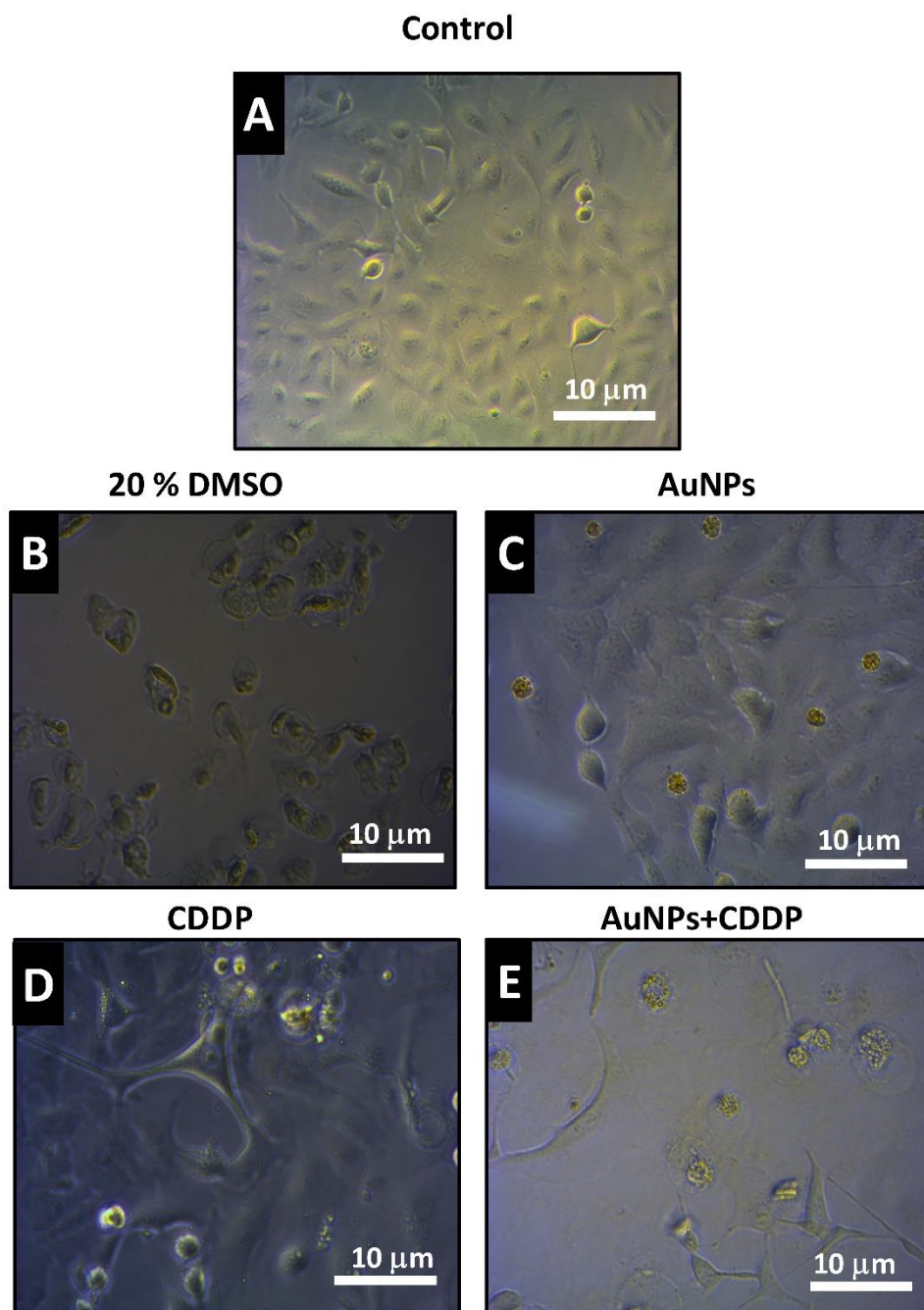


Figure 6.3. Optical image of A549 cell morphology following incubation with (A) Media only (Control), (B) 20% DMSO (positive control), (C) AuNPs (25 μg/mL), (D) CDDP (2.191 μg/mL), (E) Combination of AuNPs:CDDP. Treated cells were imaged using a Zeiss microscope with 20X magnification (Scale bar 10 μm).

6.2.4 Does AuNPs:CDDP Combination CDDP Cause Oxidative Stress?

Exposure to anticancer drugs and NPs can cause the formation of free radicals in the mitochondria [71]. Abnormal concentration of free radicals can potentially interfere with the function of the mitochondria resulting in oxidative stress that damages cell membranes, DNA, and inhibits activation of certain signaling pathways such as P13/AKT/mTOR, MAPK/ERK and Wnt [135]. The combined effect of CDDP and AuNPs on oxidative stress was determined by measuring the levels of reactive oxygen species (ROS) and intracellular glutathione in A549 cells. As can be seen in **Figure 6.4A**, the combination of CDDP and AuNPs increased the levels of ROS significantly ($p < 0.05$) compared to CDDP or AuNPs alone and control. ROS generation causes an imbalance in endogenous antioxidants such as glutathione that neutralise their oxidative damage [130]. An imbalance in intracellular glutathione homeostasis leads to the progression and development of many diseases including cancer [504]. The results in **Figure 6.4B** show that AuNPs:CDDP combination significantly ($p < 0.05$) reduced the ratio of GSH/GSSG to 9.95% compared to CDDP (12%), AuNPs (17.39%), and control (23.65%).

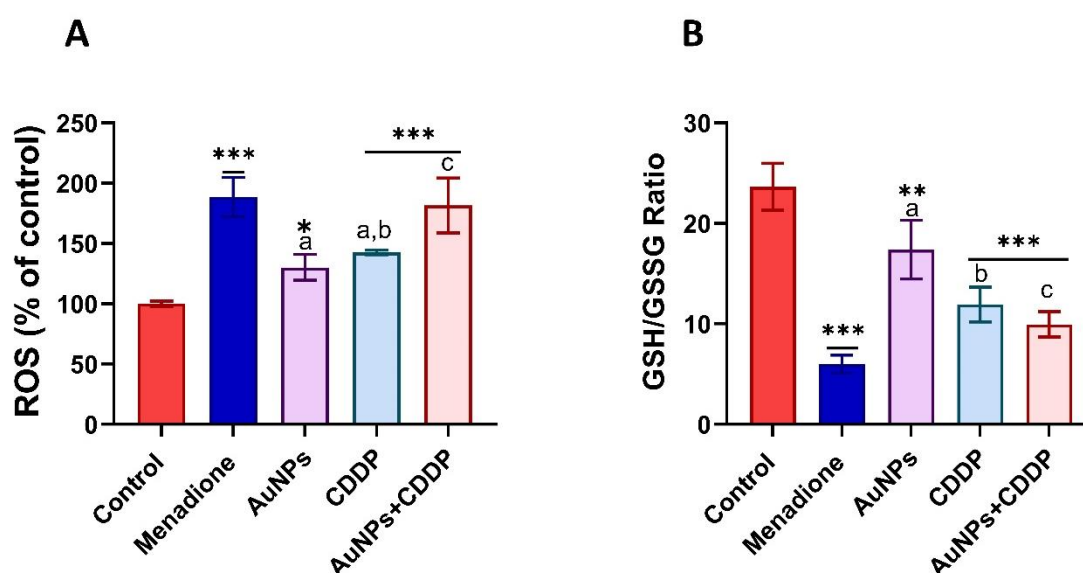


Figure 6.4: Effects of AuNPs, CDDP, and AuNPs:CDDP combination on oxidative stress

biomarkers in A549 cells after 24 h **(A)** ROS generation, **(B)** GSH/GSSG ratio. Menadione (50 μ M) was used as a positive control. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical differences (* p < 0.05, ** p < 0.01, and *** p < 0.001) compared with the control. Data with different letters above the bars represent the statistically significant differences (p < 0.05) between the combination of CDDP:AuNPs, CCDP, and AuNPs alone.

6.2.5 Does AuNPs:CDDP Combination Affect Mitochondria Membrane Potential (MMP)?

The mitochondrial membrane potential of A549 cells treated with CDDP, AuNPs and the combination of AuNPs:CDDP was measured using HCS mitochondrial health stain kit. Carbonyl cyanid-3-chlorophenylhydrazone (CCCP) was used as a positive control. The MitoHealth stain signals changes by accumulating in healthy mitochondria producing higher fluorescence intensity proportional to MMP. Therefore, as the mitochondrial membrane is compromised, low fluorescence intensity is observed. As shown in **Figure 6.5A-E**, A549 cells treated with AuNPs, CDDP and the AuNPs:CDDP combination show a lower fluorescence intensity compared to control, suggesting a loss of MMP. When the fluorescence intensity was normalised against control, the combination of AuNPs:CDDP displayed a significant (p < 0.05) decrease in normalised fluorescence intensity value (49.47%) for mitochondrial membrane potential compared to AuNPs alone (78.84%) and CDDP alone (65.56%) as shown in **Figure 6.5F**. On the other hand, the cells treated with 50 mM of carbonyl cyanid-3-chlorophenylhydrazone (CCCP) showed a high effect on mitochondrial health, as demonstrated in its lowest normalised fluorescence intensity value (46.18%) for MMP. This shows that positive control (CCCP) induced percentage mitochondrial disruption in a close range to AuNPs:CDDP treated cells.

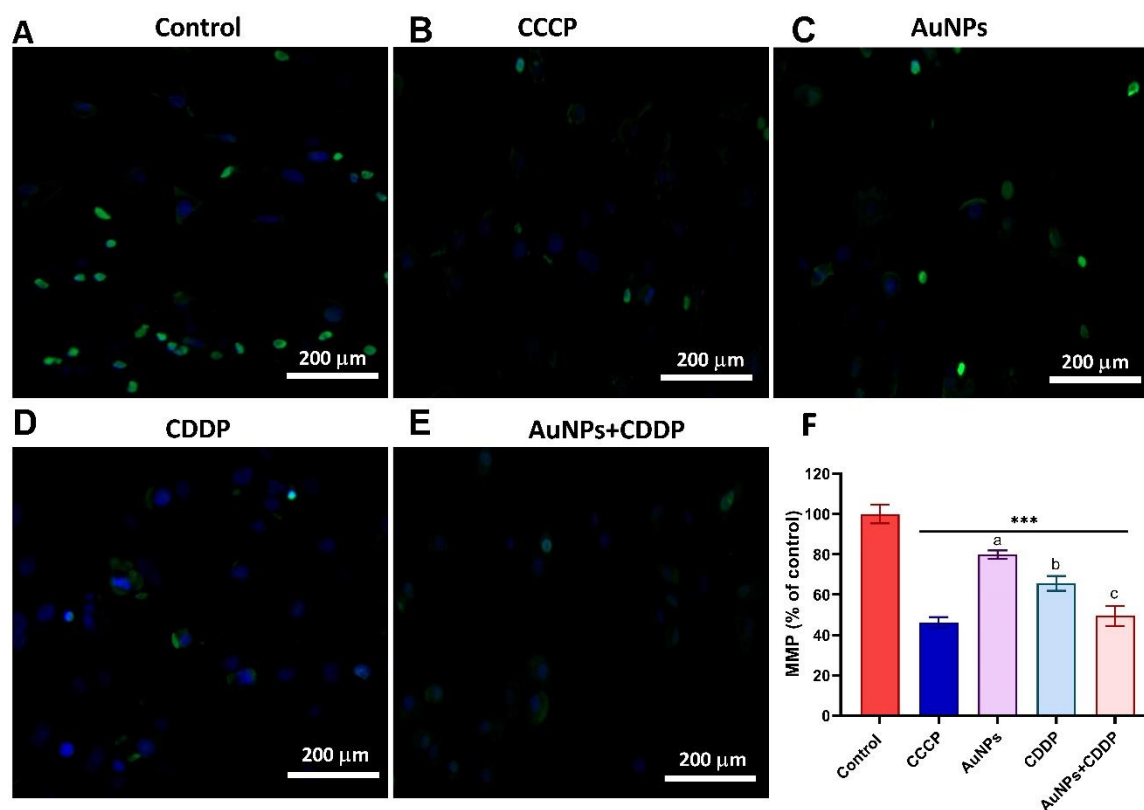


Figure 6.5. Mitochondrial Membrane Potential of A549 cells following treatment for 24 h with (A) Untreated cells, (B) 50 mM of carbonyl cyanid-3-chlorophenylhydrazone (CCCP) (positive control), (C) AuNPs, (D) CDDP (E) AuNPs:CDDP combination (F) normalised fluorescence intensity. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference ($***p < 0.001$) compared with the control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the combination of AuNPs:CDDP, CCCP, and AuNPs alone.

6.2.6 AuNPs:CDDP Combination Enhances Apoptosis

To determine whether the combination treatment of AuNPs:CDDP leads to apoptosis, the latter was measured using Annexin V/PI and Caspase 3/7 assays following 24 h treatment of cells with AuNPs, CDDP, and AuNPs:CDDP combination. Staurosporine was used as a positive control. As shown in **Figure 6.6A** combination treatment of CDDP and AuNPs enhanced

apoptosis by 22.65% more than that of AuNPs (5.25%) and CDDP (15.21%) alone. When compared with control, CDDP alone increased caspase 3/7 activity to 30% in A549 cells. Surprisingly the effect was lower than that of AuNPs alone (33%). Treatment with the AuNPs: CDDP combination significantly increased the caspase 3/7 activity by 72% compared to control (Figure 6.6B).

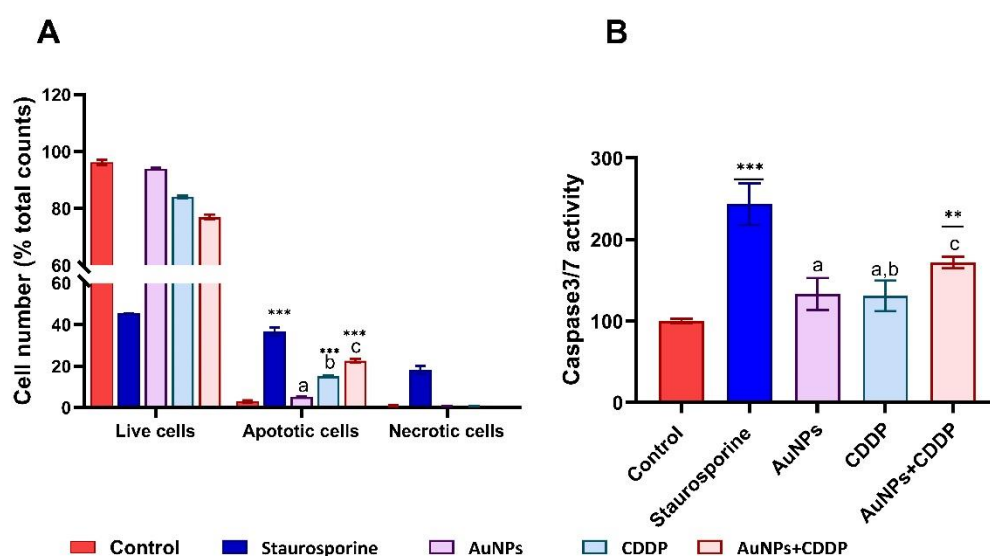


Figure 6.6: AuNPs:CDDP enhanced apoptosis in A549 cells following 24 h treatment. (A) Bar graphs analysis of apoptosis of apoptosis measured by flow cytometry, (B) Caspase-3/7 activity. 1 μ m of Staurosporine was used as a positive control for 6 h. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical differences (***) $p < 0.001$ compared to control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the combination of CDDP:AuNPs, CCDP, and AuNPs alone.

6.2.7 Effects of AuNPs:CDDP Combination on Molecular Chaperone Heat-Shock Proteins (HSPs)

Expression of HSPs is involved in the control of cell survival, metastasis, resistance to therapy, and avoidance of apoptotic cell death in many cancers including A549 lung carcinoma. It is therefore important to understand the effects of our combination therapy as targeting these proteins might be an effective strategy in cancer therapy [277]. A549 cells were exposed to AuNPs, CDDP, and AuNPs:CDDP combination for 24 h and the expression levels of HSPs were assessed. It was found that the protein expression of HSP40, HSP60, HSP70, and HSP90 were downregulated in A549 cells when treated with AuNPs:CDDP combination compared to control (**Figure 6.7A**). On the other hand, when the relative proteins expression was semi-quantified using ImageJ software, it was noted that the relative proteins expression of all the HSPs significantly ($p < 0.05$) reduced with AuNPs:CDDP compared to treatment with AuNPs or CDDP alone (**Figure 6.7B**). Likewise, cells treatment with a 10 $\mu\text{g/mL}$ of quercetin (positive control) showed similar effects on the relative protein expression of HSPs.

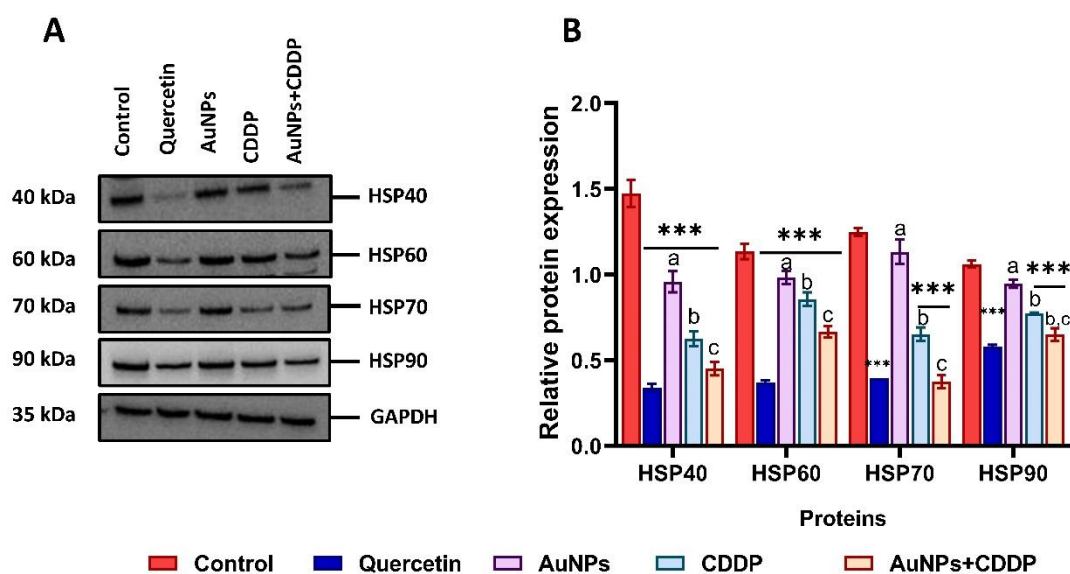


Figure 6.7. Effects of AuNPs, CDDP and AuNPs:CDDP combination on the expression of HSPs (A) Protein expression of HSP40, HSP60, HSP70, and HSP90 detected by western blotting, (B) Relative protein expression of HSP40, HSP60, HSP70, and HSP90 semi-quantified by ImageJ Software. Quercetin (10 µg/mL) was used as a positive control. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference ($***p < 0.001$) compared to control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the combination of CDDP:AuNPs, CDDP, and AuNPs alone.

6.2.8 Effects of AuNPs:CDDP Combination on P13K/AKT/mTOR Pathway

The combination of HSPs with AKT protein form the HSP-AKT complex which is essential for the functional stabilisation of P13K/AKT/mTOR signaling pathway [458]. To evaluate whether HSPs are involved in the phosphorylation of the AKT pathway, the expression of the AKT protein, and its downstream target (such as MTOR and GSK-3 β) involved in cell metabolism, survival, and progression were analysed upon treatment with AuNPs, CDDP and the AuNPs:CDDP combination after 24 h. As shown in **Figure 6.8A**, the western blot analysis data shows that, the expression level of AKT and mTOR proteins were downregulated while the expression of PTEN and GSK-3 β were upregulated with AuNPs:CDDP compared to AuNPs and CDDP alone. Quercetin treatment (positive control) showed the same pattern in terms of downregulation and upregulation of these proteins. When the relative protein expression was semi-quantified with ImageJ software, the AuNPs: CDDP combination showed a significant decrease ($p < 0.05$) in the relative protein expression of AKT protein compared to control, AuNPs and CDDP alone as shown in **Figure 6.8B**. Next, the relative protein expression of PTEN a tumour suppressor protein that is implicated in A549 cells by withholding the

phosphorylation of AKT protein; was checked [505]. PTEN expression was found to be significantly ($p < 0.05$) upregulated with AuNPs:CDDP combination compared to AuNPs and CDDP alone. To further evaluate the effect of the combination therapy on the AKT pathway, the expression of mammalian target rapamycin complex (mTOR) was semi-quantified. mTOR is a downstream target of the AKT pathway that regulates protein synthesis, gene transcription, apoptosis, and autophagy [506]. The level of mTOR expression was significantly ($p < 0.05$) downregulated when treated with the AuNPs:CDDP combination. Additionally, CDDP alone also reduced ($p < 0.001$) the expression of mTOR compared to the control and AuNPs alone. Moreover, all the treatments were found to upregulate the expression of GSK-3 β ; a downstream target of the P13K/AKT/mTOR pathway that is negatively regulated by AKT overactivation.

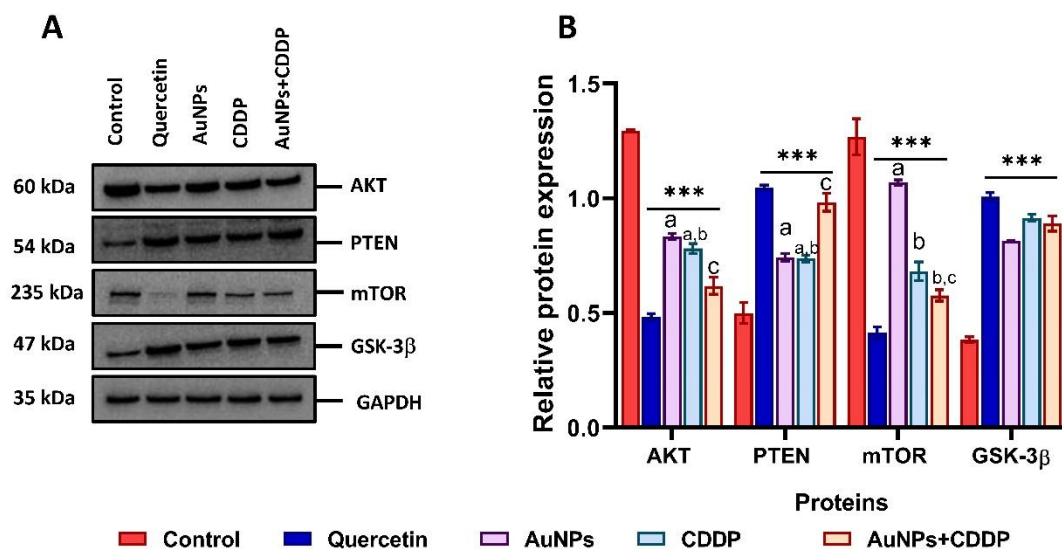


Figure 6.8. Effects of AuNPs, CDDP and the AuNPs:CDDP combination on the expression of P13K/AKT/mTOR pathway proteins (**A**) protein expression of AKT, PTEN, mTOR, and GSK-3 β detected by western blotting, (**B**) Relative protein expression of AKT, PTEN, mTOR, and GSK-3 β semi-quantified by ImageJ software. Quercetin (10 μ g/mL) was used as a positive control.

Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (***) $p < 0.001$ compared to control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the combination of CDDP:AuNPs, CDDP, and AuNPs alone.

6.2.9 Effects of AuNPs: CDDP Combination on Apoptosis Related Proteins

P13K/AKT/mTOR pathway overactivation blocks apoptosis by preventing the release of cytochrome C from the mitochondrial inner space, inactivating the cysteine aspartyl proteases (caspases), and phosphorylating MDM2 which inhibits the tumour suppressor protein p53 [507]. In order to understand if the downregulation of P13K/AKT/mTOR pathway proteins upregulate apoptosis-related proteins, the expression of PARP, Caspase-3, Caspase-9, and MDM2 were studied following exposure to CDDP, AuNPs alone and AuNPs:CDDP combination for 24 h. 10 $\mu\text{g/mL}$ of quercetin was used as a positive control for 24 h as it induced mitochondrial-related apoptosis by increasing the expression of caspases [203]. As shown in **Figure 6.9A**, treatment of A549 cells with AuNPs:CDDP upregulated the expression of PARP, caspase-3, caspase-9 and downregulated the expression of MDM2. Treatment with 10 $\mu\text{g/mL}$ of quercetin showed a similar pattern in increasing the expression of PARP, caspase-3, caspase-9, and downregulation of MDM2. When the relative protein expression was semi-quantified with ImageJ, a significant ($p < 0.05$) increase in the relative expression of PARP, Caspase-3, and Caspase-9 was observed with AuNPs:CDDP combination compared to AuNPs and CDDP alone (**Figure 6.9B**). In addition, the expression of MDM2 protein was significantly downregulated with AuNPs:CDDP combination ($p < 0.05$) compared to AuNPs and CDDP alone.

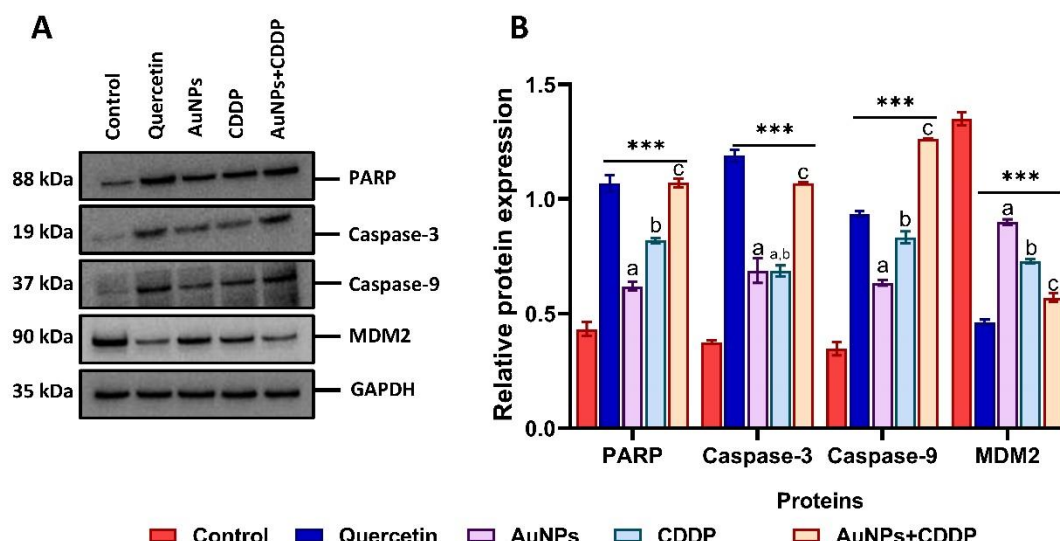


Figure 6.9. Effects of AuNPs, CDDP and AuNPs:CDDP combination on the expression of mitochondrial related-proteins (A) protein expression of PARP, Caspase-3, Caspase-9, and MDM2 detected by western blotting, (B) Relative protein expression of PARP, Caspase-3, Caspase-9, and MDM2 semi-quantified by ImageJ Software. Quercetin (10 $\mu\text{g/mL}$) was used as a positive control. Plotted graphs represent the means $\pm$ SD of three independent experiments. Bars with an asterisk (*) show statistical difference (***) $p < 0.001$ compared with the control. Data with different letters above the bars represent the statistically significant difference ($p < 0.05$) between the combination of CDDP:AuNPs, CDDP, and AuNPs alone.

6.3 Discussion

In this chapter, the effects of combined therapy of citrate functionalised AuNPs (10 nm) with the anticancer drug cisplatin (CDDP) were evaluated. As highlighted in Chapters 3 and 5, monodispersed AuNPs with an average size distribution of 8-14 nm were used.

The effects of different concentrations of CDDP, AuNPs on cell viability were assessed using the mLDH and MTT assays. IC_{50} values were found to be 2.191 $\mu\text{g/mL}$ for CDDP and 38.92

$\mu\text{g/mL}$ for AuNPs. This was also highlighted by, Guemei et al., [508] who found that CDDP significantly reduced A549 cell viability compared to AuNPs. In addition, AuNPs did not show any cytotoxicity to A549 cells at all the concentrations tested (0.002-5 $\mu\text{g/mL}$) [508]. Based on our cell viability analysis, the IC_{50} value of CDDP (2.191 $\mu\text{g/mL}$) and 25 $\mu\text{g/mL}$ of AuNPs were used to study the effect of the AuNPs:CDDP combination on A549 cell viability. Interestingly, the cytotoxicity assays demonstrated that the AuNPs:CDDP combination elicits a greater cytotoxicity than monotherapies (CDDP and AuNPs alone). In line with our results, a previous study showed that the combination of CDDP and AuNPs had a greater potential of inhibiting the growth of HeLa (cervical), AGS (human hyperdiploid), and C6 (glioma) cell lines compared to CDDP and AuNPs alone [509]. Likewise, combination of CDDP and AuNPs have been reported to reduce the proliferation of different human ovarian cancer (A2780, OVCAR5, and SKOV3-iP) at low IC_{50} values compared to CDDP alone [510]. In addition, the cell morphology data (Section 6.2.3) suggested greater cytotoxicity with AuNPs:CDDP combination. The high cytotoxicity of combination therapy could be mediated by the ability of AuNPs to reduce tumour stress and decompose its vessels to improve CDDP delivery [38, 511]. To gain a deeper understanding of the mechanisms of cytotoxicity, the effects of the combination therapy on oxidative stress makers was first studied. The results revealed that AuNPs:CDDP combination induced oxidative stress by increasing ROS and reducing the intracellular glutathione levels. Oxidative stress arises because of an imbalance between the production of free radicals in the cells and the inability of antioxidants to neutralise them [172]. In addition, the generation of ROS induces a loss in MMP and release of mitochondrial proteins such as cytochrome C from the inner space into the cytosol which is considered to trigger caspase-dependent apoptosis [512, 513]. In this work, AuNPs: CDDP combination was found to decrease MMP by attenuating the mitochondrial fluorescence intensity in A549 cells. Some studies suggest that

the generation of ROS coupled with a decrease in MMP could lead to the activation of caspase-dependent mitochondrial apoptosis in the A549 tumour cells [131, 399, 514]. Interestingly, our results for Annexin V and caspase 3/7 activity assays demonstrated similar findings. This suggests that apoptosis is another mechanism by which the combination therapy selectively destroyed A549 cancer cells. In agreement with our study, Zhao et al., [38] reported that CDDP conjugation to AuNPs potentiates the antitumour effects of CDDP through apoptosis induction.

HSPs have been recently recognised as a novel therapeutic target in cancer due to their roles in cell growth, differentiation, immune regulation, and proteolytic degradation [515]. These proteins are ubiquitously overexpressed in various malignant cells including A549 cells and their overexpression has been linked to metastasis and resistance to chemotherapeutic drugs [311]. Inhibitors of HSPs are clinically limited due to their off-target toxicity and lack of *in vivo* data [516]. Therefore, in this work effect of AuNPs:CDDP combination on HSP40, HSP60, HSP70, and HSP90 proteins was studied in A549 cells. It was observed that exposure to combination therapy downregulated the expression of all the proteins. Semi-quantification of band intensity by ImageJ software demonstrated a significant decrease in the relative expression of the proteins ($p < 0.05$) compared with the control, CDDP and AuNPs alone. Although the effect of AuNPs or combination of drugs and NPs has never been studied against HSPs at the time of this study, previous research showed that ibuprofen enhances the anticancer potential of CDDP by suppressing the expression of HSP70 and HSF-1 in A549 cells [517]. Studies have suggested that dysregulation of P13K/AKT/mTOR signalling pathway in cancer patients is mainly through HSPs and the UPS [232, 518]. Thus, it is likely that overexpression of HSPs mediates the expression of Akt proteins and its downstream target in A549 cells by forming the HSP-AKT complex, thereby promoting cell survival [455, 458]. It was

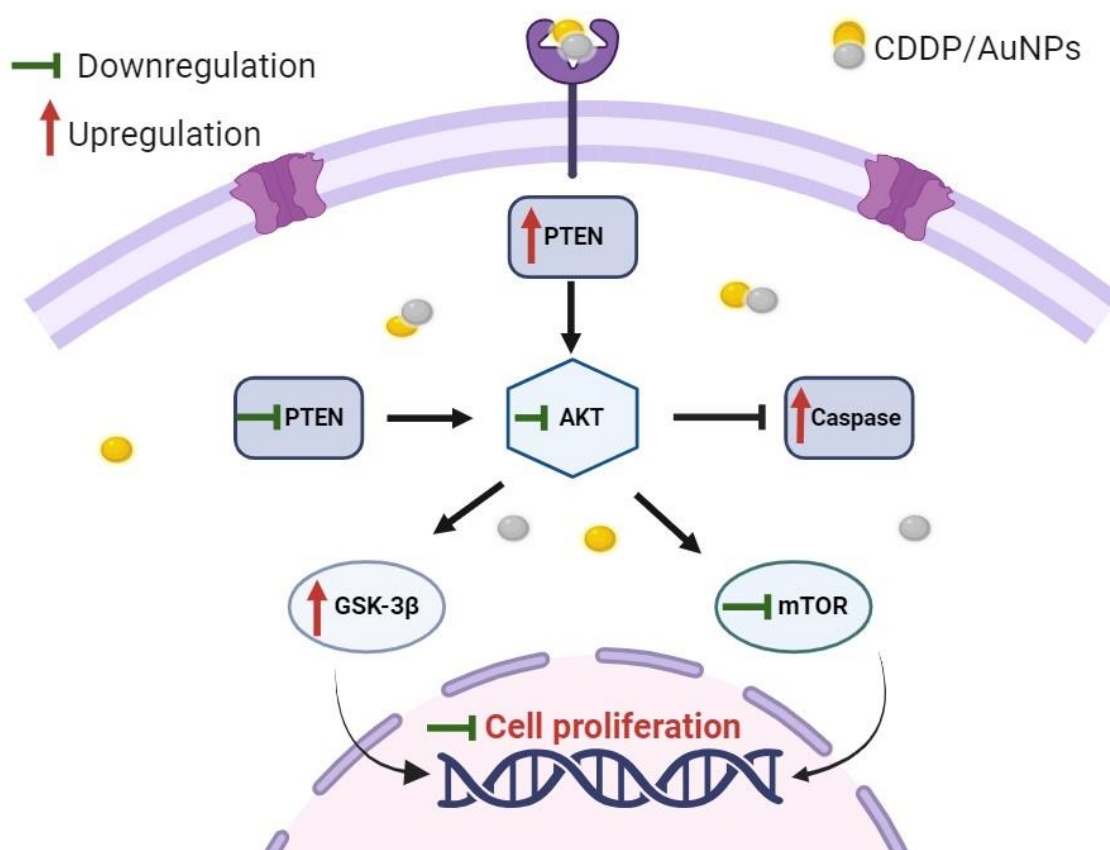
shown that AuNPs:CDDP combination significantly downregulated the expression of AKT and mTOR proteins to a greater extent than the control. Unlike the AuNPs:CDDP combination, AuNPs alone showed no effects on the expression of either AKT or mTOR proteins. In addition, it was shown that AuNPs:CDDP combination upregulated the expression of PTEN and GSK-3 β proteins, suggesting that the combination therapy reduced the proliferation of A549 cells. This finding is consistent with others who showed that lowering the expression of HSPs triggers the downregulation of P13K/AKT/mTOR pathway proteins in tumour cells [510, 519].

Next, the molecular mechanisms of mitochondrial cell death were studied, since both HSPs and AKT overexpression provides a protective effect against A549 apoptosis by the inhibition of apoptosome or forming a complex with MDM2 proteins [520-522]. A significant upregulation of mitochondrial apoptotic proteins (PARP, caspase-3, and caspase-9) was observed in A549 cells when incubated with all the treatments. However, the upregulation was lower with AuNPs alone. Furthermore, the decrease in the expression of MDM2 proteins suggest the activation of mitochondrial apoptosis. This is in agreement with what others have found in which they showed an association between HSPs downregulations in A549 cells and activation of mitochondrial-dependent apoptosis [517].

6.4 Conclusion

In conclusion, the study provides evidence that AuNPs might potentiate the anticancer properties of CDDP in A549 cells through the downregulation of HSPs, P13K/AKT/mTOR pathway, and upregulation of mitochondrial related proteins. The results of the study showed that combination of CDDP with AuNPs exerted synergistic or an additive effect in A549 cells, resulting in increased anticancer effects of CDDP compared to single treatment. The finding

may also suggest further opportunities for the development of a novel strategy to increase the effectiveness of anticancer drugs as well novel targets for cancer therapies.



Schematic 6.1: Schematic illustration summarising the findings of this chapter and the effects of CDDP and AuNPs on HSPs, P13K/AKT/mTOR and apoptosis in A549 cells. The green arrow indicates the proteins that were downregulated while the red arrow indicates the proteins that were downregulated upon exposure to AuNPs:CDDP. The scheme was created with BioRender.com

Abbreviations: Cisplatin (CDDP), Gold nanoparticles (AuNPs), Phosphatase and tension homolog (PTEN), Serine 473 (Ser473), Protein kinase B (AKT), Glycogen synthase kinase-3β (GSK-3β), Glycogen synthase kinase (GSK), Mammalian target rapamycin complex (mTOR), Heat shock proteins (HSPs).

7.1 General Discussion

In the Chapter one, the effects of different sizes AuNPs on ubiquitin proteasome system in A549 cells was studied with a particular focus on deubiquitinating enzymes such as USP7, USP8, USP10, and UCHL-1. A549 cells were used in this work because lungs represent the first portal to the body after prolonged inhalation of NMs and therefore can be used for studying AuNPs' cytotoxicity on one type of lung cells. In the last few years drugs such as Bortezomib, Carfilzomib, D395, and MG132 have shown to reduce tumour cell viability by inhibiting the ubiquitin proteasome system as highlighted by different researchers [523-526]. Nonetheless, extensive rate of peripheral neuropathy, poor rate of delivery, rapid clearance, and low biocompatibility have limited their clinical application in cancer [527, 528]. This chapter characterised different size AuNPs in ultrapure water (UPW) and complete culture media (CCM) to investigate the changes in their physicochemical properties before performing cytotoxicity studies. All the different sizes of AuNPs tested have shown a greater hydrodynamic diameter in CCM compared to UPW in both at 0 and 24 h. The TEM images demonstrated well-dispersed spherically shaped AuNPs in UPW and had negative zeta potential. Regarding cytotoxicity, AuNPs cytotoxicity assessed by MTT and LDH released indicated that 80 nm AuNPs were less cytotoxic ($IC_{50}=18.30 \mu\text{g/mL}$) compared to 10 nm ($IC_{50}=16.22 \mu\text{g/mL}$) and 5 nm ($IC_{50}=12.61 \mu\text{g/mL}$) AuNPs. The potent higher cytotoxic effect of small-size AuNPs may be due to their higher surface area to volume ratio. The higher surface area of small-size AuNPs gives them more available space to interact with the cellular components such as nucleic acid, carbohydrates, proteins and fatty acid and results in toxicity [529, 530]. The toxicity of the small-size AuNPs perhaps may also be related to the fact that

there are more particle numbers at the highest concentration (40 µg/mL) of 5 nm AuNPs (3.17×10^{13} NPs/mL) than for the equivalent mass concentration (40 µg/mL) of 10 nm AuNPs (2.5×10^{12} NPs/mL) and 80 nm AuNPs (8.36×10^9 NPs/mL). However, this is not always true as the normalisation results by particles number concentration suggest different outcomes as illustrated in the supplementary chapter (**Table S3.2**) where by 80 nm AuNPs could be regarded as more cytotoxic on a particle number basis since it required less amount of particle number to inhibit cell viability. Internalisation of AuNPs is also another factor that is linked to toxicity of smaller size AuNPs compared to their counterparts [531, 532]. Small-size AuNPs can be internalised into the cells in large number easily without requiring any active energy compared to larger AuNPs as suggested by others [79, 533]. The toxicity assays agreed with previous studies that revealed size-dependent cytotoxicity of AuNPs [79, 380, 534]. Low uptake of larger AuNPs was expected due to their lower toxicity to the cells but surprisingly the opposite was true with 80 nm AuNPs showing the highest uptake as revealed by ICP-MS and flow cytometry. The higher uptake of large size (80 nm) AuNPs can be linked to the amount of energy needed for wrapping the AuNPs, and the kinetics of diffusion of the receptors to the binding sites in the biological membrane [69]. For the uptake of small size AuNPs only few receptors are needed for the wrapping of AuNPs, and consequently less free energy will be produced to wrap the AuNPs on the surface of biological membrane. In the case of larger AuNPs more receptors are needed to wrap AuNPs, this in turn affects the kinetics of diffusion of the receptors to the binding sites [532, 535]. It also suggests that there might be an optimal AuNPs size for the active cellular uptake; for instance in HeLa cells treated with different sizes of AuNPs (14, 30, 50, 74 and 100 nm), 50 nm AuNPs had been observed to have higher uptake efficiency compared to other small sizes AuNPs [73]. In addition, a dose-dependent cellular uptake of AuNPs was noted based on their mass concentration. Flow

cytometry confirmed the same results which were comparable to those of ICP-MS. The cells were imaged using TEM, which showed that all the AuNPs were localised in the endosome after 24 h exposure. Moreover, all AuNPs were internalised through clathrin- and caveolae-mediated endocytosis as identified by ICP-MS and flow cytometry. Previous researchers demonstrated that AuNPs can be localised within endosomes following uptake through clathrin- and caveolae-mediated endocytosis [536, 537]. Further studies, including application of higher resolution transmission X-ray microscopy (TXM), 3D confocal microscopy and energy dispersive X-ray (EDX) are however needed to validate the internalisation of AuNPs in the endosome. Moreover, as observed in the work presented in Chapter 3, the cellular internalisation of AuNPs led to the destabilization of the cell membrane as well as generation of intracellular reactive oxygen species (ROS), depletion in intracellular glutathione and triggering of mitochondrial related apoptosis. In addition, small size AuNPs displayed greater percentage of toxicity compared to 10 and 80 nm, this may be related to higher dissolution of 5 nm AuNPs and release of Au^+ that ultimately cause toxicity [538]. Other studies agree with these findings; for instance, Khaing et al., [539] reported that exposure to 15 nm AuNPs led to significant increase in ROS generation compared to 66 and 100 nm AuNPs. Chakraborty et al., [456] showed that 46 nm AuNPs, enhanced the rate of ROS production and induced apoptosis by disrupting the mitochondrial membrane potential of osteosarcoma (MG63) cells compared to 60 nm AuNPs [456]. However, some studies have suggested that the toxicity of NPs depend on their rapid clearance from the cells rather than size [540, 541]. When internalised into the cells NPs circulate in the blood until there are cleared from circulation and eliminated through renal or hepatobiliary elimination mechanisms [542, 543]. For instance, it has shown that increased in AuNPs size (14, 50 and 74nm) decrease the rate of AuNPs clearance by the HeLa cells thereby by inducing more cytotoxic effects [544]. An

important finding in this Chapter was that AuNPs similar to MG132 were able to downregulate the expression level of overexpressed USP7, USP8, USP10, and UCHL-1 in a dose-dependent manner, implying that AuNPs could be used to target the proteasome system. Downregulation of these proteins was also observed to be size and concentration dependent with small-size (5 nm) having a significant effect on the protein's expression. Decrease in the percentage of cell viability and ROS generation observed correlates with a decrease in the expression of the protein. Furthermore, the inhibition of deubiquitinating enzymes resulted in the induction of mitochondrial apoptosis via caspase-3, caspase-9, and PARP upregulation. Based on the results of this chapter 80 nm has showed lower toxicity to A549 compared to 5 and 10 nm AuNPs in all concentrations used for the study and it could therefore be an ideal candidate to be used in pharmaceutical industry for the treatment of cancer and drug delivery.

The key findings from the first experimental Chapter were taken into consideration in order to investigate the effect of functionalisation on the ubiquitin proteasome system. This Chapter highlighted the feasibility of using AuNPs functionalised with tannic acid, citrate, and PVP. AuNPs characterisation revealed that AuNPs are well dispersed and spherical in shape with a hydrodynamic diameter similar to that reported by the Suppliers. The non-cytotoxic AuNPs concentrations were determined by the mLDH and MTT assays and were used for other cytotoxicity assays. The cytotoxicity mechanisms associated with AuNPs such as ROS, MMP, and GSH demonstrated that PVP functionalised AuNPs induced greater cytotoxicity compared to tannic acid & citrate functionalised AuNPs functionalised ($p < 0.05$). In agreement with these results, other studies have also revealed how the nature of surface functionalisation dictate the degree of toxicity; for instance, It was reported that AuNPs functionalised with citrate can inhibit the proliferation rate of A549 cells associated with the

concentration and time of NPs incubation compared to cetyltrimethylammonium bromide (CTAB) [545]. Another study showed that citrate functionalised AuNPs were also cytotoxic to breast cancer (MCF-7) and prostate cancer (PC-3) compared to starch and gymnemic acid (GA) functionalised AuNPs [546]. Together they suggest that the toxicity of citrate functionalised AuNPs could be due to the acidic nature of the citrate. Contrarily to these, others have shown that functionalisation of AuNPs only improved the stability and delivery of AuNPs by forming a protective layer on the surface of AuNPs to prevent their aggregation in the biological fluids instead of increasing the rate of toxicity [547, 548]. Polyethylene glycol (PEG) functionalisation has been reported to efficiently mediate the intracellular delivery of less than 30 nm AuNPs [548]. Again, when researchers studied the toxic effects of PEG, citrate, and PVP AuNPs, they reported that surface functionalisation has no effects on toxicity [94, 398, 549]. However, this is not always true as cell viability assay of the functionalisation agents suggest that there are positive correlations between the functionalisation agents in the supplementary chapter (**Figure S4.2**). The concentrations and the type of functionalisation agents used during the AuNPs synthesis determine the rate of their cytotoxicity [545]. Interestingly downregulation in the expression of USP7, USP8, USP10, and UCHL-1 was observed at 10 $\mu\text{g/mL}$ for all functionalised AuNPs in this chapter. In addition, the downregulation of these DUBs was observed be functionalisation and time dependent. This perhaps supports the evidence that functionalisation agents play a critical role in enhancing AuNPs toxicity. However, other studies including studying the effects of functionalisation agents and other mechanisms of cytotoxicity are necessarily needed to understand how the functionalising agents alone enhance the rate of cytotoxicity. PI3K/AKT/mTOR, and Wnt signalling pathways are crucial in regulating the rate of cell proliferation and development [550, 551]. Overexpression of PI3K/AKT/mTOR required the activity of deubiquitinating

enzymes (DUBs) to remove the K63 linked polyubiquitin chain on AKT to allow to recycle in tumour cells [550]. Therefore, inhibition of PI3K/AKT/mTOR and Wnt pathway required further investigation after inhibition of DUBs. AKT, p-AKT, mTOR, GSK3 β , β -catenin, and p- β -catenin were found to be downregulated at 48 h when incubated with different functionalised AuNPs. Additionally, it was further observed that the inhibition of PI3K/AKT/mTOR and Wnt signalling pathways mediate mitochondrial apoptosis through the upregulation of caspase-3, caspase-9, and PARP. Based on the results of this chapter 5 nm Tannic acid functionalised AuNPs have demonstrated lower toxicity to A549 compared to citrate and PVP functionalised AuNPs in all concentrations used for the study and it could therefore be an ideal candidate to be used in pharmaceutical industry for the treatment of cancer and drug delivery.

The third experimental described the effects of AgNPs and AuNPs on molecular chaperones heat shock proteins (HSPs) in A549 cells. HSPs inhibitors such as RP101, Quercetin, radicicol, and geldanamycin have unfortunately been suspended in Phase 1 clinical trials due to their structural instability, *in vitro* and *in vivo* cytotoxicity, and poor biocompatibility [325, 552, 553]. Therefore, the development of other HSPs inhibitors is urgently needed as a feasible therapeutic target in different tumour cells. In an attempt to contribute to this, the effects of citrate functionalised AgNPs and AuNPs on molecular chaperone HSPs were studied. The NPs were first characterised in UPW and spherical dispersed particles with hydrodynamic size distribution close to the hydrodynamic diameter was reported by the suppliers. The dispersion of particles in complete culture media (CCM) create a new biological identity (protein corona) that changes the hydrodynamic diameter and polydispersity index (PDI) of both the AgNPs and AuNPs, indicating NPs interactions with the biomolecules in the CCM [554, 555]. The presence of these biomolecules in the CCM also affected the surface charge of both AgNPs and AuNPs as seen in the zeta potential results, which became apparently

lower in CCM than in UPW due to serum protein absorption [554]. When exposed to a mass concentrations ranging of 1.25-35 $\mu\text{g/mL}$, AuNPs ($\text{IC}_{50}=28.05 \text{ } \mu\text{g/mL}$) demonstrated less cytotoxic effects compared to AgNPs ($\text{IC}_{50}=13.87 \text{ } \mu\text{g/mL}$). The higher cytotoxicity of AgNPs compared to AuNPs could be potentiated with release of Ag^+ which could bind to the proteins in the cell membrane structure and affect the membrane integrity [469, 556]. This is confirmed by the dissolution measurement in the **Figure 5.2** that shows higher dissolution of Ag^+ compared to Au^+ dissolution. In accordance with these results, AgNPs have been reported to be highly toxic to tumour cells, more so than AuNPs: for instance Pem et al., [556] report that 10 nm AgNPs decrease the percentage of cell viability of murine fibroblast (L929) by more than 50% at a dose 5 times lower than AuNPs. Considering this was related to proteins, concentrations (1.25-10 $\mu\text{g/mL}$) less than the IC_{50} were used for further experiments to avoid affecting protein concentration in the samples. It was further revealed that AuNPs were less cytotoxic to 3D tumour spheroids compared to AgNPs as assessed by the Live/Dead assays. It was also found that AgNPs prompted a higher degree of ROS and apoptosis in A549 cells compared to AuNPs which prompted a minimal level of ROS intensity as well as apoptosis. These further demonstrate the safety profile of AuNPs compared to AgNPs at lower dose levels. Similar findings regarding the low or non-toxicity of AuNPs in comparison to AgNPs have been reported in earlier investigations. For example, using the same size (31 nm) at a concentration range of 2-10 $\mu\text{g/mL}$, AuNPs showed minimal ROS generation and higher percentage of apoptosis induction in human colorectal adenocarcinoma (HT29) compared to AgNPs [557]. Similarly, Haase et al., [84] reported that 20 nm AuNPs triggers less oxidative stress and apoptosis in human macrophage (THP-1) even at a concentration range of 10-200 $\mu\text{g/mL}$ compared 20 nm AgNPs. Together, they suggest that the relatively lower cytotoxicity

of AuNPs could be due to their inert nature, lower cellular internalisation, and biocompatibility.

Western blotting results have shown that AgNPs significantly downregulated the highly conserved expression of HSP40, HSP60, HSP70 and HSP90 ($p < 0.001$) compared to control. On the other hand, AuNPs did not show any effect on the expression level of HSPs throughout the incubation time. As reported, HSPs interact with the P13K/AKT/mTOR signalling pathway to increase the proliferation of tumour cells [458]. Since the inhibition of HSPs correlates with the downregulation of AKT, mTOR, GSK3 β and β -catenin proteins, this was investigated and the inhibitions of HSPs were found to downregulate these proteins upon treatment with only AgNPs. Having established that AgNPs can modulate the overexpressed P13K/AKT/mTOR and Wnt related proteins the study further investigated if the downregulation of P13K/AKT/mTOR and Wnt results in upregulation of PARP, caspase-3 and caspase-9 because there are the well-known indicators of mitochondrial related apoptosis [558, 559]. The results in this chapter suggested that the inhibition of AKT and β -catenin may lead to the upregulation of caspase-3, caspase-9, and PARP. Based on the results of this chapter AuNPs showed no profound effect to A549 compared to AgNPs in all concentrations used for the study and it could therefore be an ideal candidate to be used in pharmaceutical industry for the treatment of cancer and drug delivery. Based on the results of this chapter, AuNPs were used to investigate the effects of AuNPs combination with a well-known platinum-containing anticancer drug (Cisplatin) on molecular chaperone HSPs in A549, which was presented in the chapter 4.

10 nm citrate functionalised AuNPs was used in fourth experimental chapter to study if the combination of AuNPs and CDDP affects the expression of molecular chaperone HSPs in A549 cells. The results revealed that AuNPs:CDDP combination caused profound effects on A549

cells through a decrease in percentage cell viability, accumulation of ROS, depletion of glutathione and loss of MMP compared to individual treatments of each alone. It was also found that AuNPs:CDDP combination decreased the expression of HSPs (HSP40, HSP60, HSP70, and HSP90), AKT, mTOR, MDM2, and upregulated the expression of PTEN, GSK-3 β as well as mitochondrial related apoptosis proteins (PARP, caspase-3, and caspase-9). This was therefore the first study to investigate the effects of the combination of AuNPs and CDDP on molecular chaperone HSPs in A549 cells. Other work, however, has shown that that combination of two drugs (ibuprofen and cisplatin) suppressed the overexpression of HSP in A549 cells which led to the induction of mitochondrial mediated apoptosis compared to individual treatments of the two drugs [560]. The work in this Chapter highlighted the potential importance of AuNPs combination with CDDP to enhance the anticancer efficacy of CDDP through the downregulation of HSPs and P13K/AKT/mTOR signalling.

7.2 Conclusion

In conclusion, this thesis investigated how different sized (5, 10 and 80 nm) and functionalised AuNPs (tannic acid, citrate and PVP) induced cytotoxicity, oxidative stress, mitochondrial damage, and apoptosis in A549 cells. The study also shed light on how AuNPs can be used to reduce the proliferation of cancer cells through the inhibition of DUBs, P13K/AKT/mTOR and Wnt pathway related proteins. The importance of inhibition of HSPs proteins by NPs (AgNPs and AuNPs) in A549 cells was also studied and the effects of their inhibition on suppressing the overexpressed P13K/AKT/mTOR related proteins as well as enabling the induction of mitochondrial related apoptosis were investigated. This thesis provides first evidence that AuNPs might enhance the therapeutic effect of the anticancer drug cisplatin through the inhibition of HSPs. In addition, the study provides first evidence of the targeting of ubiquitin

proteasome system and HSPs by NPs in A549 cells. The findings of this thesis may lead to the development of novel therapeutic modalities to decrease cancer cells proliferation by targeting DUBs and molecular chaperone HSPs.

7.3 Future work or recommendations

While this thesis demonstrated that ubiquitin proteasome system and molecular heat shock proteins can be targeted in A549 cells using NPs, it was also found that size, surface functionalised, concentration and incubation time are major contributors in the cytotoxicity of NPs. Therefore, future work should focus on:

- Comparing the effects of AgNPs and dissolved Ag on molecular chaperone heat shock proteins to determine whether the inhibition is induced by the dissolved Ag or AgNPs
- The use of other target approach such as target activation, conjugation, or E-ligases enzymes to identify other ways of inhibiting ubiquitin proteasome using AuNPs and AgNPs.
- Further investigation to understand if AuNPs and AgNPs can be used as proteasome and molecular chaperone inhibitors i.e., a study of their effects on ubiquitin proteasome system and molecular chaperone HSPs in different cell types could be considered.
- The current study demonstrated that inhibition of molecular chaperone HSPs and UPS enzymes can affect the protein expression of P13K/AKT/mTOR and Wnt pathway-related proteins. More in-depth investigation is needed to investigate how AgNPs and AuNPs alter the P13K/AKT/mTOR and Wnt intracellular signalling pathway.
- Cell viability assay from a developed 3D spheroid model demonstrated that AgNPs and AuNPs have a minimal impact on 3D cell culture after 24 h exposure. Other

mechanisms of cytotoxicity such as ROS production, genotoxicity, apoptosis, and internalisation are recommended to compare the effects of NPs in 2D, and 3D model.

- Although the current study demonstrated that combining AuNPs with CDDP can enhance CDDP's cytotoxicity on HSPs. Further studies to investigate the effects of AuNPs combination with CDDP at various concentrations and exposure conditions can be considered in A549 cells as well as other types of cells. The effects of combining AuNPs with other platinum-based compounds such as oxaliplatin, carboplatin and lobaplatin could also be considered as a potential strategy for overcoming tumour cell drug resistance.
- Additional cytotoxicity of combined therapy of AuNPs with CDDP on ubiquitin-proteasome system in A549 cells could be explored. The assessment can also be extended to both cancerous and non-cancerous cell lines.

References

1. Fytianos, G., A. Rahdar, and G.Z. Kyzas, *Nanomaterials in Cosmetics: Recent Updates*. Nanomaterials (Basel), 2020. **10**(5).
2. McIntyre, R.A., *Common nano-materials and their use in real world applications*. Sci Prog, 2012. **95**(Pt 1): p. 1-22.
3. Abdelhamid, H.N. and G. Badr, *Nanobiotechnology as a platform for the diagnosis of COVID-19: a review*. Nanotechnology for Environmental Engineering, 2021. **6**(1).
4. Hochella, M.F., Jr., et al., *Natural, incidental, and engineered nanomaterials and their impacts on the Earth system*. Science, 2019. **363**(6434).
5. Sharma, V.K., et al., *Natural inorganic nanoparticles--formation, fate, and toxicity in the environment*. Chem Soc Rev, 2015. **44**(23): p. 8410-23.
6. Lespes, G., S. Faucher, and V.I. Slaveykova, *Natural Nanoparticles, Anthropogenic Nanoparticles, Where Is the Frontier?* Frontiers in Environmental Science, 2020. **8**.
7. Bandala, E.R. and M. Berli, *Engineered nanomaterials (ENMs) and their role at the nexus of Food, Energy, and Water*. Materials Science for Energy Technologies, 2019. **2**(1): p. 29-40.
8. Yokel, R.A. and R.C. Macphail, *Engineered nanomaterials: exposures, hazards, and risk prevention*. J Occup Med Toxicol, 2011. **6**: p. 7.
9. Hu, W., et al., *Graphene-based antibacterial paper*. ACS Nano, 2010. **4**(7): p. 4317-23.
10. Patel, K.D., R.K. Singh, and H.-W. Kim, *Carbon-based nanomaterials as an emerging platform for theranostics*. Materials Horizons, 2019. **6**(3): p. 434-469.
11. Rastogi, A., et al., *Impact of Metal and Metal Oxide Nanoparticles on Plant: A Critical Review*. Front Chem, 2017. **5**: p. 78.

12. Yoon, Y., et al., *Metal-Oxide Nanomaterials Synthesis and Applications in Flexible and Wearable Sensors*. ACS Nanoscience Au, 2021. **2**(2): p. 64-92.
13. Sanka, R.V.S.P., et al., *Soft Self-Healing Nanocomposites*. Frontiers in Materials, 2019. **6**.
14. Herizchi, R., et al., *Current methods for synthesis of gold nanoparticles*. Artif Cells Nanomed Biotechnol, 2016. **44**(2): p. 596-602.
15. Panzarini, E., et al., *Intracellular Transport of Silver and Gold Nanoparticles and Biological Responses: An Update*. Int J Mol Sci, 2018. **19**(5).
16. Sani, A., C. Cao, and D. Cui, *Toxicity of gold nanoparticles (AuNPs): A review*. Biochem Biophys Rep, 2021. **26**: p. 100991.
17. Slepicka, P., et al., *Methods of Gold and Silver Nanoparticles Preparation*. Materials (Basel), 2019. **13**(1).
18. Quintero-Quiroz, C., et al., *Optimization of silver nanoparticle synthesis by chemical reduction and evaluation of its antimicrobial and toxic activity*. Biomater Res, 2019. **23**: p. 27.
19. Wiley, B., et al., *Shape-controlled synthesis of metal nanostructures: the case of silver*. Chemistry, 2005. **11**(2): p. 454-63.
20. Piella, J., N.G. Bastús, and V. Puntes, *Size-Controlled Synthesis of Sub-10-nanometer Citrate-Stabilized Gold Nanoparticles and Related Optical Properties*. Chemistry of Materials, 2016. **28**(4): p. 1066-1075.
21. Genc, R., et al., *Green synthesis of gold nanoparticles using glycerol-incorporated nanosized liposomes*. Langmuir, 2011. **27**(17): p. 10894-900.

22. Bhattarai, B., Y. Zaker, and T.P. Bigioni, *Green synthesis of gold and silver nanoparticles: Challenges and opportunities*. Current Opinion in Green and Sustainable Chemistry, 2018. **12**: p. 91-100.
23. Katas, H., et al., *Biosynthesis and Potential Applications of Silver and Gold Nanoparticles and Their Chitosan-Based Nanocomposites in Nanomedicine*. Journal of Nanotechnology, 2018. **2018**: p. 1-13.
24. Shankar, S.S., et al., *Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes*. Journal of Materials Chemistry, 2003. **13**(7).
25. Ashraf, J.M., et al., *Green synthesis of silver nanoparticles and characterization of their inhibitory effects on AGEs formation using biophysical techniques*. Sci Rep, 2016. **6**: p. 20414.
26. Suman, T.Y., et al., *The Green synthesis of gold nanoparticles using an aqueous root extract of Morinda citrifolia L*. Spectrochim Acta A Mol Biomol Spectrosc, 2014. **118**: p. 11-6.
27. Hammami, I., et al., *Gold nanoparticles: Synthesis properties and applications*. Journal of King Saud University - Science, 2021. **33**(7).
28. Li, X., et al., *Biosynthesis of Nanoparticles by Microorganisms and Their Applications*. Journal of Nanomaterials, 2011. **2011**: p. 1-16.
29. Mafuné, F., et al., *Formation of Gold Nanoparticles by Laser Ablation in Aqueous Solution of Surfactant*. The Journal of Physical Chemistry B, 2001. **105**(22): p. 5114-5120.
30. Levy, A., et al., *Surface Chemistry of Gold Nanoparticles Produced by Laser Ablation in Pure and Saline Water*. Langmuir, 2021. **37**(19): p. 5783-5794.

31. Chugh, H., et al., *Role of gold and silver nanoparticles in cancer nano-medicine*. Artif Cells Nanomed Biotechnol, 2018. **46**(sup1): p. 1210-1220.
32. Yamada, M., M. Foote, and T.W. Prow, *Therapeutic gold, silver, and platinum nanoparticles*. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2015. **7**(3): p. 428-45.
33. Sun, H., et al., *Gold Nanoparticle-Induced Cell Death and Potential Applications in Nanomedicine*. Int J Mol Sci, 2018. **19**(3).
34. Sztandera, K., M. Gorzkiewicz, and B. Klajnert-Maculewicz, *Gold Nanoparticles in Cancer Treatment*. Mol Pharm, 2019. **16**(1): p. 1-23.
35. Kalepu, S. and V. Nekkanti, *Insoluble drug delivery strategies: review of recent advances and business prospects*. Acta Pharm Sin B, 2015. **5**(5): p. 442-53.
36. Hussein, H.A. and M.A. Abdullah, *Novel drug delivery systems based on silver nanoparticles, hyaluronic acid, lipid nanoparticles and liposomes for cancer treatment*. Applied Nanoscience, 2021.
37. Wilczewska, A.Z., et al., *Nanoparticles as drug delivery systems*. Pharmacological Reports, 2012. **64**(5): p. 1020-1037.
38. Zhao, X., et al., *Gold nanoparticles enhance cisplatin delivery and potentiate chemotherapy by decompressing colorectal cancer vessels*. Int J Nanomedicine, 2018. **13**: p. 6207-6221.
39. Gouyau, J., et al., *Investigation of Nanoparticle Metallic Core Antibacterial Activity: Gold and Silver Nanoparticles against Escherichia coli and Staphylococcus aureus*. Int J Mol Sci, 2021. **22**(4).
40. Mohamed, M.M., et al., *Antibacterial effect of gold nanoparticles against Corynebacterium pseudotuberculosis*. Int J Vet Sci Med, 2017. **5**(1): p. 23-29.

41. Jiang, H.S., et al., *Interaction between Silver Nanoparticles and Two Dehydrogenases: Role of Thiol Groups*. *Small*, 2019. **15**(27): p. e1900860.
42. Korani, S., et al., *Evaluation of Antimicrobial and Wound Healing Effects of Gold Nanoparticles Containing Abelmoschus esculentus (L.) Aqueous Extract*. *Bioinorg Chem Appl*, 2021. **2021**: p. 7019130.
43. Kim, T.-S., J.-R. Cha, and M.-S. Gong, *Investigation of the antimicrobial and wound healing properties of silver nanoparticle-loaded cotton prepared using silver carbamate*. *Textile Research Journal*, 2017. **88**(7): p. 766-776.
44. Tessaro, L., et al., *A systematic review on gold nanoparticles based-optical biosensors for Influenza virus detection*. *Sensors and Actuators Reports*, 2021. **3**.
45. Wang, J., et al., *Gold nanoparticles in virus detection: Recent advances and potential considerations for SARS-CoV-2 testing development*. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 2022. **14**(1): p. e1754.
46. Reina, G., et al., *Hard Nanomaterials in Time of Viral Pandemics*. *ACS Nano*, 2020. **14**(8): p. 9364-9388.
47. Pramanik, A., et al., *The rapid diagnosis and effective inhibition of coronavirus using spike antibody attached gold nanoparticles*. *Nanoscale Adv*, 2021. **3**(6): p. 1588-1596.
48. Almanza-Reyes, H., et al., *Evaluation of silver nanoparticles for the prevention of SARS-CoV-2 infection in health workers: In vitro and in vivo*. *PLoS One*, 2021. **16**(8): p. e0256401.
49. Foroozandeh, P. and A.A. Aziz, *Insight into Cellular Uptake and Intracellular Trafficking of Nanoparticles*. *Nanoscale Res Lett*, 2018. **13**(1): p. 339.
50. Harayama, T. and H. Riezman, *Understanding the diversity of membrane lipid composition*. *Nat Rev Mol Cell Biol*, 2018. **19**(5): p. 281-296.

51. Contini, C., et al., *Nanoparticle–membrane interactions*. Journal of Experimental Nanoscience, 2017. **13**(1): p. 62-81.
52. Abdelkhaliq, A., et al., *Impact of nanoparticle surface functionalization on the protein corona and cellular adhesion, uptake and transport*. J Nanobiotechnology, 2018. **16**(1): p. 70.
53. Kim, S. and I.H. Choi, *Phagocytosis and endocytosis of silver nanoparticles induce interleukin-8 production in human macrophages*. Yonsei Med J, 2012. **53**(3): p. 654-7.
54. Behzadi, S., et al., *Cellular uptake of nanoparticles: journey inside the cell*. Chem Soc Rev, 2017. **46**(14): p. 4218-4244.
55. Gustafson, H.H., et al., *Nanoparticle Uptake: The Phagocyte Problem*. Nano Today, 2015. **10**(4): p. 487-510.
56. Richards, D.M. and R.G. Endres, *The mechanism of phagocytosis: two stages of engulfment*. Biophys J, 2014. **107**(7): p. 1542-53.
57. Kuhn, D.A., et al., *Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages*. Beilstein J Nanotechnol, 2014. **5**: p. 1625-36.
58. Krpetic, Z., et al., *Phagocytosis of biocompatible gold nanoparticles*. Langmuir, 2010. **26**(18): p. 14799-805.
59. Claudia, M., et al., *Comparison of fluorescence-based methods to determine nanoparticle uptake by phagocytes and non-phagocytic cells in vitro*. Toxicology, 2017. **378**: p. 25-36.
60. Lin, J., et al., *Penetration of lipid membranes by gold nanoparticles: insights into cellular uptake, cytotoxicity, and their relationship*. ACS Nano, 2010. **4**(9): p. 5421-9.
61. Pelkmans, L. and A. Helenius, *Endocytosis via caveolae*. Traffic, 2002. **3**(5): p. 311-20.

62. Kiss, A.L. and E. Botos, *Endocytosis via caveolae: alternative pathway with distinct cellular compartments to avoid lysosomal degradation?* J Cell Mol Med, 2009. **13**(7): p. 1228-37.
63. 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**: p. 23.
64. Hao, X., et al., *Caveolae-mediated endocytosis of biocompatible gold nanoparticles in living Hela cells.* J Phys Condens Matter, 2012. **24**(16): p. 164207.
65. Li, Z., et al., *Shape Effect of Glyco-Nanoparticles on Macrophage Cellular Uptake and Immune Response.* ACS Macro Lett, 2016. **5**(9): p. 1059-1064.
66. dos Santos, T., et al., *Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines.* PLoS One, 2011. **6**(9): p. e24438.
67. Lu, R., D.G. Drubin, and Y. Sun, *Clathrin-mediated endocytosis in budding yeast at a glance.* J Cell Sci, 2016. **129**(8): p. 1531-6.
68. Xie, X., et al., *The Effect of shape on Cellular Uptake of Gold Nanoparticles in the forms of Stars, Rods, and Triangles.* Sci Rep, 2017. **7**(1): p. 3827.
69. Chithrani, B.D. and W.C. Chan, *Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes.* Nano Lett, 2007. **7**(6): p. 1542-50.
70. Ng, C.T., et al., *Clathrin-mediated endocytosis of gold nanoparticles in vitro.* Anat Rec (Hoboken), 2015. **298**(2): p. 418-27.
71. Akter, M., et al., *A systematic review on silver nanoparticles-induced cytotoxicity: Physicochemical properties and perspectives.* J Adv Res, 2018. **9**: p. 1-16.

72. Ali-Boucetta, H., et al., *Cellular uptake and cytotoxic impact of chemically functionalized and polymer-coated carbon nanotubes*. Small, 2011. **7**(22): p. 3230-8.
73. Chithrani, B.D., A.A. Ghazani, and W.C. Chan, *Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells*. Nano Lett, 2006. **6**(4): p. 662-8.
74. McShan, D., P.C. Ray, and H. Yu, *Molecular toxicity mechanism of nanosilver*. J Food Drug Anal, 2014. **22**(1): p. 116-127.
75. Peretz, V., et al., *The Effect of Nanoparticle Size on Cellular Binding Probability*. Journal of Atomic, Molecular, and Optical Physics, 2012. **2012**: p. 1-7.
76. Marchesano, V., et al., *Imaging inward and outward trafficking of gold nanoparticles in whole animals*. ACS Nano, 2013. **7**(3): p. 2431-42.
77. Hoshyar, N., et al., *The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction*. Nanomedicine (Lond), 2016. **11**(6): p. 673-92.
78. 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**: p. 11.
79. Kumar, D., et al., *Cytotoxicity and cellular uptake of different sized gold nanoparticles in ovarian cancer cells*. Nanotechnology, 2017. **28**(47): p. 475101.
80. Liu, W., et al., *Impact of silver nanoparticles on human cells: effect of particle size*. Nanotoxicology, 2010. **4**(3): p. 319-30.
81. Neshatian, M., et al., *Determining the Size Dependence of Colloidal Gold Nanoparticle Uptake in a Tumor-like Interface (Hypoxic)*. Colloids and Interface Science Communications, 2014. **1**: p. 57-61.

82. Jiang, Y., et al., *The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 nm Gold Nanoparticles*. ACS Nano, 2015. **9**(10): p. 9986-93.
83. Xia, Q., et al., *Size- and cell type-dependent cellular uptake, cytotoxicity and in vivo distribution of gold nanoparticles*. Int J Nanomedicine, 2019. **14**: p. 6957-6970.
84. Haase, A., et al., *Toxicity of silver nanoparticles in human macrophages: uptake, intracellular distribution and cellular responses*. Journal of Physics: Conference Series, 2011. **304**.
85. Kettler, K., et al., *Exploring the effect of silver nanoparticle size and medium composition on uptake into pulmonary epithelial 16HBE14o-cells*. J Nanopart Res, 2016. **18**: p. 182.
86. Wu, M., et al., *Size-dependent cellular uptake and localization profiles of silver nanoparticles*. Int J Nanomedicine, 2019. **14**: p. 4247-4259.
87. Nguyen, K.C., et al., *Comparison of toxicity of uncoated and coated silver nanoparticles*. Journal of Physics: Conference Series, 2013. **429**.
88. Pozzi, D., et al., *Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells*. Nanoscale, 2014. **6**(5): p. 2782-92.
89. Jo, D.H., et al., *Size, surface charge, and shape determine therapeutic effects of nanoparticles on brain and retinal diseases*. Nanomedicine, 2015. **11**(7): p. 1603-11.
90. Boyles, M.S., et al., *Chitosan functionalisation of gold nanoparticles encourages particle uptake and induces cytotoxicity and pro-inflammatory conditions in phagocytic cells, as well as enhancing particle interactions with serum components*. J Nanobiotechnology, 2015. **13**: p. 84.

91. Wang, L., et al., *Surface chemistry of gold nanorods: origin of cell membrane damage and cytotoxicity*. *Nanoscale*, 2013. **5**(18): p. 8384-91.
92. Frohlich, E., *The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles*. *Int J Nanomedicine*, 2012. **7**: p. 5577-91.
93. Ayala, V., et al., *Effect of surface charge on the colloidal stability and in vitro uptake of carboxymethyl dextran-coated iron oxide nanoparticles*. *J Nanopart Res*, 2013. **15**(8): p. 1874.
94. Fraga, S., et al., *Influence of the surface coating on the cytotoxicity, genotoxicity and uptake of gold nanoparticles in human HepG2 cells*. *J Appl Toxicol*, 2013. **33**(10): p. 1111-9.
95. Huhn, D., et al., *Polymer-coated nanoparticles interacting with proteins and cells: focusing on the sign of the net charge*. *ACS Nano*, 2013. **7**(4): p. 3253-63.
96. Srijampa, S., et al., *Effects of Gold Nanoparticles with Different Surface Charges on Cellular Internalization and Cytokine Responses in Monocytes*. *BioNanoScience*, 2019. **9**(3): p. 580-586.
97. Makama, S., et al., *Effects of Systematic Variation in Size and Surface Coating of Silver Nanoparticles on Their In Vitro Toxicity to Macrophage RAW 264.7 Cells*. *Toxicol Sci*, 2018. **162**(1): p. 79-88.
98. Sabourian, P., et al., *Effect of Physico-Chemical Properties of Nanoparticles on Their Intracellular Uptake*. *Int J Mol Sci*, 2020. **21**(21).
99. Yue, J., et al., *Gold Nanoparticle Size and Shape Effects on Cellular Uptake and Intracellular Distribution of siRNA Nanoconstructs*. *Bioconjug Chem*, 2017. **28**(6): p. 1791-1800.

100. Lee, Y.J., E.Y. Ahn, and Y. Park, *Shape-dependent cytotoxicity and cellular uptake of gold nanoparticles synthesized using green tea extract*. *Nanoscale Res Lett*, 2019. **14**(1): p. 129.
101. Kulkarni, S.A. and S.S. Feng, *Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery*. *Pharm Res*, 2013. **30**(10): p. 2512-22.
102. Ma, N., et al., *Influence of nanoparticle shape, size, and surface functionalization on cellular uptake*. *J Nanosci Nanotechnol*, 2013. **13**(10): p. 6485-98.
103. Nambara, K., et al., *Reverse Size Dependences of the Cellular Uptake of Triangular and Spherical Gold Nanoparticles*. *Langmuir*, 2016. **32**(47): p. 12559-12567.
104. Cho, E.C., et al., *The effects of size, shape, and surface functional group of gold nanostructures on their adsorption and internalization by cells*. *Small*, 2010. **6**(4): p. 517-22.
105. Graf, C., et al., *Shape-Dependent Dissolution and Cellular Uptake of Silver Nanoparticles*. *Langmuir*, 2018. **34**(4): p. 1506-1519.
106. Anderson, C.R., et al., *Modulation of cell responses to Ag-(MeO2 MA-co-OEGMA): Effects of nanoparticle surface hydrophobicity and serum proteins on cellular uptake and toxicity*. *J Biomed Mater Res A*, 2018. **106**(4): p. 1061-1071.
107. Fleischer, C.C. and C.K. Payne, *Nanoparticle-cell interactions: molecular structure of the protein corona and cellular outcomes*. *Acc Chem Res*, 2014. **47**(8): p. 2651-9.
108. Maiorano, G., et al., *Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response*. *ACS Nano*, 2010. **4**(12): p. 7481-91.

109. Cheng, X., et al., *Protein Corona Influences Cellular Uptake of Gold Nanoparticles by Phagocytic and Nonphagocytic Cells in a Size-Dependent Manner*. ACS Appl Mater Interfaces, 2015. **7**(37): p. 20568-75.
110. Monteiro-Riviere, N.A., et al., *Protein binding modulates the cellular uptake of silver nanoparticles into human cells: implications for in vitro to in vivo extrapolations?* Toxicol Lett, 2013. **220**(3): p. 286-93.
111. Albanese, A. and W.C. Chan, *Effect of gold nanoparticle aggregation on cell uptake and toxicity*. ACS Nano, 2011. **5**(7): p. 5478-89.
112. Lesniak, A., et al., *Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells*. ACS Nano, 2012. **6**(7): p. 5845-57.
113. Shannahan, J.H., et al., *Formation of a protein corona on silver nanoparticles mediates cellular toxicity via scavenger receptors*. Toxicol Sci, 2015. **143**(1): p. 136-46.
114. Liao, C., Y. Li, and S.C. Tjong, *Bactericidal and Cytotoxic Properties of Silver Nanoparticles*. Int J Mol Sci, 2019. **20**(2).
115. Talarska, P., M. Boruckowski, and J. Zurawski, *Current Knowledge of Silver and Gold Nanoparticles in Laboratory Research-Application, Toxicity, Cellular Uptake*. Nanomaterials (Basel), 2021. **11**(9).
116. Geethalakshmi, R. and D.V. Sarada, *Gold and silver nanoparticles from Trianthema decandra: synthesis, characterization, and antimicrobial properties*. Int J Nanomedicine, 2012. **7**: p. 5375-84.
117. Benn, T., et al., *The release of nanosilver from consumer products used in the home*. J Environ Qual, 2010. **39**(6): p. 1875-82.

118. Pourzahedi, L., M. Vance, and M.J. Eckelman, *Life Cycle Assessment and Release Studies for 15 Nanosilver-Enabled Consumer Products: Investigating Hotspots and Patterns of Contribution*. Environ Sci Technol, 2017. **51**(12): p. 7148-7158.
119. Tripathi, D.K., et al., *Uptake, Accumulation and Toxicity of Silver Nanoparticle in Autotrophic Plants, and Heterotrophic Microbes: A Concentric Review*. Front Microbiol, 2017. **8**: p. 07.
120. Uboldi, C., et al., *Gold nanoparticles induce cytotoxicity in the alveolar type-II cell lines A549 and NCIH441*. Part Fibre Toxicol, 2009. **6**: p. 18.
121. Li, B. and M. Tang, *Research progress of nanoparticle toxicity signaling pathway*. Life Sci, 2020. **263**: p. 118542.
122. Ferdous, Z. and A. Nemmar, *Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure*. Int J Mol Sci, 2020. **21**(7).
123. Baez, D.F., et al., *The Influence of Size and Chemical Composition of Silver and Gold Nanoparticles on in vivo Toxicity with Potential Applications to Central Nervous System Diseases*. Int J Nanomedicine, 2021. **16**: p. 2187-2201.
124. Foldbjerg, R., et al., *PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes*. Toxicol Lett, 2009. **190**(2): p. 156-62.
125. Jiang, X., et al., *Influences of simulated gastrointestinal environment on physicochemical properties of gold nanoparticles and their implications on intestinal epithelial permeability*. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2019. **37**(2): p. 116-131.

126. Hiatt, R., *Introduction to free radical chemistry (Pryor, William A.)*. Journal of Chemical Education, 1967. **44**(5).
127. Lobo, V., et al., *Free radicals, antioxidants and functional foods: Impact on human health*. Pharmacogn Rev, 2010. **4**(8): p. 118-26.
128. Gupta, R.K., et al., *Oxidative stress and antioxidants in disease and cancer: a review*. Asian Pac J Cancer Prev, 2014. **15**(11): p. 4405-9.
129. Birben, E., et al., *Oxidative stress and antioxidant defense*. World Allergy Organ J, 2012. **5**(1): p. 9-19.
130. Mironczuk-Chodakowska, I., A.M. Witkowska, and M.E. Zujko, *Endogenous non-enzymatic antioxidants in the human body*. Adv Med Sci, 2018. **63**(1): p. 68-78.
131. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: how are they linked?* Free Radic Biol Med, 2010. **49**(11): p. 1603-16.
132. Zahra, K.F., et al., *The Involvement of the Oxidative Stress Status in Cancer Pathology: A Double View on the Role of the Antioxidants*. Oxid Med Cell Longev, 2021. **2021**: p. 9965916.
133. Ray, P.D., B.W. Huang, and Y. Tsuji, *Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling*. Cell Signal, 2012. **24**(5): p. 981-90.
134. Tangestani Fard, M. and C. Stough, *A Review and Hypothesized Model of the Mechanisms That Underpin the Relationship Between Inflammation and Cognition in the Elderly*. Front Aging Neurosci, 2019. **11**: p. 56.
135. Zarkovic, N., *Roles and Functions of ROS and RNS in Cellular Physiology and Pathology*. Cells, 2020. **9**(3).
136. Guerrero-Florez, V., et al., *Gold nanoparticle-mediated generation of reactive oxygen species during plasmonic photothermal therapy: a comparative study for different*

- particle sizes, shapes, and surface conjugations*. J Mater Chem B, 2020. **8**(14): p. 2862-2875.
137. Holmila, R.J., et al., *Silver Nanoparticles Induce Mitochondrial Protein Oxidation in Lung Cells Impacting Cell Cycle and Proliferation*. Antioxidants (Basel), 2019. **8**(11).
 138. Galluzzi, L., et al., *Mitochondrial control of cellular life, stress, and death*. Circ Res, 2012. **111**(9): p. 1198-207.
 139. Kroemer, G., G. Marino, and B. Levine, *Autophagy and the integrated stress response*. Mol Cell, 2010. **40**(2): p. 280-93.
 140. Karatas, O.F., et al., *Interaction of gold nanoparticles with mitochondria*. Colloids Surf B Biointerfaces, 2009. **71**(2): p. 315-8.
 141. Prasad, A., et al., *Low-dose exposure to phytosynthesized gold nanoparticles combined with glutamine deprivation enhances cell death in the cancer cell line HeLa via oxidative stress-mediated mitochondrial dysfunction and G0/G1 cell cycle arrest*. Nanoscale, 2022. **14**(29): p. 10399-10417.
 142. Li, J., et al., *The crosstalk between DRP1-dependent mitochondrial fission and oxidative stress triggers hepatocyte apoptosis induced by silver nanoparticles*. Nanoscale, 2021. **13**(28): p. 12356-12369.
 143. Li, J., et al., *Silver nanoparticles modulate mitochondrial dynamics and biogenesis in HepG2 cells*. Environ Pollut, 2020. **256**: p. 113430.
 144. Liang, L., et al., *Nanoparticles' interference in the evaluation of in vitro toxicity of silver nanoparticles*. RSC Advances, 2015. **5**(82): p. 67327-67334.
 145. Gallud, A., et al., *Cationic gold nanoparticles elicit mitochondrial dysfunction: a multi-omics study*. Sci Rep, 2019. **9**(1): p. 4366.

146. Jin, X., et al., *Surface charge-dependent mitochondrial response to similar intracellular nanoparticle contents at sublethal dosages*. Part Fibre Toxicol, 2021. **18**(1): p. 36.
147. Costa, C.S., et al., *In vitro effects of silver nanoparticles on the mitochondrial respiratory chain*. Mol Cell Biochem, 2010. **342**(1-2): p. 51-6.
148. Chen, H., A. Chomyn, and D.C. Chan, *Disruption of fusion results in mitochondrial heterogeneity and dysfunction*. J Biol Chem, 2005. **280**(28): p. 26185-92.
149. Piao, M.J., et al., *Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis*. Toxicol Lett, 2011. **201**(1): p. 92-100.
150. Thierry, A.R., et al., *Origins, structures, and functions of circulating DNA in oncology*. Cancer Metastasis Rev, 2016. **35**(3): p. 347-76.
151. Berger, M.F. and E.R. Mardis, *The emerging clinical relevance of genomics in cancer medicine*. Nat Rev Clin Oncol, 2018. **15**(6): p. 353-365.
152. Stratton, M.R., P.J. Campbell, and P.A. Futreal, *The cancer genome*. Nature, 2009. **458**(7239): p. 719-24.
153. Chatterjee, N. and G.C. Walker, *Mechanisms of DNA damage, repair, and mutagenesis*. Environ Mol Mutagen, 2017. **58**(5): p. 235-263.
154. Jackson, S.P. and J. Bartek, *The DNA-damage response in human biology and disease*. Nature, 2009. **461**(7267): p. 1071-8.
155. Nallanthighal, S., et al., *Differential effects of silver nanoparticles on DNA damage and DNA repair gene expression in Ogg1-deficient and wild type mice*. Nanotoxicology, 2017. **11**(8): p. 996-1011.

156. Tian, L., T.M. Cronin, and Y. Weizmann, *Enhancing-effect of gold nanoparticles on DNA strand displacement amplifications and their application to an isothermal telomerase assay*. Chem. Sci., 2014. **5**(11): p. 4153-4162.
157. AshaRani, P.V., et al., *Cytotoxicity and genotoxicity of silver nanoparticles in human cells*. ACS Nano, 2009. **3**(2): p. 279-90.
158. Li, J.J., et al., *Gold Nanoparticles Induce Oxidative Damage in Lung Fibroblasts In Vitro*. Advanced Materials, 2008. **20**(1): p. 138-142.
159. Di Bucchianico, S., et al., *Aneuploidogenic effects and DNA oxidation induced in vitro by differently sized gold nanoparticles*. Int J Nanomedicine, 2014. **9**: p. 2191-204.
160. Foldbjerg, R., D.A. Dang, and H. Autrup, *Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549*. Arch Toxicol, 2011. **85**(7): p. 743-50.
161. Lebedova, J., et al., *Size-dependent genotoxicity of silver, gold and platinum nanoparticles studied using the mini-gel comet assay and micronucleus scoring with flow cytometry*. Mutagenesis, 2018. **33**(1): p. 77-85.
162. Nelson, B.C., et al., *NIST gold nanoparticle reference materials do not induce oxidative DNA damage*. Nanotoxicology, 2013. **7**(1): p. 21-9.
163. Perde-Schrepler, M., et al., *Size-Dependent Cytotoxicity and Genotoxicity of Silver Nanoparticles in Cochlear Cells In Vitro*. Journal of Nanomaterials, 2019. **2019**: p. 1-12.
164. Xia, Q., et al., *The effect of particle size on the genotoxicity of gold nanoparticles*. J Biomed Mater Res A, 2017. **105**(3): p. 710-719.
165. Jan, R. and G.E. Chaudhry, *Understanding Apoptosis and Apoptotic Pathways Targeted Cancer Therapeutics*. Adv Pharm Bull, 2019. **9**(2): p. 205-218.

166. Xu, X., Y. Lai, and Z.C. Hua, *Apoptosis and apoptotic body: disease message and therapeutic target potentials*. Biosci Rep, 2019. **39**(1).
167. Elmore, S., *Apoptosis: a review of programmed cell death*. Toxicol Pathol, 2007. **35**(4): p. 495-516.
168. Loreto, C., et al., *The role of intrinsic pathway in apoptosis activation and progression in Peyronie's disease*. Biomed Res Int, 2014. **2014**: p. 616149.
169. Rowinsky, E.K., *Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents*. J Clin Oncol, 2005. **23**(36): p. 9394-407.
170. Loreto, C., et al., *Apoptosis in displaced temporomandibular joint disc with and without reduction: an immunohistochemical study*. J Oral Pathol Med, 2011. **40**(1): p. 103-10.
171. Loreto, C., et al., *Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptor (DR5) in Peyronie's disease. A biomolecular study of apoptosis activation*. J Sex Med, 2011. **8**(1): p. 109-15.
172. Zhang, X.F. and S. Gurunathan, *Combination of salinomycin and silver nanoparticles enhances apoptosis and autophagy in human ovarian cancer cells: an effective anticancer therapy*. Int J Nanomedicine, 2016. **11**: p. 3655-75.
173. Gurunathan, S., et al., *Nanoparticle-Mediated Combination Therapy: Two-in-One Approach for Cancer*. Int J Mol Sci, 2018. **19**(10).
174. Ahmadian, E., et al., *Effect of silver nanoparticles in the induction of apoptosis on human hepatocellular carcinoma (HepG2) cell line*. Mater Sci Eng C Mater Biol Appl, 2018. **93**: p. 465-471.

175. Bin-Jumah, M., et al., *Effects of Green Silver Nanoparticles on Apoptosis and Oxidative Stress in Normal and Cancerous Human Hepatic Cells in vitro*. Int J Nanomedicine, 2020. **15**: p. 1537-1548.
176. Rohde, M.M., et al., *The mechanism of cell death induced by silver nanoparticles is distinct from silver cations*. Part Fibre Toxicol, 2021. **18**(1): p. 37.
177. Zhu, B., et al., *Silver Nanoparticles Induce HePG-2 Cells Apoptosis Through ROS-Mediated Signaling Pathways*. Nanoscale Res Lett, 2016. **11**(1): p. 198.
178. Bao, J., et al., *Silver nanoparticles induce mitochondria-dependent apoptosis and late non-canonical autophagy in HT-29 colon cancer cells*. Nanotechnology Reviews, 2022. **11**(1): p. 1911-1926.
179. Yuan, Y.G., et al., *Silver Nanoparticles Potentiates Cytotoxicity and Apoptotic Potential of Camptothecin in Human Cervical Cancer Cells*. Oxid Med Cell Longev, 2018. **2018**: p. 6121328.
180. Lee, Y.S., et al., *Silver nanoparticles induce apoptosis and G2/M arrest via PKCzeta-dependent signaling in A549 lung cells*. Arch Toxicol, 2011. **85**(12): p. 1529-40.
181. Selim, M.E. and A.A. Hendi, *Gold nanoparticles induce apoptosis in MCF-7 human breast cancer cells*. Asian Pac J Cancer Prev, 2012. **13**(4): p. 1617-20.
182. Jawaid, P., et al., *Small size gold nanoparticles enhance apoptosis-induced by cold atmospheric plasma via depletion of intracellular GSH and modification of oxidative stress*. Cell Death Discov, 2020. **6**: p. 83.
183. Enea, M., et al., *Gold Nanoparticles Induce Oxidative Stress and Apoptosis in Human Kidney Cells*. Nanomaterials (Basel), 2020. **10**(5).

184. Zhang, C., et al., *Insights into the distinguishing stress-induced cytotoxicity of chiral gold nanoclusters and the relationship with GSTP1*. *Theranostics*, 2015. **5**(2): p. 134-49.
185. Ke, S., et al., *Gold nanoparticles enhance TRAIL sensitivity through Drp1-mediated apoptotic and autophagic mitochondrial fission in NSCLC cells*. *Int J Nanomedicine*, 2017. **12**: p. 2531-2551.
186. Oladimeji, O., et al., *Modified Gold Nanoparticles for Efficient Delivery of Betulinic Acid to Cancer Cell Mitochondria*. *Int J Mol Sci*, 2021. **22**(10).
187. Lunova, M., et al., *Targeting the mTOR Signaling Pathway Utilizing Nanoparticles: A Critical Overview*. *Cancers (Basel)*, 2019. **11**(1).
188. Lee, E.R., et al., *Interplay between PI3K/Akt and MAPK signaling pathways in DNA-damaging drug-induced apoptosis*. *Biochim Biophys Acta*, 2006. **1763**(9): p. 958-68.
189. Cao, Z., et al., *AKT and ERK dual inhibitors: The way forward?* *Cancer Lett*, 2019. **459**: p. 30-40.
190. Millis, S.Z., et al., *Landscape of Phosphatidylinositol-3-Kinase Pathway Alterations Across 19784 Diverse Solid Tumors*. *JAMA Oncol*, 2016. **2**(12): p. 1565-1573.
191. Shariati, M. and F. Meric-Bernstam, *Targeting AKT for cancer therapy*. *Expert Opin Investig Drugs*, 2019. **28**(11): p. 977-988.
192. Davies, B.R., et al., *Tumors with AKT1E17K Mutations Are Rational Targets for Single Agent or Combination Therapy with AKT Inhibitors*. *Mol Cancer Ther*, 2015. **14**(11): p. 2441-51.
193. Shrestha Bhattarai, T., et al., *AKT mutant allele-specific activation dictates pharmacologic sensitivities*. *Nat Commun*, 2022. **13**(1): p. 2111.

194. Porta, C., C. Paglino, and A. Mosca, *Targeting PI3K/Akt/mTOR Signaling in Cancer*. Front Oncol, 2014. **4**: p. 64.
195. Bhanumathi, R., et al., *Drug-Carrying Capacity and Anticancer Effect of the Folic Acid- and Berberine-Loaded Silver Nanomaterial To Regulate the AKT-ERK Pathway in Breast Cancer*. ACS Omega, 2018. **3**(7): p. 8317-8328.
196. Jiao, Z.H., et al., *Hormesis effects of silver nanoparticles at non-cytotoxic doses to human hepatoma cells*. PLoS One, 2014. **9**(7): p. e102564.
197. Yuan, Y.G., Q.L. Peng, and S. Gurunathan, *Silver nanoparticles enhance the apoptotic potential of gemcitabine in human ovarian cancer cells: combination therapy for effective cancer treatment*. Int J Nanomedicine, 2017. **12**: p. 6487-6502.
198. Hsin, Y.H., et al., *The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells*. Toxicol Lett, 2008. **179**(3): p. 130-9.
199. Kovacs, D., et al., *Silver nanoparticles defeat p53-positive and p53-negative osteosarcoma cells by triggering mitochondrial stress and apoptosis*. Sci Rep, 2016. **6**: p. 27902.
200. Xue, Y., et al., *Comparative cytotoxicity and apoptotic pathways induced by nanosilver in human liver HepG2 and L02 cells*. Hum Exp Toxicol, 2018. **37**(12): p. 1293-1309.
201. Jiang, X., et al., *Nanotoxicity of Silver Nanoparticles on HEK293T Cells: A Combined Study Using Biomechanical and Biological Techniques*. ACS Omega, 2018. **3**(6): p. 6770-6778.
202. Mahmoud, N.N., et al., *Gold Nanocomplex Strongly Modulates the PI3K/Akt Pathway and Other Pathways in MCF-7 Breast Cancer Cell Line*. Int J Mol Sci, 2020. **21**(9).

203. Balakrishnan, S., et al., *Gold nanoparticles-conjugated quercetin induces apoptosis via inhibition of EGFR/PI3K/Akt-mediated pathway in breast cancer cell lines (MCF-7 and MDA-MB-231)*. Cell Biochem Funct, 2017. **35**(4): p. 217-231.
204. De Araujo, R.F., Jr., et al., *Apoptosis in human liver carcinoma caused by gold nanoparticles in combination with carvedilol is mediated via modulation of MAPK/Akt/mTOR pathway and EGFR/FAAD proteins*. Int J Oncol, 2018. **52**(1): p. 189-200.
205. Tiloke, C., et al., *Moringa oleifera Gold Nanoparticles Modulate Oncogenes, Tumor Suppressor Genes, and Caspase-9 Splice Variants in A549 Cells*. J Cell Biochem, 2016. **117**(10): p. 2302-14.
206. Comfort, K.K., et al., *Interference of silver, gold, and iron oxide nanoparticles on epidermal growth factor signal transduction in epithelial cells*. ACS Nano, 2011. **5**(12): p. 10000-8.
207. Noel, C., J.C. Simard, and D. Girard, *Gold nanoparticles induce apoptosis, endoplasmic reticulum stress events and cleavage of cytoskeletal proteins in human neutrophils*. Toxicol In Vitro, 2016. **31**: p. 12-22.
208. Liu, M., et al., *Gold nanoparticles trigger apoptosis and necrosis in lung cancer cells with low intracellular glutathione*. Journal of Nanoparticle Research, 2013. **15**(8).
209. MacDonald, B.T., K. Tamai, and X. He, *Wnt/beta-catenin signaling: components, mechanisms, and diseases*. Dev Cell, 2009. **17**(1): p. 9-26.
210. Nusse, R. and H. Clevers, *Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities*. Cell, 2017. **169**(6): p. 985-999.
211. Wiese, K.E., R. Nusse, and R. van Amerongen, *Wnt signalling: conquering complexity*. Development, 2018. **145**(12).

212. MacDonald, B.T. and X. He, *Frizzled and LRP5/6 receptors for Wnt/beta-catenin signaling*. Cold Spring Harb Perspect Biol, 2012. **4**(12).
213. Bian, J., et al., *Transcriptional Regulation of Wnt/beta-Catenin Pathway in Colorectal Cancer*. Cells, 2020. **9**(9).
214. He, X., et al., *LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way*. Development, 2004. **131**(8): p. 1663-77.
215. Mukherjee, A., et al., *Understanding How Wnt Influences Destruction Complex Activity and β -Catenin Dynamics*. iScience, 2018. **6**: p. 13-21.
216. van Kappel, E.C. and M.M. Maurice, *Molecular regulation and pharmacological targeting of the beta-catenin destruction complex*. Br J Pharmacol, 2017. **174**(24): p. 4575-4588.
217. Daniels, D.L. and W.I. Weis, *Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation*. Nat Struct Mol Biol, 2005. **12**(4): p. 364-71.
218. Franiak-Pietryga, I., et al., *Blockage of Wnt/beta-Catenin Signaling by Nanoparticles Reduces Survival and Proliferation of CLL Cells In Vitro-Preliminary Study*. Macromol Biosci, 2017. **17**(11).
219. Krishnamurthy, N. and R. Kurzrock, *Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors*. Cancer Treat Rev, 2018. **62**: p. 50-60.
220. Stamos, J.L. and W.I. Weis, *The beta-catenin destruction complex*. Cold Spring Harb Perspect Biol, 2013. **5**(1): p. a007898.
221. Zhang, Y. and X. Wang, *Targeting the Wnt/beta-catenin signaling pathway in cancer*. J Hematol Oncol, 2020. **13**(1): p. 165.

222. Cooper, R.J., et al., *Involvement of beta-catenin in cytoskeleton disruption following adult neural stem cell exposure to low-level silver nanoparticles*. Neurotoxicology, 2019. **71**: p. 102-112.
223. Surapaneni, S.K., S. Bashir, and K. Tikoo, *Gold nanoparticles-induced cytotoxicity in triple negative breast cancer involves different epigenetic alterations depending upon the surface charge*. Sci Rep, 2018. **8**(1): p. 12295.
224. Hershko, A., *Ubiquitin: Roles in protein modification and breakdown*. Cell, 1983. **34**(1): p. 11-12.
225. Morrow, J.K., et al., *Targeting ubiquitination for cancer therapies*. Future Med Chem, 2015. **7**(17): p. 2333-50.
226. Ye, Y. and M. Rape, *Building ubiquitin chains: E2 enzymes at work*. Nat Rev Mol Cell Biol, 2009. **10**(11): p. 755-64.
227. Komander, D. and M. Rape, *The ubiquitin code*. Annu Rev Biochem, 2012. **81**: p. 203-29.
228. Jana, N.R., *Protein homeostasis and aging: role of ubiquitin protein ligases*. Neurochem Int, 2012. **60**(5): p. 443-7.
229. Zhao, C., et al., *A novel nickel complex works as a proteasomal deubiquitinase inhibitor for cancer therapy*. Oncogene, 2016. **35**(45): p. 5916-5927.
230. Tian, Z., et al., *A novel small molecule inhibitor of deubiquitylating enzyme USP14 and UCHL5 induces apoptosis in multiple myeloma and overcomes bortezomib resistance*. Blood, 2014. **123**(5): p. 706-16.
231. Huang, H., et al., *Platinum-containing compound platinum pyrithione suppresses ovarian tumor proliferation through proteasome inhibition*. J Exp Clin Cancer Res, 2017. **36**(1): p. 79.

232. Luo, Y., et al., *UCH-L1 promotes invasion of breast cancer cells through activating Akt signaling pathway*. J Cell Biochem, 2018. **119**(1): p. 691-700.
233. Zheng, S., et al., *Heterogeneous expression and biological function of ubiquitin carboxy-terminal hydrolase-L1 in osteosarcoma*. Cancer Lett, 2015. **359**(1): p. 36-46.
234. Fan, Y.H., et al., *USP7 inhibitor P22077 inhibits neuroblastoma growth via inducing p53-mediated apoptosis*. Cell Death Dis, 2013. **4**: p. e867.
235. Chen, C., et al., *Synthesis and biological evaluation of thiazole derivatives as novel USP7 inhibitors*. Bioorg Med Chem Lett, 2017. **27**(4): p. 845-849.
236. Ding, F., et al., *The role of the ubiquitin-proteasome pathway in cancer development and treatment*. Front Biosci (Landmark Ed), 2014. **19**(6): p. 886-95.
237. Graner, E., et al., *The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer*. Cancer Cell, 2004. **5**(3): p. 253-261.
238. Priolo, C., et al., *The isopeptidase USP2a protects human prostate cancer from apoptosis*. Cancer Res, 2006. **66**(17): p. 8625-32.
239. Qu, Q., et al., *USP2 promotes cell migration and invasion in triple negative breast cancer cell lines*. Tumour Biol, 2015. **36**(7): p. 5415-23.
240. Colland, F., et al., *Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells*. Mol Cancer Ther, 2009. **8**(8): p. 2286-95.
241. Turnbull, A.P., et al., *Molecular basis of USP7 inhibition by selective small-molecule inhibitors*. Nature, 2017. **550**(7677): p. 481-486.
242. Qin, D., et al., *CDDO-Me reveals USP7 as a novel target in ovarian cancer cells*. Oncotarget, 2016. **7**(47): p. 77096-77109.
243. Park, J., J. Cho, and E.J. Song, *Ubiquitin-proteasome system (UPS) as a target for anticancer treatment*. Arch Pharm Res, 2020. **43**(11): p. 1144-1161.

244. Shen, M., et al., *Targeting the ubiquitin-proteasome system for cancer therapy*. Expert Opin Ther Targets, 2013. **17**(9): p. 1091-108.
245. Luise, C., et al., *An atlas of altered expression of deubiquitinating enzymes in human cancer*. PLoS One, 2011. **6**(1): p. e15891.
246. Qi, S., et al., *Targeting E2 ubiquitin-conjugating enzyme UbcH5c by small molecule inhibitor suppresses pancreatic cancer growth and metastasis*. Mol Cancer, 2022. **21**(1): p. 70.
247. Qie, S. and J.A. Diehl, *Cyclin D degradation by E3 ligases in cancer progression and treatment*. Semin Cancer Biol, 2020. **67**(Pt 2): p. 159-170.
248. Di Costanzo, A., et al., *The Ubiquitin Proteasome System in Hematological Malignancies: New Insight into Its Functional Role and Therapeutic Options*. Cancers (Basel), 2020. **12**(7).
249. Morgan, J.J. and L.J. Crawford, *The Ubiquitin Proteasome System in Genome Stability and Cancer*. Cancers (Basel), 2021. **13**(9).
250. Fhu, C.W. and A. Ali, *Dysregulation of the Ubiquitin Proteasome System in Human Malignancies: A Window for Therapeutic Intervention*. Cancers (Basel), 2021. **13**(7).
251. Jayaweera, S.P.E., et al., *Carfilzomib: A Promising Proteasome Inhibitor for the Treatment of Relapsed and Refractory Multiple Myeloma*. Front Oncol, 2021. **11**: p. 740796.
252. Roelofs, J., *Proteasome inhibition by bortezomib: A left hook and a right punch*. EBioMedicine, 2015. **2**(7): p. 619-20.
253. Ding, Q., et al., *Discovery of RG7388, a potent and selective p53-MDM2 inhibitor in clinical development*. J Med Chem, 2013. **56**(14): p. 5979-83.

254. Oliner, J.D., A.Y. Saiki, and S. Caenepeel, *The Role of MDM2 Amplification and Overexpression in Tumorigenesis*. Cold Spring Harb Perspect Med, 2016. **6**(6).
255. Huang, T., et al., *Inhibition of Skp2 sensitizes lung cancer cells to paclitaxel*. Onco Targets Ther, 2017. **10**: p. 439-446.
256. Gluschnaider, U., et al., *beta-TrCP inhibition reduces prostate cancer cell growth via upregulation of the aryl hydrocarbon receptor*. PLoS One, 2010. **5**(2): p. e9060.
257. Gao, Y., et al., *Cdc20 inhibitor apcin inhibits the growth and invasion of osteosarcoma cells*. Oncol Rep, 2018. **40**(2): p. 841-848.
258. Aliabadi, F., et al., *Ubiquitin-proteasome system and the role of its inhibitors in cancer therapy*. Open Biol, 2021. **11**(4): p. 200390.
259. Kobayashi, E., et al., *Inhibition of UCH-L1 Deubiquitinating Activity with Two Forms of LDN-57444 Has Anti-Invasive Effects in Metastatic Carcinoma Cells*. Int J Mol Sci, 2019. **20**(15).
260. Chow, P.M., et al., *The UCHL5 inhibitor b-AP15 overcomes cisplatin resistance via suppression of cancer stemness in urothelial carcinoma*. Mol Ther Oncolytics, 2022. **26**: p. 387-398.
261. Davis, M.I., et al., *Small Molecule Inhibition of the Ubiquitin-specific Protease USP2 Accelerates cyclin D1 Degradation and Leads to Cell Cycle Arrest in Colorectal Cancer and Mantle Cell Lymphoma Models*. J Biol Chem, 2016. **291**(47): p. 24628-24640.
262. He, J., et al., *Inhibition of USP2 eliminates cancer stem cells and enhances TNBC responsiveness to chemotherapy*. Cell Death Dis, 2019. **10**(4): p. 285.
263. Li, Y., et al., *Ubiquitin-specific protease 4 inhibits breast cancer cell growth through the upregulation of PDCD4*. Int J Mol Med, 2016. **38**(3): p. 803-11.

264. Chauhan, D., et al., *A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance*. *Cancer Cell*, 2012. **22**(3): p. 345-58.
265. Reverdy, C., et al., *Discovery of specific inhibitors of human USP7/HAUSP deubiquitinating enzyme*. *Chem Biol*, 2012. **19**(4): p. 467-77.
266. Kushwaha, D., et al., *USP9X inhibition promotes radiation-induced apoptosis in non-small cell lung cancer cells expressing mid-to-high MCL1*. *Cancer Biol Ther*, 2015. **16**(3): p. 392-401.
267. Liu, H., et al., *WP1130 increases doxorubicin sensitivity in hepatocellular carcinoma cells through usp9x-dependent p53 degradation*. *Cancer Lett*, 2015. **361**(2): p. 218-25.
268. Hu, C., et al., *The USP10-HDAC6 axis confers cisplatin resistance in non-small cell lung cancer lacking wild-type p53*. *Cell Death Dis*, 2020. **11**(5): p. 328.
269. Yang, J., et al., *Inhibition of the deubiquitinase USP10 induces degradation of SYK*. *Br J Cancer*, 2020. **122**(8): p. 1175-1184.
270. Chitta, K., et al., *Targeted inhibition of the deubiquitinating enzymes, USP14 and UCHL5, induces proteotoxic stress and apoptosis in Waldenstrom macroglobulinaemia tumour cells*. *Br J Haematol*, 2015. **169**(3): p. 377-90.
271. Ellis, R.J., *Protein misassembly: macromolecular crowding and molecular chaperones*. *Adv Exp Med Biol*, 2007. **594**: p. 1-13.
272. Shimamura, T. and G.I. Shapiro, *Heat shock protein 90 inhibition in lung cancer*. *J Thorac Oncol*, 2008. **3**(6 Suppl 2): p. S152-9.
273. Whitley, D., S.P. Goldberg, and W.D. Jordan, *Heat shock proteins: A review of the molecular chaperones*. *Journal of Vascular Surgery*, 1999. **29**(4): p. 748-751.

274. Wu, J., et al., *Heat Shock Proteins and Cancer*. Trends Pharmacol Sci, 2017. **38**(3): p. 226-256.
275. Shrestha, L., et al., *Heat Shock Protein (HSP) Drug Discovery and Development: Targeting Heat Shock Proteins in Disease*. Curr Top Med Chem, 2016. **16**(25): p. 2753-64.
276. Yang, S., H. Xiao, and L. Cao, *Recent advances in heat shock proteins in cancer diagnosis, prognosis, metabolism and treatment*. Biomed Pharmacother, 2021. **142**: p. 112074.
277. Chatterjee, S. and T.F. Burns, *Targeting Heat Shock Proteins in Cancer: A Promising Therapeutic Approach*. Int J Mol Sci, 2017. **18**(9).
278. Hoter, A. and H.Y. Naim, *Heat Shock Proteins and Ovarian Cancer: Important Roles and Therapeutic Opportunities*. Cancers (Basel), 2019. **11**(9).
279. Miller, D.J. and P.E. Fort, *Heat Shock Proteins Regulatory Role in Neurodevelopment*. Front Neurosci, 2018. **12**: p. 821.
280. Neckers, L. and P. Workman, *Hsp90 molecular chaperone inhibitors: are we there yet?* Clin Cancer Res, 2012. **18**(1): p. 64-76.
281. Calderwood, S.K. and J. Gong, *Heat Shock Proteins Promote Cancer: It's a Protection Racket*. Trends Biochem Sci, 2016. **41**(4): p. 311-323.
282. Calderwood, S.K. and A. Murshid, *Molecular Chaperone Accumulation in Cancer and Decrease in Alzheimer's Disease: The Potential Roles of HSF1*. Front Neurosci, 2017. **11**: p. 192.
283. Ciocca, D.R. and S.K. Calderwood, *Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications*. Cell Stress Chaperones, 2005. **10**(2): p. 86-103.

284. Hendrick, J.P. and F.U. Hartl, *Molecular chaperone functions of heat-shock proteins*. Annu Rev Biochem, 1993. **62**: p. 349-84.
285. Lang, B.J., et al., *The functions and regulation of heat shock proteins; key orchestrators of proteostasis and the heat shock response*. Arch Toxicol, 2021. **95**(6): p. 1943-1970.
286. Webster, J.M., et al., *Small Heat Shock Proteins, Big Impact on Protein Aggregation in Neurodegenerative Disease*. Front Pharmacol, 2019. **10**: p. 1047.
287. Voll, E.A., et al., *Heat shock protein 27 regulates human prostate cancer cell motility and metastatic progression*. Oncotarget, 2014. **5**(9): p. 2648-63.
288. Xu, L., S. Chen, and R.C. Bergan, *MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer*. Oncogene, 2006. **25**(21): p. 2987-98.
289. Castro, G.N., et al., *Hsp27 (HSPB1): a possible surrogate molecular marker for loss of heterozygosity (LOH) of chromosome 1p in oligodendrogliomas but not in astrocytomas*. Cell Stress Chaperones, 2012. **17**(6): p. 779-90.
290. Yang, L., et al., *Hsp27: a novel therapeutic target for pediatric M4/M5 acute myeloid leukemia*. Oncol Rep, 2013. **29**(4): p. 1459-66.
291. Zhang, S., et al., *Heat shock protein 27 promotes cell proliferation through activator protein-1 in lung cancer*. Oncol Lett, 2015. **9**(6): p. 2572-2576.
292. Hsu, H.S., et al., *Chemoresistance of lung cancer stemlike cells depends on activation of Hsp27*. Cancer, 2011. **117**(7): p. 1516-28.
293. Batra, J., et al., *Human Heat shock protein 40 (Hsp40/DnaJB1) promotes influenza A virus replication by assisting nuclear import of viral ribonucleoproteins*. Sci Rep, 2016. **6**: p. 19063.

294. Li, Y., et al., *DNAJC12 promotes lung cancer growth by regulating the activation of betacatenin*. Int J Mol Med, 2021. **47**(6).
295. Huang, L., et al., *HSP40 interacts with pyruvate kinase M2 and regulates glycolysis and cell proliferation in tumor cells*. PLoS One, 2014. **9**(3): p. e92949.
296. Oka, M., et al., *Autoantibody to heat shock protein Hsp40 in sera of lung cancer patients*. Jpn J Cancer Res, 2001. **92**(3): p. 316-20.
297. Singh, P., et al., *Heat shock protein 60 is a disease-associated sialoglycoprotein in human non-small cell lung cancer*. Biol Chem, 2020. **401**(8): p. 969-983.
298. Desmetz, C., et al., *Proteomics-based identification of HSP60 as a tumor-associated antigen in early stage breast cancer and ductal carcinoma in situ*. J Proteome Res, 2008. **7**(9): p. 3830-7.
299. Castilla, C., et al., *Immunohistochemical expression of Hsp60 correlates with tumor progression and hormone resistance in prostate cancer*. Urology, 2010. **76**(4): p. 1017 e1-6.
300. Zhang, K., et al., *HSP70 regulates cell proliferation and apoptosis in actinomycin-D-treated lung cancer cells*. Transl Cancer Res, 2020. **9**(2): p. 1167-1173.
301. Yang, X., et al., *Hsp70 promotes chemoresistance by blocking Bax mitochondrial translocation in ovarian cancer cells*. Cancer Lett, 2012. **321**(2): p. 137-43.
302. Wong, H.R., et al., *Increased expression of heat shock protein-70 protects A549 cells against hyperoxia*. Am J Physiol, 1998. **275**(4): p. L836-41.
303. Zhang, J., N. Fan, and Y. Peng, *Heat shock protein 70 promotes lipogenesis in HepG2 cells*. Lipids Health Dis, 2018. **17**(1): p. 73.

304. Sharma, A., A.S. Meena, and M.K. Bhat, *Hyperthermia-associated carboplatin resistance: differential role of p53, HSF1 and Hsp70 in hepatoma cells*. Cancer Sci, 2010. **101**(5): p. 1186-93.
305. Gao, M., X.-P. Geng, and H.-P. Xiang, *HSP90 and SIRT3 expression in hepatocellular carcinoma and their effect on invasive capability of human hepatocellular carcinoma cells*. Asian Pacific Journal of Tropical Medicine, 2015. **8**(4): p. 305-308.
306. Liu, K., et al., *BJ-B11, an Hsp90 Inhibitor, Constrains the Proliferation and Invasion of Breast Cancer Cells*. Front Oncol, 2019. **9**: p. 1447.
307. Marrugal, A., et al., *Impact of Heat Shock Protein 90 Inhibition on the Proteomic Profile of Lung Adenocarcinoma as Measured by Two-Dimensional Electrophoresis Coupled with Mass Spectrometry*. Cells, 2019. **8**(8).
308. Hauser, P., et al., *Expression and prognostic examination of heat shock proteins (HSP 27, HSP 70, and HSP 90) in medulloblastoma*. J Pediatr Hematol Oncol, 2006. **28**(7): p. 461-6.
309. Magwenyane, A.M., et al., *Heat Shock Protein 90 (HSP90) Inhibitors as Anticancer Medicines: A Review on the Computer-Aided Drug Discovery Approaches over the Past Five Years*. Comput Math Methods Med, 2022. **2022**: p. 2147763.
310. Chen, S.F., et al., *Quercetin suppresses drug-resistant spheres via the p38 MAPK-Hsp27 apoptotic pathway in oral cancer cells*. PLoS One, 2012. **7**(11): p. e49275.
311. Mittal, S. and M.S. Rajala, *Heat shock proteins as biomarkers of lung cancer*. Cancer Biol Ther, 2020. **21**(6): p. 477-485.
312. Chen, X., et al., *Suppression of HSP27 increases the antitumor effects of quercetin in human leukemia U937 cells*. Mol Med Rep, 2016. **13**(1): p. 689-96.

313. Fahrig, R., et al., *RP101 improves the efficacy of chemotherapy in pancreas carcinoma cell lines and pancreatic cancer patients*. *Anticancer Drugs*, 2006. **17**(9): p. 1045-56.
314. Trinh, D.L., A.N. Elwi, and S.W. Kim, *Direct interaction between p53 and Tid1 proteins affects p53 mitochondrial localization and apoptosis*. *Oncotarget*, 2010. **1**(6): p. 396-404.
315. Sharma, A., A.K. Upadhyay, and M.K. Bhat, *Inhibition of Hsp27 and Hsp40 potentiates 5-fluorouracil and carboplatin mediated cell killing in hepatoma cells*. *Cancer Biol Ther*, 2009. **8**(22): p. 2106-13.
316. Xu, D., et al., *Inhibition of mutant Kras and p53-driven pancreatic carcinogenesis by atorvastatin: Mainly via targeting of the farnesylated DNAJA1 in chaperoning mutant p53*. *Mol Carcinog*, 2019. **58**(11): p. 2052-2064.
317. Cho, K.F., et al., *Chaperone mediated detection of small molecule target binding in cells*. *Nat Commun*, 2020. **11**(1): p. 465.
318. Fortugno, P., et al., *Regulation of survivin function by Hsp90*. *Proc Natl Acad Sci U S A*, 2003. **100**(24): p. 13791-6.
319. Cheung, C.H., et al., *Targeting Hsp90 with small molecule inhibitors induces the over-expression of the anti-apoptotic molecule, survivin, in human A549, HONE-1 and HT-29 cancer cells*. *Mol Cancer*, 2010. **9**: p. 77.
320. Zhu, S., et al., *Design, Synthesis, and Biological Evaluation of HSP90 Inhibitor-SN38 Conjugates for Targeted Drug Accumulation*. *J Med Chem*, 2020. **63**(10): p. 5421-5441.
321. Jegu, G., et al., *Targeting heat shock proteins in cancer*. *Cancer Lett*, 2013. **332**(2): p. 275-85.

322. Jensen, M.R., et al., *NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models*. Breast Cancer Res, 2008. **10**(2): p. R33.
323. Mayor-Lopez, L., et al., *Comparative Study of 17-AAG and NVP-AUY922 in Pancreatic and Colorectal Cancer Cells: Are There Common Determinants of Sensitivity?* Transl Oncol, 2014. **7**(5): p. 590-604.
324. Maziasz, T., et al., *Predictive toxicology approaches for small molecule oncology drugs*. Toxicol Pathol, 2010. **38**(1): p. 148-64.
325. Supko, J.G., et al., *Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent*. Cancer Chemother Pharmacol, 1995. **36**(4): p. 305-15.
326. Quevedo, A.C., I. Lynch, and E. Valsami-Jones, *Silver nanoparticle induced toxicity and cell death mechanisms in embryonic zebrafish cells*. Nanoscale, 2021. **13**(12): p. 6142-6161.
327. Alimov, I., et al., *Bile acid analogues are activators of pyrin inflammasome*. J Biol Chem, 2019. **294**(10): p. 3359-3366.
328. Dalzon, B., et al., *How reversible are the effects of silver nanoparticles on macrophages? A proteomic-instructed view*. Environmental Science: Nano, 2019. **6**(10): p. 3133-3157.
329. Costa, E.C., et al., *Optimization of liquid overlay technique to formulate heterogenic 3D co-cultures models*. Biotechnol Bioeng, 2014. **111**(8): p. 1672-85.
330. Eilenberger, C., et al., *Effect of Spheroidal Age on Sorafenib Diffusivity and Toxicity in a 3D HepG2 Spheroid Model*. Sci Rep, 2019. **9**(1): p. 4863.
331. Wang, Y.N., et al., *CPT1A-mediated fatty acid oxidation promotes colorectal cancer cell metastasis by inhibiting anoikis*. Oncogene, 2018. **37**(46): p. 6025-6040.

332. Banerjee, H., et al., *A role for apoptosis-inducing factor in T cell development*. J Exp Med, 2012. **209**(9): p. 1641-53.
333. Yamada, Y., et al., *Mitochondrial Delivery of an Anticancer Drug Via Systemic Administration Using a Mitochondrial Delivery System That Inhibits the Growth of Drug-Resistant Cancer Engrafted on Mice*. J Pharm Sci, 2020. **109**(8): p. 2493-2500.
334. Li, J., et al., *Real-time detection of CTL function reveals distinct patterns of caspase activation mediated by Fas versus granzyme B*. J Immunol, 2014. **193**(2): p. 519-28.
335. Simonian, M.H. and J.A. Smith, *Spectrophotometric and colorimetric determination of protein concentration*. Curr Protoc Mol Biol, 2006. **Chapter 10**: p. Unit 10 1A.
336. Peng, J. and X. Liang, *Progress in research on gold nanoparticles in cancer management*. Medicine (Baltimore), 2019. **98**(18): p. e15311.
337. Vines, J.B., et al., *Gold Nanoparticles for Photothermal Cancer Therapy*. Front Chem, 2019. **7**: p. 167.
338. Jain, S., D.G. Hirst, and J.M. O'Sullivan, *Gold nanoparticles as novel agents for cancer therapy*. Br J Radiol, 2012. **85**(1010): p. 101-13.
339. Kong, F.Y., et al., *Unique Roles of Gold Nanoparticles in Drug Delivery, Targeting and Imaging Applications*. Molecules, 2017. **22**(9).
340. Singh, P., et al., *Gold Nanoparticles in Diagnostics and Therapeutics for Human Cancer*. Int J Mol Sci, 2018. **19**(7).
341. Abu-Dief, A.M., M. Salaheldeen, and T. El-Dabea, *Recent Advances in Development of Gold Nanoparticles for Drug Delivery Systems*. Journal of Modern Nanotechnology, 2021. **1**(1).
342. Afzal, O., et al., *Nanoparticles in Drug Delivery: From History to Therapeutic Applications*. Nanomaterials (Basel), 2022. **12**(24).

343. Pradhan, D., et al., *Recent Advancement in Nanotechnology-Based Drug Delivery System Against Viral Infections*. AAPS PharmSciTech, 2021. **22**(1): p. 47.
344. Dykman, L.A., *Gold nanoparticles for preparation of antibodies and vaccines against infectious diseases*. Expert Rev Vaccines, 2020. **19**(5): p. 465-477.
345. Mateu Ferrando, R., L. Lay, and L. Polito, *Gold nanoparticle-based platforms for vaccine development*. Drug Discov Today Technol, 2020. **38**: p. 57-67.
346. Sportelli, M.C., et al., *Can Nanotechnology and Materials Science Help the Fight against SARS-CoV-2?* Nanomaterials (Basel), 2020. **10**(4).
347. Carnovale, C., et al., *Identifying Trends in Gold Nanoparticle Toxicity and Uptake: Size, Shape, Capping Ligand, and Biological Corona*. ACS Omega, 2019. **4**(1): p. 242-256.
348. Alaqad, K. and T.A. Saleh, *Gold and Silver Nanoparticles: Synthesis Methods, Characterization Routes and Applications towards Drugs*. Journal of Environmental & Analytical Toxicology, 2016. **6**(4).
349. Alkilany, A.M. and C.J. Murphy, *Toxicity and cellular uptake of gold nanoparticles: what we have learned so far?* J Nanopart Res, 2010. **12**(7): p. 2313-2333.
350. Chueh, P.J., et al., *Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines*. J Hazard Mater, 2014. **264**: p. 303-12.
351. Muthukumarasamyvel, T., et al., *Role of Surface Hydrophobicity of Dicationic Amphiphile-Stabilized Gold Nanoparticles on A549 Lung Cancer Cells*. ACS Omega, 2017. **2**(7): p. 3527-3538.
352. Zheng, Y., et al., *Gold nano particles synthesized from Magnolia officinalis and anticancer activity in A549 lung cancer cells*. Artif Cells Nanomed Biotechnol, 2019. **47**(1): p. 3101-3109.

353. Ashokkumar, T., et al., *Apoptosis in liver cancer (HepG2) cells induced by functionalized gold nanoparticles*. Colloids Surf B Biointerfaces, 2014. **123**: p. 549-56.
354. Ramalingam, V., et al., *Biogenic gold nanoparticles induce cell cycle arrest through oxidative stress and sensitize mitochondrial membranes in A549 lung cancer cells*. RSC Advances, 2016. **6**(25): p. 20598-20608.
355. Henson, J.C., et al., *PEGylated Gold Nanoparticle Toxicity in Cardiomyocytes: Assessment of Size, Concentration, and Time Dependency*. IEEE Trans Nanobioscience, 2022. **21**(3): p. 387-394.
356. Llewellyn, S.V., et al., *Deducing the cellular mechanisms associated with the potential genotoxic impact of gold and silver engineered nanoparticles upon different lung epithelial cell lines in vitro*. Nanotoxicology, 2022. **16**(1): p. 52-72.
357. Eldridge, A.G. and T. O'Brien, *Therapeutic strategies within the ubiquitin proteasome system*. Cell Death Differ, 2010. **17**(1): p. 4-13.
358. Sha, Z., J. Zhao, and A.L. Goldberg, *Measuring the Overall Rate of Protein Breakdown in Cells and the Contributions of the Ubiquitin-Proteasome and Autophagy-Lysosomal Pathways*. Methods Mol Biol, 2018. **1844**: p. 261-276.
359. Chen, X., et al., *Metal-based proteasomal deubiquitinase inhibitors as potential anticancer agents*. Cancer Metastasis Rev, 2017. **36**(4): p. 655-668.
360. Fang, S. and A.M. Weissman, *A field guide to ubiquitylation*. Cell Mol Life Sci, 2004. **61**(13): p. 1546-61.
361. Wang, M., et al., *The USP7 Inhibitor P5091 Induces Cell Death in Ovarian Cancers with Different P53 Status*. Cell Physiol Biochem, 2017. **43**(5): p. 1755-1766.
362. Cartel, M., et al., *Inhibition of ubiquitin-specific protease 7 sensitizes acute myeloid leukemia to chemotherapy*. Leukemia, 2021. **35**(2): p. 417-432.

363. Das, T., et al., *Regulation of Deubiquitinating Enzymes by Post-Translational Modifications*. Int J Mol Sci, 2020. **21**(11).
364. Ding, Y., et al., *Deubiquitinase inhibitor b-AP15 activates endoplasmic reticulum (ER) stress and inhibits Wnt/Notch1 signaling pathway leading to the reduction of cell survival in hepatocellular carcinoma cells*. Eur J Pharmacol, 2018. **825**: p. 10-18.
365. Xia, X., et al., *Targeting proteasome-associated deubiquitinases as a novel strategy for the treatment of estrogen receptor-positive breast cancer*. Oncogenesis, 2018. **7**(9): p. 75.
366. Crawford, L.J., B. Walker, and A.E. Irvine, *Proteasome inhibitors in cancer therapy*. J Cell Commun Signal, 2011. **5**(2): p. 101-10.
367. Zucker, R.M., et al., *Detection of silver nanoparticles in cells by flow cytometry using light scatter and far-red fluorescence*. Cytometry A, 2013. **83**(10): p. 962-72.
368. Wu, Y., et al., *Improving the Flow Cytometry-based Detection of the Cellular Uptake of Gold Nanoparticles*. Anal Chem, 2019. **91**(22): p. 14261-14267.
369. Ivanov, M.R., H.R. Bednar, and A.J. Haes, *Investigations of the mechanism of gold nanoparticle stability and surface functionalization in capillary electrophoresis*. ACS Nano, 2009. **3**(2): p. 386-94.
370. Liu, Z., et al., *Effects of internalized gold nanoparticles with respect to cytotoxicity and invasion activity in lung cancer cells*. PLoS One, 2014. **9**(6): p. e99175.
371. Subtil, A., et al., *Acute cholesterol depletion inhibits clathrin-coated pit budding*. Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6775-80.
372. Harrigan, J.A., et al., *Deubiquitylating enzymes and drug discovery: emerging opportunities*. Nat Rev Drug Discov, 2018. **17**(1): p. 57-78.

373. Dreaden, E.C., et al., *The golden age: gold nanoparticles for biomedicine*. Chem Soc Rev, 2012. **41**(7): p. 2740-79.
374. Yang, X., et al., *Gold Nanomaterials at Work in Biomedicine*. Chem Rev, 2015. **115**(19): p. 10410-88.
375. Falagan-Lotsch, P., E.M. Grzincic, and C.J. Murphy, *One low-dose exposure of gold nanoparticles induces long-term changes in human cells*. Proc Natl Acad Sci U S A, 2016. **113**(47): p. 13318-13323.
376. Lima, T., et al., *Understanding the Lipid and Protein Corona Formation on Different Sized Polymeric Nanoparticles*. Sci Rep, 2020. **10**(1): p. 1129.
377. Sabuncu, A.C., et al., *Probing nanoparticle interactions in cell culture media*. Colloids Surf B Biointerfaces, 2012. **95**: p. 96-102.
378. Senut, M.C., et al., *Size-Dependent Toxicity of Gold Nanoparticles on Human Embryonic Stem Cells and Their Neural Derivatives*. Small, 2016. **12**(5): p. 631-46.
379. Avalos, A., et al., *Effects of silver and gold nanoparticles of different sizes in human pulmonary fibroblasts*. Toxicol Mech Methods, 2015. **25**(4): p. 287-95.
380. Pan, Y., et al., *Gold nanoparticles of diameter 1.4 nm trigger necrosis by oxidative stress and mitochondrial damage*. Small, 2009. **5**(18): p. 2067-76.
381. Augustine, R., et al., *Cellular uptake and retention of nanoparticles: Insights on particle properties and interaction with cellular components*. Materials Today Communications, 2020. **25**.
382. Engstrom, A.M., et al., *Size-Dependent Interactions of Lipid-Coated Gold Nanoparticles: Developing a Better Mechanistic Understanding Through Model Cell Membranes and in vivo Toxicity*. Int J Nanomedicine, 2020. **15**: p. 4091-4104.

383. Martinez-Torres, A.C., et al., *Chitosan gold nanoparticles induce different ROS-dependent cell death modalities in leukemic cells*. Int J Nanomedicine, 2019. **14**: p. 7173-7190.
384. Schlinkert, P., et al., *The oxidative potential of differently charged silver and gold nanoparticles on three human lung epithelial cell types*. J Nanobiotechnology, 2015. **13**: p. 1.
385. Mohammadinejad, R., et al., *Necrotic, apoptotic and autophagic cell fates triggered by nanoparticles*. Autophagy, 2019. **15**(1): p. 4-33.
386. Cardoso, F., et al., *Targeting the ubiquitin-proteasome pathway in breast cancer*. Clin Breast Cancer, 2004. **5**(2): p. 148-57.
387. Chen, X., et al., *Inhibition of Proteasomal Deubiquitinase by Silver Complex Induces Apoptosis in Non-Small Cell Lung Cancer Cells*. Cell Physiol Biochem, 2018. **49**(2): p. 780-797.
388. Dang, L., et al., *Proteasome inhibitor MG132 inhibits the proliferation and promotes the cisplatin-induced apoptosis of human esophageal squamous cell carcinoma cells*. Int J Mol Med, 2014. **33**(5): p. 1083-8.
389. Guo, N. and Z. Peng, *MG132, a proteasome inhibitor, induces apoptosis in tumor cells*. Asia Pac J Clin Oncol, 2013. **9**(1): p. 6-11.
390. Wu, J. and H. Xie, *Effects of titanium dioxide nanoparticles on alpha-synuclein aggregation and the ubiquitin-proteasome system in dopaminergic neurons*. Artif Cells Nanomed Biotechnol, 2016. **44**(2): p. 690-4.
391. Khan, O.M., et al., *Proteasomal degradation of the tumour suppressor FBW7 requires branched ubiquitylation by TRIP12*. Nat Commun, 2021. **12**(1): p. 2043.

392. Pandit, B. and A.L. Gartel, *Proteasome inhibitors induce p53-independent apoptosis in human cancer cells*. Am J Pathol, 2011. **178**(1): p. 355-60.
393. Ramalingam, V., et al., *Gold nanoparticle induces mitochondria-mediated apoptosis and cell cycle arrest in nonsmall cell lung cancer cells*. Gold Bulletin, 2017. **50**(2): p. 177-189.
394. Zhang, Y., et al., *Effect of Size, Shape, and Surface Modification on Cytotoxicity of Gold Nanoparticles to Human HEp-2 and Canine MDCK Cells*. Journal of Nanomaterials, 2012. **2012**: p. 1-7.
395. Mieszawska, A.J., et al., *Multifunctional gold nanoparticles for diagnosis and therapy of disease*. Mol Pharm, 2013. **10**(3): p. 831-47.
396. Gerber, A., et al., *Gold nanoparticles: recent aspects for human toxicology*. J Occup Med Toxicol, 2013. **8**(1): p. 32.
397. Zhao, P., et al., *Differential toxicity mechanism of gold nanoparticles in HK-2 renal proximal tubular cells and 786-0 carcinoma cells*. Nanomedicine, 2020. **15**(11): p. 1079-1096.
398. Ozcicek, I., et al., *The effects of surface functionality and size of gold nanoparticles on neuronal toxicity, apoptosis, ROS production and cellular/suborgan biodistribution*. Mater Sci Eng C Mater Biol Appl, 2021. **128**: p. 112308.
399. Zhao, M.X., et al., *The Apoptosis Effect on Liver Cancer Cells of Gold Nanoparticles Modified with Lithocholic Acid*. Nanoscale Res Lett, 2018. **13**(1): p. 304.
400. Mohan, J.C., et al., *Functionalised gold nanoparticles for selective induction of in vitro apoptosis among human cancer cell lines*. Journal of Experimental Nanoscience, 2013. **8**(1): p. 32-45.

401. Vales, G., et al., *Size, Surface Functionalization, and Genotoxicity of Gold Nanoparticles In Vitro*. Nanomaterials (Basel), 2020. **10**(2).
402. Haiss, W., et al., *Determination of size and concentration of gold nanoparticles from UV-vis spectra*. Anal Chem, 2007. **79**(11): p. 4215-21.
403. Kumar, V. and S.K. Yadav, *Characterisation of gold nanoparticles synthesised by leaf and seed extract of Syzygium cumini L.* Journal of Experimental Nanoscience, 2012. **7**(4): p. 440-451.
404. Pashkov, D.M., et al., *Quantitative Analysis of the UV-Vis Spectra for Gold Nanoparticles Powered by Supervised Machine Learning*. The Journal of Physical Chemistry C, 2021. **125**(16): p. 8656-8666.
405. Alhadlaq, H.A., M.J. Akhtar, and M. Ahamed, *Different cytotoxic and apoptotic responses of MCF-7 and HT1080 cells to MnO₂ nanoparticles are based on similar mode of action*. Toxicology, 2019. **411**: p. 71-80.
406. Aquilano, K., S. Baldelli, and M.R. Ciriolo, *Glutathione: new roles in redox signaling for an old antioxidant*. Front Pharmacol, 2014. **5**: p. 196.
407. Misawa, M. and J. Takahashi, *Generation of reactive oxygen species induced by gold nanoparticles under x-ray and UV Irradiations*. Nanomedicine, 2011. **7**(5): p. 604-14.
408. El-Seedi, H.R., et al., *Metal nanoparticles fabricated by green chemistry using natural extracts: biosynthesis, mechanisms, and applications*. RSC Advances, 2019. **9**(42): p. 24539-24559.
409. Riaz Ahmed, K.B., et al., *Silver nanoparticles: Significance of physicochemical properties and assay interference on the interpretation of in vitro cytotoxicity studies*. Toxicol In Vitro, 2017. **38**: p. 179-192.

410. Bobyk, L., et al., *Toxicity and chemical transformation of silver nanoparticles in A549 lung cells: dose-rate-dependent genotoxic impact*. Environmental Science: Nano, 2021. **8**(3): p. 806-821.
411. Leibrock, L., et al., *Nanoparticle induced barrier function assessment at liquid-liquid and air-liquid interface in novel human lung epithelia cell lines*. Toxicol Res (Camb), 2019. **8**(6): p. 1016-1027.
412. Cohen, J.M., J.G. Teeguarden, and P. Demokritou, *An integrated approach for the in vitro dosimetry of engineered nanomaterials*. Part Fibre Toxicol, 2014. **11**: p. 20.
413. Lowry, G.V., et al., *Guidance to improve the scientific value of zeta-potential measurements in nanoEHS*. Environmental Science: Nano, 2016. **3**(5): p. 953-965.
414. Goodman, C.M., et al., *Toxicity of gold nanoparticles functionalized with cationic and anionic side chains*. Bioconjug Chem, 2004. **15**(4): p. 897-900.
415. Subramanian, K. and K. Ponnuchamy, *Gold nanoparticles tethered cinnamic acid: preparation, characterization, and cytotoxic effects on MCF-7 breast cancer cell lines*. Applied Nanoscience, 2018. **8**(5): p. 1133-1138.
416. Borgese, M., et al., *Recovery ability of human adipose stem cells exposed to cobalt nanoparticles: outcome of dissolution*. Nanomedicine (Lond), 2020. **15**(5): p. 453-465.
417. Mironava, T., et al., *Gold nanoparticles cellular toxicity and recovery: effect of size, concentration and exposure time*. Nanotoxicology, 2010. **4**(1): p. 120-37.
418. Aggarwal, V., et al., *Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements*. Biomolecules, 2019. **9**(11).
419. Xu, Z., et al., *Green Biosynthesized Silver Nanoparticles With Aqueous Extracts of Ginkgo Biloba Induce Apoptosis via Mitochondrial Pathway in Cervical Cancer Cells*. Front Oncol, 2020. **10**: p. 575415.

420. Li, Y., J. Yang, and X. Sun, *Reactive Oxygen Species-Based Nanomaterials for Cancer Therapy*. Front Chem, 2021. **9**: p. 650587.
421. Tabatabaie, F., et al., *Oxidative Damage to Mitochondria Enhanced by Ionising Radiation and Gold Nanoparticles in Cancer Cells*. Int J Mol Sci, 2022. **23**(13).
422. Khan, H.A., et al., *Effect of gold nanoparticles on glutathione and malondialdehyde levels in liver, lung and heart of rats*. Saudi J Biol Sci, 2012. **19**(4): p. 461-4.
423. Suski, J.M., et al., *Relation between mitochondrial membrane potential and ROS formation*. Methods Mol Biol, 2012. **810**: p. 183-205.
424. Schaeublin, N.M., et al., *Surface charge of gold nanoparticles mediates mechanism of toxicity*. Nanoscale, 2011. **3**(2): p. 410-20.
425. Eleftheriadis, T., et al., *Cytochrome c as a Potentially Clinical Useful Marker of Mitochondrial and Cellular Damage*. Front Immunol, 2016. **7**: p. 279.
426. Zeinizade, E., et al., *Selective apoptosis induction in cancer cells using folate-conjugated gold nanoparticles and controlling the laser irradiation conditions*. Artif Cells Nanomed Biotechnol, 2018. **46**(sup1): p. 1026-1038.
427. Kapuria, V., et al., *Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis*. Cancer Res, 2010. **70**(22): p. 9265-76.
428. Lee, C.S., et al., *Deubiquitinases: Modulators of Different Types of Regulated Cell Death*. Int J Mol Sci, 2021. **22**(9).
429. Lei, H., H. Shan, and Y. Wu, *Targeting deubiquitinating enzymes in cancer stem cells*. Cancer Cell Int, 2017. **17**: p. 101.
430. Nijman, S.M., et al., *A genomic and functional inventory of deubiquitinating enzymes*. Cell, 2005. **123**(5): p. 773-86.

431. Ibrahim, B., et al., *Gold Nanoparticles Induced Size Dependent Cytotoxicity on Human Alveolar Adenocarcinoma Cells by Inhibiting the Ubiquitin Proteasome System*. Pharmaceutics, 2023. **15**(2).
432. Yuan, T., et al., *Inhibition of Ubiquitin-Specific Proteases as a Novel Anticancer Therapeutic Strategy*. Front Pharmacol, 2018. **9**: p. 1080.
433. Byun, S., et al., *USP8 is a novel target for overcoming gefitinib resistance in lung cancer*. Clin Cancer Res, 2013. **19**(14): p. 3894-904.
434. Issaenko, O.A. and A.Y. Amerik, *Chalcone-based small-molecule inhibitors attenuate malignant phenotype via targeting deubiquitinating enzymes*. Cell Cycle, 2012. **11**(9): p. 1804-17.
435. Jeong, C.H., *Inhibition of Ubiquitin-specific Peptidase 8 Suppresses Growth of Gefitinib-resistant Non-small Cell Lung Cancer Cells by Inducing Apoptosis*. J Cancer Prev, 2015. **20**(1): p. 57-63.
436. Yang, Y., et al., *Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics*. Cancer Res, 2007. **67**(19): p. 9472-81.
437. Liao, Y., et al., *Growth arrest and apoptosis induction in androgen receptor-positive human breast cancer cells by inhibition of USP14-mediated androgen receptor deubiquitination*. Oncogene, 2018. **37**(14): p. 1896-1910.
438. Bai, D.P., et al., *Zinc oxide nanoparticles induce apoptosis and autophagy in human ovarian cancer cells*. Int J Nanomedicine, 2017. **12**: p. 6521-6535.
439. Chan, F.K., K. Moriwaki, and M.J. De Rosa, *Detection of necrosis by release of lactate dehydrogenase activity*. Methods Mol Biol, 2013. **979**: p. 65-70.
440. Huang, Y., et al., *Comparative study of the effects of gold and silver nanoparticles on the metabolism of human dermal fibroblasts*. Regen Biomater, 2020. **7**(2): p. 221-232.

441. Xue, Y., et al., *Cytotoxicity and apoptosis induced by silver nanoparticles in human liver HepG2 cells in different dispersion media*. J Appl Toxicol, 2016. **36**(3): p. 352-60.
442. Chen, B., M.E. Feder, and L. Kang, *Evolution of heat-shock protein expression underlying adaptive responses to environmental stress*. Mol Ecol, 2018. **27**(15): p. 3040-3054.
443. Macario, A.J. and E. Conway de Macario, *Molecular chaperones: multiple functions, pathologies, and potential applications*. Front Biosci, 2007. **12**: p. 2588-600.
444. Haslbeck, M. and E. Vierling, *A first line of stress defense: small heat shock proteins and their function in protein homeostasis*. J Mol Biol, 2015. **427**(7): p. 1537-48.
445. Jee, H., *Size dependent classification of heat shock proteins: a mini-review*. J Exerc Rehabil, 2016. **12**(4): p. 255-9.
446. Jaattela, M., *Heat shock proteins as cellular lifeguards*. Ann Med, 1999. **31**(4): p. 261-71.
447. Jin, H.O., et al., *Induction of HSP27 and HSP70 by constitutive overexpression of Redd1 confers resistance of lung cancer cells to ionizing radiation*. Oncol Rep, 2019. **41**(5): p. 3119-3126.
448. Seigneuric, R., et al., *Heat shock proteins as danger signals for cancer detection*. Front Oncol, 2011. **1**: p. 37.
449. Landriscina, M., et al., *Heat shock proteins, cell survival and drug resistance: the mitochondrial chaperone TRAP1, a potential novel target for ovarian cancer therapy*. Gynecol Oncol, 2010. **117**(2): p. 177-82.
450. Saini, J. and P.K. Sharma, *Clinical, Prognostic and Therapeutic Significance of Heat Shock Proteins in Cancer*. Curr Drug Targets, 2018. **19**(13): p. 1478-1490.

451. Antonova, A., et al., *Heat-Shock Protein 90 Controls the Expression of Cell-Cycle Genes by Stabilizing Metazoan-Specific Host-Cell Factor HCFC1*. Cell Rep, 2019. **29**(6): p. 1645-1659 e9.
452. Pechan, P.M., *Heat shock proteins and cell proliferation*. FEBS Letters, 1991. **280**(1): p. 1-4.
453. Shimamura, T., et al., *Ganetespib (STA-9090), a nongeldanamycin HSP90 inhibitor, has potent antitumor activity in in vitro and in vivo models of non-small cell lung cancer*. Clin Cancer Res, 2012. **18**(18): p. 4973-85.
454. Wang, L., et al., *Novel Hsp90 Inhibitor C086 Potently Inhibits Non-Small Cell Lung Cancer Cells As A Single Agent Or In Combination With Gefitinib*. Cancer Manag Res, 2019. **11**: p. 8937-8945.
455. Das, J.K., et al., *Heat Shock Proteins in Cancer Immunotherapy*. J Oncol, 2019. **2019**: p. 3267207.
456. Chakraborty, A., et al., *Size-dependent apoptotic activity of gold nanoparticles on osteosarcoma cells correlated with SERS signal*. J Photochem Photobiol B, 2020. **203**: p. 111778.
457. Khan, M.I., et al., *Induction of ROS, mitochondrial damage and autophagy in lung epithelial cancer cells by iron oxide nanoparticles*. Biomaterials, 2012. **33**(5): p. 1477-88.
458. Basso, A.D., et al., *Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function*. J Biol Chem, 2002. **277**(42): p. 39858-66.

459. Fujiwara, H., et al., *IC101 induces apoptosis by Akt dephosphorylation via an inhibition of heat shock protein 90-ATP binding activity accompanied by preventing the interaction with Akt in L1210 cells*. J Pharmacol Exp Ther, 2004. **310**(3): p. 1288-95.
460. Sato, S., N. Fujita, and T. Tsuruo, *Modulation of Akt kinase activity by binding to Hsp90*. Proc Natl Acad Sci U S A, 2000. **97**(20): p. 10832-7.
461. Lee, S.H. and B.H. Jun, *Silver Nanoparticles: Synthesis and Application for Nanomedicine*. Int J Mol Sci, 2019. **20**(4).
462. Nel, A.E., et al., *Understanding biophysicochemical interactions at the nano-bio interface*. Nat Mater, 2009. **8**(7): p. 543-57.
463. Hansen, U. and A.F. Thunemann, *Characterization of Silver Nanoparticles in Cell Culture Medium Containing Fetal Bovine Serum*. Langmuir, 2015. **31**(24): p. 6842-52.
464. Singh, P., et al., *Pharmacological importance, characterization and applications of gold and silver nanoparticles synthesized by Panax ginseng fresh leaves*. Artif Cells Nanomed Biotechnol, 2017. **45**(7): p. 1415-1424.
465. Wang, W., et al., *Zeta-potential data reliability of gold nanoparticle biomolecular conjugates and its application in sensitive quantification of surface absorbed protein*. Colloids Surf B Biointerfaces, 2016. **148**: p. 541-548.
466. Loza, K., et al., *The dissolution and biological effects of silver nanoparticles in biological media*. J Mater Chem B, 2014. **2**(12): p. 1634-1643.
467. Mbanga, O., E. Cukrowska, and M. Gulumian, *Dissolution of citrate-stabilized, polyethylene glycol-coated carboxyl and amine-functionalized gold nanoparticles in simulated biological fluids and environmental media*. Journal of Nanoparticle Research, 2021. **23**(1).

468. Mbanga, O., E. Cukrowska, and M. Gulumian, *Dissolution kinetics of silver nanoparticles: Behaviour in simulated biological fluids and synthetic environmental media*. Toxicology Reports, 2022. **9**: p. 788-796.
469. Parveen, A. and S. Rao, *Cytotoxicity and Genotoxicity of Biosynthesized Gold and Silver Nanoparticles on Human Cancer Cell Lines*. Journal of Cluster Science, 2014. **26**(3): p. 775-788.
470. Lewinski, N., V. Colvin, and R. Drezek, *Cytotoxicity of nanoparticles*. Small, 2008. **4**(1): p. 26-49.
471. He, C., et al., *Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles*. Biomaterials, 2010. **31**(13): p. 3657-66.
472. Iswarya, V., et al., *Surface capping and size-dependent toxicity of gold nanoparticles on different trophic levels*. Environ Sci Pollut Res Int, 2016. **23**(5): p. 4844-58.
473. Costa, E.C., et al., *3D tumor spheroids: an overview on the tools and techniques used for their analysis*. Biotechnol Adv, 2016. **34**(8): p. 1427-1441.
474. Kwak, B., et al., *Mass fabrication of uniform sized 3D tumor spheroid using high-throughput microfluidic system*. J Control Release, 2018. **275**: p. 201-207.
475. Wang, D., et al., *Roles of ROS and cell cycle arrest in the genotoxicity induced by gold nanorod core/silver shell nanostructure*. Nanoscale Res Lett, 2020. **15**(1): p. 224.
476. Ullah, I., et al., *Green-Synthesized Silver Nanoparticles Induced Apoptotic Cell Death in MCF-7 Breast Cancer Cells by Generating Reactive Oxygen Species and Activating Caspase 3 and 9 Enzyme Activities*. Oxid Med Cell Longev, 2020. **2020**: p. 1215395.
477. El-Hussein, A., et al., *Comparative study between the photodynamic ability of gold and silver nanoparticles in mediating cell death in breast and lung cancer cell lines*. J Photochem Photobiol B, 2015. **153**: p. 67-75.

478. Patlolla, A.K., D. Hackett, and P.B. Tchounwou, *Silver nanoparticle-induced oxidative stress-dependent toxicity in Sprague-Dawley rats*. Mol Cell Biochem, 2015. **399**(1-2): p. 257-68.
479. Chairuangkitti, P., et al., *Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways*. Toxicology in Vitro, 2013. **27**(1): p. 330-338.
480. Ma, W., et al., *Silver nanoparticle exposure induced mitochondrial stress, caspase-3 activation and cell death: amelioration by sodium selenite*. Int J Biol Sci, 2015. **11**(8): p. 860-7.
481. Han, J.W., et al., *Oxidative stress mediated cytotoxicity of biologically synthesized silver nanoparticles in human lung epithelial adenocarcinoma cell line*. Nanoscale Res Lett, 2014. **9**(1): p. 459.
482. Lin, T., et al., *Heat Shock Protein 90 Family Isoforms as Prognostic Biomarkers and Their Correlations with Immune Infiltration in Breast Cancer*. Biomed Res Int, 2020. **2020**: p. 2148253.
483. Sheng, B., et al., *Increased HSP27 correlates with malignant biological behavior of non-small cell lung cancer and predicts patient's survival*. Sci Rep, 2017. **7**(1): p. 13807.
484. Zackova, M., et al., *Hsp90 - a potential prognostic marker in CML*. Blood Cells Mol Dis, 2013. **50**(3): p. 184-9.
485. Banerji, U., et al., *Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies*. J Clin Oncol, 2005. **23**(18): p. 4152-61.
486. Su, T.R., et al., *Proteomic investigation of anti-tumor activities exerted by sinularin against A2058 melanoma cells*. Electrophoresis, 2012. **33**(7): p. 1139-52.

487. Lall, R.K., V.M. Adhami, and H. Mukhtar, *Dietary flavonoid fisetin for cancer prevention and treatment*. Mol Nutr Food Res, 2016. **60**(6): p. 1396-405.
488. Xin, L., et al., *Increased oxidative stress and activated heat shock proteins in human cell lines by silver nanoparticles*. Hum Exp Toxicol, 2015. **34**(3): p. 315-23.
489. Ahamed, M., et al., *Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in Drosophila melanogaster*. Toxicol Appl Pharmacol, 2010. **242**(3): p. 263-9.
490. Deng, W., et al., *Heat shock protein 27 downstream of P38-PI3K/Akt signaling antagonizes melatonin-induced apoptosis of SGC-7901 gastric cancer cells*. Cancer Cell Int, 2016. **16**: p. 5.
491. Kanagasabai, R., et al., *Hsp27 protects adenocarcinoma cells from UV-induced apoptosis by Akt and p21-dependent pathways of survival*. Mol Cancer Res, 2010. **8**(10): p. 1399-412.
492. Lee, H., et al., *Ganetespib induces G2/M cell cycle arrest and apoptosis in gastric cancer cells through targeting of receptor tyrosine kinase signaling*. Int J Oncol, 2017. **51**(3): p. 967-974.
493. Pirali, M., et al., *Artesunate, as a HSP70 ATPase activity inhibitor, induces apoptosis in breast cancer cells*. Int J Biol Macromol, 2020. **164**: p. 3369-3375.
494. Saleh, A., et al., *Negative regulation of the Apaf-1 apoptosome by Hsp70*. Nat Cell Biol, 2000. **2**(8): p. 476-83.
495. Zheng, L., et al., *Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages*. J Immunol, 2004. **173**(10): p. 6319-26.

496. Riaz, M.A., et al., *Metformin enhances the radiosensitizing effect of cisplatin in non-small cell lung cancer cell lines with different cisplatin sensitivities*. Sci Rep, 2019. **9**(1): p. 1282.
497. Shen, M., et al., *Inhibition of ATM reverses EMT and decreases metastatic potential of cisplatin-resistant lung cancer cells through JAK/STAT3/PD-L1 pathway*. J Exp Clin Cancer Res, 2019. **38**(1): p. 149.
498. Dasari, S. and P.B. Tchounwou, *Cisplatin in cancer therapy: molecular mechanisms of action*. Eur J Pharmacol, 2014. **740**: p. 364-78.
499. Nonnekens, J. and J.H. Hoeijmakers, *After surviving cancer, what about late life effects of the cure?* EMBO Mol Med, 2017. **9**(1): p. 4-6.
500. Duan, X., et al., *Nanoparticle formulations of cisplatin for cancer therapy*. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2016. **8**(5): p. 776-91.
501. Li, Z., et al., *Synergistic effects of silver nanoparticles and cisplatin in combating inflammation and hyperplasia of airway stents*. Bioact Mater, 2022. **9**: p. 266-280.
502. Iram, S., et al., *Cisplatin bioconjugated enzymatic GNPs amplify the effect of cisplatin with acquiescence*. Sci Rep, 2019. **9**(1): p. 13826.
503. Min, Y., et al., *Combating the drug resistance of cisplatin using a platinum prodrug based delivery system*. Angew Chem Int Ed Engl, 2012. **51**(27): p. 6742-7.
504. Ballatori, N., et al., *Glutathione dysregulation and the etiology and progression of human diseases*. Biol Chem, 2009. **390**(3): p. 191-214.
505. Lu, X.X., et al., *PTEN Inhibits Cell Proliferation, Promotes Cell Apoptosis, and Induces Cell Cycle Arrest via Downregulating the PI3K/AKT/hTERT Pathway in Lung Adenocarcinoma A549 Cells*. Biomed Res Int, 2016. **2016**: p. 2476842.

506. Paquette, M., L. El-Houjeiri, and A. Pause, *mTOR Pathways in Cancer and Autophagy*. Cancers (Basel), 2018. **10**(1).
507. Rascio, F., et al., *The Pathogenic Role of PI3K/AKT Pathway in Cancer Onset and Drug Resistance: An Updated Review*. Cancers (Basel), 2021. **13**(16).
508. Guemei, A., et al., *Study of the Efficacy and Multidrug Resistance Using Gold Nanoparticles-Based Drug Delivery Versus Conventional Chemotherapy in Non-Small-Cell Lung Cancer Cell Line*. Research in Oncology, 2021. **17**(2): p. 42-50.
509. González-López, M.A., et al., *Reducing the effective dose of cisplatin using gold nanoparticles as carriers*. Cancer Nanotechnology, 2020. **11**(1).
510. Xiong, X., et al., *Sensitization of ovarian cancer cells to cisplatin by gold nanoparticles*. Oncotarget, 2014. **5**(15): p. 6453-65.
511. Moradpoor, R., et al., *Enhancement of Cisplatin Efficacy by Gold Nanoparticles or Microwave Hyperthermia? An In Vitro Study on a Melanoma Cell Line*. Iranian Journal of Cancer Prevention, 2017. **In Press**(In Press).
512. Chien, C.C., et al., *Roles of reactive oxygen species, mitochondrial membrane potential, and p53 in evodiamine-induced apoptosis and G2/M arrest of human anaplastic thyroid carcinoma cells*. Chin Med, 2021. **16**(1): p. 134.
513. Marchi, S., et al., *Mitochondria-ros crosstalk in the control of cell death and aging*. J Signal Transduct, 2012. **2012**: p. 329635.
514. Redza-Dutordoir, M. and D.A. Averill-Bates, *Activation of apoptosis signalling pathways by reactive oxygen species*. Biochim Biophys Acta, 2016. **1863**(12): p. 2977-2992.
515. Albakova, Z., et al., *Extracellular heat shock proteins and cancer: New perspectives*. Transl Oncol, 2021. **14**(2): p. 100995.

516. Den, R.B. and B. Lu, *Heat shock protein 90 inhibition: rationale and clinical potential*. Ther Adv Med Oncol, 2012. **4**(4): p. 211-8.
517. Endo, H., et al., *Ibuprofen enhances the anticancer activity of cisplatin in lung cancer cells by inhibiting the heat shock protein 70*. Cell Death Dis, 2014. **5**: p. e1027.
518. Moc, C., et al., *Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy*. Cardiovasc Res, 2015. **105**(2): p. 160-70.
519. Yin, L., et al., *Heat Shock Protein 90 Triggers Multi-Drug Resistance of Ovarian Cancer via AKT/GSK3beta/beta-Catenin Signaling*. Front Oncol, 2021. **11**: p. 620907.
520. Mayo, L.D. and D.B. Donner, *A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus*. Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11598-603.
521. Peng, Y., et al., *Inhibition of MDM2 by hsp90 contributes to mutant p53 stabilization*. J Biol Chem, 2001. **276**(44): p. 40583-90.
522. Xia, M., et al., *Elevated MDM2 boosts the apoptotic activity of p53-MDM2 binding inhibitors by facilitating MDMX degradation*. Cell Cycle, 2008. **7**(11): p. 1604-12.
523. Adams, J., *The development of proteasome inhibitors as anticancer drugs*. Cancer Cell, 2004. **5**(5): p. 417-421.
524. Shen, X., et al., *Anti-tumor activity of a novel proteasome inhibitor D395 against multiple myeloma and its lower cardiotoxicity compared with carfilzomib*. Cell Death Dis, 2021. **12**(5): p. 429.
525. Sun, F., et al., *Proteasome Inhibitor MG132 Enhances Cisplatin-Induced Apoptosis in Osteosarcoma Cells and Inhibits Tumor Growth*. Oncol Res, 2018. **26**(4): p. 655-664.

526. Zhang, L., et al., *In vitro and in vivo therapeutic efficacy of carfilzomib in mantle cell lymphoma: targeting the immunoproteasome*. Mol Cancer Ther, 2013. **12**(11): p. 2494-504.
527. Berenson, J.R., et al., *Replacement of bortezomib with carfilzomib for multiple myeloma patients progressing from bortezomib combination therapy*. Leukemia, 2014. **28**(7): p. 1529-36.
528. Mendez-Toro, A., C. Diaz-Brochero, and E. Acosta-Gutierrez, *Carfilzomib induced cardiotoxicity in a multiple myeloma patient*. Cardiooncology, 2020. **6**: p. 17.
529. Adewale, O.B., et al., *Toxicological Behavior of Gold Nanoparticles on Various Models: Influence of Physicochemical Properties and Other Factors*. Int J Toxicol, 2019. **38**(5): p. 357-384.
530. Shang, L., K. Nienhaus, and G.U. Nienhaus, *Engineered nanoparticles interacting with cells: size matters*. J Nanobiotechnology, 2014. **12**: p. 5.
531. Dong, X., et al., *The Size-dependent Cytotoxicity of Amorphous Silica Nanoparticles: A Systematic Review of in vitro Studies*. Int J Nanomedicine, 2020. **15**: p. 9089-9113.
532. Magogotya, M., et al., *In vitro toxicity and internalization of gold nanoparticles (AuNPs) in human epithelial colorectal adenocarcinoma (Caco-2) cells and the human skin keratinocyte (HaCaT) cells*. Mutat Res Genet Toxicol Environ Mutagen, 2022. **883-884**: p. 503556.
533. Mazumdar, S., D. Chitkara, and A. Mittal, *Exploration and insights into the cellular internalization and intracellular fate of amphiphilic polymeric nanocarriers*. Acta Pharm Sin B, 2021. **11**(4): p. 903-924.
534. Vijayakumar, S. and S. Ganesan, *Size-dependent in vitro cytotoxicity assay of gold nanoparticles*. Toxicological & Environmental Chemistry, 2013. **95**(2): p. 277-287.

535. Wang, P., et al., *Evaluating cellular uptake of gold nanoparticles in HL-7702 and HepG2 cells for plasmonic photothermal therapy*. Nanomedicine (Lond), 2018. **13**(18): p. 2245-2259.
536. Brandenberger, C., et al., *Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles*. Small, 2010. **6**(15): p. 1669-78.
537. Yang, L., L. Shang, and G.U. Nienhaus, *Mechanistic aspects of fluorescent gold nanocluster internalization by live HeLa cells*. Nanoscale, 2013. **5**(4): p. 1537-43.
538. McCarrick, S., et al., *Gold Nanoparticles Dissolve Extracellularly in the Presence of Human Macrophages*. Int J Nanomedicine, 2021. **16**: p. 5895-5908.
539. Khaing Oo, M.K., et al., *Gold nanoparticle-enhanced and size-dependent generation of reactive oxygen species from protoporphyrin IX*. ACS Nano, 2012. **6**(3): p. 1939-47.
540. Blanco, E., H. Shen, and M. Ferrari, *Principles of nanoparticle design for overcoming biological barriers to drug delivery*. Nat Biotechnol, 2015. **33**(9): p. 941-51.
541. Mitchell, M.J., et al., *Engineering precision nanoparticles for drug delivery*. Nat Rev Drug Discov, 2021. **20**(2): p. 101-124.
542. Longmire, M., P.L. Choyke, and H. Kobayashi, *Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats*. Nanomedicine (Lond), 2008. **3**(5): p. 703-17.
543. Zhu, G.H., A.B.C. Gray, and H.K. Patra, *Nanomedicine: controlling nanoparticle clearance for translational success*. Trends Pharmacol Sci, 2022. **43**(9): p. 709-711.
544. Sibuyi, N.R.S., et al., *Multifunctional Gold Nanoparticles for Improved Diagnostic and Therapeutic Applications: A Review*. Nanoscale Res Lett, 2021. **16**(1): p. 174.

545. Wang, P., et al., *Interaction of gold nanoparticles with proteins and cells*. Sci Technol Adv Mater, 2015. **16**(3): p. 034610.
546. Vijayakumar, S. and S. Ganesan, *In Vitro Cytotoxicity Assay on Gold Nanoparticles with Different Stabilizing Agents*. Journal of Nanomaterials, 2012. **2012**: p. 1-9.
547. Arvizo, R., R. Bhattacharya, and P. Mukherjee, *Gold nanoparticles: opportunities and challenges in nanomedicine*. Expert Opin Drug Deliv, 2010. **7**(6): p. 753-63.
548. Tiwari, P.M., et al., *Functionalized Gold Nanoparticles and Their Biomedical Applications*. Nanomaterials (Basel), 2011. **1**(1): p. 31-63.
549. Shahbazi, R., et al., *Modified gold-based siRNA nanotherapeutics for targeted therapy of triple-negative breast cancer*. Nanomedicine (Lond), 2017. **12**(16): p. 1961-1973.
550. Goldbraikh, D., et al., *USP1 deubiquitinates Akt to inhibit PI3K-Akt-FoxO signaling in muscle during prolonged starvation*. EMBO Rep, 2020. **21**(4): p. e48791.
551. Yang, W.L., et al., *The E3 ligase TRAF6 regulates Akt ubiquitination and activation*. Science, 2009. **325**(5944): p. 1134-8.
552. Gartner, E.M., et al., *A phase II study of 17-allylamino-17-demethoxygeldanamycin in metastatic or locally advanced, unresectable breast cancer*. Breast Cancer Res Treat, 2012. **131**(3): p. 933-7.
553. Heinrich, J.C., et al., *RP101 (brivudine) binds to heat shock protein HSP27 (HSPB1) and enhances survival in animals and pancreatic cancer patients*. J Cancer Res Clin Oncol, 2011. **137**(9): p. 1349-61.
554. Konduru, N.V., et al., *Protein corona: implications for nanoparticle interactions with pulmonary cells*. Part Fibre Toxicol, 2017. **14**(1): p. 42.
555. Montes-Burgos, I., et al., *Characterisation of nanoparticle size and state prior to nanotoxicological studies*. Journal of Nanoparticle Research, 2009. **12**(1): p. 47-53.

556. Pem, B., et al., *Toxicity and safety study of silver and gold nanoparticles functionalized with cysteine and glutathione*. Beilstein J Nanotechnol, 2019. **10**: p. 1802-1817.
557. Schneider, T., M. Westermann, and M. Glei, *In vitro uptake and toxicity studies of metal nanoparticles and metal oxide nanoparticles in human HT29 cells*. Arch Toxicol, 2017. **91**(11): p. 3517-3527.
558. Los, M., et al., *Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling*. Mol Biol Cell, 2002. **13**(3): p. 978-88.
559. Walsh, J.G., et al., *Executioner caspase-3 and caspase-7 are functionally distinct proteases*. Proc Natl Acad Sci U S A, 2008. **105**(35): p. 12815-9.
560. Endo, H., et al., *Ibuprofen enhances the anticancer activity of cisplatin in lung cancer cells by inhibiting the heat shock protein 70*. Cell Death Dis, 2014. **5**(1): p. e1027.
561. Teeguarden, J.G., et al., *Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments*. Toxicol Sci, 2007. **95**(2): p. 300-12.
562. Huk, A., et al., *Is the toxic potential of nanosilver dependent on its size? Part Fibre Toxicol*, 2014. **11**: p. 65.

Supplementary Materials

The chapter present the list of figures and tables used as supplementary materials.

Supplementary Materials Chapter 3

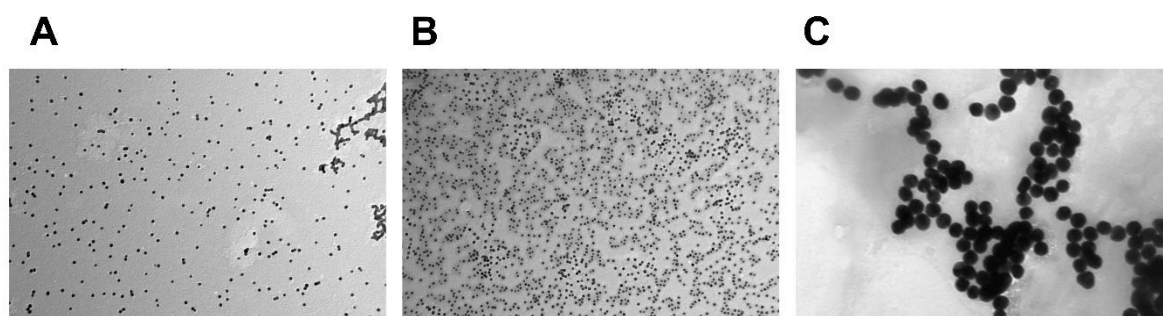


Figure S3.1. Characterisation of different sizes AuNPs dispersed in Ultrapure water by Transmission of electron microscopy (TEM): **(A)** 5 nm AuNPs **(B)** 10 nm AuNPs **(C)** 80 nm AuNPs

Surface area (SA)

The surface area of different size AuNPs corresponding to the concentrations used in the cell viability assay was calculated according to Teeguarden *et al.*, (2007) using the below equation.

Where AuNPs are assumed to be spherical or nanosphere, r is the radius of AuNPs suspended in medium determined by DLS and V is the volume of a sphere used [561].

$$V = \frac{4}{3}(\pi * r^3)$$

For instance, the surface area of 5 nm AuNPs (17.57 nm diameter measured with DLS) at a starting concentration was as follows.

Mass concentration (gm)	concentration	0.005
Diameter (nm)	r^{*2}	308.7049
Radius (r)	Diameter/2	154.35245
Density of Au (g/cm ³)	19.32	19.32
Volume (V)	mass/density	0.000258799
Volume per particle (v)	$4/3(\pi * r^3)$	15011136.78
N	V/v	1.72405E-11
SA per particles (M ²)	$4 * \pi * r^2$	299237.966
Total SA M ² /g	N*sa (m ²)	5.15901E-06

Table S3.1: Surface area (m²/g) of different 5, 10 and 80 nm AuNPs at different mass concentrations used in the cell viability assays.

Mass concentration (µg/mL)								
AuNPs	5	10	15	20	25	30	35	40
5 nm	5.13E-06	1.03E-05	1.55E-05	2.06E-05	2.58E-05	3.1E-05	3.61E-05	4.13E-05
10 nm	1.36E-06	2.73E-06	4.09E-06	5.46E-06	6.82E-06	8.18E-06	9.55E-06	1.09E-05
80 nm	1.27E-07	2.54E-07	3.81E-07	5.08E-07	6.35E-07	7.62E-07	8.89E-07	1.02E-06

Particle number concentration

The particles number concentration of AuNPs was calculated according to the formula described by Huk *et al.*, [562]. The mass (Au) concentration(mg/mL) and the particle

concentration (NPs/ml) of different size AuNPs were provided in the certificate of analysis of NPs by manufacturer (<https://tools.nanocomposix.com:48/cdn/coa/Gold/Spheres/NanoXact/AU5-NX-CIT-JRC0024.pdf?121%20956>). Using the highest concentration of AuNPs used for our study (40 µg/mL), we calculated the particle number concentration using the formula below:

$$\text{PNC} = \text{NPs mass concentration} * \text{NPs/mL/Mass Au}$$

Table S3.2: The table show the number of particles per volume (NPs/ML) for the highest concentration used in the cell viability assay and the number of viable cells remained after exposed to different size AuNPs for 24 h.

AuNPs	40 µg/mL	
	NPs/mL	% Viability
5 nm	3.17E+13	9.240198
10 nm	2.50E+12	12.9125
80 nm	8.46E+09	29.08156

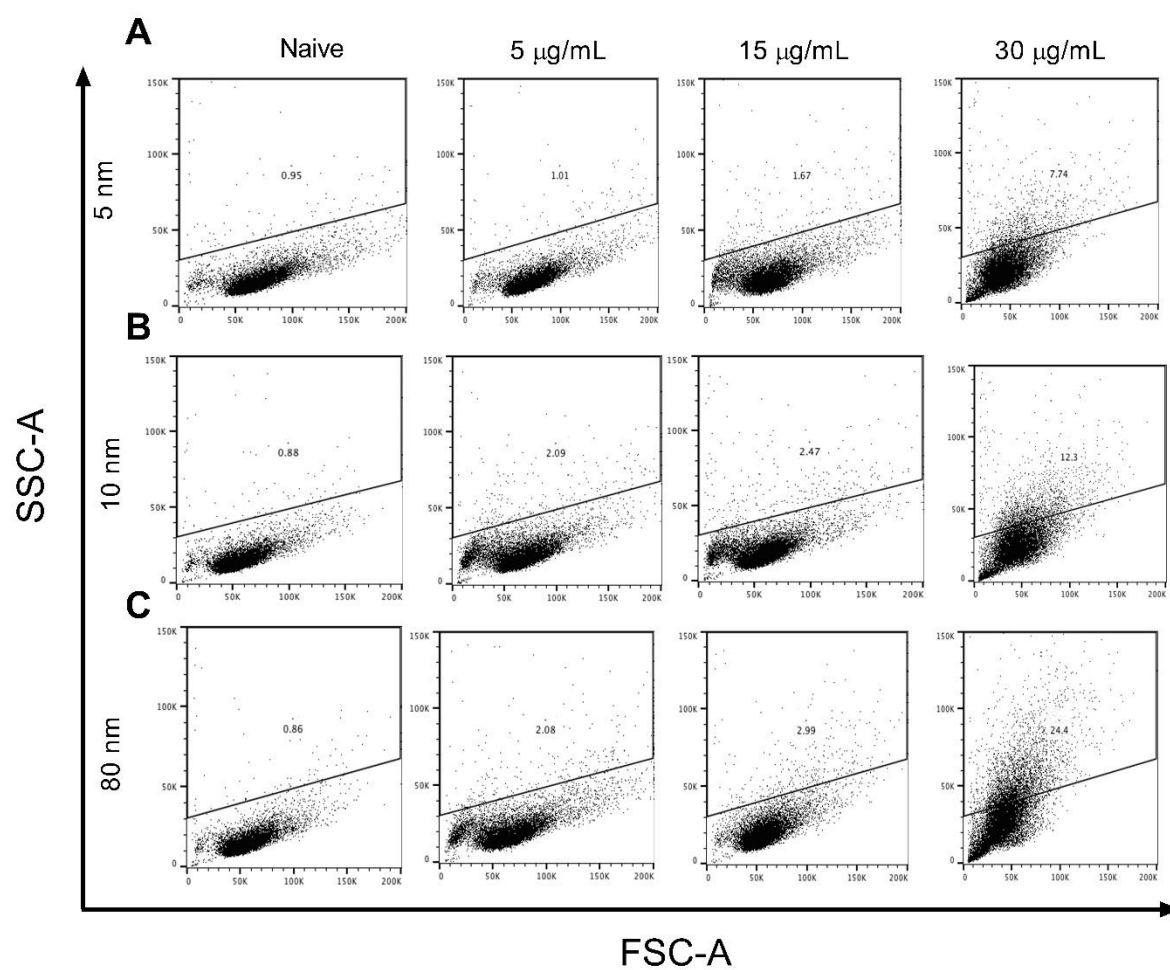


Figure S3.2. Dot plots of side scattered intensity (SSC) versus forward scattered intensity (FSC) of A549 cells treated with different concentration of AuNPs for 24 h. (A) 5 nm (B) 10 nm (C) 80 nm

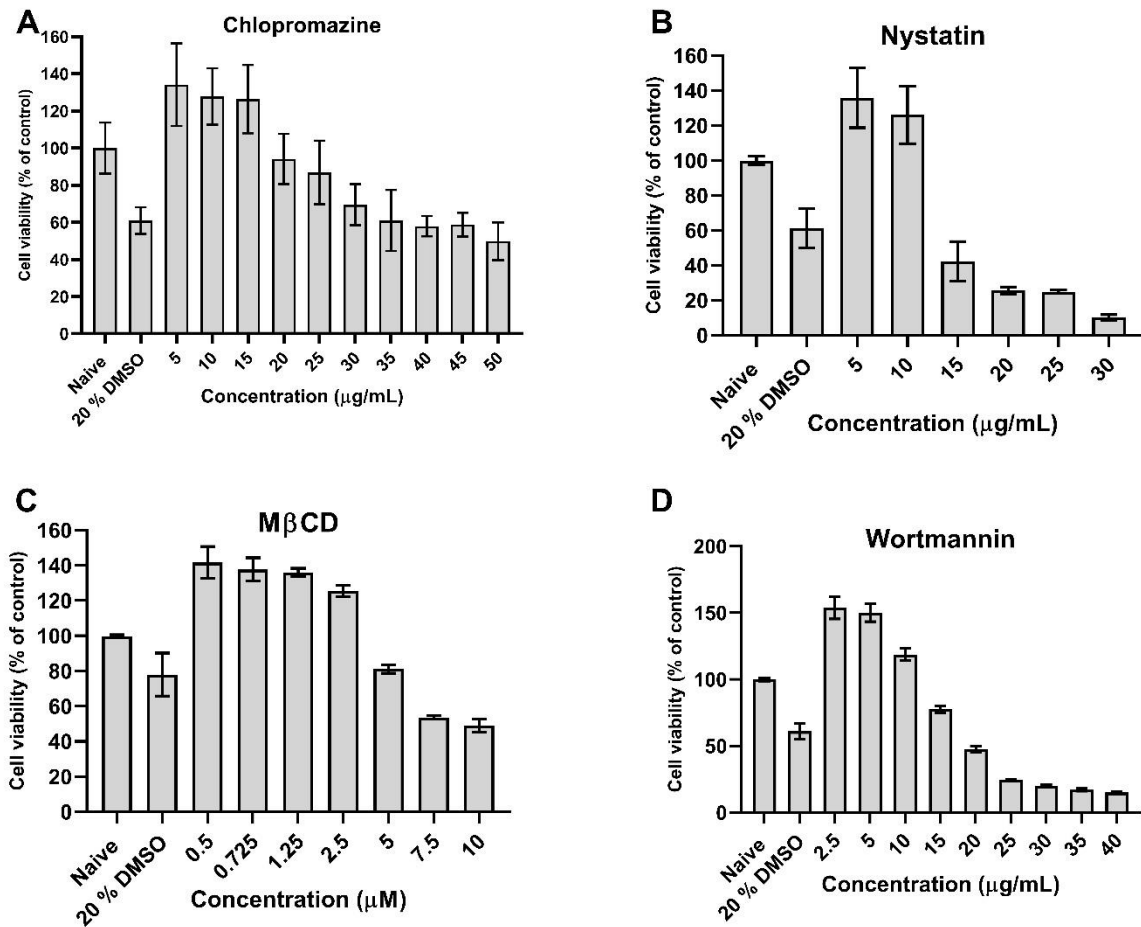


Figure S3.3. Percentage cell viability of A549 cells determined using MTT assay for pre-treated with pharmacological inhibitors **(A)** Chlorpromazine hydrochloride, inhibitor of Clathrin mediated endocytosis **(B)** Nystatin, inhibitor of Caveolae mediated endocytosis **(C)** M β CD, inhibitor of Caveolae mediated endocytosis **(D)** Wortmannin, inhibitor of macropinocytosis for different concentration after 1 h exposure. 20% DMSO was used as a positive control.

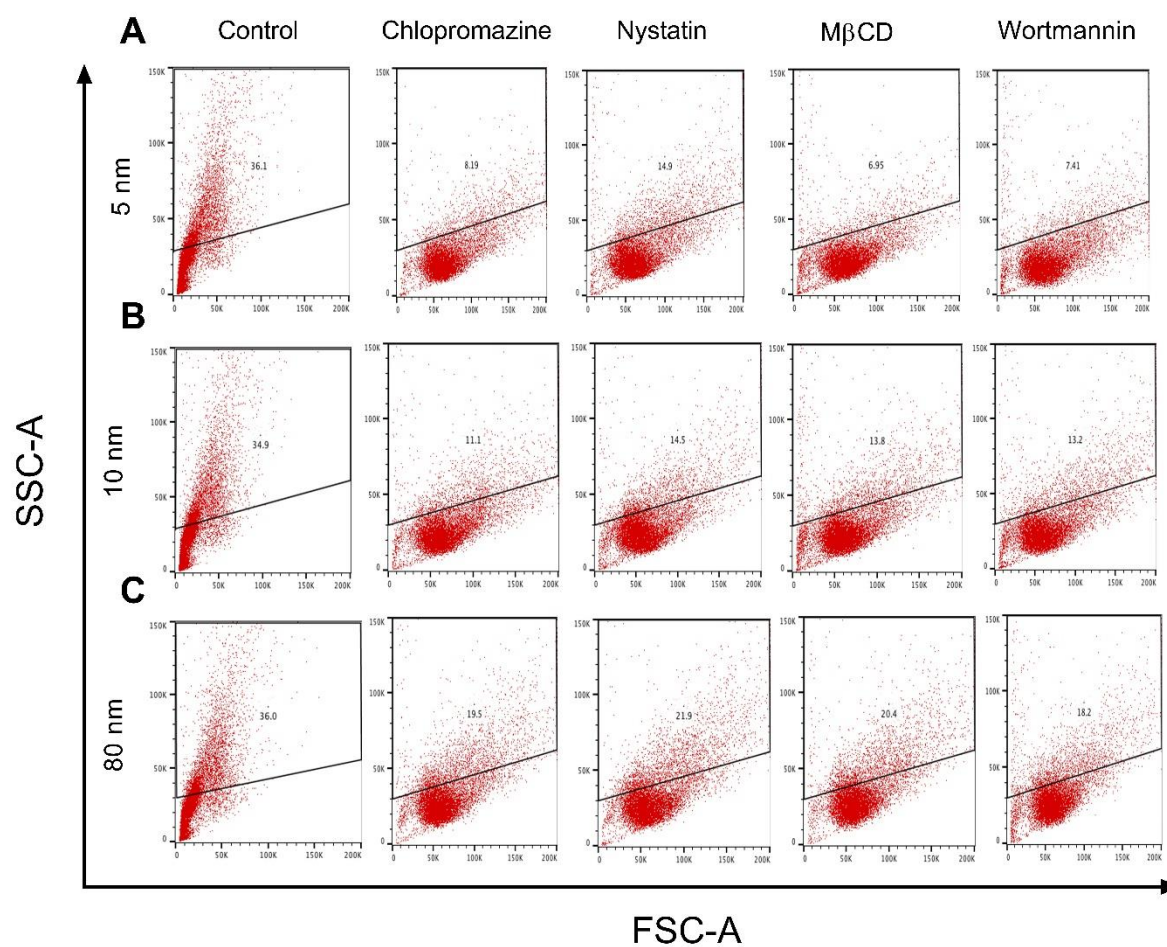


Figure S3.4: Dot plots effects of pharmacological inhibitors on the internalisation of different size AuNPs in A549 cells. Cells were separately pre-incubated with Chlorpromazine, Nystatin, methyl- β -cyclodextrin (M β CD) and Wortmannin for 1 h, followed by adding AuNPs incubation for 3 h and analysed flow cytometry. (A) 5 nm (B) 10 nm (C) 80 nm

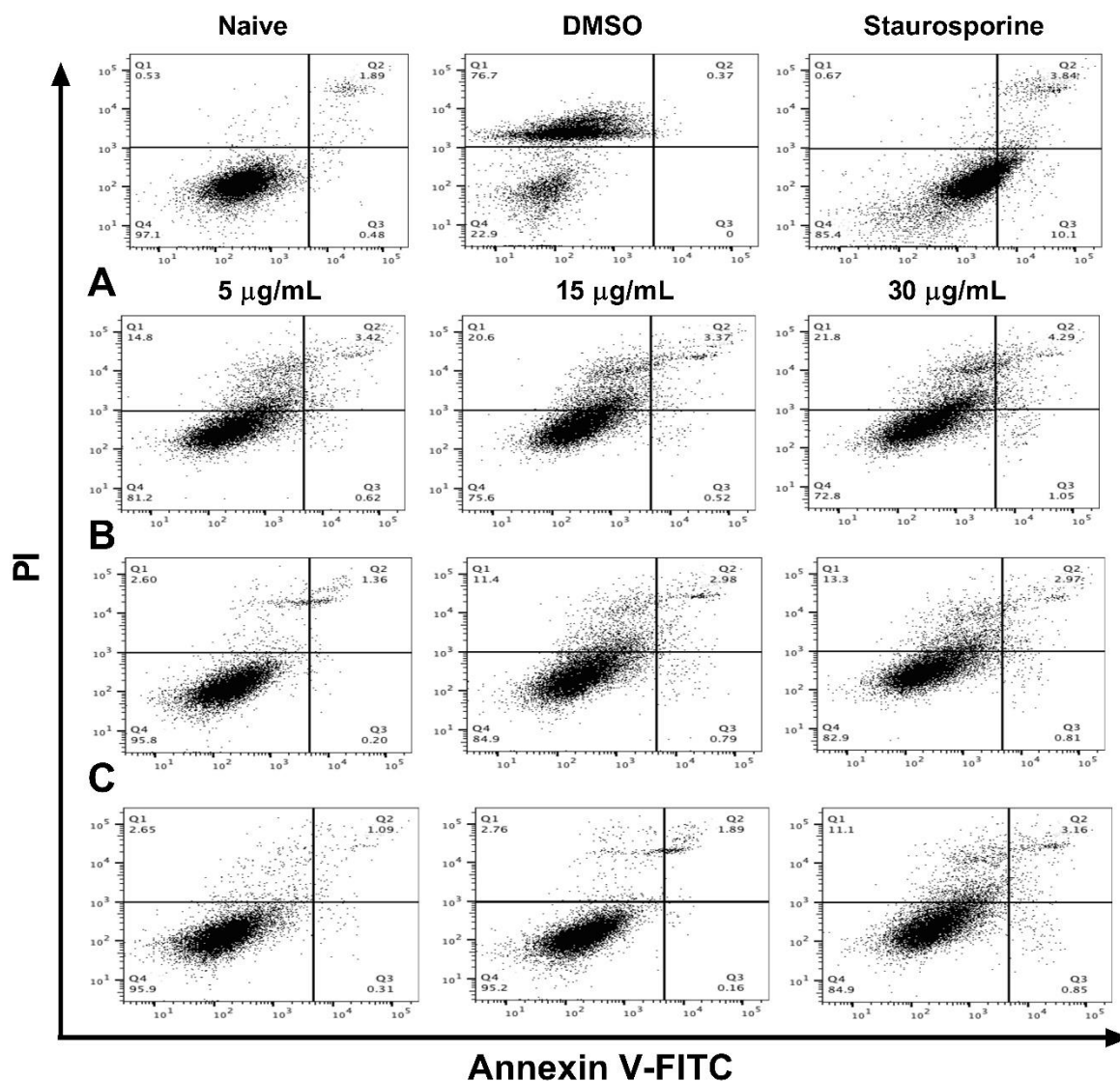


Figure S3.5. Apoptosis and necrosis analysis of A549 cells following the exposure to AuNPs of different sizes flow cytometry at of 5-30 $\mu\text{g/mL}$ for 24 h. (A) 5 nm (B) 10 nm (C) 80 nm. 1 μM Staurosporine and 10 % DMSO was used as positive control for apoptosis and necrosis for 6 h.

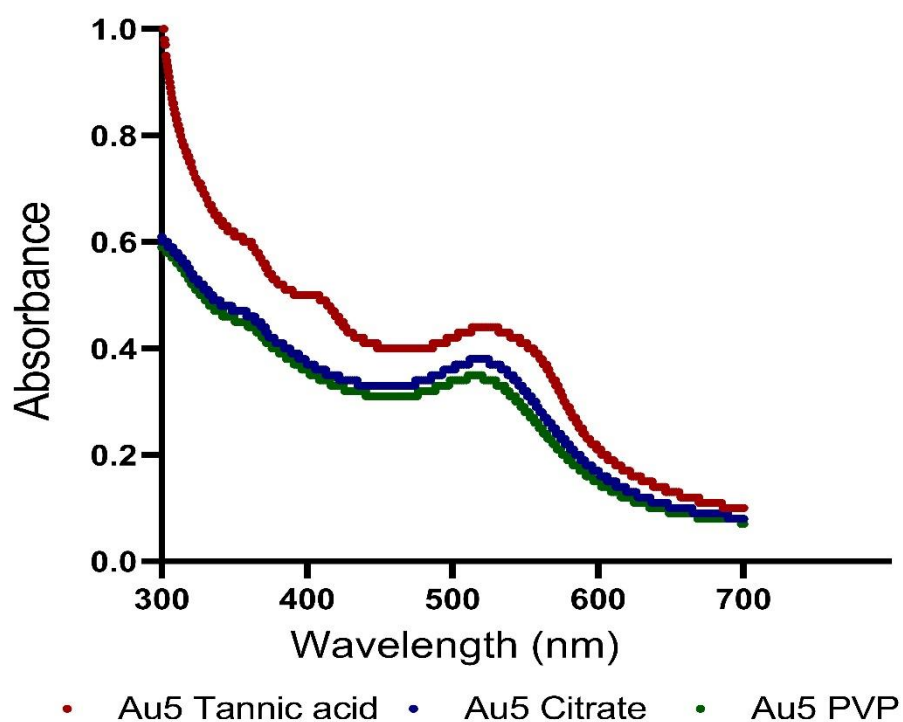


Figure S4.1: Absorption spectra of 5 nm AuNPs dispersed in UPW measured by UV-Vis.

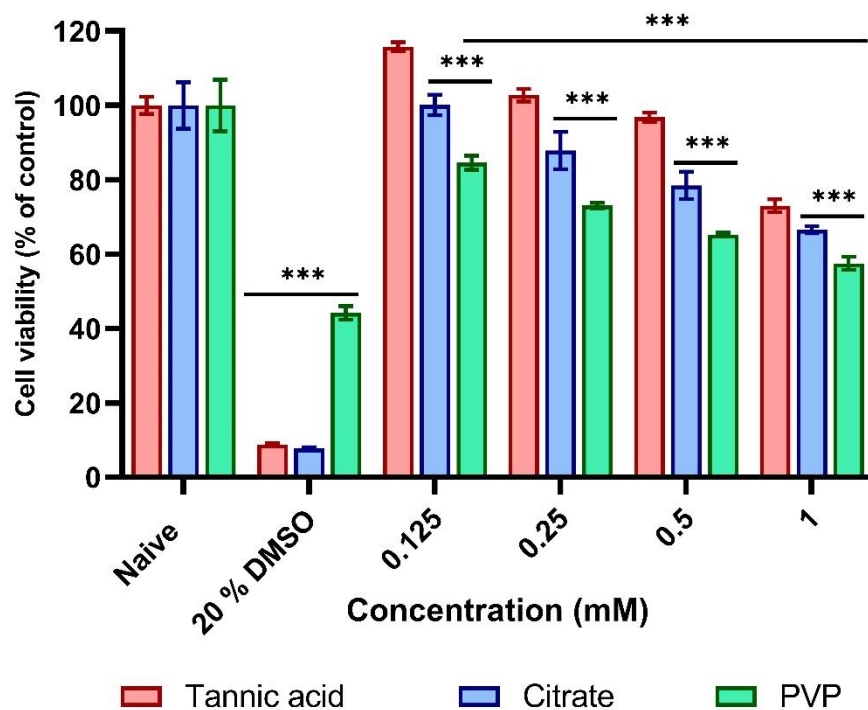


Figure S4.2: Cell viability of A549 cells exposed to stabilising agents at 0.125-1 mM for 24 h. Data represent mean + standard deviation of 3 independent experiments. Bars with asterisk (*) show statistically different of (***) $P < 0.001$ when compared with the control.

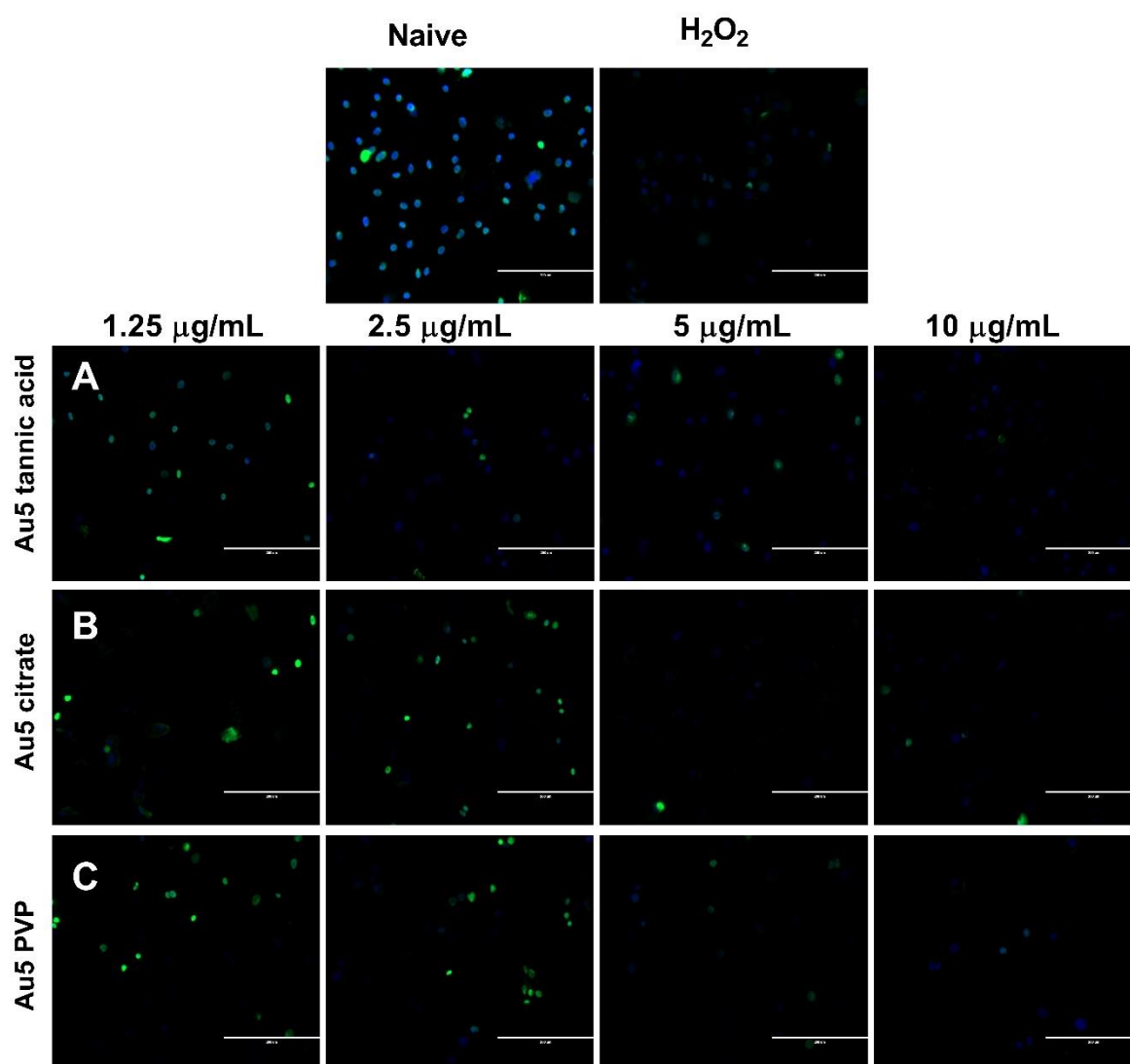


Figure S4.3: Mitochondrial membrane potential of A549 cells treated with different functionalised AuNPs at concentration of 1.25 -10 µg/mL stained with mitohealth staining kit. Blue stainin indicate cell nuclues and the grean fluorensce shows mitohealth dye in the mitochondria. Fluorescence intensity of 5 nm AuNPs capped (**A**) Tannic acid (**B**) Citrate (**C**) PVP. The images were captured with an EVOS fluorescence microscope using 20X maganification. Scale bar (200 µM).

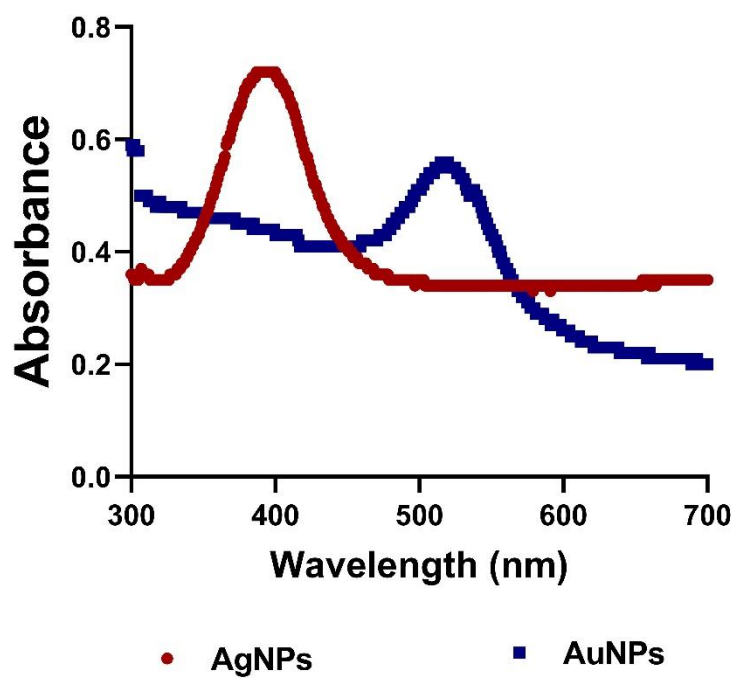


Figure S5.1: Absorption spectra of 10 nm of AgNPs and AuNPs dispersed in UPW measured by DLS.