

Design of a Smart, Stealth Nano-system for Targeted Drug Delivery in Prostate Cancer Treatment

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Declaration

I, Divesha Essa, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy by dissertation at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



____ (Signature of candidate)

30th day of August 2024 in Reading

Animal Ethics Declaration

I, Divesha Essa, hereby declare that the study entitled “Poly (lactide-co-glycolide) Based Nanoparticles for Prostate Cancer Treatment in a Nude Mouse Model” was approved by the Animal Research Ethics Committee (AREC) at the University of the Witwatersrand with the ethics approval number 2022/11/02/C (Appendix 4)

Publications

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Dedication

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Abstract

There have been several polymeric drug delivery vehicles developed on the nanoscale for chemotherapeutic applications. However, the use of polymers without the implementation of design perspectives has led to limitations in efficacy, including rapid elimination, poor biocompatibility, and high off-site delivery of the therapeutic agent. In this study we developed polymeric nanoparticle systems with enhanced cell penetrating and targeting abilities with the aid of design and optimization. The ultimate goal was to fabricate an optimized system with improved properties and performance as a nanocarrier for the chemotherapeutic drug docetaxel to prostate cancer tissue. Poly (lactic-co-glycolic) acid provided the ideal polymeric matrix to entrap hydrophobic drugs with high efficacy. Chitosan provided a positively charged surface to the system, ensuring favorable interactions with negatively charged cell membranes. Poly ethylene glycol provided a shielding effect to increase *in vivo* residence time, while folate and anti-PSMA antibody acted as targeted ligands to bind to prostate specific membrane antigen, PSMA, a cell surface receptor that is overexpressed on prostate cancer cells. Fabrication approaches compared traditional and microfluidics-based method, and we were able to prepare uniform, reproducible nanoparticles using microfluidics. We used disulfiram and the plant-based bioactive quercetin as model bio-actives, and investigated chitosan and folic acid for their reported cell penetrating properties, using a design of experiments optimization. Finally, we used the drug docetaxel, with PLGA and PEG as matrix polymers and anti-PSMA antibody as a targeting ligand for its specific ability to bind to PSMA. All systems were characterized by Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM), Thermogravimetric Analysis (TGA), and X-ray powder diffraction (XRD). Drug loading and release were conducted by ultra-violet (UV) spectroscopy and high-performance liquid chromatography (HPLC). Using the microfluidics method and an intermediate PEG density, the disulfiram encapsulated nanoparticles were prepared with a size of 179 ± 2.5 nm and maximum drug release of 70%. The targeted systems loaded with quercetin or docetaxel both displayed sustained pH dependent release profiles with maximum release of 78% quercetin and 72% of docetaxel at the tumour microenvironment-relevant acidic pH of 6. We evaluated *in vitro* toxicity of these systems on PSMA positive (LnCap) and negative (PC-3) cell lines as well as on a non-cancerous cell line (NIH-3T3), and thereafter conducted comparative fluorescence-based cellular uptake studies. Both targeted systems demonstrated higher cellular uptake in LnCap and an increase in toxicity of 66% for the quercetin-loaded and 60% for the docetaxel-loaded systems on LnCap compared to PC-3. The anti-PSMA targeted docetaxel-loaded system also selectively caused cell cycle arrest and $89.4\pm 8.4\%$ inhibition of cell migration in LnCap cells, but no inhibition on PC-3 cells, suggesting a PSMA-specific mechanism of action in these processes. Finally, we used an *in vitro* 3D LnCap multicellular tumour spheroid model to test for anti-cancer efficacy of the prepared docetaxel loaded nano-systems, and an *in vivo* nude mouse model to evaluate its biocompatibility. There was significantly more inhibition of spheroid growth by the non-targeted system ($18.3\pm 5.7\%$) and the targeted system ($50.4\pm 3.3\%$) compared to the untreated group after fifteen days of treatment, while all treatments showed no toxicity on the major organs of nude mice. Further studies are required to explore the potential of the PSMA targeted nano-system for its *in vivo* anti-cancer therapeutic potential.

Table of Contents

Declaration.....	i
Animal Ethics Declaration	ii
Publications.....	iii
Presentations.....	iv
Acknowledgements.....	v
Dedication.....	vi
Abstract.....	vii
List of Figures.....	xiii
List of Tables	xix
 CHAPTER 1 INTRODUCTION	 20
1.1 Background.....	20
1.2 Rationale and motivation for this study.....	24
1.3 Novelty.....	24
1.4 Aim and Objectives.....	25
1.5 Overview of Thesis.....	26
1.6 References	27
 CHAPTER 2 THE DESIGN OF POLY(LACTIDE-CO-GLYCOLIDE) NANOCARRIERS FOR MEDICAL APPLICATIONS	 30
2.1 Introduction	30
2.2 PLGA as a nanocarrier	34
2.2.1 Properties	34
2.2.2 Surface functionalisation.....	35
2.3 Synthetic methods of PLGA nanocarriers	38
2.3.1 Single and Double Emulsion	38
2.3.2 Spray drying.....	39
2.3.3 Coacervation.....	39
2.3.4 Salting out.....	40

2.3.5	Nanoprecipitation.....	41
2.3.6	Supercritical fluid technology.....	42
2.3.7	Microfluidics.....	43
2.3.8	Membrane extrusion emulsification	45
2.3.9	Nanoimprint lithography and the PRINT technique.....	46
2.4	Medical applications	48
2.4.1	Cancer Research	48
2.4.2	HIV Treatment	49
2.4.3	Inflammatory disorders.....	50
2.4.4	Other applications	50
2.4.5	Inclusion of PLGA formulations in the clinic.....	51
2.5	Computational modelling.....	1
2.6	Design Considerations.....	5
2.7	Disadvantages of PLGA as a nanocarrier.....	6
2.8	Conclusions and future work	6
2.9	References.....	7

CHAPTER 3 COMPARATIVE NANOFABRICATION OF PLGA-CHITOSAN-PEG SYSTEMS

EMPLOYING MICROFLUIDICS AND EMULSIFICATION SOLVENT EVAPORATION TECHNIQUES.24

3.1	Introduction.....	24
3.2	Materials and Methods.....	28
3.2.1	Materials.....	28
3.2.2	Preparation of Nanoparticles by the Emulsification Solvent Evaporation Approach	29
3.2.3	. Preparation of Nanoparticles Using the Microfluidics Approach.....	30
3.2.4	Determination of Particle Size and Zeta Potential	30
3.2.5	Determination of the Chemical and Structural Integrity of the Nano-systems.....	31
3.2.6	Investigation of the Thermal Properties of the Nano-systems.....	31
3.2.7	Determination of the Crystallinity of the Nano-systems	32
3.2.8	Investigation of Surface Morphology Using Scanning Electron Microscopy (SEM)	32
3.2.9	UV Spectrophotometric Analysis to Determine Entrapment Efficiency of Disulfiram.....	32
3.2.10	In vitro Disulfiram Release Studies.....	33
3.2.11	Statistical Analysis.....	33
3.3	Results and Discussion	33
3.3.1	Size and Stability Analysis of Formulations Prepared by Both Methods.....	33
3.3.2	FTIR Spectral Analysis Showing Adsorption of Polymer Layers.....	38
3.3.3	Comparative Thermogravimetric Analysis and Differential Scanning Calorimetry of Disulfiram, Blank and Loaded Nanoparticles Prepared by Both Methods	39

3.3.4	XRD Spectral Analysis Using Powder Samples of Pristine Polymers and Formulations Indicating Dominance of Blank Nanoparticle and PEG Surface Properties	41
3.3.5	Scanning Electron Microscopy of Lyophilized Samples Showing Spherical Morphology and Nanoparticle Adhesion in Microfluidics Prepared Formulations.....	42
3.3.6	Drug Release Kinetics of Disulfiram and Emulsification Solvent Evaporation and Microfluidic Formulations Prepared Showing Higher Relative Release of Microfluidics-based Nanoparticles	43
3.4	Conclusions and Future Work	45
3.5	References.....	46

CHAPTER 4 DESIGN OF CHITOSAN-COATED, QUERCETIN-LOADED PLGA NANOPARTICLES

FOR ENHANCED PSMA-SPECIFIC ACTIVITY ON LNCAP PROSTATE CANCER CELLS.....51

4.1	Introduction	52
4.2	Materials and Methods	55
4.2.1	Materials	55
4.2.2	Preparation of Nanoparticles.....	55
4.2.3	Particle size, polydispersity index and zeta potential analysis using dynamic light scattering	57
4.2.4	Determination of Folic Acid Content by Ultra-Violet (UV) Spectrophotometry.....	57
4.2.5	Characterisation using Fourier Transform Infra-Red (FTIR).....	57
4.2.6	Investigation of thermal degradation by thermogravimetric analysis (TGA)	58
4.2.7	Surface and crystallinity experiments using powder X-ray diffraction (XRD).....	58
4.2.8	. Phase transition studies employing differential scanning calorimetry (DSC).....	58
4.2.9	Scanning Electron Microscopy (SEM) of Sample Suspensions.....	58
4.2.10	UV Spectrophotometric Analysis of Quercetin Loading in Nanoparticle Systems	58
4.2.11	In vitro release of Quercetin	59
4.2.12	Cell Culture Conditions and Cytotoxicity Studies.....	59
4.2.13	Cellular uptake of Nanoparticles.....	59
4.2.14	Statistical Analysis.....	60
4.3	Results.....	60
4.3.1	Optimized formulation results using DOE.....	60
4.3.2	Size and potential data show spherical coated particles with positive surface charge.....	64
4.3.3	Molecular Structure by FTIR shows adsorption interactions and XRD show amorphous nanoparticle structure	65
4.3.4	Thermal Degradation and Phase Transition show retention of polymer properties.....	66
4.3.5	Increased cytotoxicity of quercetin in nanoparticle system	68
4.3.6	Greater cellular uptake of targeted nanoparticles in a PSMA positive cell line	70
4.4	Discussion	71

4.5	Conclusions.....	76
4.6	References	76

CHAPTER 5 OPTIMIZED ACTIVE TARGETED PLGA-PEG NANOPARTICLES FUNCTIONALIZED WITH ANTI-PSMA ANTIBODY PROVIDE CONTROLLED RELEASE OF DOCETAXEL AND INCREASED UPTAKE AND CYTOTOXICITY IN PROSTATE CANCER CELLS 86

5.1	Introduction.....	86
5.2	Materials and Methods	88
5.2.1	Materials.....	88
5.2.2	Design of Experiments and Preparation of Nanoparticles	89
5.2.3	Conjugation of antibody.....	90
5.2.4	Particle size diameter, polydispersity index and zeta potential analysis using dynamic light scattering.....	91
5.2.5	Determination of Antibody Conjugation Efficiency by the Pierce Bradford Assay	91
5.2.6	Fourier Transform Infra-Red (FTIR) analysis shows characteristic peaks.....	91
5.2.7	Investigation of thermal degradation by thermogravimetric analysis (TGA).....	92
5.2.8	Powder X-ray diffraction (XRD) to examine crystallinity and surface properties	92
5.2.9	Differential scanning calorimetry (DSC) to measure temperature of phase changes	92
5.2.10	Scanning Electron Microscopy (SEM) of Sample Suspensions	92
5.2.11	HPLC Analysis of Docetaxel Loading in Nanoparticle Systems.....	93
5.2.12	In vitro release of Docetaxel.....	93
5.2.13	Cell Culture Conditions	93
5.2.14	Cytotoxicity Using the MTT Assay	93
5.2.15	Investigation of Cellular Uptake Using FITC-loaded Nanoparticles.....	94
5.2.16	Statistical Analysis	94
5.3	Results and Discussion.....	94
5.3.1	Optimization of Targeted Nano-system using a Face Centred Cubic Design.....	94
5.3.2	Efficient preparation of nanoparticles and conjugation of antibody.....	98
5.3.3	Physical Characterisation of Nanosystems Shows Conjugation of Antibody.....	100
5.3.4	Examination of Thermal Properties Show Phase Changes and Corresponding Mass Degradation.....	102
5.3.5	In-vitro Kinetic Study of Nanoparticle Systems Shows pH Dependent Release of Docetaxel	103
5.3.6	Targeted Nano-system shows increased cytotoxicity on LnCap and PC-3 Cell Lines.....	104
5.3.7	Increased Cellular Uptake of Targeted Nano-system on LnCap Cell Line	108
5.4	Conclusions.....	109
5.5	References	109

CHAPTER 6	EVALUATION OF DOCETAXEL-LOADED ANTI-PSMA-FUNCTIONALISED PLGA-PEG NANOPARTICLES IN LNCAP PROSTATE CANCER CELLS AND SPHEROIDS AND BIOCOMPATIBILITY ANALYSIS IN NUDE MF1 MICE	117
6.1	Introduction	117
6.2	Materials and Methods	118
6.2.1	Materials.....	118
6.2.2	Preparation and Characterization of Nanoparticles.....	118
6.2.3	Cell Culture Conditions.....	119
6.2.4	In vivo Biocompatibility Studies on Male Nude Mice.....	123
6.2.5	Statistical Analysis	123
6.3	Results and Discussion.....	123
6.3.1	Assessment of Nanosystem Therapeutic Efficacy by Cancer Cell Scratch Assay	123
6.3.2	Apoptotic Effect of Treatments	127
6.3.3	3.4 Cell Cycle Arrest.....	131
6.3.4	ROS Activation	134
6.3.5	Histopathological Analysis of Major Organs of Nude MF1 mice after treatments.....	141
6.4	Conclusions.....	142
6.5	References	142
CHAPTER 7	CONCLUSIONS AND FUTURE PERSPECTIVES.....	156
7.1	Conclusions.....	156
7.2	Future Recommendations	158
Appendix 1 Supplementary Information for Chapter 3: Comparative Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and Emulsification Solvent Evaporation Techniques.....		159
Appendix 2 Supplementary Information for Chapter 4: Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells.....		166
Appendix 3 Supplementary Information for Chapter 5: Optimized Active Targeted PLGA-PEG Nanoparticles Functionalized with Anti-PSMA Antibody Provide Controlled Release of Docetaxel and Increased Uptake and Cytotoxicity in Prostate Cancer Cells		169
Appendix 4 Animal Ethics Clearance Certificate		172

List of Abbreviations

Abbreviations

ADT - Androgen Deprivation Therapy

FDA – Food and Drug Administration

DSF - Disulfiram

PLGA – poly lactic co glycolic acid

PEG – polyethylene glycol

PSMA – Prostate Specific Membrane Antigen

RES – Reticulo Endothelial System

TAT- Transactivator of Transcription

5-ASA – 5 Aminosalicylic Acid

GROMACS – GRONingen MACHine for Chemical Simulations

LAMMPS – Large scale Atomic/Molecular Massively Parallel Simulator

PLA – Poly lactic acid

EPR – Enhanced Permeability and Retention

CHI - Chitosan

PVA – Poly Vinyl Alcohol

PSD – Particle Size Diameter

PDI – Poly Dispersity Index

API – Active Pharmaceutical Ingredient

PBS – Phosphate Buffered Saline

FITC – Fluorescein Isothiocyanate

LHRH – Luteinizing Hormone-Releasing Hormone

MPF – Maturation-Promoting Factor

ROS – Reactive Oxygen Species

List of Figures

Figure 1.1 Mechanistic absorption approaches for cell membrane permeation based on (A) targeted nanoparticles and (B) positively charged nanoparticles. Adapted from (Salatin & Yari Khosroushahi, 2017).....	22
Figure 1.2 (A) The large extracellular domain of PSMA, exposed for ligand recognition and binding; (B) the structure of PSMA showing zinc binding sites in red, ligand in green and surrounding amino acids; adapted from (Gourni & Henriksen, 2017) and (Wüstemann et al., 2019)	23
Figure 2.1 The synthesis and physiological breakdown of PLGA, adapted from (Sun et al., 2017)	32
Figure 2.2 Favorable characteristics of PLGA as a nano delivery system, reproduced from (Mir et al., 2017)	33
Figure 2.3 Surface functionalised PLGA nanoparticles for active targeting and cellular binding reproduced from (Yoo et al., 2019).....	37
Figure 2.4 Formation of PLGA nanoparticles by double (W/O/W) emulsion, spray drying and phase separation (coacervation) methods, adapted from (Ding & Zhu, 2018)	40
Figure 2.5 Illustration of the salting out method of preparation, reproduced from (Crucho & Barros, 2017)	41
Figure 2.6 The nanoprecipitation method, adapted from (Crucho & Barros, 2017)	42
Figure 2.7 Simple setup of a supercritical fluid technology method, reproduced from (Jog & Burgess, 2017).....	43
Figure 2.8 I - Schematic of the microfluidics method and the three most common microchannel geometries. (A) - T junction, (B) – flow focussing (C) - continuous flow. Reproduced from (Shembekar et al., 2016)-published by the Royal Society of Chemistry. II - (D) and (E) – SEM images of PLGA particles formed by this technique, adapted from (Xu et al., 2009)....	44
Figure 2.9 (A) - Direct- and premix- two common processes using membrane extrusion; (B) SEM image of an emulsion formed by this method (C) SEM images of nanoparticles formed by premix ME, reproduced with permission from (P. Guo et al., 2018)	46
Figure 2.10 Schematic showing PRINT procedure, reprinted with permission from (Perry et al., 2011). Copyright (2019). American Chemical Society	47
Figure 2.11 SEM Images of PLGA PRINT particles. (A), (B) and (C) – cylinders of different dimensions. (D) – spheres. (E) – ridged cubes. (F) [bottom right]– particles with centre fenestrations, reprinted with permission from (Enlow et al., 2011). Copyright (2019)	48
Figure 2.12 Functionalised PLGA nanocarriers and their medical applications, reproduced from (Mir et al., 2017).....	1

Figure 2.13 Molecular dynamics simulation showing (A) [left] 12% w/w and (B) [right] 24% itraconazole loading in a PLGA nanoparticle, adapted with permission from (Wilkosz et al., 2018). Copyright (2019) American Chemical Society.	5
Figure 3.1 Image representing different arrangements of PEG molecules in a nanoparticle. Adapted with permission from (Rabanel, Hildgen & Banquy 2014) (B) - Different conformations on nanoparticle surface. Representations of the mushroom (C) and brush (D) conformations, showing surface densities, reproduced from (Jokerst et al. 2011) with permission from Nanomedicine as agreed by Future Medicine Ltd. (E) - schematic representation of the PLGA-chitosan-PEG nanoparticles prepared in this study. Reproduced with permission from (Parveen & Sahoo 2011). (F) - Nanoparticle surface showing detection of surface charge and PEG chains in both mushroom and brush conformations. Adapted with permission from (Rabanel et al. 2014).	27
Figure 3.2 A) - Mean particle size obtained for formulations using acetone as the organic phase, and different concentrations of PVA in the aqueous phase. (B) - Mean particle size obtained for formulations containing PLGA, chitosan and PEG with either chloroform o	34
Figure 3.3 (A) and (B) – FTIR spectra of the formulations prepared using solvent evaporation (bulk). (C) and (D) – Formulations prepared using microfluidics (nano).	39
Figure 3.4 Graphs showing thermal degradation profiles of disulfiram, blank and loaded nanoparticles: (A) - blank formulations. (B) - microfluidic formulations. Relative DSC curves are shown with offset heat flow values for comparison: (C) - emulsification solvent evaporation formulations. (D) - microfluidic formulations.	40
Figure 3.5 (A) and (B): X-ray diffraction spectra of the polymers and the loaded nanoparticles (2 % PEG) prepared by the emulsification solvent evaporation method (bulk/B); (C) and (D): diffraction spectra of the microfluidic formulations (nano/N).	41
Figure 3.6 Scanning electron micrographs of nanoparticles prepared by emulsification solvent evaporation (A) and microfluidic (B) methods.	42
Figure 3.7 A: 24-hour and B: 72-hour cumulative in vitro release profiles of native disulfiram, emulsification solvent evaporation and microfluidic nanoparticles.	44
Figure 4.1 Fabrication process of uncoated (PLGA) and coated (PLGA-chitosan-folic acid) nanoparticles	54
Figure 4.2 Response surface plots showing quercetin loading (A), charge B), and folic acid content (C), variation with different quercetin and chitosan amounts. Amount of folic acid was kept constant at the intermediate value of 30 mg. Obtained from JMP V17.	61
Figure 4.3 Prediction profiler showing optimized solution with maximum desirability – from JMP V17.....	63
Figure 4.4 Scanning Electron Micrographs of A- uncoated nanoparticles and B- coated nanoparticles.....	64

Figure 4.5 A- FTIR spectra of uncoated and coated nps and pristine polymers where Fol = folic acid, Chi = Chitosan and Que = Quercetin. B- XRD spectra of Quercetin and uncoated and coated nanoparticles.....	65
Figure 4.6 A- DSC and B- TGA thermograms of loaded and unloaded coated nanoparticles where Que = Quercetin.....	66
Figure 4.7 Release curves of quercetin from coated and uncoated nanoparticles A- 24 hours and B- 168 hours.	67
Figure 4.8 <i>In-vitro</i> cell viability of A- LnCap and B- PC-3 cells after being treated for 72 hours with free quercetin, uncoated and coated nanoparticles (*p value <0.05).	69
Figure 4.9 LnCap cells and B – PC-3 cells treated with coated (1) and uncoated (2) nanoparticles.....	71
Figure 5.1 Response surface plots showing yield (A), drug loading (B), and antibody conjugation efficiency (C) variation with different PLGA: PLGA-PEG ratios, initial docetaxel amounts, and initial antibody amounts. Obtained from JMP V17.	96
Figure 5.2 Prediction profiler showing optimized solution with maximum desirability Of 0,877 – from JMP V17.	97
Figure 5.3 Scanning Electron Micrographs of A1 and A2 - unloaded and loaded non targeted nanoparticles (NTN) and B1 and B2 – unloaded and loaded actively targeted nanoparticles (ATN).....	98
Figure 5.4 FTIR spectra of A- unloaded non targeted nanoparticles (NTN), unloaded actively targeted nanoparticles (ATN) and their constituent pristine polymers, PLGA and PLGA-PEG. B– comparison of the FTIR spectra of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC)	100
Figure 5.5 XRD spectra of A- Docetaxel (DOC) and pristine polymers PLGA and PLGA-PEG (PP) and B– comparison of the XRD spectra of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC)	101
Figure 5.6 A- DSC thermograms comparing the phase changes of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC). B– TGA analysis of the the unloaded and loaded NTN and ATN systems.	102
Figure 5.7 Docetaxel in-vitro release patterns from NTN and ATN systems at ph 7.4 and 6 from (A) 0-24H and (B) 0-168H.	103
Figure 5.8 Cytotoxicity of docetaxel and NTN and ATN systems on PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 48- and 72H of treatment, showing dose dependent toxicity of docetaxel and increased efficacy of NTN's and ATN's (* p<0.05) compared to free docetaxel on LnCap cells.....	105

Figure 5.9 Comparison of effective concentrations (cell viability of 50%) of docetaxel and NTN and ATN systems on PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 48- and 72H of treatment	106
Figure 5.10 Fluorescent images of PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 24H of treatment with FITC-loaded NTN's and ATN's. Nuclei are stained blue with DAPI and FITC is in green.....	108
Figure 6.1 . Workflow of the scratch assay conducted on PC-3 and LnCap cells, reproduced from (Grada et al., 2017).	120
Figure 6.2 Time scale of growth and treatment of LnCap multicellular tumour spheroids, adapted from (Friedrich et al., 2009)	123
Figure 6.3 Wound healing of PC-3 cells treated with growth media (control), and effective concentrations of 8 ng/mL docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate.	124
Figure 6.4 Wound healing of LnCap cells treated with growth media (control), and effective concentrations of 8 ng/mL docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate.	125
Figure 6.5 . Percentage Wound Closure and Wound Closure Inhibition relative to control of PC-3 (A and B) and LnCap cells (C and D) after being treated for 24. 48 and 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).	126
Figure 6.6 Apoptosis profiles – representative dot plots showing cell densities of PC-3 cells after being treated for 24. 48 and 72 hours with A- growth media (control), and 8 ng/mL of docetaxel in C-free docetaxel (DOC), B-non-targeted nanoparticles (NTN) and D-targeted nanoparticles (ATN). In each profile, lower left quadrant shows live cells, lower right quadrant shows early apoptotic cells, upper right quadrant shows late apoptotic cells and upper left quadrant shows dead cells. Experiments were conducted in triplicate.	128
Figure 6.7 Apoptosis profiles – representative dot plots showing cell densities of LnCap cells after being treated for 24. 48 and 72 hours with A- growth media (control), and 8 ng/mL of docetaxel in C-free docetaxel (DOC), B-non-targeted nanoparticles (NTN) and D- targeted nanoparticles (ATN). In each profile, lower left quadrant shows live cells, lower right quadrant shows early apoptotic cells, upper right quadrant shows late apoptotic cells and upper left quadrant shows dead cells. Experiments were conducted in triplicate.	130
Figure 6.8 Profiles showing apoptotic populations of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC),	

non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate	131
Figure 6.9 Profiles showing apoptotic populations of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate	132
Figure 6.10 Comparison of populations in the different cell cycle phases of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).	133
Figure 6.11 Activation of reactive oxygen species of PC-3 and LnCap cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).	135
Figure 6.12 Dependence of untreated spheroid morphology and size on initial seeding density of LnCap cells. Experiments were conducted in quadruplicate for the 5000 and 2500 cells/well density and duplicate for the 1250 cells/well density.	Error! Bookmark not defined.
Figure 6.13 Bright field images showing 17-day growth of LnCap multicellular tumour cell spheroids treated with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in quadruplicate	Error! Bookmark not defined.
Figure 6.14 Spheroid volume increase and percentage growth inhibition relative to control of spheroids treated with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).....	Error! Bookmark not defined.
Figure 6.15 Specimens of major organs of Nude MF1 mice treated with saline (cont), docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). ...	141

List of Tables

Table 2.1 Properties of some prepared PLGA nanocarriers, their methods of formation and biological targets	52
Table 2.2 Molecular simulations of PLGA and their significance to nanocarrier drug delivery properties	4
Table 3.1 Size, dispersity, surface charge and entrapment efficiency of bulk and microfluidic prepared formulations with varying amounts of PEG coatings.	35
Table 4.1 Formulations generated by JMP software with varying amounts of quercetin (Q), chitosan (Chi) and folic acid (Fol).	56
Table 4.2 Responses obtained using varying amounts of quercetin, chitosan and folic acid, where QL= quercetin loading, Z Potential = zeta potential, FA = folic acid content.	60
Table 4.3 Responses obtained using varying amounts of quercetin, chitosan and folic acid	63
Table 4.4 Summary of dynamic light scattering measurements of uncoated and coated nanoparticles	64
Table 4.5 Fluorescent intensities of LnCap and PC-3 cells treated with coated and uncoated nanoparticles	71
Table 4.6 Comparison of fabricated PLGA-Chitosan Folic Acid System with previously reported results	74
Table 5.1 Formulations generated by JMP software with a range of ratios of PLGA:PLGA-PEG (P:PP ratio), amount of docetaxel (Doc) and volume of antibody (AB).	89
Table 5.2 Responses obtained using varying amounts of PLGA: PEG, docetaxel and antibody, where Yield = percent of total nanoparticle product, DL = drug loading percent, ACE = percent antibody conjugation efficiency. Levels -1, 0 and 1 correspond to parameters in Table 5.1 .	95
Table 5.3 Comparison of actual results vs predicted results using optimized formulation parameters of PLGA: PLGA-PEG ratios, docetaxel, and antibody amounts.....	98
Table 5.4 Size, Stability and Drug Loading Properties of Non-targeted and targeted nanoparticles where PDI = polydispersity index and DL = drug loading	98

1.1 Background

Several polymeric nanoparticle-based systems have been developed as drug delivery systems due to their ease of preparation and the fact that they can be made from biocompatible polymers that can be tuned to provide desirable entrapment and release of bio-actives (Hsu et al., 2023). Prostate cancer is the most common diagnosis of male carcinoma worldwide, with the odds being one in seven men (above the age of 76), among countries with a high sociodemographic index (Fitzmaurice et al., 2018). Aside from radiation and primary curative surgery, androgen-deprivation therapy (ADT) is the current first-line therapeutic strategy for prostate cancer. However, approximately 37% - 48% of prostate cancer patients experience biochemical recurrence or progression to metastatic castration-resistance (Merseburger, Alcaraz, von Klot, & therapy, 2016). This androgen-independence presents a challenge for disease management under current medications (Nader, El Amm, & Aragon-Ching, 2018) and highlights the necessity for the development of alternate treatment options (Iljin et al., 2009).

The costs of producing new anti-cancer drugs with FDA approval are substantial due to the use of intricate and expensive procedures required to test safety. Therefore, significant efforts have been made in repurposing and redirecting FDA approved drugs and plant derived compounds as anti-cancer agents (Ashburn & Thor, 2004). Disulfiram (DSF) is one such drug, which has been used for the treatment of alcohol addiction for many decades and has been investigated for its additional therapeutic effects (Sauna, Shukla, & Ambudkar, 2005). In recent years, various studies have demonstrated DSF's anti-tumour activity in breast, colon, lung and prostate cancer (Zhuo et al., 2018), and, DSF was shown to have an efficient and selective inhibitory effect on prostate cancer cell growth (Iljin et al., 2009). Quercetin is a plant-derived flavonoid that has shown several medicinal properties, including anti-cancer activity and marked inhibition on prostate cancer cell growth (Saavedra-Leos et al., 2021; Sharma et al., 2022)

Despite the recognized clinical pharmacology and activity of DSF, its potential as a chemotherapeutic is restricted by the instability of its currently available oral formulation in acidic gastric conditions and its immediate metabolism into less effective molecules in the circulatory system (Wang et al., 2017), which would result in insufficient quantities of the drug at the target cells to cause apoptosis. Similarly, the application of quercetin as an anti-cancer drug is limited due to its low bioavailability and instability in aqueous solutions (Vinayak and Maurya, 2019; Ghafouri-Fard et al., 2021). Therefore the pharmacological profiles of both DSF and quercetin can be improved by integrating them into drug delivery systems that protects them from degradation in the bloodstream (He, Markoutsas, Li, & Xu, 2018). Additionally, their size and bioavailability restrictions are similar to those of many cancer drugs (Zang et al., 2021), and hence they can also

be used as a model therapeutic in preliminary studies for the development of drug delivery systems for more toxic chemotherapeutic drugs, such as docetaxel, which is part of current treatment regimens for prostate cancer (Zhao et al., 2023) .

Synthetic polymers are a popular choice for the fabrication of nanocarriers and poly lactide-co-glycolide especially, has been widely used for a variety of applications in nanomedicine. It is a synthetic polymer but is biodegradable and biocompatible – it is metabolized completely, with its waste product being excreted by the lungs (Semete et al., 2010). PLGA is also highly versatile as the ratios and weights of its co-polymers can easily be varied to produce the drug delivery characteristics desired for the nanocarrier being designed (Mittal, Sahana, Bhardwaj, & Ravi Kumar, 2007). Nanoparticles formed with PLGA as the hydrophobic core has been shown to reduce leaking of lipophilic drug cargo during preparation and hence has demonstrated higher encapsulation efficiencies and increased stability due to the favorable hydrophobic interactions between drug and polymer (Barichello, Morishita, Takayama, & Nagai, 1999).

Chitosan is a biodegradable, biocompatible natural polymer that has shown desirable mucoadhesive properties for cell membrane penetration when incorporated in nano-systems (Susana Rodrigues, Dionísio, López, & Grenha, 2012). Uncharged hydrophobic nanoparticles are unstable in solution, as they aggregate in order to expose a minimum surface area to the solution molecules. However, if the nanoparticles carry sufficient charge, the repulsive forces between the charged particles stabilize them in aqueous solution. (Suk, Xu, Kim, Hanes, & Ensign, 2016). The amino end groups on chitosan create strongly positively charged surface chemistry and therefore impart more stable cationic and membrane permeating properties to the entire system, as shown by Figure 1.1(B). Several studies of the chemotherapeutic activity of chitosan-based nanoparticles have demonstrated increased cellular uptake (Garg, Chauhan, Nagaich, & Jain, 2019; Mohammed, Syeda, Wasan, & Wasan, 2017) as compared to treatments with native cargo and furthermore, these delivery systems have exhibited reduced toxicity to healthy cells (Rizeq, Younes, Rasool, & Nasrallah, 2019), as well as enhanced chemotherapeutic activity (S. Rodrigues, Dionisio, Lopez, & Grenha, 2012).

Polyethylene glycol (PEG) is a synthetic polymer that is approved by the FDA for use in drug delivery systems (D'souza & Shegokar, 2016). Conventional hydrophobic nanocarriers are immediately recognized *in vivo* by the reticulo-endothelial system and are opsonized and subsequently eliminated by the liver or spleen. To circumvent this, polyethylene glycol groups are covalently attached or adsorbed onto their surface (PEGylation), and this confers “stealth” surface properties to the system by disrupting interactions at the nanoparticle interface with the bloodstream (Veronese & Pasut, 2005). It has been shown that the optimization of the content and extent of PEG end groups on the surface of nanoparticles can increase the circulation time of these

systems by several orders of magnitude, while also decreasing immunogenicity and still maintaining therapeutic activity (Suk et al., 2016). This employs passive targeting, where the increased residence time of the nanoparticles are able to take advantage of the enhanced permeation and retention effect, where the leaky tumour vasculature enables increased uptake of the nanoparticles compared to normal cells, while the inadequate lymphatic drainage maintains high therapeutic concentrations at the target site (Greish, 2010).

Another strategy is active targeting, which involves a further modification of the nanoparticle surface with ligands that have affinities to specific receptors on the target cell, as shown in Figure 1.1 (A). These ligands promote binding to the cell surface receptors and subsequent cellular internalization via receptor mediated endocytosis (Muhamad, Plengsuriyakarn, & Na-Bangchang, 2018). However, the incorporation of targeting ligands on the nanoparticle surface have been shown to disrupt the “stealth” activity provided by polyethylene glycol groups (Gu et al., 2008). The degree of ligand conjugation should also be optimized since the targeting of receptors can only be achieved if the nanoparticle is able to escape clearance from the circulatory system by opsonin proteins (Suk et al., 2016).

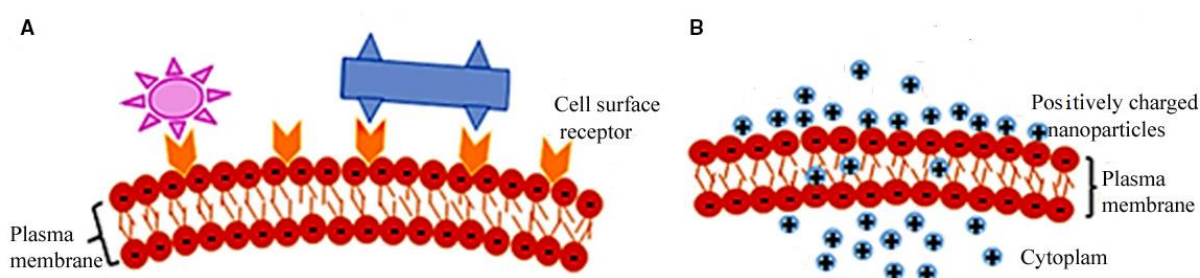


Figure 1.1 Mechanistic absorption approaches for cell membrane permeation based on (A) targeted nanoparticles and (B) positively charged nanoparticles. Adapted from (Salatin & Yari Khosroushahi, 2017)

Prostate specific membrane antigen (PSMA) is a type II transmembrane protein that is found on both healthy and cancerous prostate cells. However, it is overexpressed 1000 fold on prostate cancer cells and its degree of expression has been found to correlate directly to the state of the carcinoma (Wüstemann, Haberkorn, Babich, & Mier, 2019). Furthermore, it has been reported that while on healthy cells, PSMA is located primarily near the prostatic ducts in the cytoplasm, it is found closer to the cell surface membrane on metastatic cancer cells (Eiber et al., 2017), exposing a large extracellular domain for binding (Figure 1.2 A), and hence making it a suitable target for site specific drug delivery. The role of PSMA in prostatic tissue is unclear but it has been associated with the binding of glutamates and folates. Upon binding, the moiety is internalized via receptor

mediated endocytosis (Sanna et al., 2017), making PSMA an ideal target for chemotherapeutics. The binding site of PSMA consists of a tunnel containing two zinc ions (Figure 1.2 B), which can be exploited in the design of targeted prostate cancer therapeutics with glutamate or folate binding moieties as ligands.

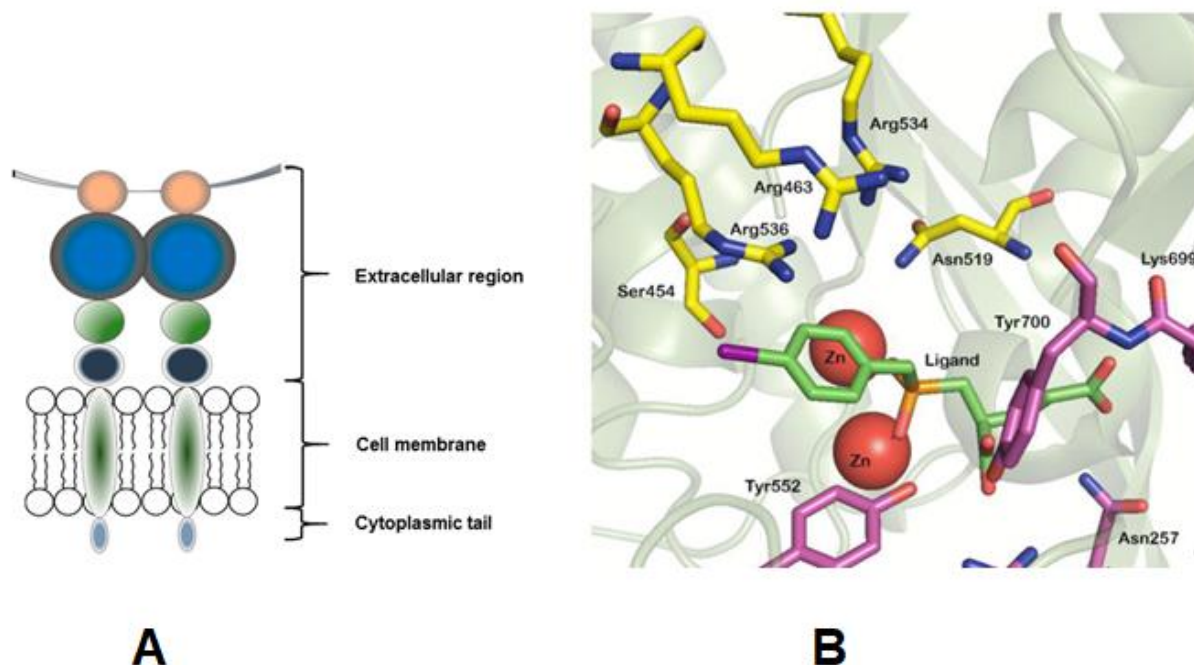


Figure 1.2 (A) The large extracellular domain of PSMA, exposed for ligand recognition and binding; (B) the structure of PSMA showing zinc binding sites in red, ligand in green and surrounding amino acids; adapted from (Gourni & Henriksen, 2017) and (Wüstemann et al., 2019)

In this study, we explored the importance of precision engineering and how design considerations can include the implementation of innovative preparation techniques and the use of optimization methods to tailor the delivery vehicle for its specific application. We proposed the design and evaluation of a targeted nano-system for the delivery of the chemotherapeutic drug docetaxel to prostate cancer cells. Starting with PLGA as a base for the polymeric matrix, we explored different polymers and ligands for their cell penetrating, shielding and targeting properties, as well as two model active compounds.

The inclusion of a chitosan coating increased the surface charge of the nanoparticles, and rendered it cationic, hence increasing the membrane permeation ability of the system while polyethylene glycol chains provided a hydrophilic shield, and would thereby increase the circulation time of the system. Attaching targeting ligands to bind to prostate specific membrane antigen served to increase the cellular uptake of the nanoparticles at the target site. The efficacy of the formulations was evaluated *in vitro* for cytotoxicity on healthy cells, chemotherapeutic activity on prostate cancer cells, and growth inhibition on a 3D spheroid model. The specificity of the targeted

formulation was compared by cellular uptake studies, while the *in vivo* biocompatibility was investigated using a nude MF1 mouse model.

1.2 Rationale and motivation for this study

There have been numerous polymer-based nanocarriers developed over the years for chemotherapeutic drug delivery. However, they possess major limitations, including toxicity, low biocompatibility and biodegradability, as well as limited cellular uptake and inadequate therapeutic concentrations at the target site. An ideal chemotherapeutic delivery system should exhibit the following characteristics; (a) to provide and maintain favourable interactions with the cargo for high drug entrapment, (b) to shield the drug from degradation under physiological conditions, (c) to deliver the cargo to the cancer site with minimal off target losses (d) to control and localize the release of incorporated drug, and (e) to be eliminated from the system with minimal toxicity and side effects. Furthermore, there are properties that need to be enhanced or maintained in the combination of polymers forming the nano-system. These are controlled biodegradation, thermostability, controlled solubility and surface shielding properties. Achieving these characteristics requires the use of different combinations of starting polymers in order to fabricate systems with optimal therapeutic functions.

The current study focuses on these properties which translate to optimal biomaterial performances. The goal of an optimal chemotherapeutic delivery system for docetaxel can be achieved by the use of : a) PLGA, a biodegradable FDA approved polymer that undergoes favourable hydrophobic interactions when incorporating pharmaceutically active compounds in its polymeric matrix, b) PEG, a hydrophilic shielding moiety that increases *in vivo* residence time, c) chitosan, a mucoadhesive cationic polymer that imparts cell penetrating properties, d) folate, a biocompatible folic acid derivative that has shown promise for PSMA binding and e) Anti-PSMA antibody, an antibody that specifically binds to PSMA.

Of particular interest in this study is the enhancement of the physicochemical, physico-mechanical and biological performances of the delivery system, as well as the incorporation of a biocompatible ligands as a targeting moieties. The system will be composed of different combinations of polymers and different targeting ligands. The ultimate goal is to fabricate a system with improved cell penetrating properties for site specific delivery of docetaxel as a prostate cancer therapeutic.

1.3 Novelty

The novelty of the study lies within the functionality of the developed systems. The polymeric platforms have the ability to act as either a cationic nanoparticulate system, a passive targeting and an active targeting system. This functionality not only enhances the application and efficacy

of the system, but also presents the potential of producing novel data that may contribute towards knowledge in the field of targeted chemotherapeutics.

The use of effective targeting ligands for PSMA is a development in the design of future targeted systems. Being biocompatible and biodegradable, folic acid has shown to be internalized when bound to different receptors, and therefore demonstrates potential as a viable alternative to synthetically complex ligands that have been rationally designed to bind PSMA. Antibody conjugation on the surface of the docetaxel-loaded nanoparticles is a strategy used to design the novel docetaxel delivery system for enhanced cellular uptake, which is beneficial for exposing cancer cells to sustained concentrations of the therapeutic agent. Since all the components are biocompatible and biodegradable, they are expected to be easily and safely eliminated *in vivo*.

1.4 Aim and Objectives

The aim of this study is to develop a novel docetaxel encapsulated NP system, employing site specific active targeting, and to investigate its anti-cancer *in vitro* and *in vivo* characteristics. This will be compared to native docetaxel, evaluating the selectivity of the targeted NP to PSMA expressing cells. Additionally, systems containing superior biomaterial constituents with mucoadhesive properties and biomimetic targeting abilities will be designed and evaluated *in vitro*, assessing their relative targeting and chemotherapeutic properties.

Objectives:

1. Fabrication and evaluation of a PLGA-chitosan-PEG nanosystem for the entrapment of disulfiram, comparing microfluidics as a fabrication method to a traditional 'bulk' solvent evaporation method.
2. Preparation, optimization, characterization and *in-vitro* evaluation of a quercetin loaded model PSMA targeted system, employing PLGA, chitosan and folic acid as a PSMA-targeting moiety.
3. The use of microfluidics and design of experiments to fabricate an optimized PSMA-targeted nano-system using PLGA, PEG and anti-PSMA antibody and loading it with docetaxel.
4. Detailed evaluation of *in-vitro* activity of the antibody targeted system including cellular toxicity and uptake studies, cell migratory inhibition, apoptosis induction and cell cycle arrest.
5. Investigation of the inhibitory effect of the non-targeted and antibody targeted nano-system on a 3D *in vitro* multicellular tumour spheroid model derived from PSMA- positive LnCap cells

6. Evaluation of the biocompatibility of the systems and free docetaxel by histopathological analysis of the major organs of nude MF1 mice after a single 72-hour dose of docetaxel and nano-systems.

1.5 Overview of Thesis

Chapter One – The introduction to the study, detailing the background, choice of materials and rationale behind the investigations. It includes an overview of the dissertation.

Chapter Two – This chapter is composed of the published review article entitled “The Design of Poly (lactic-co-glycolic) Acid Nanocarriers for Medical Applications”. This chapter contains information on PLGA nanoparticles from a nanoparticle design perspective and highlights the importance of the choice of fabrication methods and the effects it can have on nanoparticle characteristics.

Chapter Three – This chapter is the published research article entitled “Comparative Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and Emulsification Solvent Evaporation Techniques”. It details the fabrication and comparison of the popular solvent evaporation method of preparation of nanoparticles with a more recently developed microfluidics-based method of preparation.

Chapter Four – This chapter is the published research article entitled “Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells” and reports on the preparation, optimization and evaluation of a folic acid targeted nano-system to target PSMA positive LnCap cells.

Chapter Five – This chapter has been prepared to be submitted for publication to the journal ACS Omega and is entitled “Optimized Active Targeted PLGA-PEG Nanoparticles Functionalized with Anti-PSMA Antibody Provide Controlled Release of Docetaxel and Increased Uptake and Cytotoxicity in PSMA-positive LnCap Prostate Cancer Cells”. It details the microfluidics-based optimization, preparation and conjugation of PLGA-PEG with anti-PSMA antibody. It also reports on the characterization and comparative cytotoxic activity of the targeted and non-targeted nano-systems.

Chapter Six – This chapter will be prepared as part of a future publication. It is entitled “Evaluation of Docetaxel-Loaded Anti-PSMA-Functionalised PLGA-PEG Nanoparticles in LnCap Prostate Cancer Cells and Spheroids and Biocompatibility Analysis in Nude MF1 Mice”. It reports on the optimized nano-system from chapter 5 and its inhibitory activity on cell migration, cell cycle and LnCap spheroid growth, as well as the biocompatibility and organ toxicity of the treatments on nude MF1 mice.

Chapter Seven – This is the concluding chapter. It summarises the work accomplished by the PhD study and implications for possible clinical translation. Future recommendations are proposed on how to move the research forward.

1.6 References

- Ashburn, T. T., & Thor, K. B. J. N. r. D. d. (2004). Drug repositioning: identifying and developing new uses for existing drugs. 3(8), 673-683.
- Barichello, J. M., Morishita, M., Takayama, K., & Nagai, T. (1999). Encapsulation of Hydrophilic and Lipophilic Drugs in PLGA Nanoparticles by the Nanoprecipitation Method. *Drug Development and Industrial Pharmacy*, 25(4), 471-476. doi:10.1081/DDC-100102197
- D'souza, A. A., & Shegokar, R. J. E. o. o. d. d. (2016). Polyethylene glycol (PEG): a versatile polymer for pharmaceutical applications. 13(9), 1257-1275.
- Dranoff, G. J. N. R. I. (2012). Experimental mouse tumour models: what can be learnt about human cancer immunology? , 12(1), 61-66.
- Eiber, M., Fendler, W. P., Rowe, S. P., Calais, J., Hofman, M. S., Maurer, T., . . . Giesel, F. L. (2017). Prostate-Specific Membrane Antigen Ligands for Imaging and Therapy. 58(Supplement 2), 67S-76S. doi:10.2967/jnumed.116.186767
- Fitzmaurice, C., Akinyemiju, T. F., Al Lami, F. H., Alam, T., Alizadeh-Navaei, R., Allen, C., . . . Anderson, B. O. J. J. o. (2018). Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: a systematic analysis for the global burden of disease study. 4(11), 1553-1568.
- Garg, U., Chauhan, S., Nagaich, U., & Jain, N. (2019). Current Advances in Chitosan Nanoparticles Based Drug Delivery and Targeting. *Advanced pharmaceutical bulletin*, 9(2), 195-204. doi:10.15171/apb.2019.023
- Gourni, E., & Henriksen, G. J. M. (2017). Metal-based PSMA radioligands. 22(4), 523.
- Greish, K. (2010). Enhanced permeability and retention (EPR) effect for anticancer nanomedicine drug targeting. *Methods Mol Biol*, 624, 25-37. doi:10.1007/978-1-60761-609-2_3
- Gu, F., Zhang, L., Teply, B. A., Mann, N., Wang, A., Radovic-Moreno, A. F., . . . Farokhzad, O. C. (2008). Precise engineering of targeted nanoparticles by using self-assembled biointegrated block copolymers. 105(7), 2586-2591. doi:10.1073/pnas.0711714105 %J Proceedings of the National Academy of Sciences
- He, H., Markoutsas, E., Li, J., & Xu, P. J. A. b. (2018). Repurposing disulfiram for cancer therapy via targeted nanotechnology through enhanced tumor mass penetration and disassembly. 68, 113-124.

Iljin, K., Ketola, K., Vainio, P., Halonen, P., Kohonen, P., Fey, V., . . . Kallioniemi, O. J. C. C. R. (2009). High-throughput cell-based screening of 4910 known drugs and drug-like small molecules identifies disulfiram as an inhibitor of prostate cancer cell growth. *15*(19), 6070-6078.

Merseburger, A. S., Alcaraz, A., von Klot, C. A. J. O., & therapy. (2016). Androgen deprivation therapy as backbone therapy in the management of prostate cancer. *9*, 7263.

Mittal, G., Sahana, D. K., Bhardwaj, V., & Ravi Kumar, M. N. (2007). Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. *J Control Release*, *119*(1), 77-85. doi:10.1016/j.jconrel.2007.01.016

Mohammed, M. A., Syeda, J. T. M., Wasan, K. M., & Wasan, E. K. (2017). An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery. *Pharmaceutics*, *9*(4), 53. doi:10.3390/pharmaceutics9040053

Muhamad, N., Plengsuriyakarn, T., & Na-Bangchang, K. (2018). Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: a systematic review. *International journal of nanomedicine*, *13*, 3921-3935. doi:10.2147/ijn.S165210

Nader, R., El Amm, J., & Aragon-Ching, J. B. J. A. j. o. a. (2018). Role of chemotherapy in prostate cancer. *20*(3), 221.

Rizeq, B. R., Younes, N. N., Rasool, K., & Nasrallah, G. K. (2019). Synthesis, Bioapplications, and Toxicity Evaluation of Chitosan-Based Nanoparticles. *International journal of molecular sciences*, *20*(22), 5776. doi:10.3390/ijms20225776

Rodrigues, S., Dionisio, M., Lopez, C. R., & Grenha, A. (2012). Biocompatibility of chitosan carriers with application in drug delivery. *Journal of functional biomaterials*, *3*(3), 615-641. doi:10.3390/jfb3030615

Rodrigues, S., Dionísio, M., López, C. R., & Grenha, A. (2012). Biocompatibility of chitosan carriers with application in drug delivery. *Journal of functional biomaterials*, *3*(3), 615-641. doi:10.3390/jfb3030615

Salatin, S., & Yari Khosroushahi, A. (2017). Overviews on the cellular uptake mechanism of polysaccharide colloidal nanoparticles. *J Cell Mol Med*, *21*(9), 1668-1686. doi:10.1111/jcmm.13110

Sanna, V., Pintus, G., Roggio, A. M., Punzoni, S., Posadino, A. M., Arca, A., . . . Sechi, M. J. J. o. m. c. (2011). Targeted biocompatible nanoparticles for the delivery of (–)-epigallocatechin 3-gallate to prostate cancer cells. *54*(5), 1321-1332.

- Sanna, V., Singh, C. K., Jashari, R., Adhami, V. M., Chamcheu, J. C., Rady, I., . . . Siddiqui, I. A. (2017). Targeted nanoparticles encapsulating (–)-epigallocatechin-3-gallate for prostate cancer prevention and therapy. *Scientific Reports*, 7(1), 41573. doi:10.1038/srep41573
- Sauna, Z. E., Shukla, S., & Ambudkar, S. V. J. M. b. (2005). Disulfiram, an old drug with new potential therapeutic uses for human cancers and fungal infections. 1(2), 127-134.
- Savjani, K. T., Gajjar, A. K., & Savjani, J. K. J. I. p. (2012). Drug solubility: importance and enhancement techniques. 2012.
- Semete, B., Booyesen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., & Swai, H. S. (2010). In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. *Nanomedicine*, 6(5), 662-671. doi:10.1016/j.nano.2010.02.002
- Suk, J. S., Xu, Q., Kim, N., Hanes, J., & Ensign, L. M. (2016). PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Advanced Drug Delivery Reviews*, 99(Pt A), 28-51. doi:10.1016/j.addr.2015.09.012
- Veronese, F. M., & Pasut, G. J. D. d. t. (2005). PEGylation, successful approach to drug delivery. 10(21), 1451-1458.
- Wang, Z., Tan, J., McConville, C., Kannappan, V., Tawari, P. E., Brown, J., . . . Medicine. (2017). Poly lactic-co-glycolic acid controlled delivery of disulfiram to target liver cancer stem-like cells. 13(2), 641-657.
- Wüstemann, T., Haberkorn, U., Babich, J., & Mier, W. (2019). Targeting prostate cancer: Prostate-specific membrane antigen based diagnosis and therapy. 39(1), 40-69. doi:10.1002/med.21508
- Zhuo, X., Lei, T., Miao, L., Chu, W., Li, X., Luo, L., . . . science, i. (2018). Disulfiram-loaded mixed nanoparticles with high drug-loading and plasma stability by reducing the core crystallinity for intravenous delivery. 529, 34-43.
- Zou, Y., Wei, J., Xia, Y., Meng, F., Yuan, J., Zhong, Z. J. S. t., & therapy, t. (2018). Targeted chemotherapy for subcutaneous and orthotopic non-small cell lung tumors with cyclic RGD-functionalized and disulfide-crosslinked polymersomal doxorubicin. 3(1), 1-8.



The Design of Poly(lactide-co-glycolide) Nanocarriers for Medical Applications

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Abstract

Polymeric biomaterials have found widespread applications in nanomedicine, and poly(lactide-co-glycolide), (PLGA) in particular has been successfully implemented in numerous drug delivery formulations due to its synthetic malleability and biocompatibility. However, the need for preconception in these formulations is increasing, and this can be achieved by selection and elimination of design variables in order for these systems to be tailored for their specific applications. The starting materials and preparation methods have been shown to influence various parameters of PLGA-based nanocarriers and their implementation in drug delivery systems, while the implementation of computational simulations as a component of formulation studies can provide valuable information on their characteristics. This review provides a critical summary of the synthesis and applications of PLGA-based systems in bio-medicine and outlines experimental and computational design considerations of these systems.

2.1 Introduction

The design of novel delivery systems using nanomaterials has experienced substantial growth since the application of nanotechnology to biomedical applications established the field of nanomedicine. As a result of the ongoing discovery of numerous new pharmaceutically active compounds which have shown excellent efficacy but inadequate clinical translation, there is a growing need to fill the gap between the formulations available and their successful inclusion into active treatment. This has urged scientists to investigate alternate forms of delivery to the biological target in order to overcome the hurdles associated with conventional drug delivery, such as poor

drug entrapment, inadequate bioavailability and pharmacokinetics, as well as systemic toxicity and side effects. These novel delivery systems all strive for the “magic bullet” effect (Bosch & Rosich, 2008) which is a vehicle that can form favorable interactions with a lipophilic or hydrophilic drug to facilitate high drug loading (Aravind et al., 2013; Bauer, Situ, Griswold, & Samia, 2016), can shield the drug from physiological conditions, deliver it to the biological target with minimal loss, and then can release it at the site in a sustained manner and at therapeutic concentrations (Al-Jamal et al., 2016; Almoustafa, Alshawsh, & Chik, 2017). Moreover, the carrier is ideally biodegradable, biocompatible and non-immunogenic, with low systemic toxicity (Alshamsan, 2014; Ananta, Paulmurugan, & Massoud, 2016). Nanomaterials are a befitting source to meet these requirements because they can be tailored to a vast range of sizes and shapes and can suit various delivery mechanisms, while the interactions between the carrier and the physiological medium can be controlled by adapting the surface properties of the carrier (Kakkar, Traverso, Farokhzad, Weissleder, & Langer, 2017). This has given rise to the widespread implementation of nanomaterials as pharmaceutical carriers for medical diagnostics and therapeutics (theranostics) (Berthet, Gauthier, Lacroix, Verrier, & Monge, 2017; A. P. Singh, Biswas, Shukla, & Maiti, 2019). Nanostructures can be fabricated from organic, inorganic, metallic or non-metallic sources. Examples include carbon nanotubes (N. K. Singh et al., 2013), dendrimers, liposomes, micelles and solid lipid nanoparticles (Lombardo, Kiselev, & Caccamo, 2019; Mishra et al., 2014).

Polymeric nanoparticles are commonly implemented as components of drug delivery systems and the use of synthetic polymers in particular can enable the design of carriers in a well-controlled and reproducible manner in order to suit the desired application (Lai, Daeer, Löbenberg, Prenner, & Biointerfaces, 2014). Polymer-based nanoparticles act as drug delivery vehicles by encapsulating the active agent inside its polymeric matrix, by conjugating to the agent or by adsorbing it onto the surface of the polymer (Mahapatro & Singh, 2011) (Ansary, Awang, & Rahman, 2014). Polymers can be constructed to be linear, branched or globular and their size and their properties can be modulated by the choice of synthetic process (Panyam & Labhasetwar, 2012) (Y. Chen et al., 2013). Biodegradable polymers are suitable as nanocarriers as they often self-assemble and are easily sourced. Natural biodegradable polymers are chitosan (Ali & Ahmed, 2018), alginate (Jana, Kumar Sen, & Gandhi, 2016) and inorganic ceramic hydroxyapatite composites (Turon, del Valle, Alemán, & Puiggalí, 2017). Synthetic polymers such as poly lactide-co-glycolide (PLGA) are an attractive alternative as they can be precisely engineered from monomers to suit the target and physiological environment they are intended for (Middleton & Tipton, 2000).

PLGA has become ubiquitous in the bio-medical field for many reasons. Firstly, it is a synthetic, biodegradable polymer that is easily broken down *in vivo* by hydrolysis into lactic acid and glycolic acid. These monomers are biocompatible and are physiologically metabolized by the tricarboxylic acid cycle for final excretion in the lungs (Boitumelo Semete et al., 2010; Sequeira, Santos, Serra,

Veiga, & Ribeiro, 2018), as shown by figure 2.1 (Sun, Xu, Wu, Ye, & Wang, 2017). Hence, PLGA as a nanocarrier is considered to produce minimal systemic toxicity when used for biomedical applications (Kumari, Yadav, Yadav, & biointerfaces, 2010) and has been used in various formulations including membranes, sponges and gels (Sun et al., 2017).

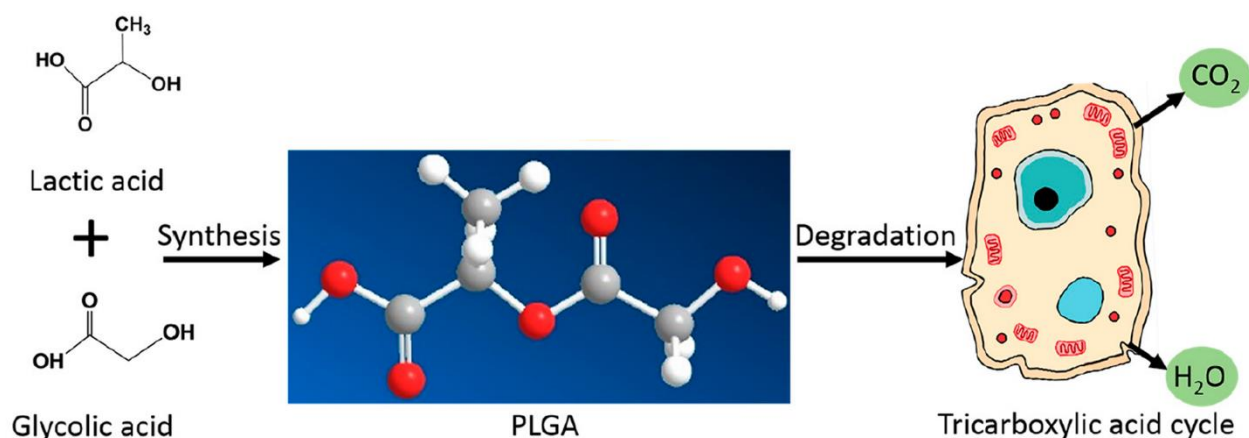


Figure 2.1 The synthesis and physiological breakdown of PLGA, adapted from (Sun et al., 2017)

The appeal of PLGA also lies in the fact that its properties can be manipulated and adapted to modify the encapsulation profile and drug release kinetics of the nanostructure to suit the desired application (Mittal, Sahana, Bhardwaj, & Ravi Kumar, 2007). PLGA is overall a hydrophobic polymer and is therefore detected by the reticulo-endothelial system (RES) and if unmodified, is bound by phagocytes for elimination by the liver or spleen and eliminated before delivering its payload to the target site (Danhier et al., 2012). To circumvent this, surface modification of PLGA is necessary. One such modification is the coating of hydrophilic poly ethylene glycol (PEG) groups on the surface of PLGA to shield the hydrophobic end groups from the RES, resulting in an amphiphilic di-block co-polymer (Salmaso & Caliceti, 2013). Other polymers used as surface modifiers include chitosan (Lu, Lv, & Le, 2019), polaxamer and poloxamines (Redhead, Davis, & Illum, 2001) which work by altering the electrostatic and hydrophobic surface properties of the PLGA block co-polymer. To increase the therapeutic efficacy, the surface of the PLGA nanocarrier can be decorated with targeting ligands such as small molecules, antibodies and aptamers. These molecules selectively bind to receptors on the target cell and guide the vehicle to the site of action (Jahan, Sadat, Walliser, & Haddadi, 2017). Targeting moieties such as aptamers, have been shown to increase retention time at the site of action (Dinarvand, Sepehri, Manoochehri, Rouhani, & Atyabi, 2011).

The use of PLGA in biomedicine dates back to the 1970s when it was used as a component of biodegradable sutures and implants. With the advent of nanomedicine, it has found application as

nanocarrier in various areas of medical research, including chemotherapeutics, immunology and biomechanics (Swider et al., 2018). Numerous studies have also reported successful applications in antibiotics, antiseptics, imaging, wound healing and as nano scaffolds (Sharma, Parmar, Kori, & Sandhir, 2016). The suitability and adaptability of PLGA as a nanocarrier is illustrated by figure 2.2 (Mir, Ahmed, ur Rehman, & Biointerfaces, 2017).

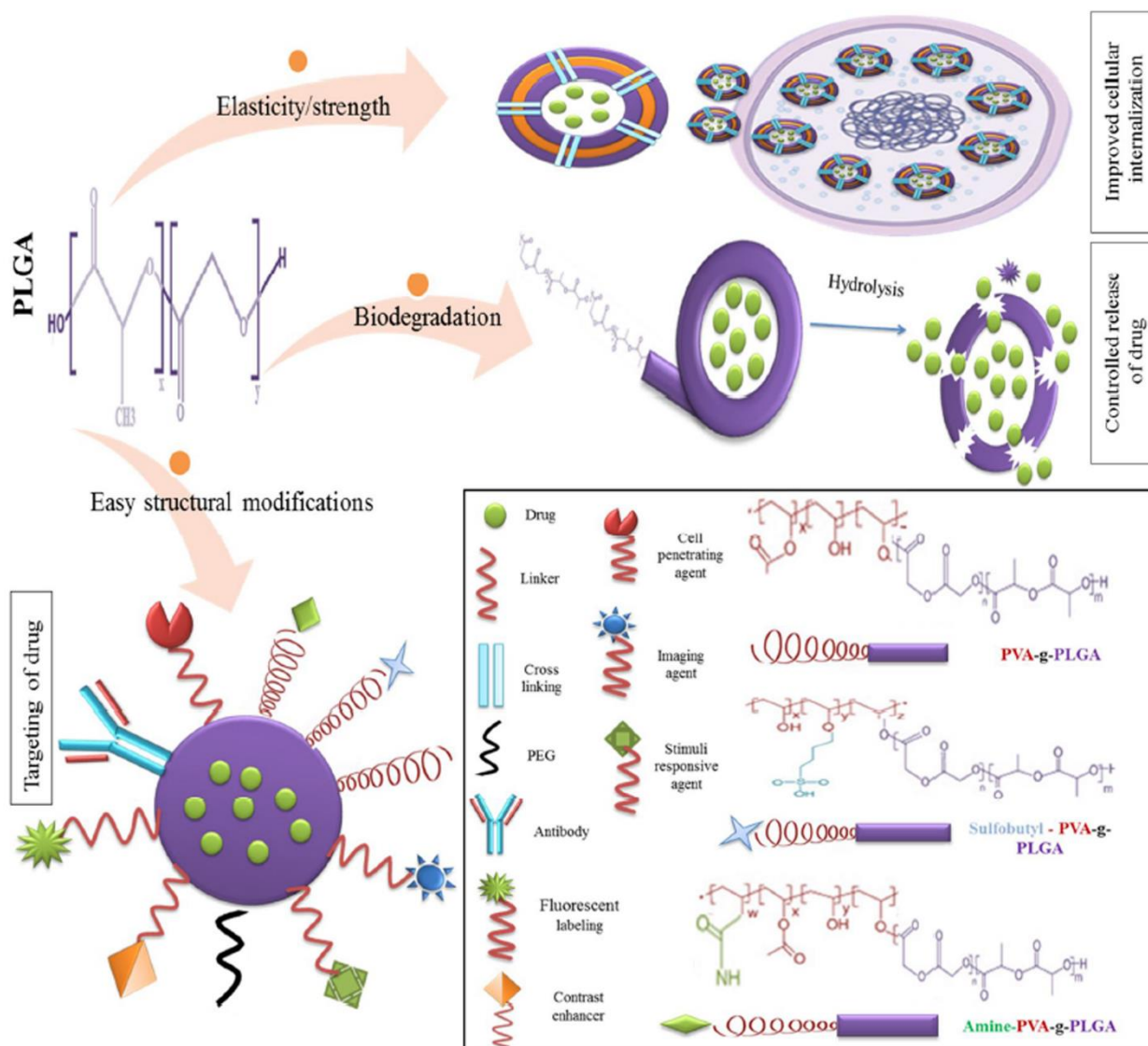


Figure 2.2 Favorable characteristics of PLGA as a nano delivery system, reproduced from (Mir et al., 2017)

Optimizing the synthetic procedure by changing the parameters can affect other properties of nanocarriers and therefore a great deal of forethought should go into the design of the system for the particular application (Rezvantlab et al., 2018). During synthesis, parameters such as particle size, surface behavior, degree of crystallinity, degradation rate and molecular weight can be modified to adapt the nanocarrier for desired dosage and site-specific action (Mittal et al., 2007). Bio-nano interactions are important considerations in design as they determine the suitability of the nanostructure for the intended application as well as the undesired toxicity that may result from

the engineering process. Previous research has indicated accumulation of PLGA in the liver when used as nanocarriers and therefore there could be toxicity challenges caused by dose dumping (Makadia & Siegel, 2011). While there are numerous reviews on PLGA based nano-delivery systems in general, this work considers the literature from a design perspective. Schrur's nano-toxicology editorial states "few studies offer consistent results that are of value, and it is difficult to compare studies because they are often carried out using poorly characterized nanomaterials and arbitrary experimental conditions." (Schrurs & Lison, 2012). With these considerations in mind, in silico design, which is an expanding field in drug delivery, could be used to model numerous parameters, including polymer degradation, drug loading and toxicity and hereby provide insight into the structure-behavior relationships of PLGA-based nanocarriers (Ramezanpour et al., 2016). The aim of this review is to collate research on PLGA based delivery vehicles that have been studied for common medical applications, to compare the choices of starting materials and synthetic methods on the properties and functions of the final polymer-drug systems, and to explore how computational investigations can assist in the design of these systems.

2.2 PLGA as a nanocarrier

2.2.1 Properties

PLGA is synthesized from its constituent monomers (Sun et al., 2017) and can be obtained commercially in varying ratios of these monomers (Sadat Tabatabaei Mirakabad et al., 2014). Each constituent has its own physical characteristics that it brings to the co-polymer. PLGA retains properties of both copolymers and can be customized using these properties, which are stiff, hydrophobic and slowly degrading lactic acid vs malleable, less hydrophobic and faster degrading glycolic acid (Engineer, Parikh, Raval, & Organs, 2011). For example, poly-D L-lactic acid has a methyl group, as shown in figure 2.1, and is therefore more hydrophobic than poly glycolic acid. Hence, adjusting the concentration of poly-lactic acid in PLGA varies the solubility of the final polymer (Makadia & Siegel, 2011). A study investigating the rate of hydrolysis of PLGA demonstrated that increasing glycolic acid to lactic acid ratio increases the hydrophilicity of the PLGA co-polymer and hence leads to faster degradation (Keles, Naylor, Clegg, Sammon, & stability, 2015), while a separate study quantified the degradation constant to be 1.3 times higher for glycolic units than for lactic units in the PLGA co-polymers investigated (Vey et al., 2011). It has been shown that PLGA co polymer ratios can be varied to adapt the degradation rate from months to years (Sun et al., 2017). In general, the higher the glycolic acid content of the PLGA polymer, the more amorphous it is and the faster it degrades due to it being more hydrophilic. An exception is PLGA 50:50 lactic: glycolic units, which has exhibited the fastest degradation rate (Lü et al., 2009). It has been shown that increasing the glycolic acid ratio increases the wettability of PLGA for thin film applications (Ayyoob & Kim, 2018) and that increasing the lactic acid content has application in designing PLGA carriers for sustained release (Li & Biomaterials, 1999). PLGA co-polymers with lactic acid content less than 70% have been characterized as amorphous and

suitable for drug delivery applications (Habraken, Wolke, Mikos, & Jansen, 2006). As expected, the higher the molecular weight of PLGA, the more structural integrity it exhibits and the longer it has shown to degrade *in vivo* (Anderson & Shive, 2012). The PLGA co-polymer can be end-capped with different functional groups which have shown to affect the degradation kinetics of the delivery system. For example ester end-capped polymers exhibit a slower degradation rate than acid end-capped polymers and are therefore suitable for slower release applications (Gentile, Chiono, Carmagnola, & Hatton, 2014). Apart from degradation rate, it is also possible to control solubility and glass transition temperature of the PLGA system by varying the molecular weight, lactic/glycolic ratios and end-cap functional groups of the starting material (Gentile et al., 2014).

PLGA is also soluble in a variety of organic solvents including acetone, dichloromethane, chloroform, ethyl acetate and THF (S. Sharma, Parmar, Kori, & Sandhir, 2016) and therefore is relatively simple to work with as carriers for both hydrophobic and hydrophilic drugs (K. Zhang et al., 2014).

2.2.2 Surface functionalisation

2.2.2.1 Shielding

In order to avoid elimination by the RES, a stealth coating around the hydrophobic PLGA nanoparticle surface has been achieved by incorporation of co-polymers with desired properties. The most frequently used co-polymer is polyethylene glycol (PEG) as it is biocompatible and easily grafted or adsorbed onto the surface of PLGA. The hydrophilic PEG shields the PLGA carrier from being taken up by opsonins (Vllasaliu, Fowler, & Stolnik, 2014) and it has been shown that the PEG shield dramatically increases the blood circulation half-life of the nanocarrier (Owens & Peppas, 2006). Some studies have shown that the nanoparticle *in vivo* residence time is dependent on the surface density of the PEG chains (Bertrand et al., 2017). PEGylation has also been associated with enhanced drug loading and tunable carrier degradation (N. M. Khalil et al., 2013). Chitosan, a natural polymer that is formed by partial deacetylation of chitin, is also commonly grafted onto the surface of PLGA based systems to increase biocompatibility. It is biodegradable and has mucoadhesive properties as it carries a positive charge and can efficiently bind to negatively charged cell membranes (Bruinsmann et al., 2019). Hence, a coating of chitosan on the PLGA nanostructure shields it from opsonins and promotes stronger cellular interaction and retention (Lima et al., 2018). Collagen is a highly hydrophilic protein that also increases cellular interaction and when blended with PLGA, can form a delivery system with superior hybrid properties such as increased biological compatibility and mechanical strength (Sadeghi-Avalshahr, Nokhasteh, Molavi, Khorsand-Ghayeni, & Mahdavi-Shahri, 2017). Heparin, a biocompatible material that can be obtained both naturally and synthetically, has been used to impart specific binding properties to delivery systems when combined with polymers (Rodriguez-Torres, Acosta-Torres, & Diaz-Torres, 2018). It is a sulfated glycosaminoglycan with high binding affinity for

various growth factors and has been used in sustained delivery applications by immobilization on the surface of PLGA delivery systems (Chung, Kim, Yoon, & Park, 2006).

2.2.2.2 Surfactants

One of the strategies employed in the nanofabrication process to increase colloidal stability is the use of surfactants. These are agents which usually have amphiphilic properties and reduce the interfacial tension between the hydrophobic and hydrophilic components, hence increasing miscibility and dispersion (Heinz et al., 2017) and preventing particle aggregation (Shkodra-Pula et al., 2019). A commonly used agent is polyvinyl alcohol (PVA), which is a hydrophilic polymeric surfactant that has been shown to decrease the size and increase the uniformity of PLGA nanocarriers, but is also associated with hypertension and central nervous system depression in animal studies (Menon et al., 2012). The use of Polysorbate 80, 60 and 20 has also shown increased residence time and enhanced permeation of the blood brain barrier (S. Sharma et al., 2016). However, it has shown in some cases to cause anaphylactoid reactions (Coors, Seybold, Merk, Mahler, & Immunology, 2005) and long-term infertility (Gajdova, Jakubovsky, Valky, & toxicology, 1993). Polaxamer is a thermo-reversible, non-toxic coating that has been used when encapsulating hydrophobic drugs and has been shown to preferentially target cancer cells. However, it has shown rapid erosion times and it is associated with hyperlipidemia and hypercholesterolemia (Miller & Drabik, 1984). Poloxamine is an amphiphilic block co-polymer and therefore has been used to stabilize hydrophobic drugs while increasing circulatory residence time (Alvarez-Lorenzo, Rey-Rico, Sosnik, Taboada, & Concheiro, 2010). Vitamin E TGPS is a water-soluble form of vitamin E and is used as a solubilizing and emulsifying agent in nano drug delivery. It is commonly used to enhance drug loading (Z. Zhang & Feng, 2006) and nanoparticle degradation rates (Jalali et al., 2011).

2.2.2.3 Active targeting

In active targeting, the surface of the nanoparticle is further decorated with ligands that specifically bind to receptors on the cells of interest and enables the carrier to enter the cell by receptor mediated endocytosis (Muhamad, Plengsuriyakarn, & Na-Bangchang, 2018). These targeted delivery systems are designed to localize drug release at the disease site (Danhier, Feron, & Pr  at, 2010). There are various different kinds of targeting ligands such as small molecules, peptides, antibodies, aptamers and polysaccharides, as shown in figure 2.3 (Yoo, Park, Yi, Lee, & Koo, 2019). These ligands can be either conjugated or adsorbed onto the surface of the nanocarrier after formation or can be linked to one of the components of the carrier before nanoparticle formation (Yoo et al., 2019). It has been shown that increasing the conjugation density of targeting ligands has an effect on the targeting ability of the nanocarrier.

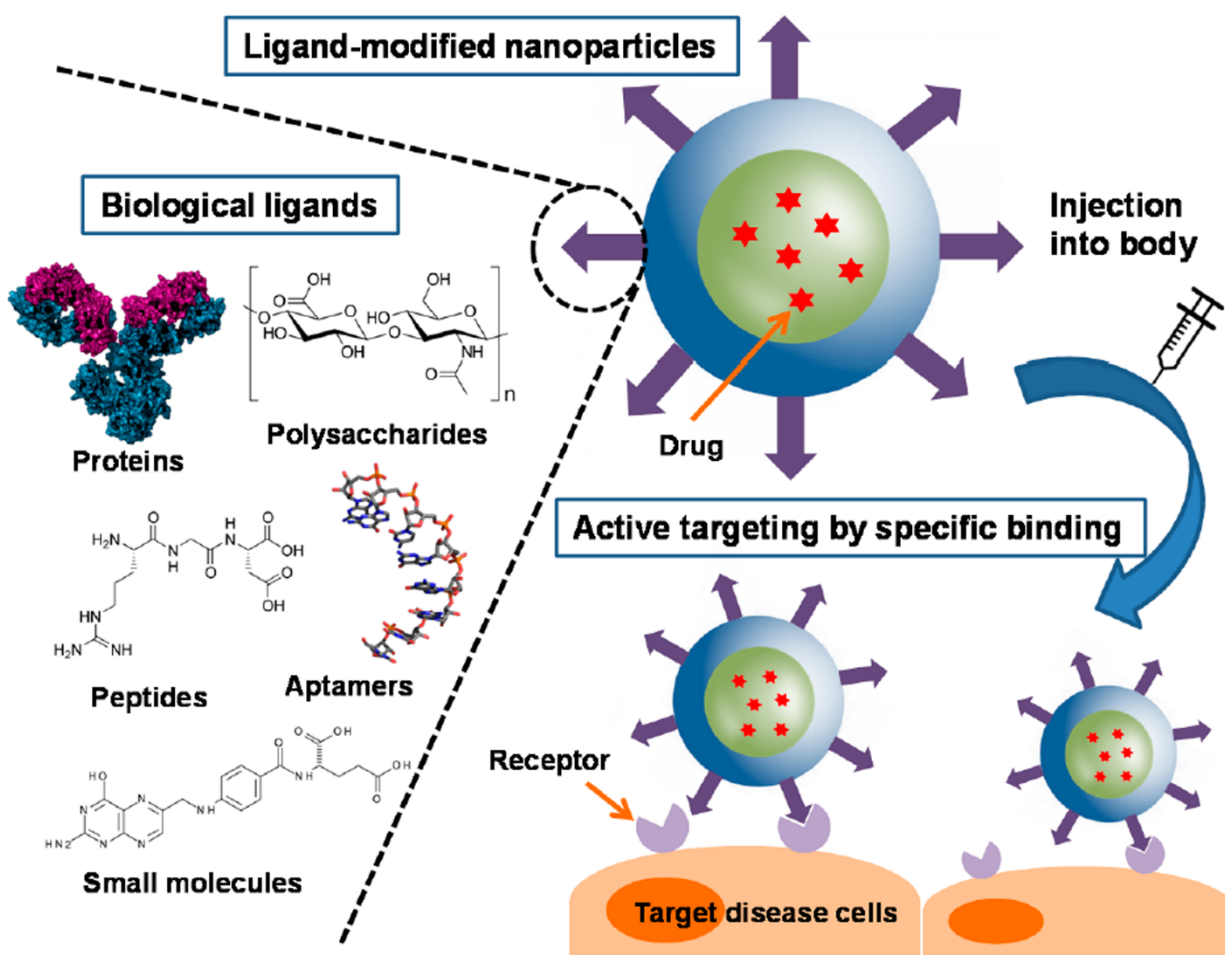


Figure 2.3 Surface functionalised PLGA nanoparticles for active targeting and cellular binding reproduced from (Yoo et al., 2019)

Monoclonal antibodies have had a long history as targeting ligands (Friedman, Claypool, & Liu, 2013) since they have complementarity determining regions that enable them to bind to receptors on cell surfaces with high specificity and affinity (Carter, Mulholland, & Chester, 2016). However, since they are large molecules, their conjugation density capacity on the nanocarrier is substantially decreased (Yoo et al., 2019) compared to other ligands, and furthermore, they raise immunogenicity concerns (Karra & Benita, 2012). Compared to antibodies, peptides have the advantage of smaller size and non-immunogenicity but they still are able to retain target specificity (Zhao et al., 2007). Aptamers are short strands of nucleic acids that can be synthetically designed to bind specific biological targets. They are non-immunogenic and non-toxic but their synthesis can be costly (Friedman et al., 2013). Despite their advantages for *in vivo* targeting, both peptides and aptamers are prone to enzymatic degradation (Yoo et al., 2019). Polysaccharides are advantageous because they are biocompatible and can be used as structural components of the nanocarrier (Choi et al., 2011) as well as to target carbohydrate binding receptors on cell surfaces. However, some polysaccharides could have solubility challenges and modification of the

carbohydrate structure could result in unintended toxicity (Peng, Yang, Tong, & Ma, 2018). Small molecules form a class of targeting ligands that comprise of synthetic compounds that are designed to target certain domains on cell surface receptors. They are usually chosen because of ease and control of synthesis but they often do not bind with high specificity and some target receptors can be expressed in healthy cells (Yoo et al., 2019), resulting in unintentional cell binding. Even though active targeting strategies provide an attractive avenue for site specific drug delivery, there are many challenges in this area, such as receptor accessibility and off-target binding. Particularly during different stages of tumor development, certain receptors can be up or down regulated, which provides an additional challenge for the use of targeting ligands in chemotherapeutic drug delivery (Vhora et al., 2014).

2.3 Synthetic methods of PLGA nanocarriers

PLGA nanocarriers may be fabricated by different methods and the choice of method has shown to affect properties such as particle size, colloidal stability, drug loading/encapsulation efficiency and release behavior of the final product (Swider et al., 2018). Depending on the process of preparation, the structural organization may also be different. The drug is either encapsulated inside the carrier or adsorbed on the surface (Danhier et al., 2012). There are several methods that can be employed for the preparation of PLGA nanocarriers, and the following provides a brief overview on both the well-established and relatively recently developed techniques. The traditional methods based on emulsions are illustrated by figure 2.4 (Ding & Zhu, 2018).

2.3.1 Single and Double Emulsion

The emulsion methods have been the most frequently used methods of synthesis and they are suitable for a wide range of drugs with varying solubilities (Wang, Li, Truong-Dinh Tran, Zhang, & Kong, 2016). The single emulsion (oil in water or O/W) method is suitable for hydrophobic drugs. PLGA and the drug is dissolved in a small volume of suitable volatile organic solvent and added dropwise to the aqueous phase containing a stabilizer, usually PVA. The mixture is sometimes sonicated and then stirred, often under sheer stress for a fixed amount of time to allow the organic solvent to evaporate. The double emulsion method is used when the active agent to be entrapped is hydrophilic, such as proteins and peptides. The active is dissolved in an aqueous phase and then added to PLGA which is dissolved in the organic phase, and this forms a primary water in oil (W/O) emulsion. This is then added to another aqueous phase containing a stabilizer and allowed to mix under stress, allowing the organic solvent to evaporate. The nanoparticles are therefore formed by a water in oil in water (W/O/W) emulsion (Makadia & Siegel, 2011). The product is isolated by centrifugation or ultrafiltration and washed to remove unreacted products. Thereafter it is freeze dried and can be stable for several months to years. Recently, a single emulsion method was used to successfully entrap proteins for vaccine application (Ospina-Villa, Gómez-Hoyos, Zuluaga-Gallego, & Triana-Chávez, 2019) and a PLGA-PEG nanocarrier was formulated using the

double emulsion method for intraperitoneal insulin delivery (Haggag et al., 2018). The emulsion methods can be adjusted by changing the drug to PLGA ratio, the organic solvent, the stabilizer concentration in the aqueous phase and the stirring speed and can hereby be adapted to control the size range of the nanocarriers to some extent. However, there are often batch to batch variation with these methods and the carriers prepared by this method for protein-based drugs have limited stability due to degradation of proteins at the aqueous interface and the sheer stress of homogenization leading to unfolding of the protein sheets (Ding & Zhu, 2018).

2.3.2 *Spray drying*

This method involves the preparation of water in oil or solid in oil emulsions, which are sprayed in a thin stream of heated air. The type of drug (hydrophilic or hydrophobic) would determine the solvent used in the emulsion (Makadia & Siegel, 2011). Recently, spray drying was used in the preparation of a PLGA nano-formulation for sustained treatment of tuberculosis (TB) (Kalombo et al., 2019) and in the fabrication of a carrier for antibiotic coating of dental implants (Baghdan et al., 2019). This method is highly advantageous because it is suitable for hydrophobic and hydrophilic drugs and can be used for sensitive compounds since the conditions are mild. It is also a rapid method (Nie, Lee, Tong, & Wang, 2008) which can be suitable for industrial scale-up due to the minimal processing parameters involved (Ding & Zhu, 2018). The main drawback of this technique is the wastage caused by inaccessible product that adheres to the inside of the nano-sprayer (Wang et al., 2016). Parameters such as orientation of jets, temperature, and solvent choice can all affect the properties of the final nanoparticles (Berkland, Pollauf, Pack, & Kim, 2004).

2.3.3 *Coacervation*

With coacervation or phase separation, the polymer and drug are prepared as O/W for hydrophobic drugs and W/O/W for hydrophilic drugs, and then a non-solvent, e.g. silicon oil is added dropwise under stirring (Verma, Gulati, Kaul, Mukherjee, & Nagaich, 2018). This reduces the solubility of PLGA in the organic solvent and results in the formation of a polymer-rich phase in which PLGA surrounds the drug molecules to form microdroplets (coacervates). These are rapidly quenched in a non-soluble medium to form the solid product (Wang et al., 2016). Parameters such as starting polymer, solvent choice, stirring rate and temperature can be varied to control the properties of the particles. Coacervation usually forms micrometer sized particles (S. Sharma et al., 2016) but has been used in protein nanoparticle preparation (Verma et al., 2018).

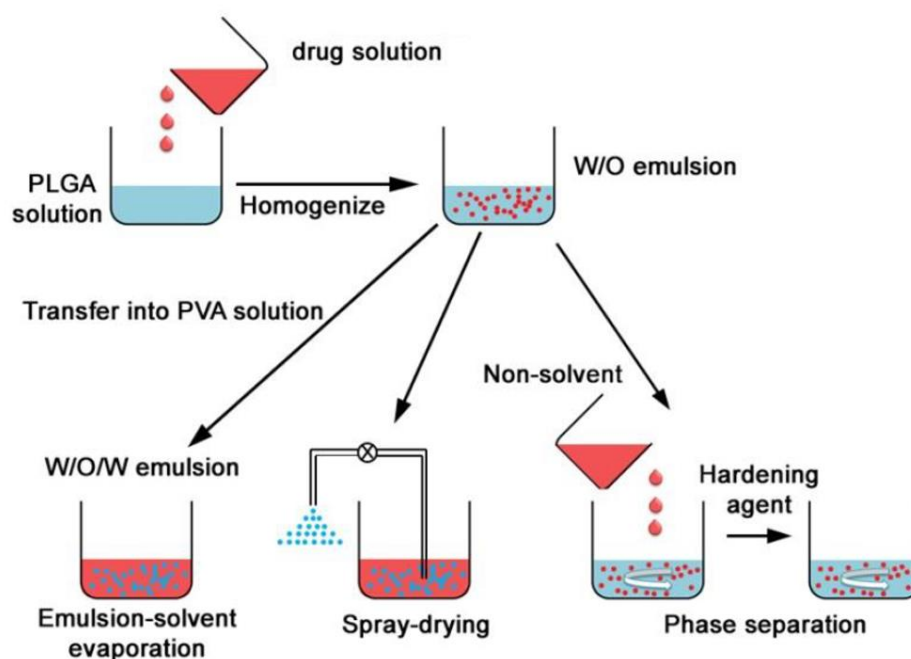


Figure 2.4 Formation of PLGA nanoparticles by double (W/O/W) emulsion, spray drying and phase separation (coacervation) methods, adapted from (Ding & Zhu, 2018)

2.3.4 Salting out

In the salting out method, drug and PLGA are dissolved in a miscible organic solvent and added to the aqueous phase containing stabilizer and a salt under sheer mechanical force. The salt usually used is magnesium chloride hexahydrate or magnesium acetate tetrahydrate (Wang et al., 2016) and is used at a ratio of 1:3 PLGA:salt (Eley, Pujari, & McLane, 2004). Upon addition of water, the organic solvent diffuses into the aqueous phase, causing the formation of PLGA-drug nanoparticles, illustrated by figure 2.5 (Crucho & Barros, 2017). This method is not suitable for lipophilic drugs and can be time intensive since isolation of the product involves several washing steps to remove reagents. However, it would suit drugs which are very temperature sensitive since heat is not required (Nagavarma, Yadav, Ayaz, Vasudha, & Shivakumar, 2012). This method is robust and is suitable for nanoparticles with high polymer concentrations since the size of the particle is not generally affected by the amount of polymer (Swider et al., 2018).

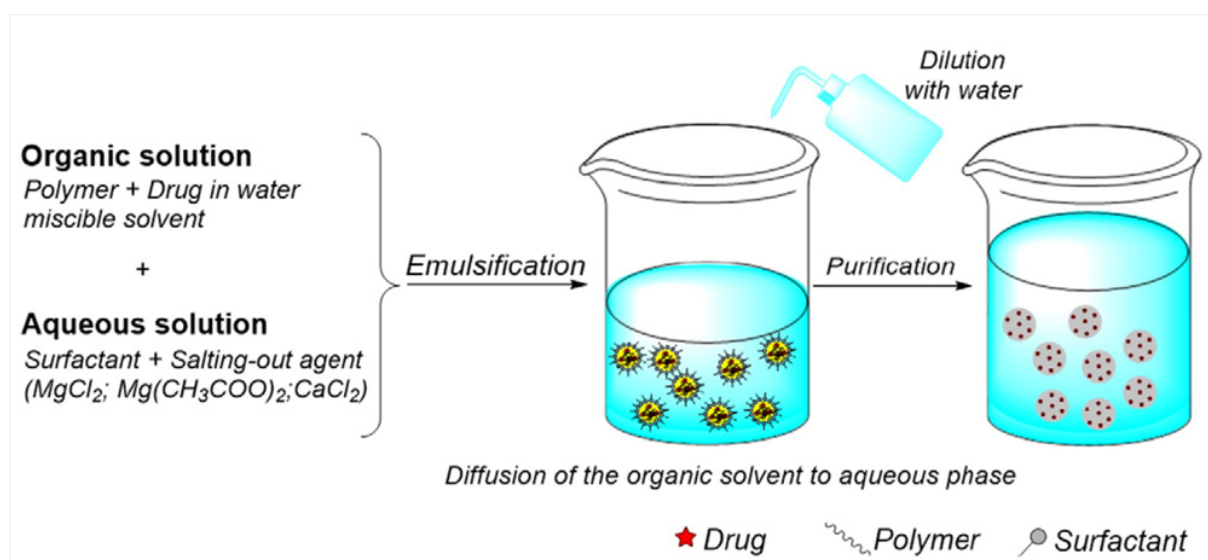


Figure 2.5 Illustration of the salting out method of preparation, reproduced from (Crucho & Barros, 2017)

2.3.5 Nanoprecipitation

In this method, PLGA and the drug is dissolved in a polar, water miscible solvent and added dropwise to the aqueous phase, which may contain a surfactant. The product is formed by rapid diffusion of the water miscible solvent into the aqueous phase, resulting in precipitation of the PLGA-drug nanoparticles, as shown in figure 2.6 (Crucho & Barros, 2017). The properties of the nanocarrier are controlled by PLGA content and molecular weight, PLGA to drug ratio and choice of solvent (Wang et al., 2016). Recently, an optimised nanoprecipitation method was developed for the preparation of PLGA encapsulated alendronate sodium, a drug for osteoporosis (Oz, Küçüktürkmen, Devrim, Saka, & Bozkir, 2019) and a modified procedure was reported for a PLGA hybrid nanocarrier for simvastatin (M. Zhang, He, Zhang, & Liu, 2018). Nanoprecipitation can be used to prepare particles in the 100nm size range and is advantageous because of the absence of sheer stress (Fessi, Puisieux, Devissaguet, Ammoury, & Benita, 1989). However, the unmodified nanoprecipitation method does not usually work well for hydrophilic drugs as they do not form favourable interactions with PLGA in a water miscible solvent (Govender, Stolnik, Garnett, Illum, & Davis, 1999).

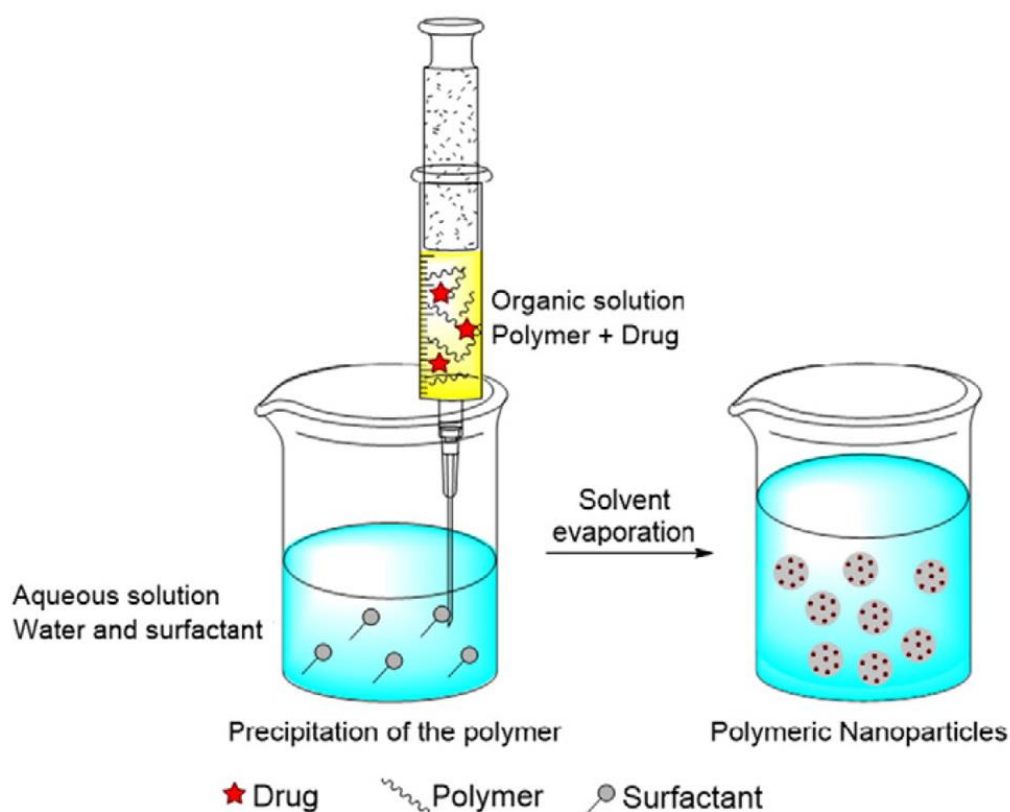


Figure 2.6 The nanoprecipitation method, adapted from (Crucho & Barros, 2017)

2.3.6 Supercritical fluid technology

Supercritical fluid technology, illustrated by figure 2.7 (Jog & Burgess, 2017), can provide an environmentally friendly method of generating nanoparticles since it reduces, and in some cases, eliminates the use of organic solvents (Koushik & Kompella, 2004). In this method, the polymer and drug are dissolved in a supercritical fluid which is then rapidly expanded and depressurized. The resultant mixture is then passed through a fine nozzle or capillary, resulting in supersaturation and formation of nanoparticles, which are collected separately (Mishima, 2008). This is an attractive method as it is highly tuneable but the kind of nanoparticle products are restricted since not all starting materials are compatible with the supercritical fluid (Soh & Lee, 2019).

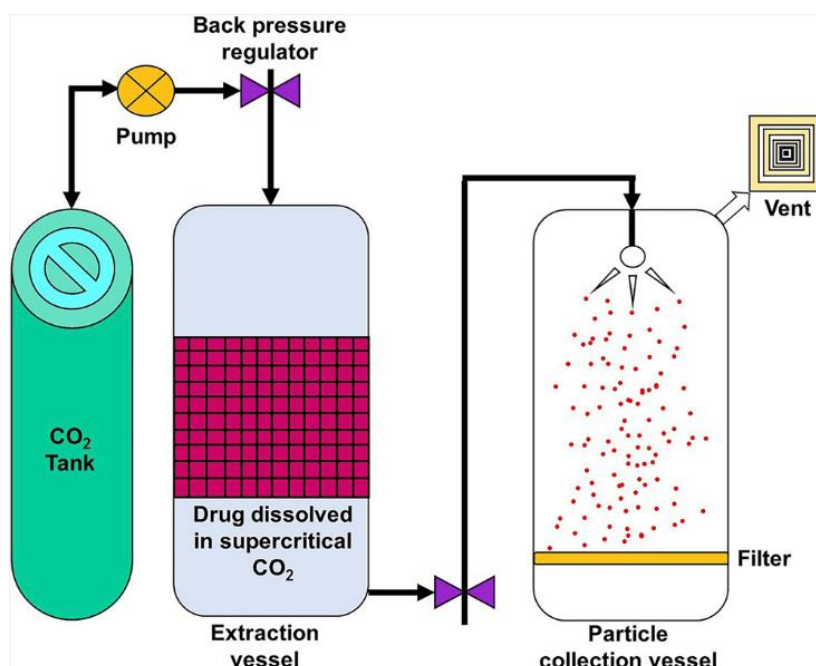


Figure 2.7 Simple setup of a supercritical fluid technology method, reproduced from (Jog & Burgess, 2017)

2.3.7 Microfluidics

The area of microfluidics deals with channels of the micrometre size range that are used to control and manipulate the movement of volumes of fluid from the nanolitre size range and below. When working at the nanoscale, the conditions of flow can be precisely controlled and constant laminar flow is maintained, which is impossible when conducting reactions at the macroscale level (Chiesa et al., 2018). Therefore this technique has lent itself to the synthesis of nanoparticles by the formation of emulsions using droplet microfluidics. In this method, the polymer and drug are combined and the emulsion is formed in the microfluidic mixer, which can have different channel architectures (Collins, Neild, deMello, Liu, & Ai, 2015). The most commonly used geometries for droplet based PLGA nanoparticle formation are the t-junction, flow focusing, and continuous flow microchannels, as shown in figure 2.8 I (Shembekar, Chaipan, Utharala, & Merten, 2016). In the t-junction geometry, channels are perpendicular to each other. The dispersed phase (aqueous) flows through one channel while the continuous phase (oil) flows through the other and droplets are formed at the junction. In the flow focusing system, the aqueous phase flows through a square capillary where shear force is provided on either side of it by the flow of the oil phase. The emulsion then flows through a narrow capillary and droplets are formed in a collection chamber. With continuous flow geometry channels, the aqueous phase flows through a capillary that resides in another capillary through which the oil flows in the same direction. Droplets begin to form once the two phases mix (Shembekar et al., 2016). SEM images of particles formed by this method are shown in Figure 2.8 II D-E (Xu et al., 2009).

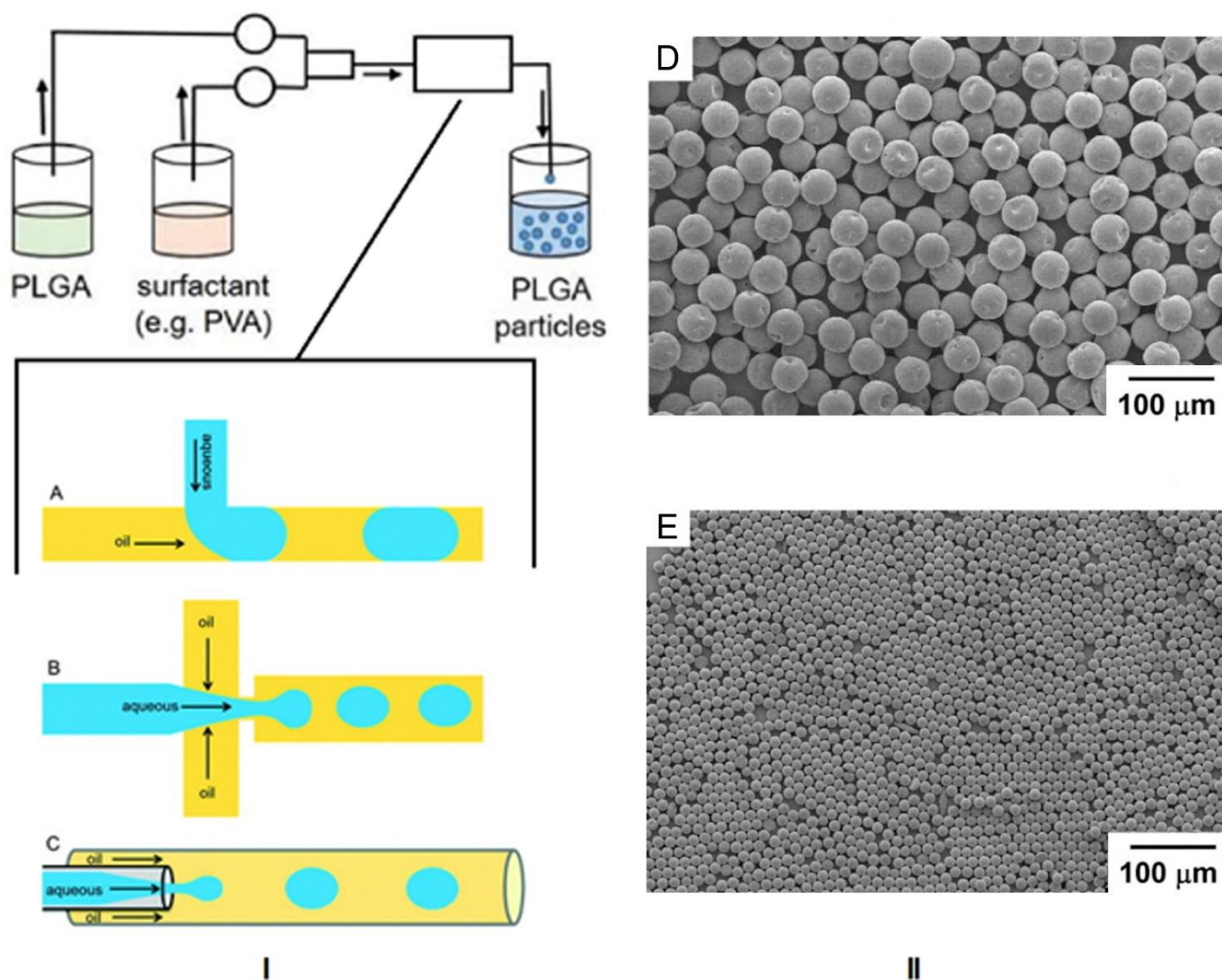


Figure 2.8 I - Schematic of the microfluidics method and the three most common microchannel geometries. (A) - T junction, (B) – flow focussing (C) - continuous flow. Reproduced from (Shembekar et al., 2016)-published by the Royal Society of Chemistry. **II** - (D) and (E) – SEM images of PLGA particles formed by this technique, adapted from (Xu et al., 2009)

These microchannels are used for O/W emulsions, but can be adapted for double (W/O/W) emulsions by using a combination of channels. Recently, a microfluidics method was developed for the encapsulation of cell penetrating peptides (Streck et al., 2019) and targeted delivery of taxanes (C. Martins & Sarmiento, 2020). There are numerous advantages of microfluidics for nanoparticle synthesis. With this technique, the chemical composition of the final product can be preselected according to the desired application. The synthetic parameters can be controlled to the extent that there is a much larger particle size homogeneity compared to bulk methods, and there are also smaller volumes of solvent needed. However, the scale of nanoparticle production is limited and the microchannels are also susceptible to blockage and contamination. The time and temperature of mixing, flow rate, choice of solvents and payload type determine the properties of the final nanoparticles (K. T. Kim, Lee, Kim, Yoon, & Cho, 2019).

2.3.8 *Membrane extrusion emulsification*

With this technique, single or double emulsions of PLGA and drug are either initially prepared or formed when extruded through a membrane of predetermined pore size. There are two ways to do this - direct and pre-mix membrane extrusion (ME), as illustrated by figure 2.9 (A) (P. Guo et al., 2018), and a characteristic emulsion is shown in figure 2.9 (B). In direct ME, the membrane emulsifies the dispersed phase into nanosized droplets, while in premix ME, the emulsion is formed via a conventional method and thereafter extruded through the membrane, which downsizes the coarse emulsion into uniform nanosized droplets. This method can be used for hydrophobic or hydrophilic drugs and is advantageous because the size of product can be controlled by varying the nano-porous membrane pore size to create particles of required dimensions, resulting in a large size homogeneity. Premix ME in particular has been shown to have a higher uniformity in dispersity of final nanoparticles as shown by figure 2.9 (C), compared to direct ME. In general, it is a mild procedure with low energy requirements and can be easily scaled up. However, it is not suitable for emulsions with high viscosity (P. Guo et al., 2018).

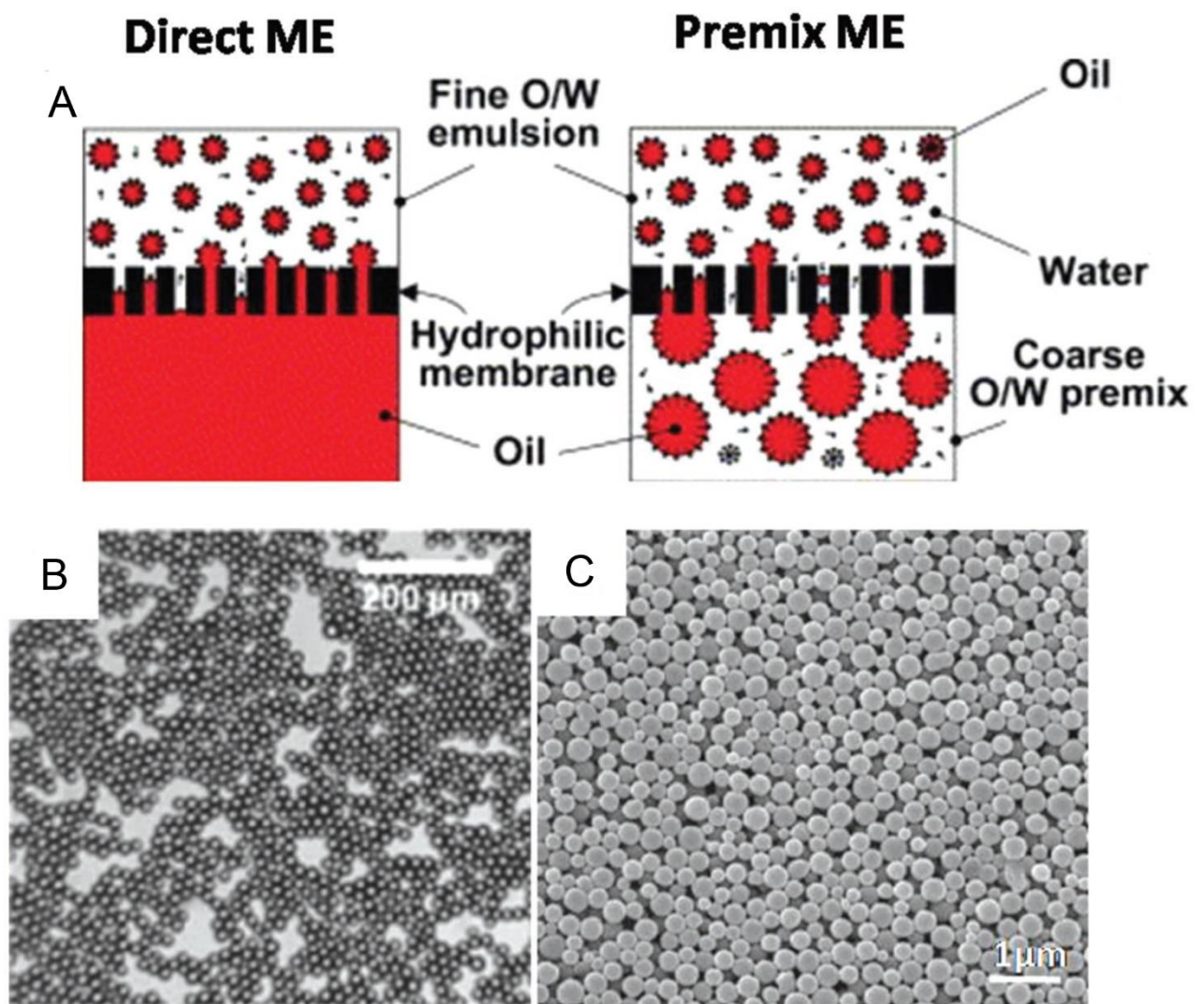


Figure 2.9 (A) - Direct- and premix- two common processes using membrane extrusion; (B) SEM image of an emulsion formed by this method (C) SEM images of nanoparticles formed by premix ME, reproduced with permission from (P. Guo et al., 2018)

2.3.9 Nanoimprint lithography and the PRINT technique

Nanoimprint lithography is used to form nanoparticles from a nanostructure template that is placed over a layer of precursor material which is heated to above the glass transition temperature of the polymer. Thereby, the malleable precursor material is molded into the desired size and shape, which is retained upon cooling. The template is then removed, leaving the product on the substrate base. Su and co-workers have successfully used this method for the nanofabrication of submicron PLGA grooves for the control of the length and direction of retraction fibres during cell division (Su et al., 2015). The major drawback of this method is the residual interconnecting layer on the substrate base that prevents the formation of isolated nanostructures (Fu et al., 2018). The PRINT (particle replication in non-wetting template) technique involves the preparation of the PLGA-drug solution matrix and casting it on a delivering sheet. Thereafter a mold with nanosized cavities is

placed over the delivering tray and it is passed through a nip and separated so that the polymeric material fills the mold cavities. The particles are then solidified and placed on a high energy adhesive layer and passed through the nip without separation. After the mold is removed, the nanoparticles are collected by washing with a solvent that dissolves the adhesive (Perry, Herlihy, Napier, & Desimone, 2011). The method is automated with a high degree of control over the individual parameters, and can be used for a wide variety of cargos including hydrophobic and hydrophilic drugs, vaccines, and proteins. However, it is a multistep process that can be labour intensive (Swider et al., 2018). The desired particle size, surface properties and composition can be preset and controlled in the initial step. The PRINT process is illustrated in figure 2.10 (Perry et al., 2011), while figure 2.11 shows SEM micrographs of the different shapes of PLGA nanoparticles that have been prepared by this method. Enlow and colleagues have reported the PRINT process whereby PLGA micro- and nanoparticles were prepared, with cylindrical, spherical, ridged and fenestrated morphologies. These particles demonstrated >40% drug loading and >90% encapsulation efficiencies of docetaxel (Enlow et al., 2011).

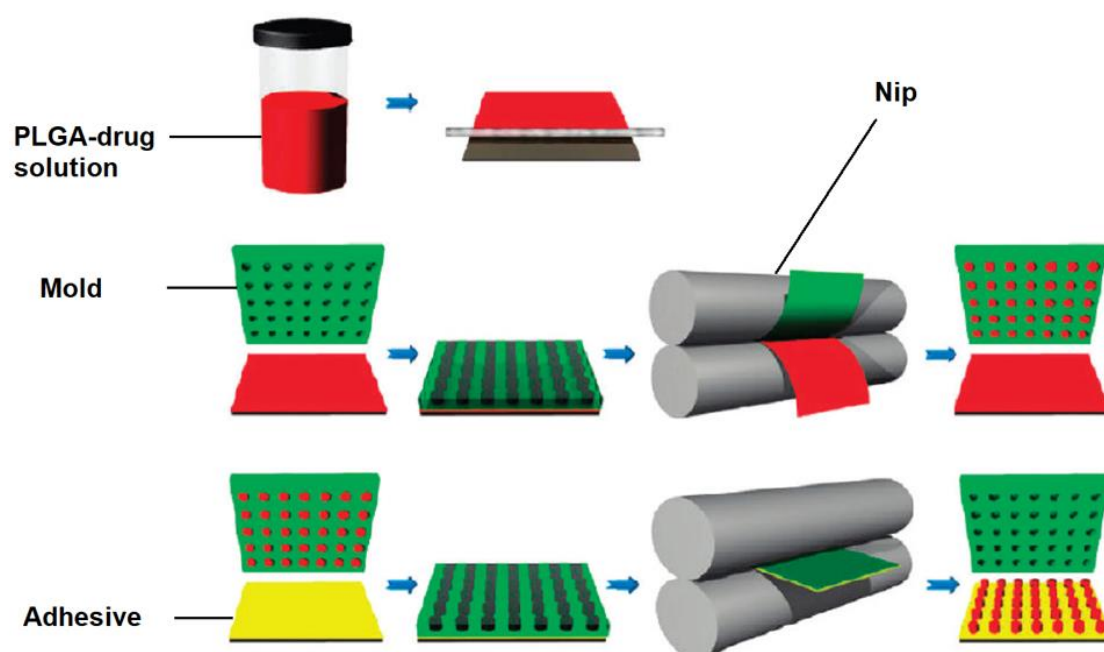


Figure 2.10 Schematic showing PRINT procedure, reprinted with permission from (Perry et al., 2011). Copyright (2019). American Chemical Society

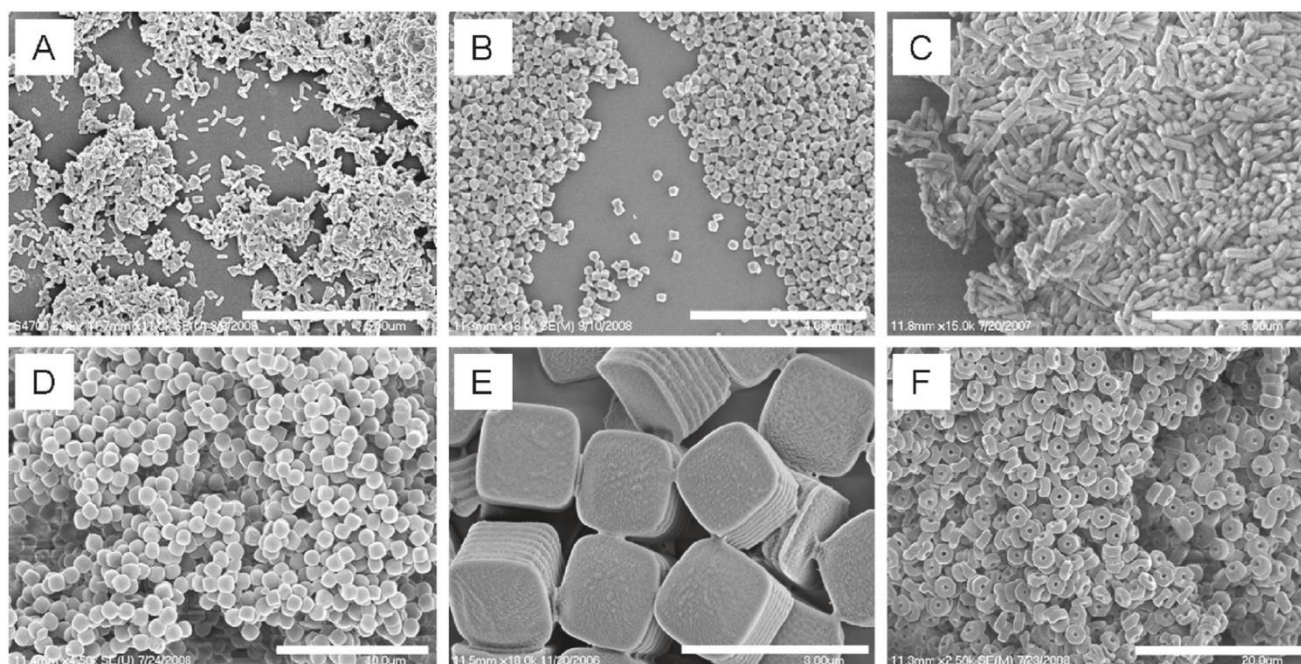


Figure 2.11 SEM Images of PLGA PRINT particles. (A), (B) and (C) – cylinders of different dimensions. (D) – spheres. (E) – ridged cubes. (F) [bottom right]– particles with centre fenestrations, reprinted with permission from (Enlow et al., 2011). Copyright (2019)

2.4 Medical applications

2.4.1 Cancer Research

2.4.1.1 Actively targeted chemotherapeutics

In the United States of America alone, 1,762,450 new cancer diagnoses and 606,880 cancer related deaths are expected to occur in 2019 (Siegel, Miller, & Jemal, 2019). Despite the ubiquity of this disease, treatment options are challenging due to the complex pathology of the different cancers. Current chemotherapy often leaves debilitating and life altering side effects since most drugs on the market that target the rapidly dividing cancer cells also inadvertently damage cells that are vital for normal life processes (Rizvi & Saleh, 2018). Actively targeting PLGA nanoparticles are able to circumvent this; Moku and colleagues have shown increased drug loading and efficacy against lung cancer by using trans-activator of transcription (TAT) peptide ligands to target mesenchymal stem cells (Moku et al., 2019) while Ganipineni and co-workers found that magnetically targeted paclitaxel- and SPIO-loaded PLGA-based nanoparticles effected increased cellular uptake in glioblastoma cells compared to the non-targeted carriers (Ganipineni et al., 2019). Another type of nanoparticle targeting is the use of ‘smart’ carriers that are engineered to respond to a stimulus (Kapoor et al., 2015). Recently, a pH dependent aptamer functionalized PLGA nanocarrier system was reported to increase anti-cancer activity of doxorubicin to human lung cancer cells, with reduced toxicity to healthy cells (Saravanakumar et al., 2019) and a

superparamagnetic iron oxide encapsulated nanocarrier for docetaxel demonstrated favorable pharmacokinetics and a greater degree of uptake in breast cancer cells (Panda et al., 2019).

2.4.1.2 Immunotherapy

Since Allison and Honjo were awarded the 2018 Nobel prize in Physiology and Medicine “for their discovery of cancer therapy by inhibition of negative immune regulation” (Z. S. Guo, 2018), the area of nanomedical research into cancer immunotherapy has received substantial attention. This approach involves the use of pharmaceutical agents to activate a patient’s immune system to fight cancers as opposed to traditional chemotherapy which involves directly drugging the cancer cells (D. N. Khalil, Smith, Brentjens, & Wolchok, 2016). Chen and co-workers have described PLGA nanocarriers equipped with an immunostimulant and photothermal agent, and this formulation showed increased activation of the immune system of BALB mice compared to the free agent (Q. Chen et al., 2016). More recently a sustained controlled release PLGA nano-system was developed to activate the anti-tumor immune response in mice bearing melanoma and colon cancer (Yin, 2019) and a PLGA system containing an immune adjuvant together with an enzyme that increased the efficacy of radiation therapy demonstrated the feasibility of combination immunotherapy and targeted radiotherapy in BALB mice (Q. Chen et al., 2019).

2.4.1.3 Imaging and diagnostics

PLGA has applications for tumor diagnostics as it is able to deliver imaging agents to cancer cells with specificity and controlled biodistribution. Advances in nano-theranostics, which is the incorporation of imaging and therapeutic agents in one nanocarrier, have shown promise for real time imaging throughout a patient’s treatment course (Chapman et al., 2013). A novel theranostic PLGA nanocarrier with a near infrared imaging agent, further decorated with gold nanoparticles has been synthesized and shown to have increased activity and photodynamic properties in tumor grafted BALB mice (Xi et al., 2018) and a targeted PLGA-based nanobubble was designed with an ultrasound contrast agent, and demonstrated specificity and imaging capabilities to breast cancer in BALB mice (Du et al., 2018). More recently an image guided photothermal PLGA nanocarrier for doxorubicin showed promise for real time photoacoustic imaging in tumor bearing nude mice (Shen et al., 2019) and a near infra-red dye loaded PEGylated PLGA nanocarrier was also able to provide information on the circulation and distribution of the nanoparticles in nude mice (Kumar, Van Treuren, Ranjan, Chaudhary, & Vishwanatha, 2019).

2.4.2 HIV Treatment

The delivery of anti-retro viral drugs faces many of the general limitations of conventional drug delivery and therefore biomaterials with low toxicity such as PLGA based nanocarriers are being implemented in formulations to treat HIV. Mannosylated PLGA nanoparticle carriers have shown promise for targeted delivery of anti-retro viral drugs to the brain (Patel, Parikh, & Patel, 2018) and

the use of microfluidics technology enabled the novel synthesis of efiravine loaded PLGA nanoparticles (Cláudia Martins et al., 2019). A recent study reported a PLGA based nanocarrier for the combination of the anti-retro virals griffithsin and dapirivine which showed a long acting treatment profile (H. Yang et al., 2019) and a separate proof of concept study showed promise for a long acting bictegavir encapsulated PLGA nanocarrier (Mandal, Prathipati, Belshan, & Destache, 2019).

2.4.3 Inflammatory disorders

Many current treatments have proven to be inadequate at treating or alleviating symptoms of inflammation. The specific delivery of anti-inflammatory agents to the target site could potentially increase their therapeutic concentration in the inflamed tissue with reduced side effects (Gendelman et al., 2015), and the use of PLGA is particularly suitable to this application because of its favorable biodegradability and non-immunogenicity (Lamprecht et al., 2001). Davoudi and co-workers described a carrier within a carrier system using intestinal organoids to transport 5-ASA encapsulated PLGA nanoparticle to treat inflammatory bowel disease (Davoudi et al., 2018), and Perreira's research involved the development of a metformin loaded nano-formulation that showed efficacy against periodontal inflammation in diabetic rats (Pereira et al., 2018). Gholizadeh and colleagues have formulated a dactolisib-PLGA nanoparticle that showed activity against inflamed endothelial cells (Gholizadeh, Kamps, Hennink, & Kok, 2018) and more recently, Yang's group reported the synthesis of a crocetin-loaded nanoparticles that reduced the level of pro-inflammatory cytokines in renal tissue and therefore shows potential for the treatment of diabetes induced nephropathy (X. Yang, 2019).

2.4.4 Other applications

PLGA has been adapted to treat conditions in many fields of biomedicine, as shown by Figure 2.12 (Mir, Ahmed, ur Rehman, & Biointerfaces, 2017). A hyaluronic acid functionalized PLGA based nanocarrier for methatrexate has been developed for targeted treatment of rheumatoid arthritis (Trujillo-Nolasco et al., 2019), a PLGA nanoparticle with protease inhibitor has shown to overcome gastro-intestinal limitations of oral insulin delivery in rats (Faheem, Abdelkader, Osman, McCarron, & El-Gizawy, 2019), a PLGA-chitosan based nanocarrier has been synthesized and shown to be selective for human antigen presenting cells (Durán et al., 2019), a potential DNA vaccine delivery system has been designed using a PLGA based nanocarrier (Besumbes et al., 2019), and a Vitamin D encapsulated PLGA based delivery system has recently shown activity against various markers for Alzheimer's disease in mice (Jeon et al., 2019). Gonzalez and colleagues have prepared an optimized fluoromethalone-PLGA nanoparticle that demonstrated increased efficacy in treating ocular inflammation compared to the commercial formula (Gonzalez-Pizarro et al., 2018). The use of some of the available methods in PLGA nanocarrier synthesis and their applications are summarized in Table 1.

2.4.5 *Inclusion of PLGA formulations in the clinic*

The biocompatibility, biodegradability and versatility of PLGA has made it suitable for a wide range of clinical applications. PLGA was commercialized in the 1970's as a suture material under the trade name Vicryl® (Kamaly, Yameen, Wu, & Farokhzad, 2016). Other sutures include Dolphin Sutures®, and Polysorb® which are both currently approved. PLGA-containing chemotherapeutic formulations approved for clinical use include Lupron Depot®, for sustained release of leuprolide, which has application in the management of prostate cancer (Swider et al., 2018), Trelstar®, a triptorelin-containing suspension for the treatment of prostate cancer and Zoladex, a goserelin-containing implant used in the treatment of breast and prostate cancer and endometriosis. Formulations approved for other applications include Risperdal® Consta®(risperidone), Vivitrol®(naltrexone) and Arestin®(minocycline) for the treatment of schizophrenia, opioid dependence and periodontal disease respectively (Jain et al., 2016). A promising direction for clinical development is the engineering of PLGA based systems with imaging agents to monitor disease progress and/or relapse patterns using magnetic resonance imaging (MRI). Studies have shown these structures to be non-invasive and cost effective, with excellent safety profiles (Strohbehn et al., 2015). Furthermore, there are a number of PLGA based systems have been used in clinical trials that are ongoing or have been recently concluded.

Table 2.1 Properties of some prepared PLGA nanocarriers, their methods of formation and biological targets

Type of nanocarrier	Route of administration	LA:GA in PLGA	Mw of PLGA	Synthetic Method	Particle size range	Medical application	Reference
PLGA nanoparticle embedded in a microsphere	Inhalation	50:50	38.5K	Single emulsion	~200nm	Lung cancer treatment	(X. Guo et al., 2014)
PLGA-HDL hybrid nanoparticle	injectable	50:50	30-60K	microfluidics	~100nm	Cardiovascular disease treatment	(Sanchez-Gaytan et al., 2015)
Dye loaded PLGA nanoparticles	intravenous	50:50	13.5K	Spray drying	~500nm	Photoacoustic imaging	(Kohl et al., 2011)
Paclitaxel loaded PLGA nanoparticle	Injectable	75:25	694K	Membrane extrusion	~300nm	Malignant melanoma treatment	(Liang et al., 2013)
Insulin loaded plga nanoparticle	oral	50:50	10K	Nano precipitation	~100nm	Diabetes treatment	(Chopra et al., 2017)
Nimodipine loaded PLGA nanoparticle	intravenous	85:15	-	Modified nano precipitation	~200nm	sustained release for cerebral vasospasm treatment	(Mehta, Yadav, & Sawant, 2007)
Alendronate loaded PLGA nanoparticle	intravenous	50:50	40-75K	Double emulsion	~300nm	Restenosis inhibition	(Cohen-Sela, Chorny, Koroukhov, Danenberg, & Golomb, 2009)
Docetaxel loaded PLGA nanoparticles	injectable	50:50	33K	PRINT	~300nm	Cancer treatment	(Enlow et al., 2011)

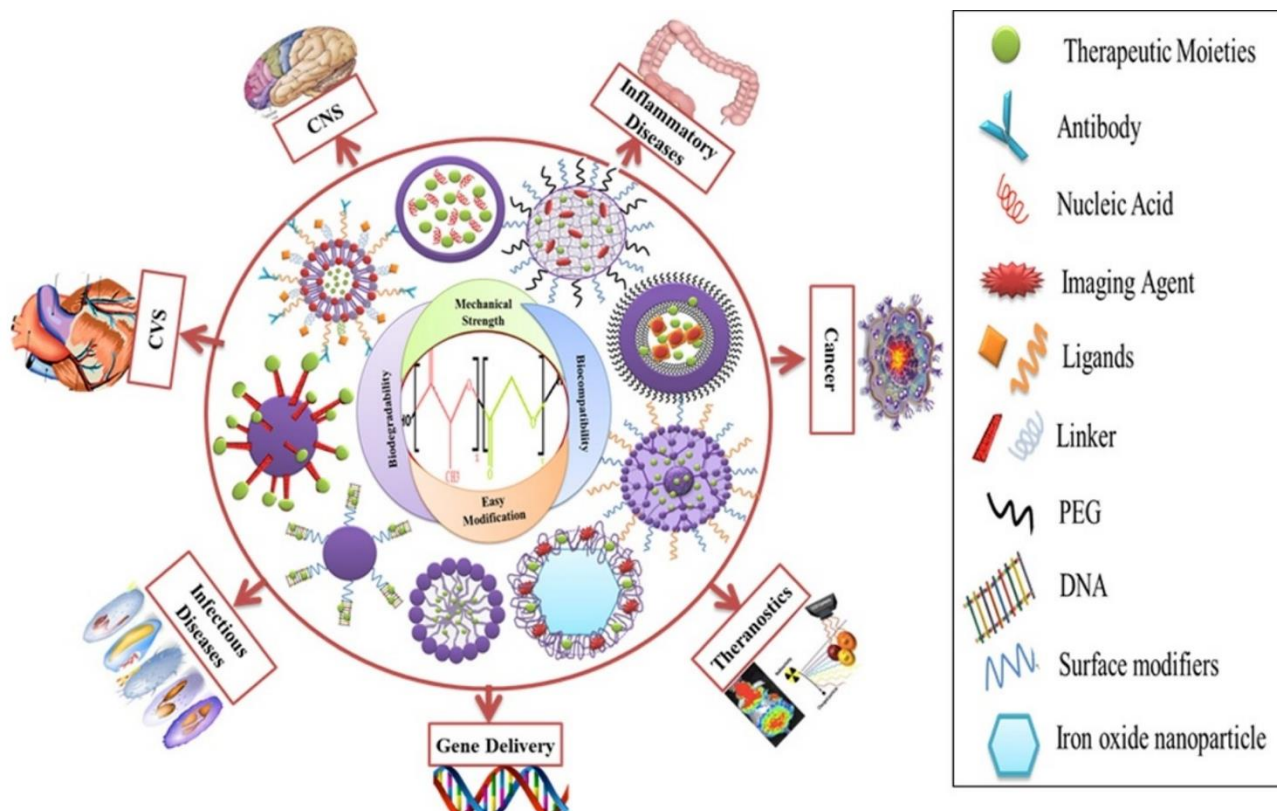


Figure 2.12 Functionalised PLGA nanocarriers and their medical applications, reproduced from (Mir et al., 2017)

2.5 Computational modelling

Despite the numerous formulations and methods available for PLGA synthesis, there is a large discrepancy between *in vitro*, *in vivo* and clinical results. One of the reasons for this could be the fact that it is difficult to obtain mechanistic insight into nano-formulation behavior in the various systems by evaluation of results based solely on experimental methods (Huynh, Neale, Pomès, & Allen, 2012). Because of the ubiquity of PLGA across so many biomedical fields of research, there is an abundance of data at our fingertips for computational modelling (*in silico*). There are various levels of detail that can be used in computational simulations. The approach used most widely for nanoparticle drug delivery systems is molecular dynamics (MD). This technique uses the motion of the molecules in the system to predict its behavior. The parameters it uses are the bonds, bond angles and dihedrals, and here the atoms are treated as point charges. If the degree of detail of atomistic interaction is not required, a coarse grained (CG) model can be used. Here, atoms are grouped into molecular fragments and their behavior in the system is modelled (Frenkel & Smit, 2001). Density functional theory is a model based on electronic density around atoms in the system and measures these interactions within the system of interest (Geerlings, De Proft, & Langenaeker, 2003). Computational

simulations at these levels can give insight into polymer interactions, drug-carrier miscibility, drug loading, drug release and complex stability (Ramezanpour et al., 2016). Mathematical modelling such as finite element analysis and computational flow dynamics are particular useful when studying polymeric nanoparticle formation (Lince, Marchisio, & Barresi, 2011), diffusion and degradation (Kojic, Milosevic, Simic, Stojanovic, & Uskokovic, 2017). Hence, coupled with experimental methods, they can be powerful tools in the rational design of PLGA nanocarriers for biomedical applications. A study of PLGA binding to curcumin was conducted using molecular dynamics with the GROningen MAchine for Chemical Simulations (GROMACS) program, compared to laboratory findings and collated with the experimental results of ten other PLGA-drug formulations. This study also predicted that PLGA could entrap curcumin with a higher encapsulation efficiency than tripalmitin, a lipid-based carrier, and this prediction was confirmed experimentally (Metwally & Hathout, 2015). A study on the drug release of the anti-cancer drug oxaliplatin in a PLGA matrix was conducted using the Large-scale Atomic/Molecular Massively Parallel Simulator (LAMMPS) program (Lange et al., 2016). A detailed insight into PLGA “patchy particles”, which are particles made up of PLGA and lipid-polymer groups, was obtained from computational fluid dynamics, molecular dynamics and coarse grain simulations (Salvador-Morales et al., 2016). A study involving the simulation of PLGA-PEG co-polymer with the hydrophobic drug itraconazole, as shown in figure 2.13, provided information about the drug loading limitations of this system (Wilkosz et al., 2018). A molecular dynamics simulation of the peptide Melittin showed that it constituted a more stable formulation with PLGA than PLA (Asadzadeh & Moosavi, 2019). The level of these studies as well as the information they provide is summarized in table 2.

Another area of expansion of computational modeling on PLGA nano-systems is be the study of the transport of these nanocarriers in the circulatory system. One of the limitations of the clinical translation of nano-systems in general is the poor correlation between *in vitro* and *in vivo* results. The use of mathematical and computational methods to model the interactions between the drug, carrier, biological transport system and tumor vasculature can be used to gain insight into these complexities (Curtis, Wu, Lowengrub, Decuzzi, & Frieboes, 2015). Finite element analysis can be employed to model the dynamics of a nanoparticles within a channel, hence simulating transport in a blood vessel while continuum models can also be used to simulate nanoparticles in a vascular network generated by physical input parameters (Liu, Shah, & Tan, 2012).

While computational simulations can provide valuable information on the molecular interactions in various PLGA nanocarrier systems, they can be limited by computational cost and time intensive calculations (Ramezanpour et al., 2016). It has been proposed that a

minimum reporting standard be instituted where researchers are required to present their results with enough information to make it useful for in silico modelling and future work (Faria et al., 2018).

Computational modelling could be immensely useful once it reaches a level where it can be used to select or eliminate certain experimental variables before laboratory research is conducted. Currently, even though simulation time scales are appropriate for the modelling of several nano-systems, the detailed investigation of the formation of nanoparticles by new methods, for example microfluidics, is beyond the abilities of current computational technology. The majority of studies conducted thus far involve the modelling of individual systems; however, more data is needed so that we can move away from specific systems to create profiles to generalize these delivery systems for rational design. (Ramezanzpour et al., 2016)

Optimizing nano-formulations especially with PLGA polymers which have numerous possible combinations of lactic acid to glycolic acid ratio, molecular weight, endcaps and surface functionalization, could be very time consuming, expensive and in some cases not experimentally feasible. Since computational simulations give a molecular insight to macroscopic properties (Huynh et al., 2012), it could provide a platform to model these initial parameters in order to narrow down the possibilities in a specific study.

Table 2.2 Molecular simulations of PLGA and their significance to nanocarrier drug delivery properties

Simulation type	Cargo	Property modelled	Experimental validation	Information obtained	Reference
Density functional theory	Doxorubicin, daunorubicin	Physicochemical properties, binding energy	qualitative	Carrier-drug affinity, surface modification effects	(Rahimi, Bahlake, Chamani, & Bagheri, 2012)
Molecular dynamics	Curcumin, prednisolone, resveratrol	Binding energy	>85%	Carrier–drug affinity	(Metwally & Hathout, 2015)
Molecular dynamics	oxaliplatin	Density, Glass transition temperature	>96% >95%	Polymer degradation, drug release	(Lange et al., 2016)
Molecular dynamics, computational fluid dynamics, coarse grain	none	Shear stress, lipid-PLGA interaction energy,	qualitative	Loading capacity, release kinetics	(Salvador-Morales et al., 2016)
Finite element analysis	Rhodamine B	Diffusion, drug release	qualitative	Polymer degradation, drug release	(Milosevic et al., 2018)
Molecular dynamics	Itraconazole	Binding energy	qualitative	Drug loading ability of carrier	(Wilkoosz et al., 2018)
Molecular dynamcs	Melittin	Binding energy, radial distribution function	none	Protein-peptide interactions, encapsulation affinity	(Asadzadeh & Moosavi, 2019)

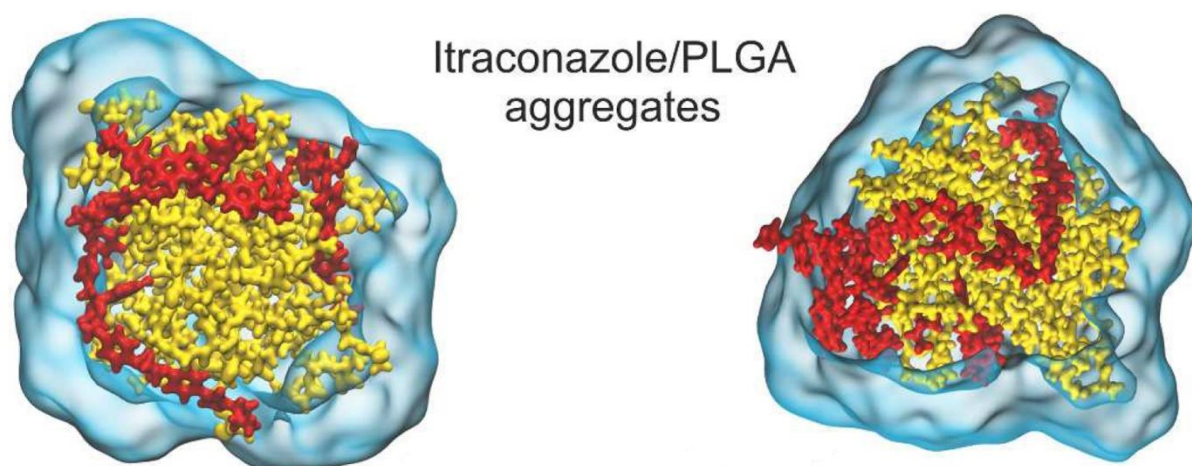


Figure 2.13 Molecular dynamics simulation showing (A) [left] 12% w/w and (B) [right] 24% itraconazole loading in a PLGA nanoparticle, adapted with permission from (Wilkosz et al., 2018). Copyright (2019) American Chemical Society.

2.6 Design Considerations

Several studies have demonstrated the increase in particle size and decrease in drug release rate with increase in molecular weight and lactide:glycolide ratios (Dinarvand et al., 2011; Song et al., 2008). Recently, Lu and colleagues designed a 75:25 lactide:glycolide PLGA nanocarrier for the sustained release of paclitaxel (Lu et al., 2019). Surface functionalization is a component that needs to factor in when designing nanocarriers. Gu and co-workers conducted a study that optimized the in-vitro release rate of docetaxel in PLGA, and additionally found that they could reduce the size of the nanoparticles from ~291nm to ~160nm by shortening the length of the PEG chains that were used for surface functionalization (Gu et al., 2008), while Bertrand and colleagues found that up to a point, increasing the density of PEG surface functionalization increased the blood circulation time of their nano-formulations (Bertrand et al., 2017). Gu's group also investigated an optimum targeting ligand density in order to provide maximum targeting ability without inhibiting the shielding effect of the PEG corona (Gu et al., 2008), while Lu's group found that increasing the density of the chitosan coating in their formulation increased the particle size from ~133nm to ~173nm (Lu et al., 2019).

Since the choice of fabrication methods and processes can determine the physicochemical characteristics of the resulting system, it is important to select an approach that is associated with the desired nanoparticle properties for the system of interest. For example Kim and co-workers reported that even though the preparation of their entacavir-loaded system by spray

drying produced larger particle size diameters compared to emulsion techniques, the spray dried system showed a much more favorable drug loading and release profiles and hence was the better performing delivery system (S. R. Kim, Ho, Choi, & Kang, 2019). Krishnamoorthy described a multi-criteria decision making approach to the synthesis of polymeric nanoparticles which concluded that nanoprecipitation would be the best suited preparation method for a camphothecin-loaded system (Krishnamoorthy & Mahalingam, 2015), and an adapted approach could be implemented in the selection of synthetic methods for specific PLGA-based systems.

2.7 Disadvantages of PLGA as a nanocarrier

Even though the versatility of PLGA makes it an attractive option as a nanocarrier, it does present several challenges in nanomedicine. PLGA co-polymers are usually readily commercially available, but to obtain it in a high purity, and the specificity required for different molecular weights, lactic/glycolic acid ratios and end capped options can make it very costly (Danhier et al., 2012). Many formulations show poor drug loading and therefore would require large doses in order to achieve therapeutic concentrations of cargo at the target site. Furthermore, these systems often exhibit burst release kinetics, which would result in off target *in vivo* delivery. The degradation rate of PLGA is often unpredictable and the acidic degradation products have shown to affect the activity of the encapsulated drug (S. Sharma et al., 2016), and despite its biodegradability, reports have shown that the use of PLGA in medical devices may produce localized reaction at the site of delivery (Makadia & Siegel, 2011). Even though targeted PLGA based carriers theoretically have more efficient site specific delivery properties, the targeting moieties in these nano-systems can induce additional immunogenicity (Danhier et al., 2010). There are various *in vivo* physiological barriers and up- and down-regulation of cell surface receptors and other targets can also decrease the efficacy of the targeting agents in these systems. The adaptability of PLGA as a polymer for its specific application has resulted in it being used in many delivery systems and therefore it is difficult to make comprehensive predictions at this stage about its general behavior and toxicity (S. Sharma et al., 2016).

2.8 Conclusions and future work

Even though PLGA is a polymer with many desirable features, there are various areas in which research can be conducted to improve the viability of PLGA based nanocarriers for clinical translation. Since PLGA has been developed in drug delivery systems for such a wide berth of applications, it should be precisely designed in terms of cargo suitability, particle size, drug entrapment and degradation kinetics, for its specific target. This would determine the choice of starting materials and in some cases, the method of preparation and would therefore

remove some of the uncertainty present in several trial-and-error attempts in previous drug delivery systems. The innovative fabrication techniques mentioned above could also be attempted to increase control over homogeneity of the products. The use of in silico modelling for PLGA nanoparticles as an element of experimental design and could have tremendous implications for the future of nanoparticle design.

2.9 References

- . Retrieved from <https://clinicaltrials.gov/ct2/results?cond=&term=plga&cntry=&state=&city=&dist=>
- Al-Jamal, K. T., Bai, J., Wang, J. T.-W., Protti, A., Southern, P., Bogart, L., . . . Asker, D. J. N. I. (2016). Magnetic drug targeting: preclinical in vivo studies, mathematical modeling, and extrapolation to humans. *16*(9), 5652-5660.
- Ali, A., & Ahmed, S. J. I. j. o. b. m. (2018). A review on chitosan and its nanocomposites in drug delivery. *109*, 273-286.
- Almoustafa, H. A., Alshawsh, M. A., & Chik, Z. J. I. j. o. p. (2017). Technical aspects of preparing PEG-PLGA nanoparticles as carrier for chemotherapeutic agents by nanoprecipitation method. *533*(1), 275-284.
- Alshamsan, A. J. S. P. J. (2014). Nanoprecipitation is more efficient than emulsion solvent evaporation method to encapsulate cucurbitacin I in PLGA nanoparticles. *22*(3), 219-222.
- Alvarez-Lorenzo, C., Rey-Rico, A., Sosnik, A., Taboada, P., & Concheiro, A. J. F. B. (2010). Poloxamine-based nanomaterials for drug delivery. *2*, 424-440.
- Ananta, J. S., Paulmurugan, R., & Massoud, T. F. J. N. r. (2016). Temozolomide-loaded PLGA nanoparticles to treat glioblastoma cells: a biophysical and cell culture evaluation. *38*(1), 51-59.
- Anderson, J. M., & Shive, M. S. J. A. d. d. r. (2012). Biodegradation and biocompatibility of PLA and PLGA microspheres. *64*, 72-82.
- Ansary, R. H., Awang, M. B., & Rahman, M. M. J. T. J. o. P. R. (2014). Biodegradable poly (D, L-lactic-co-glycolic acid)-based micro/nanoparticles for sustained release of protein drugs-A review. *13*(7), 1179-1190.
- Aravind, A., Nair, R., Raveendran, S., Veerananarayanan, S., Nagaoka, Y., Fukuda, T., . . . Materials, M. (2013). Aptamer conjugated paclitaxel and magnetic fluid loaded fluorescently tagged PLGA nanoparticles for targeted cancer therapy. *344*, 116-123.

Asadzadeh, H., & Moosavi, A. J. M. R. E. (2019). Investigation of the interactions between Melittin and the PLGA and PLA polymers: molecular dynamic simulation and binding free energy calculation. 6(5), 055318.

Ayyoob, M., & Kim, Y. J. P. (2018). Effect of Chemical Composition Variant and Oxygen Plasma Treatments on the Wettability of PLGA Thin Films, Synthesized by Direct Copolycondensation. 10(10), 1132.

Bauer, L. M., Situ, S. F., Griswold, M. A., & Samia, A. C. S. J. N. (2016). High-performance iron oxide nanoparticles for magnetic particle imaging-guided hyperthermia (hMPI). 8(24), 12162-12169.

Berthet, M., Gauthier, Y., Lacroix, C., Verrier, B., & Monge, C. J. T. i. b. (2017). Nanoparticle-based dressing: the future of wound treatment? , 35(8), 770-784.

Bertrand, N., Grenier, P., Mahmoudi, M., Lima, E. M., Appel, E. A., Dormont, F., . . . Farokhzad, O. C. J. N. c. (2017). Mechanistic understanding of in vivo protein corona formation on polymeric nanoparticles and impact on pharmacokinetics. 8(1), 777.

Besumbes, E. S., Fornaguera, C., Monge, M., García-Celma, M. J., Carrión, J., Solans, C., & Dols-Perez, A. J. E. P. J. (2019). PLGA cationic nanoparticles, obtained from nano-emulsion templating, as potential DNA vaccines. 109229.

Bisht, R., Jaiswal, J. K., Oliver, V. F., Eurtivong, C., Reynisson, J., & Rupenthal, I. D. (2017). Preparation and evaluation of PLGA nanoparticle-loaded biodegradable light-responsive injectable implants as a promising platform for intravitreal drug delivery. *Journal of Drug Delivery Science and Technology*, 40, 142-156. doi:<https://doi.org/10.1016/j.jddst.2017.06.006>

Bosch, F., & Rosich, L. J. P. (2008). The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. 82(3), 171-179.

Bruinsmann, F. A., Pigana, S., Aguirre, T., Dadalt Souto, G., Garrastazu Pereira, G., Bianchera, A., . . . Sonvico, F. (2019). Chitosan-Coated Nanoparticles: Effect of Chitosan Molecular Weight on Nasal Transmucosal Delivery. *Pharmaceutics*, 11(2), 86. doi:10.3390/pharmaceutics11020086

Carter, T., Mulholland, P., & Chester, K. (2016). Antibody-targeted nanoparticles for cancer treatment. *Immunotherapy*, 8(8), 941-958. doi:10.2217/imt.16.11

Chapman, S., Dobrovolskaia, M., Farahani, K., Goodwin, A., Joshi, A., Lee, H., . . . Rao, J. J. N. t. (2013). Nanoparticles for cancer imaging: The good, the bad, and the promise. *8*(5), 454-460.

Chen, J., Wu, Q., Luo, L., Wang, Y., Zhong, Y., Dai, H. B., . . . Wang, G. X. (2017). Dual tumor-targeted poly(lactic-co-glycolic acid)-polyethylene glycol-folic acid nanoparticles: a novel biodegradable nanocarrier for secure and efficient antitumor drug delivery. *International journal of nanomedicine*, *12*, 5745-5760. doi:10.2147/ijn.S136488

Chen, Q., Chen, J., Yang, Z., Xu, J., Xu, L., Liang, C., . . . Liu, Z. J. A. M. (2019). Nanoparticle-Enhanced Radiotherapy to Trigger Robust Cancer Immunotherapy. *31*(10), 1802228.

Chen, Q., Xu, L., Liang, C., Wang, C., Peng, R., & Liu, Z. (2016). Photothermal therapy with immune-adjuvant nanoparticles together with checkpoint blockade for effective cancer immunotherapy. *Nature Communications*, *7*(1), 13193. doi:10.1038/ncomms13193

Chen, Y., Yang, Z., Liu, C., Wang, C., Zhao, S., Yang, J., . . . Song, C. J. I. j. o. n. (2013). Synthesis, characterization, and evaluation of paclitaxel loaded in six-arm star-shaped poly(lactic-co-glycolic acid). *8*, 4315.

Chiesa, E., Dorati, R., Pisani, S., Conti, B., Bergamini, G., Modena, T., & Genta, I. (2018). The Microfluidic Technique and the Manufacturing of Polysaccharide Nanoparticles. *Pharmaceutics*, *10*(4), 267. doi:10.3390/pharmaceutics10040267

Choi, K. Y., Yoon, H. Y., Kim, J. H., Bae, S. M., Park, R. W., Kang, Y. M., . . . Park, J. H. (2011). Smart nanocarrier based on PEGylated hyaluronic acid for cancer therapy. *ACS Nano*, *5*(11), 8591-8599. doi:10.1021/nn202070n

Chopra, S., Bertrand, N., Lim, J. M., Wang, A., Farokhzad, O. C., & Karnik, R. (2017). Design of Insulin-Loaded Nanoparticles Enabled by Multistep Control of Nanoprecipitation and Zinc Chelation. *ACS Appl Mater Interfaces*, *9*(13), 11440-11450. doi:10.1021/acsami.6b16854

Chung, H. J., Kim, H. K., Yoon, J. J., & Park, T. G. (2006). Heparin Immobilized Porous PLGA Microspheres for Angiogenic Growth Factor Delivery. *Pharm Res*, *23*(8), 1835-1841. doi:10.1007/s11095-006-9039-9

Cohen-Sela, E., Chorny, M., Koroukhov, N., Danenberg, H. D., & Golomb, G. (2009). A new double emulsion solvent diffusion technique for encapsulating hydrophilic molecules in PLGA nanoparticles. *J Control Release*, *133*(2), 90-95. doi:10.1016/j.jconrel.2008.09.073

- Collins, D. J., Neild, A., deMello, A., Liu, A.-Q., & Ai, Y. (2015). The Poisson distribution and beyond: methods for microfluidic droplet production and single cell encapsulation. *Lab on a Chip*, 15(17), 3439-3459. doi:10.1039/C5LC00614G
- Coors, E. A., Seybold, H., Merk, H. F., Mahler, V. J. A. o. A., Asthma, & Immunology. (2005). Polysorbate 80 in medical products and nonimmunologic anaphylactoid reactions. 95(6), 593-599.
- Crucho, C. I. C., & Barros, M. T. (2017). Polymeric nanoparticles: A study on the preparation variables and characterization methods. *Mater Sci Eng C Mater Biol Appl*, 80, 771-784. doi:10.1016/j.msec.2017.06.004
- Curtis, L. T., Wu, M., Lowengrub, J., Decuzzi, P., & Frieboes, H. B. (2015). Computational Modeling of Tumor Response to Drug Release from Vasculature-Bound Nanoparticles. *PLoS one*, 10(12), e0144888-e0144888. doi:10.1371/journal.pone.0144888
- Danhier, F., Ansorena, E., Silva, J. M., Coco, R., Le Breton, A., & Préat, V. J. J. o. c. r. (2012). PLGA-based nanoparticles: an overview of biomedical applications. 161(2), 505-522.
- Danhier, F., Feron, O., & Préat, V. (2010). To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *Journal of Controlled Release*, 148(2), 135-146. doi:<https://doi.org/10.1016/j.jconrel.2010.08.027>
- Davoudi, Z., Peroutka-Bigus, N., Bellaire, B., Wannemuehler, M., Barrett, T. A., Narasimhan, B., & Wang, Q. (2018). Intestinal organoids containing poly(lactic-co-glycolic acid) nanoparticles for the treatment of inflammatory bowel diseases. *Journal of biomedical materials research. Part A*, 106(4), 876-886. doi:10.1002/jbm.a.36305
- Dinarvand, R., Sepehri, N., Manoochehri, S., Rouhani, H., & Atyabi, F. (2011). Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents. *International journal of nanomedicine*, 6, 877-895. doi:10.2147/IJN.S18905
- Ding, D., & Zhu, Q. (2018). Recent advances of PLGA micro/nanoparticles for the delivery of biomacromolecular therapeutics. *Mater Sci Eng C Mater Biol Appl*, 92, 1041-1060. doi:10.1016/j.msec.2017.12.036
- Du, J., Li, X.-Y., Hu, H., Xu, L., Yang, S.-P., & Li, F.-H. J. S. r. (2018). Preparation and Imaging Investigation of Dual-targeted C 3 F 8-filled PLGA Nanobubbles as a Novel Ultrasound Contrast Agent for Breast Cancer. 8(1), 3887.

Durán, V., Yasar, H., Becker, J., Thiyagarajan, D., Loretz, B., Kalinke, U., . . . Medicine. (2019). Preferential uptake of chitosan-coated PLGA nanoparticles by primary human antigen presenting cells. *21*, 102073.

Eley, J. G., Pujari, V. D., & McLane, J. (2004). Poly (lactide-co-glycolide) nanoparticles containing coumarin-6 for suppository delivery: in vitro release profile and in vivo tissue distribution. *Drug Deliv*, *11*(4), 255-261. doi:10.1080/10717540490467384

Engineer, C., Parikh, J., Raval, A. J. T. i. B., & Organs, A. (2011). Review on Hydrolytic Degradation Behavior of Biodegradable Polymers from Controlled Drug Delivery System. *25*(2).

Enlow, E. M., Luft, J. C., Napier, M. E., & DeSimone, J. M. (2011). Potent Engineered PLGA Nanoparticles by Virtue of Exceptionally High Chemotherapeutic Loadings. *Nano Letters*, *11*(2), 808-813. doi:10.1021/nl104117p

Faheem, A. M., Abdelkader, D., Osman, M. A., McCarron, P. A., & El-Gizawy, S. A. J. B. J. o. P. (2019). Oral Insulin Delivery in Diabetic Rats by PLGA Nanoparticles Combined with a Protease Inhibitor (N-Ethylmaleimide). *4*(1).

Friedman, A. D., Claypool, S. E., & Liu, R. (2013). The smart targeting of nanoparticles. *Current pharmaceutical design*, *19*(35), 6315-6329. doi:10.2174/13816128113199990375

Fu, X., Cai, J., Zhang, X., Li, W.-D., Ge, H., & Hu, Y. (2018). Top-down fabrication of shape-controlled, monodisperse nanoparticles for biomedical applications. *Advanced Drug Delivery Reviews*, *132*, 169-187. doi:<https://doi.org/10.1016/j.addr.2018.07.006>

Gajdova, M., Jakubovsky, J., Valky, J. J. F., & toxicology, c. (1993). Delayed effects of neonatal exposure to Tween 80 on female reproductive organs in rats. *31*(3), 183-190.

Ganipineni, L. P., Ucakar, B., Joudiou, N., Riva, R., Jérôme, C., Gallez, B., . . . Préat, V. J. J. o. d. t. (2019). Paclitaxel-loaded multifunctional nanoparticles for the targeted treatment of glioblastoma. *27*(5-6), 614-623.

Geerlings, P., De Proft, F., & Langenaeker, W. (2003). Conceptual density functional theory. *Chem Rev*, *103*(5), 1793-1873. doi:10.1021/cr990029p

Gendelman, H. E., Anantharam, V., Bronich, T., Ghaisas, S., Jin, H., Kanthasamy, A. G., . . . Mallapragada, S. K. (2015). Nanoneuromedicines for degenerative, inflammatory, and infectious nervous system diseases. *Nanomedicine*, *11*(3), 751-767. doi:10.1016/j.nano.2014.12.014

Gentile, P., Chiono, V., Carmagnola, I., & Hatton, P. J. I. j. o. m. s. (2014). An overview of poly (lactic-co-glycolic) acid (PLGA)-based biomaterials for bone tissue engineering. *15*(3), 3640-3659.

Gholizadeh, S., Kamps, J. A. A. M., Hennink, W. E., & Kok, R. J. (2018). PLGA-PEG nanoparticles for targeted delivery of the mTOR/PI3kinase inhibitor dactolisib to inflamed endothelium. *Int J Pharm*, *548*(2), 747-758. doi:<https://doi.org/10.1016/j.ijpharm.2017.10.032>

Gonzalez-Pizarro, R., Silva-Abreu, M., Calpena, A. C., Egea, M. A., Espina, M., & Garcia, M. L. (2018). Development of fluorometholone-loaded PLGA nanoparticles for treatment of inflammatory disorders of anterior and posterior segments of the eye. *Int J Pharm*, *547*(1-2), 338-346. doi:10.1016/j.ijpharm.2018.05.050

Grabowski, N., Hillaireau, H., Vergnaud-Gauduchon, J., Nicolas, V., Tsapis, N., Kerdine-Romer, S., & Fattal, E. (2016). Surface-Modified Biodegradable Nanoparticles' Impact on Cytotoxicity and Inflammation Response on a Co-Culture of Lung Epithelial Cells and Human-Like Macrophages. *J Biomed Nanotechnol*, *12*(1), 135-146. doi:10.1166/jbn.2016.2126

Grabowski, N., Hillaireau, H., Vergnaud, J., Tsapis, N., Pallardy, M., Kerdine-Romer, S., & Fattal, E. (2015). Surface coating mediates the toxicity of polymeric nanoparticles towards human-like macrophages. *Int J Pharm*, *482*(1-2), 75-83. doi:10.1016/j.ijpharm.2014.11.042

Gu, F., Zhang, L., Teply, B. A., Mann, N., Wang, A., Radovic-Moreno, A. F., . . . Farokhzad, O. C. (2008). Precise engineering of targeted nanoparticles by using self-assembled biointegrated block copolymers. *105*(7), 2586-2591. doi:10.1073/pnas.0711714105 %J Proceedings of the National Academy of Sciences

Guo, L., Chen, B., Liu, R., Xia, G., Wang, Y., Li, X., . . . Jiang, H. (2015). Biocompatibility Assessment of Polyethylene Glycol-Poly L-Lysine-Poly Lactic-Co-Glycolic Acid Nanoparticles In Vitro and In Vivo. *J Nanosci Nanotechnol*, *15*(5), 3710-3719. doi:10.1166/jnn.2015.9509

Guo, P., Huang, J., Zhao, Y., Martin, C. R., Zare, R. N., & Moses, M. A. (2018). Nanomaterial Preparation by Extrusion through Nanoporous Membranes. *Small*, *14*(18), e1703493. doi:10.1002/smll.201703493

Guo, X., Zhang, X., Ye, L., Zhang, Y., Ding, R., Hao, Y., . . . Zhang, Y. (2014). Inhalable microspheres embedding chitosan-coated PLGA nanoparticles for 2-methoxyestradiol. *J Drug Target*, *22*(5), 421-427. doi:10.3109/1061186x.2013.878944

Guo, Z. S. (2018). The 2018 Nobel Prize in medicine goes to cancer immunotherapy (editorial for BMC cancer). *BMC cancer*, *18*(1), 1086-1086. doi:10.1186/s12885-018-5020-3

Habraken, W., Wolke, J., Mikos, A., & Jansen, J. J. J. o. B. S., Polymer Edition. (2006). Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics. 17(9), 1057-1074.

Haggag, Y. A., Faheem, A. M., Tambuwala, M. M., Osman, M. A., El-Gizawy, S. A., O'Hagan, B., . . . McCarron, P. A. (2018). Effect of poly(ethylene glycol) content and formulation parameters on particulate properties and intraperitoneal delivery of insulin from PLGA nanoparticles prepared using the double-emulsion evaporation procedure. *Pharmaceutical Development and Technology*, 23(4), 370-381. doi:10.1080/10837450.2017.1295066

Heinz, H., Pramanik, C., Heinz, O., Ding, Y., Mishra, R. K., Marchon, D., . . . Ziolo, R. F. (2017). Nanoparticle decoration with surfactants: Molecular interactions, assembly, and applications. *Surface Science Reports*, 72(1), 1-58. doi:<https://doi.org/10.1016/j.surfrep.2017.02.001>

<https://clinicaltrials.gov/ct2/results?cond=&term=plga&cntry=&state=&city=&dist=>.

Huynh, L., Neale, C., Pomès, R., & Allen, C. (2012). Computational approaches to the rational design of nanoemulsions, polymeric micelles, and dendrimers for drug delivery. *Nanomedicine: Nanotechnology, Biology and Medicine*, 8(1), 20-36. doi:<https://doi.org/10.1016/j.nano.2011.05.006>

Jahan, S. T., Sadat, S. M. A., Walliser, M., & Haddadi, A. (2017). Targeted Therapeutic Nanoparticles: An Immense Promise to Fight against Cancer. *Journal of drug delivery*, 2017, 9090325-9090325. doi:10.1155/2017/9090325

Jain, A., Kunduru, K. R., Basu, A., Mizrahi, B., Domb, A. J., & Khan, W. (2016). Injectable formulations of poly(lactic acid) and its copolymers in clinical use. *Advanced Drug Delivery Reviews*, 107, 213-227. doi:<https://doi.org/10.1016/j.addr.2016.07.002>

Jalali, N., Moztaarzadeh, F., Mozafari, M., Asgari, S., Motevalian, M., Alhosseini, S. N. J. C., . . . Aspects, E. (2011). Surface modification of poly (lactide-co-glycolide) nanoparticles by d- α -tocopheryl polyethylene glycol 1000 succinate as potential carrier for the delivery of drugs to the brain. 392(1), 335-342.

Jana, S., Kumar Sen, K., & Gandhi, A. J. C. p. d. (2016). Alginate based nanocarriers for drug delivery applications. 22(22), 3399-3410.

Jeon, S. G., Cha, M.-Y., Kim, J.-i., Hwang, T. W., Kim, K. A., Kim, T. H., . . . Medicine. (2019). Vitamin D-binding protein-loaded PLGA nanoparticles suppress Alzheimer's disease-related pathology in 5XFAD mice. 17, 297-307.

- Jesus, S., Schmutz, M., Som, C., Borchard, G., Wick, P., & Borges, O. (2019). Hazard Assessment of Polymeric Nanobiomaterials for Drug Delivery: What Can We Learn From Literature So Far. 7(261). doi:10.3389/fbioe.2019.00261
- Jog, R., & Burgess, D. J. (2017). Pharmaceutical Amorphous Nanoparticles. *Journal of Pharmaceutical Sciences*, 106(1), 39-65. doi:<https://doi.org/10.1016/j.xphs.2016.09.014>
- Kakkar, A., Traverso, G., Farokhzad, O. C., Weissleder, R., & Langer, R. J. N. R. C. (2017). Evolution of macromolecular complexity in drug delivery systems. 1(8), 0063.
- Kamaly, N., Yameen, B., Wu, J., & Farokhzad, O. C. (2016). Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chem Rev*, 116(4), 2602-2663. doi:10.1021/acs.chemrev.5b00346
- Kapoor, D. N., Bhatia, A., Kaur, R., Sharma, R., Kaur, G., & Dhawan, S. (2015). PLGA: a unique polymer for drug delivery. *Ther Deliv*, 6(1), 41-58. doi:10.4155/tde.14.91
- Karra, N., & Benita, S. (2012). The ligand nanoparticle conjugation approach for targeted cancer therapy. *Curr Drug Metab*, 13(1), 22-41.
- Keles, H., Naylor, A., Clegg, F., Sammon, C. J. P. d., & stability. (2015). Investigation of factors influencing the hydrolytic degradation of single PLGA microparticles. 119, 228-241.
- Khalil, D. N., Smith, E. L., Brentjens, R. J., & Wolchok, J. D. (2016). The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nature Reviews Clinical Oncology*, 13, 273. doi:10.1038/nrclinonc.2016.25
- Khalil, N. M., do Nascimento, T. C., Casa, D. M., Dalmolin, L. F., de Mattos, A. C., Hoss, I., . . . Mainardes, R. M. (2013). Pharmacokinetics of curcumin-loaded PLGA and PLGA-PEG blend nanoparticles after oral administration in rats. *Colloids Surf B Biointerfaces*, 101, 353-360. doi:10.1016/j.colsurfb.2012.06.024
- Kim, K. T., Lee, J. Y., Kim, D. D., Yoon, I. S., & Cho, H. J. (2019). Recent Progress in the Development of Poly(lactic-co-glycolic acid)-Based Nanostructures for Cancer Imaging and Therapy. *Pharmaceutics*, 11(6). doi:10.3390/pharmaceutics11060280
- Kim, S. R., Ho, M. J., Choi, Y. W., & Kang, M. J. (2019). Improved Drug Loading and Sustained Release of Entecavir-loaded PLGA Microsphere Prepared by Spray Drying Technique. 40(4), 306-312. doi:10.1002/bkcs.11682
- Kohl, Y., Kaiser, C., Bost, W., Stracke, F., Fournelle, M., Wischke, C., . . . Lemor, R. (2011). Preparation and biological evaluation of multifunctional PLGA-nanoparticles designed for

photoacoustic imaging. *Nanomedicine: Nanotechnology, Biology and Medicine*, 7(2), 228-237. doi:<https://doi.org/10.1016/j.nano.2010.07.006>

Kojic, M., Milosevic, M., Simic, V., Stojanovic, D., & Uskokovic, P. J. J. S. S. C. M. (2017). A radial 1D finite element for drug release from drug loaded nanofibers. *11*, 82-93.

Koushik, K., & Kompella, U. B. (2004). Preparation of large porous deslorelin-PLGA microparticles with reduced residual solvent and cellular uptake using a supercritical carbon dioxide process. *Pharm Res*, 21(3), 524-535. doi:10.1023/B:PHAM.0000019308.25479.a4

Krishnamoorthy, K., & Mahalingam, M. (2015). Selection of a suitable method for the preparation of polymeric nanoparticles: multi-criteria decision making approach. *Advanced pharmaceutical bulletin*, 5(1), 57-67. doi:10.5681/apb.2015.008

Kumar, P., Van Treuren, T., Ranjan, A. P., Chaudhary, P., & Vishwanatha, J. K. J. N. (2019). In vivo imaging and biodistribution of near infrared dye loaded brain-metastatic-breast-cancer-cell-membrane coated polymeric nanoparticles. *30*(26), 265101.

Kumari, A., Yadav, S. K., Yadav, S. C. J. C., & biointerfaces, s. B. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *75*(1), 1-18.

Lai, P., Daeer, W., Löbenberg, R., Prenner, E. J. J. C., & Biointerfaces, S. B. (2014). Overview of the preparation of organic polymeric nanoparticles for drug delivery based on gelatine, chitosan, poly (d, l-lactide-co-glycolic acid) and polyalkylcyanoacrylate. *118*, 154-163.

Lamprecht, A., Ubrich, N., Yamamoto, H., Schafer, U., Takeuchi, H., Maincent, P., . . . Lehr, C. M. (2001). Biodegradable nanoparticles for targeted drug delivery in treatment of inflammatory bowel disease. *J Pharmacol Exp Ther*, 299(2), 775-781.

Lange, J., de Souza, F. G., Nele, M., Tavares, F. W., Segtovich, I. S. V., da Silva, G. C. Q., . . . Simulations. (2016). Molecular Dynamic Simulation of Oxaliplatin Diffusion in Poly (lactic acid-co-glycolic acid). Part A: Parameterization and Validation of the Force-Field CVFF. *25*(1), 45-62.

Li, S. J. J. o. B. M. R. A. O. J. o. T. S. f. B., The Japanese Society for Biomaterials,, & Biomaterials, T. A. S. f. (1999). Hydrolytic degradation characteristics of aliphatic polyesters derived from lactic and glycolic acids. *48*(3), 342-353.

Liang, R., Wang, J., Wu, X., Dong, L., Deng, R., Wang, K., . . . Zhu, J. (2013). Multifunctional biodegradable polymer nanoparticles with uniform sizes: generation and in vitro anti-melanoma activity. *Nanotechnology*, 24(45), 455302. doi:10.1088/0957-4484/24/45/455302

- Lima, I. A. d., Khalil, N. M., Tominaga, T. T., Lechanteur, A., Sarmento, B., & Mainardes, R. M. (2018). Mucoadhesive chitosan-coated PLGA nanoparticles for oral delivery of ferulic acid. *Artificial Cells, Nanomedicine, and Biotechnology*, 46(sup2), 993-1002. doi:10.1080/21691401.2018.1477788
- Lince, F., Marchisio, D. L., & Barresi, A. A. (2011). A comparative study for nanoparticle production with passive mixers via solvent-displacement: Use of CFD models for optimization and design. *Chemical Engineering and Processing: Process Intensification*, 50(4), 356-368. doi:<https://doi.org/10.1016/j.cep.2011.02.015>
- Liu, Y., Shah, S., & Tan, J. (2012). Computational Modeling of Nanoparticle Targeted Drug Delivery. *Reviews in Nanoscience and Nanotechnology*, 1(1), 66-83. doi:10.1166/rnn.2012.1014
- Lombardo, D., Kiselev, M. A., & Caccamo, M. T. J. J. o. N. (2019). Smart nanoparticles for drug delivery application: development of versatile nanocarrier platforms in biotechnology and nanomedicine. 2019.
- Lu, B., Lv, X., & Le, Y. (2019). Chitosan-Modified PLGA Nanoparticles for Control-Released Drug Delivery. *Polymers*, 11(2), 304. doi:10.3390/polym11020304
- Lü, J.-M., Wang, X., Marin-Muller, C., Wang, H., Lin, P. H., Yao, Q., & Chen, C. J. E. r. o. m. d. (2009). Current advances in research and clinical applications of PLGA-based nanotechnology. 9(4), 325-341.
- Mahapatro, A., & Singh, D. K. J. J. o. n. (2011). Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines. 9(1), 55.
- Makadia, H. K., & Siegel, S. J. J. P. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. 3(3), 1377-1397.
- Mandal, S., Prathipati, P. K., Belshan, M., & Destache, C. J. J. A. r. (2019). A potential long-acting bictegrovir loaded nano-drug delivery system for HIV-1 infection: A proof-of-concept study. 167, 83-88.
- Martins, C., Araújo, F., Gomes, M. J., Fernandes, C., Nunes, R., Li, W., . . . Biopharmaceutics. (2019). Using microfluidic platforms to develop CNS-targeted polymeric nanoparticles for HIV therapy. 138, 111-124.
- Martins, C., & Sarmento, B. (2020). Microfluidic Manufacturing of Multitargeted PLGA/PEG Nanoparticles for Delivery of Taxane Chemotherapeutics. *Methods Mol Biol*, 2059, 213-224. doi:10.1007/978-1-4939-9798-5_11

- Mehta, A. K., Yadav, K. S., & Sawant, K. K. (2007). Nimodipine loaded PLGA nanoparticles: formulation optimization using factorial design, characterization and in vitro evaluation. *Curr Drug Deliv*, 4(3), 185-193. doi:10.2174/156720107781023929
- Menon, J. U., Kona, S., Wadajkar, A. S., Desai, F., Vadla, A., & Nguyen, K. T. (2012). Effects of surfactants on the properties of PLGA nanoparticles. *Journal of biomedical materials research. Part A*, 100(8), 1998-2005. doi:10.1002/jbm.a.34040
- Metwally, A. A., & Hathout, R. M. J. M. p. (2015). Computer-assisted drug formulation design: novel approach in drug delivery. 12(8), 2800-2810.
- Middleton, J. C., & Tipton, A. J. J. B. (2000). Synthetic biodegradable polymers as orthopedic devices. 21(23), 2335-2346.
- Miller, S. C., & Drabik, B. R. J. I. j. o. p. (1984). Rheological properties of poloxamer vehicles. 18(3), 269-276.
- Milosevic, M., Stojanovic, D., Simic, V., Milicevic, B., Radisavljevic, A., Uskokovic, P., & Kojic, M. (2018). A Computational Model for Drug Release from PLGA Implant. *Materials (Basel)*, 11(12). doi:10.3390/ma11122416
- Mir, M., Ahmed, N., ur Rehman, A. J. C., & Biointerfaces, S. B. (2017). Recent applications of PLGA based nanostructures in drug delivery. 159, 217-231.
- Mishima, K. (2008). Biodegradable particle formation for drug and gene delivery using supercritical fluid and dense gas. *Advanced Drug Delivery Reviews*, 60(3), 411-432. doi:<https://doi.org/10.1016/j.addr.2007.02.003>
- Mishra, A., Singh, S. K., Dash, D., Aswal, V. K., Maiti, B., Misra, M., & Maiti, P. J. A. b. (2014). Self-assembled aliphatic chain extended polyurethane nanobiohybrids: Emerging hemocompatible biomaterials for sustained drug delivery. 10(5), 2133-2146.
- Mittal, G., Sahana, D. K., Bhardwaj, V., & Ravi Kumar, M. N. (2007). Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo. *J Control Release*, 119(1), 77-85. doi:10.1016/j.jconrel.2007.01.016
- Moku, G., Layek, B., Trautman, L., Putnam, S., Panyam, J., & Prabha, S. J. C. (2019). Improving Payload Capacity and Anti-Tumor Efficacy of Mesenchymal Stem Cells Using TAT Peptide Functionalized Polymeric Nanoparticles. 11(4), 491.
- Muhamad, N., Plengsuriyakarn, T., & Na-Bangchang, K. (2018). Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in

cancer therapy: a systematic review. *International journal of nanomedicine*, 13, 3921-3935. doi:10.2147/ijn.S165210

Nagavarma, B., Yadav, H. K., Ayaz, A., Vasudha, L., & Shivakumar, H. J. A. J. P. C. R. (2012). Different techniques for preparation of polymeric nanoparticles-a review. 5(3), 16-23.

Ospina-Villa, J. D., Gómez-Hoyos, C., Zuluaga-Gallego, R., & Triana-Chávez, O. J. J. o. m. m. (2019). Encapsulation of proteins from *Leishmania panamensis* into PLGA particles by a single emulsion-solvent evaporation method. 162, 1-7.

Owens, D. E., 3rd, & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *Int J Pharm*, 307(1), 93-102. doi:10.1016/j.ijpharm.2005.10.010

Panda, J., Satapathy, B. S., Majumder, S., Sarkar, R., Mukherjee, B., Tudu, B. J. J. o. M., & Materials, M. (2019). Engineered polymeric iron oxide nanoparticles as potential drug carrier for targeted delivery of docetaxel to breast cancer cells. 485, 165-173.

Panyam, J., & Labhasetwar, V. J. A. d. d. r. (2012). Biodegradable nanoparticles for drug and gene delivery to cells and tissue. 64, 61-71.

Patel, B. K., Parikh, R. H., & Patel, N. J. I. j. o. n. (2018). Targeted delivery of mannosylated-PLGA nanoparticles of antiretroviral drug to brain. 13(T-NANO 2014 Abstracts), 97.

Peng, P., Yang, K., Tong, G., & Ma, L. (2018). Polysaccharide Nanoparticles for Targeted Cancer Therapies. *Curr Drug Metab*, 19(9), 781-792. doi:10.2174/1389200219666180511153403

Pereira, A. d. S. B. F., Brito, G. A. d. C., Lima, M. L. d. S., Silva Júnior, A. A. d., Silva, E. D. S., de Rezende, A. A., . . . Araújo, A. A. d. (2018). Metformin Hydrochloride-Loaded PLGA Nanoparticle in Periodontal Disease Experimental Model Using Diabetic Rats. *International journal of molecular sciences*, 19(11), 3488. doi:10.3390/ijms19113488

Perry, J. L., Herlihy, K. P., Napier, M. E., & Desimone, J. M. (2011). PRINT: a novel platform toward shape and size specific nanoparticle theranostics. *Acc Chem Res*, 44(10), 990-998. doi:10.1021/ar2000315

Platel, A., Carpentier, R., Becart, E., Mordacq, G., Betbeder, D., & Nessler, F. (2016). Influence of the surface charge of PLGA nanoparticles on their in vitro genotoxicity, cytotoxicity, ROS production and endocytosis. *J Appl Toxicol*, 36(3), 434-444. doi:10.1002/jat.3247

Rahimi, F., Bahlake, A., Chamani, Z., & Bagheri, S. J. E. J. E. B. (2012). Theoretical Study on the Conjugation of PLGA and PLGA-PEG Carriers to Doxorubicin and Daunorubicin. 2, 2055-2060.

Ramezanzpour, M., Leung, S., Delgado-Magnero, K., Bashe, B., Thewalt, J., & Tieleman, D. J. B. e. B. A.-B. (2016). Computational and experimental approaches for investigating nanoparticle-based drug delivery systems. 1858(7), 1688-1709.

Redhead, H. M., Davis, S. S., & Illum, L. (2001). Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: in vitro characterisation and in vivo evaluation. *J Control Release*, 70(3), 353-363. doi:10.1016/s0168-3659(00)00367-9

Rezvantab, S., Drude, N. I., Moraveji, M. K., Güvener, N., Koons, E. K., Shi, Y., . . . Kiessling, F. (2018). PLGA-Based Nanoparticles in Cancer Treatment. *Frontiers in pharmacology*, 9, 1260-1260. doi:10.3389/fphar.2018.01260

Rizvi, S. A. A., & Saleh, A. M. (2018). Applications of nanoparticle systems in drug delivery technology. *Saudi Pharmaceutical Journal*, 26(1), 64-70. doi:<https://doi.org/10.1016/j.jsps.2017.10.012>

Rodriguez-Torres, M. d. P., Acosta-Torres, L. S., & Diaz-Torres, L. A. (2018). Heparin-Based Nanoparticles: An Overview of Their Applications %J Journal of Nanomaterials. 2018, 8. doi:10.1155/2018/9780489

Sadat Tabatabaei Mirakabad, F., Nejati-Koshki, K., Akbarzadeh, A., Yamchi, M. R., Milani, M., Zarghami, N., . . . Joo, S. W. (2014). PLGA-based nanoparticles as cancer drug delivery systems. *Asian Pac J Cancer Prev*, 15(2), 517-535. doi:10.7314/apjcp.2014.15.2.517

Sadeghi-Avalshahr, A., Nokhasteh, S., Molavi, A. M., Khorsand-Ghayeni, M., & Mahdavi-Shahri, M. (2017). Synthesis and characterization of collagen/PLGA biodegradable skin scaffold fibers. *Regenerative biomaterials*, 4(5), 309-314. doi:10.1093/rb/rbx026

Salmaso, S., & Caliceti, P. J. J. o. d. d. (2013). Stealth properties to improve therapeutic efficacy of drug nanocarriers. 2013.

Salvador-Morales, C., Brahmbhatt, B., Márquez-Miranda, V., Araya-Duran, I., Canan, J., Gonzalez-Nilo, F., . . . Zweit, J. (2016). Mechanistic Studies on the Self-Assembly of PLGA Patchy Particles and Their Potential Applications in Biomedical Imaging. *Langmuir*, 32(31), 7929-7942. doi:10.1021/acs.langmuir.6b02177

Sanchez-Gaytan, B. L., Fay, F., Lobatto, M. E., Tang, J., Ouimet, M., Kim, Y., . . . Mulder, W. J. M. (2015). HDL-Mimetic PLGA Nanoparticle To Target Atherosclerosis Plaque Macrophages. *Bioconjugate Chemistry*, 26(3), 443-451. doi:10.1021/bc500517k

Saravanakumar, K., Hu, X., Shanmugam, S., Chelliah, R., Sekar, P., Oh, D.-H., . . . biophysics. (2019). Enhanced cancer therapy with pH-dependent and aptamer functionalized doxorubicin loaded polymeric (poly D, L-lactic-co-glycolic acid) nanoparticles. 671, 143-151.

Schrurs, F., & Lison, D. (2012). Join the dialogue. *Nature Nanotechnology*, 7(9), 545-545. doi:10.1038/nnano.2012.150

Semete, B., Booyesen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., & Swai, H. S. (2010). In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. *Nanomedicine*, 6(5), 662-671. doi:10.1016/j.nano.2010.02.002

Semete, B., Booyesen, L., Lemmer, Y., Kalombo, L., Katata, L., Verschoor, J., . . . Medicine. (2010). In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. 6(5), 662-671.

Sequeira, J. A. D., Santos, A. C., Serra, J., Veiga, F., & Ribeiro, A. J. (2018). Chapter 10 - Poly(lactic-co-glycolic acid) (PLGA) matrix implants. In A. M. Grumezescu (Ed.), *Nanostructures for the Engineering of Cells, Tissues and Organs* (pp. 375-402): William Andrew Publishing.

Sharma, M., Sharma, S., Sharma, V., Sharma, K., Yadav, S. K., Dwivedi, P., . . . Rawat, A. K. S. (2017). Oleanolic-bioenhancer coloaded chitosan modified nanocarriers attenuate breast cancer cells by multimode mechanism and preserve female fertility. *Int J Biol Macromol*, 104(Pt A), 1345-1358. doi:10.1016/j.ijbiomac.2017.06.005

Sharma, S., Parmar, A., Kori, S., & Sandhir, R. (2016). PLGA-based nanoparticles: A new paradigm in biomedical applications. *TrAC Trends in Analytical Chemistry*, 80, 30-40. doi:<https://doi.org/10.1016/j.trac.2015.06.014>

Shembekar, N., Chaipan, C., Utharala, R., & Merten, C. A. (2016). Droplet-based microfluidics in drug discovery, transcriptomics and high-throughput molecular genetics. *Lab Chip*, 16(8), 1314-1331. doi:10.1039/c6lc00249h

Shen, X., Li, T., Chen, Z., Xie, X., Zhang, H., Feng, Y., . . . Wu, C. J. M. p. (2019). NIR-light-triggered anticancer strategy for dual-modality imaging-guided combination therapy via a bioinspired hybrid PLGA nanoplatfrom. 16(3), 1367-1384.

- Shkodra-Pula, B., Grune, C., Traeger, A., Vollrath, A., Schubert, S., Fischer, D., & Schubert, U. S. (2019). Effect of surfactant on the size and stability of PLGA nanoparticles encapsulating a protein kinase C inhibitor. *Int J Pharm*, 566, 756-764. doi:<https://doi.org/10.1016/j.ijpharm.2019.05.072>
- Siegel, R. L., Miller, K. D., & Jemal, A. (2019). Cancer statistics, 2019. *CA Cancer J Clin*, 69(1), 7-34. doi:10.3322/caac.21551
- Singh, A. P., Biswas, A., Shukla, A., & Maiti, P. (2019). Targeted therapy in chronic diseases using nanomaterial-based drug delivery vehicles. *Signal Transduction and Targeted Therapy*, 4(1), 33. doi:10.1038/s41392-019-0068-3
- Singh, N. K., Singh, S. K., Dash, D., Gonugunta, P., Misra, M., & Maiti, P. J. T. J. o. P. C. C. (2013). CNT Induced β -phase in polylactide: unique crystallization, biodegradation, and biocompatibility. *117*(19), 10163-10174.
- Singh, R. P., & Ramarao, P. (2013). Accumulated polymer degradation products as effector molecules in cytotoxicity of polymeric nanoparticles. *Toxicol Sci*, 136(1), 131-143. doi:10.1093/toxsci/kft179
- Soh, S. H., & Lee, L. Y. J. P. (2019). Microencapsulation and nanoencapsulation using supercritical fluid (SCF) techniques. *11*(1), 21.
- Song, X., Zhao, Y., Wu, W., Bi, Y., Cai, Z., Chen, Q., . . . Hou, S. (2008). PLGA nanoparticles simultaneously loaded with vincristine sulfate and verapamil hydrochloride: systematic study of particle size and drug entrapment efficiency. *Int J Pharm*, 350(1-2), 320-329. doi:10.1016/j.ijpharm.2007.08.034
- Streck, S., Clulow, A. J., Nielsen, H. M., Rades, T., Boyd, B. J., McDowell, A. J. J. o. c., & science, i. (2019). The distribution of cell-penetrating peptides on polymeric nanoparticles prepared using microfluidics and elucidated with small angle X-ray scattering. 555, 438-448.
- Strohbehn, G., Coman, D., Han, L., Ragheb, R. R. T., Fahmy, T. M., Huttner, A. J., . . . Zhou, J. (2015). Imaging the delivery of brain-penetrating PLGA nanoparticles in the brain using magnetic resonance. *Journal of Neuro-Oncology*, 121(3), 441-449. doi:10.1007/s11060-014-1658-0
- Su, Y. H., Chiang, P. C., Cheng, L. J., Lee, C. H., Swami, N. S., & Chou, C. F. (2015). High aspect ratio nanoimprinted grooves of poly(lactic-co-glycolic acid) control the length and direction of retraction fibers during fibroblast cell division. *Biointerphases*, 10(4), 041008. doi:10.1116/1.4936589

- Sun, X., Xu, C., Wu, G., Ye, Q., & Wang, C. J. P. (2017). Poly (lactic-co-glycolic acid): Applications and future prospects for periodontal tissue regeneration. 9(6), 189.
- Swider, E., Koshkina, O., Tel, J., Cruz, L. J., de Vries, I. J. M., & Srinivas, M. (2018). Customizing poly(lactic-co-glycolic acid) particles for biomedical applications. *Acta Biomaterialia*, 73, 38-51. doi:<https://doi.org/10.1016/j.actbio.2018.04.006>
- Trujillo-Nolasco, R. M., Morales-Avila, E., Ocampo-García, B. E., Ferro-Flores, G., Gibbens-Bandala, B. V., Escudero-Castellanos, A., . . . C, E. (2019). Preparation and in vitro evaluation of radiolabeled HA-PLGA nanoparticles as novel MTX delivery system for local treatment of rheumatoid arthritis. 103, 109766.
- Tulinska, J., Kazimirova, A., Kuricova, M., Barancokova, M., Liskova, A., Neubauerova, E., . . . Dusinska, M. (2015). Immunotoxicity and genotoxicity testing of PLGA-PEO nanoparticles in human blood cell model. *Nanotoxicology*, 9 Suppl 1, 33-43. doi:10.3109/17435390.2013.816798
- Turon, P., del Valle, L., Alemán, C., & Puiggali, J. J. A. S. (2017). Biodegradable and biocompatible systems based on hydroxyapatite nanoparticles. 7(1), 60.
- Verma, D., Gulati, N., Kaul, S., Mukherjee, S., & Nagaich, U. (2018). Protein Based Nanostructures for Drug Delivery. *Journal of pharmaceutics*, 2018, 9285854-9285854. doi:10.1155/2018/9285854
- Vey, E., Rodger, C., Booth, J., Claybourn, M., Miller, A. F., Saiani, A. J. P. d., & stability. (2011). Degradation kinetics of poly (lactic-co-glycolic) acid block copolymer cast films in phosphate buffer solution as revealed by infrared and Raman spectroscopies. 96(10), 1882-1889.
- Vhora, I., Patil, S., Bhatt, P., Gandhi, R., Baradia, D., & Misra, A. (2014). Receptor-targeted drug delivery: current perspective and challenges. *Ther Deliv*, 5(9), 1007-1024. doi:10.4155/tde.14.63
- Vllasaliu, D., Fowler, R., & Stolnik, S. (2014). PEGylated nanomedicines: recent progress and remaining concerns. *Expert Opin Drug Deliv*, 11(1), 139-154. doi:10.1517/17425247.2014.866651
- Wang, Y., Li, P., Truong-Dinh Tran, T., Zhang, J., & Kong, L. (2016). Manufacturing Techniques and Surface Engineering of Polymer Based Nanoparticles for Targeted Drug Delivery to Cancer. *Nanomaterials (Basel, Switzerland)*, 6(2), 26. doi:10.3390/nano6020026

- Wilkosz, N., Łazarski, G., Kovacik, L., Gargas, P., Nowakowska, M., Jamróz, D., & Kepczynski, M. J. T. J. o. P. C. B. (2018). Molecular insight into drug-loading capacity of PEG–PLGA nanoparticles for itraconazole. *122*(28), 7080-7090.
- Xi, J., Wang, W., Da, L., Zhang, J., Fan, L., Gao, L. J. A. B. S., & Engineering. (2018). Au-PLGA Hybrid Nanoparticles with Catalase-Mimicking and near-Infrared Photothermal Activities for Photoacoustic Imaging-Guided Cancer Therapy. *4*(3), 1083-1091.
- Xu, Q., Hashimoto, M., Dang, T. T., Hoare, T., Kohane, D. S., Whitesides, G. M., . . . Anderson, D. G. (2009). Preparation of monodisperse biodegradable polymer microparticles using a microfluidic flow-focusing device for controlled drug delivery. *Small*, *5*(13), 1575-1581. doi:10.1002/sml.200801855
- Yang, H., Li, J., Patel, S. K., Palmer, K. E., Devlin, B., & Rohan, L. C. J. P. (2019). Design of Poly (lactic-co-glycolic Acid)(PLGA) Nanoparticles for Vaginal Co-Delivery of Griffithsin and Dapivirine and Their Synergistic Effect for HIV Prophylaxis. *11*(4), 184.
- Yang, X. (2019). Design and optimization of crocetin loaded PLGA nanoparticles against diabetic nephropathy via suppression of inflammatory biomarkers: a formulation approach to preclinical study. *Drug Deliv*, *26*(1), 849-859. doi:10.1080/10717544.2019.1642417
- Yin, Q. (2019). Abstract B142: Activation of endogenous anergic self-specific CD8+ T-cells by polymeric nanoparticles for enhanced cancer immunotherapy. In: AACR.
- Yoo, J., Park, C., Yi, G., Lee, D., & Koo, H. (2019). Active Targeting Strategies Using Biological Ligands for Nanoparticle Drug Delivery Systems. *Cancers*, *11*(5), 640. doi:10.3390/cancers11050640
- Zhang, K., Tang, X., Zhang, J., Lu, W., Lin, X., Zhang, Y., . . . He, H. J. J. o. C. r. (2014). PEG–PLGA copolymers: Their structure and structure-influenced drug delivery applications. *183*, 77-86.
- Zhang, Z., & Feng, S.-S. (2006). The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)–tocopheryl polyethylene glycol succinate nanoparticles. *Biomaterials*, *27*(21), 4025-4033. doi:<https://doi.org/10.1016/j.biomaterials.2006.03.006>
- Zhao, P., Grabinski, T., Gao, C., Skinner, R. S., Giambernardi, T., Su, Y., . . . Cao, B. (2007). Identification of a Met-Binding Peptide from a Phage Display Library. *Clinical Cancer Research*, *13*(20), 6049. doi:10.1158/1078-0432.CCR-07-0035

Chapter 3 Comparative Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and Emulsification Solvent Evaporation Techniques



polymers



Article

Comparative Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and Emulsification Solvent Evaporation Techniques

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Special Issue

[Advances in Polymer-Based Materials and Fabrication Processes for Microfluidic Applications](#)

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Abstract

Poor circulation stability and inadequate cell membrane penetration are significant impediments in the implementation of nanocarriers as delivery systems for therapeutic agents with low bioavailability. This research discusses the fabrication of a biocompatible poly(lactide-co-glycolide) based nanocarrier with cationic and hydrophilic surface properties provided by natural polymer chitosan and coating polymer polyethylene glycol for the entrapment of the hydrophobic drug disulfiram. The traditional emulsification solvent evaporation method was compared to a microfluidics-based method of fabrication, with the optimisation of the parameters for each method, and the PEGylation densities on the experimental nanoparticle formulations were varied. The size and surface properties of the intermediates and products were characterised and compared by dynamic light scattering, scanning electron microscopy and X-ray diffraction, while the thermal properties were investigated using thermogravimetric analysis and differential scanning calorimetry. Results showed optimal particle properties with an intermediate PEG density and a positive surface charge for greater biocompatibility, with nanoparticle surface characteristics shielding physical interaction of the entrapped drug with the exterior. The formulations prepared using the microfluidic method displayed superior surface charge, entrapment and drug release properties. The final system shows potential as a component of a biocompatible nanocarrier for poorly soluble drugs.

3.1 Introduction

Nanostructured materials have demonstrated efficiency at improving bioavailability and reducing toxicity of bio-actives when implemented in the design of drug delivery systems. Synthetic polymeric nanocarriers are the simplest delivery systems because of their facile preparation, their ability to efficiently incorporate bio-actives within their matrices, and thereafter provide controlled release either by erosion or diffusion mechanisms (Senapati et al. 2018). In particular, biodegradable poly(lactic-co-glycolic) acid (PLGA) has been commonly used. PLGA can be prepared via various routes (Gentile et al. 2014) from the co-polymers glycolic acid and lactic acid, which is derived from green and renewable resources such as corn (Dahman 2012). It is a material well suited to be implemented in the design of synthetic polymeric nanocarriers due to its malleable structure that can be optimized by tuning the ratios of its constituent co-polymers to suit various applications (Makadia & Siegel 2011). However, the hydrophobicity of PLGA activates the reticulo-endothelial system (RES), which is responsible for elimination of administered nanocarriers by opsonin proteins (Rezvantlab et al. 2018). Hence a hydrophilic interface is critical in order to decrease this elimination (called opsonisation) (Suk et al. 2016).

Naturally occurring polymers such as chitosan can mimic the extracellular matrix of biological systems (Kravanja et al. 2019; Villemin et al. 2019; Wang et al. 2020). Chitosan is a cationic polymer, formed by the deacetylation of chitin from the exoskeletons of crustaceans. When used as a coating in the design of nano-systems, it confers numerous benefits such as stealth, muco-adhesion, increased cell and tissue penetration, controlled drug release and increase in bioavailability and efficacy of the drug load (Frank et al. 2020). Chitosan is biodegradable, biocompatible, non-immunogenic, and has displayed anti-tumoural, antimicrobial and antibacterial activity. It is the second most renewable biomaterial after cellulose (Kravanja et al. 2019) and is approved by the FDA for use in biomedical systems (Wang et al. 2020).

In the design of advanced nanocarriers there are generally two main regions of the nanoparticle structure that influences the biocompatibility, i.e. the nanoparticle core and the corona (Jokerst et al. 2011). The core is considered to have no interaction with circulatory elements while the corona (typically 10 nm of the particle surface), exhibits properties that determines the response of the nanoparticles to the RES. RES-associated proteins (opsonins) bind efficiently to the surfaces of hydrophobic particles, and removes them from circulation, resulting in their elimination in the liver or spleen (Jokerst et al. 2011). Studies have shown that this can be circumvented by coating such nanoparticles with chitosan (Amoozgar et al. 2012; Abouelmagd, Ku & Yeo 2015). Low molecular weight chitosan serves as a hydrophilic coating and provides a degree of stealth to PLGA nanoparticles (Amoozgar et al. 2012). A further increase in the degree of biocompatibility can be achieved by coating with polyethylene glycol (PEG) which is widely used for the surface modification of amphiphilic nanoparticles due to the strong hydrophilicity provided by the repeating glycol units within the PEG structural backbone (Suk et al. 2016). Furthermore, previous studies have proven that PEG provides stealth properties by non-specific interactions with circulatory proteins (Wu et al. 2014; Guerrini, Alvarez-Puebla & Pazos-Perez 2018; Zhou et al. 2018).

PEG chains also introduce a degree of steric repulsion which further protects the nano-system from the RES (Parveen & Sahoo 2011). Hereby, PEGylation provides an anti-fouling (or shielding) effect, and increases the nanoparticle circulation time. In passively targeted nano-systems for chemotherapy, this increased residence time can enable the system to access the enhanced permeation and retention (EPR) effect at the tumour site. The EPR effect takes advantage of the 'leaky vasculature' of tumour cells which have fenestrations >400 nm, allowing for preferential nanoparticle penetration with typically impaired tumoural lymphatic

drainage resulting in nanoparticle accumulation within the cancerous cells rather than their escape back into circulation (Suk et al. 2016). However, there is no current consensus on the optimal surface PEGylation density of nanoparticles that would induce and sustain favourable surface properties. Studies have shown that the structure of PEG occurs in two main conformations i.e. mushroom and brush (Cruje & Chithrani 2014) (Figure 3.1) with freedom of conformational mobility of PEG chains from attachment point on the core to the surface of nanoparticles in the brush conformation, while there is more lateral interaction in the mushroom configuration (Suk et al. 2016).

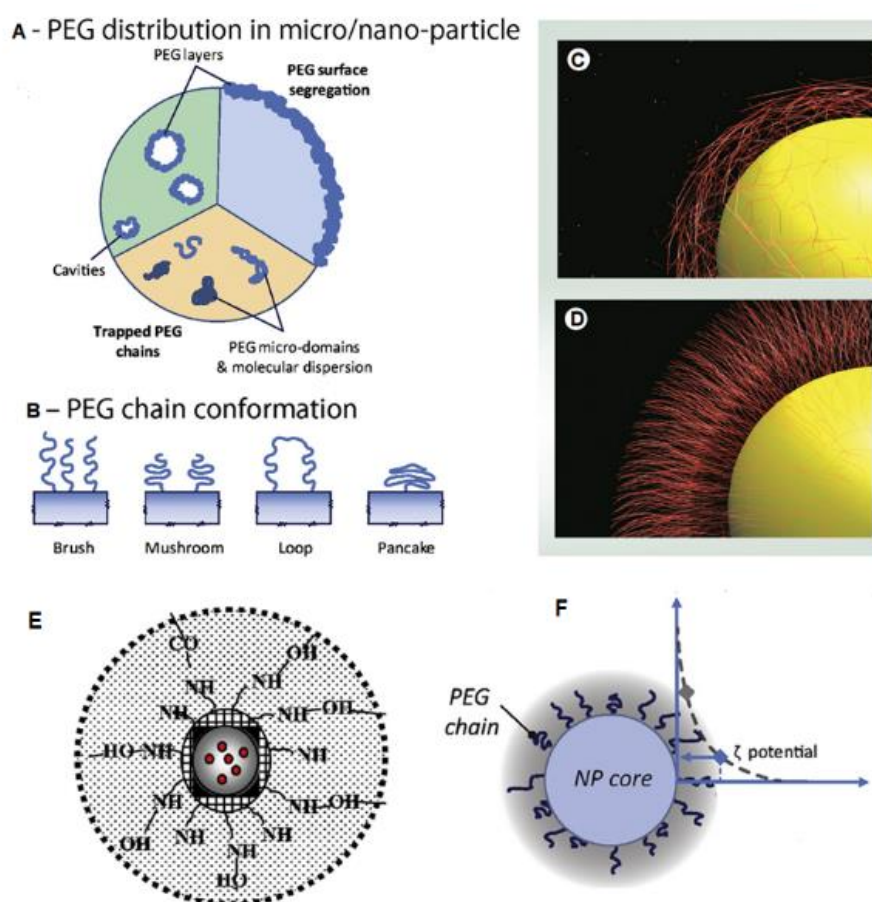


Figure 3.1 Image representing different arrangements of PEG molecules in a nanoparticle. Adapted with permission from (Rabanel, Hildgen & Banquy 2014) (B) - Different conformations on nanoparticle surface. Representations of the mushroom (C) and brush (D) conformations, showing surface densities, reproduced from (Jokerst et al. 2011) with permission from Nanomedicine as agreed by Future Medicine Ltd. (E) - schematic representation of the PLGA-chitosan-PEG nanoparticles prepared in this study. Reproduced with permission from (Parveen & Sahoo 2011). (F) - Nanoparticle surface showing detection of surface charge and PEG chains in both mushroom and brush conformations. Adapted with permission from (Rabanel et al. 2014).

In order to fully exploit the individual properties of PLGA, chitosan and PEG for the design of an optimised nano-enabled drug delivery system, the particle size, dispersity, surface charge and structural organisation need to be precisely controlled. These characteristics are influenced in part by the preparation methods used, and we have discussed previously how fabrication techniques can determine the overall functionality of these nano-systems (Essa et al. 2020), even though the majority of PLGA-based nanoparticles are prepared via emulsion methods (Wang et al. 2016). A less explored approach (but one with significant benefit) is the use of microfluidics for specialised nanoparticle synthesis (Chiesa et al. 2018). Microfluidics is a relatively recently developed method that uses microchannel technology to blend mix phases under constant laminar flow, which cannot be performed using solvent emulsion. This approach has been previously reported to produce nanoparticles with more precise uniformity in particle size and dispersity and with a high degree of reproducibility (Chiesa et al. 2018). In addition, microfluidics is a scalable technology (Kim et al. 2019) and therefore has promising applications in specialized drug delivery.

In this study, we compared two methods for the synthesis of PLGA-chitosan-PEG nanoparticles to encapsulate the model hydrophobic drug disulfiram (DSF) which is used clinically for the treatment of alcohol abuse (Sauna, Shukla & Ambudkar 2005) and, more recently, has been explored as a potential treatment for several types of cancer (Ekinici et al. 2019). However, when tested as a chemotherapeutic, it has shown systemic instability (He et al. 2018) resulting in poor bioavailability. In this study, we prepared nano-systems incorporating PLGA as a hydrophobic core to encourage favourable interactions with disulfiram, cationic chitosan for preferential cellular penetration, and PEG as an outer layer for stealth properties. Microfluidics was used to provide reproducible nanoparticle synthesis with precisely controlled parameters (low variation) and was compared to a conventional emulsification method that typically displays large batch to batch variation (Ding & Zhu 2018). In addition, previous PLGA-based nano-systems prepared using microfluidics have explored only one or two polymer components (Donno et al. 2017; Streck et al. 2019). However, in this study a novel approach using a benchtop NanoAssemblr™ was used to attempt the synthesis using three polymers and a surfactant. The nanoparticles fabricated by both methods were characterised based on size, surface properties, thermal and molecular profiles.

3.2 Materials and Methods

3.2.1 Materials

Polymers, namely poly(*D,L*-lactide-co-glycolide) (PLGA) (lactide/glycolide ratio of 50:50, Mw= 7000-17 000 Da with acidic end groups, low molecular weight chitosan (CHT), polyvinyl alcohol (PVA) (Mw=13000-20 000 Da), disulfiram (model drug), dialysis tubing (MWCO 14 KDa), PBS buffer tablets (pH 7.2), tween80 and mannitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyethylene glycol (PEG2000), glacial acetic acid, acetone, chloroform, dichloromethane, and acetonitrile were purchased from Merck (Pty) Ltd., (Estate South, Modderfontein, South Africa). All reagents were of analytical grade.

3.2.2 Preparation of Nanoparticles by the Emulsification Solvent Evaporation Approach

Various nanoparticle formulations were prepared using a single-emulsion oil in water (O/W) solvent evaporation approach (Parveen & Sahoo 2011). Four initial formulation sets were prepared varying the solvent, surfactant concentration and sonication parameters, with an unmodified, unloaded PLGA formulation, an unmodified PLGA formulation loaded with disulfiram, a drug loaded PLGA formulation coated with chitosan (CHT) and a drug loaded PLGA formulation coated with chitosan and PEG.

For each CHT-based formulation, 25 mg of CHT was agitated in 1% (v/v) glacial acetic acid to produce a 0.25% (w/v) concentration as the aqueous phase. The PEG-based formulations were prepared using 2% PEG and the PVA-based formulations were prepared using either 0.5, 1 or 2% PVA. These constituted the aqueous phase and were prepared prior to the addition of the organic phase. The organic phase in each case was prepared by dissolving 100 mg of PLGA in 1 mL of either acetone, chloroform or dichloromethane and added dropwise under stirring to the aqueous phase. For the DSF-loaded formulations, 10 mg of DSF was added to the organic phase before addition to the aqueous phase. These concentrations and ratios were optimal for PLGA and DSF as previously reported (Madala et al. 2018). The emulsion was left to agitate under magnetic stirring for 8 hours to allow complete evaporation of the organic solvent.

For the sonicated formulations, the emulsion was sonicated for 2 minutes using a probe sonicator at 130 W with 40% amplitude. (Sonics Vibra Cell, Newtown, CT, USA) before the evaporation step. Thereafter the formulation was centrifuged (Universal 320, Hettich Lab Tech., Tuttlingen, Germany) for 30 minutes at 5000 rpm at 4 °C and washed thrice with distilled water to remove any untrapped drug. The resultant pellets were redispersed in 4 mL of distilled water and then sonicated for 10 minutes to produce free-flowing nanoparticles that were frozen at -80 °C and then lyophilized (Freezone 12 lyophilizer, Labcono, Kansas City,

USA) for 24 hours. A portion of sample of each formulation was mixed with 5% (w/v) mannitol to observe the effect of cryopreservation.

3.2.3 . Preparation of Nanoparticles Using the Microfluidics Approach

For the microfluidics approach, polymeric nanoparticles were prepared using a benchtop Nanoassemblr (Precision Nanosystems Inc., Canada). The defining characteristics of the nanoparticles produced by this approach were compared to those of the emulsification solvent evaporation method. The Nanoassemblr utilizes microchannels in a cartridge that allows for the mixing of organic and aqueous phase in precisely controlled speeds and ratios under laminar flow. The organic phase comprised 10 mg/mL PLGA and 1 mg/mL disulfiram dissolved in acetonitrile using the favourable ratio of polymer to drug (10:1) described earlier, and a concentration of organic phase polymer which was shown to produce relatively low particle size using this instrument (Garg et al. 2016).

Formulations were prepared using CHT (0.25% w/v), PEG (2% w/v) and PVA (2% w/v) in the aqueous phase. The particle size and stability were compared using flow rate ratios of 1:1, 1:3 and 1:5 and flow rates of 8, 12 and 15 mLmin⁻¹. Thereafter, DSF-loaded and native nanoparticles were prepared using CHT (0.25% w/v) and PEG (2% w/v) and loaded nanoparticles were prepared with 0.25% (w/v) chitosan only, and 0.25% (w/v) chitosan with 4%, 6% and 8% (w/v) PEG, using a flow rate of 8 mLmin⁻¹. Materials were weighed and dissolved before the fabrication process. Using 1 mL luer lock syringes, 300 µL of PLGA solution and 800 µL of aqueous phase (consisting of chitosan, PEG and PVA in varying ratios as described above), were loaded into the NanoAssemblr benchtop. Nanoparticles were formed using a flow rate of 8 mLmin⁻¹, and the suspensions were collected using 15 mL falcon tubes. The downstream processing protocol of centrifugation, washing, and lyophilization was followed as per the emulsification solvent evaporation method.

3.2.4 Determination of Particle Size and Zeta Potential

The nanoparticles were dissolved (1 mg of each formulation in 1 mL of Millipore water) and analysed using dynamic light scattering and photon correlation spectroscopy at a fixed angle of 90° on a ZetaSizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) particle size analyzer. Samples were diluted and sonicated before measurement, with the sample temperature maintained at 25 °C throughout the analyses. The particle size, polydispersity index and zeta potential values were used to screen formulations and select the most favourable preparation parameters such as the organic solvent, the concentration of stabilizer

and sonication for the emulsification solvent evaporation approach. The flow rate ratio and total flow rate was used to screen formulations prepared by the microfluidics-based approach using the NanoAssemblr.

3.2.5 Determination of the Chemical and Structural Integrity of the Nano-systems

The native polymers and different nanoparticle formulations were subjected to Fourier Transform Infra-Red (FTIR) analysis to assess the chemical integrity and stability of nanoparticles produced from the two approaches. Characteristic peaks were compared to confirm the molecular vibrations of the polymers in the formulations. The FTIR spectra were recorded using a PerkinElmer (Waltham, MA, USA) spectrometer equipped with a single reflection diamond MIRTGS detector. Samples were processed by a universal attenuated total reflectance (ATR) polarization accessory at a resolution of 4 cm^{-1} with a constant pressure of 110 psi. The chemical structures of each nanoparticle formulation prepared by either the emulsification solvent evaporation and microfluidic methods were compared.

3.2.6 Investigation of the Thermal Properties of the Nano-systems

In order to characterize the thermal properties of the nanoparticles, thermogravimetric analysis (TGA) was undertaken using a TG analyser (PerkinElmer, TGA 4000, Llantrisant, Wales, UK), whereby the temperature range at which the formulation samples degraded were determined. Native DSF and DSF-loaded nanoparticle samples were exposed to $30\text{ }^{\circ}\text{C}$ and then heated at a rate of $10\text{ to }800\text{ }^{\circ}\text{Cmin}^{-1}$. The samples were maintained in an inert environment by constant nitrogen purging throughout the experiment. Thermograms were plotted as percentage mass against temperature. The phase transitions of the fabricated loaded and blank nanoparticles and native polymers were investigated using a differential scanning calorimeter (DSC) (Mettler Toledo, DSC, STAReSystem, Schwerzenback, ZH, Switzerland). These analyses provide insight about the thermally induced phase changes that the samples undergo, and hence characterizes the variation in thermal and physical properties with change in the constituent materials of the samples. The nanoparticles and drug samples of $\sim 5\text{ mg}$ were weighed into aluminium crucibles which were sealed and then heated over a temperature range of $0\text{ to }300\text{ }^{\circ}\text{C}$, at a heating rate of $10\text{ }^{\circ}\text{Cmin}^{-1}$. The samples were maintained in an inert N_2 gas atmosphere. Calorimetry spectra were recorded as a function of heat flow against temperature.

3.2.7 Determination of the Crystallinity of the Nano-systems

Lyophilized DSF-loaded samples and the native polymers were finely milled. PVA crystals were dissolved in distilled water and dried to form a film. Each sample was finely packed and smoothed into a sample holder for analysis using a benchtop MiniFlex 600 (Rigaku, Japan) powder X-ray diffractometer. CuK α radiation at 40 kV and 15 mA was employed and data was recorded using a 2 θ scan range of 10 - 60° at a scan rate of 10 °min⁻¹. Powder X-Ray Diffractometry (X-RD) analyses indicate the degree of crystallinity and amorphous nature of the polymers and nanoparticles and hence provides insight on their surface properties and behaviour.

3.2.8 Investigation of Surface Morphology Using Scanning Electron Microscopy (SEM)

Lyophilized samples were re-dispersed in distilled water and a drop of each sample was placed on aluminium stubs and dried for 48 hours. Solid samples were mounted on double sided carbon tape. In order to induce electrical conduction, samples were coated in a vacuum with a fine layer of gold using a sputter coater. Each sample was then analysed on a ZEISS SIGMA 03-39 Field Emission Scanning Electron Microscope at 5 – 15 kV acceleration voltage under an argon atmosphere.

3.2.9 UV Spectrophotometric Analysis to Determine Entrapment Efficiency of Disulfiram

Due to the hydrophobic nature of disulfiram a UV spectrophotometric method of analysis was adapted from (Nikhil P. Kothawade & Suvarna A. Katti 2016). Briefly, disulfiram standards were prepared using a ratio of 1:1 PBS:methanol, hereafter referred to as specialized buffer matrix (SBM). A linear calibration curve was constructed using the Nanophotometer UV/Vis spectrophotometer NP80 (Implen, Germany). 2 μ L of sample was pipetted onto the nanophotometer head and the absorbance was read at 216 nm. A combination approach was developed to extract the drug into a medium that would allow for analysis. 5 mg of nanoparticles were added to 2 mL of SBM. The mixture was then stirred at 37 °C for 48 hours using an orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd, Hahistradrut, Holon, Israel) at 50 rpm. The resultant solutions were centrifuged at 4500 rpm for 10 minutes and the supernatant analysed by UV spectrophotometry for disulfiram content. Entrapment

efficiencies of each formulation was calculated from the absorbance and were based on the initial mass of disulfiram used in the fabrication method, as shown by equations 1 and 2 below.

$$DL = \text{mass of disulfiram} / \text{mass of nanoparticles} \dots\dots \text{Equation 3.1}$$

$$EE = \frac{DL \times \text{mass of nanoparticles}}{\text{mass of disulfiram initially added}} \times 100\% \dots\dots\dots \text{Equation 3.2}$$

3.2.10 In vitro Disulfiram Release Studies

In vitro release of DSF from the nanoparticle formulations were determined using a dialysis tubing method[30]. The tests were performed in PBS (pH 7.2; 37 °C). For the comparative release study between nanoparticles produced from the emulsification solvent evaporation and microfluidic approaches, 5 mg of both formulations and pure DSF were suspended in 2 mL of PBS and transferred into the dialysis tubing immersed in 50 mL of outer medium containing 0.5% (v/v) tween80 and agitated for 72 hours at 37 °C. At predetermined time intervals (15 minutes, 30 minutes as well as 1, 2, 4, 8, 12, 24, 48 and 72 hours), 1 mL of the external release medium was withdrawn and replaced with an equal volume of drug-free medium to maintain sink conditions. Parameters were kept constant for all formulations assessed and samples were filtered prior to DSF analysis at 216 nm.

3.2.11 Statistical Analysis

Data for preparation and characterization of nanoparticles and drug release studies were processed and analyzed by Origin software (version 8.5.0 SR1, OriginLab Corporation, U.S.). All results were represented by the mean $\pm$ standard error of three experiments.

3.3 Results and Discussion

3.3.1 Size and Stability Analysis of Formulations Prepared by Both Methods

Even though the abnormal tumour vasculature allows the penetration of particles <400 nm in size (Bamrungsap et al. 2012), particles >200 nm have been shown to accumulate in the liver or spleen (Blanco, Shen & Ferrari 2015). Therefore, both methods were used with the ultimate goal of fabricating nanoparticles <200 nm in size, with a dispersity index of ~0.2.

3.3.1.1. Dichloromethane as the Solvent of Choice in the Emulsification Method

Solvent evaporation using acetone in the presence of no stabilizing agent were the parameters initially used for the preparation of the nanoparticles. While this method produced nanoparticles in a desired size range for the unloaded and disulfiram loaded PLGA nanoparticles, as previously reported (Madala et al. 2018), the sizes and dispersity were erratic once the complexity of the formulations increased to include chitosan and PEG, and even when different concentrations of stabilizer were added, the size and dispersity did not improve, as shown by Figure 3.2 below, and Table 1S1 in Appendix 1.

When chloroform, dichloromethane and sonication were used, the best formulation (with a size of 221 nm and a PDI of 0.028) was obtained with 1 %(w/v) PVA, sonication time of 2 minutes, and dichloromethane as the organic phase, as shown in Figure 3.4. For a full list of results, refer to Table 1S1 in Appendix 1.

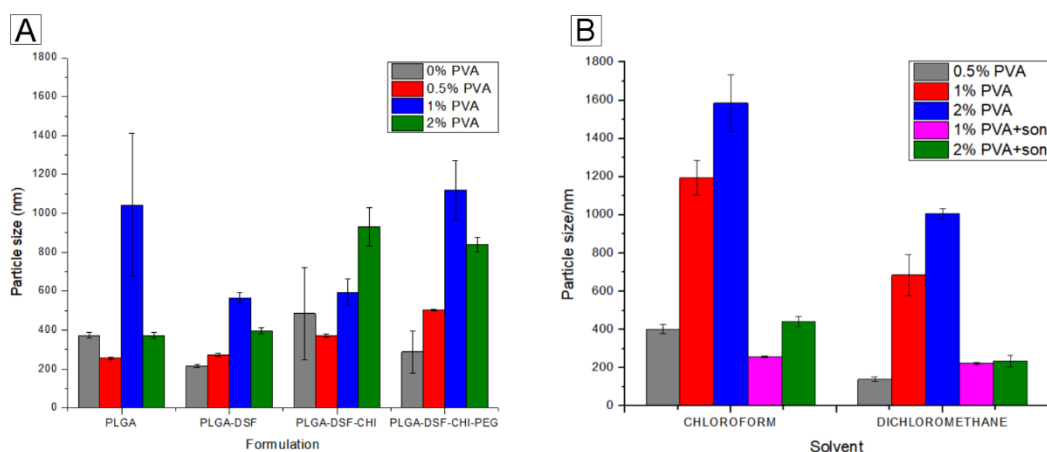


Figure 3.2 A) - Mean particle size obtained for formulations using acetone as the organic phase, and different concentrations of PVA in the aqueous phase. (B) - Mean particle size obtained for formulations containing PLGA, chitosan and PEG with either chloroform or

3.3.1.2 Microfluidics-Prepared Formulations

The parameters were optimized using an aqueous phase of 2% (w/v) PVA, 2% (w/v) PEG and 0.25% (w/v) chitosan, and an organic phase of 10 mg/mL PLGA, with a PLGA:DSF ratio of 10:1 in acetonitrile. The best formulation (with a size of 218 nm and PDI of 0.165) was obtained using a flow rate ratio of 3:1 aqueous:organic phase and a total flow rate of 8 mL per minute, as shown by Table 1S2 in Appendix 1.

3.3.1.3 Analysis of Emulsification Solvent Evaporation and Microfluidics Data Showing Dependence of Properties on PEG Surface Density

The emulsification solvent evaporation formulations were prepared using dichloromethane as a solvent, 1% (w/v) PVA and sonication time of 2 minutes. The microfluidic formulations were prepared using a flow rate of 3:1 and a total flow rate of 8 mLmin⁻¹. In this method, the mixing of the two phases were automated and controlled by the machine and hence the results obtained were highly reproducible. The uniformity of the dispersions was much better compared to the solvent emulsions method across all formulations as shown by the relatively low PDI values (0.191-0.258) and size disparity (218-330 nm) of the microfluidic preparations in Table 1S2, compared to the large variation in size and dispersity of the solvent emulsion prepared formulations in Table 1S1. The microfluidics-prepared formulations also required fewer experiments before acceptable results were achieved.

Both emulsification solvent evaporation and microfluidic prepared formulations showed optimal properties when prepared with 6% (w/v) PEG in the aqueous phase, as shown by Table 3.1 below and Figure 1S1(A) and (B) in Appendix 1. In the solvent emulsion method, the formulation with 4% (w/v) PEG displayed similar characteristics as that of the 6% (w/v) PEG. In formulations prepared by both methods, the uncoated PLGA nanoparticles have negative zeta potentials. The emulsification solvent evaporation uncoated nanoparticles display a larger negative surface charge, which is due to the larger relative amount of PLGA used in this formulation. When coated with chitosan, there is an increase in surface charge of both formulations, while there is an initial decrease when PEG (2% (w/v)) is added, then an increase until the PEG concentration reaches 6% (w/v), and a decrease once again when the PEG concentration is increased to 8% (w/v). The entrapment efficiencies of both formulations are high with PEG concentrations of 4 and 6% (w/v) and peak when the concentration of PEG is 6% (w/v). The entrapment efficiency of the PEG 6% formulations prepared by the microfluidics method (78.7%) was 8.4% higher than those of the solvent evaporation method (72.6%).

Table 3.1 Size, dispersity, surface charge and entrapment efficiency of bulk and microfluidic prepared formulations with varying amounts of PEG coatings.

Formulation	CHI /%	PEG /%	PSD /nm	PDI	ζpotential /mV	Entrapment Efficiency/%
Bulk	0	0	278±1.0	0.100±0.033	-17.2±0.2	-
	0.25	0	245±2.4	0.156±0.000	14.0±0.9	-
	0.25	2	221±4.1	0.028±0.020	3.09±0.2	16.5±1.0
	0.25	4	143±3.7	0.183±0.013	20.3±0.9	72.1±0.9

	0.25	6	184±2.6	0.189±0.011	21.3±3.5	72.6±0.2
	0.25	8	162±10	0.155±0.037	11.5±1.4	26.5±0.4
Microfluidic	0	0	95.8±4.6	0.303±0.031	-6.92±0.7	-
	0.25	0	235±1.9	0.292±0.002	14.9±1.0	-
	0.25	2	171±14	0.284±0.012	8.24±1.8	29.9±0.7
	0.25	4	220±8.1	0.263±0.001	19.2±1.5	74.6±1.0
	0.25	6	179±2.5	0.247±0.003	32.3±0.1	78.7±1.1
	0.25	8	204±1.7	0.200±0.006	18.3±0.5	50.2±0.2

Acetone was not a suitable solvent for this system in this study, despite being the optimal solvent for a previously prepared disulfiram-incorporated PLGA-PEG system (Madala et al. 2018). This could be due to the chitosan in the aqueous phase, which increased its viscosity, making it difficult for the emulsion to break into smaller droplets (Mohammadi-Samani & Taghipour 2015). Furthermore, acetone is a water miscible solvent, hence it yielded particles with much larger sizes than dichloromethane, which is immiscible in water. This could be due to the faster rate of diffusion of acetone into the aqueous phase because of its miscibility, instead of the slow rate of diffusion which is required for the formation of smaller particles using the solvent evaporation method. The size and stability results are consistent with literature reports (Mohammadi-Samani & Taghipour 2015; Abd El Hady et al. 2019) which cite dichloromethane as a favourable solvent for PLGA nanoparticle formation by solvent evaporation. The formulations prepared by the microfluidics methods displayed a more uniform dispersity, as shown by Table 3.1, a more positive surface charge and greater entrapment efficiency than their solvent evaporation prepared counterparts. The more positive charge could be due in part to the larger amount of chitosan present in these formulations. The particle uniformity and stability could be due to the precise control of mixing rate and ratio that this method provides compared to the emulsification solvent evaporation solvent evaporation method.

The uncoated PLGA nanoparticles prepared by both methods displayed negative surface charges due to the carboxylic acid end groups on the PLGA. Chitosan becomes soluble in acidic media as the amine groups are protonated (Villemin et al. 2019); thereafter the hydrophilic polymer swells in aqueous solution, increasing the viscosity of the layer of water at the polymer surface, which explains the larger size of the nanoparticles coated only with chitosan. The positive zeta potential of the chitosan coated nanoparticles is due to the amino groups on the chitosan surface and its magnitude can be influenced by the surface area of the nanoparticles.

In theory, the larger the surface area, the more amino groups are present and therefore the more positive the zeta potential of the nanoparticle. The high surface charge of the nanoparticles also prevents particle aggregation in aqueous solution due to electrostatic repulsion (Al-Nemrawi et al. 2018). The increase in zeta potential upon the coating of PEG may be due to multilayer deposition of PEG polymer during nanoparticle formation (Parveen & Sahoo 2011), which might cause interactions with the PLGA layer, exposing more of the cationic chitosan charge on the nanoparticle surface, as shown in Figure 3.1A. There are two major conformations of PEG on the surface of nanoparticles, with the brush conformation (present at higher PEG densities,) providing a higher degree of steric mobility than the mushroom conformation (found at lower PEG densities), as shown by Figure 3.1B. In this system, the optimal density of 6% (w/v) PEG resulted in particles with the highest entrapment efficiencies, suggesting a stabilizing effect on the amphiphilic structure, leading to a larger amount of hydrophobic drug-polymer interactions within the PLGA core.

The optimal entrapment efficiencies were similar for the formulations prepared by both methods, and this could be due to the optimal PLGA:disulfiram ratio of 10:1 (Fasehee et al. 2017; Madala et al. 2018) that was maintained in both methods, and demonstrates the dependence of drug entrapment properties on the compatibility of cargo with the core forming block (Wilkosz et al. 2018).

Reports describe low PEG surface density in a mushroom conformation and as PEG density increases, the PEG chains reconfigure into a brush conformation (de GENNES 2003; Wu 2014). With low PEG densities, the mushroom conformation allows free chains to move around on the chitosan surface and form multiple interactions, such as the intermolecular hydrogen bonding and electrostatic interactions between electropositive amine groups on chitosan and electronegative ether oxygen atoms on PEG (Jiang & Han 1998). This results in more shielding of the positive charge on chitosan, hence neutralising it to a large degree.

With higher concentrations of PEG, the conformations change from mushroom to brush, with steric repulsions between PEG chains causing reduced lateral interactions between PEG chains and chitosan surface, allowing for end chain PEG interactions only, as shown by Figure 3.1 C and 3.1 D. However, the conformational states of PEG on the nanoparticle surface can be complex due to multilayer depositions and interactions between these layers, as shown by Figure 3.1 A. Therefore, there could be a concentration of PEG close to the mushroom to brush transition density, that would form the optimal level of interactions between the polymers, utilizing the steric repulsion of the brush conformation and the topographical inhibition of protein binding provided by the mushroom conformation. In the systems prepared in this study, this optimal concentration (6% PEG) provides the desired solubility and stealth

effects due to the PEG shield, while the cationic properties of chitosan are still retained by the entire system, as shown by Table 3.1 and Figure 3.1 F.

3.3.2 FTIR Spectral Analysis Showing Adsorption of Polymer Layers

Figure 3.3 shows the spectra of the starting materials and formulations with increasing complexity prepared by both methods. The peaks in the PLGA spectra corresponded to bending of single carbon-hydrogen bonds at $500\text{--}1500\text{ cm}^{-1}$, stretching of carbonyl groups at 1775 cm^{-1} , as well as stretching of single carbon-hydrogen (C-H), methylene (CH_2), and methyl (CH_3) bonds between 2885 cm^{-1} and 3000 cm^{-1} . A methylene stretching peak due to ester bonds is also observed at 2955 cm^{-1} (Abd El Hady et al. 2019). In the PVA spectrum, a broad peak due to hydroxyl groups is found at 3300 cm^{-1} , a peak corresponding to methyl groups at 2800 cm^{-1} , a carbonyl stretching peak at 1775 cm^{-1} , and broad peaks due to C-H bending from 600 cm^{-1} to 1400 cm^{-1} . The disulfiram spectrum shows a peak due to methyl stretching at 2975 cm^{-1} , a peak at 1500 cm^{-1} due to C-H stretching, a peak at 1250 cm^{-1} due to C=S stretching and 1150 cm^{-1} – 1200 cm^{-1} due to C-C stretching. Peaks at 950 cm^{-1} and 800 cm^{-1} were due to C-N and C-S stretching respectively, while peaks at 500 cm^{-1} – 625 cm^{-1} were due to S-S bending (Jana 2015). In the chitosan spectrum, there is a broad peak at $\sim 3400\text{ cm}^{-1}$ due to hydroxyl and amine stretching, and a characteristic amide peak at 1550 cm^{-1} (Lu, Lv & Le 2019). In the PEG spectrum, a short, broad hydroxyl peak at $3250\text{--}3500\text{ cm}^{-1}$, a strong peak at 2800 cm^{-1} due to C-H stretching, and a characteristic peak at 1125 cm^{-1} can be observed due to C-O-C stretching vibrations (Gao et al. 2012).

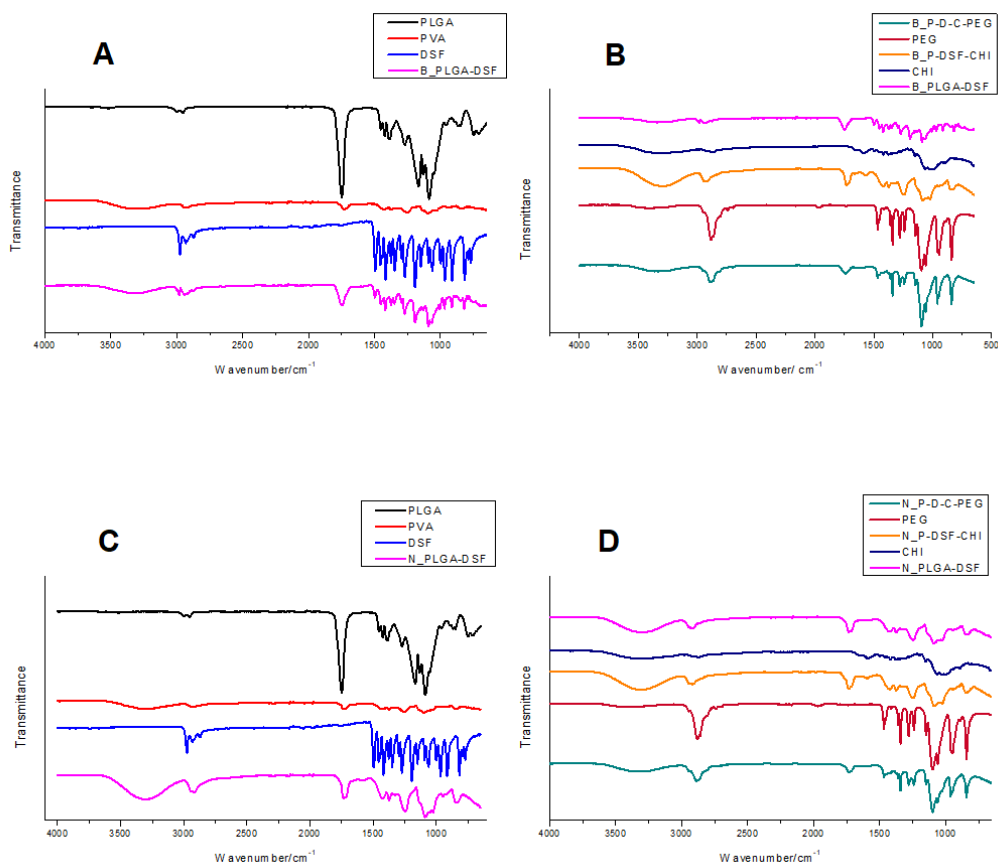


Figure 3.3 (A) and (B) – FTIR spectra of the formulations prepared using solvent evaporation (bulk). (C) and (D) – Formulations prepared using microfluidics (nano).

The FTIR spectra show no new peaks on nanoparticles as coatings are added, and the spectra are superimpositions of the starting materials, with no peak shifts or new peaks, indicating no formation of new covalent bonds. This confirms that the coatings interact by physical adsorption only (Lu et al. 2019). The nanoparticles prepared by both methods have very similar spectra, indicating that the manner in which the materials combine is the same for both methods.

3.3.3 Comparative Thermogravimetric Analysis and Differential Scanning Calorimetry of Disulfiram, Blank and Loaded Nanoparticles Prepared by Both Methods

The degradation of disulfiram occurred at 225 °C and degraded completely by 250 °C. The thermal degradation profiles of the formulations prepared by both methods were very similar. The blank nanoparticles prepared by both methods began degrading at 275 °C, with a slow degradation step until 410 °C, at which temperature 30% of the blank and 40% of the loaded nanoparticles had degraded, and a second step from 410 °C until complete degradation at

440 °C. There was still 8 % of residue of the loaded nanoparticles by the end of the experiment. The loaded nanoparticles followed the degradation profile of the blank nanoparticles closely, as shown in Figure 3.4.

The thermogram for disulfiram show characteristic endotherms at 74 °C and 190 – 250 °C corresponding to melting and degradation events, respectively (Tyukova et al. 2020). The thermograms for blank and loaded nanoparticles prepared by both methods were almost identical (Figure 4), with a characteristic endotherm for the glass transition temperature for PLGA nanoparticles shown at 55 °C (Zhu et al. 2013).

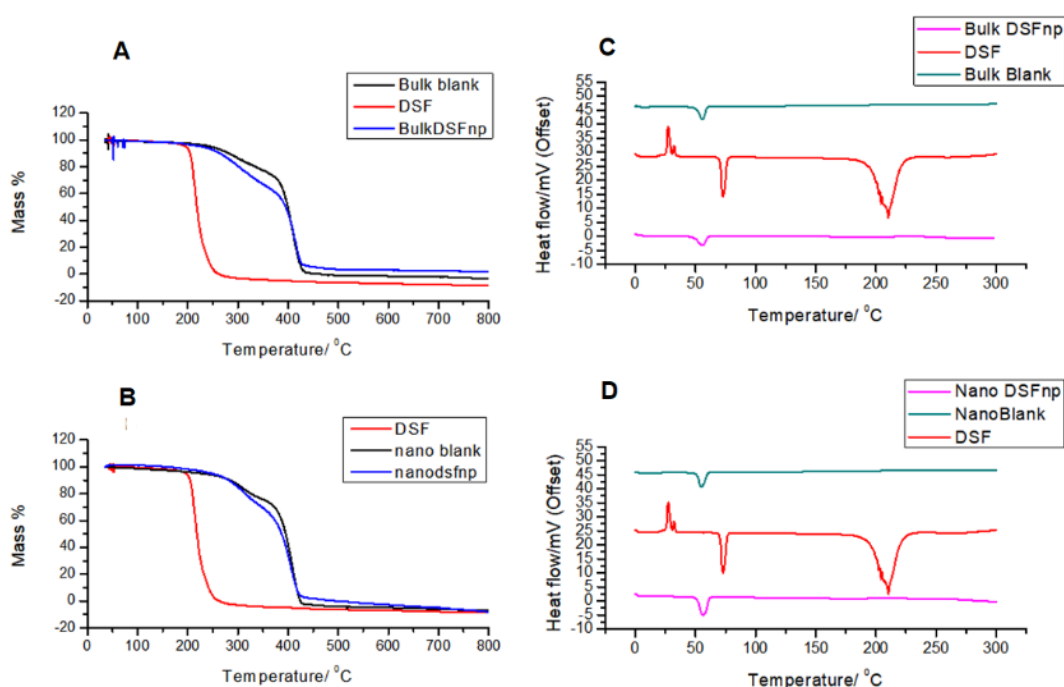


Figure 3.4 Graphs showing thermal degradation profiles of disulfiram, blank and loaded nanoparticles: (A) - blank formulations. (B) - microfluidic formulations. Relative DSC curves are shown with offset heat flow values for comparison: (C) - emulsification solvent evaporation formulations. (D) - microfluidic formulations.

The thermal and physical state of the nanoparticles influence its drug loading and release characteristics. The thermogravimetric analysis and differential scanning calorimetry data show that all the thermal events due to disulfiram were not displayed by any of the nanoparticle formulations, indicating that the loaded nanoparticles have adopted the thermal characteristics of the blank nanoparticles and that disulfiram is found physically dispersed in the nanoparticle system. The similarity of the formulations prepared by both methods indicate that they have similar physical states, which could be another reason for the similar entrapment efficiency observed in Table 3.1.

3.3.4 XRD Spectral Analysis Using Powder Samples of Pristine Polymers and Formulations Indicating Dominance of Blank Nanoparticle and PEG Surface Properties

As shown by Figure 3.5, PLGA and PVA display amorphous surface characteristics while there are several crystalline peaks observed for disulfiram. The loaded nanoparticles show no crystalline peaks until coated by PEG. The PEG (2%) coated nanoparticles displayed small crystalline peaks at $2\theta = 19^\circ$ and 24° , which increased with increased amount of PEG coating, and this can be seen in Figure 1S2 in Appendix 1, which demonstrates the relatively larger crystalline peaks at $2\theta = 19^\circ$ and 24° , due to the higher concentration (6%) of PEG in these formulations.

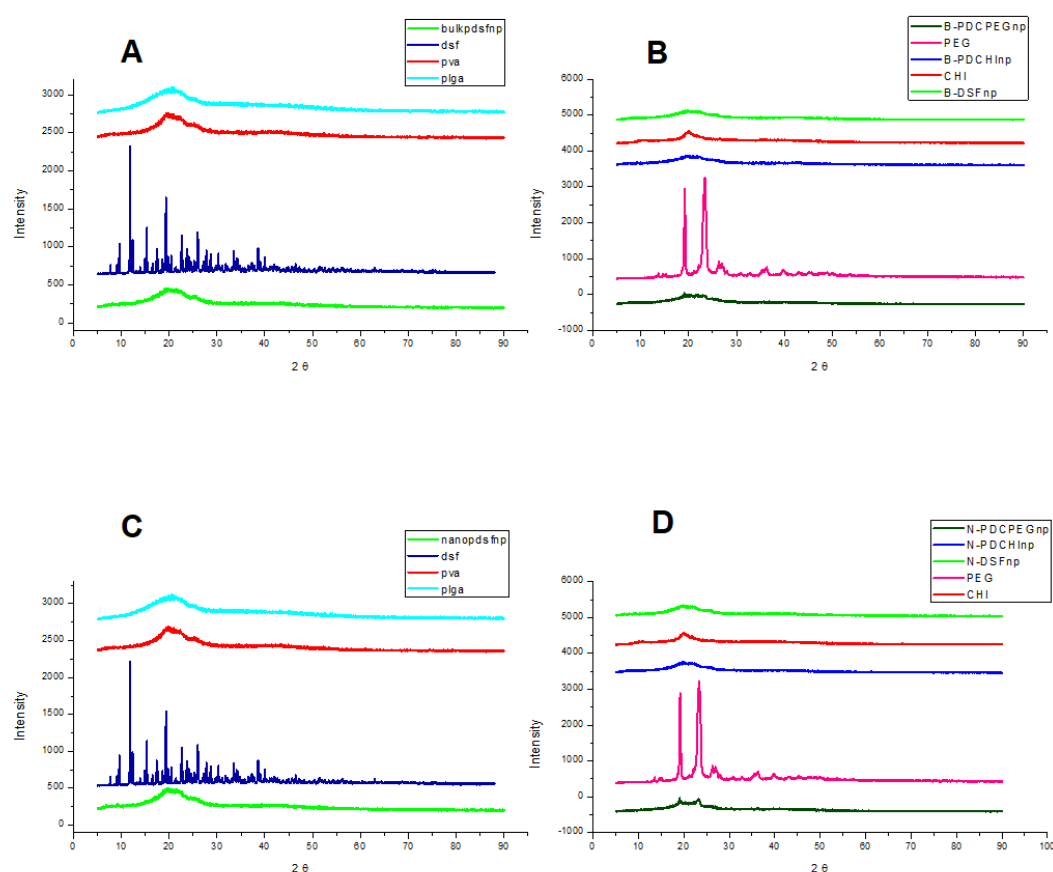


Figure 3.5 (A) and (B): X-ray diffraction spectra of the polymers and the loaded nanoparticles (2 % PEG) prepared by the emulsification solvent evaporation method (bulk/B); (C) and (D): diffraction spectra of the microfluidic formulations (nano/N).

XRD provides information of surface characteristics of nanoparticles, which is important for interactions within the circulatory system (Arya, Das & Sahoo 2018). As illustrated by Figure 3.8, disulfiram is crystalline and its surface properties are not found when encapsulated in the nanoparticle formulations. The loaded nanoparticles' surface is perceived in the same as that of the blank nanoparticles, suggesting that disulfiram is only present in an amorphous state within the nanoparticle. The surface properties show some crystalline peaks of PEG in Figure 3.8 B and D, suggesting that PEG shielding should be in effect in these formulations.

3.3.5 Scanning Electron Microscopy of Lyophilized Samples Showing Spherical Morphology and Nanoparticle Adhesion in Microfluidics Prepared Formulations

The use of mannitol to cryopreserve the samples had no effect except dilution and the samples that were evaporated on stubs were unstable and prone to cracking under the microscope. Hence all images were captured using solid lyophilized samples with no cryoprotectant. Figure 3.6 shows the spherical morphology of the nanoparticles prepared by both methods. There is a notable increase in nanoparticle adhesion observed in the particles prepared by the microfluidic method compared to those prepared by the emulsification solvent evaporation method, as the former was prepared with a relatively larger amount of chitosan in the aqueous phase.

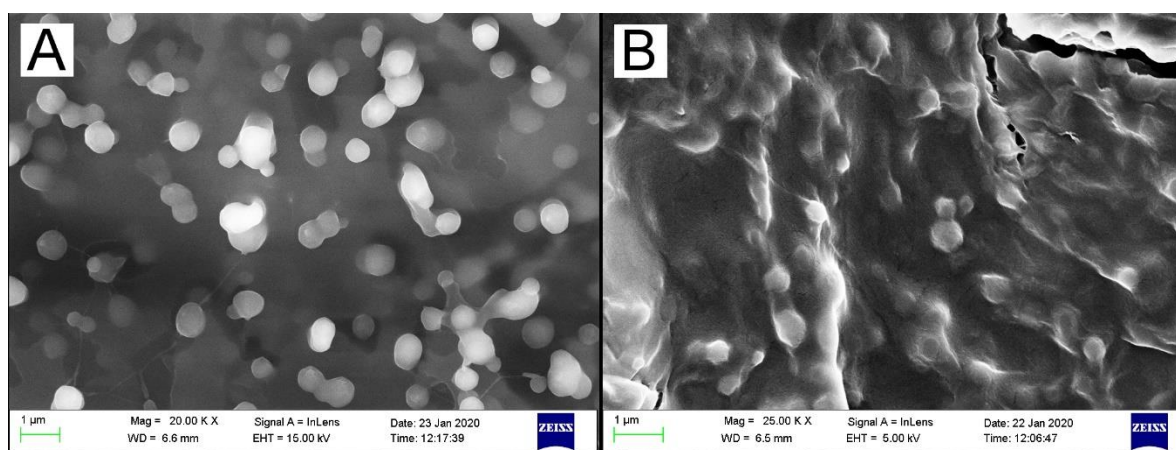


Figure 3.6 Scanning electron micrographs of nanoparticles prepared by emulsification solvent evaporation (A) and microfluidic (B) methods.

Both methods of preparation produced particles with spherical morphologies. The chitosan coating provides a positive charge which favours increased interaction with negative cell

membranes. As previously reported (Lu et al. 2019), when the chitosan:PLGA ratio is greater than 0.4, there is a relative increase of chitosan on the surface of the nanoparticles which causes adhesion and this can be seen in the SEM micrographs of the nanoparticles prepared by the microfluidic method which had a chitosan:PLGA concentration of ~0.8, while the solvent evaporation method had a chitosan:PLGA concentration of 0.25. The degree of nanoparticle adhesion corresponds to the amount of chitosan present in the formulation and it has been demonstrated that higher ratios of chitosan:PLGA leads to increased cancer cell uptake of the nanoparticles which in turn results in higher cytotoxicity[44]. This result supports the relative zeta potentials of the particles prepared by the microfluidic method that were more positive than those prepared by solvent evaporation, which is another indication of the higher chitosan concentration.

3.3.6 Drug Release Kinetics of Disulfiram and Emulsification Solvent Evaporation and Microfluidic Formulations Prepared Showing Higher Relative Release of Microfluidics-based Nanoparticles

The drug release profile of the emulsification solvent evaporation and microfluidics prepared nanoparticles compared to native disulfiram is shown in Figure 3.7. By 4 hours, disulfiram had released 10% of its concentration into the buffer, with negligible release thereafter. Both nanoparticle formulations demonstrated biphasic release patterns, with initial burst release followed by sustained release. The nanoparticles prepared by the emulsification solvent evaporation and microfluidic methods released maxima of 40% by 8 hours, and 70% by 12 hours, of their disulfiram content respectively.

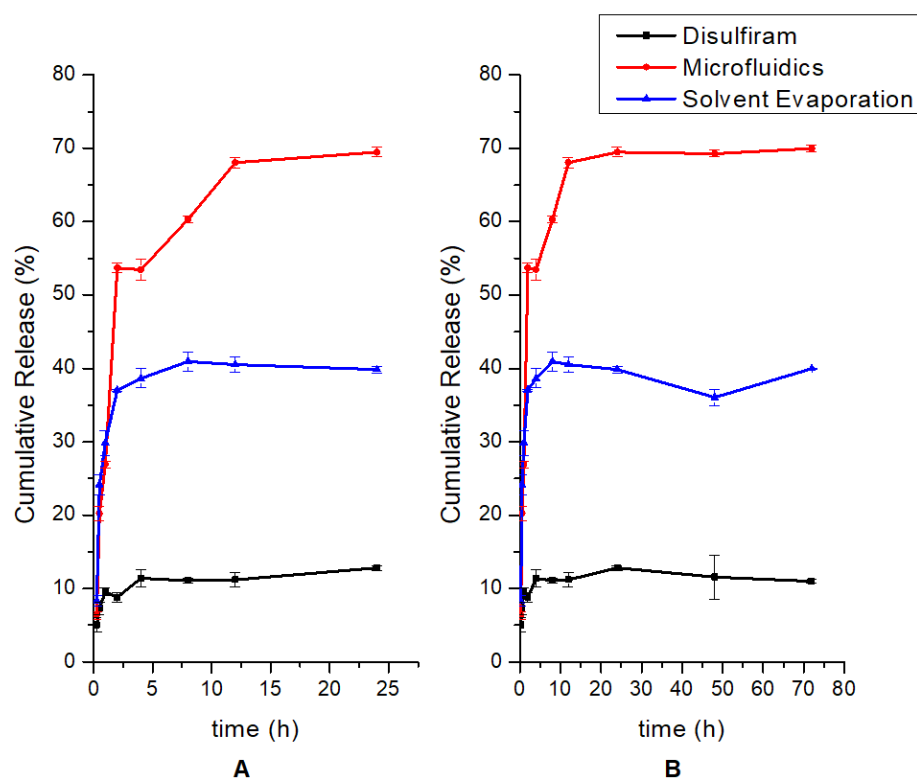


Figure 3.7 A: 24-hour and B: 72-hour cumulative in vitro release profiles of native disulfiram, emulsification solvent evaporation and microfluidic nanoparticles.

Release behaviour is biphasic, with initial burst release caused by drug that is poorly bound by polymeric matrix or adsorbed on nanoparticle surface, while subsequent sustained release from PLGA core is further controlled by chitosan and PEG surface layers. PLGA degrades into its constituent co-polymers, lactic acid and glycolic acid, by hydrolysis of its ester linkages, while chitosan swells, providing a hydrogel-like layer which controls drug diffusion from the surface (Al-Nemrawi et al. 2018). The profile of the microfluidic formulation displayed a slower release from 4 hours to 12 hours compared to the emulsification solvent evaporation prepared formulation. The dominant factor for the faster initial release of drug from the solvent emulsification preparation could be larger amount of drug adsorbed onto the surface of these particles, possibly due to larger surface pores of these types of particles compared to those prepared by microfluidics, as previously reported (Brzeziński, Socka & Kost 2019). The microfluidic formulation also displayed a higher cumulative release over the 72-hour period and a sustained release after 12 hours. It has been demonstrated that microfluidics methods produce particles with a more uniform distribution of drug within the matrix compared to conventional emulsification methods (Brzeziński et al. 2019), and this could be the reason for the more favourable release kinetics of the microfluidics preparation in Figure 7. The higher cumulative release of disulfiram from the microfluidic formulation could be due to a uniform

structural organisation (compared to the solvent emulsion formulation) of the drug molecules within the polymer matrix, resulting in more reversible interactions, while the drug molecules could have been more tightly bound to the core in the solvent emulsion formulation, resulting in lower amounts of free drug released into the medium. It is worthy to note that although the entrapment efficiencies are in the same range for both formulations, the release characteristics of the microfluidic formulation displayed higher availability of the disulfiram content. The level of free disulfiram detected in solution was relatively low, which demonstrates its limited solubility in aqueous media that has been previously reported (Shergill et al. 2016).

3.4 Conclusions and Future Work

We have designed and prepared polymeric nano-systems with a hydrophobic core, a cationic component and surface density of PEG chains that have resulted in favourable size, dispersity, surface charge and entrapment efficiencies. The hydrophobic PLGA core encouraged a high degree of carrier-drug interactions due to favourable drug: polymer ratio, while the chitosan and PEG coatings provided cationic and hydrophilic properties which are important for enhanced biocompatibility. The formulations prepared by solvent evaporation and microfluidics technique were physically, thermally and chemically similar. However, the difference in their physical surface properties was due in part to the difference in polymer concentrations which was an outcome of the method parameters. The formulations prepared by both methods entrapped the drug in an amorphous state, and did not retain any of its thermal or chemical properties, suggesting that the entrapped drug was efficiently shielded. The formulations also displayed cationic properties while maintaining the PEG groups on the nanoparticle surface. However, the microfluidics-based formulations displayed more desirable characteristics in terms of more positive surface charge which is associated with higher cancer cell uptake and toxicity, high entrapment efficiency with favourable drug binding and release properties, and higher cumulative and sustained release. These properties would make it highly effective for the delivery of drugs with low bioavailability, and could have applications for enhanced membrane permeation while retaining the greater benefits of antifouling effects in a passively targeted nanocarrier. Future directions of study with these formulations could include comparison of the hydrophilic properties that the different density of PEG shields convey to the delivery system, investigation of protein adsorption to the surface of the nanoparticles, and cytotoxicity and cellular uptake assays to investigate biocompatibility and therapeutic activity.

3.5 References

- Abd El Hady, W.E., Mohamed, E.A., Soliman, O.A.E.-A. & Sabbagh, H.M. EL, 2019, 'In vitro–in vivo evaluation of chitosan-PLGA nanoparticles for potentiated gastric retention and anti-ulcer activity of diosmin', *International Journal of Nanomedicine*, Volume 14, 7191–7213.
- Abouelmagd, S.A., Ku, Y.J. & Yeo, Y., 2015, 'Low molecular weight chitosan-coated polymeric nanoparticles for sustained and pH-sensitive delivery of paclitaxel', *Journal of Drug Targeting*, 23(7–8), 725–735.
- Al-Nemrawi, N., Alshraideh, N., Zayed, A. & Altaani, B., 2018, 'Low Molecular Weight Chitosan-Coated PLGA Nanoparticles for Pulmonary Delivery of Tobramycin for Cystic Fibrosis', *Pharmaceuticals*, 11(1), 28.
- Amoozgar, Z., Park, J., Lin, Q. & Yeo, Y., 2012, 'Low Molecular-Weight Chitosan as a pH-Sensitive Stealth Coating for Tumor-Specific Drug Delivery', *Molecular Pharmaceutics*, 9(5), 1262–1270.
- Arya, G., Das, M. & Sahoo, S.K., 2018, 'Evaluation of curcumin loaded chitosan/PEG blended PLGA nanoparticles for effective treatment of pancreatic cancer', *Biomedicine & Pharmacotherapy*, 102, 555–566.
- Bamrungsap, S., Zhao, Z., Chen, T., Wang, L., Li, C., Fu, T. & Tan, W., 2012, 'Nanotechnology in therapeutics: a focus on nanoparticles as a drug delivery system', *Nanomedicine*, 7(8), 1253–1271.
- Blanco, E., Shen, H. & Ferrari, M., 2015, 'Principles of nanoparticle design for overcoming biological barriers to drug delivery', *Nature Biotechnology*, 33(9), 941–951.
- Brzeziński, M., Socka, M. & Kost, B., 2019, 'Microfluidics for producing polylactide nanoparticles and microparticles and their drug delivery application', *Polymer International*, 68(6), 997–1014.
- Chiesa, E., Dorati, R., Pisani, S., Conti, B., Bergamini, G., Modena, T. & Genta, I., 2018, 'The Microfluidic Technique and the Manufacturing of Polysaccharide Nanoparticles', *Pharmaceutics*, 10(4), 267.
- Cruje, C. & Chithrani, D.B., 2014, 'Polyethylene Glycol Functionalized Nanoparticles for Improved Cancer Treatment', *Reviews in Nanoscience and Nanotechnology*, 3(1), 20–30.

Dahman, Y., 2012, 'Poly (Lactic Acid): Green and Sustainable Plastics', *Fermentation Technology*, 01(05).

Ding, D. & Zhu, Q., 2018, 'Recent advances of PLGA micro/nanoparticles for the delivery of biomacromolecular therapeutics', *Materials Science and Engineering: C*, 92, 1041–1060.

Donno, R., Gennari, A., Lallana, E., La Rosa, J.M.R. De, d'Arcy, R., Treacher, K., Hill, K., Ashford, M. & Tirelli, N., 2017, 'Nanomanufacturing through microfluidic-assisted nanoprecipitation: Advanced analytics and structure-activity relationships', *International Journal of Pharmaceutics*, 534(1–2), 97–107.

Ekinci, E., Rohondia, S., Khan, R. & Dou, Q.P., 2019, 'Repurposing Disulfiram as An Anti-Cancer Agent: Updated Review on Literature and Patents', *Recent Patents on Anti-Cancer Drug Discovery*, 14(2), 113–132.

Essa, D., Kondiah, P.P.D., Choonara, Y.E. & Pillay, V., 2020, 'The Design of Poly(lactide-co-glycolide) Nanocarriers for Medical Applications', *Frontiers in Bioengineering and Biotechnology*, 8.

Fasehee, H., Ghavamzadeh, A., Alimoghaddam, K., Ghaffari, S.-H. & Faghihi, S., 2017, 'A Comparative Cytotoxic Evaluation of Disulfiram Encapsulated PLGA Nanoparticles on MCF-7 Cells.', *International journal of hematology-oncology and stem cell research*, 11(2), 102–107.

Frank, L.A., Onzi, G.R., Morawski, A.S., Pohlmann, A.R., Guterres, S.S. & Contri, R.V., 2020, 'Chitosan as a coating material for nanoparticles intended for biomedical applications', *Reactive and Functional Polymers*, 147, 104459.

Gao, D., Tang, Duan & Tong, 2012, 'Oleanolic acid liposomes with polyethylene glycol modification: promising antitumor drug delivery', *International Journal of Nanomedicine*, 3517.

Garg, S., Heuck, G., Ip, S. & Ramsay, E., 2016, 'Microfluidics: a transformational tool for nanomedicine development and production', *Journal of Drug Targeting*, 24(9), 821–835.

GENNES, P.G. de, 2003, 'POLYMERS AT AN INTERFACE: A SIMPLIFIED VIEW', *Simple Views on Condensed Matter*, pp. 270–291, WORLD SCIENTIFIC.

Gentile, P., Chiono, V., Carmagnola, I. & Hatton, P., 2014, 'An Overview of Poly(lactic-co-glycolic) Acid (PLGA)-Based Biomaterials for Bone Tissue Engineering', *International Journal of Molecular Sciences*, 15(3), 3640–3659.

Guerrini, L., Alvarez-Puebla, R. & Pazos-Perez, N., 2018, 'Surface Modifications of Nanoparticles for Stability in Biological Fluids', *Materials*, 11(7), 1154.

- He, H., Markoutsas, E., Li, J. & Xu, P., 2018, 'Repurposing disulfiram for cancer therapy via targeted nanotechnology through enhanced tumor mass penetration and disassembly', *Acta Biomaterialia*, 68, 113–124.
- Jana, S., 2015, 'Spectroscopic Characterization of Disulfiram and Nicotinic Acid after Biofield Treatment', *Journal of Analytical & Bioanalytical Techniques*, 6(5).
- Jiang, W.H. & Han, S.J., 1998, 'Study of interaction between polyethylene glycol and chitosan by viscosity method', *Journal of Polymer Science Part B: Polymer Physics*, 36(8), 1275–1281.
- Jokerst, J. V, Lobovkina, T., Zare, R.N. & Gambhir, S.S., 2011, 'Nanoparticle PEGylation for imaging and therapy', *Nanomedicine*, 6(4), 715–728.
- Kim, K.-T., Lee, J.-Y., Kim, D.-D., Yoon, I.-S. & Cho, H.-J., 2019, 'Recent Progress in the Development of Poly(lactic-co-glycolic acid)-Based Nanostructures for Cancer Imaging and Therapy', *Pharmaceutics*, 11(6), 280.
- Kravanja, G., Primožič, M., Knez, Ž. & Leitgeb, M., 2019, 'Chitosan-Based (Nano)Materials for Novel Biomedical Applications', *Molecules*, 24(10), 1960.
- Lu, B., Lv, X. & Le, Y., 2019, 'Chitosan-Modified PLGA Nanoparticles for Control-Released Drug Delivery', *Polymers*, 11(2), 304.
- Madala, H.R., Punganuru, S.R., Ali-Osman, F., Zhang, R. & Srivenugopal, K.S., 2018, 'Brain- and brain tumor-penetrating disulfiram nanoparticles: Sequence of cytotoxic events and efficacy in human glioma cell lines and intracranial xenografts', *Oncotarget*, 9(3), 3459–3482.
- Makadia, H.K. & Siegel, S.J., 2011, 'Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier', *Polymers*, 3(3), 1377–1397.
- Mohammadi-Samani, S. & Taghipour, B., 2015, 'PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches', *Pharmaceutical Development and Technology*, 20(4), 385–393.
- Nikhil P. Kothawade & Suvarna A. Katti, 2016, 'UV METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF DISULFIRAM IN MARKETED TABLET PREPARATION', *INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACY AND CHEMISTRY*, 6(3), 2231–2781.
- Parveen, S. & Sahoo, S.K., 2011, 'Long circulating chitosan/PEG blended PLGA nanoparticle for tumor drug delivery', *European Journal of Pharmacology*, 670(2–3), 372–383.
- Rabanel, J.-M., Hildgen, P. & Banquy, X., 2014, 'Assessment of PEG on polymeric particles surface, a key step in drug carrier translation', *Journal of Controlled Release*, 185, 71–87.

Rezvantab, S., Drude, N.I., Moraveji, M.K., Güvener, N., Koons, E.K., Shi, Y., Lammers, T. & Kiessling, F., 2018, 'PLGA-Based Nanoparticles in Cancer Treatment', *Frontiers in Pharmacology*, 9.

Sauna, Z.E., Shukla, S. & Ambudkar, S. V., 2005, 'Disulfiram, an old drug with new potential therapeutic uses for human cancers and fungal infections', *Molecular BioSystems*, 1(2), 127.

Senapati, S., Mahanta, A.K., Kumar, S. & Maiti, P., 2018, 'Controlled drug delivery vehicles for cancer treatment and their performance', *Signal Transduction and Targeted Therapy*, 3(1), 7.

Shergill, M., Patel, M., Khan, S., Bashir, A. & McConville, C., 2016, 'Development and characterisation of sustained release solid dispersion oral tablets containing the poorly water soluble drug disulfiram', *International Journal of Pharmaceutics*, 497(1–2), 3–11.

Streck, S., Clulow, A.J., Nielsen, H.M., Rades, T., Boyd, B.J. & McDowell, A., 2019, 'The distribution of cell-penetrating peptides on polymeric nanoparticles prepared using microfluidics and elucidated with small angle X-ray scattering', *Journal of Colloid and Interface Science*, 555, 438–448.

Suk, J.S., Xu, Q., Kim, N., Hanes, J. & Ensign, L.M., 2016, 'PEGylation as a strategy for improving nanoparticle-based drug and gene delivery', *Advanced Drug Delivery Reviews*, 99, 28–51.

Tyukova, V.S., Kedik, S.A., Panov, A. V., Zhavoronok, E.S., Mendelev, D.I., Senchikhin, I.N., Fursova, A.Zh., Rumyantseva, Yu. V. & Kolosova, N.G., 2020, 'Synthesis of a Disulfuram Inclusion Complex with Hydroxypropyl- β -Cyclodextrin and Its Effect on Cataract Development in Rats', *Pharmaceutical Chemistry Journal*, 53(12), 1158–1163.

Villemin, E., Ong, Y.C., Thomas, C.M. & Gasser, G., 2019, 'Polymer encapsulation of ruthenium complexes for biological and medicinal applications', *Nature Reviews Chemistry*, 3(4), 261–282.

Wang, W., Meng, Q., Li, Q., Liu, J., Zhou, M., Jin, Z. & Zhao, K., 2020, 'Chitosan Derivatives and Their Application in Biomedicine', *International Journal of Molecular Sciences*, 21(2), 487.

Wang, Y., Li, P., Truong-Dinh Tran, T., Zhang, J. & Kong, L., 2016, 'Manufacturing Techniques and Surface Engineering of Polymer Based Nanoparticles for Targeted Drug Delivery to Cancer', *Nanomaterials*, 6(2), 26.

Wilkosz, N., Łazarski, G., Kovacik, L., Gargas, P., Nowakowska, M., Jamróz, D. & Kepczynski, M., 2018, 'Molecular Insight into Drug-Loading Capacity of PEG–PLGA Nanoparticles for Itraconazole', *The Journal of Physical Chemistry B*, 122(28), 7080–7090.

Wu, C., 2014, 'How does a polymer brush repel proteins?', *Chinese Journal of Polymer Science*, 32(11), 1575–1580.

Wu, J., Zhao, C., Lin, W., Hu, R., Wang, Q., Chen, H., Li, L., Chen, S. & Zheng, J., 2014, 'Binding characteristics between polyethylene glycol (PEG) and proteins in aqueous solution', *Journal of Materials Chemistry B*, 2(20), 2983.

Zhou, H., Fan, Z., Li, P.Y., Deng, J., Arhontoulis, D.C., Li, C.Y., Bowne, W.B. & Cheng, H., 2018, 'Dense and Dynamic Polyethylene Glycol Shells Cloak Nanoparticles from Uptake by Liver Endothelial Cells for Long Blood Circulation', *ACS Nano*, 12(10), 10130–10141.

Zhu, G., Wang, F., Gao, Q. & Liu, Y., 2013, 'Physicochemical Properties of Poly(lactic acid-co-glycolic acid) Film Modified via Blending with Poly(butyl acrylate-co-methyl methacrylate)', *Polímeros Ciência e Tecnologia*, 23(5), 619–623.

Chapter 4 Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells



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Article

Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells

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Abstract

Nanoparticles are designed to entrap drugs at a high concentration, escape clearance by the immune system, be selectively taken up by cancer cells, and to release bioactives in a rate-modulated manner. In this study quercetin-loaded PLGA nanoparticles were prepared and optimized to determine whether coating with chitosan would increase the cellular uptake of the nanoparticles and if the targeting ability of a folic acid as a ligand can provide selective toxicity and enhanced uptake in model LnCap prostate cancer cells, which express high levels of the receptor prostate specific membrane antigen (PSMA), compared to PC-3 cells, that have relatively low PSMA expression. A design of experiments approach was used to optimize the PLGA nanoparticles to have the maximum quercetin loading, optimal cationic charge and folic acid coating. The *in-vitro* release of quercetin and comparative cytotoxicity and cellular uptake of the optimized PLGA nanoparticles were examined, and it was revealed that the targeted nano-system provided sustained, pH-dependent quercetin release, and higher cytotoxicity and cellular uptake, compared to the non-targeted nano-system on LnCap cells. There was no significant difference in the cytotoxicity or cellular uptake between the targeted and non-targeted nano-systems on PC-3 cells (featured by low levels of PSMA), pointing to a PSMA-specific mechanism of action of the targeted nano-system. The findings suggest that the nano-system can be used as an efficient nanocarrier for the targeted delivery and release of quercetin (and other similar chemotherapeutics) against prostate cancer cells.

Keywords: PLGA nanoparticles; chitosan; folic acid; prostate specific membrane antigen; quercetin; active targeting

4.1 Introduction

Globally, prostate cancer is a major cause of male mortality, associated with 7% of male cancer-related deaths and 14% of cancer diagnoses (Sung et al., 2021). Current therapy includes, surgery, radiation and conventional chemotherapy, with each route resulting in a range of undesirable side effects (Lehto et al., 2017), highlighting the need for alternative treatment options. The advantages of nanotechnology in designing advanced drug delivery systems have been explored extensively since the first nano-formulation, Doxil a liposomal system carrying the cancer drug doxorubicin, was clinically approved for cancer treatment in 1995 (Wolfram and Ferrari, 2019). Nanotechnology in medicine has been used as a tool to minimize off- target losses of the active pharmaceutical ingredient (API) (Ventola, 2017; Han et al., 2022) , improve biodistribution (Mitchell et al., 2021) and minimize side effects and systemic exposure (Riccardi et al., 2021), hereby enhancing overall efficacy, safety, and pharmacokinetic and pharmacodynamic profile of the API (Naqvi et al., 2020). Nanoparticle-based treatments achieve this by the precision engineering of their components to improve site specific targeting and penetration, and to increase solubility and bioavailability of the payload (Rizvi and Saleh, 2018).

Quercetin is a plant-derived flavonoid that is found abundantly in fruits and vegetables. It has numerous medicinal properties, including anti-cancer activity but it's application as an anti-cancer drug is hampered by poor aqueous solubility, low bioavailability and chemical instability (Vinayak and Maurya, 2019; Ghafouri-Fard et al., 2021). Therefore, it is an ideal candidate as the API in a nanoparticle-based system, and such studies have been conducted and tested on several different cancers, including prostate cancer (Saavedra-Leos et al. 2021; Sharma et al. 2022) Additionally, its solubility and bioavailability limitations are characteristic of many cancer drugs (Zang et al., 2021) and therefore can also be used as a model therapeutic in preliminary studies for more toxic chemotherapeutic drugs.

Chitosan, a natural polymer derived from the shells of crustaceans, has been widely used for drug delivery applications because of its high biocompatibility. It has a strong positive charge in solution, which can increase cancer cell uptake through interactions with the negatively charged cell membrane (Nan et al., 2018). There are also several studies on chitosan nanoparticle systems that demonstrate its ability to release its payload more efficiently at an acidic pH (Popat et al., 2012; Tıǧlı Aydın and Pulat, 2012a; Shahid et al., 2022), which is

relevant for cancer treatment as the tumour microenvironment is reported to lie in the acidic pH range of 5.6 - 6.8 (Feng et al., 2018a; Lin et al., 2019).

The “magic bullet” effect is a term coined by nobel prizewinner Paul Ehrlich in 1909 (Xiong et al., 2020) describing the ability of a nano-system to target cancer cells for therapeutic effect while causing relatively no harm to surrounding healthy tissue. This can include active targeting of the cancer cells, which involves attaching a targeting ligand to the nanoparticle surface to specifically bind receptors highly expressed on cancer cells but not on healthy cells. Thereafter, the cancer cell receptors allow only the targeted nanoparticles to enter the cell, where it can release the API (Tewabe et al., 2021). Prostate specific membrane antigen (PSMA) is a cell surface receptor that expresses highly on prostate cancer cells but minimally on non-malignant cells. This makes it an excellent biomarker for prostate cancer, and various reports detail the use of antibodies, aptamers, and small molecules attached to nanoparticles in order to target PSMA on prostate cancer cells (Donin and Reiter, 2018). Folic acid is well-known for its action as a targeting agent for the folate cell surface receptors, and various reports demonstrate its targeting efficiency in nano-systems (Cheng et al., 2017; Angelopoulou et al., 2019; Bellotti et al., 2021). It was used it as a targeting ligand to expand on the limited studies detailing its binding ability for PSMA (Tewabe et al., 2021; Donin and Reiter, 2018) choosing LnCap and PC-3, as PSMA positive and negative cell lines respectively. Importantly, both cell lines do not express high levels of folate (Tewabe et al., 2021), eliminating the possibility of competitive binding for folate. Therefore, the folic acid moiety in this system was used to specifically bind to PSMA, to allow for the nanoparticles to preferentially enter LnCap cells via receptor mediated endocytosis, wherein the entrapped payload could be released in a controlled manner (Hattori and Maitani, 2004).

This system included poly(lactic-co-glycolic) acid (PLGA), which is a biodegradable, biocompatible FDA approved polymer. PLGA is widely used in drug delivery systems and is useful in the system of nanoparticle formation with the hydrophobic quercetin component, as it forms non-polar interactions with the molecule and stabilizes the matrix by maintaining quercetin in the core of the nanoparticle, separate from the surrounding aqueous medium [24]. (Wischke and Schwendeman, 2008; Wilkosz et al., 2018; Essa et al., 2020). Here, PLGA was used to entrap quercetin, forming nanoparticles which were then coated with chitosan and folic acid, as shown in Figure 4.1.

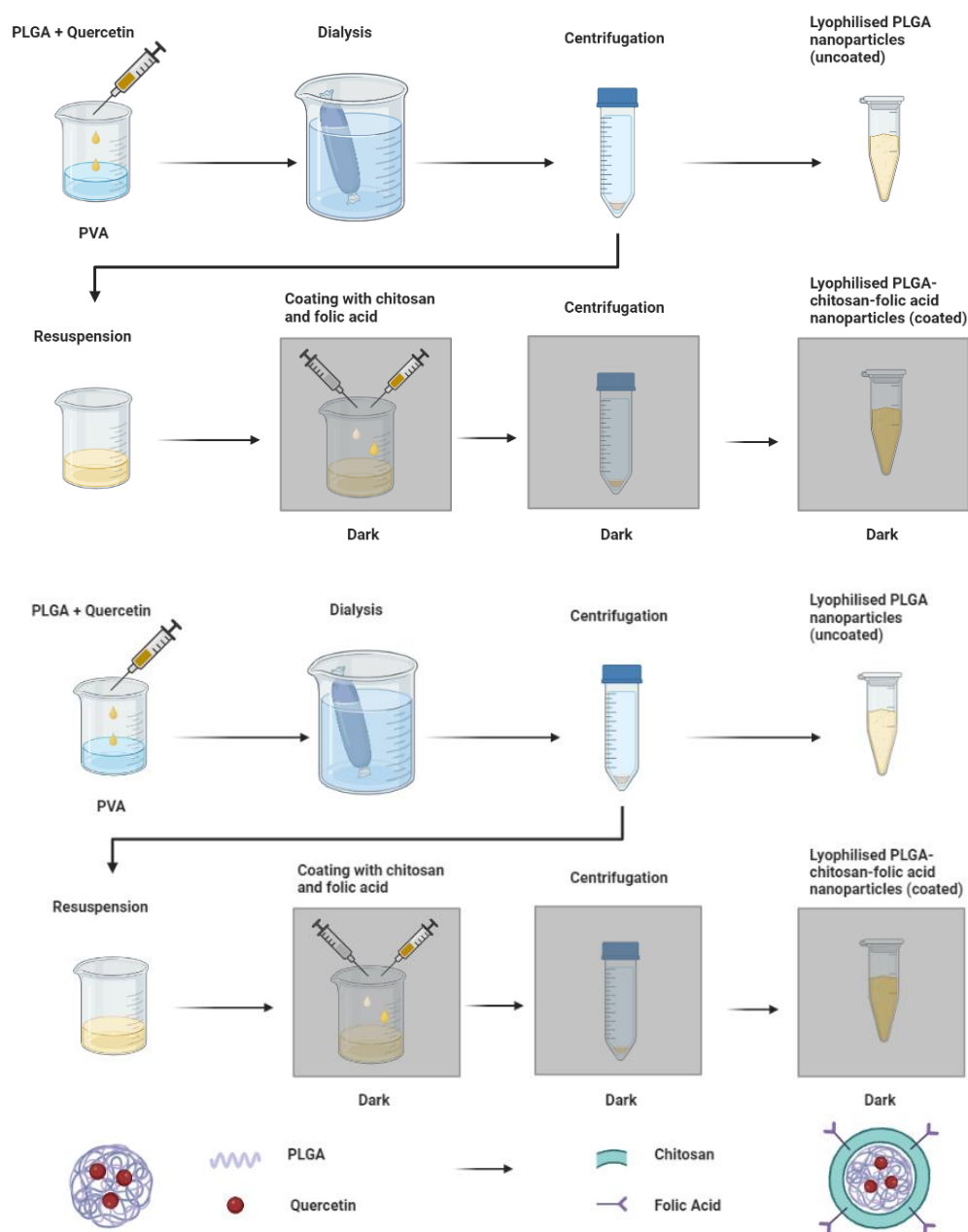


Figure 4.1 Fabrication process of uncoated (PLGA) and coated (PLGA-chitosan-folic acid) nanoparticles

Quercetin-entrapped nanoparticles have been previously prepared with PLGA alone (Yadav et al., 2022), in combination with PLGA and folic acid (Gupta et al., 2015), and with chitosan (Baksi et al., 2018) but not with all three components. Nano-systems prepared for drug delivery have been focused on highly positive (cationic) or highly negative (anionic) surface charge for formulation stability and increased interaction with cell membranes (Wu et al., 2011) even though nanoparticles that are too positively charged could damage the cell membrane and

anionic particles might be repelled by the cell membrane which is also negatively charged. Furthermore, highly charged nanoparticles are rapidly cleared by the immune system, while more neutral nanoparticles exhibit longer clearance times (Mitchell et al., 2021). Therefore, the activity of slightly positively charged nanoparticles was investigated, which a literature search has provided scarce previous data about.

Design of experiments is a tool used in formulation science to analyse the data obtained from a set of experiments and make predictions on how to tailor the fabrication process to formulate a product with the most desired properties (Tavares Luiz et al., 2021). The aim to use this methodology to fabricate nanoparticles optimized for the highest loading of quercetin, a positive surface charge as close to neutral as possible, and the highest folic acid content to maximize targeting efficiency.

4.2 Materials and Methods

4.2.1 Materials

Poly(D,L-lactide-co-glycolide)(PLGA) (lactide/glycolide ratio of 50:50, molecular weight 7 000-17 000 with acid end groups, low molecular weight chitosan, anhydrous dimethyl sulfoxide (DMSO), folic acid, sodium hydroxide pellets (NaOH), glacial acetic acid, polyvinyl alcohol (MW 23 000 - 28 000) (PVA), quercetin hydrate, dialysis tubing (MWCO 14KDa), sodium carbonate, methanol, PBS buffer tablets (pH 7.4), fluorescein iso-thiocyanate (FITC) and tween80 were purchased from Merck (Pty) Ltd, Estate South, Modderfontein, Gauteng, SA.

4.2.2 Preparation of Nanoparticles

Nanoparticles were prepared by the nanoprecipitation method, modified from (Fasehee et al., 2016) , where PLGA was dissolved in 1 mL of DMSO and added dropwise under stirring to the aqueous phase, consisting of 0.5% PVA. For the quercetin- and FITC- loaded nano-systems, quercetin or FITC were also dissolved in the organic phase before the addition. The solution was then dialysed for 24 hours to remove DMSO, and the PVA was subsequently removed by centrifugation for 30 minutes at 12 000 rpm (3 times). These nanoparticles were designated uncoated. For the “coated” nanoparticles, the centrifugal pellet was resuspended and incubated in the dark, at room temperature, for 1.5 hours, with solutions of chitosan and folic acid. The chitosan solutions were prepared by dissolving chitosan in 1% glacial acetic acid and were subsequently filtered, while the folic acid solutions were prepared by dissolving folic acid in 0.4 M NaOH (Nguyen et al., 2019). After the incubation period, the nanoparticles were separated by centrifugation and frozen at -80 °C for 24 hours. Frozen samples were then lyophilized (Freezone 12 lyophilizer, Labcono, Kansas City, USA) for 24 hours. Henceforth,

the PLGA nanoparticles are referred to as “uncoated nps” and the PLGA/chitosan-folic acid nanoparticles are referred to as “coated nps”.

4.2.2.1. Optimization by Design of Experiments

For the optimization of the nanoparticles, design of experiments was implemented, where the mass of quercetin, mass of chitosan and mass of folic acid were chosen as the independent variables while the zeta potential, quercetin loading and folic acid content were measured as the dependent response variables. In preliminary experiments, the mass of quercetin influenced drug loading, the mass of chitosan influence surface charge and the mass of folic acid influenced folic acid content. Therefore, these quantities were varied using data from previous studies (Lu et al., 2019a; Nguyen et al., 2019; Dandamudi et al., 2021) and their maximum solubility as upper limits. Loading of quercetin was maximized in order to entrap the maximum amount of bioactive within the nanosystem. The smallest positive charge was set as the target for the zeta potential. This was to fabricate nanoparticles that retain the longer residence time characteristic of neutral particles, while providing electrostatic interaction with the negative cell membrane for enhanced permeation. Folic acid content was maximized in order to create nanoparticles with the highest number of targeting moieties to efficiently target PSMA. Using JMP 17 software, a face central composite design, with two centre points was used to generate 16 formulations, listed in Table 4.1. These formulations were then prepared by the above method.

Table 4.1 Formulations generated by JMP software with varying amounts of quercetin (Q), chitosan (Chi) and folic acid (Fol).

Formulation	Parameters	Q/mg	Chi/mg	Fol/mg
P1	(1,0,0)	30	35	30
P2	(-1,-1,-1)	10	10	15
P3	(0,0,-1)	20	35	10
P4	(1,1,1)	30	60	45
P5	(0,0,0)	20	35	30
P6	(0,1,0)	20	60	30
P7	(-1,1,-1)	10	60	15
P8	(1,1,-1)	30	60	15
P9	(1,-1,-1)	30	10	15

P10	(1,-1,1)	30	10	45
P11	(0,-1,0)	20	10	30
P12	(0,0,0)	20	35	30
P13	(-1,-1,1)	10	10	45
P14	(0,0,1)	20	35	45
P15	(-1,1,1)	10	60	45
P16	(-1,0,0)	10	35	30

4.2.3 Particle size, polydispersity index and zeta potential analysis using dynamic light scattering

The nanoparticles were dissolved (1 mg in 1 mL of distilled water) and analysed using dynamic light scattering (DLS) on a ZetaSizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) particle size analyser. Samples were diluted with distilled water before measurement in capillary cells (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The temperature of the samples was maintained at 25 °C throughout the analyses.

4.2.4 Determination of Folic Acid Content by Ultra-Violet (UV) Spectrophotometry

The folic acid content was determined by a method modified from (Musalli et al., 2020). Briefly, 5 mg of nanoparticles were dispersed in 1 mL of 0.2 M sodium carbonate and vortexed for 1 min. The dispersions were then centrifuged at 3500 rpm for 15 minutes and the absorbance of the supernatants were read at 283 nm using the Nanophotometer UV/Vis spectrophotometer NP80 (Implen, Germany). The folic acid content was assayed against standards of known concentrations of folic acid dissolved in 0.2 M sodium carbonate, and was used to determine the percentage of folic acid in the nanoparticle formulations

4.2.5 Characterisation using Fourier Transform Infra-Red (FTIR)

The starting materials and nanoparticle systems were subjected to FTIR analysis and characteristic peaks were compared to confirm the structure of the pristine polymers and the nano-systems. The spectra were recorded using a PerkinElmer Inc. (Waltham, Massachusetts, USA) spectrometer, with a single reflection diamond MIRTGS detector. Samples were processed by a universal attenuated total reflectance (ATR) polarization accessory, at a resolution of 4cm⁻¹, with a constant pressure of 110 psi.

4.2.6 *Investigation of thermal degradation by thermogravimetric analysis (TGA)*

The temperature ranges with which the samples degrade, were studied using a thermogravimetric analyser (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Starting materials and samples were allowed to reach 30 °C and then heated at a rate of 10 °Cmin⁻¹ to 800 °C. An inert environment was maintained for the samples by constant purging of nitrogen gas for the duration of the run.

4.2.7 *Surface and crystallinity experiments using powder X-ray diffraction (XRD)*

The lyophilized samples and pristine polymers were crushed to form fine powders which were then loaded and smoothed onto a sample holder for analysis. X-ray diffraction spectra were generated on a benchtop MiniFlex 600 (Rigaku, Japan) powder diffractometer. CuK α radiation at 40 kV and 15 mA were set as the parameters for all experiments. Data were recorded using a 2 θ scan range of 10-60 degrees at a scan rate of 10 °min⁻¹. These powder X-ray diffraction analyses indicates the degree of crystallinity and amorphous nature of the polymers and nanoparticles and hence provides information about their surface properties and behavior.

4.2.8 *Phase transition studies employing differential scanning calorimetry (DSC)*

The thermal properties of the loaded and unloaded nano-systems and starting materials were investigated using a differential scanning calorimeter (DSC) (Mettler Toledo, DSC, STAReSystem, Schwerzenback, ZH, Switzerland). DSC measurements provide information about the thermal and phase changes of the samples, and is used in this case to compare how these properties change once quercetin is loaded into the nano-system. The nanoparticle and quercetin samples of ~5 mg were weighed into aluminum crucibles which were sealed and then heated over a temperature range of 0 to 400°C, at a heating rate of 10 °Cmin⁻¹. The samples were maintained in an inert N₂ gas atmosphere.

4.2.9 *Scanning Electron Microscopy (SEM) of Sample Suspensions*

Suspensions of lyophilized samples in distilled water were diluted and dropped on to aluminium stubs and dried for 48 hours under vacuum. In order to induce electrical conduction, samples were coated with a fine layer of gold under vacuum, using a sputter coater. Coated samples were analyzed on a ZEISS SIGMA 03-39 Field Emission Scanning Electron Microscope at 5 – 15kV acceleration voltage under an argon atmosphere.

4.2.10 *UV Spectrophotometric Analysis of Quercetin Loading in Nanoparticle Systems*

Nanoparticles were dispersed in PBS (pH 7.4) at a concentration of 10 mg/mL and incubated at 37 °C for 2 hours using an Orbit shaker incubator (LM-530-2, MRC Laboratory Instruments

Ltd, Hahistradrut, Holon, Israel) at 50 rpm. Thereafter, 50µL of the solution was added to 50µL of DMSO and 900µL of methanol, a method modified from (Fasehee et al., 2016). The mixture was vortexed for 30 seconds and centrifuged at 14 000rpm for 5 minutes and the absorbance of the supernatant read at 371 nm (Chaudhari et al., 2014). Quercetin standards in methanol were used to construct a calibration curve, and samples were analysed using the Nanophotometer UV/Vis spectrophotometer NP80 (Implen, Germany). Quercetin loading was represented as the percentage of quercetin per mg of nanoparticles.

4.2.11 In vitro release of Quercetin

In vitro release studies of the nanoparticle systems were performed in phosphate buffered saline (PBS) at pH 7.4 at 37 °C, to simulate physiological pH and at pH 6.0 to simulate the pH of the tumour microenvironment (Feng et al., 2018b). The method was adapted from (Anwer et al., 2016). 10 mg of nanoparticles were suspended in 2 mL of PBS in a dialysis bag placed in 50 mL of PBS containing 0.5% (v/v) tween80 and stirred for 72 hours at 37 °C. Samples were prepared in triplicate and compared with unloaded nanoparticles. At specific time points, 0.5 mL of release medium was withdrawn and replaced with an equal volume of the fresh medium. Samples were diluted and centrifuged and quercetin concentration was analysed spectrophotometrically at 371 nm.

4.2.12 Cell Culture Conditions and Cytotoxicity Studies

3T3-NIH mouse fibroblast cells and PC-3 and LnCap prostate carcinoma cells were obtained from Cellonex (Johannesburg, South Africa). Cells were confirmed to be free of mycoplasma. 3T3-NIH and PC-3 cells were grown in RPMI culture medium and LnCap cells were grown in DMEM/F12 culture medium. The media were supplemented with 10% FBS and 1% penicillin/streptomycin as per cell culture protocols. 3T3-NIH, LnCap and PC-3 cells were used at passage numbers 5-11. When confluent, PC-3 and LnCap cells were seeded in 96 well plates at a cell density of 5×10^3 cells/well and post cell attachment were treated with quercetin and quercetin-loaded nanoparticles. 3T3-NIH cells were seeded at a density of 3×10^3 cells/well and treated with unloaded nanoparticles as a toxicity control. 72 hours after treatment, cell viability was evaluated using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay.

4.2.13 Cellular uptake of Nanoparticles

PC-3 and LnCap cells were seeded onto coverslips in 6 well plates at a cell density of 1×10^5 cells/well and treated with FITC-loaded nanoparticles. 24 hours post treatment, cells were washed several times with PBS, fixed with 4% paraformaldehyde and stained with DAPI. The coverslips were then mounted onto glass slides for fluorescence microscopy, using an

Olympus BX41 Fluorescence Microscope (Olympus Corporation, Tokyo, Japan). Fluorescence intensity of images was calculated using ImageJ software.

4.2.14 Statistical Analysis

Measured data were calculated as the average of three experiments and represented with the standard error of the mean. Data between different experimental groups were compared using one way analysis of variance (ANOVA) on Origin V8.5 software. P values of less than 0.05 were considered significant.

4.3 Results

4.3.1 Optimized formulation results using DOE

4.3.1.1. Quercetin Loading, Size and Potential and Folic Acid Conjugation of Formulations

There was a size increase after coating nanoparticles with chitosan and folic acid (Table S1). A response surface design was used to create a quadratic model for the formulations and the order of experiments listed in Table 4.2. The limits of quercetin, chitosan and folic acid were based on their solubility and previous studies (Lu et al., 2019a; Nguyen et al., 2019; Dandamudi et al., 2021).

Table 4.2 Responses obtained using varying amounts of quercetin, chitosan and folic acid, where QL= quercetin loading, Z Potential = zeta potential, FA = folic acid content.

Formulation	Parameters	QL/%	Z Potential	FA/%
P1	(1,0,0)	9,04 ±2,15	-1,60 ±1,45	1,90 ±0,15
P2	(-1,-1,-1)	3,01 ±1,50	+2,33 ±0,87	0,37 ±0,15
P3	(0,0,-1)	3,90 ±1,05	+2,40 ±0,08	0,77 ±0,26
P4	(1,1,1)	8,27 ±1,30	-5,03 ±0,67	1,20 ±0,25
P5	(0,0,0)	2,78 ±1,71	+3,79 ±1,21	0,44 ±0,06
P6	(0,1,0)	1,83 ±1,30	-2,17 ±0,63	0,21 ±0,17
P7	(-1,1,-1)	1,71 ±2,86	+3,83 ±1,34	0,18 ±0,15
P8	(1,1,-1)	6,72 ±1,30	-4,93 ±0,17	0,66 ±0,46

P9	(1,-1,-1)	8,94 ±1,30	+1.07 ±0,35	0,91 ±0,10
P10	(1,-1,1)	14,2 ±2,39	-3,37 ±0,31	1,48 ±0,59
P11	(0,-1,0)	5,18 ±0,80	+1,58 ±1,56	0,61 ±0,21
P12	(0,0,0)	2,45 ±1,67	+3,15 ±0,47	0,54 ±0,17
P13	(-1,-1,1)	7,01 ±1,05	-1,20 ±0,61	0,95 ±0,25
P14	(0,0,1)	6,12 ±1,30	+2,39 ±0,85	1,13 ±0,21
P15	(-1,1,1)	6,05 ±0,80	+3,28 ±0,36	0,95 ±0,15
P16	(-1,0,0)	3,42 ±1,30	+2,26 ±0,29	0,70 ±0,25

4.3.1.2. Analysis of responses

The analysis showed that the quercetin and chitosan quadratic factors and the interaction between the amount of quercetin and the amount of chitosan had significant effects ($p < 0.05$) on all the measured responses, while the amount of folic acid did not have a significant effect ($p > 0.05$) on the responses (Figure 2S3, Appendix 2). The data was shown to fit the quadratic model with R^2 values between 0.85 and 0.97 (Fig 2S1, Appendix 2) with a normal distribution (Fig 2S2, Appendix 2). The interaction between the amounts of quercetin and chitosan and its effect on quercetin loading, surface charge and folic acid conjugation is shown in Figure 4.2.

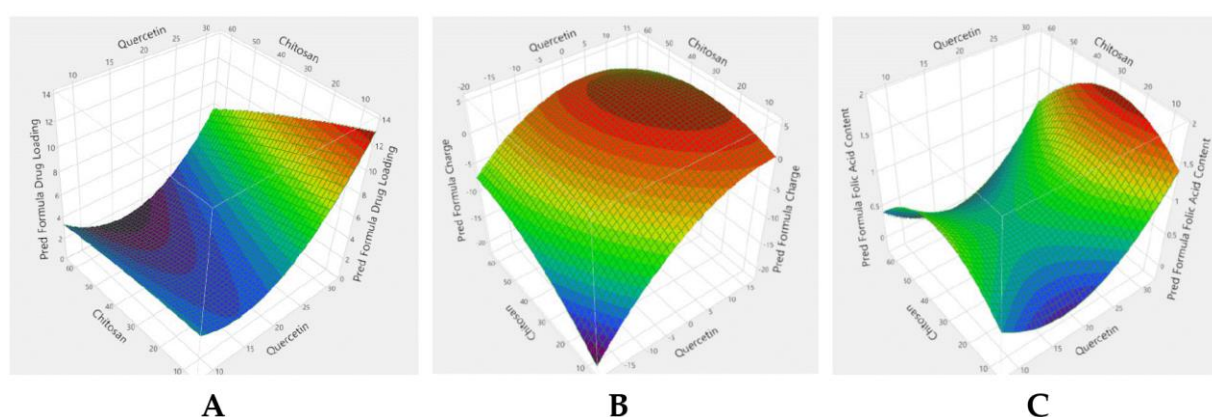


Figure 4.2 Response surface plots showing quercetin loading (A), charge B), and folic acid content (C), variation with different quercetin and chitosan amounts. Amount of folic acid was kept constant at the intermediate value of 30 mg. Obtained from JMP V17.

Effects of the interaction between the quercetin and chitosan factors - when both quercetin and chitosan are low, there are intermediate quercetin loading values and when quercetin is high and chitosan is low, there are high quercetin loading values. This is because of the higher total amount of quercetin in the formulation, up until the point where the maximum amount of quercetin is entrapped and adding more quercetin does not increase the loading. Increasing the initial amount of chitosan decreases the quercetin loading of in an almost linear fashion. This is because of the lower fraction of quercetin in the formulation as increasing the chitosan amount increases the total amount of product (Table 2S1, Appendix 2).

The amount of quercetin inversely affects the surface charge of the nanoparticles. This could be because quercetin is negatively charged and therefore reduces the total charge. The amount of chitosan, which is positively charged, increases the surface charge of the nanoparticles, up until the maximum amount of chitosan coating that could be achieved by this method. The folic acid conjugation increases with the amount of quercetin in the system, this could be because as quercetin decreases the surface charge of the uncoated nanoparticles, it increases the amount of chitosan coating and therefore increases the amount of negatively charged folic acid molecules that can adsorb to the positive charge. This also explains why increasing the chitosan amount increases folic acid conjugation until the value of 30 mg, which could be at the maximum amount of chitosan coating, and any further increase in chitosan leads to neutralization of the folic acid by the uncoated chitosan in solution.

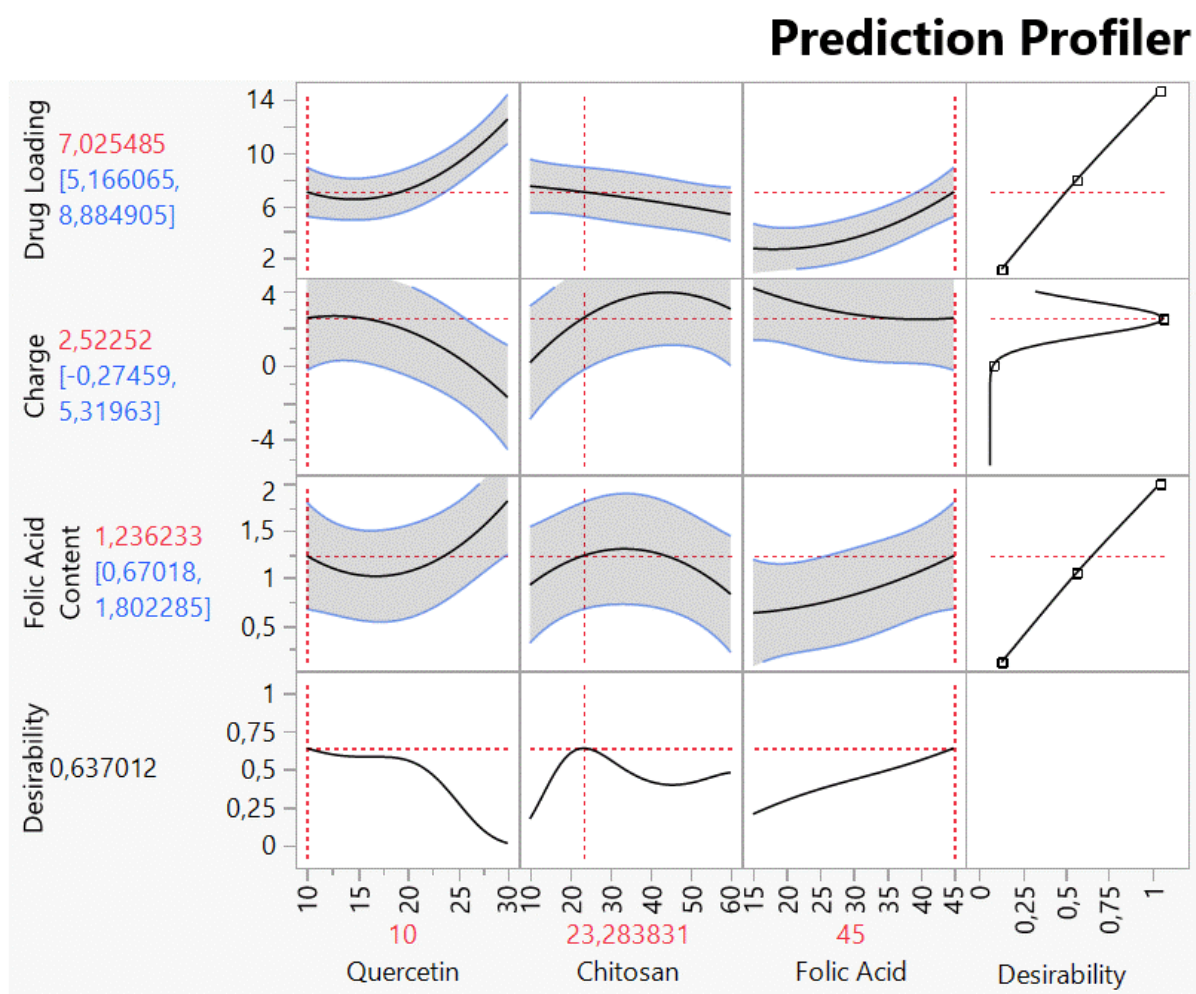


Figure 4.3 Prediction profiler showing optimized solution with maximum desirability – from JMP V17.

Table 4.3 Responses obtained using varying amounts of quercetin, chitosan and folic acid

Response	Predicted	Actual	Bias
Quercetin Loading	7,02	7,11 ±1,60	+0,0126
Surface Charge	+2,52	+1,84 ±0,40	-0,370
Folic Acid Content	1,24	1,61 ±0,35	+0,236

The optimized solution shown by Figure 4.3, was the input of 10 mg of quercetin, 23 mg of chitosan and 45 mg of folic acid. The actual versus predicted responses shown in Table 4.3 reflects values within the limits of the predicted solution (shown in Figure 4.4). This formulation was used in all further experiments.

4.3.2 Size and potential data show spherical coated particles with positive surface charge

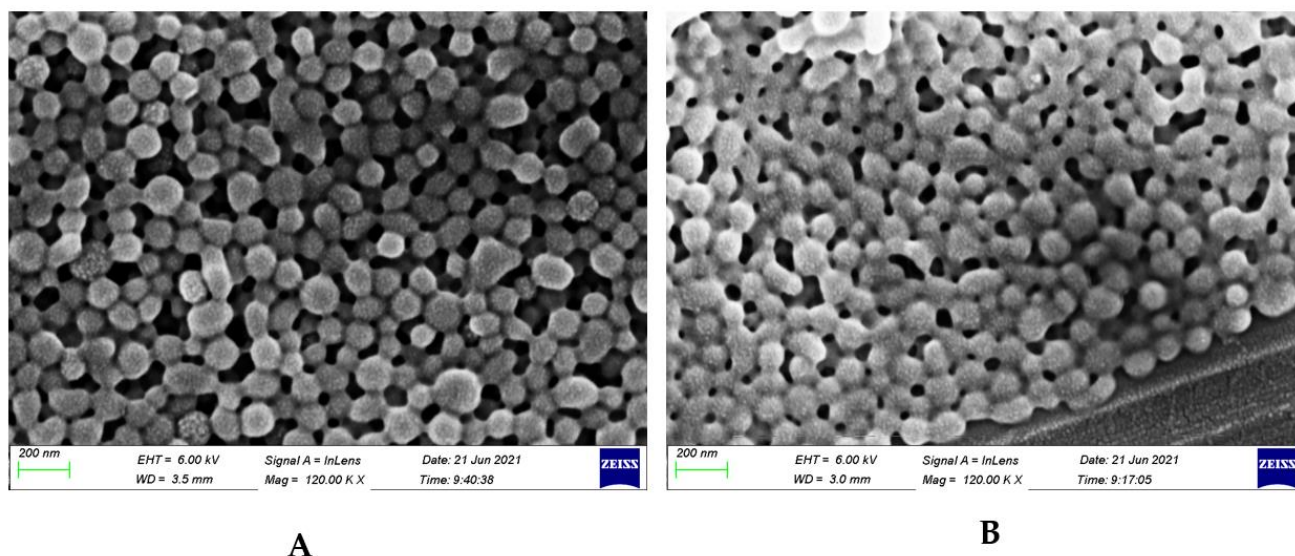


Figure 4.4 Scanning Electron Micrographs of A- uncoated nanoparticles and B- coated nanoparticles.

Table 4.4 Summary of dynamic light scattering measurements of uncoated and coated nanoparticles

Formulation	Size/nm	PDI	z Potential/mV
Uncoated nps	159,8 $\pm$ 2,0	0,068 $\pm$ 0,01	-21,0 $\pm$ 1,6
Coated nps	206,2 $\pm$ 1,7	0,069 $\pm$ 0,002	+1,84 $\pm$ 0,4

The particle size diameter of the uncoated nanoparticles increased from 159,8 $\pm$ 2,0 to 206,2 $\pm$ 1,7 nm upon coating with chitosan and folic acid (Figure S4A- and B-1 in Appendix 2 and Table 4.4). The polydispersity indices were very low (Table 4.4), suggesting precise, uniform particles. The particle uniformity can also be seen in the scanning electron micrographs (Figure 4.4A- and B-3), which show spherical particle morphology. The SEM images also suggest slightly smaller diameters (using the scale bar of 200 nm) than the dynamic light scattering measurements in Figure S4A- and B-1, which could be due to the fact that the DLS technique measures the hydrodynamic diameter of the particles in solution whereas scanning electron microscopy examines the particles in solid form, where the hydrated polymeric shell has collapsed during drying and under the vacuum of the SEM chamber (Fissan et al., 2014; Lu et al., 2019b; Hernández-Giottonini et al., 2020). Figure 4.4 B3 also show some adhesion

between the individual nanoparticles which could be due to the interaction between the moieties of the chitosan component of the coating (Lu et al., 2019b). The zeta potential results show that the surface charge of the uncoated nanoparticles increased from $-21,0 \pm 1,6$ to $+1,84 \pm 0,4$ mV after coating with chitosan and folic acid (Figure S4 A- and B-2 in Appendix 2 and Table 4.4). The negative charge of the uncoated nanoparticles is due to the carboxylic acid end groups in the PLGA polymer, whereas the small positive charge of the coated nanoparticles is due to the highly positive charge of the amine groups on the chitosan moiety and the negative charge of the acid end groups on the folic acid moiety.

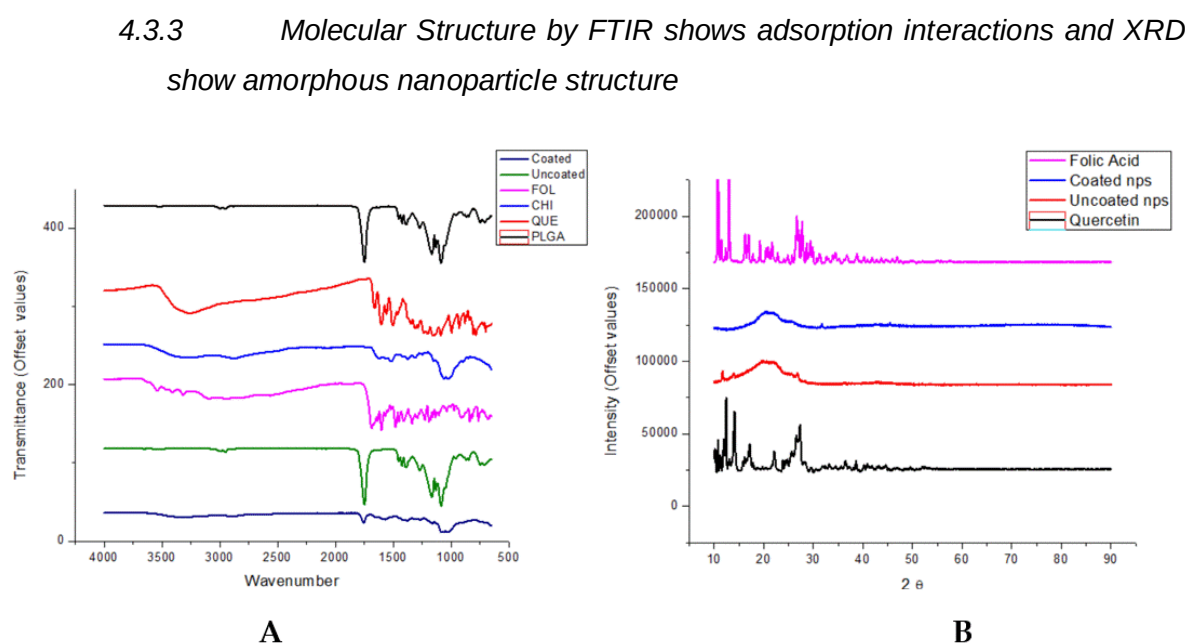


Figure 4.5 A- FTIR spectra of uncoated and coated nps and pristine polymers where Fol = folic acid, Chi = Chitosan and Que = Quercetin. B- XRD spectra of Quercetin and uncoated and coated nanoparticles.

FTIR spectra of all reagents and products are shown in Figure 4.5A. The PLGA spectrum shows characteristic strong bands at 1750 cm^{-1} and 1094 cm^{-1} due to C=O and C–O–C stretching respectively, C–H bending at $1200\text{--}1400\text{ cm}^{-1}$, and small bands due to linear C–H stretching vibrations at ~ 2990 and 3000 cm^{-1} . In the spectrum of chitosan, bands at $\sim 1110\text{ cm}^{-1}$ can be observed due to amine stretching and bands at $1600\text{--}1700\text{ cm}^{-1}$ due to amide bonds. Bands due to C–H bending at 1400 cm^{-1} and C–H stretching at 2900 cm^{-1} can also be observed. The spectrum for folic acid shows a number of bands between 2800 and 3600 cm^{-1} due to N–H and OH stretching, with a sharp band at 1700 cm^{-1} due to C=O amide stretching. A band due to the OH phenyl group is also found at 1400 cm^{-1} . These observations were consistent with previous findings (Al-Nemrawi et al., 2018; Abd El Hady et al., 2019; Lu et al., 2019b).

The characteristic bands of chitosan can be seen in the spectrum of the coated nanoparticles but not in that of the uncoated nanoparticles, while the strong characteristic PLGA band at 1750 cm^{-1} can be seen in both uncoated and coated nanoparticles spectra. This indicates that the chitosan had been successfully coated onto the surface of the PLGA nanoparticles. The specific bands of folic acid are not clearly visible in the spectra of the coated nanoparticles. This could be due to the larger relative concentrations of the PLGA and chitosan in the nanoparticle system, which could overwhelm the bands due to the folic acid constituent. The spectra of the nanoparticles indicate that the components interact by physical adsorption alone, since there are no new bands, and therefore no formation of new chemical bonds in the nanoparticle systems (Nguyen et al., 2019).

XRD (Figure 4.5B) shows crystallinity of quercetin and folic acid showing several sharp bands, while the nanoparticles are more amorphous. The spectrum of the uncoated nanoparticles shows more crystallinity, with bands corresponding to those of quercetin, which could be because there is some quercetin at the surface of the nanoparticles. In the spectrum of the coated nanoparticles, these peaks are indistinct and less intense, indicating that quercetin is entrapped more completely, within the core of these nanoparticles (Al-Nemrawi et al., 2022).

4.3.4 Thermal Degradation and Phase Transition show retention of polymer properties

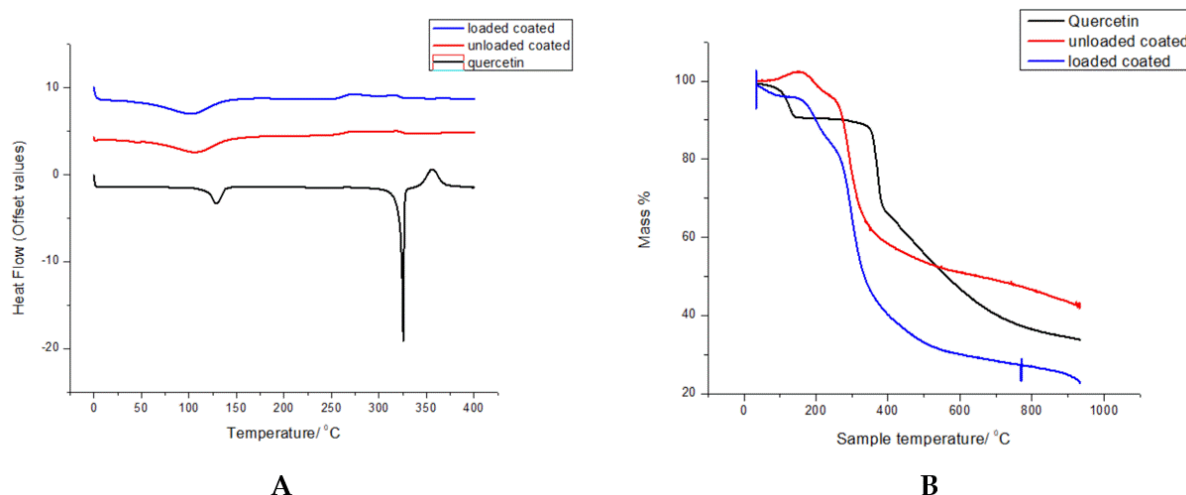


Figure 4.6 A- DSC and B- TGA thermograms of loaded and unloaded coated nanoparticles where Que = Quercetin.

The DSC thermograms are shown in Figure 4.6A, where native quercetin showed endothermic peaks at 125 and 325 °C, corresponding to its melting and decomposition temperatures

respectively (Li et al., 2013). The thermograms of loaded coated nanoparticles is similar to that of the unloaded nanoparticles, with a broad endothermic peak at 100 °C, corresponding to a characteristic dehydration peak of chitosan (Abd El Hady et al., 2019), but no definite peaks due to melting or degradation. The sharp peaks in the quercetin thermogram are not visible, indicated that quercetin is entrapped within the nanoparticle in an amorphous state (Hassan et al., 2017).

For the TGA (Figure 4.6B), weight loss of the nanoparticle systems can be divided into the stages where weight loss is due to the water loss from the chitosan component at 0 – 300 °C (Dandamudi et al., 2021) and the thermal degradation stage, until 400 °C for the unloaded nanoparticles and 450 °C for the loaded nanoparticles. Quercetin undergoes thermal oxidation at 100 – 200 °C and thereafter, degradation at 400 until 800 °C (Jardim et al., 2022). The weight loss steps observed correspond with the temperatures of the degradation steps observed by the DSC thermograms (Figure 4.7A). The loaded nanoparticles exhibit a greater thermal stability compared to both the unloaded nanoparticles and pristine polymers, probably due to nanoparticle interactions and intact structural conformation, which is consistent with previous studies with the individual components (Gathirwa et al. 2014; Hu et al. 2019; Dandamudi et al. 2021)

4.3.5. *In-vitro* kinetic study of nanoparticle systems shows efficient quercetin release

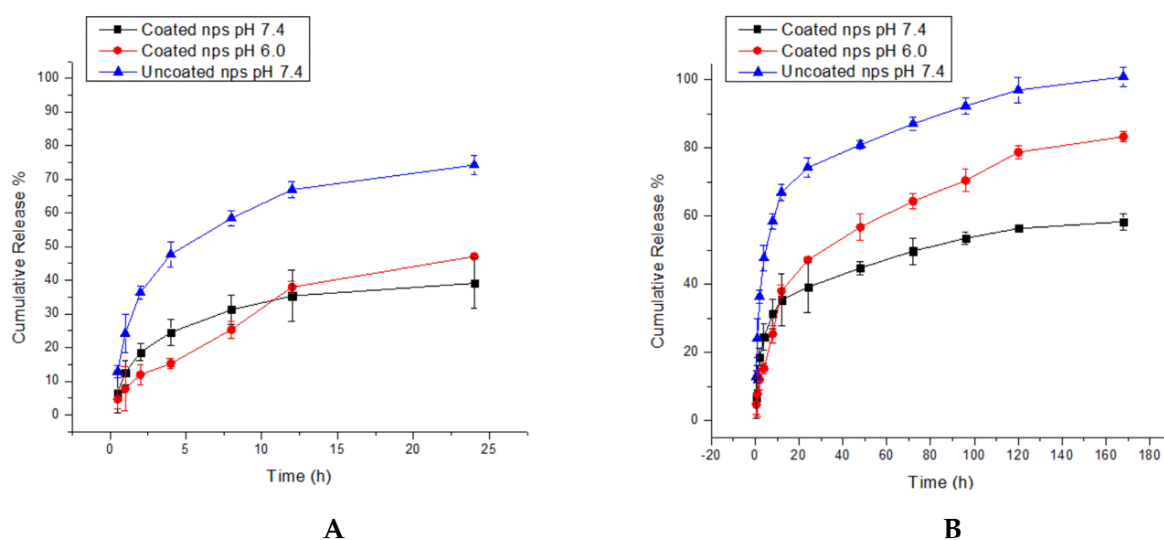


Figure 4.7 Release curves of quercetin from coated and uncoated nanoparticles A- 24 hours and B- 168 hours.

At physiological pH of 7.4, both coated and uncoated nanoparticles displayed a biphasic release pattern, as shown by Figure 7 A and B, with an initial burst release, followed by a

slower release. The uncoated nanoparticles showed a markedly higher burst release, with 48 and 68% release by 4 and 12 hours respectively, compared to 25 and 35% release by the coated nanoparticles at the same time points. The burst release is caused by the adsorption of quercetin molecules on the surface of the nanoparticles, allowing for rapid desorption and diffusion in solution due to the weak interactions between the quercetin and polymer layers at the nanoparticle surface (Lima et al., 2018). In the case of the coated nanoparticles, the slower burst release suggests strong physical interactions between quercetin and the coating polymer layer, possibly due to the polysaccharidic chitosan component. However, the maximum release at pH 7.4 was only 55% for the coated nanoparticles compared to 98 % for the uncoated nanoparticles after 168 hours. This could be due to the swelling of the chitosan layer in solution, forming a physical barrier to the diffusion of quercetin out of the nanoparticle core (Chronopoulou et al., 2013)

There is a much shorter burst release by the coated nanoparticles at pH 6.0 (compared to both of the nano-systems tested at pH 7.4) of 12% at 2 hours then a constant release until 36%, where it matches the cumulative release of the coated nanoparticles at 12 H. This could be due to the rapid swelling in nanoparticle size due to the protonation of the amino groups in the chitosan layer, which increases the time taken for the quercetin molecules to diffuse into bulk solution (Tıǧlı Aydın and Pulat, 2012b). Thereafter, once the nanoparticles reach equilibrium swelling, the low pH allows for increased solubility of the chitosan layer (since the pH of 6.0 is lower than the pKa of chitosan, which is 6.3), resulting in faster and more complete release of 78% of the quercetin molecules from the nanoparticle core (Chronopoulou et al. 2013; Lu et al. 2019b) Importantly, the dissolution of the chitosan layer results in a gradual decrease in the thickness of the chitosan coating around the core, and therefore the coated nanoparticle system displays a more controlled and sustained release profile than that of the uncoated nanoparticles (Chronopoulou et al., 2013).

4.3.5 *Increased cytotoxicity of quercetin in nanoparticle system*

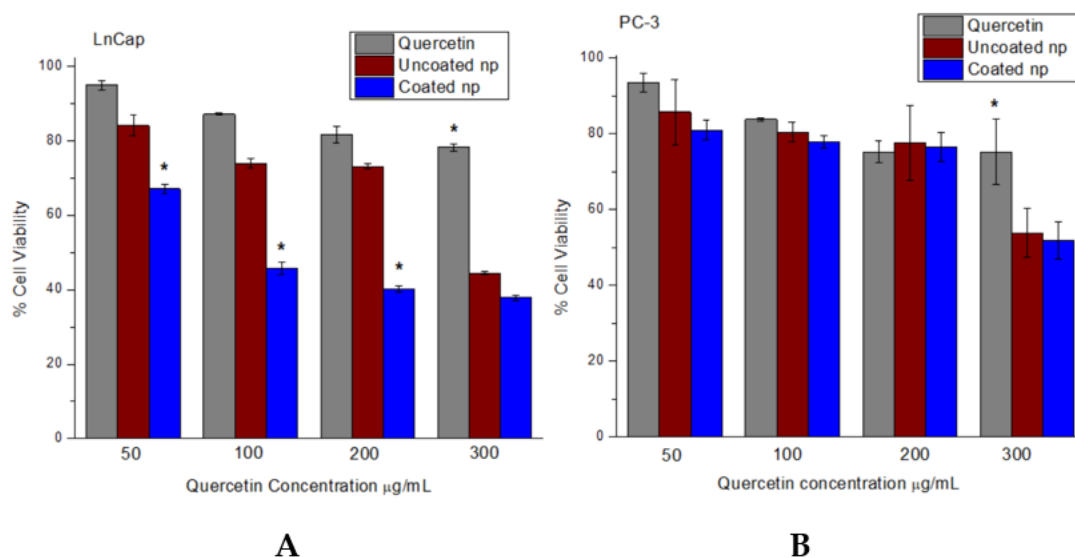


Figure 4.8 *In-vitro* cell viability of A- LnCap and B- PC-3 cells after being treated for 72 hours with free quercetin, uncoated and coated nanoparticles (*p value <0.05).

The unloaded nano-systems showed no toxicity on the 3T3-NIH fibroblast cell lines, with viabilities greater than 90% at all concentrations tested (Fig 2S5 in Appendix 2). As shown by Fig 4.8, after 72 hours, both cell lines showed a general dose-dependent decrease in viability with increasing concentration of quercetin. The nanoparticle systems showed significantly ($p < 0.05$) more cytotoxicity than free quercetin on the PC-3 cell line (Fig 4.8 B) only at a concentration of 300 µg/mL (50 – 52% for the nanoparticles vs 75% for free quercetin). Moreover, at all concentrations, there was no significant difference in the cytotoxicity of the coated and uncoated nanoparticles on this cell line. However, the coated nanoparticles were significantly more cytotoxic than both free quercetin and uncoated nanoparticles on the LnCap cell line at all concentrations except for 300 µg/mL, where the nanoparticle systems were not significantly different from each other but both were more cytotoxic than free quercetin (38% for the coated nanoparticles vs 44% for the uncoated nanoparticles and 78% for free quercetin). This corresponds to the concentration that the nanoparticle systems were significantly different to quercetin in the PC-3 cell line (noted above). The coated nanoparticles were also more cytotoxic on the LnCap cell line at lower concentrations than the PC-3 cell line with viability of 48, 40 and 38% for LnCap compared to 77, 70 and 50% for PC-3 at concentrations of 100, 200 and 300 µg/mL respectively. The selective cytotoxicity of the coated nanoparticles on the LnCap cell line could indicate a greater nanoparticle cellular uptake by this cell line compared to the PC-3 cell line. This could be due to the PSMA receptors on the surface of the LnCap cells (but not on PC-3 cells) that allow the coated nanoparticles to enter the cells through the binding of the folic acid moiety, hereby increasing intra-cellular

quercetin concentration and causing inhibition of cell growth (Homayouni Tabrizi 2022). However, the lack of significance of the toxicity difference between the coated and uncoated nano-systems on both cell lines at a concentration of 300 µg/mL indicates that this could be the concentration at which the nanoparticles are able to enter the cells by some mechanism other than active targeting of the PSMA receptor.

4.3.6 *Greater cellular uptake of targeted nanoparticles in a PSMA positive cell line*

PSMA-positive LnCap and PSMA-negative PC-3 cells were treated with uncoated and coated nanoparticles that had FITC entrapped within their core. As shown by Figure 4.9, there is a definite increase in cell association of FITC moieties (shown as green) around the nuclei (shown as blue) in the LnCap cell line when treated with the coated nanoparticles (Figure 4.9-A1). There is also more green fluorescence in the LnCap cell line that was treated with uncoated nanoparticles (Figure 4.9-A2) compared to the PC-3 cell line treated with either coated or uncoated nanoparticles (Figure 4.9- B1 and 2). As expected, there is no significant difference in fluorescence between the coated and uncoated nanoparticle treatments on the PC-3 cell line (Figure 4.9 – B1 and 2) and Table 4.5. This suggests a greater selective uptake of the coated nanoparticles by the PSMA-positive LnCap cell line compared with the PSMA-negative PC-3 cell line, which could be facilitated by the binding of the PSMA receptors on the LnCap cells by the folic acid moieties on the surface of the coated nanoparticles, allowing them to enter the cells by receptor-mediated endocytosis (Flores et al. 2017a)

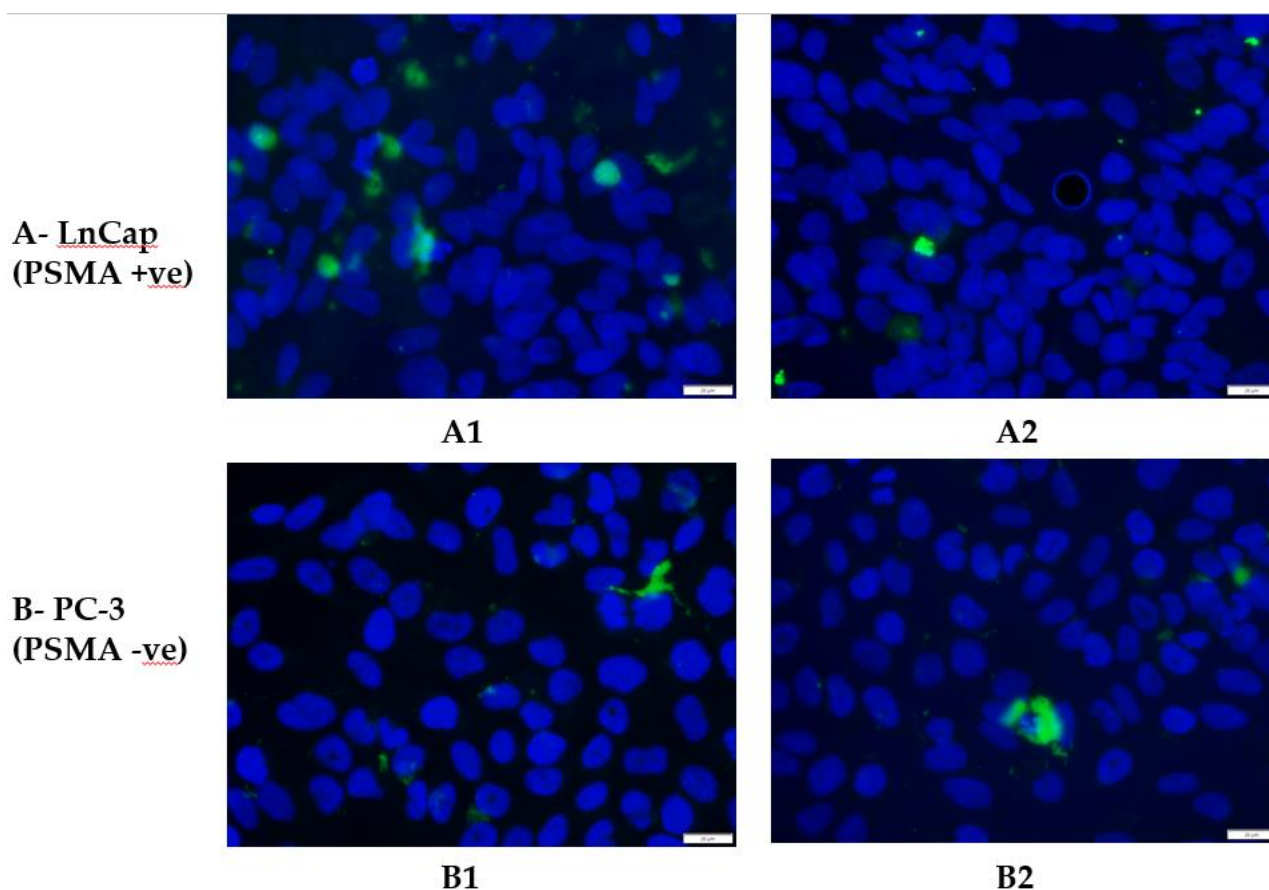


Figure 4.9 LnCap cells and B – PC-3 cells treated with coated (1) and uncoated (2) nanoparticles.

Table 4.5 Fluorescent intensities of LnCap and PC-3 cells treated with coated and uncoated nanoparticles

Cell line	Treatment	% Fluorescence
LnCap	Coated nps	2,79±0,50
	Uncoated nps	1,86±0,22
PC-3	Coated nps	1,72±0,37
	Uncoated nps	1,99±0,39

4.4 Discussion

Response surface methodology in JMP V17 with a face-centred cubic design was used to generate 16 formulation parameter combinations shown in Table 4.1. This design was

selected as the solubility limits of the materials used were pre-determined or known. Response surface methodology aims to investigate how changing certain input parameters in a process affects properties of interest in the product of that process (Bezerra et al., 2008). The amounts of quercetin, chitosan, and folic acid were varied, and expected effects were on the quercetin loading, surface charge and folic acid content properties of the nanoparticles. These properties were measured and related mathematically to the input parameters using analysis of variance (ANOVA), which also identified which input variable or combination of variables produced statistically significant changes in the measurements, within predefined confidence levels of 95% (Lee, 2019). This mathematical relationship provided us with a model to predict what input values would maximize the quercetin loading, obtain the smallest positive surface charge, and maximize the folic acid content. The optimization process included a desirability function that was used to predict the combination of input values of 10 mg of quercetin, 23 mg of chitosan and 45 mg of folic acid that would result in a product with the best possible response values of 7.02% quercetin loading, +2.52 mV surface charge and 1.24% folic acid content. The experimental values matched the predicted values closely, indicating that the model was good. However, the R^2 value of 0.85 for folic acid content (Fig 2S1.C in Appendix 2) indicates that 15% of the data is unexplained by the model, and could suggest that this response was affected by a variable that was not accounted for in this study.

Design of experiments has been used successfully in the optimization of numerous polymeric nanoparticle systems using input parameters that had the most influence on the responses of interest. The most common measured responses have been mean particle size, polydispersity index, zeta potential and entrapment efficiency (Dhas et al., 2015; Duranoğlu et al., 2018; Pandey et al., 2019). In this study quercetin loading was maximized, since it is very well tolerated, with an LD50 of 160 mg/kg bodyweight (Chen et al., 2014). It is more difficult to achieve high drug loading than high entrapment efficiency for most nano-systems (Shen et al., 2017), even though high drug loading has been associated with higher efficacy and better control over release properties (Yang et al., 2023). This focus on achieving maximum drug loading was also because this property has been found to be more significant on therapeutic effects and metabolism in *in-vivo* studies (Shen et al., 2017; Liu et al., 2020). The aim was for a small positive charge on the nanoparticles in order to promote attractive interactions with the negative cell membrane (Fröhlich, 2012) and increase cellular penetration [65,66]. Folic acid content was also maximized in order to increase the likelihood of these moieties binding to the PSMA antigen on the cell surface (Duskey and Rice, 2014; Yoo et al., 2019; Mesquita et al., 2022), thereby enabling the nanoparticles to enter cancer cells more easily.

Chu and co-workers (Chu et al., 2013) have described how nanofabrication techniques have hydrophobic drug loading limitations of approximately 10%, and a report by Lestari's group

(Lestari et al., 2022) analyzed differently sized silica nanoparticles at drug loadings of 8.9 and 10%. They showed that there was a controlled, pH dependent release of quercetin from the nanoparticle system, which is consistent with the results observed here. However, after 72 hours there was a maximum cumulative release of only 7% in acidic pH, indicating a much lower bioavailability than of what measured here (65% at 72 hours). Li and team (Li et al., 2021) have reported on a pegylated nanoliposomal system with 8.5% drug loading that displayed a sustained release pattern with a much more favourable maximum release, while similar sustained and maximum cumulative release was recorded by Davarnejad and co-workers (Davarnejad et al., 2022) who used mixed nanomicelles with a relatively low drug loading of 2.3%. However, these systems were tested at pH 7.4, modeling the release in normal physiological pH but no information can be extrapolated about the release in the characteristically acidic pH of the tumour microenvironment. Another study by Nan and team (Nan et al., 2018) describes quercetin entrapped chitosan nanoparticles with a relatively high drug loading of 13.2% for topical applications. No drug release experiments were conducted in solution, but this work also highlighted the enhanced cellular uptake of the nanoparticles due to the chitosan component. As expected, the quercetin loading measurements for all the systems tested in Table 4.2 fall in the range of the above literature reports, while the charge, drug release and biological activity of the final system is compared with reports in Table 4.6.

Baksi and team (Baksi et al., 2018) prepared quercetin entrapped chitosan nanoparticles which showed increased cytotoxicity on breast cancer cells compared to free quercetin. Interestingly, the *in-vitro* kinetics data revealed that there were much higher percentages of drug release than those observed here after 12 hours (their final time point) - they reported 67 and 76% at pH 7.4 and 5.3 respectively, compared to the values of 35 and 38% obtained here respectively at pH values of 7.4 and 6.0, indicating a prolonged release by this system. Gupta's group optimized folic acid targeted PLGA nanoparticles for skin cancer (Gupta et al., 2015), and as expected, they found similar release patterns for quercetin at pH 7.5 and 5.6. Yadav and co-workers (Yadav et al., 2022) reported on PLGA nanoparticles that caused more destruction of cellular morphology and an increased cytotoxicity on cervical and breast cancer cell lines, when compared to free quercetin. A similar trend was observed in this study regarding cytotoxicity in the PC-3 and more especially in the LnCap cell lines when comparing the non-targeted (uncoated) nanosystem to free quercetin.

Other smart nanosystems that display controlled preferential drug release at the target cancer site have been previously described (Kaskooli et al., 2020; Gu et al., 2023). Additionally, Liu's group synthesized DNA-modified nanoparticles, intercalated with doxorubicin and reported an increase of doxorubicin release from 5% to 38% after triggering the release by infra-red irradiation (Liu et al., 2023), showing promise for remotely controlled

drug release, while Barve and co-workers described polymeric micelles with enzyme responsive peptides that displayed a substantial increase in cabazitaxel release from ~10 to ~80% in the respective absence and presence of matrix metalloproteinase 2 (MMP 2), which is an enzyme that is overexpressed in the tumour microenvironment of prostate cancer (Barve et al., 2020).

Table 4.6 Comparison of fabricated PLGA-Chitosan Folic Acid System with previously reported results

System	z Potential/mV	Drug Release	Biological Activity
Silica nanoparticles	varied	7 % after 72 h	Not specified
Pegylated quercetin liposomes	-13.1	85% after 96h	Increased cytotoxicity in cervical cancer cells and greater reduction of tumour size in a mouse model compared to free quercetin
Nanomicelles	Not specified	83.6% after 120h	Increased cytotoxicity on breast cancer cells compared to free quercetin
Chitosan-quercetin nps	+22.53	76% after 12h	Increased cytotoxicity on lung and breast cancer cells and greater reduction in tumour volume in a mouse model compared to free quercetin
SPIONS	varied	Not applicable	Increased protein binding of positively charged particles
Chitosan nps	+9	100% after 24h	Not specified
Folate-linked nps	+19	70% after 4h	PSMA binding on LnCap cells

Folic Acid conjugated nps	Not specified	90% after 24h	Increased cytotoxicity of therapeutic peptide and greater cellular uptake in LnCap cells
Folic Acid minicells	Not specified	Not applicable	Increased cellular uptake in LnCap cells
PLGA-quercetin chitosan folic acid nps	+1.84	78% after 168h	Increased cytotoxicity of quercetin and greater cellular uptake in LnCap cells

Using chitosan, nanoparticles were formulated with a slightly positive surface charge of +1.84 mV, intended to minimize opsonization commonly found with highly charged nanoparticles (Xiao et al., 2011), to control release (Yadav et al., 2020), and to enhance cellular uptake (Fröhlich, 2012). However, in a study involving highly positive, slightly positive (+6 mV) and negatively charged SPIONS, an array of proteins preferentially bound to both the slightly and highly positive particles rather than the negatively charged particles (Sakulku et al., 2015). Slightly more positive (+9 mV) chitosan nanoparticles were also prepared by (Bozkir and Saka, 2004) and investigated for their drug release of plasmid DNA. The study showed complete release by 24 hours. More recently, Subramaniam and co-workers (Subramaniam et al., 2022) aimed to fabricate chitosan nanoparticles with a charge between +1 and +10 mV for increased cellular penetration. The prepared nanoparticles were +19 mV and it achieved 70% release of its payload after 4 hours at pH 7.2. Several recent studies have demonstrated the controlled drug release behaviour and enhanced cellular uptake and cytotoxicity of chitosan-based nanoparticles on cancer cells (Wang et al., 2020; Yadav et al., 2020; Escareño et al., 2021). Even though the chitosan component in the optimized nano-system in this study did provide a controlled, pH dependent release, a significant increase in cellular uptake or cytotoxicity was not observed between the uncoated and coated nanoparticles on the PSMA-negative PC-3 cell line. This could be due to the fact that all the comparable studies have been done on chitosan nanoparticles with a highly positive charge which could promote their entry into the cancer cells where they could deliver their payload in a sustained manner and thereby cause increased cytotoxicity (Foroozandeh and Aziz, 2018).

The results in this study also suggest that the increased cytotoxicity and cellular uptake observed in the PSMA positive LnCap cell line is due to the presence of the folic acid

component in the coated nanoparticles, which was maximized to 1.61%. Yao et al (Yao et al., 2010) found that folic acid was able to inhibit the enzymatic functions of PSMA, suggesting competitive binding of folic acid to PSMA, and Hattori et al (Hattori and Maitani, 2004) used folate-linked nanoparticles for DNA delivery to LnCap cells via the binding of PSMA, proposing folic acid as a ligand to target PSMA. Flores and co-workers (Flores et al., 2017b) validated the binding and internalization of folic acid using a folate conjugated fluorescently labelled probe. In agreement with the results observed in this study, they reported an increase in cytotoxicity and cellular uptake in the PSMA positive LnCap cell line compared to the PSMA negative PC-3 cell line when using a folic-acid conjugated nano-system. Jivrajani and co-workers (Jivrajani and Nivsarkar, 2016) used folic acid targeted bacterial minicells of siRNA delivery and showed a large degree of uptake using the folate targeted minicells and no observable uptake of the non-targeted minicells, but no comparison was done on a cell line without folic acid binding receptors. The results in this study used LnCap and PC-3 cell lines, both of which have very low levels of folate receptors (Hattori and Maitani, 2004) and therefore the difference in uptake can be equated to the level of PSMA expression in each cell line. There is also no significant difference in either the uptake or cytotoxicity between the uncoated (non-targeted) and coated (targeted) nano-system on the PC-3 cell line, since this cell line has low levels of PSMA expression (Hattori and Maitani, 2004) and cannot selectively take up the targeted nano-system by PSMA binding. The inverse is true about the LnCap cell line, where high PSMA-specific cytotoxicity and cellular uptake is observed.

4.5 Conclusions

This research presents the design, optimization and evaluation of a folic acid targeted nano-delivery system for prostate cancer. It was demonstrated that the optimized nano-system displayed a sustained, pH dependent release profile and increase in cancer cell uptake and toxicity, when compared to free quercetin and the corresponding non-targeted system. This system shows potential as an actively targeted carrier for prostate cancer drug delivery. Future work should include the testing of this nano-system for particle stability over time, biocompatibility and possibly another optimization process investigating the best surface charge for nanoparticles to simultaneously minimize opsonization and maximize cellular uptake.

4.6 References

Abd El Hady, W.E., Mohamed, E.A., Soliman, O.A.E.-A. & Sabbagh, H.M. el, 2019, 'In vitro–in vivo evaluation of chitosan-PLGA nanoparticles for potentiated gastric retention and anti-ulcer activity of diosmin', *International Journal of Nanomedicine*, Volume 14, 7191–7213.

Al-Nemrawi, N., Alshraideh, N., Zayed, A. & Altaani, B., 2018, 'Low Molecular Weight Chitosan-Coated PLGA Nanoparticles for Pulmonary Delivery of Tobramycin for Cystic Fibrosis', *Pharmaceuticals*, 11(1), 28.

Al-Nemrawi, N.K., Altawabeyeh, R.M. & Darweesh, R.S., 2022, 'Preparation and Characterization of Docetaxel-PLGA Nanoparticles Coated with Folic Acid-chitosan Conjugate for Cancer Treatment', *Journal of Pharmaceutical Sciences*, 111(2), 485–494.

Angelopoulou, A., Kolokithas-Ntoukas, A., Fytas, C. & Avgoustakis, K., 2019, 'Folic Acid-Functionalized, Condensed Magnetic Nanoparticles for Targeted Delivery of Doxorubicin to Tumor Cancer Cells Overexpressing the Folate Receptor', *ACS Omega*, 4(26), 22214–22227.

Anwer, M.K., Al-Mansoor, M.A., Jamil, S., Al-Shdefat, R., Ansari, M.N. & Shakeel, F., 2016, 'Development and evaluation of PLGA polymer based nanoparticles of quercetin', *International Journal of Biological Macromolecules*, 92.

Baksi, R., Singh, D.P., Borse, S.P., Rana, R., Sharma, V. & Nivsarkar, M., 2018, 'In vitro and in vivo anticancer efficacy potential of Quercetin loaded polymeric nanoparticles', *Biomedicine & Pharmacotherapy*, 106, 1513–1526.

Bellotti, E., Cascone, M.G., Barbani, N., Rossin, D., Rastaldo, R., Giachino, C. & Cristallini, C., 2021, 'Targeting Cancer Cells Overexpressing Folate Receptors with New Terpolymer-Based Nanocapsules: Toward a Novel Targeted DNA Delivery System for Cancer Therapy', *Biomedicines*, 9(9), 1275.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S. & Escaleira, L.A., 2008, 'Response surface methodology (RSM) as a tool for optimization in analytical chemistry', *Talanta*, 76(5), 965–977.

Bozkir, A. & Saka, O.M., 2004, 'Chitosan Nanoparticles for Plasmid DNA Delivery: Effect of Chitosan Molecular Structure on Formulation and Release Characteristics', *Drug Delivery*, 11(2), 107–112.

Chaudhari, S.P., Bangar, J. v., Akuskar, G.K. & Ratnaparkhi, M.P., 2014, 'Development and validation of UV spectrophotometric method for simultaneous estimation of rutin and quercetin in niosome formulation', *Der Pharmacia Lettre*, 6(3).

Chen, R., Lin, Jingan, Hong, J., Han, D., Zhang, A.D., Lan, R., Fu, L., Wu, Z., Lin, Jianhua, Zhang, W., Wang, Z., Chen, W., Chen, C. & Zhang, H., 2014, 'Potential toxicity of quercetin: The repression of mitochondrial copy number via decreased POLG expression and excessive TFAM expression in irradiated murine bone marrow', *Toxicology Reports*, 1, 450–458.

Cheng, L., Ma, H., Shao, M., Fan, Q., Lv, H., Peng, J., Hao, T., Li, D., Zhao, C. & Zong, X., 2017, 'Synthesis of folate-chitosan nanoparticles loaded with ligustrazine to target folate receptor positive cancer cells', *Molecular Medicine Reports*, 16(2), 1101–1108.

Chronopoulou, L., Massimi, M., Giardi, M.F., Cametti, C., Devirgiliis, L.C., Dentini, M. & Palocci, C., 2013, 'Chitosan-coated PLGA nanoparticles: A sustained drug release strategy for cell cultures', *Colloids and Surfaces B: Biointerfaces*, 103, 310–317.

Chu, K.S., Schorzman, A.N., Finniss, M.C., Bowerman, C.J., Peng, L., Luft, J.C., Madden, A.J., Wang, A.Z., Zamboni, W.C. & DeSimone, J.M., 2013, 'Nanoparticle drug loading as a design parameter to improve docetaxel pharmacokinetics and efficacy', *Biomaterials*, 34(33), 8424–8429.

Dandamudi, M., McLoughlin, P., Behl, G., Rani, S., Coffey, L., Chauhan, A., Kent, D. & Fitzhenry, L., 2021, 'Chitosan-Coated PLGA Nanoparticles Encapsulating Triamcinolone Acetonide as a Potential Candidate for Sustained Ocular Drug Delivery', *Pharmaceutics*, 13(10), 1590.

Davarnejad, R., Layeghy, K., Soleymani, M. & Ayazi, A., 2022, 'Encapsulation of Quercetin in a Mixed Nanomicellar System to Enhance its Cytotoxicity against Breast Cancer Cells', *Chemical Engineering & Technology*, 45(6), 1100–1105.

Dhas, N.L., Ige, P.P. & Kudarha, R.R., 2015, 'Design, optimization and in-vitro study of folic acid conjugated-chitosan functionalized PLGA nanoparticle for delivery of bicalutamide in prostate cancer', *Powder Technology*, 283, 234–245.

Donin, N.M. & Reiter, R.E., 2018, 'Why Targeting PSMA Is a Game Changer in the Management of Prostate Cancer', *Journal of Nuclear Medicine*, 59(2), 177–182.

Duranoğlu, D., Uzunoglu, D., Mansuroglu, B., Arasoglu, T. & Derman, S., 2018, 'Synthesis of hesperetin-loaded PLGA nanoparticles by two different experimental design methods and biological evaluation of optimized nanoparticles', *Nanotechnology*, 29(39), 395603.

Duskey, J.T. & Rice, K.G., 2014, 'Nanoparticle Ligand Presentation for Targeting Solid Tumors', *AAPS PharmSciTech*, 15(5), 1345–1354.

Escareño, N., Hassan, N., Kogan, M.J., Juárez, J., Topete, A. & Daneri-Navarro, A., 2021, 'Microfluidics-assisted conjugation of chitosan-coated polymeric nanoparticles with antibodies: Significance in drug release, uptake, and cytotoxicity in breast cancer cells', *Journal of Colloid and Interface Science*, 591, 440–450.

Essa, D., Kondiah, P.P.D., Choonara, Y.E. & Pillay, V., 2020, 'The Design of Poly(lactide-co-glycolide) Nanocarriers for Medical Applications', *Frontiers in Bioengineering and Biotechnology*, 8.

Fasehee, H., Dinarvand, R., Ghavamzadeh, A., Esfandyari-Manesh, M., Moradian, H., Faghihi, S. & Ghaffari, S.H., 2016, 'Delivery of disulfiram into breast cancer cells using folate-receptor-targeted PLGA-PEG nanoparticles: In vitro and in vivo investigations', *Journal of Nanobiotechnology*, 14(1).

Feng, L., Dong, Z., Tao, D., Zhang, Y. & Liu, Z., 2018a, 'The acidic tumor microenvironment: a target for smart cancer nano-theranostics', *National Science Review*, 5(2), 269–286.

Feng, L., Dong, Z., Tao, D., Zhang, Y. & Liu, Z., 2018b, 'The acidic tumor microenvironment: a target for smart cancer nano-theranostics', *National Science Review*, 5(2), 269–286.

Fissan, H., Ristig, S., Kaminski, H., Asbach, C. & Eppe, M., 2014, 'Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization', *Analytical Methods*, 6(18), 7324.

Flores, O., Santra, S., Kaftanis, C., Bassiouni, R., Khaled, A.S., Khaled, A.R., Grimm, J. & Perez, J.M., 2017a, 'PSMA-Targeted Theranostic Nanocarrier for Prostate Cancer', *Theranostics*, 7(9), 2477–2494.

Flores, O., Santra, S., Kaftanis, C., Bassiouni, R., Khaled, A.S., Khaled, A.R., Grimm, J. & Perez, J.M., 2017b, 'PSMA-Targeted Theranostic Nanocarrier for Prostate Cancer', *Theranostics*, 7(9), 2477–2494.

Foroozandeh, P. & Aziz, A.A., 2018, 'Insight into Cellular Uptake and Intracellular Trafficking of Nanoparticles', *Nanoscale Research Letters*, 13(1), 339.

Fröhlich, E., 2012, 'The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles', *International Journal of Nanomedicine*, 5577.

Gathirwa, J.W., Omwoyo, W., Ogutu, B., Oloo, F., Swai, H., Kalombo, L., Melariri, P. & Maroa, G., 2014, 'Preparation, characterization, and optimization of primaquine-loaded solid lipid nanoparticles', *International Journal of Nanomedicine*, 3865.

Ghafouri-Fard, S., Shabestari, F.A., Vaezi, S., Abak, A., Shoorei, H., Karimi, A., Taheri, M. & Basiri, A., 2021, 'Emerging impact of quercetin in the treatment of prostate cancer', *Biomedicine & Pharmacotherapy*, 138, 111548.

Gupta, A., Kaur, C.D., Saraf, Shailendra & Saraf, Swarnlata, 2015, 'Formulation, characterization, and evaluation of ligand-conjugated biodegradable quercetin nanoparticles for active targeting', *Artificial Cells, Nanomedicine, and Biotechnology*, 1–11.

Han, Y., Wen, P., Li, J. & Kataoka, K., 2022, 'Targeted nanomedicine in cisplatin-based cancer therapeutics', *Journal of Controlled Release*, 345, 709–720.

Hassan, M., Farid, D., Mahdi, A., Masood, K., Hossein, A. & Morteza, K., 2017, 'Methotrexate-Loaded PLGA Nanoparticles: Preparation, Characterization and their Cytotoxicity Effect on Human Glioblastoma U87MG Cells', *International Journal of Medical Nano Research*, 4(1).

Hattori, Y. & Maitani, Y., 2004, 'Enhanced in vitro DNA transfection efficiency by novel folate-linked nanoparticles in human prostate cancer and oral cancer', *Journal of Controlled Release*, 97(1), 173–183.

Hernández-Giottonini, K.Y., Rodríguez-Córdova, R.J., Gutiérrez-Valenzuela, C.A., Peñuñuri-Miranda, O., Zavala-Rivera, P., Guerrero-Germán, P. & Lucero-Acuña, A., 2020, 'PLGA nanoparticle preparations by emulsification and nanoprecipitation techniques: effects of formulation parameters', *RSC Advances*, 10(8), 4218–4231.

Homayouni Tabrizi, M., 2022, 'Fabrication of folic acid-conjugated chitosan-coated PLGA nanoparticles for targeted delivery of Peganum harmala smoke extract to breast cancer cells', *Nanotechnology*, 33(49), 495101.

Hu, F., Liu, W., Yan, L., Kong, F. & Wei, K., 2019, 'Optimization and characterization of poly(lactic-co-glycolic acid) nanoparticles loaded with astaxanthin and evaluation of anti-photodamage effect *in vitro*', *Royal Society Open Science*, 6(10), 191184.

Jardim, K.V., Siqueira, J.L.N., Bão, S.N. & Parize, A.L., 2022, 'In vitro cytotoxic and antioxidant evaluation of quercetin loaded in ionic cross-linked chitosan nanoparticles', *Journal of Drug Delivery Science and Technology*, 74, 103561.

Jivrajani, M. & Nivsarkar, M., 2016, 'Ligand-targeted bacterial minicells: Futuristic nano-sized drug delivery system for the efficient and cost effective delivery of shRNA to cancer cells', *Nanomedicine: Nanotechnology, Biology and Medicine*, 12(8), 2485–2498.

Lee, R., 2019, 'Statistical Design of Experiments for Screening and Optimization', *Chemie Ingenieur Technik*, 91(3), 191–200.

Lehto, U.-S., Tenhola, H., Taari, K. & Aromaa, A., 2017, 'Patients' perceptions of the negative effects following different prostate cancer treatments and the impact on psychological well-being: a nationwide survey', *British Journal of Cancer*, 116(7), 864–873.

- Lestari, W.A., Wahyuningsih, S., Gomez-Ruiz, S. & Wibowo, F.R., 2022, 'Drug loading ability and release study of various size small mesoporous silica nanoparticle as drug carrier', *Journal of Physics: Conference Series*, 2190(1), 012032.
- Li, J., Li, Z., Gao, Y., Liu, S., Li, K., Wang, S., Gao, L., Shi, M., Liu, Z., Han, Z. & Qiu, Y., 2021, 'Effect of a Drug Delivery System Made of Quercetin Formulated into PEGylation Liposomes on Cervical Carcinoma In Vitro and In Vivo', *Journal of Nanomaterials*, 2021, 1–12.
- Li, X.-Y., Li, Y.-C., Yu, D.-G., Liao, Y.-Z. & Wang, X., 2013, 'Fast Disintegrating Quercetin-Loaded Drug Delivery Systems Fabricated Using Coaxial Electrospinning', *International Journal of Molecular Sciences*, 14(11), 21647–21659.
- Lima, I.A. de, Khalil, N.M., Tominaga, T.T., Lechanteur, A., Sarmiento, B. & Mainardes, R.M., 2018, 'Mucoadhesive chitosan-coated PLGA nanoparticles for oral delivery of ferulic acid', *Artificial Cells, Nanomedicine, and Biotechnology*, 46(sup2), 993–1002.
- Lin, B., Chen, H., Liang, D., Lin, W., Qi, X., Liu, H. & Deng, X., 2019, 'Acidic pH and High-H₂O₂ Dual Tumor Microenvironment-Responsive Nanocatalytic Graphene Oxide for Cancer Selective Therapy and Recognition', *ACS Applied Materials & Interfaces*, 11(12), 11157–11166.
- Liu, Y., Yang, G., Jin, S., Xu, L. & Zhao, C., 2020, 'Development of High-Drug-Loading Nanoparticles', *ChemPlusChem*, 85(9), 2143–2157.
- Lu, B., Lv, X. & Le, Y., 2019a, 'Chitosan-Modified PLGA Nanoparticles for Control-Released Drug Delivery', *Polymers*, 11(2), 304.
- Lu, B., Lv, X. & Le, Y., 2019b, 'Chitosan-Modified PLGA Nanoparticles for Control-Released Drug Delivery', *Polymers*, 11(2), 304.
- Mesquita, B.S., Fens, M.H.A.M., Maggio, A. di, Bosman, E.D.C., Hennink, W.E., Heger, M. & Oliveira, S., 2022, 'The Impact of Nanobody Density on the Targeting Efficiency of PEGylated Liposomes', *International Journal of Molecular Sciences*, 23(23), 14974.
- Mitchell, M.J., Billingsley, M.M., Haley, R.M., Wechsler, M.E., Peppas, N.A. & Langer, R., 2021, 'Engineering precision nanoparticles for drug delivery', *Nature Reviews Drug Discovery*, 20(2), 101–124.
- Musalli, A.H., Talukdar, P.D., Roy, P., Kumar, P. & Wong, T.W., 2020, 'Folate-induced nanostructural changes of oligochitosan nanoparticles and their fate of cellular internalization by melanoma', *Carbohydrate Polymers*, 244.

Nan, W., Ding, L., Chen, H., Khan, F.U., Yu, L., Sui, X. & Shi, X., 2018, 'Topical Use of Quercetin-Loaded Chitosan Nanoparticles Against Ultraviolet B Radiation', *Frontiers in Pharmacology*, 9.

Naqvi, S., Panghal, A. & Flora, S.J.S., 2020, 'Nanotechnology: A Promising Approach for Delivery of Neuroprotective Drugs', *Frontiers in Neuroscience*, 14.

Nguyen, C.N., Tran, B.N., Thi, Hoa Nguyen, Huu, P.P. & Thi, Huong Nguyen, 2019, 'Physical absorption of folic acid and chitosan on dihydroartemisinin-loaded poly-lactic-co-glycolic acid nanoparticles via electrostatic interaction for their enhanced uptake and anticancer effect', *Journal of Nanomaterials*, 2019.

Pandey, P., Chellappan, D.K., Tambuwala, M.M., Bakshi, H.A., Dua, K. & Dureja, H., 2019, 'Central composite designed formulation, characterization and in vitro cytotoxic effect of erlotinib loaded chitosan nanoparticulate system', *International Journal of Biological Macromolecules*, 141, 596–610.

Popat, A., Liu, J., Lu, G.Q. (Max) & Qiao, S.Z., 2012, 'A pH-responsive drug delivery system based on chitosan coated mesoporous silica nanoparticles', *Journal of Materials Chemistry*, 22(22), 11173.

Riccardi, C., Napolitano, F., Montesarchio, D., Sampaolo, S. & Melone, M.A.B., 2021, 'Nanoparticle-Guided Brain Drug Delivery: Expanding the Therapeutic Approach to Neurodegenerative Diseases', *Pharmaceutics*, 13(11), 1897.

Rizvi, S.A.A. & Saleh, A.M., 2018, 'Applications of nanoparticle systems in drug delivery technology', *Saudi Pharmaceutical Journal*, 26(1), 64–70.

Saavedra-Leos, M.Z., Jordan-Alejandre, E., López-Camarillo, C., Pozos-Guillen, A., Leyva-Porras, C. & Silva-Cázares, M.B., 2021, 'Nanomaterial Complexes Enriched With Natural Compounds Used in Cancer Therapies: A Perspective for Clinical Application', *Frontiers in Oncology*, 11.

Sabourian, P., Yazdani, G., Ashraf, S.S., Frounchi, M., Mashayekhan, S., Kiani, S. & Kakkar, A., 2020, 'Effect of Physico-Chemical Properties of Nanoparticles on Their Intracellular Uptake', *International Journal of Molecular Sciences*, 21(21), 8019.

Sakulkhu, U., Mahmoudi, M., Maurizi, L., Coullerez, G., Hofmann-Antenbrink, M., Vries, M., Motazacker, M., Rezaee, F. & Hofmann, H., 2015, 'Significance of surface charge and shell material of superparamagnetic iron oxide nanoparticle (SPION) based core/shell nanoparticles on the composition of the protein corona', *Biomaterials Science*, 3(2), 265–278.

Shahid, N., Erum, A., Zaman, M., Tulain, U.R., Shoaib, Q., Malik, N.S., Kausar, R., Rashid, A. & Rehman, U., 2022, 'Synthesis and evaluation of chitosan based controlled release nanoparticles for the delivery of ticagrelor', *Designed Monomers and Polymers*, 25(1), 55–63.

Sharma, T., Singh, D., Mahapatra, A., Mohapatra, P., Sahoo, S. & Sahoo, S.K., 2022, 'Advancements in clinical translation of flavonoid nanoparticles for cancer treatment', *OpenNano*, 8, 100074.

Shen, S., Wu, Y., Liu, Y. & Wu, D., 2017, 'High drug-loading nanomedicines: progress, current status, and prospects', *International Journal of Nanomedicine*, Volume 12, 4085–4109.

Subramaniam, B., Leonick, N., Gade, V., Frey, D.M., Zhang, F., Linhardt, R.J. & Gross, R.A., 2022, 'Ionically Complexed Nanoparticles for Heparin Oral Delivery', *Precision Nanomedicine*, 5(3).

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F., 2021, 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA: A Cancer Journal for Clinicians*, 71(3), 209–249.

Tavares Luiz, M., Santos Rosa Viegas, J., Palma Abriata, J., Viegas, F., Testa Moura de Carvalho Vicentini, F., Lopes Badra Bentley, M.V., Chorilli, M., Maldonado Marchetti, J. & Tapia-Blácido, D.R., 2021, 'Design of experiments (DoE) to develop and to optimize nanoparticles as drug delivery systems', *European Journal of Pharmaceutics and Biopharmaceutics*, 165, 127–148.

Tewabe, A., Abate, A., Tamrie, M., Seyfu, A. & Abdela Siraj, E., 2021, 'Targeted Drug Delivery — From Magic Bullet to Nanomedicine: Principles, Challenges, and Future Perspectives', *Journal of Multidisciplinary Healthcare*, Volume 14, 1711–1724.

Tiğli Aydın, R.S. & Pulat, M., 2012a, '5-Fluorouracil Encapsulated Chitosan Nanoparticles for pH-Stimulated Drug Delivery: Evaluation of Controlled Release Kinetics', *Journal of Nanomaterials*, 2012, 1–10.

Tiğli Aydın, R.S. & Pulat, M., 2012b, '5-Fluorouracil Encapsulated Chitosan Nanoparticles for pH-Stimulated Drug Delivery: Evaluation of Controlled Release Kinetics', *Journal of Nanomaterials*, 2012, 1–10.

Ventola, C.L., 2017, 'Progress in Nanomedicine: Approved and Investigational Nanodrugs.', *P & T: a peer-reviewed journal for formulary management*, 42(12), 742–755.

- Vinayak, M. & Maurya, A.K., 2019, 'Quercetin Loaded Nanoparticles in Targeting Cancer: Recent Development', *Anti-Cancer Agents in Medicinal Chemistry*, 19(13), 1560–1576.
- Wang, F., Li, J., Tang, X., Huang, K. & Chen, L., 2020, 'Polyelectrolyte three layer nanoparticles of chitosan/dextran sulfate/chitosan for dual drug delivery', *Colloids and Surfaces B: Biointerfaces*, 190, 110925.
- Wilkosz, N., Łazarski, G., Kovacik, L., Gargas, P., Nowakowska, M., Jamróz, D. & Kepczynski, M., 2018, 'Molecular Insight into Drug-Loading Capacity of PEG–PLGA Nanoparticles for Itraconazole', *The Journal of Physical Chemistry B*, 122(28), 7080–7090.
- Wischke, C. & Schwendeman, S.P., 2008, 'Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles', *International Journal of Pharmaceutics*, 364(2), 298–327.
- Wolfram, J. & Ferrari, M., 2019, 'Clinical cancer nanomedicine', *Nano Today*, 25, 85–98.
- Wu, L., Zhang, J. & Watanabe, W., 2011, 'Physical and chemical stability of drug nanoparticles', *Advanced Drug Delivery Reviews*, 63(6), 456–469.
- Xiao, K., Li, Y., Luo, J., Lee, J.S., Xiao, W., Gonik, A.M., Agarwal, R.G. & Lam, K.S., 2011, 'The effect of surface charge on in vivo biodistribution of PEG-oligocholeic acid based micellar nanoparticles', *Biomaterials*, 32(13), 3435–3446.
- Xiong, G.M., Venkatraman, K. & Venkatraman, S., 2020, 'The magic bullet as cancer therapeutic—has nanotechnology failed to find its mark?', *Progress in Biomedical Engineering*, 2(4), 042004.
- Yadav, A.S., Radharani, N.N.V., Gorain, M., Bulbule, A., Shetti, D., Roy, G., Baby, T. & Kundu, G.C., 2020, 'RGD functionalized chitosan nanoparticle mediated targeted delivery of raloxifene selectively suppresses angiogenesis and tumor growth in breast cancer', *Nanoscale*, 12(19), 10664–10684.
- Yadav, N., Tripathi, A.K. & Parveen, A., 2022, 'PLGA-Quercetin Nano-Formulation Inhibits Cancer Progression via Mitochondrial Dependent Caspase-3,7 and Independent FoxO1 Activation with Concomitant PI3K/AKT Suppression', *Pharmaceutics*, 14(7), 1326.
- Yang, G., Liu, Y., Jin, S., Hui, Y., Wang, X., Xu, L., Chen, D., Weitz, D. & Zhao, C., 2023, 'Phase separation-induced nanoprecipitation for making polymer nanoparticles with high drug loading', *Aggregate*.
- Yao, V., Berkman, C.E., Choi, J.K., O'Keefe, D.S. & Bacich, D.J., 2010, 'Expression of prostate-specific membrane antigen (PSMA), increases cell folate uptake and proliferation and

suggests a novel role for PSMA in the uptake of the non-polyglutamated folate, folic acid', *The Prostate*, 70(3), 305–316.

Yoo, J., Park, C., Yi, G., Lee, D. & Koo, H., 2019, 'Active Targeting Strategies Using Biological Ligands for Nanoparticle Drug Delivery Systems', *Cancers*, 11(5), 640.

Zang, X., Cheng, M., Zhang, X. & Chen, X., 2021, 'Quercetin nanoformulations: a promising strategy for tumor therapy', *Food & Function*, 12(15), 6664–6681.

Chapter 5 Optimized Active Targeted PLGA-PEG Nanoparticles Functionalized with Anti-PSMA Antibody Provide Controlled Release of Docetaxel and Increased Uptake and Cytotoxicity in Prostate Cancer Cells

Abstract

Polymeric nanoparticles have been well researched as drug delivery systems for chemotherapeutic drugs as their properties are tuneable for the entrapment and controlled release of many active pharmaceutical ingredients. Here, the development of an active- and passively targeted nano-system for the delivery of docetaxel is described. The system was composed of poly (lactic -co-glycolic) acid, poly (ethylene glycol) and an antibody that effectively targets prostate specific membrane antigen, a receptor highly expressed on the surface of the majority of prostate cancer cells. The ratio of polymers, amount of docetaxel and amount of antibody was varied, while the yield of production, drug loading and antibody conjugation efficiency was measured. The optimized system showed a yield of $63,2 \pm 1,1\%$, a drug loading of $11,5 \pm 0,66\%$ and antibody conjugation efficiency of $74,3 \pm 4,6\%$. The system also demonstrated uniform spherical morphology and favourable size and stability characteristics, while the surface and chemical analysis show efficient entrapment of docetaxel within the nanoparticle core. The in-vitro release kinetic study showed a pH dependent $72,2 \pm 5,5\%$ maximum release of docetaxel after 72H, while the *in-vitro* toxicity and uptake assays showed a high selectivity of the targeted system towards LnCap, a prostate cancer cell line that highly expresses PSMA. There was a 43% and 71% increase in toxicity by the targeted compared to the non-targeted system and free docetaxel, respectively, on LnCap and a 60% increase in toxicity compared with a cancer cell line that expresses low levels of PSMA (PC-3). The optimized nano-system's favourable pharmacological profile shows promise for the development of a more effective targeted delivery vehicle for docetaxel in prostate cancer.

5.1 Introduction

Prostate cancer is one of the primary causes of cancer related deaths, and according to a recent report of 1 414 259 new cases globally, it is also one of the most frequently diagnosed malignancies (Sung et al. 2021). Treatment includes radiation, surgery and chemotherapy (Moradi et al. 2022), with docetaxel being one of the most commonly prescribed FDA – approved drugs for prostate cancer. As a conventional chemotherapeutic, the anti-cancer effect of docetaxel is primarily based on the difference in proliferation rate between cancer cells and non-malignant cells (Sazonova et al. 2021) . However, this approach results in

significant toxicity to healthy tissue and a large reduction in the therapeutic index of the drug (Dostalova et al. 2016). The systemic toxicity and reported side effects of docetaxel, which include cardiac disorders, bone marrow suppression, alopecia and peripheral neuropathy (Sanna et al. 2011; Autio et al. 2018) , limit the dose and duration of treatment (Sanna et al. 2011) and therefore, are detrimental to the clinical effectiveness of the drug (Hrkach et al. 2012). Additionally, the clinical formulation of docetaxel includes an ethanol/tween80 solvent system to counter the poor water solubility of the drug. However, this formulation is reported to cause hypersensitivity reactions and further reduces the efficacy and increases off-target losses of the drug (Sanna et al. 2011) .

There has been increasing research into the role of nanoparticles for chemotherapeutic drug delivery to tackle the challenges of low efficacy, safety and specificity (Moradi et al. 2022). These nano-sized carriers work as vehicles to improve the solubility, reduce the side effects and increase the proportion of drug that reaches the cancer site. The small size of these nanoparticles enables “passive targeting”, allowing for increased access to tumour tissue via its irregularly dilated vasculature (Dostalova et al. 2016) , while the high surface to volume ratio of the nanoparticles is exploited to increase interactions with cancer cells and control drug release (CHENG et al. 2007; Moradi et al. 2022). Poly(lactic-co-glycolic) acid (PLGA) is a FDA approved polymer that has been used in numerous formulations of polymeric nano-delivery systems due to its favourable biocompatibility and biodegradability and its ability to increase the physicochemical stability and release profile of poorly soluble drugs (Badran et al. 2018) . Poly(ethylene) glycol (PEG) is another polymer that has been used in nano-systems to improve solubility and increase circulation time. It imparts “stealth” properties to the system, shielding it from immune proteins, which would otherwise recognize the naked nanoparticles, and quickly opsonize them, removing them from circulation, for elimination in the liver (Gu et al. 2008; Rafiei & Haddadi 2017).

Smart nanoparticles are designed to function as nano-delivery systems that respond to certain stimuli and thereafter aggregate at target sites before releasing drug (Sun et al. 2023) and this design is used to increase the efficacy and direct delivery of drug to cancer cells, while minimizing the effect on surrounding healthy tissue. The attachment of targeting ligands such as antibodies, peptides or small molecules to the surface of nano-carriers enables “active targeting”, whereby the targeting moiety selectively binds to receptors found on cancer cells, and thereafter the nano-system is permitted to enter the cell where it releases its drug payload (Gu et al. 2008; Dostalova et al. 2016). The use of antibodies as targeting ligands in biomaterial-based nano-carriers is an effective strategy as the affinity, specificity, and activity of antibodies has proved to be superior to other targeting agents (Lee et al. 2021) . Moreover, the advancements in large scale production of antibodies have made them a feasible option

as targeting moieties for clinical applications (Rivero-Buceta et al. 2019; Lee et al. 2021) . However, for the successful clinical translation of nano-sized drug delivery systems, the biophysicochemical properties as well as the fabrication process parameters of the nano-systems should fall within a narrow margin for reproducibility and precision (Gu et al. 2008) . Design of experiments is a useful tool to tackle the optimization of key parameters in nano-formulation, especially since many of these parameters are interdependent and impact each other in a complex manner (Hrkach et al. 2012). The design of experiments approach uses statistical tools to evaluate the interactions between the parameters and makes it possible to balance out their effects on each other (Gu et al. 2008) and to calculate an optimal solution that maximizes the “desirability” of the system, with preset formulation goals, while reducing the investments of substantial time and money which are usually required with the conventional “one factor at a time” approach (Rafiei & Haddadi 2017).

Previous studies used prostate specific membrane antigen (PSMA) as a biological target for prostate cancer, as it is highly expressed in prostate cancer cells but minimally expressed in healthy tissue (Dai et al. 2021; Zhang et al. 2022; Essa et al. 2023). The microfluidics method of fabrication (Chapter 3) also proved to be superior to a popular traditional method of nanoparticle preparation for a passively targeted nano-system (Essa et al. 2020). In this study, a system designed for both passive and active targeting of prostate cancer cells is reported, with PLGA as a controlled release polymer for the chemotherapeutic drug docetaxel, PEG as a hydrophilic anti-biofouling agent and an anti-PSMA antibody as a ligand to target PSMA. A strategy is presented for the optimization of this system based on the variation of its particle makeup while setting the yield of production, drug loading, and antibody conjugation efficiency to experimentally relevant targets. The optimized formulation was then characterised in terms of morphology, size and stability, surface and thermal properties. Finally, the system was evaluated for its safety on non-malignant cells and its antiproliferative activity using *in-vitro* toxicity assays, as well as fluorescence-based cellular uptake studies on prostate cancer cell lines PC-3, which expresses low levels of PSMA, and LnCap, a line that highly expresses PSMA.

5.2 Materials and Methods

5.2.1 Materials

The polymeric materials Poly(D,L-lactide-co-glycolide)(PLGA) (lactide/glycolide ratio of 50:50), molecular weight 7 000-17 000 with acid end groups, carboxylic acid polyethylene glycol PLGA (carboxylPEGPLGA), Pluronic F127, MES free acid, TRIS, EDC, NHSS, Anti-PSMA antibody, hydroxylamine hydrochloride, sodium hydroxide pellets (NaOH), hydrochloric acid, dialysis tubing (MWCO 14KDa), PBS buffer tablets (pH 7.4), tween80, acetonitrile,

Bradford reagent and fluorescein iso-thiocyanate (FITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Pty) Ltd, Estate South, Modderfontein, Gauteng, SA. Docetaxel was obtained from DLD Scientific, Durban South Africa. HPLC grade acetonitrile and methanol were purchased from Microsep.

5.2.2 *Design of Experiments and Preparation of Nanoparticles*

The targeted nano-system was optimized by design of experiments, using the ratio of polymers PLGA: PLGA-PEG, the amount of docetaxel, and the volume of antibody as input factors, while the measured responses were the total yield of nanoparticles, the drug loading, and the antibody conjugation efficiency. The yield of production and antibody conjugation efficiency were chosen as responses to be maximized while a target of 10% drug loading (Lu et al. 2020) was set using JMP V17 software's response surface design with an on face central composite design and two centre points to generate 16 formulations, listed in Table 5.1.

Table 5.1. Formulations generated by JMP software with a range of ratios of PLGA:PLGA-PEG (P:PP ratio), amount of docetaxel (Doc) and volume of antibody (AB).

Table 5.1 Formulations generated by JMP software with a range of ratios of PLGA:PLGA-PEG (P:PP ratio), amount of docetaxel (Doc) and volume of antibody (AB).

Formulation	Parameters	P:PP ratio	Doc/mg	AB/ μ L
P1	(0,-1,0)	1,5	3	10
P2	(1,-1,1)	2	3	15
P3	(1,0,0)	2	4,5	10
P4	(-1,1,1)	1	6	15
P5	(0,1,0)	1,5	6	10
P6	(1,1,-1)	2	6	5
P7	(1,-1,-1)	2	3	5
P8	(0,0,1)	1,5	4,5	15
P9	(-1,-1,1)	1	3	15
P10	(0,0,-1)	1,5	4,5	5
P11	(-1,1,-1)	1	6	5

P12	(-1,-1,-1)	1	3	5
P13	(0,0,0)	1,5	4,5	10
P14	(1,1,1)	2	6	15
P15	(-1,0,0)	1	4,5	10
P16	(0,0,0)	1,5	4,5	10

The nanoparticles were prepared using a method modified previously in chapter 3 (Essa et al. 2020). Briefly, the NanoAssemblr Benchtop (Precision Nanosystems Inc., Vancouver, BC, Canada), with a flow rate of 8mL/min and a flow rate ratio of 3:1 aqueous to organic phase was used, with the organic phase being composed of either PLGA only, or a mixture of PLGA: PLGA-PEG-COOH (at a concentration of 10 mg/mL of total polymer) and docetaxel in acetonitrile. The aqueous phase comprised of 0.1% Pluronic F127 solution. After nanoparticle formation, the mixture was evaporated at room temperature for 4 hours after which the unreacted materials including excess drug were removed from the dispersion by 3 x centrifugation at 12 000rpm for 30 minutes each, washing with Millipore water after each step. The nanoparticle pellets formed with PLGA-docetaxel only, designated “non-targeting nanoparticles” (NTN) were frozen at -80 °C for 24 hours and then freeze dried (Freezone 12 lyophilizer, Labcono, Kansas City, USA) for 24 hours. The PLGA: PLGA-PEG-COOH-docetaxel nanoparticle pellets were used for antibody conjugation, detailed below.

5.2.3 Conjugation of antibody

The attachment of the antibody to the surface of the nanoparticles was conducted according to a method modified from (Gu et al. 2008). PLGA: PLGA-PEG-COOH pellets were re-dispersed in MES buffer (pH 6), centrifuged and then washed and resuspended three times in this buffer. 8 mg and 1.1 mg equivalents of EDC and hydroxysulfosuccinimide in water and MES respectively were prepared and added to the nanoparticle suspension, which was vortexed and subsequently incubated for 12 hours at room temperature on a rotary wheel. Thereafter the suspension was washed thrice with MES buffer and resuspended in 990 uL of MES. 10 uL of anti-PSMA antibody was added and stirred overnight at room temperature. The reaction was quenched with hydroxylamine (final concentration 10mM) for 30 min. The reaction mixture was then centrifuged and the supernatant kept to assay the remaining antibody after conjugation. The nanoparticles were resuspended in TRIS blocking buffer (pH 8) and incubated for 8 H at room temperature and thereafter washed three times with TRIS buffer, and three times with millipore water consecutively. Samples were then frozen at -80°C

and then freeze dried (Freezone 12 lyophilizer, Labcono, Kansas City, USA) for 24 hours. These PLGA: PLGA-PEG-COO-Ab-docetaxel nanoparticles were designated “Active targeting nanoparticles” (ATN).

5.2.4 *Particle size diameter, polydispersity index and zeta potential analysis using dynamic light scattering*

Nanoparticle suspensions were prepared by adding distilled water to lyophilized product. Sample size, dispersity, and zeta potential were analyzed using the ZetaSizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) particle size analyser. Plastic cuvettes were used to measure particle size diameter and polydispersity index, while folded capillary cells were used for zeta potential measurements (Malvern Instruments Ltd., Malvern, Worcestershire, UK)

5.2.5 *Determination of Antibody Conjugation Efficiency by the Pierce Bradford Assay*

The antibody conjugation efficiency was determined using the Bradford assay, modified from (Rivero-Buceta et al. 2019). The supernatant obtained after centrifuging the antibody-conjugated nanoparticles were tested for protein content. Briefly, 150 μ L of samples and standards were aliquoted into a 96 well plate and treated with Bradford reagent. Thereafter the absorbance was read at 595nm (Spectramax ID3, Molecular Devices LLC, San Jose, USA) and the protein concentration in the supernatant was calculated using a calibration curve. The antibody conjugation efficiency was calculated as follows:

$$(Ab_{conc} \text{ initially added} - Ab_{conc} \text{ in supernatant}) / Ab_{conc} \text{ initially added} \times 100\%.$$

Where Ab_{conc} = concentration of anti-PSMA antibody

5.2.6 *Fourier Transform Infra-Red (FTIR) analysis shows characteristic peaks*

The spectra of the starting materials and nanoparticle systems were recorded and analyzed using a PerkinElmer Inc. (Waltham, Massachusetts, USA) spectrometer, with a single reflection diamond MIRTGS detector. A universal attenuated total reflectance (ATR) polarization accessory, set at a resolution of 4 cm^{-1} , with a constant pressure of 110 psi was used to generate the graphs. The characteristic peaks of the nano-systems and starting materials were compared to confirm structures.

5.2.7 *Investigation of thermal degradation by thermogravimetric analysis (TGA)*

Samples in the weight range of 5-10 mg were loaded into a thermogravimetric analyser (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Samples were permitted to reach 30 °C and then heated at a rate of 10 °Cmin⁻¹ to 950°C. A nitrogen gas environment was maintained for the samples by constant purging for the duration of the analysis. The spectra provided information about the temperature ranges of mass degradation of the nano-systems and polymers.

5.2.8 *Powder X-ray diffraction (XRD) to examine crystallinity and surface properties*

Samples were ground into fine powders using a mortar and pestle and were then onto a sample holder for analysis on a benchtop MiniFlex 600 (Rigaku, Japan) powder diffractometer, with CuK α radiation at 40 kV and 15 mA. A 2 θ scan range of 10-90 degrees at a scan rate of 10 °min⁻¹ was used to record the spectra. The data from these spectra provided insight about the degree of crystallinity or amorphousness of the starting materials and nano-systems and information about their surface structure.

5.2.9 *Differential scanning calorimetry (DSC) to measure temperature of phase changes*

Samples in the weight range of 3-5 mg were placed in aluminium crucibles and crimped shut. An opening was pierced on the top of the pan for pressure release and the samples were then sequentially loaded into a differential scanning calorimeter (DSC) (Mettler Toledo, DSC, STAReSystem, Schwerzenback, ZH, Switzerland). The samples were heated from 0 to 400°C, at a heating rate of 10 °Cmin⁻¹, being maintained in an inert atmosphere by constant purging of nitrogen gas. These analyses provided information about the temperature at which phase changes occur and the energy dynamics involved in those changes. Hereby, the differences in the thermal properties of the nano-systems and starting materials were evaluated.

5.2.10 *Scanning Electron Microscopy (SEM) of Sample Suspensions*

Freshly prepared nanoparticle suspensions were diluted and dropped onto aluminium stubs and dried overnight under vacuum. Once dry, they were sputter coated with a fine layer of gold. SEM images were recorded using a ZEISS SIGMA 03-39 Field Emission Scanning Electron Microscope at 5 – 15kV acceleration voltage under an inert argon atmosphere.

5.2.11 *HPLC Analysis of Docetaxel Loading in Nanoparticle Systems*

Nanoparticles were dissolved in 0.5 mL acetonitrile and 1 mL methanol was then added to precipitate the polymers. The samples were centrifuged at 15 000 rpm for 5 min and the supernatant read using HPLC with a Waters 1525 Binary Pump and UV/Visible detector and paclitaxel as an internal standard, using a method adapted from (Kim et al. 2017). A C18 column of 250 mm in length with 4.6mm id and 10µm particle size, and a mobile phase of 55:45 Acetonitrile:Water was used. The flow rate was 1mL/min and wavelength set at 232nm for docetaxel. Drug loading was calculated as follows:

$$DL = \text{mass of docetaxel} / \text{mass of sample} \times 100\% \dots \text{Equation 5.1}$$

5.2.12 *In vitro release of Docetaxel*

In order to mimic the normal biological pH and the pH of the tumour microenvironment, drug release studies were performed at pH 7.4 and 6.0 respectively (Feng et al. 2018). 10 mg of nanoparticles were suspended in 2 mL of PBS, loaded into a dialysis bag which was then placed in 50 mL of PBS containing 0.5% (v/v) tween80 and incubated in an orbital shaker at 37 °C. At specific time points, 1 mL of release medium was removed and replenished with an equal volume of the fresh medium. Samples were diluted with methanol, spiked with paclitaxel, and filtered before analysis on HPLC as above.

5.2.13 *Cell Culture Conditions*

NIH-3T3 fibroblasts, PC-3 and LnCap prostate carcinoma cells were purchased from Cellonex (Johannesburg, South Africa). Cells were confirmed to be free of mycoplasma by the supplier. 3T3-NIH and PC-3 cells were cultured in RPMI, while LnCap cells were grown in DMEM/F12 culture medium. The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

5.2.14 *Cytotoxicity Using the MTT Assay*

When confluent, PC-3 and LnCap cells were seeded in 96 well plates at a cell density of 5×10^3 cells/well. After 24 hours, the cells were confirmed to be attached to the culture plate and were treated with docetaxel, and docetaxel-loaded NTN's and ATN's. 3T3-NIH cells were seeded at a density of 3×10^3 cells/well and treated with unloaded nanoparticles as a toxicity control.

Cell viability was measured spectrophotometrically using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay, 48 and 72 hours after treatment.

5.2.15 *Investigation of Cellular Uptake Using FITC-loaded Nanoparticles*

PC-3 and LnCap cells were seeded in 6 well plates at a cell density of 1×10^5 cells/well on sterile coverslips. NTN's and ATN's were prepared with FITC in a 1:1 docetaxel:FITC ratio. Post attachment, both cell lines were treated with FITC-loaded- NTN's and ATN's. Untreated cells and cells treated with systems with no FITC were used as controls. Twenty-four hours after treatment, cells were rinsed thrice with PBS and fixed with 4 % paraformaldehyde. The nuclei of the cells were stained with DAPI. Cells were then mounted on glass slides and stored in the dark before viewing and imaging on a Leica DMI8 Inverted Microscope (Leica Microsystems GmbH, Wetzlar, Germany).

5.2.16 *Statistical Analysis*

All experiments for preparation and characterization of nanoparticles by dynamic light scattering, drug loading and release, as well as cytotoxicity were conducted in triplicate and the data obtained were processed and analyzed by Origin software (version 8.5.0 SR1, OriginLab Corporation, U.S.). Results were presented by the mean $\pm$ standard error. Differences in results between data sets of experimental groups were deemed significant with a p value $< 0,05$, using Origin's one way analysis of variance (ANOVA).

5.3 Results and Discussion

5.3.1 *Optimization of Targeted Nano-system using a Face Centred Cubic Design*

Using the formulations listed in Table 5.1, the yield, drug loading and antibody conjugation efficiency responses were measured and the percentages are shown in Table 5.2. The amount of drug added, the P:PP ratio and the amount of antibody added were all shown to have significant ($p > 0,05$) interactions with each other, as reflected in Figure 3S1 in Appendix 3. The actual vs predicted plots show a high degree of correlation, with R^2 values between 0.82 and 0.9 (Figure 3S2, Appendix 3) and the residual plots demonstrate the data follow a normal distribution (Figure 3S3, Appendix 3).

Table 5.2 Responses obtained using varying amounts of PLGA: PEG, docetaxel and antibody, where Yield = percent of total nanoparticle product, DL = drug loading percent, ACE = percent antibody conjugation efficiency. Levels -1, 0 and 1 correspond to parameters in Table 5.1

Formulation	Parameters	Yield/ %	DL/ %	ACE/ %
P1	(0,-1,0)	68,1	10,8	74,7
P2	(1,-1,1)	41,2	10,8	40,0
P3	(1,0,0)	58,5	11,0	50,2
P4	(-1,1,1)	33,0	13,6	35,8
P5	(0,1,0)	70,8	10,8	66,0
P6	(1,1,-1)	41,1	19,3	62,5
P7	(1,-1,-1)	38,2	10,5	61,4
P8	(0,0,1)	81,7	11,8	50,7
P9	(-1,-1,1)	31,3	12,8	38,8
P10	(0,0,-1)	43,8	11,0	69,4
P11	(-1,1,-1)	34,7	13,7	55,4
P12	(-1,-1,-1)	27,2	13,2	66,8
P13	(0,0,0)	70,0	11,3	77,6
P14	(1,1,1)	61,4	13,2	34,8
P15	(-1,0,0)	37,8	11,5	73,0
P16	(0,0,0)	73,0	11,8	82,4

5.3.1.1. Analysis of Responses Showing Effects of Factors on Nano-system Characteristics

The variations in drug loading, yield and antibody conjugation efficiency with the change in input factors (initial amount of drug, P:PP ratio and initial amount of antibody) are shown in Figure 5.2. The increase in drug loading is directly impacted by the initial amount of drug added, as this factor increases, so does the drug loading in an almost linear fashion. The drug loading also decreases as the P:PP ratio increases, and this could be due to the fact that as the P:PP ratio increases, the number of hydrophilic poly(ethylene) end groups decreases leading to a reduction in the stability of the amphiphilic structure in solution, and consequently,

a lower amount of drug in the hydrophobic core (Zhu 2013; Bose et al. 2021). The drug loading also decreases with increasing amount of antibody added, as this adds to the total mass of nanoparticle yield, and therefore decreases the percentage of drug in the total system. The yield of the nanoparticle preparation increases at first with increasing P:PP ratio. This could be due to the fact that the product containing a larger ratio of PLGA to PEG is less soluble and therefore a larger amount can be precipitated in solution (Fasehee et al. 2016; Sanna et al. 2017). However, as the ratio increases from 1.5 to 2, the yield decreases, and, again, this could be due to the decrease in structure stability. The yield also increases marginally with increasing amounts of drug and antibody added, and this is due to the increase of the total mass of product. Interestingly, the antibody conjugation efficiency increases with increasing P:PP ratio, which is unexpected as the decrease in PEG groups would, in theory, decrease the number of sites for the antibody to conjugate to. However, the solubility of the nano-system could be a contributing factor, as the maximum antibody conjugation efficiency is achieved with a 1.5 ratio of P:PP, which is very close to the maximum yield obtained with a ratio of 1.6. As expected, increasing the antibody amount increases the conjugation efficiency until a point where it can be concluded that the maximum number of conjugation sites are occupied.

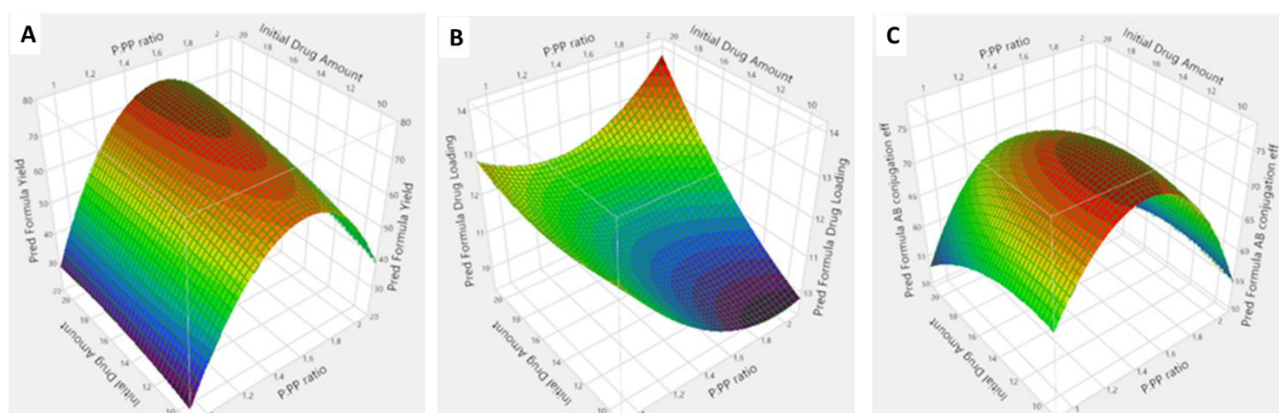


Figure 5.1 Response surface plots showing yield (A), drug loading (B), and antibody conjugation efficiency (C) variation with different PLGA: PLGA-PEG ratios, initial docetaxel amounts, and initial antibody amounts. Obtained from JMP V17.

In this study, the drug loading was optimized at an intermediate level of 10% per mass, to achieve the balance between feasible administration (Liu et al. 2020) and particle stability properties (CHENG et al. 2007). Yield was maximized for the most efficient preparation method and antibody conjugation was maximised so that the most targeting moieties would be available to bind to the PSMA receptors on the prostate cancer cell surface.

Using the prediction profiler in JMP V17, the desirability was maximized using these targets and a predicted optimized formulation shown in Figure 5.3 was obtained. Using these parameters, the optimized formulation was prepared, and the values for yield, drug loading and antibody conjugation efficiency (shown by Table 3) were contained within the limits in the prediction profiler (Figure 3). Additionally, the bias for each predicted response was less than $\pm 8\%$ (Table 3), showing the close correlation of the predicted model to the actual responses of the nanoparticle system. This optimized formulation of actively targeted nanoparticles (ATN) was used for all further experiments.

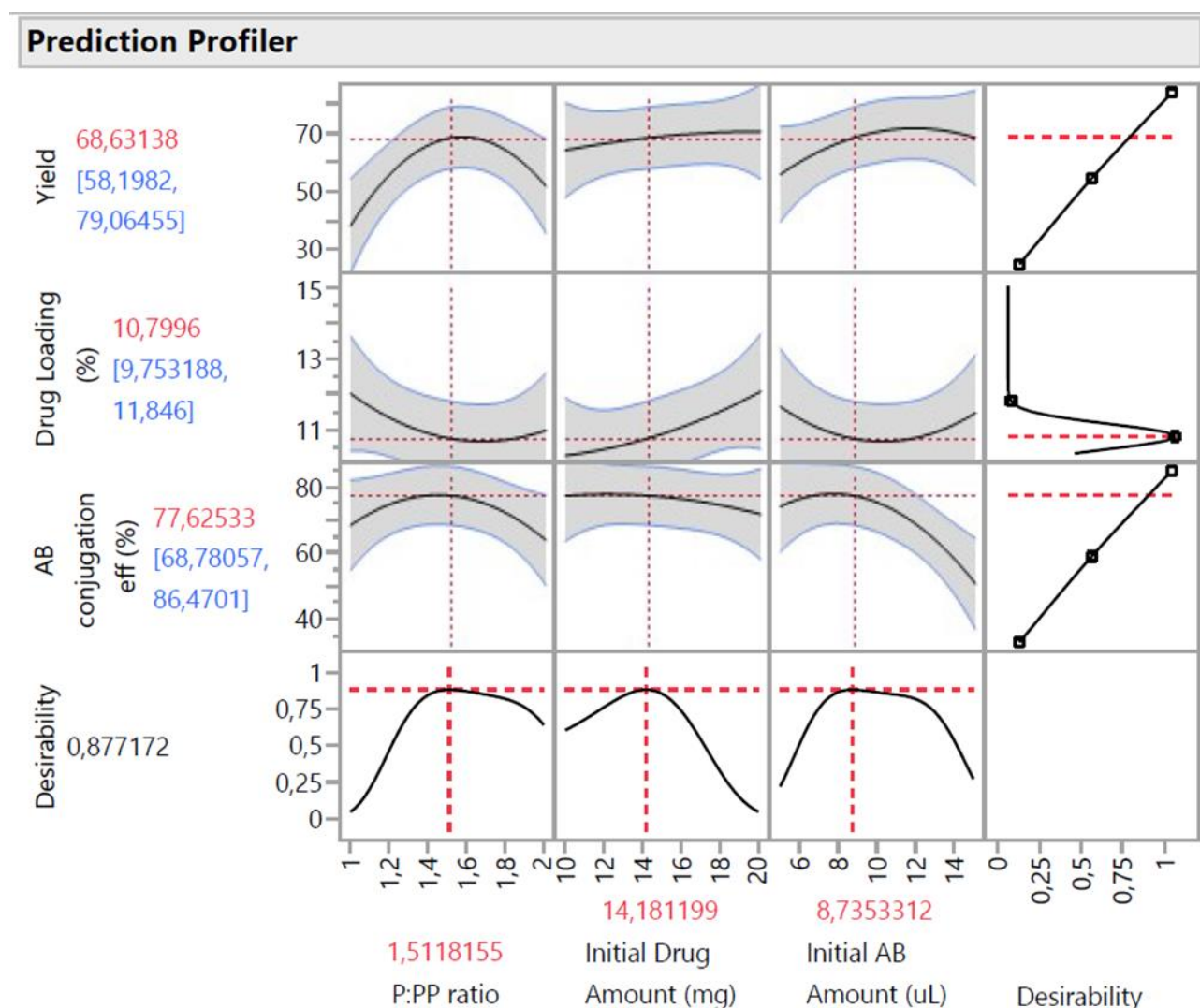


Figure 5.2 Prediction profiler showing optimized solution with maximum desirability Of 0,877 – from JMP V17.

Table 5.3 Comparison of actual results vs predicted results using optimized formulation parameters of PLGA: PLGA-PEG ratios, docetaxel, and antibody amounts.

Response	Predicted/%	Actual/%	Bias/%
Yield	68,6	63,2 $\pm$ 1,1	+7,9
Drug Loading	10,8	11,5 $\pm$ 0,66	-6,5
AB Conjugation Eff	77,6	74,3 $\pm$ 4,55	+4,3

5.3.2 Efficient preparation of nanoparticles and conjugation of antibody

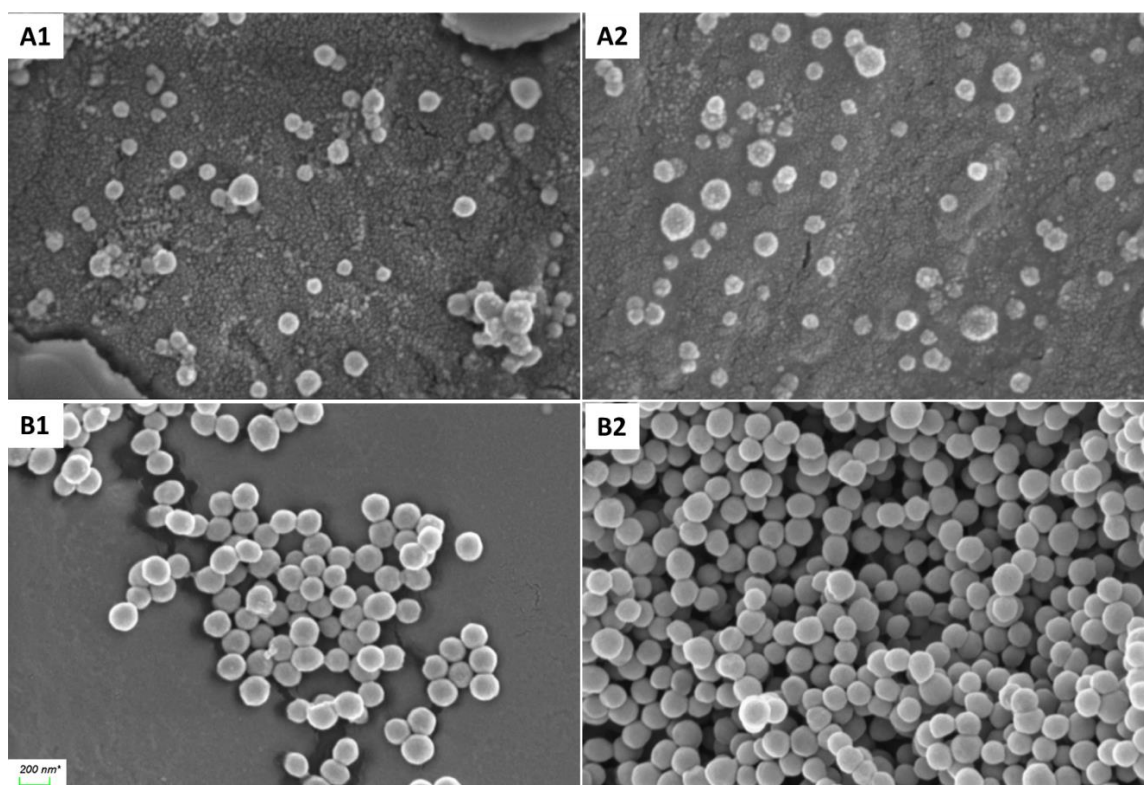


Figure 5.3 Scanning Electron Micrographs of A1 and A2 - unloaded and loaded non targeted nanoparticles (NTN) and B1 and B2 – unloaded and loaded actively targeted nanoparticles (ATN)

Table 5.4 Size, Stability and Drug Loading Properties of Non-targeted and targeted nanoparticles where PDI = polydispersity index and DL = drug loading

System	Size/nm	PDI	z Potential/mV	DL/%
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Unloaded NTNs	102,7 ±0,26	0,132 ±0,02	-19,0 ±1,15	-
Unloaded ATNs	146,0 ±1,4	0,056 ±0,01	-36,0 ±0,31	-
Loaded NTNs	120,4 ±1,8	0,196 ±0,01	-27,6 ±0,2	11,0 ±0,36
Loaded ATNs	160,5 ±4,2	0,130±0,05	-28,1 ±1,3	11,5 ±0,66

The PLGA acid termini were activated with EDC for conjugation with bifunctional PEG. After the preparation of the nanoparticles, the acid end groups on the PEG chain were activated in order to conjugate the amine groups on the antibody. It was done this way instead of activating the acid end groups on the antibody, which could result in internal conjugation of the antibody before reacting with the nanoparticle surface (Lu et al. 2021) . Antibody conjugation efficiency was calculated to be 74,3 ±4,55%, indicating an efficient activation and coupling process.

Table 5.4 shows the hydrodynamic size, dispersity, surface potential, and, in the case of the loaded nano-systems, the drug loading properties of the different types of nanoparticles prepared. There was an increase in size upon loading the nanoparticles with docetaxel, due to the inclusion of the drug molecules within the particle core (Table 5.4), which corresponds to the scanning electron micrographs of the loaded systems in Figure 3S4 in Appendix 3. There was also an increase in size between the NTN and ATN nanosystems, and this can be attributed to the end groups of PEG and antibody on the ATN system, adding to the size of the NTN system. The ζ potential values of both systems are negative (Table 5.4 and Figure S5 in Appendix 3), due to the negatively charged carboxylic acid end groups of PLGA on the NTN system and the negatively charged antibody on the surface of the ATN system. The ζ potential values of both loaded and unloaded ATN particles were -28 to -37mV, generally classified as moderately to highly stable with regard to colloid stability (Bhattacharjee 2016). The SEM images (Figure 5.4) shows a spherical particle morphology with < 200nm diameter, which correlates with the dynamic light scattering measurements in Table 5.4. Figure 4.4 also reveals that the both the loaded and unloaded ATN system self-assembled into more uniform particles than the NTN system, and this could be due to the increased stability imparted to the ATN system by the hydrophilic PEG groups (Betancourt et al. 2009) . The microfluidics parameters were also previously optimized for the amphiphilic structure of the ATN system, and this method has been reported to produce particles with a very uniform size distribution (Choi et al. 2009; Bezelya, Küçüktürkmen & Bozkır 2023).

Zumaya and co-workers have also reported measureable size increases after antibody conjugation of PLGA nanoparticles loaded with oxaliplatin for colorectal cancer (Zumaya et al. 2022), while Arslan's group have reported both size and surface charge increase after

conjugating CD56 antibody for small cell lung cancer (Arslan et al. 2023). However, they were not able to reproduce the uniform particle distribution of their unconjugated nanoparticles, while in this study, low dispersity was conserved (Table 5.4) and particle uniformity was improved (Figure 5.4) after nanoparticle conjugation. This can be attributed to the precise method of preparation (Bezelya et al. 2023) and the optimization of the particle parameters (Rafiei & Haddadi 2017).

5.3.3 Physical Characterisation of Nanosystems Shows Conjugation of Antibody

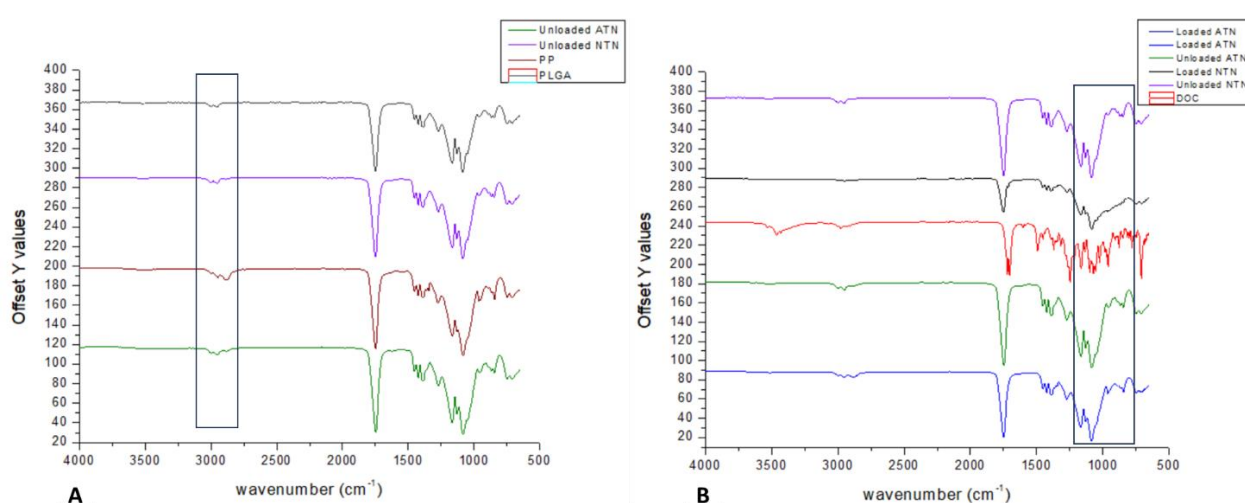


Figure 5.4 FTIR spectra of A- unloaded non targeted nanoparticles (NTN), unloaded actively targeted nanoparticles (ATN) and their constituent pristine polymers, PLGA and PLGA-PEG. B– comparison of the FTIR spectra of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC)

The PLGA spectrum shows characteristic strong bands at 1750 cm^{-1} and 1094 cm^{-1} due to C=O of the carboxylic acid end groups and C–O–C stretching of the ether backbone, respectively. C–H bending at $1200\text{--}1400\text{ cm}^{-1}$, and small bands due to linear C–H stretching vibrations at ~ 2990 and 3000 cm^{-1} . The PLGA-PEG spectrum reflects the structural similarity to PLGA, exhibiting the same bands at the same positions. There is an extra small band around 2860 cm^{-1} due to the asymmetric C-H stretching found in the poly(ethylene)glycol constituent of this polymer(Shitole et al. 2020) . The spectrum of the unloaded NTN system is exactly the same as PLGA, its constituent polymer while the spectrum of the unloaded ATN system shows a small band at 2860 cm^{-1} due to the PLGA-PEG constituent. The size of the band is consistent with the fact that it is a minor component in the formulation of this nano-system. This corresponds with previous reports on similar systems(Ingalls 2019; Tran et al.

2021) . Figure 5.5B shows that the loaded ATN has exactly the same spectrum as that of the unloaded ATN while the loaded NTN shows some obscured peaks in the fingerprint region of $750 - 1100\text{ cm}^{-1}$, possibly due to some interference by the loaded docetaxel which shows characteristic bands in this region.

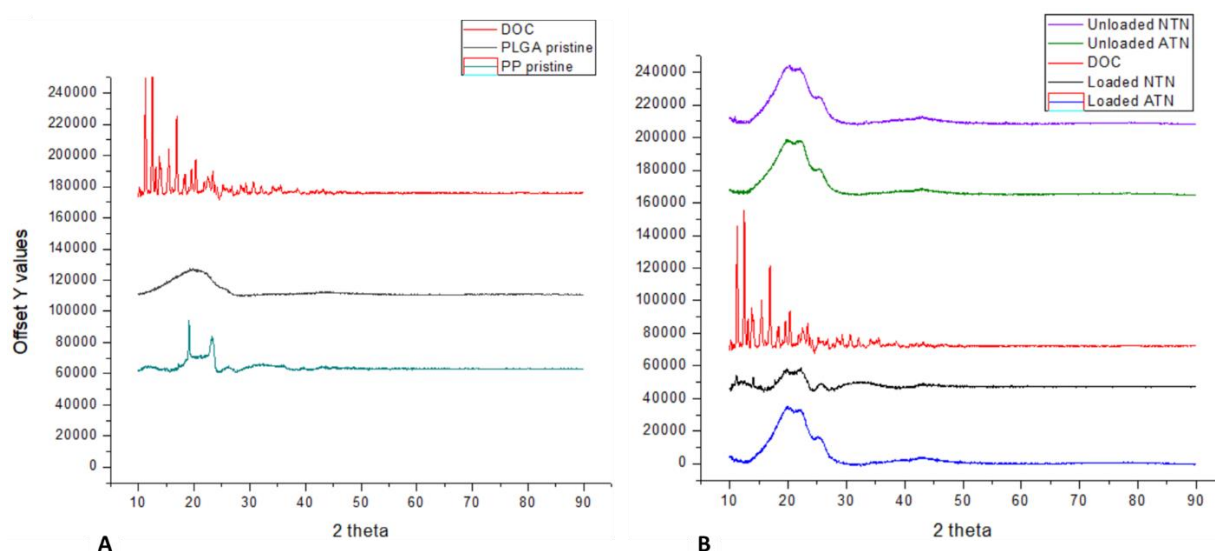


Figure 5.5 XRD spectra of A- Docetaxel (DOC) and pristine polymers PLGA and PLGA-PEG (PP) and B- comparison of the XRD spectra of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC)

The XRD spectra of the pristine starting materials show that docetaxel has a crystalline structure, displaying several sharp bands between 2θ of 10-25, characteristic of docetaxel's self-lattice arrangement (Ren et al. 2020) . The PLGA diffractogram shows a smooth curve, indicating a completely amorphous structure (Giannouli et al. 2018) , while the PLGA-PEG diffractogram also indicates a predominantly amorphous structure with 2 peaks only due to the polyethylene glycol groups in this material. As expected, the unloaded nano-systems show amorphous diffraction characteristics. However, the loaded NTN system does show some bands that can be attributed to docetaxel. These are present in a lower intensity than pristine docetaxel, indicating that while most of the drug is incorporated into the nano-system in an amorphous state, some crystalline docetaxel might be bound to the surface of these nanoparticles (Al-Nemrawi et al. 2018) . No crystalline bands can be observed in the loaded ATN system as this spectrum is the same as the unloaded spectrum, indicating that the docetaxel is completely entrapped within the nanoparticle core, hence shielded from the surface in the loaded ATN system. These results are consistent with previously conducted studies on docetaxel-loaded systems (Chen et al. 2008; Kulhari et al. 2014; Park et al. 2014).

5.3.4 Examination of Thermal Properties Show Phase Changes and Corresponding Mass Degradation

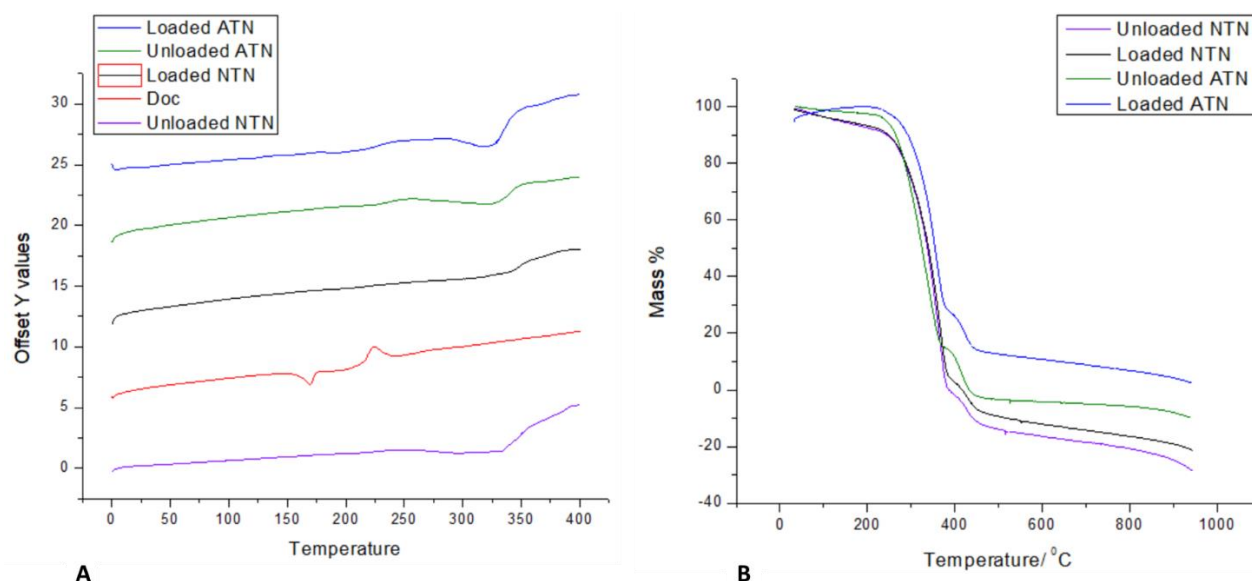


Figure 5.6 A- DSC thermograms comparing the phase changes of unloaded and loaded NTN and ATN, as well as the drug docetaxel (DOC). B– TGA analysis of the the unloaded and loaded NTN and ATN systems.

The thermogram of docetaxel shows an endothermic melting peak at 160 – 170°C and an exothermic decomposition peak at 215 - 230°C. All the prepared nano-systems have broad decomposition peaks at 310 - 340°C and, notably, the drug-loaded systems do not exhibit any of the characteristic melting and decomposition peaks associated with docetaxel. This is another indication of the loss of crystallinity of docetaxel in the loaded nano-systems(Mei et al. 2009) , corresponding to the more amorphous XRD profiles of the drug-loaded systems, compared to free drug in Figure 5.6.

The nano-systems were investigated further using thermo-gravimetric analysis to examine the weight loss associated with the phase changes. The data obtained correlated closely with the differential scanning calorimetry data, as the thermal decomposition of all systems began at 300°C with a 90% mass loss until 400°C (Campos et al. 2019) . The TGA spectra also revealed a second decomposition step for the nano-systems from 400 – 415°C, which accounted for the last 10% weight loss. The thermal phase changes and mass degradation agreed with previous reports on the degradation of PLGA and docetaxel-based nano-systems(Tao et al. 2013; Goodarzi et al. 2014; Park et al. 2014; Jose et al. 2019; da Rocha et al. 2020; Bortolotto Degregori et al. 2023) .

5.3.5 *In-vitro Kinetic Study of Nanoparticle Systems Shows pH Dependent Release of Docetaxel*

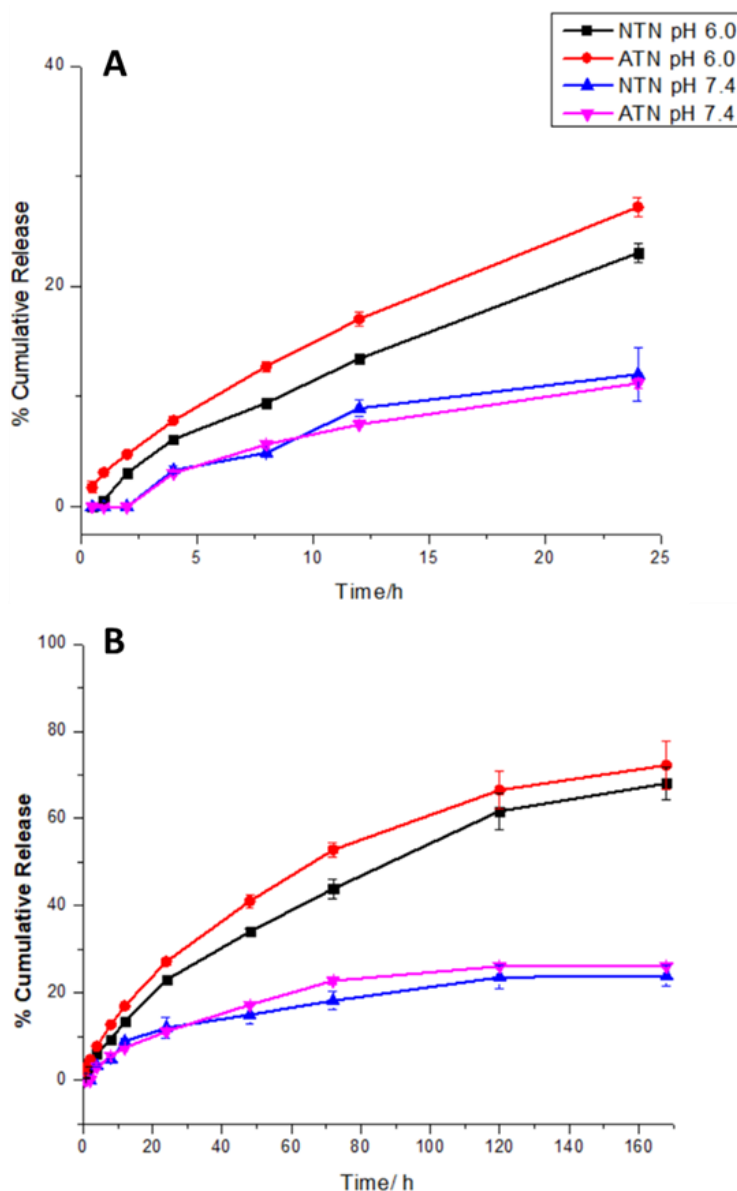


Figure 5.7 Docetaxel in-vitro release patterns from NTN and ATN systems at pH 7.4 and 6 from (A) 0-24H and (B) 0-168H.

The release patterns of docetaxel from the NTN and ATN systems are shown in Figure 5.8. At pH 7.4, the NTN's and ATN's exhibit $12,0 \pm 2,4\%$ and $11,2 \pm 0,41\%$ release after 24 hours and $24,0 \pm 2,4\%$ and $26,2 \pm 0,49\%$ release respectively after 168 hours. The drug release rates and patterns for both systems are very similar at neutral pH. However, at an acidic pH of 6, characteristic of tumour tissue (Lin et al. 2019), there was a significantly greater and faster release of docetaxel. At this pH, The NTN's and ATN's released $23,0 \pm 0,83\%$ and $27,2 \pm 0,86\%$, respectively, after 24 hours and $68,1 \pm 3,8\%$ and $72,2 \pm 5,5\%$, respectively, after 168 hours. The mechanism of drug release from PLGA nano-systems has been accepted as

polymer erosion drug diffusion from the carrier matrix (Musumeci et al. 2006; Poltavets et al. 2019; Saniee et al. 2021) , and in this case, the enhanced release of docetaxel at lower pH could be due to the faster degradation of PLGA in acidic media (Emami et al. 2023) . Until 72 hours, the release rate of ATN's is marginally higher than NTN. This could be due to the presence of PEG chains in the ATN system, which are more hydrophilic than PLGA and, therefore, causes an increased rate of hydration of the nanoparticle surface. This, in turn, results in a faster overall release of drug from the PEG-containing ATN nano-system compared to the NTN system, which only has PLGA moieties.

This phenomenon has been reported in previous studies comparing the in-vitro drug release of PLGA and PEG containing nanoparticles (Locatelli & Comes Franchini 2012; Rafiei & Haddadi 2017) .Emami et al. prepared EGFR and PDL1-targeted PLGA nanoparticles, reporting a 30 % and 60 % release of docetaxel from their non-targeted system at pH 7.4 and 5.5, respectively (Emami et al. 2023), which is very similar to results obtained here, *shown* in Figure 8. No release curves were provided for the targeted systems and it was assumed that the release profiles were the same as the unconjugated nanoparticles. Interestingly, Sui's group reported 50 – 70 % release of docetaxel from their folate targeted pH responsive PEG conjugated nanoparticles after 48 hours, at pH's 6.5 and 5.5, and <20 % release at pH 7.4 (Sui et al. 2023), demonstrating the marked effect that the PEG layer can have on docetaxel release at lower pH's.

In this study, the drug release rates between the ATN and NTN systems are not significantly different after 72 hours at pH 6, and this could be that there is complete hydration of the PEG moieties on the ATN system after 72 hours, and thereafter, the drug release is affected only by the PLGA moieties on both the ATN and NTN systems. The in vitro release pattern of both systems indicate that they would release the drug more efficiently at the pH of tumour sites, and it has been postulated that such controlled systems would have favourable in-vivo kinetics (Patel et al. 2018; Poltavets et al. 2019) as there would be minimal release of drug in blood and healthy tissue and optimal and extended release in tumour tissue (Li et al. 2019; Yurt et al. 2023). Additionally, the PEG moieties would impart stealth properties and increased stability to the ATN systems at a physiological pH (Shitole et al. 2020) .

5.3.6 *Targeted Nano-system shows increased cytotoxicity on LnCap and PC-3 Cell Lines*

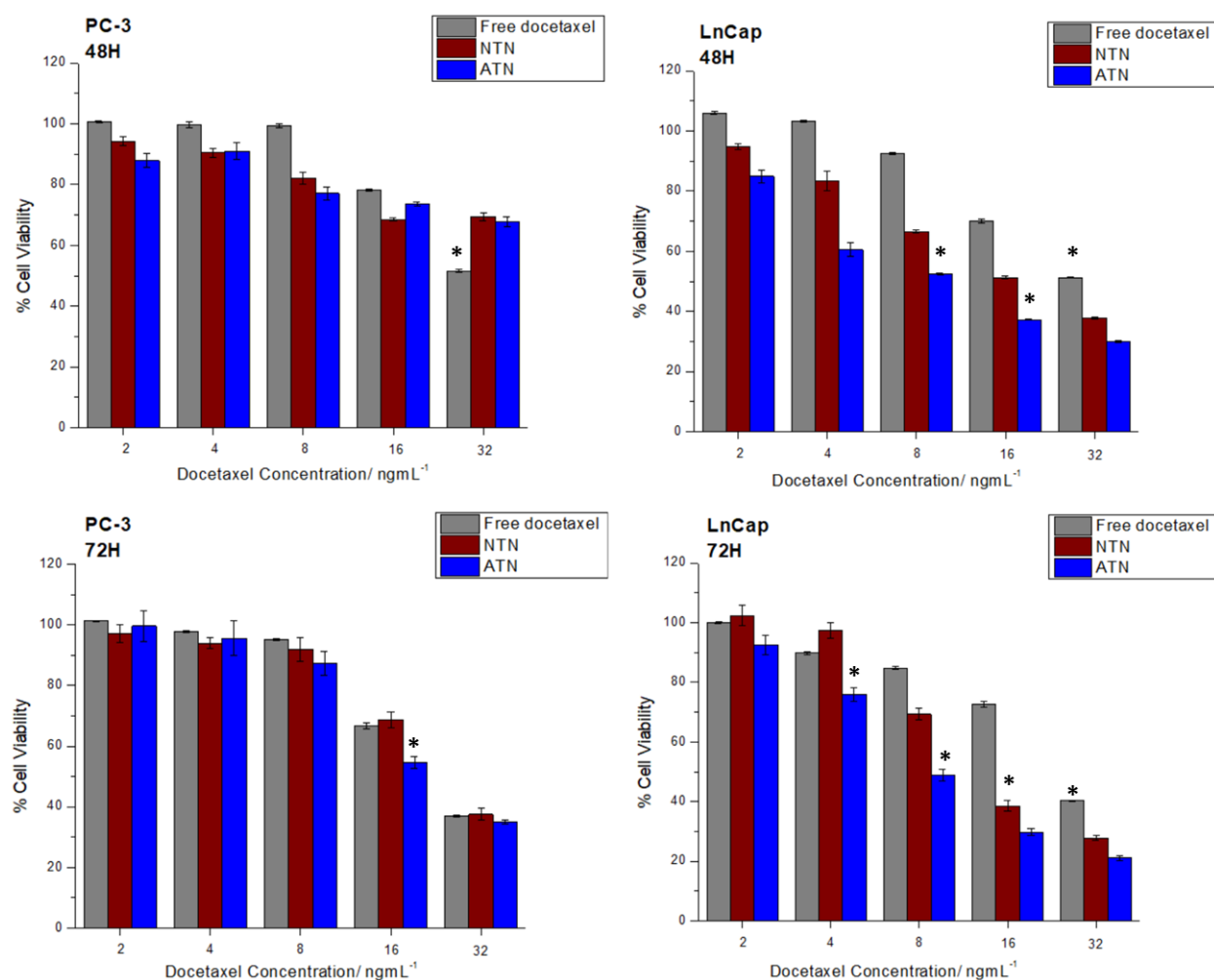


Figure 5.8 Cytotoxicity of docetaxel and NTN and ATN systems on PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 48- and 72H of treatment, showing dose dependent toxicity of docetaxel and increased efficacy of NTN's and ATN's (* p<0.05) compared to free docetaxel on LnCap cells.

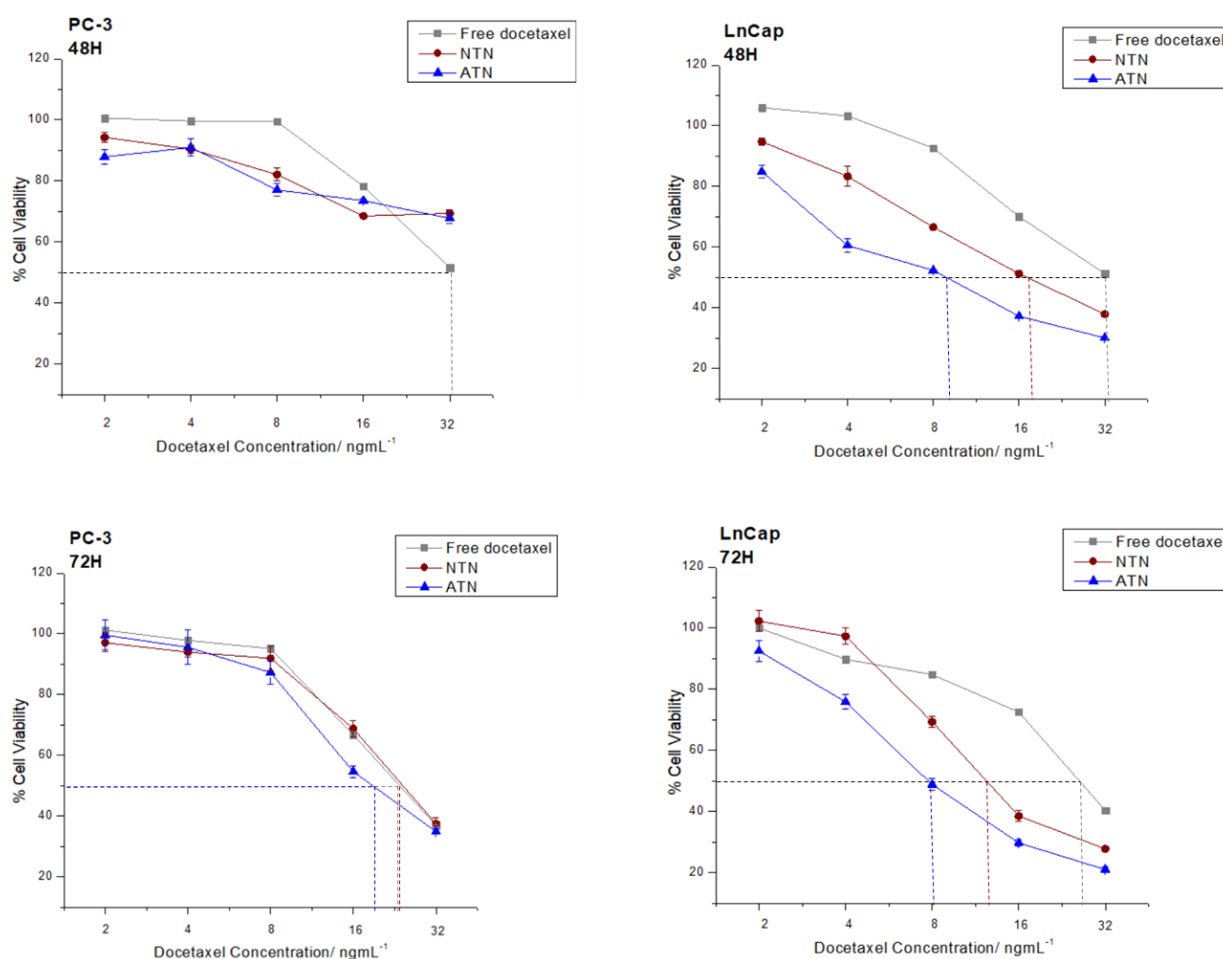


Figure 5.9 Comparison of effective concentrations (cell viability of 50%) of docetaxel and NTN and ATN systems on PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 48- and 72H of treatment

The unloaded NTN and ATN systems were tested on 3T3-NIH cells and showed >90% cell viability in concentrations up to 100µg/mL (Figure S6 in Appendix 3). This indicates that the systems have favourable biocompatibility profiles (Afsharzadeh et al. 2020) and would be relatively non-toxic to non-cancerous cells. As shown by Figures 5.9 and 5.10, both nano-systems were not effective at reducing the viability of PC-3 cells after 48 hours of treatment. Only free docetaxel was able to reduce the viability of the cells by 50% at 32 ng/mL. This could be due to the greater non-specific cellular uptake of the free docetaxel in the media due to passive diffusion and greater contact area with the cell membranes (Chen et al. 2016) since all the docetaxel molecules would be in solution. By 72 hours, the effective concentrations (concentration of drug required to reduce cell viability by 50%) of docetaxel, NTN and ATN were 25, 25,5 and 20 ng/mL respectively. This suggests that the entrapped docetaxel was available to act on the cells at this time point. The lower effective concentration for ATN at this

time point can be attributed to the slightly higher percentage of docetaxel released from this system compared to the NTN at this time point, as shown by Figure 5.8b.

There was a general dose dependent response in cell viability to free docetaxel and both nano-systems for the LnCap cells at both time points, as shown in Figures 5.9 and 5.10. At both time points, the effective concentrations decreased from free docetaxel to the NTN system and finally the ATN system, with the lowest concentrations being 28, 13,5 and 8 ng/mL for free docetaxel, NTN and ATN respectively, measured at 72 hours after treatment. There is a 50% decrease in the effective concentration between free docetaxel and the NTN nano-system, which could be due to the greater uptake of the nanoparticle system by membrane mobile transport (Chen et al. 2016) while free docetaxel is only taken in by the cell through passive diffusion and could be pumped back out of the cell by p-glycoprotein pumps (Sanna et al. 2011). There was a 41% decrease in effective concentration between the NTN and ATN system in LnCap cells. This suggests a larger effect than that due only to the increase in amount of docetaxel released. This effect could be due to interactions between the antibody on the the nanoparticle surface of the ATN system and the PSMA receptors on the surface of the LnCap cells, causing greater cellular uptake through receptor mediated endocytosis in addition to membrane mobile transport (Chen et al. 2016). Moreover, the transport mechanisms of the nanosystems allows them to escape being eliminated by p glycoprotein pumps and therefore would result in accumulation within the cell (Sanna et al. 2011; Shitole et al. 2020), allowing for a longer time for intracellular release and action of docetaxel. Here the favourable pharmacokinetic profile of the nano-systems, demonstrated in Figure 5.8, would allow for sustained exposure of the docetaxel to the cells, allowing for greater drug efficacy. The ATN nanosystem activity is in agreement with several studies that have reported the increased cytotoxicity of actively targeted nanosystems on LnCap cells though specific binding of a high – affinity targeting moeity to PSMA receptors, resulting in greater cellular uptake (Chen et al. 2016; Rivero-Buceta et al. 2019; Saniee et al. 2021).

The efficacy of docetaxel and both nano-systems increased from 48 to 72 hours, consistent with previous reports (Luo et al. 2010; Sanna et al. 2011) of docetaxel and docetaxel loaded systems being more effective at longer incubation times due to the mechanism of action of the drug, which requires cell division to operate. Sanna et al. demonstrated this by showing a 20 % increase of growth inhibition on PC-3 cells between 48 and 72 hours with both free docetaxel and loaded PLGA-polycaprolactone nanoparticles (Sanna et al. 2011), while Chen and co-workers (Chen et al. 2016) reported an average increase of 42% in inhibition of LnCap cells between 24 and 48 hours of treatment with docetaxel, non-targeted- and aptamer targeted loaded nanoparticles.

Shitole and co-workers also reported a 16 % and 40 % lower IC₅₀ on PC-3 and LnCap cells respectively, after 48 hours of treatment with their LHRH conjugated docetaxel loaded nanoparticles compared to free docetaxel (Shitole et al. 2020). In this study, the effective concentration of the ATN system was 71% lower than that of free docetaxel in LnCap cells after 72 hours of treatment. This could have implications for dosage modifications using targeted docetaxel loaded nano-systems to treat aggressive malignancies, while still reducing exposure of healthy tissue to high doses of free drug (Rivero-Buceta et al. 2019).

5.3.7 *Increased Cellular Uptake of Targeted Nano-system on LnCap Cell Line*

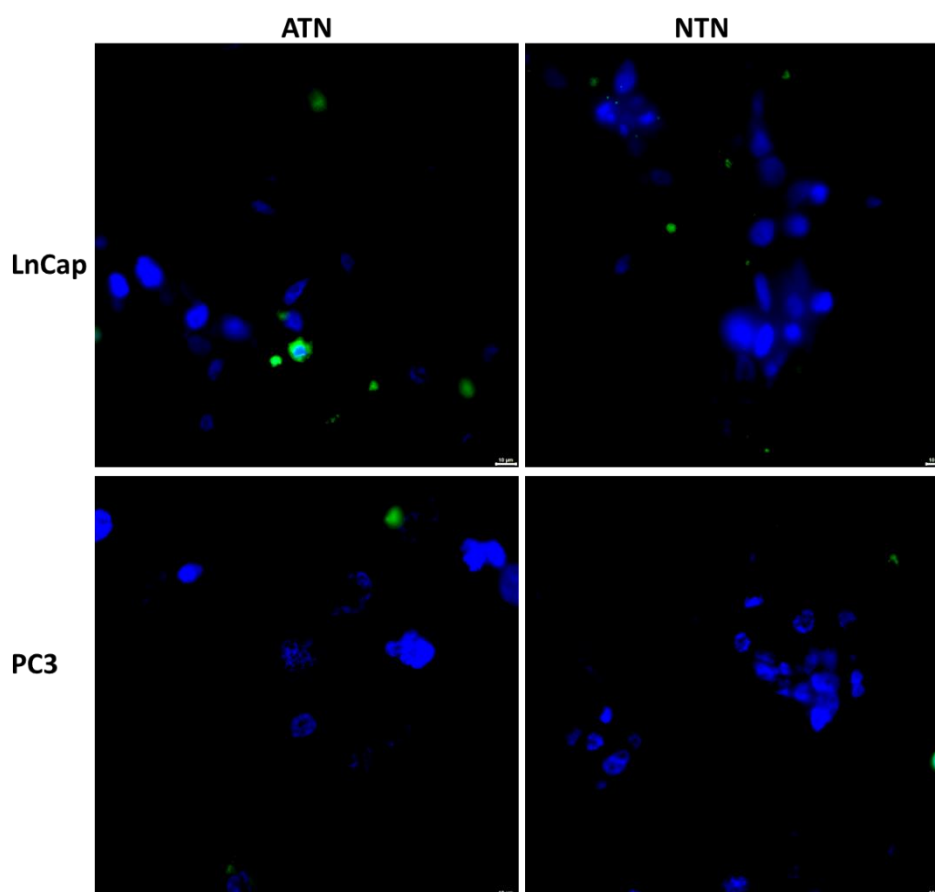


Figure 5.10 Fluorescent images of PC-3 (PSMA -ve) and LnCap (PSMA +ve) cell lines after 24H of treatment with FITC-loaded NTN's and ATN's. Nuclei are stained blue with DAPI and FITC is in green

As shown in Figure 5.11, there is a greater amount of green fluorescence corresponding to cellular uptake of the actively targeted nano-system (ATN) in the LnCap (PSMA +ve) cell line compared to the non-targeted nano-system (NTN). There is also minimal cell associated fluorescence from both nano-systems in the PC-3 (PSMA -ve) cell line. Additionally, there is

a clear localization of green fluorescence due to ATN's in LnCap cells around the nucleus, as captured by Figure 5.11 A, and this can be attributed to the binding of the anti-PSMA antibody on the surface of the ATN's to the overexpressed PSMA receptors in the LnCap cell line, forming an antibody-receptor conjugate, and allowing for the increased and selective uptake of the targeted system into the cell by receptor-mediated endocytosis (Rivero-Buceta et al. 2019). As expected, there is minimal to no uptake of NTN as this system does not have targeting ligands and therefore cannot bind to the PSMA receptors, and additionally a similar low uptake is observed for both systems on the PC-3 cell line as these cells do not have PSMA surface receptors to bind to (Dhar et al. 2008). The difference in uptake also correlates with the cytotoxicity results in Figures 9 and 10, where there is no significant difference in cytotoxicity between ATN and NTN treatments in PSMA -ve PC-3 cells, while there is a marked and significantly higher toxicity of the ATN treatment in PSMA +ve LnCap cells compared to the NTN treatment. Similar observations of increased uptake of targeted nano-systems by LnCap cells were reported by Sanna et al (Sanna et al. 2017) and Chandran et al (Chandran et al. 2008), who used small molecule targeting ligands. Wang et al. (Wang et al. 2008) and Dhar et al. (Dhar et al. 2008) used aptamer functionalised nanoparticles, and more recently, Hara's group (Hara et al. 2021) and Rivero-Buceta's group (Rivero-Buceta et al. 2019) used antibodies to target PSMA. In agreement with *the results obtained here*, these studies concluded that cellular uptake in LnCap cells is mediated by the binding of the targeting moiety to the PSMA receptors on the cell surface, while uptake is limited in PSMA -ve PC-3 cells.

5.4 Conclusions

This work details the fabrication, optimization and resulting comparative investigation of a docetaxel -loaded PLGA-PEG system functionalized with a targeting antibody for specific binding to the PSMA receptors expressed highly on prostate cancer cells. It was shown that the system displays a sustained, pH dependent release profile and enhanced selective cytotoxicity on prostate cancer cells, while displaying minimal toxicity on non-cancerous cells. Furthermore, the targeted system shows promise as a site-specific delivery system for chemotherapeutic agents to prostate cancer tissue, due to its increased cellular uptake. Therefore, future studies could investigate specific single-cell internalization, selective mechanisms of action and pre-clinical in-vivo studies.

5.5 References

Afsharzadeh, M., Hashemi, M., Babaei, M., Abnous, K. & Ramezani, M., 2020, 'PEG-PLA nanoparticles decorated with small-molecule PSMA ligand for targeted delivery of galbanic

acid and docetaxel to prostate cancer cells', *Journal of Cellular Physiology*, 235(5), 4618–4630.

Al-Nemrawi, N., Alshraideh, N., Zayed, A. & Altaani, B., 2018, 'Low Molecular Weight Chitosan-Coated PLGA Nanoparticles for Pulmonary Delivery of Tobramycin for Cystic Fibrosis', *Pharmaceuticals*, 11(1), 28.

Arslan, F.B., Öztürk, K., Tavukçuoğlu, E., Öztürk, S.C., Esendağlı, G. & Çalış, S., 2023, 'A novel combination for the treatment of small cell lung cancer: Active targeted irinotecan and static co-loaded PLGA nanoparticles', *International Journal of Pharmaceutics*, 632, 122573.

Autio, K.A., Dreicer, R., Anderson, J., Garcia, J.A., Alva, A., Hart, L.L., Milowsky, M.I., Posadas, E.M., Ryan, C.J., Graf, R.P., Dittamore, R., Schreiber, N.A., Summa, J.M., Youssoufian, H., Morris, M.J. & Scher, H.I., 2018, 'Safety and Efficacy of BIND-014, a Docetaxel Nanoparticle Targeting Prostate-Specific Membrane Antigen for Patients With Metastatic Castration-Resistant Prostate Cancer', *JAMA Oncology*, 4(10), 1344.

Badran, M.M., Alomrani, A.H., Harisa, G.I., Ashour, A.E., Kumar, A. & Yassin, A.E., 2018, 'Novel docetaxel chitosan-coated PLGA/PCL nanoparticles with magnified cytotoxicity and bioavailability', *Biomedicine & Pharmacotherapy*, 106, 1461–1468.

Betancourt, T., Byrne, J.D., Sunaryo, N., Crowder, S.W., Kadapakkam, M., Patel, S., Casciato, S. & Brannon-Peppas, L., 2009, 'PEGylation strategies for active targeting of PLA/PLGA nanoparticles', *Journal of Biomedical Materials Research Part A*, 91A(1), 263–276.

Bezelya, A., Küçüktürkmen, B. & Bozkır, A., 2023, 'Microfluidic Devices for Precision Nanoparticle Production', *Micro*, 3(4), 822–866.

Bhattacharjee, S., 2016, 'DLS and zeta potential – What they are and what they are not?', *Journal of Controlled Release*, 235, 337–351.

Bortolotto Degregori, E., Corbellini Henckes, N.A., Franco, N., Luz, H., Maurmann, N., Viana, A.R., Rohden, F., Loureiro dos Santos, L.A., Cirne Lima, E.O., Terraciano, P.B., Oliveira, F. dos S. & Contesini, E.A., 2023, 'Interaction between adipoderivated mesenchymal stem cells and PLGA/PI epox scaffold with possible use in tissue engineering: in vitro study', *International Journal of Polymeric Materials and Polymeric Biomaterials*, 72(14), 1132–1141.

Bose, A., Roy Burman, D., Sikdar, B. & Patra, P., 2021, 'Nanomicelles: Types, properties and applications in drug delivery', *IET Nanobiotechnology*, 15(1), 19–27.

Campos, M.S.T., Fialho, S.L., Pereira, B.G., Yoshida, M.I., Mussel, W.N., Junior, A.S.C. & Oliveira, M.A., 2019, 'Development and chemical characterization of biodegradable polymeric

implants containing sirolimus for the treatment of malignant solid tumors', *Die Pharmazie*, 74(4), 221–226.

Chandran, S.S., R. Banerjee, S., C. Mease, R., Pomper, M.G. & R. Denmeade, S., 2008, 'Characterization of a targeted nanoparticle functionalized with a urea-based inhibitor of prostate-specific membrane antigen (PSMA)', *Cancer Biology & Therapy*, 7(6), 974–982.

Chen, J., Qiu, L., Hu, M., Jin, Y. & Han, J., 2008, 'Preparation, Characterization and In Vitro Evaluation of Solid Dispersions Containing Docetaxel', *Drug Development and Industrial Pharmacy*, 34(6), 588–594.

Chen, Z., Tai, Z., Gu, F., Hu, C., Zhu, Q. & Gao, S., 2016, 'Aptamer-mediated delivery of docetaxel to prostate cancer through polymeric nanoparticles for enhancement of antitumor efficacy', *European Journal of Pharmaceutics and Biopharmaceutics*, 107, 130–141.

CHENG, J., TEPLY, B., SHERIFI, I., SUNG, J., LUTHER, G., GU, F., LEVYNISSENBAUM, E., RADOVICMORENO, A., LANGER, R. & FAROKHZAD, O., 2007, 'Formulation of functionalized PLGA–PEG nanoparticles for in vivo targeted drug delivery', *Biomaterials*, 28(5), 869–876.

Choi, C.-H., Jung, J.-H., Hwang, T.-S. & Lee, C.-S., 2009, 'In situ microfluidic synthesis of monodisperse PEG microspheres', *Macromolecular Research*, 17(3), 163–167.

Dai, L., Shen, G., Wang, Y., Yang, P., Wang, H. & Liu, Z., 2021, 'PSMA-targeted melanin-like nanoparticles as a multifunctional nanoplatform for prostate cancer theranostics', *Journal of Materials Chemistry B*, 9(4), 1151–1161.

Dhar, S., Gu, F.X., Langer, R., Farokhzad, O.C. & Lippard, S.J., 2008, 'Targeted delivery of cisplatin to prostate cancer cells by aptamer functionalized Pt(IV) prodrug-PLGA–PEG nanoparticles', *Proceedings of the National Academy of Sciences*, 105(45), 17356–17361.

Dostalova, S., Cerna, T., Hynek, D., Koudelkova, Z., Vaculovic, T., Kopel, P., Hrabeta, J., Heger, Z., Vaculovicova, M., Eckschlager, T., Stiborova, M. & Adam, V., 2016, 'Site-Directed Conjugation of Antibodies to Apoferritin Nanocarrier for Targeted Drug Delivery to Prostate Cancer Cells', *ACS Applied Materials & Interfaces*, 8(23), 14430–14441.

Emami, F., Duwa, R., Banstola, A., Woo, S.M., Kwon, T.K. & Yook, S., 2023, 'Dual receptor specific nanoparticles targeting EGFR and PD-L1 for enhanced delivery of docetaxel in cancer therapy', *Biomedicine & Pharmacotherapy*, 165, 115023.

Essa, D., Choonara, Y.E., Kondiah, P.P.D. & Pillay, V., 2020, 'Comparative Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and Emulsification Solvent Evaporation Techniques', *Polymers*, 12(9), 1882.

Essa, D., Kondiah, P.P.D., Kumar, P. & Choonara, Y.E., 2023, 'Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells', *Biomedicines*, 11(4).

Fasehee, H., Dinarvand, R., Ghavamzadeh, A., Esfandyari-Manesh, M., Moradian, H., Faghihi, S. & Ghaffari, S.H., 2016, 'Delivery of disulfiram into breast cancer cells using folate-receptor-targeted PLGA-PEG nanoparticles: in vitro and in vivo investigations', *Journal of Nanobiotechnology*, 14(1), 32.

Feng, L., Dong, Z., Tao, D., Zhang, Y. & Liu, Z., 2018, 'The acidic tumor microenvironment: a target for smart cancer nano-theranostics', *National Science Review*, 5(2), 269–286.

Giannouli, M., Karagkiozaki, V., F.Pappa, Moutsios, I., C.Gravalidis & Logothetidis, S., 2018, 'Fabrication of quercetin-loaded PLGA nanoparticles via electrohydrodynamic atomization for cardiovascular disease', *Materials Today: Proceedings*, 5(8), 15998–16005.

Goodarzi, N., Ghahremani, M.H., Amini, M., Atyabi, F., Ostad, S.N., Shabani Ravari, N., Nateghian, N. & Dinarvand, R., 2014, '<sc>CD</sc> 44-Targeted Docetaxel Conjugate for Cancer Cells and Cancer Stem-Like Cells: A Novel Hyaluronic Acid-Based Drug Delivery System', *Chemical Biology & Drug Design*, 83(6), 741–752.

Gu, F., Zhang, L., Teply, B.A., Mann, N., Wang, A., Radovic-Moreno, A.F., Langer, R. & Farokhzad, O.C., 2008, 'Precise engineering of targeted nanoparticles by using self-assembled biointegrated block copolymers', *Proceedings of the National Academy of Sciences*, 105(7), 2586–2591.

Hara, D., Tao, W., Totiger, T.M., Pourmand, A., Dogan, N., Ford, J.C., Shi, J. & Pollack, A., 2021, 'Prostate Cancer Targeted X-Ray Fluorescence Imaging via Gold Nanoparticles Functionalized With Prostate-Specific Membrane Antigen (PSMA)', *International Journal of Radiation Oncology*Biophysics*, 111(1), 220–232.

Hrkach, J., Hoff, D. Von, Ali, M.M., Andrianova, E., Auer, J., Campbell, T., Witt, D. De, Figa, M., Figueiredo, M., Horhota, A., Low, S., McDonnell, K., Peeke, E., Retnarajan, B., Sabnis, A., Schnipper, E., Song, J.J., Song, Y.H., Summa, J., Tompsett, D., Troiano, G., Geen Hoven, T. Van, Wright, J., LoRusso, P., Kantoff, P.W., Bander, N.H., Sweeney, C., Farokhzad, O.C., Langer, R. & Zale, S., 2012, 'Preclinical Development and Clinical Translation of a PSMA-

Targeted Docetaxel Nanoparticle with a Differentiated Pharmacological Profile', *Science Translational Medicine*, 4(128).

Ingalls, G.G., 2019, *Targeted PLGA Nanoparticles for the Sustained Release of Hypertensive Drugs* – PhD thesis, Harvard Extension School, Cambridge .

Jose, A., Sebastian, H., A., Durazzo, Lucarini, Santini & Souto, 2019, 'Transferrin-Conjugated Docetaxel-PLGA Nanoparticles for Tumor Targeting: Influence on MCF-7 Cell Cycle', *Polymers*, 11(11), 1905.

Kim, D.W., Yousaf, A.M., Li, D.X., Kim, J.O., Yong, C.S., Cho, K.H. & Choi, H.-G., 2017, 'Development of RP-HPLC method for simultaneous determination of docetaxel and curcumin in rat plasma: Validation and stability', *Asian Journal of Pharmaceutical Sciences*, 12(1), 105–113.

Kulhari, H., Pooja, D., Shrivastava, S., V.G.M, N. & Sistla, R., 2014, 'Peptide conjugated polymeric nanoparticles as a carrier for targeted delivery of docetaxel', *Colloids and Surfaces B: Biointerfaces*, 117, 166–173.

Lee, N.K., Wang, C.-P.J., Lim, J., Park, W., Kwon, H.-K., Kim, S.-N., Kim, T.-H. & Park, C.G., 2021, 'Impact of the conjugation of antibodies to the surfaces of polymer nanoparticles on the immune cell targeting abilities', *Nano Convergence*, 8(1), 24.

Li, S., Li, X., Ding, J., Han, L. & Guo, X., 2019, '<p>Anti-tumor efficacy of folate modified PLGA-based nanoparticles for the co-delivery of drugs in ovarian cancer</p>', *Drug Design, Development and Therapy*, Volume 13, 1271–1280.

Lin, B., Chen, H., Liang, D., Lin, W., Qi, X., Liu, H. & Deng, X., 2019, 'Acidic pH and High-H₂O₂ Dual Tumor Microenvironment-Responsive Nanocatalytic Graphene Oxide for Cancer Selective Therapy and Recognition', *ACS Applied Materials & Interfaces*, 11(12), 11157–11166.

Liu, Y., Yang, G., Jin, S., Xu, L. & Zhao, C., 2020, 'Development of High-Drug-Loading Nanoparticles', *ChemPlusChem*, 85(9), 2143–2157.

Locatelli, E. & Comes Franchini, M., 2012, 'Biodegradable PLGA-b-PEG polymeric nanoparticles: synthesis, properties, and nanomedical applications as drug delivery system', *Journal of Nanoparticle Research*, 14(12), 1316.

Lu, L., Duong, V.T., Shalash, A.O., Skwarczynski, M. & Toth, I., 2021, 'Chemical Conjugation Strategies for the Development of Protein-Based Subunit Nanovaccines', *Vaccines*, 9(6), 563.

Lu, X., Yang, F., Chen, Dexi, Zhao, Q., Chen, Dong, Ping, H. & Xing, N., 2020, 'Quercetin reverses docetaxel resistance in prostate cancer via androgen receptor and PI3K/Akt signaling pathways', *International Journal of Biological Sciences*, 16(7), 1121–1134.

Luo, Y., Ling, Y., Guo, W., Pang, J., Liu, W., Fang, Y., Wen, X., Wei, K. & Gao, X., 2010, 'Docetaxel loaded oleic acid-coated hydroxyapatite nanoparticles enhance the docetaxel-induced apoptosis through activation of caspase-2 in androgen independent prostate cancer cells', *Journal of Controlled Release*, 147(2), 278–288.

Mei, L., Zhang, Y., Zheng, Y., Tian, G., Song, C., Yang, D., Chen, H., Sun, H., Tian, Y., Liu, K., Li, Z. & Huang, L., 2009, 'A Novel Docetaxel-Loaded Poly (ϵ -Caprolactone)/Pluronic F68 Nanoparticle Overcoming Multidrug Resistance for Breast Cancer Treatment', *Nanoscale Research Letters*, 4(12), 1530.

Moradi, A., Abdi haji, M., Kouchaksaraie, S.B., Alkinani, T.A., Mahmoudi, A., Davoudi, A., Dashtmiani, W., Ghezeljeh, S.M., Aghajani, S., Ghasemian, R., Taramsari, S.M., Majlesi, A., Niyaki, Z.M. & Salehzadeh, A., 2022, 'Synthesize of Bi₂O₃/Gln-TSC nanoparticles and evaluation of their toxicity on prostate cancer cells and expression of CASP8, BAX, and Bcl-2 genes', *Scientific Reports*, 12(1), 21245.

Musumeci, T., Ventura, C.A., Giannone, I., Ruozzi, B., Montenegro, L., Pignatello, R. & Puglisi, G., 2006, 'PLA/PLGA nanoparticles for sustained release of docetaxel', *International Journal of Pharmaceutics*, 325(1–2), 172–179.

Park, J.-H., Lee, J.-Y., Termsarasab, U., Yoon, I.-S., Ko, S.-H., Shim, J.-S., Cho, H.-J. & Kim, D.-D., 2014, 'Development of poly(lactic-co-glycolic) acid nanoparticles-embedded hyaluronic acid–ceramide-based nanostructure for tumor-targeted drug delivery', *International Journal of Pharmaceutics*, 473(1–2), 426–433.

Patel, J., Amrutiya, J., Bhatt, P., Javia, A., Jain, M. & Misra, A., 2018, 'Targeted delivery of monoclonal antibody conjugated docetaxel loaded PLGA nanoparticles into EGFR overexpressed lung tumour cells', *Journal of Microencapsulation*, 35(2), 204–217.

Poltavets, Y.I., Zhirnik, A.S., Zavarzina, V. V., Semochkina, Y.P., Shuvatova, V.G., Krashennnikova, A.A., Aleshin, S. V., Dronov, D.O., Vorontsov, E.A., Balabanyan, V.Yu. & Posypanova, G.A., 2019, 'In vitro anticancer activity of folate-modified docetaxel-loaded PLGA nanoparticles against drug-sensitive and multidrug-resistant cancer cells', *Cancer Nanotechnology*, 10(1), 2.

Rafiei, P. & Haddadi, A., 2017, 'Docetaxel-loaded PLGA and PLGA-PEG nanoparticles for intravenous application: pharmacokinetics and biodistribution profile', *International Journal of Nanomedicine*, Volume 12, 935–947.

Ren, L., Yang, X., Guo, W., Wang, J. & Chen, G., 2020, 'Inclusion Complex of Docetaxel with Sulfobutyl Ether β -Cyclodextrin: Preparation, In Vitro Cytotoxicity and In Vivo Safety', *Polymers*, 12(10), 2336.

Rivero-Buceta, E., Vidaurre-Agut, C., Vera-Donoso, C.D., Benlloch, J.M., Moreno-Manzano, V. & Botella, P., 2019, 'PSMA-Targeted Mesoporous Silica Nanoparticles for Selective Intracellular Delivery of Docetaxel in Prostate Cancer Cells', *ACS Omega*, 4(1), 1281–1291.

Rocha, M.C.O. da, Silva, P.B. da, Radicchi, M.A., Andrade, B.Y.G., Oliveira, J.V. de, Venus, T., Merker, C., Estrela-Lopis, I., Longo, J.P.F. & B  o, S.N., 2020, 'Docetaxel-loaded solid lipid nanoparticles prevent tumor growth and lung metastasis of 4T1 murine mammary carcinoma cells', *Journal of Nanobiotechnology*, 18(1), 43.

Saniee, F., Shabani Ravari, N., Goodarzi, N., Amini, M., Atyabi, F., Saeedian Moghadam, E. & Dinarvand, R., 2021, 'Glutamate-urea-based PSMA-targeted PLGA nanoparticles for prostate cancer delivery of docetaxel', *Pharmaceutical Development and Technology*, 26(4), 381–389.

Sanna, V., Roggio, A.M., Posadino, A.M., Cossu, A., Marceddu, S., Mariani, A., Alzari, V., Uzzau, S., Pintus, G. & Sechi, M., 2011, 'Novel docetaxel-loaded nanoparticles based on poly(lactide-co-caprolactone) and poly(lactide-co-glycolide-co-caprolactone) for prostate cancer treatment: formulation, characterization, and cytotoxicity studies', *Nanoscale Research Letters*, 6(1), 260.

Sanna, V., Singh, C.K., Jashari, R., Adhami, V.M., Chamcheu, J.C., Rady, I., Sechi, M., Mukhtar, H. & Siddiqui, I.A., 2017, 'Targeted nanoparticles encapsulating (–)-epigallocatechin-3-gallate for prostate cancer prevention and therapy', *Scientific Reports*, 7(1), 41573.

Sazonova, E. V., Kopeina, G.S., Imyanitov, E.N. & Zhivotovsky, B., 2021, 'Platinum drugs and taxanes: can we overcome resistance?', *Cell Death Discovery*, 7(1), 155.

Shitole, A.A., Sharma, N., Giram, P., Khandwekar, A., Baruah, M., Garnaik, B. & Koratkar, S., 2020, 'LHRH-conjugated, PEGylated, poly-lactide-co-glycolide nanocapsules for targeted delivery of combinational chemotherapeutic drugs Docetaxel and Quercetin for prostate cancer', *Materials Science and Engineering: C*, 114, 111035.

Sui, F., Fang, Z., Li, L., Wan, X., Zhang, Y. & Cai, X., 2023, 'pH-triggered "PEG" sheddable and folic acid-targeted nanoparticles for docetaxel delivery in breast cancer treatment', *International Journal of Pharmaceutics*, 644, 123293.

Sun, L., Liu, H., Ye, Y., Lei, Y., Islam, R., Tan, S., Tong, R., Miao, Y.-B. & Cai, L., 2023, 'Smart nanoparticles for cancer therapy', *Signal Transduction and Targeted Therapy*, 8(1), 418.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F., 2021, 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA: A Cancer Journal for Clinicians*, 71(3), 209–249.

Tao, W., Zeng, X., Liu, T., Wang, Z., Xiong, Q., Ouyang, C., Huang, L. & Mei, L., 2013, 'Docetaxel-loaded nanoparticles based on star-shaped mannitol-core PLGA-TPGS diblock copolymer for breast cancer therapy', *Acta Biomaterialia*, 9(11), 8910–8920.

Tran, P., Nguyen, T.N., Lee, Y., Tran, P.N. & Park, J.-S., 2021, 'Docetaxel-loaded PLGA nanoparticles to increase pharmacological sensitivity in MDA-MB-231 and MCF-7 breast cancer cells', *The Korean Journal of Physiology & Pharmacology*, 25(5), 479–488.

Wang, A.Z., Bagalkot, V., Vasilliou, C.C., Gu, F., Alexis, F., Zhang, L., Shaikh, M., Yuet, K., Cima, M.J., Langer, R., Kantoff, P.W., Bander, N.H., Jon, S. & Farokhzad, O.C., 2008, 'Superparamagnetic Iron Oxide Nanoparticle–Aptamer Bioconjugates for Combined Prostate Cancer Imaging and Therapy', *ChemMedChem*, 3(9), 1311–1315.

Yurt, F., Özel, D., Tunçel, A., Gokbayrak, O. & Aktas, S., 2023, 'Synthesis and Optimization of the Docetaxel-Loaded and Durvalumab-Targeted Human Serum Albumin Nanoparticles, In Vitro Characterization on Triple-Negative Breast Cancer Cells', *ACS Omega*, 8(29), 26287–26300.

Zhang, X., Qi, S., Liu, D., Du, J. & Jin, J., 2022, 'PSMA-Targeted Supramolecular Nanoparticles Prepared From Cucurbit[8]uril-Based Ternary Host–Guest Recognition for Prostate Cancer Therapy', *Frontiers in Chemistry*, 10.

Zhu, Z., 2013, 'Effects of amphiphilic diblock copolymer on drug nanoparticle formation and stability', *Biomaterials*, 34(38), 10238–10248.

Zumaya, A.L.V., Rimpelová, S., Štějdířová, M., Ulbrich, P., Vilčáková, J. & Hassouna, F., 2022, 'Antibody Conjugated PLGA Nanocarriers and Superparamagnetic Nanoparticles for Targeted Delivery of Oxaliplatin to Cells from Colorectal Carcinoma', *International Journal of Molecular Sciences*, 23(3), 1200.

Chapter 6 Evaluation of Docetaxel-Loaded Anti-PSMA-Functionalised PLGA-PEG Nanoparticles in LnCap Prostate Cancer Cells and Spheroids and Biocompatibility Analysis in Nude MF1 Mice

Abstract

Polymeric nanoparticles have been used in drug delivery to treat cancer by selective targeting of tumour cells and induction of programmed cell death. Previously optimized nanosystems loaded with docetaxel and functionalized with anti-PSMA antibody were used to investigate targeting of prostate cancer cells using prostate-specific membrane antigen (PSMA) as a target for receptor-mediated endocytosis and enhanced cellular uptake. Cell migration, apoptosis, cell cycle, and generation of reactive oxygen species were evaluated using *in vitro* assays on PSMA-positive LnCap and PSMA-negative PC-3 prostate cancer cells. Thereafter, the growth inhibition of 3D spheroids cultured from LnCap cells and the *in vivo* biocompatibility of the nanosystem in a MF1 nude mouse model were investigated. There was 89±8.4% and 48.1±2.7% inhibition of LnCap cell migration by the targeted and non-targeted system respectively and 30.3±3.0% and 15.8±2.8% arrest in the G2/M phase of LnCap cells by the targeted and non-targeted systems respectively, but there was no significant difference ($p<0.05$) in the effects of the treatments on the migration and cell cycle of PC-3 cells, suggesting that the increased uptake of the targeted system by the PSMA receptors on the LnCap cells (not found on PC-3 cells) could be the driving force behind the observed enhanced efficacy. There was also higher inhibition of 3D LnCap spheroids by both nanosystems than free docetaxel after seven days of culture, with 62.9±8.6% and 48.6±9.3% inhibition for the targeted and non-targeted systems respectively, and 17.6±7.6% inhibition by docetaxel. All nanosystems showed no observable *in vivo* toxicity as evidenced by the lack of microscopic pathology observed on the major organs of nude MF1 mice after one dose. The enhanced cell uptake activity of the targeted nanosystem on LnCap cells suggests its potential as a targeted nanocarrier for docetaxel in prostate cancer.

6.1 Introduction

Nanomaterials are widely adopted in the design of drug delivery systems to improve the bioavailability of drugs (Hsu et al., 2023), with cancer nanomedicine being the most studied due to its complex pathology (Mundekkad and Cho, 2022). Prostate cancer is the most common diagnosis of male carcinoma worldwide (Sung et al., 2021), and docetaxel is the anti-cancer drug of choice approved by the US FDA (Rivero-Buceta et al., 2019; Lima et al., 2021) for prostate cancer (Li et al., 2018), but it has numerous side-effects (Autio et al., 2018; El

Rassy et al., 2018) and poor bioavailability due to its physicochemical properties (Li et al., 2018).

Therefore, this research proposes the evaluation of a targeted anti-body-functionalised PLGA nanosystem to bind to a prostate cancer cell surface receptor for receptor mediated endocytosis (Muhamad et al., 2018). The characteristics of prostate-specific membrane antigen (PSMA), a type II transmembrane protein overexpressed on the majority of prostate cancer cells, (Wüstemann et al., 2019) were harnessed. The targeted nanosystem, as described and evaluated in Chapter 5 of this thesis, showed enhanced *in-vitro* efficacy in 2D cell culture. The following work further explores the comparative efficacy of the nano-systems on cancer associated intra-cellular processes such as cell cycle arrest, apoptosis induction and the activation of reactive oxygen species (ROS) in 2D cell culture. However, it has been reported that the interaction of nanoparticles with cellular compartments in the tumour microenvironment can hamper their transport to tumour cells (Chan, 2023), which can cause reduced efficacy *in vivo*. Therefore, a 3D multicellular tumour cell spheroid model was developed to better correlate with *in vivo* data (Friedrich et al., 2009) by closely mimicking the heterogenous environment of *in vivo* prostate cancer tumours (Bromma et al., 2022).

6.2 Materials and Methods

6.2.1 Materials

The polymeric materials Poly(D,L-lactide-co-glycolide)(PLGA) (lactide/glycolide ratio of 50:50), molecular weight 7 000-17 000 with acid end groups, carboxylic acid polyethylene glycol PLGA (carboxylPEGPLGA), Pluronic F127. MES free acid, TRIS, EDC, NHSS, Anti-PSMA antibody, hydroxylamine hydrochloride, sodium hydroxide pellets (NaOH), hydrochloric acid, fluorescein iso-thiocyanate (FITC), tween80. and acetonitrile, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Pty) Ltd, Estate South, Modderfontein, Gauteng, SA. Docetaxel was obtained from DLD Scientific, Durban South Africa.

6.2.2 Preparation and Characterization of Nanoparticles

The preparation and characterisation of antibody-conjugated nanoparticles was described previously (chapter 5). Briefly, PLGA, or a mixture of PLGA and PLGA-PEG-COOH, and docetaxel dissolved in acetonitrile served as the organic phase, while a 0.1% solution of Pluronic F127 in Millipore water served as the aqueous phase for fabrication of nanoparticles using the NanoAssemblr Benchtop (Precision Nanosystems Inc., Vancouver, BC, Canada). The resultant suspensions were stirred for 4 hours at room temperature and pellets were

obtained by 3x centrifugation at 12 000 rpm for 30 minutes. PLGA-docetaxel nanoparticles, were the non-targeting nanosystem (NTN) while active targeting nanoparticles (ATN) were further functionalized by conjugation of the PLGA: PLGA-PEG-COOH with anti-PSMA antibody via carbodiimide chemistry (chapter 5). The nanoparticle pellets were frozen at -80 °C and then freeze dried (Freezone 12 lyophilizer, Labcono, Kansas City, USA) for 24 hours.

6.2.3 Cell Culture Conditions

PC-3 and LnCap prostate carcinoma cells were obtained from Cellonex (Johannesburg, South Africa). Cells were confirmed by supplier to be free of mycoplasma. PC-3 cells were grown in RPMI culture medium, and LnCap cells were grown in DMEM/F12 culture medium (Gibco). The media were supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) as per cell culture protocols. The cells were cultured under sterile conditions in an incubator at 95 % humidity and 5 % CO₂. All cell culture media, supplements, antibiotics and enzymes were purchased from Thermofisher Scientific, Waltham, MA, United States.

6.2.3.1. Wound Healing (Scratch) Assay Cancer Cell Scratch Assay

PC-3 and LnCap cells were grown in RPMI and DMEM/F12 media, as prepared above and grown until 80 % confluent. Thereafter, they were harvested and seeded in 6 well plates at a density of 2.5×10^4 cells/well for PC-3 and 5×10^4 cells/well for LnCap and grown until 80% confluence. Thereafter a scratch was made across each well with a 100 μ L pipette tip, and cells were treated with whole media (control), and docetaxel, NTN and ATN, with the effective concentration of docetaxel (determined in chapter 5) being 8 ng/mL. Images were captured every 24 hours over a 72-hour period, and gap closure inhibition relative to untreated control was evaluated using ImageJ software, using equations 6.1 and 6.2. adapted from (Ruzycka-Ayoush et al., 2021). The workflow of the assay is shown in Figure 6.1.

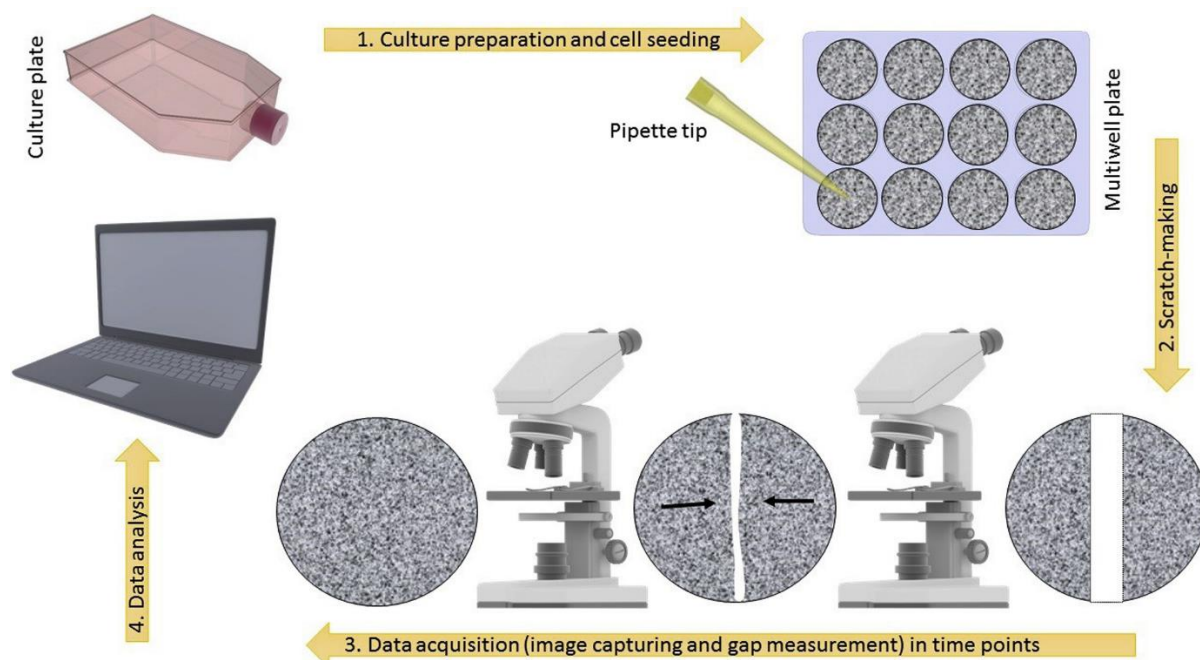


Figure 6.1 . Workflow of the scratch assay conducted on PC-3 and LnCap cells, reproduced from (Grada et al., 2017).

$$\% \text{ wound closure} = \frac{\text{wound area at time } t_0 - \text{wound area at time } t_x}{\text{wound area at time } t_0} \times 100\% \dots \dots \dots \text{Equation 6.1}$$

$$\% \text{ growth inhibition} = \frac{\% \text{ closure of control at time } t_x - \% \text{ closure at time } t_x}{\% \text{ wound closure at time } t_0} \times 100\% \dots \dots \text{Equation 6.2.}$$

Where t_0 = time of initial scratch, and t_x = time of area measurement after t_0

6.2.3.2 Analysis of Apoptotic Effect and Cell Cycle using the MUSE™ Cell Analyzer

The MUSE™ Cell Analyzer (LuminexCorp, Austin, TX, USA) was used to detect miniaturized fluorescence emission from single cell suspensions in order to provide quantitative information on the effects of the nanoparticle treatments on apoptosis and the cell cycle of PC-3 and LnCap cells. A system check and complete clean cycle were run prior to experimentation. Additionally, the quick clean function was carried out in between experiments to prevent sample cross contamination and blockage of the capillary tubes.

6.2.3.2.1 Apoptotic Effect by Annexin V and Propidium Iodide Reaction

The apoptotic effect was assayed using the MUSE™ Annexin V and Dead Cell Kit (LuminexCorp, Austin, TX, USA). PC-3 and LnCap cells were seeded at 1×10^4 cells/mL and

treated with concentrations of docetaxel equivalent to effective concentration of 8 ng/mL of native docetaxel, NTN's and ATN's. After 72 hours, cells were washed with PBS, trypsinized for 4 minutes, and diluted with culture media. Thereafter, 100 μ L samples of cell suspensions were aliquoted into microcentrifuge tubes and 100 μ L of the MUSE™ Annexin V and Dead Cell Reagent was added to each tube, which was then vortexed gently for 5-10 seconds to homogenize. Samples were stained for 20 minutes at room temperature in the dark and then analyzed by the MUSE™ Cell Analyzer by selecting the Annexin V and Dead Cell Assay.

6.2.3.2.2 *Cell Cycle Analysis*

The cell cycle was analysed using the MUSE™ Cell Cycle Kit (LuminexCorp, Austin, TX, USA). PC-3 and LnCap cells were seeded at 1×10^4 cells/mL and treated. After 72 hours, cell suspensions were centrifuged, washed with PBS and fixed with cold 70% ethanol. Samples were frozen at -20 °C for at least 4 hours. Thereafter, samples were centrifuged at 300 x g for 5 minutes, washed once with PBS and thereafter, the resulting cell pellet was aliquoted into microcentrifuge tubes. 200 μ L of the MUSE™ Cell Cycle Reagent was added to each tube, which was then vortexed. Samples were stained for 30 minutes in the dark and then analyzed by the MUSE™ Cell Analyzer by selecting the Cell Cycle Assay.

6.2.3.4. *Determination of ROS Activation*

Reactive oxygen species in cells were determined using the Fluorometric Intracellular ROS kit (Sigma-Aldrich, St. Louis, MO, USA). LnCap cells were seeded in 96 well plates at a cell density of 5×10^3 cells/well and 24 hours post cell attachment, cells were treated with 100 μ L of the fluorometric master buffer assay mix and incubated for 1 hour. Thereafter cells were treated with growth media (control) and the effective concentration of 8 ng/mL of docetaxel, NTN and ATN. Plates were read 72 hours after treatment at excitation wavelength of 475 nm and emission wavelength of 520 nm. Fluorescence intensity was taken to be proportional to ROS activation as per assay protocol.

6.2.3.5. *Spheroid Optimization Assay*

Spheroids were generated from the LnCap cell line using a method adapted from (Friedrich et al., 2009). Low attachment cell culture plates (Corning, Merck, Modderfontein, South Africa) were used to seed cells at different densities, with five replicates per density. This was to

determine the optimal density for uniform spheroid formation. Spheroids were monitored using the bright field microscopy channel on the CELENA X High Content Imaging System (Logos Biosystems, Anyang-Si, South Korea), and volumes were calculated using equation 6.3 (Jouberton et al., 2022) .

$$V = a(b^2/2) \dots\dots\dots eqn 6.3$$

Where V = volume of spheroid in μm^3 a= major diameter in μm and b = minor diameter in μm .

6.2.3.6. *Spheroid Viability Assay*

Spheroids were measured on day 4 and treated with a range of concentrations of docetaxel, NTN and ATN. Seventy-two hours later (day 7), Alamar Blue assay reagent was added to the culture medium, and culture plates were incubated for 3 hours at 37 °C. Thereafter, fluorescence readings were taken using 535 nm excitation and 595 nm wavelengths on the Viktor X3 multiplate reader (Perkin Elmer, MA, USA).

6.2.3.7. *Spheroid Growth Inhibition Assay*

Spheroids were generated from the LnCap cell line using a method adapted from (Friedrich et al., 2009). Briefly, cell culture plates were coated with 1.5% autoclaved agarose and cooled before seeding at different cell densities to determine optimal density for uniform spheroids. Spheroids were monitored using phase contrast microscopy and volumes were calculated using the software ImageJ, and equation 6.3 (Jouberton et al., 2022) .

$$V = a(b^2/2) \dots\dots\dots \text{Equation 6.3}$$

Where V = volume of spheroid in μm^3 a= major diameter in μm and b = minor diameter in μm .

Thereafter, spheroids were measured on day 4 and subsequently treated with media (control), docetaxel, NTN and ATN. Spheroids were measured 72 hours later (day 7) and treated again. Thereafter, spheroids were treated and measured every 48 hours (days 9. 11. 13), and measured again at day 15 and day 17. which was the endpoint of the assay. This schedule is outlined in Figure 6.2. adapted from (Friedrich et al., 2009).

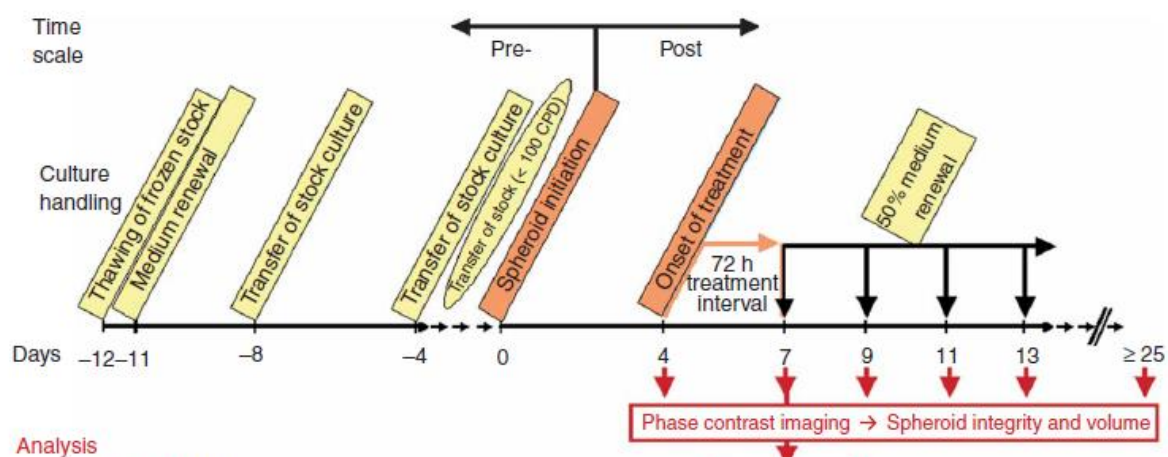


Figure 6.2 Time scale of growth and treatment of LnCap multicellular tumour spheroids, adapted from (Friedrich et al., 2009)

6.2.4 *In vivo* Biocompatibility Studies on Male Nude Mice

Twelve male nude MF1 mice at 6-8 weeks of age were housed in individual cages fitted with a filtered air supply, and maintained at 25°C. They were given food and water *ad libitum* and allowed to habituate for 7 days before intervention. Mice were injected subcutaneously with either docetaxel, NTN or ATN solutions with equivalent concentration of 20 mg/kg docetaxel (Li et al., 2018). Control animals were injected with equivalent volumes of saline. After 72 hours of treatment, mice were terminated and samples of heart, lung, liver, kidney and spleen were preserved in 10% buffered formalin for haematoxylin/eosin staining and independent histopathological analysis by IDEXX laboratories (Midrand, South Africa). All protocols were approved with the AREC ethics clearance certificate number 2022/11/02/C.

6.2.5 *Statistical Analysis*

All experimental data were processed and analyzed by Origin software (version 8.5.0 SR1, OriginLab Corporation, U.S.). All results were represented by the mean $\pm$ standard error of three experiments. Comparison of data between experimental groups was evaluated by one way analysis of variance (ANOVA) and Bonferroni adjustment, with p values less than 0.05 being designated as significant.

6.3 Results and Discussion

6.3.1 *Assessment of Nanosystem Therapeutic Efficacy by Cancer Cell Scratch Assay*

Cell migration is involved in many important cellular processes, including cell division, tumour cell invasion, angiogenesis and metastasis. The cytoskeleton of microtubules, which are tube-like intracellular structures that promote cell division, is implicated in cell migration. This cytoskeleton should be initially intact, and thereafter, the asymmetry of microtubules precedes directional migration (Wang et al., 2019). Taxanes such as docetaxel are potent microtubule stabilizers and have been found to influence migration by interacting with microtubules and interfering with their dynamics within the cell (Rodrigues-Ferreira et al., 2020).

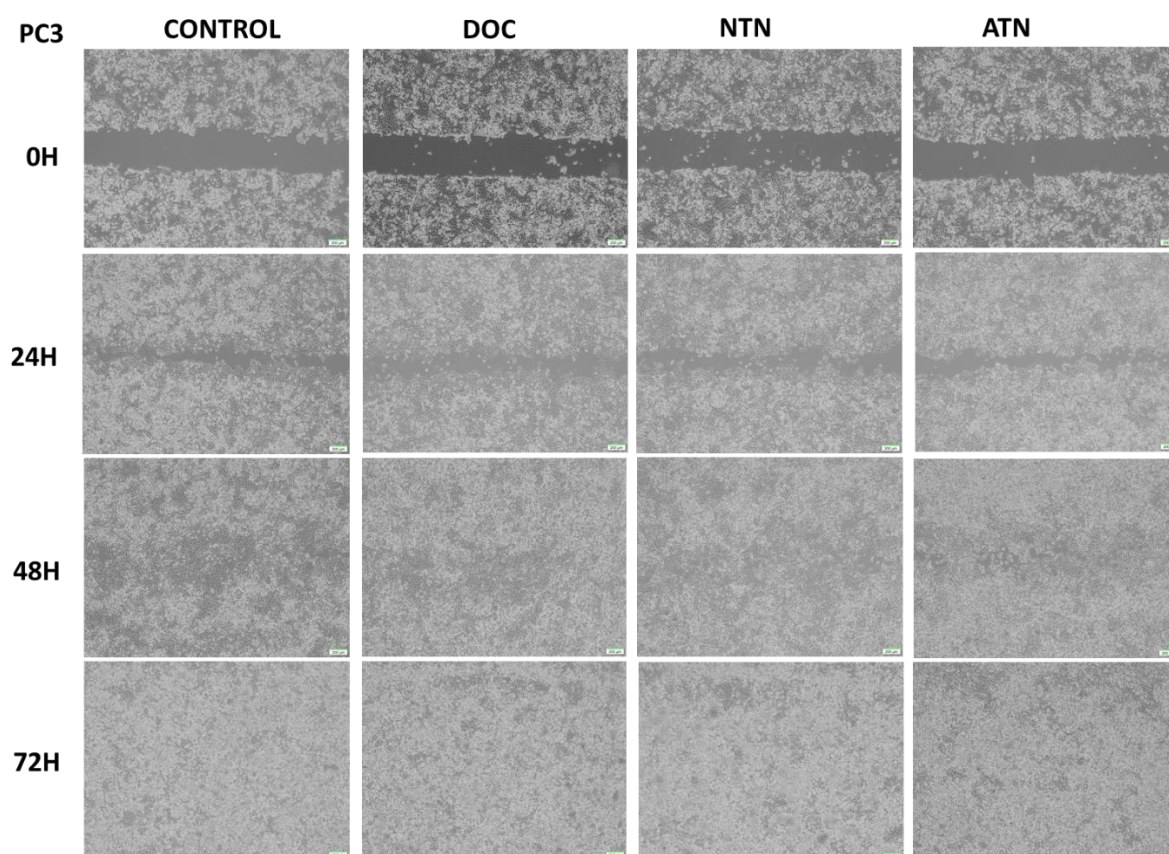


Figure 6.3 Wound healing of PC-3 cells treated with growth media (control), and effective concentrations of 8 ng/mL docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate.

Cancer cell scratch wound healing assays can provide insight into the therapeutic efficacy of cancer treatments as the deregulation of cell migration can affect the above physiological processes associated with cancer cell phenotypes. The tracking of the inhibition of wound closure of cancer cells after being exposed to effective concentrations can indicate the metastatic potential of the treatment (Bahar and Yoon, 2021). The assay is used to evaluate cancer cell metastasis by the inhibition of “wound healing” caused by the treatments and the time required for healing (Bouchalova and Bouchal, 2022).

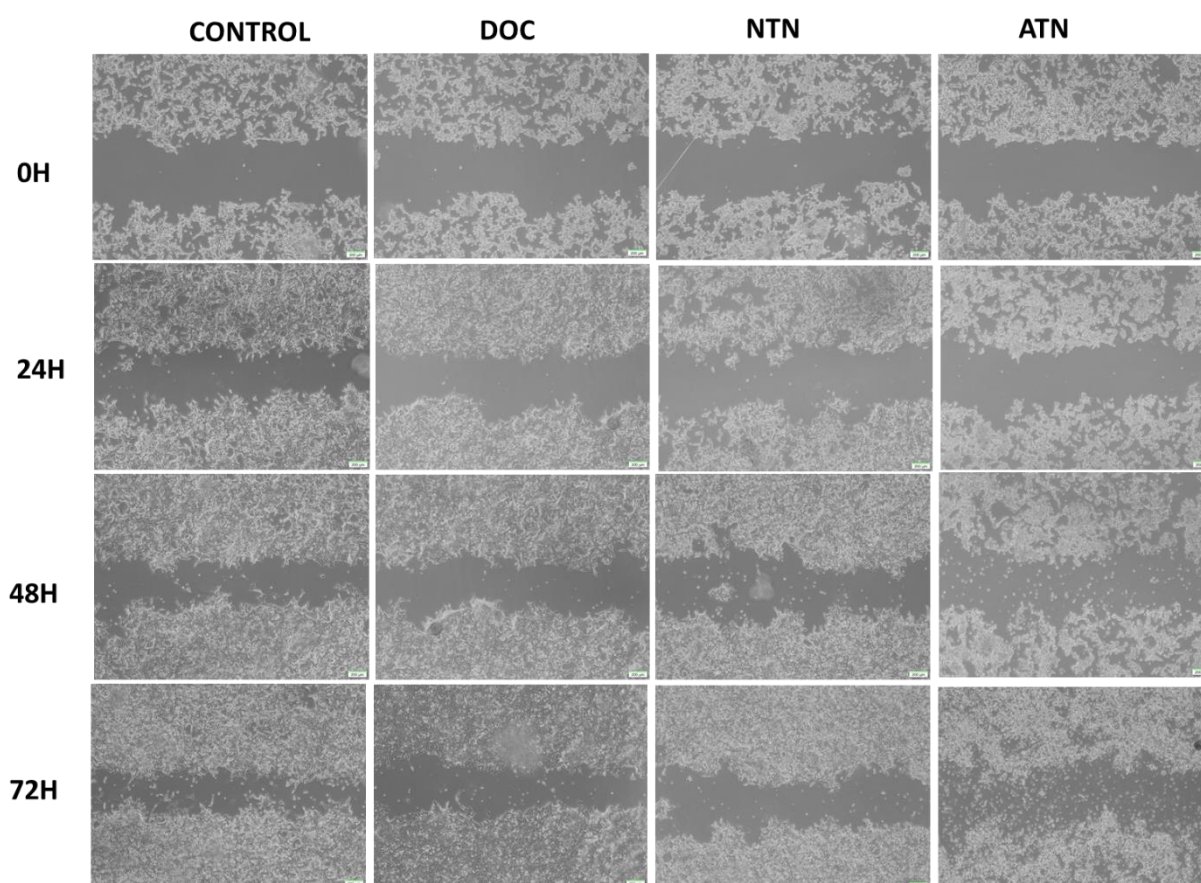


Figure 6.4 Wound healing of LnCap cells treated with growth media (control), and effective concentrations of 8 ng/mL docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate.

Images were captured at 0H to measure the initial wound area, and thereafter the change in wound area was measured and calculated by equations 6.1 and 6.2. every 24 hours for each treatment on each cell line. For the assay on PC-3 cells, all treatments had no significant difference in wound closure at 24 hours and cells migrated to fill the wound area by 48 hours (Figure 6.3). This indicated that none of the treatments had any noticeable or significant effect on wound closure (Figure 6.5). Wang and co-workers obtained similar closure results after 24 hours of treatment with 1 nM (0.81 ng/mL) docetaxel (Wang et al., 2022). However, they did recently report marked inhibition of wound closure on PC-3 cells, 24 hours after being treated with 0.1 μ M (80.8 ng/mL) of docetaxel (He et al., 2024). This suggests that docetaxel could have anti-migratory effects on PC-3 cells at higher concentrations, but since the effective concentration in this study (8ng/mL) was to the order of 10 lower than that used in He's study, the fact that there was no effect of any of the treatments on migration could be due to the relatively low effective concentration of docetaxel used.

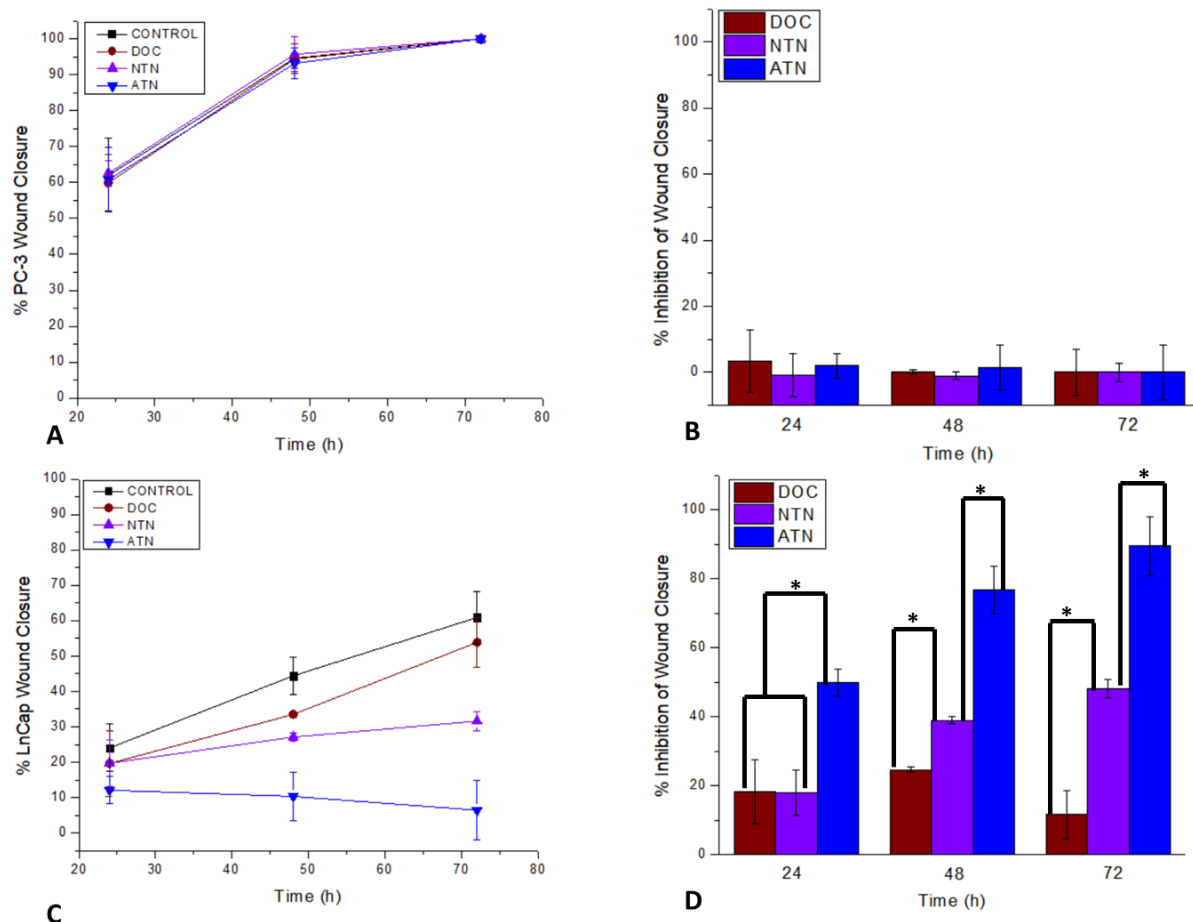


Figure 6.5 . Percentage Wound Closure and Wound Closure Inhibition relative to control of PC-3 (A and B) and LnCap cells (C and D) after being treated for 24, 48 and 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).

As can be seen by Figures 6.4 and 6.5A and C, the migration rate of both treated and control groups of LnCap cells was much slower than PC-3 cells, with the control group only reaching $61.0 \pm 7.4\%$ wound closure after 72 hours. This is consistent with a recent study by Bossio and co-workers (Bossio et al., 2023) who achieved $<75\%$ wound closure of the control group of LnCap cells after 72 hours. In this study, the NTN treated group showed significantly greater inhibition of closure than the docetaxel treated group at 48 ($39.0 \pm 1.1\%$ for NTN and $24.6 \pm 0.7\%$ for docetaxel) and 72 hours ($48.1 \pm 2.7\%$ for NTN and $11.6 \pm 7.1\%$ for docetaxel), but displayed no significantly different inhibition percentage to docetaxel at 24 hours, shown by Figure 6.5D. This could be due to the initial availability of the entire dose of docetaxel in the solution media, compared to the NTN system which showed a sustained release profile (chapter 5) and therefore caused more inhibition at the later time points.

As shown by Figure 6.5D, the inhibition of wound closure by the ATN treated LnCap cells was significantly greater ($p < 0.05$) than the other treated groups at all time points, with $49.8 \pm 3.9\%$, $76.7 \pm 6.8\%$ and $89.4 \pm 8.4\%$ inhibition at 24, 48 and 72 hours respectively. This can be attributed to the active targeting antibody moiety on the surface of the nanosystem that could have allowed for increased cellular uptake, facilitated by the PSMA receptors found on the surface of LnCap cells (Wüstemann et al., 2019), leading to the intracellular inhibition of motility. The earlier observation of no effect on wound closure of any of the treatments on PC-3 cells in Figure 6.3 and 6.5A and B was attributed mainly to the PC-3 cell line being insensitive to the concentration of docetaxel used. However, this could also be due in part to the lack of PSMA receptors on these cells (Runge et al., 2023), limiting the cellular uptake of ATN and the subsequent release of docetaxel within the cells. As shown by Figure 6.4, from 24 hours after treatment, the wound spaces of the control and docetaxel treated LnCap cells are mainly occupied by cell projections from the edge of the wound, consistent with chemotaxis, which is an important mechanism in cell migration caused by functional chemical signals (SenGupta et al., 2021). However, from the 48-hour time point, there is considerable cell shrinkage and detachment from the culture plate in the wound space of the NTN and more prominently in the ATN treated groups. This phenomenon is consistent with previous cancer cell migratory inhibition studies (Wang et al., 2019; Mishra et al., 2020), and is due to enhanced cellular uptake, toxicity and inhibition of intracellular physiological processes by the nanosystems.

Taxanes have been found to inhibit wound closure at concentrations lower than effective anti-proliferative concentrations (Wang et al., 2019; Lu et al., 2020), and this is consistent with the results shown in Figure 6.5, which still shows low inhibition of wound closure of free docetaxel on LnCap cells even though the effective concentration of native docetaxel used is much lower than the anti-proliferative concentration measured in chapter 5.

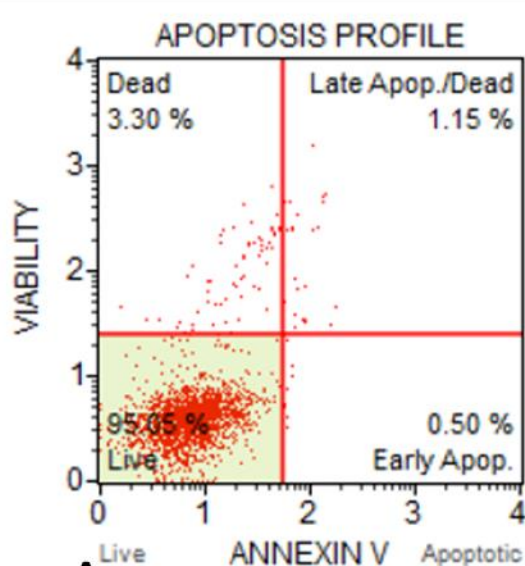
6.3.2 *Apoptotic Effect of Treatments*

Cell death can proceed through different mechanisms, the most well-known being necrosis and apoptosis. (Carneiro and El-Deiry, 2020; Liu and Jiao, 2020; Chaudhry et al., 2022). Therefore, the evaluation of apoptotic potential of treatments on cancer cells can provide information about their therapeutic efficacy (Lee and Lee, 2021).

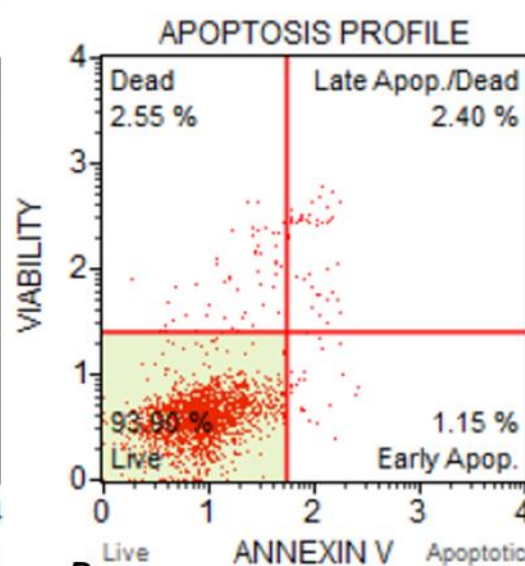
PC-3

CONT

NTN



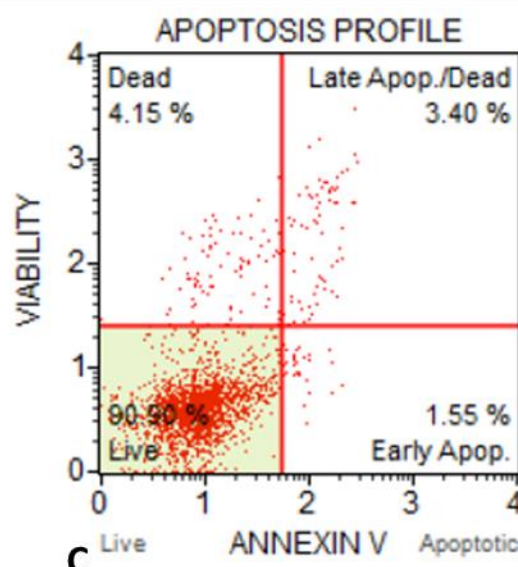
A



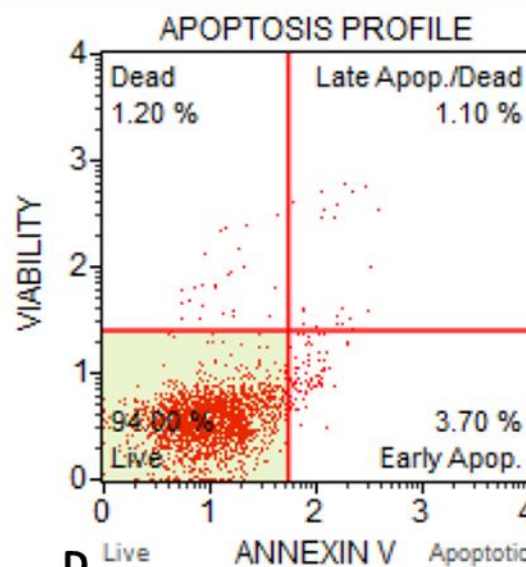
B

DOC

ATN



C



D

Figure 6.6 Apoptosis profiles – representative dot plots showing cell densities of PC-3 cells after being treated for 24, 48 and 72 hours with A- growth media (control), and 8 ng/mL of docetaxel in C-free docetaxel (DOC), B-non-targeted nanoparticles (NTN) and D-targeted nanoparticles (ATN). In each profile, lower left quadrant shows live cells, lower right quadrant shows early apoptotic cells, upper right quadrant shows late apoptotic cells and upper left quadrant shows dead cells. Experiments were conducted in triplicate.

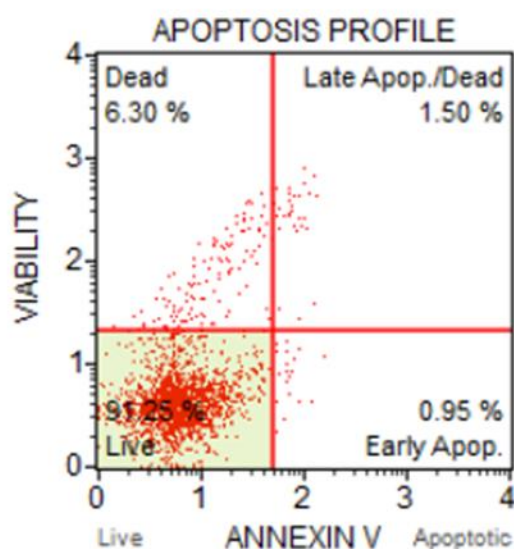
The apoptosis profiles of treated groups are reflected in Figure 6.6 for PC-3 cells and Figure 6.7 for LnCap cells. The main apoptotic pathways measured were through early and late

apoptosis, and as shown by Figure 6.8. there was no significant difference in the total apoptosis of native docetaxel ($4.83 \pm 0.4\%$), NTN ($3.4 \pm 0.80\%$) and ATN ($4.25 \pm 0.63\%$) in PC-3 cells. However, both nanosystems displayed lower necrosis profiles (top left quadrant in Figure 6.6), with $2.27 \pm 0.37\%$ for NTN and $1.26 \pm 0.07\%$ for ATN than free docetaxel ($4.12 \pm 0.30\%$). Figure 6.8 also reflects that there was no significant difference between the apoptotic profiles of both nanosystems on PC-3 cells. Yang and co-workers (Yang et al., 2019) reported similarly low response levels when treating PC-3 cells with low concentrations of docetaxel and only achieved higher apoptotic levels ($>10\%$) after treating with their measured inhibitory concentration of docetaxel. The results here, at the effective concentration of 8 ng/mL docetaxel correlates with the result of no observable inhibition of wound healing obtained with the scratch assay in Figure 6.3 and Figure 6.5A and B, suggesting that apoptosis could be involved the inhibition of cell migration and motility in PC-3 cells.

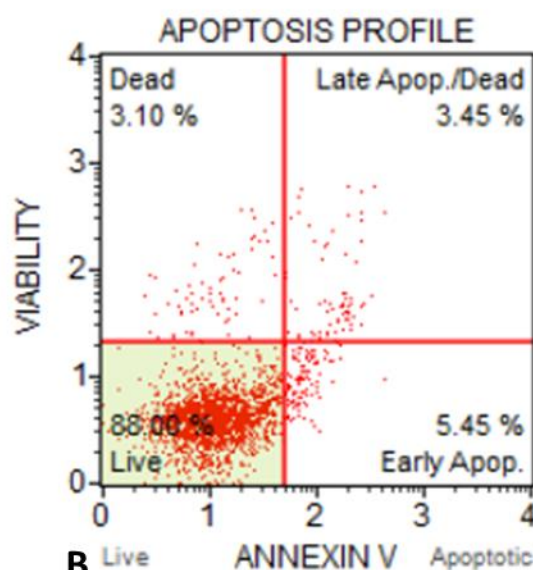
LnCap

CONT

NTN



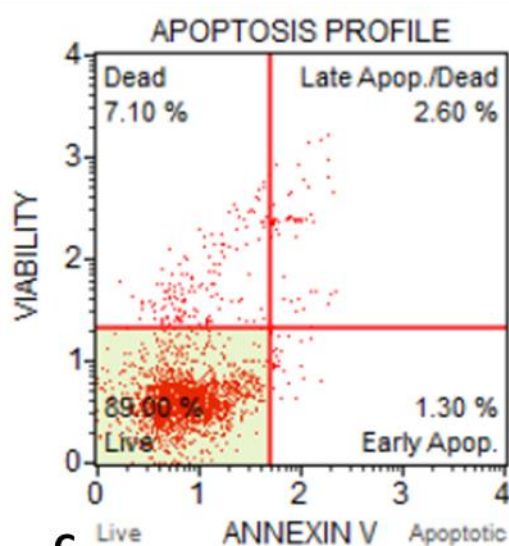
A



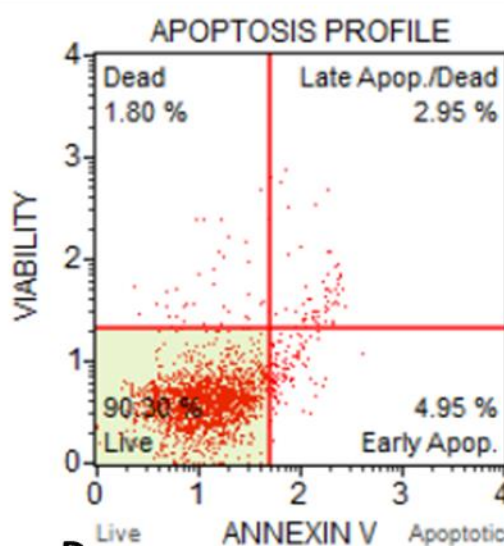
B

DOC

ATN



C



D

Figure 6.7 Apoptosis profiles – representative dot plots showing cell densities of LnCap cells after being treated for 24, 48 and 72 hours with A- growth media (control), and 8 ng/mL of docetaxel in C-free docetaxel (DOC), B-non-targeted nanoparticles (NTN) and D- targeted nanoparticles (ATN). In each profile, lower left quadrant shows live cells, lower right quadrant shows early apoptotic cells, upper right quadrant shows late apoptotic cells and upper left quadrant shows dead cells. Experiments were conducted in triplicate.

Interestingly, there was also no significant difference in the apoptotic profiles and total apoptosis of both nanosystems on LnCap cells ($8.32 \pm 0.68\%$ for NTN and 9.01 ± 0.90 for ATN), as shown by Figure 6.8. but both were significantly greater than the total apoptosis of free

docetaxel, which was $3.82 \pm 0.38\%$. The fact that there was greater apoptosis by the nanosystems compared to docetaxel in LnCap cells but not in PC-3 cells could be due to the greater sensitivity of the LnCap cell line to lower docetaxel concentrations, as shown in Chapter 5. The increase in total apoptosis of the nanosystems relative to docetaxel could be the effect of the increase in the bioavailability of docetaxel provided by both nanosystems (shown in Chapter 5), rather than any targeting ability, evidence by the lack difference in the total apoptosis between the targeted and non-targeted systems. These results are relatively consistent with several studies which show enhanced apoptotic mechanisms of nanosystems compared to free docetaxel (Chen et al., 2016; Rivero-Buceta et al., 2019; Bromma et al., 2022). In this study, the increase in apoptosis shown by both the nanosystems on LnCap cells correlates with the change in cell morphology and detachment of the monolayer observed in the scratch assay in 6.3.1 and noted in Figure 6.4 on the NTN and ATN treated groups. This suggests that apoptosis could be a contributing factor to this phenomenon, but since the result is more pronounced in the ATN treated group, there could be factors other than apoptosis, driven by effects such as cell cycle arrest and inhibition of proteins linked to metastasis (Vargas-Accarino et al., 2021) at work here.

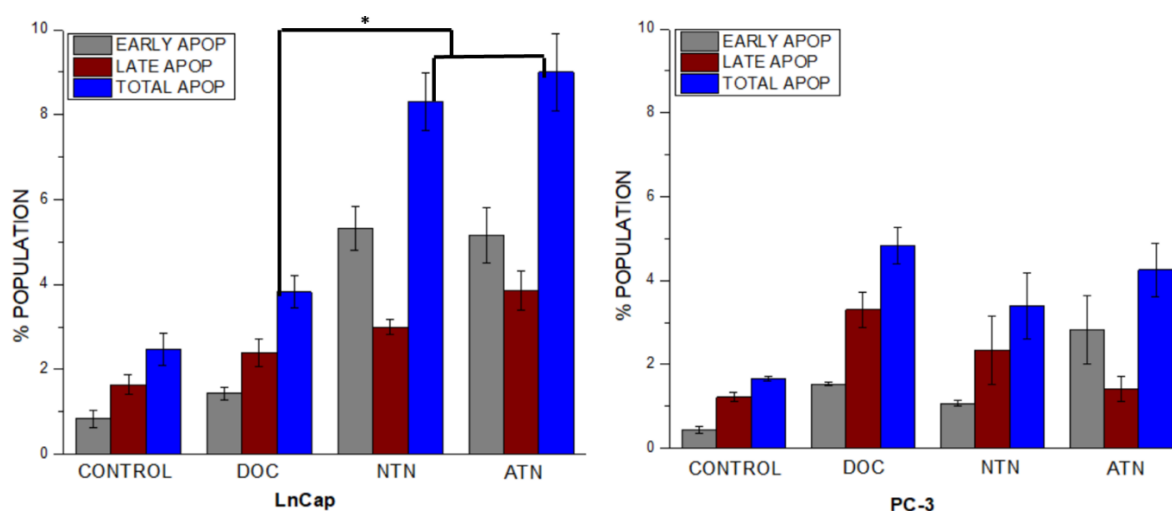


Figure 6.8 Profiles showing apoptotic populations of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. (* $p < 0.05$)

6.3.3 3.4 Cell Cycle Arrest

The anti-cancer mechanism of docetaxel is well characterised (Hashemi et al., 2023), (Imran et al., 2020). (Ehsan et al., 2022). In this study, the cell cycle profiles of both cell lines were evaluated with all treatments to compare the mechanism of action of docetaxel and the nanosystems, and their corresponding effects on the cell cycle.

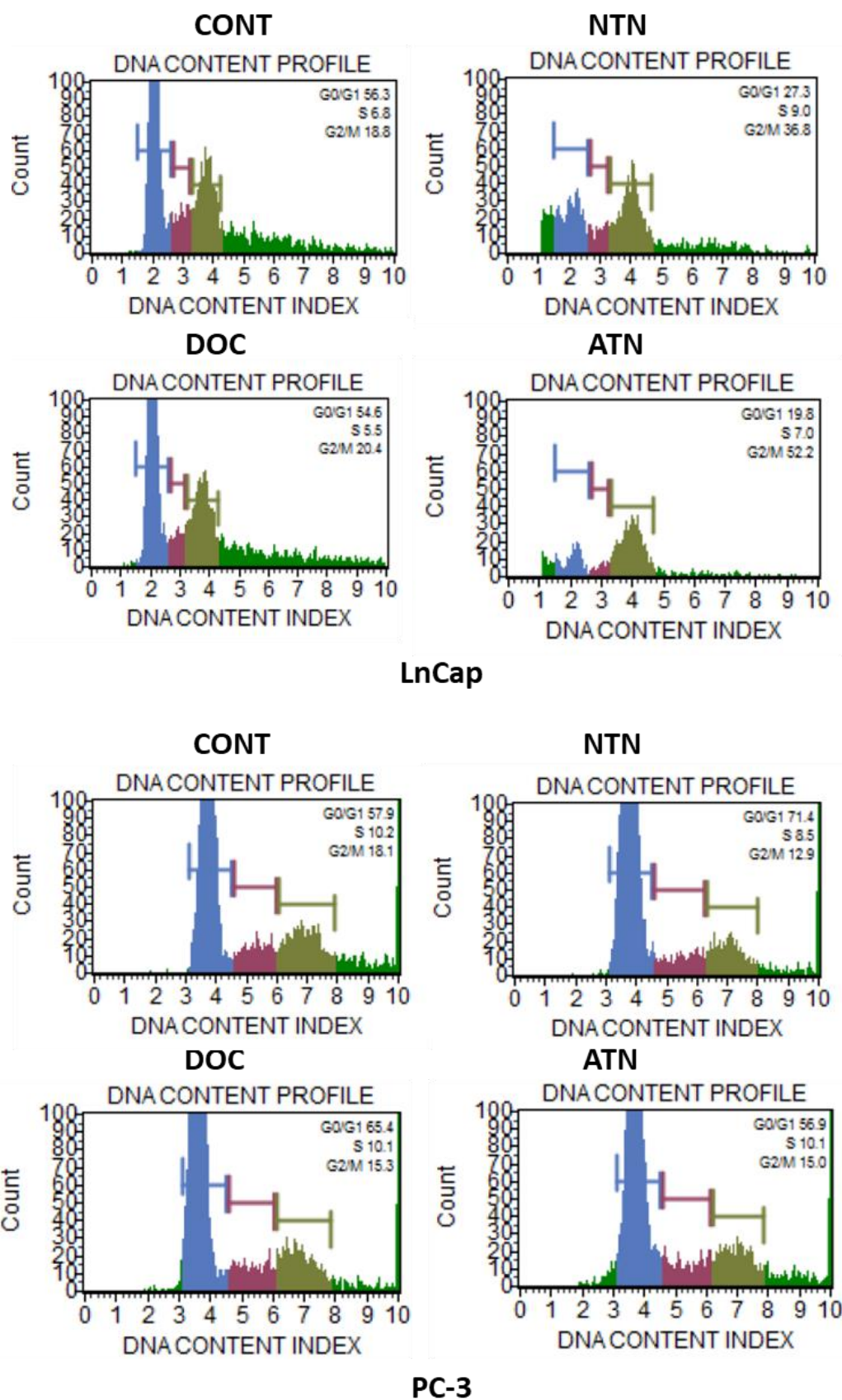


Figure 6.9 Profiles showing apoptotic populations of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC),

non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate

As suggested by Figure 6.9 and reflected in Figure 6.10, there was no significant difference in the percentages of PC-3 cells in the G2/M phase across all treatment groups, indicating that none of the treatments could cause the G2/M phase mitotic arrest that is usually associated with docetaxel. Lv and co-workers reported a modest increase from 20 to 30% in the G2/M population of PC-3 cells treated with 20 nM (16.1 ng/mL) docetaxel (Lv et al., 2017), but it was not stated if this difference was significant, while Cancino's group (Cancino-Marentes et al., 2021) and Meidavilla-Varela's group (Mediavilla-Varela et al., 2009) both reported no significant differences when treating PC-3 cells with 25 nM (20.2 ng/mL) and 3 μ M (2 424 ng/mL) docetaxel respectively. In this work, the fact that that mitotic arrest did not occur correlates with the results obtained for the wound healing assay where there was no inhibition of cell migration and wound closure across all treatments in the PC-3 cell line.

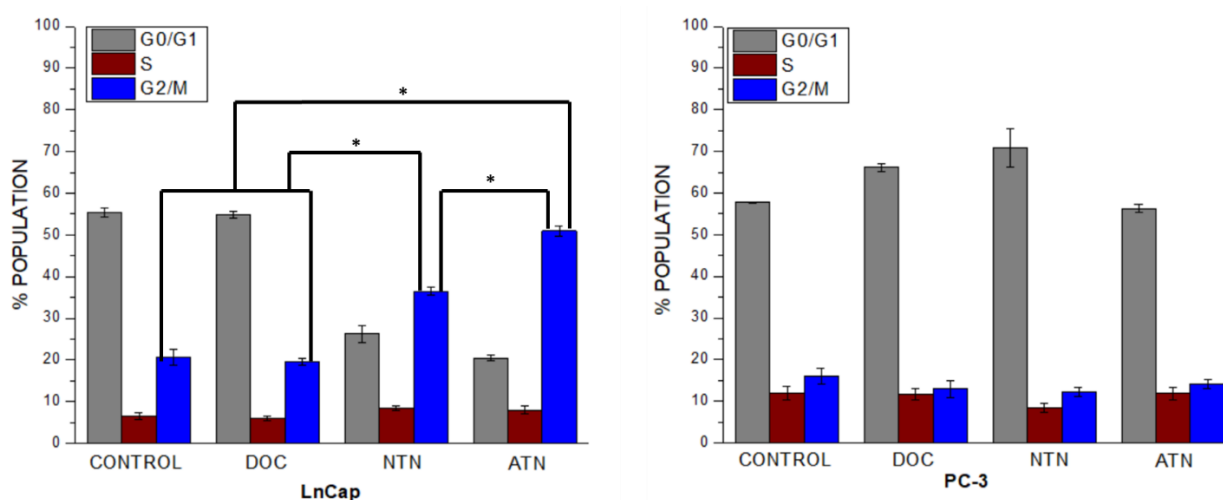


Figure 6.10 Comparison of populations in the different cell cycle phases of LnCap and PC-3 cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).

There was a significantly higher percentage of LnCap cell populations in the G2/M phase of both the groups treated with the nanosystems, compared to the untreated control and native docetaxel treated groups, as shown in Figures 6.9 and 6.10. The G2/M phase populations of the control and docetaxel treated groups which were not significantly different to each other, which suggests that at this concentration, free docetaxel was unable to cause significant

mitotic arrest. Lima's group reported a 36% increase (Lima et al., 2021) and Lv's group reported a 47% increase (Lv et al., 2017) in the LnCap G2/M phase populations, both groups having treated with 20 nM (16.1 ng/mL) docetaxel. This could suggest that in this study, the concentration of free docetaxel used (8 ng/mL) was too low to induce cell cycle arrest in the G2/M phase.

However, there were significantly higher percentages of G2/M phase populations in the NTN treated ($36.4 \pm 1.0\%$) and ATN treated ($50.9 \pm 1.2\%$) groups, as shown in Figure 6.10. Similar results were obtained by Cao and co-workers, who prepared non-targeted and targeted PLGA nanoparticles loaded with docetaxel, and reported 30% and 60% of G2/M phase populations of LnCap cells treated with the targeted and non-targeted nanosystems respectively (Cao et al., 2016). In this study, the increase in G2/M phase populations indicate the proportional induction of mitotic arrest in the NTN and ATN treated groups. The populations translated to $15.8 \pm 2.8\%$ and $30.3 \pm 3.0\%$ arrest relative to the control for NTN and ATN treated cells, respectively. The difference in efficacy can be attributed in part to the targeting ability of the ATN, allowing for increased cellular uptake via the interaction between the targeting antibody and the PSMA receptors on the surface of the LnCap cell line, whereas the NTN does not have anti-PSMA ligands on its surface and therefore is not able to take advantage of the PSMA receptors on the LnCap cell surface (chapter 5). This could explain the proportional increase (ATN>NTN>DOC) in the inhibition of cell migration observed earlier, as mitotic arrest caused by prevention of microtubule polymerisation leads to inhibition of cell motility (Nakonechnaya and Shewchuk, 2015). A contributing factor is that the cell cycle arrest would have disrupted the G2/M checkpoint that prevents cells with damaged DNA from undergoing mitosis (Ventura and Giordano, 2019). Additionally, the G2 checkpoint arrest could have inhibited the formation of maturation/mitosis promotion factor (MPF), which promotes cells from the G2 to the M phase in the cell cycle (Zhang et al., 2021), thereby preventing cell migration.

6.3.4 ROS Activation

ROS is a normal by-product of healthy physiological processes (Katerji et al., 2019). However, overproduction of ROS can trigger apoptosis and therefore the measurement of the production of ROS induced (ROS activation) caused by proposed cancer treatments can be a valuable indicator of their apoptotic potential (Zaidieh et al., 2019).

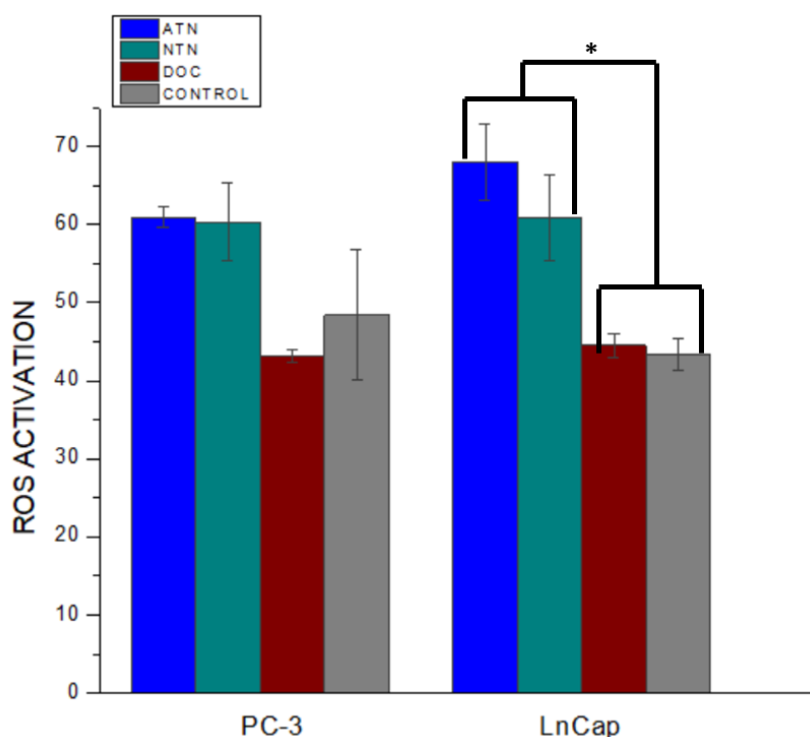


Figure 6.11 Activation of reactive oxygen species of PC-3 and LnCap cells after being treated for 72 hours with growth media (control), and 8 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Experiments were conducted in triplicate. Values reflected are the mean of three measurements with error bars showing standard error of the mean. (*p value <0.05).

The ROS activation was analysed fluorometrically and the results showed no significant difference in the ROS activation levels between any of the treated groups relative to the control in PC-3 cells, as shown in Figure 6.11. However, the ROS activation levels of both nanosystems were significantly greater than the control and docetaxel in LnCap cells, while the level of the NTN treated group was not significantly different to the ATN treated group, also reflected in Figure 6.11. This indicates no effect by the targeted system on ROS activation in LnCap cells but does suggest superior ROS activation of both nanosystems compared to docetaxel. The results of the ROS activation by both nanosystems correlate with the apoptosis results which show no significant difference between the nanosystems but more apoptosis of both nanosystems compared to docetaxel.

6.3.6.1. Inhibition of LnCap Multi-cellular Tumour Spheroid Growth

3D multicellular spheroids have been shown to mimic the characteristics of *in vivo* tumours more accurately than 2D models and there has been widespread discussion of the clinical relevance of the use of *in-vitro* 3D models in studying tumour biology (Filipiak-Duliban et al., 2022). Additionally, the 3D structure of cancer cell spheroids in assays to test efficacy of

treatments are more indicative of their projected performance *in vivo*, as the treatment is only able to penetrate outer layers of the spheroids, much like in *in vivo* tumours and unlike 2D cell culture where the treatments are available in the growth medium, to a higher percentage of the cells attached to the culture plate (Perche and Torchilin, 2012).

In this study the seeding density of LnCap cells was optimized to generate uniform spheroids, using 10 000 to 650 cells per well (Figure 6.13).

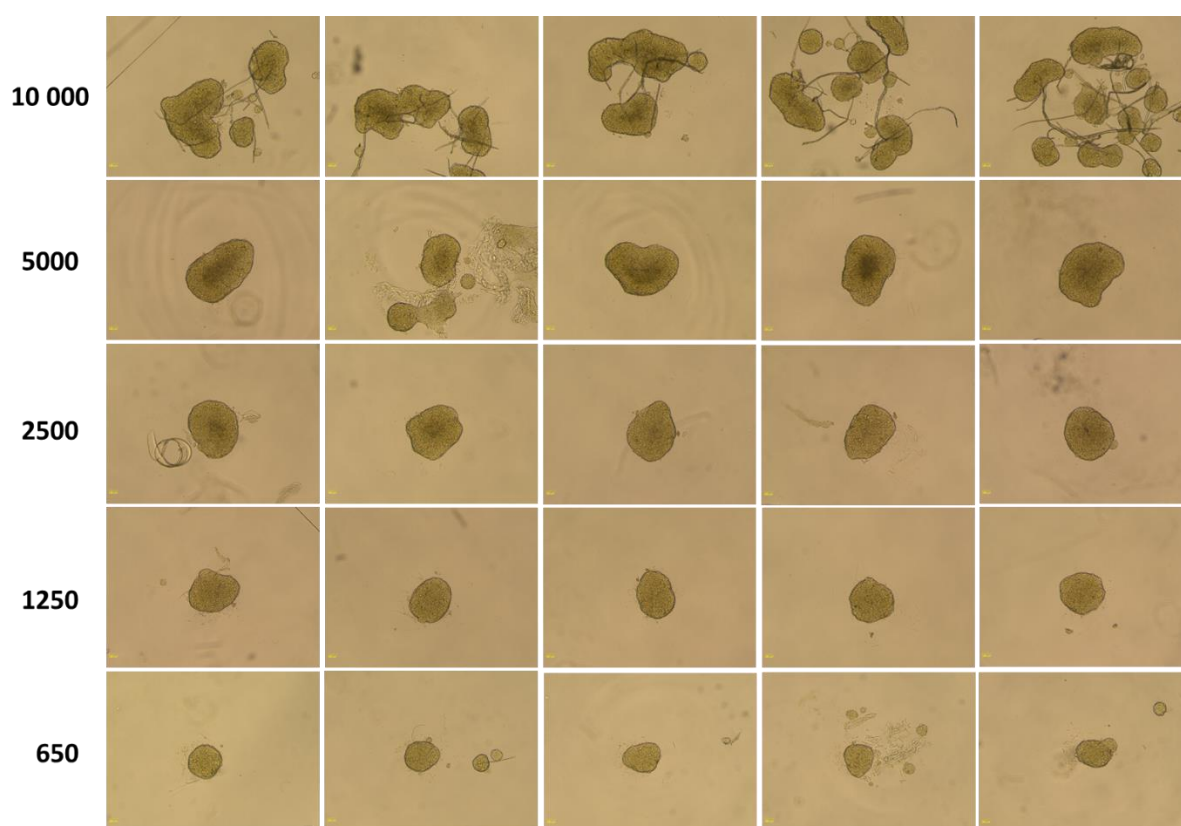


Figure 6.13. Dependence of untreated spheroid morphology and size on initial seeding density of LnCap cells shown by spheroids formed on day 4. Experiments were conducted using five replicates for each cell density.

Singular spheroids did not grow with the seeding density of 10 000 cells per well, while the spheroids that developed from the initial seeding density of 5000 cells/well displayed irregular morphology, as shown in Figure 6.13. The formation of a necrotic core can be observed in some of the spheroids initially seeded with 5000 cells/well. The necrotic zone indicates the inner areas of the spheroid which do not have access to oxygen and nutrients as the outer layers do, and therefore form a zone of toxic waste products (Dini et al., 2016). As Figure 6.13 shows, uniform spheroids were formed with a seeding density of 1250 cells/well, and their average diameter was 416 μm . This corresponds to the optimal diameter of approximately 400 μm that indicated by the protocol (Friedrich et al., 2009) and therefore this density was used to carry out all further spheroid assays.

6.3.6.2. Spheroid Viability Assay Shows Reduced Response to Treatments

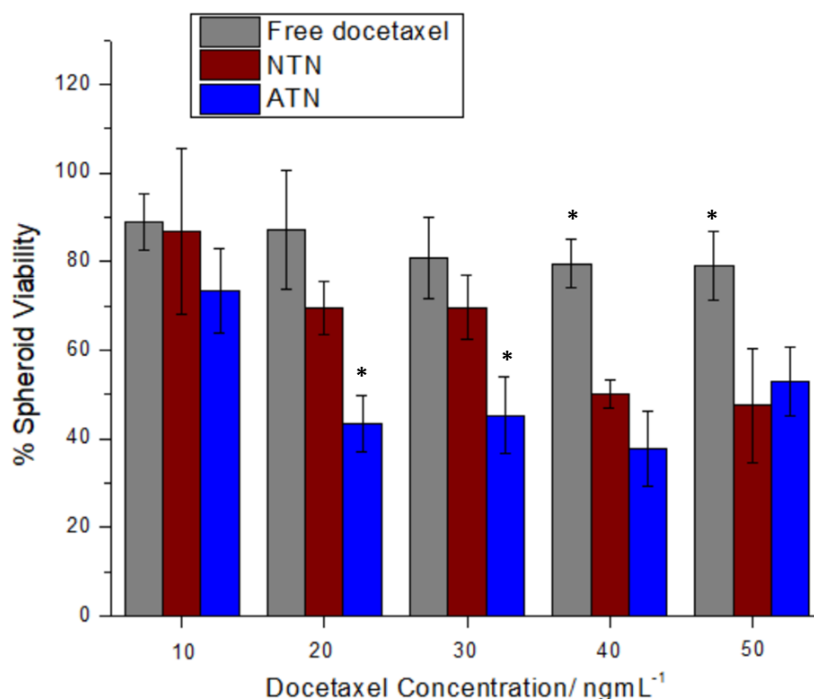


Figure 6.14: Viability of LnCap Multicellular Tumour Spheroids after being treated for 72 hours with a range of concentrations of docetaxel, NTN's and ATN's (* $p < 0.05$)

As Figure 6.14 shows, free docetaxel was not effective at reducing the viability of the spheroids at any of the tested concentrations, while the NTN treatment was significantly more effective than docetaxel alone at concentrations of 40 ng/mL and 50 ng/mL. However, the ATN treatment was significantly more effective than both treatments at 20 ng/mL and 30 ng/mL. Notably, the minimum effective concentration of the ATN treatment for spheroids is greater than that for the 2D cells, reported in Chapter 5 (20 ng/mL for spheroids compared to 8 ng/mL for 2D LnCap cells). This could be due to the inadequate penetration of the treatments through the barrier of the 3D spheroid structure (Perche and Torchilin, 2012), and also explains the lack of efficacy of free docetaxel on the spheroids shown in Figure 6.14 (80 – 90% viability at all treatment concentrations) compared to LnCap cells (50% viability at 28 ng/mL), reported on in Chapter 5. This is consistent with the research of Xu's group that required twice the concentration of docetaxel treatments to cause spheroid viability from 80 to 60% (Xu et al., 2022). For this study, 20 ng/mL was used as the effective concentration of free docetaxel and docetaxel in nano-systems for all further spheroid assays.

6.3.6.3. Spheroid Growth Inhibition Assay

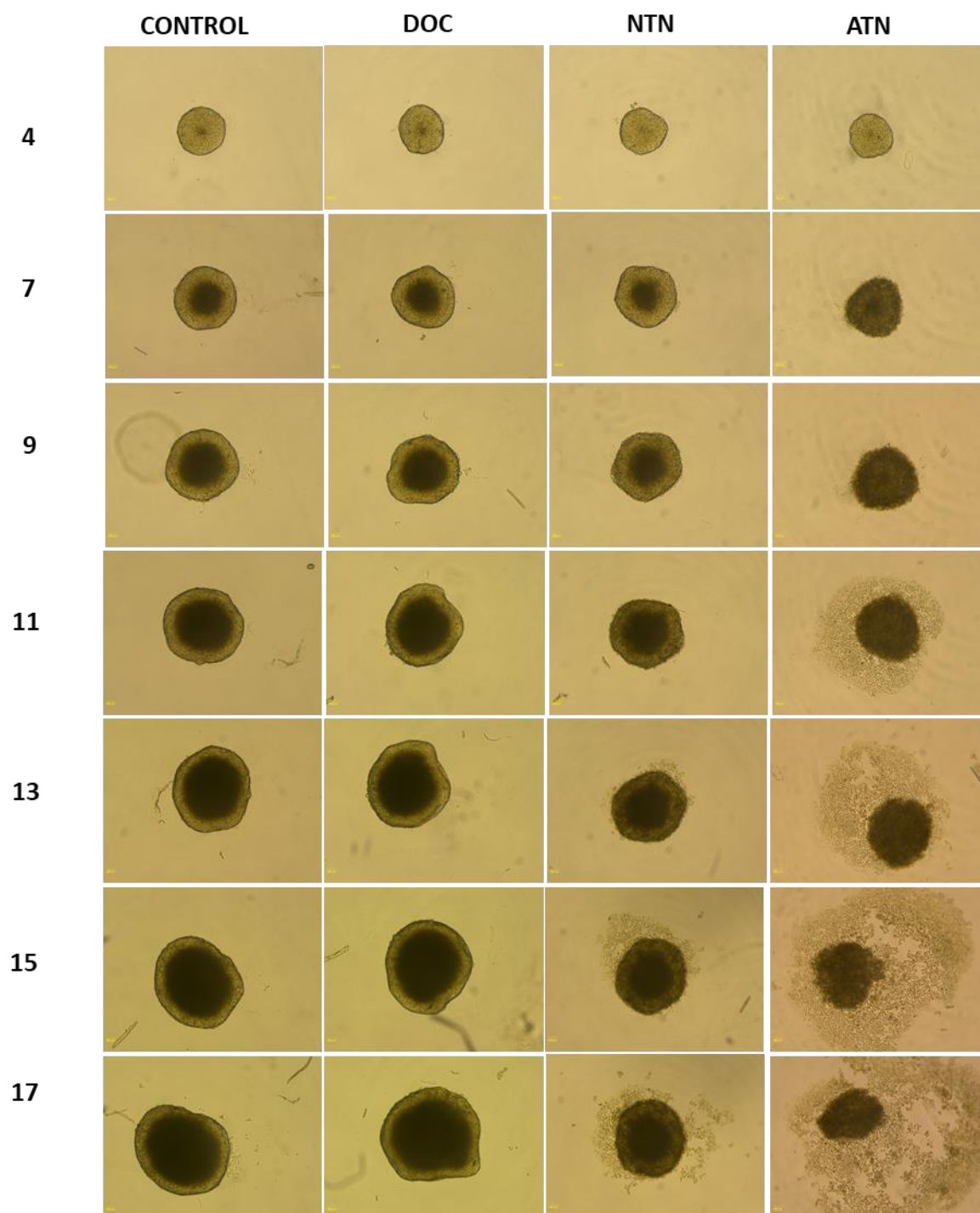


Figure 6.15. Bright field images showing 17-day growth of LnCap multicellular tumour cell spheroids treated with growth media (control), and 20 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). Five replicates were conducted for each treatment condition

Treatments were conducted as reflected in Figure 6.2. on days 4. 7. 9. 11 and 13 (Friedrich et al., 2009). As shown by Figure 6.15, there was no observable difference between the

morphology of untreated spheroids and the spheroids treated with docetaxel at all time points. Spheroids treated with the NTN showed cell death from day 11, while those treated with the ATN showed cell death from day 7. This correlates with the relative reduction in viability between the treatments, shown in Figure 6.14, and demonstrates the ability of the targeted system to penetrate the 3D spheroids more efficiently than either the NTN or docetaxel.

Figure 6.16 shows that both untreated and spheroids grew exponentially, at a much faster rate than both the nano-system treated groups after day 9. This which has been observed in previous inhibitory studies on spheroid growth (Brüningk et al., 2020; Kulesza et al., 2023) . There was also no significant difference on spheroid growth between the untreated spheroids and docetaxel treatment at all time points. This corresponds with the viability measurements in Figure 6.14, which shows no significant difference between the 20 ng/mL docetaxel treatment and the control. The endpoint of the assay was recommended to be at any point after 14 days, when the volume of the untreated spheroid reaches five times the volume before first treatment (Friedrich et al., 2009). In this study, this volume translated to 0.414 mm³ on day 15. Thereafter, one more measurement was taken at 17 days of spheroid growth.

As can be seen by Figure 6.16. there was significant inhibition of spheroid growth in the ATN treated group from day 11 (32.7±4.5%) until day 17 (67.8±5.5%) and in the NTN treated group from day 13 (21.0±3.9%) until 17 (17.6±4.6%). The earlier and higher growth inhibition of the ATN demonstrates its superior targeting ability and the inhibition by both nano-systems from day 13 points to the higher efficiency of spheroid growth inhibition of both the nano-systems compared to docetaxel at the same concentration. Additionally, Figure 6.15 shows morphology changes and necrosis, by the darkening of the ATN and NTN treated spheroids from days 7 and 11 respectively. This is earlier than the inhibition observations (days 11 and 13 respectively), indicating toxicity at the cellular level that occurs before changes to spheroid size.

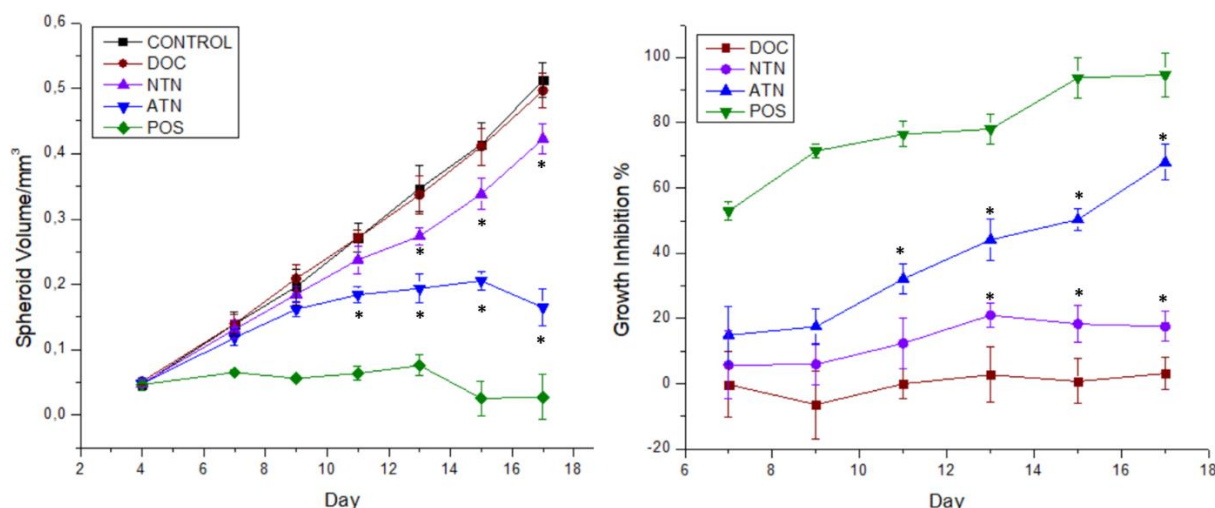


Figure 6.16. Spheroid volume increase and percentage growth inhibition relative to control of spheroids treated with growth media (control), and 20 ng/mL of docetaxel in free docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN). DMSO (5%) was used as a positive control. Five replicates were conducted per treatment condition. Values reflected are the mean of five measurements with error bars showing standard error of the mean. (*p value <0.05)

As with *in vivo* studies, the relevant dosage of treatments should be optimized for 3D culture models (Friedrich et al., 2009). In previous studies (Bäcker et al., 2016; Eder et al., 2016; Jouberton et al., 2022; Raitanen et al., 2023), cell viability studies were conducted on spheroids with ranges of concentrations in order to determine the effective inhibitory concentrations of treatments for 3D multicellular tumour spheroids inhibitory concentrations (Vinci et al., 2012). Much like 2D culture measurements, the results of cell viability assays vary greatly between studies, and there have been reports of inhibitory concentrations of docetaxel on LnCap spheroids between 70 nM (56.6 ng/mL) (Edmondson et al., 2016) and 25 μ M (Jouberton et al., 2022). These concentrations are much higher than what was used in this study (20 ng/mL), and it could be possible that this concentration was too low for docetaxel alone to have an effect on spheroid growth and health.

Previous studies on the effects of docetaxel-loaded nanosystems on LnCap spheroids are sparse. Karandish and co-workers did report on docetaxel loaded polymersomes targeted with folic acid and showed a decrease in cell viability of 88% to 55% between their non-targeted and targeted systems on LnCap spheroids (Karandish et al., 2016). They tested a lower effective docetaxel concentration of 10 nM (8.1 ng/mL), while in this study there was no significant difference between the NTN and ATN treatments at 10 ng/mL. However, at an effective concentration of 20 ng/mL, the cell viability of the spheroids was reduced from 69,5 $\pm$ 6.0% to 43.4 $\pm$ 6.4%, indicating a similar improvement of the targeted system. In

Karendish's study there was no report of spheroid growth volume inhibitory studies to compare with.

6.3.5 *Histopathological Analysis of Major Organs of Nude MF1 mice after treatments*

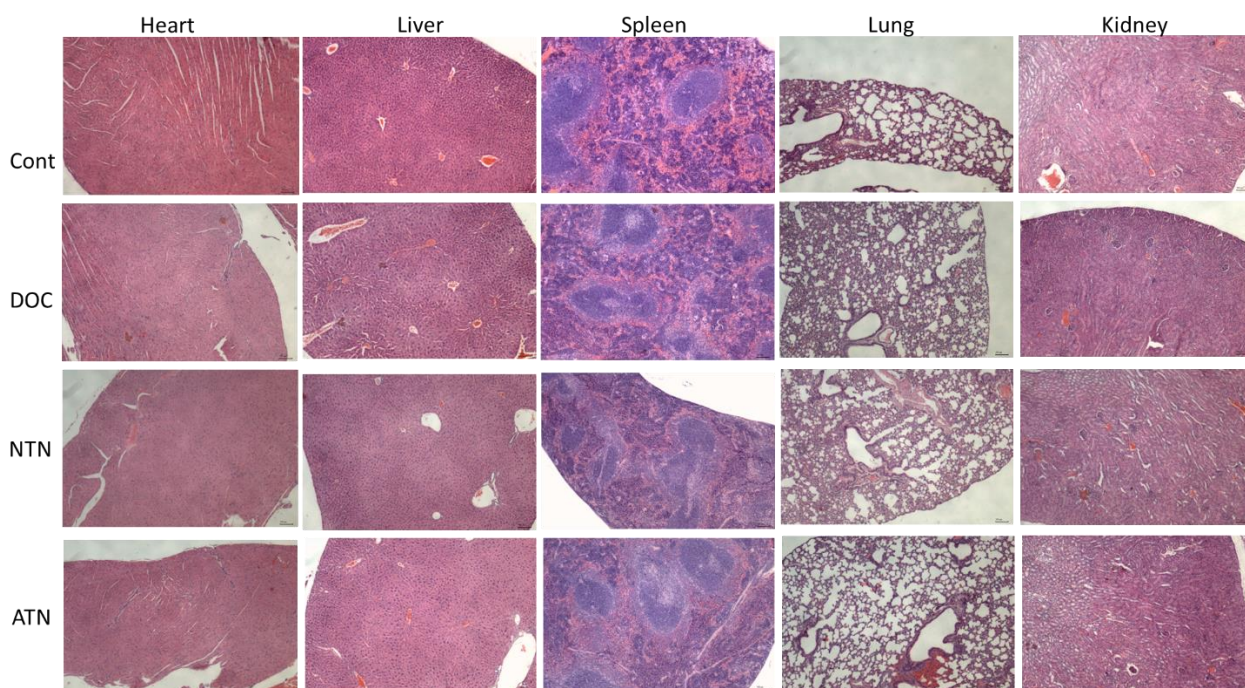


Figure 6.12 Specimens of major organs of Nude MF1 mice treated with saline (cont), docetaxel (DOC), non-targeted nanoparticles (NTN) and targeted nanoparticles (ATN).

Histopathology was independently performed by IDEXX laboratories (Midrand, South Africa). All mouse organs displayed mostly normal pathology. There was no observable microscopic pathology found in the kidneys or hearts of the mice with any of the treatments or control. The livers from mice treated with docetaxel and NTN did show mild micro-vesicular vacuolar change in some of the centrilobular hepatocytes, shown in Figure 16. However, this is a sub-lethal, nonspecific change which could be induced by several possibilities including hypoxic injury or toxicities, and the extent of the change is unlikely to be associated with clinical symptoms and is very mild.

There was some mononuclear leukostasis present in the vascular capillaries of the lungs of all treated and control groups, as well as mild alveolar hemorrhage in the lungs of mice treated with NTN and ATN, but this is a very common agonal change noted in mice, regardless of whether death is caused by euthanasia or other reasons. The spleens of mice in all groups,

including the control, showed some white pulp hyperplasia with expansion of the periarteriolar lymphoid sheaths and prominent marginal zones, but this may well fall within the normal spectrum for these mice. Therefore, there was no detectable histological indications of toxicity in these analyses of organs.

In this study, it was attempted to determine if a single dose of treatment would have an effect on the major organs after 72 hours of dosage. Similar results were observed by Bromma's group (Bromma et al., 2022) who found no observable effect on major organs after 72 hours of dosing of 6 mg/kg docetaxel. Ren and co-workers (Ren et al., 2022) also reported limited toxicity on normal tissues after 10 days of alternate-day dosing of 10 mg/kg docetaxel and docetaxel-loaded nanoparticles. Several studies have evaluated effects on major organs after the induction of xenograft tumours and subsequent treatments with docetaxel (Li et al., 2018; Ghassami et al., 2019; Di Gregorio et al., 2022). In these studies, tumours were allowed to grow until specified maximum volumes and thereafter, treatments were administered repeatedly before euthanasia, tumour and organ harvesting. This allowed for the evaluation of the effects of repeated dosing of treatment formulations on the tumours and major organs compared to free docetaxel. Relevant noteworthy results were obtained by Ghassami and co-workers (Ghassami et al., 2019), who used dosage equivalents of 5 mg/kg docetaxel every 3 days for 2 weeks and observed reduced toxicity by their targeted nanosystem compared to docetaxel on all organs except for the spleen.

6.4 Conclusions

In this study, the optimized nanosystem described in Chapter 5 was assessed for its *in-vitro* effect on prostate cancer cell mechanisms, the inhibition of LnCap spheroid growth, and *in vivo* biocompatibility in nude MF1 mice. The targeted nanosystem preferentially inhibited cell migration and cell cycle progression on LnCap cells compared to PC-3 cells. Significantly higher inhibition at Day 7 on 3D LnCap spheroid growth was noted compared to free docetaxel and none of the treatments showed any short-term detrimental effects on the major organs of nude MF1 mice.

6.5 References

Autio, K. A., Dreicer, R., Anderson, J., Garcia, J. A., Alva, A., Hart, L. L., et al. (2018). Safety and Efficacy of BIND-014, a Docetaxel Nanoparticle Targeting Prostate-Specific Membrane Antigen for Patients With Metastatic Castration-Resistant Prostate Cancer. *JAMA Oncol* 4, 1344. doi: 10.1001/jamaoncol.2018.2168

- Bäcker, A., Göppert, B., Sturm, S., Abaffy, P., Sollich, T., and Gruhl, F. J. (2016). Impact of adjustable cryogel properties on the performance of prostate cancer cells in 3D. *Springerplus* 5, 902. doi: 10.1186/s40064-016-2629-z
- Bahar, E., and Yoon, H. (2021). Modeling and Predicting the Cell Migration Properties from Scratch Wound Healing Assay on Cisplatin-Resistant Ovarian Cancer Cell Lines Using Artificial Neural Network. *Healthcare* 9, 911. doi: 10.3390/healthcare9070911
- Bossio, S., Perri, A., Gallo, R., De Bartolo, A., Rago, V., La Russa, D., et al. (2023). Alpha-Lipoic Acid Reduces Cell Growth, Inhibits Autophagy, and Counteracts Prostate Cancer Cell Migration and Invasion: Evidence from In Vitro Studies. *Int J Mol Sci* 24, 17111. doi: 10.3390/ijms242317111
- Bouchalova, P., and Bouchal, P. (2022). Current methods for studying metastatic potential of tumor cells. *Cancer Cell Int* 22, 394. doi: 10.1186/s12935-022-02801-w
- Bromma, K., Dos Santos, N., Barta, I., Alexander, A., Beckham, W., Krishnan, S., et al. (2022). Enhancing nanoparticle accumulation in two dimensional, three dimensional, and xenograft mouse cancer cell models in the presence of docetaxel. *Sci Rep* 12, 13508. doi: 10.1038/s41598-022-17752-5
- Brüningk, S. C., Rivens, I., Box, C., Oelfke, U., and ter Haar, G. (2020). 3D tumour spheroids for the prediction of the effects of radiation and hyperthermia treatments. *Sci Rep* 10, 1653. doi: 10.1038/s41598-020-58569-4
- Cancino-Marentes, M. E., Hernández-Flores, G., Ortiz-Lazareno, P. C., Villaseñor-García, M. M., Orozco-Alonso, E., Sierra-Díaz, E., et al. (2021). Sensitizing the cytotoxic action of Docetaxel induced by Pentoxifylline in a PC3 prostate cancer cell line. *BMC Urol* 21, 38. doi: 10.1186/s12894-021-00807-6
- Cao, L.-B., Zeng, S., and Zhao, W. (2016). Highly Stable PEGylated Poly(lactic-co-glycolic acid) (PLGA) Nanoparticles for the Effective Delivery of Docetaxel in Prostate Cancers. *Nanoscale Res Lett* 11, 305. doi: 10.1186/s11671-016-1509-3
- Carneiro, B. A., and El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. *Nat Rev Clin Oncol* 17, 395–417. doi: 10.1038/s41571-020-0341-y

- Chaudhry, G.-S., Md Akim, A., Sung, Y. Y., and Sifzizul, T. M. T. (2022). Cancer and apoptosis: The apoptotic activity of plant and marine natural products and their potential as targeted cancer therapeutics. *Front Pharmacol* 13. doi: 10.3389/fphar.2022.842376
- Chen, Z., Tai, Z., Gu, F., Hu, C., Zhu, Q., and Gao, S. (2016). Aptamer-mediated delivery of docetaxel to prostate cancer through polymeric nanoparticles for enhancement of antitumor efficacy. *European Journal of Pharmaceutics and Biopharmaceutics* 107, 130–141. doi: 10.1016/j.ejpb.2016.07.007
- Di Gregorio, E., Romiti, C., Di Lorenzo, A., Cavallo, F., Ferrauto, G., and Conti, L. (2022). RGD_PLGA Nanoparticles with Docetaxel: A Route for Improving Drug Efficiency and Reducing Toxicity in Breast Cancer Treatment. *Cancers (Basel)* 15, 8. doi: 10.3390/cancers15010008
- Dini, S., Binder, B. J., Fischer, S. C., Mattheyer, C., Schmitz, A., Stelzer, E. H. K., et al. (2016). Identifying the necrotic zone boundary in tumour spheroids with pair-correlation functions. *J R Soc Interface* 13, 20160649. doi: 10.1098/rsif.2016.0649
- Eder, T., Weber, A., Neuwirt, H., Grünbacher, G., Ploner, C., Klocker, H., et al. (2016). Cancer-Associated Fibroblasts Modify the Response of Prostate Cancer Cells to Androgen and Anti-Androgens in Three-Dimensional Spheroid Culture. *Int J Mol Sci* 17, 1458. doi: 10.3390/ijms17091458
- Edmondson, R., Adcock, A. F., and Yang, L. (2016). Influence of Matrices on 3D-Cultured Prostate Cancer Cells' Drug Response and Expression of Drug-Action Associated Proteins. *PLoS One* 11, e0158116. doi: 10.1371/journal.pone.0158116
- Ehsan, I., Kumari, L., Sen, R., Al Hoque, A., Mukherjee, B., Mukherjee, A., et al. (2022). J591 functionalized paclitaxel-loaded PLGA nanoparticles successfully inhibited PSMA overexpressing LNCaP cells. *J Drug Deliv Sci Technol* 75, 103689. doi: 10.1016/j.jddst.2022.103689
- El Rassy, E., Ghosn, M., Farhat, F., Bakouny, Z., Assi, T., Chahine, G., et al. (2018). Toxicities Associated with Docetaxel-Based Regimens in the Adjuvant Treatment of Early-Stage Breast Cancer: A Multicenter Prospective Real-Life Experience. *Breast Care* 13, 119–123. doi: 10.1159/000481339
- Filipiak-Duliban, A., Brodaczewska, K., Kajdasz, A., and Kieda, C. (2022). Spheroid Culture Differentially Affects Cancer Cell Sensitivity to Drugs in Melanoma and RCC Models. *Int J Mol Sci* 23, 1166. doi: 10.3390/ijms23031166

- Friedrich, J., Seidel, C., Ebner, R., and Kunz-Schughart, L. A. (2009). Spheroid-based drug screen: considerations and practical approach. *Nat Protoc* 4, 309–324. doi: 10.1038/nprot.2008.226
- Ghassami, E., Varshosaz, J., Minaiyan, M., Nasirikenari, M., and Hoseini, S. M. (2019). Biodistribution, Safety and Organ Toxicity of Docetaxel-Loaded in HER-2 Aptamer Conjugated Ecoflex® Nanoparticles in a Mouse Xenograft Model of Ovarian Cancer. *Recent Pat Nanotechnol* 13, 49–58. doi: 10.2174/1872210513666181128162403
- Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z., and Falanga, V. (2017). Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *Journal of Investigative Dermatology* 137, e11–e16. doi: 10.1016/j.jid.2016.11.020
- Hashemi, M., Zandieh, M. A., Talebi, Y., Rahmanian, P., Shafiee, S. S., Nejad, M. M., et al. (2023). Paclitaxel and docetaxel resistance in prostate cancer: Molecular mechanisms and possible therapeutic strategies. *Biomedicine & Pharmacotherapy* 160, 114392. doi: 10.1016/j.biopha.2023.114392
- He, T., Li, N.-X., Pan, Z.-J., Zou, Z.-H., Chen, J.-C., Yu, S.-Z., et al. (2024). Serine/threonine kinase 36 induced epithelial-mesenchymal transition promotes docetaxel resistance in prostate cancer. *Sci Rep* 14, 729. doi: 10.1038/s41598-024-51360-9
- Hsu, C.-Y., Rheima, A. M., Kadhim, M. M., Ahmed, N. N., Mohammed, S. H., Abbas, F. H., et al. (2023). An overview of nanoparticles in drug delivery: Properties and applications. *S Afr J Chem Eng* 46, 233–270. doi: 10.1016/j.sajce.2023.08.009
- Imran, M., Saleem, S., Chaudhuri, A., Ali, J., and Baboota, S. (2020). Docetaxel: An update on its molecular mechanisms, therapeutic trajectory and nanotechnology in the treatment of breast, lung and prostate cancer. *J Drug Deliv Sci Technol* 60, 101959. doi: 10.1016/j.jddst.2020.101959
- Jouberton, E., Voissiere, A., Penault-Llorca, F., Cachin, F., and Miot-Noirault, E. (2022). Multicellular tumor spheroids of LNCaP-Luc prostate cancer cells as in vitro screening models for cytotoxic drugs. *Am J Cancer Res* 12, 1116–1128.
- Karandish, F., Haldar, M. K., You, S., Brooks, A. E., Brooks, B. D., Guo, B., et al. (2016). Prostate-Specific Membrane Antigen Targeted Polymersomes for Delivering Mocetinostat and Docetaxel to Prostate Cancer Cell Spheroids. *ACS Omega* 1, 952–962. doi: 10.1021/acsomega.6b00126

- Katerji, M., Filippova, M., and Duerksen-Hughes, P. (2019). Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field. *Oxid Med Cell Longev* 2019, 1–29. doi: 10.1155/2019/1279250
- Kulesza, J., Paluszkiewicz, E., and Augustin, E. (2023). Cellular Effects of Selected Unsymmetrical Bisacridines on the Multicellular Tumor Spheroids of HCT116 Colon and A549 Lung Cancer Cells in Comparison to Monolayer Cultures. *Int J Mol Sci* 24, 15780. doi: 10.3390/ijms242115780
- Lee, S.-H., and Lee, Y.-J. (2021). Synergistic anticancer activity of resveratrol in combination with docetaxel in prostate carcinoma cells. *Nutr Res Pract* 15, 12. doi: 10.4162/nrp.2021.15.1.12
- Li, C., Wang, Z., Wang, Q., Ka Yan Ho, R. L., Huang, Y., Chow, M. S. S., et al. (2018). Enhanced anti-tumor efficacy and mechanisms associated with docetaxel-piperine combination- *in vitro* and *in vivo* investigation using a taxane-resistant prostate cancer model. *Oncotarget* 9, 3338–3352. doi: 10.18632/oncotarget.23235
- Lima, T. S., Iglesias-Gato, D., Souza, L. D. O., Stenvang, J., Lima, D. S., Røder, M. A., et al. (2021). Molecular Profiling of Docetaxel-Resistant Prostate Cancer Cells Identifies Multiple Mechanisms of Therapeutic Resistance. *Cancers (Basel)* 13, 1290. doi: 10.3390/cancers13061290
- Liu, Z., and Jiao, D. (2020). Necroptosis, tumor necrosis and tumorigenesis. *Cell Stress* 4, 1–8. doi: 10.15698/cst2020.01.208
- Lu, X., Yang, F., Chen, D., Zhao, Q., Chen, D., Ping, H., et al. (2020). Quercetin reverses docetaxel resistance in prostate cancer via androgen receptor and PI3K/Akt signaling pathways. *Int J Biol Sci* 16, 1121–1134. doi: 10.7150/ijbs.41686
- Lv, G., Sun, D., Zhang, J., Xie, X., Wu, X., Fang, W., et al. (2017). Lx2-32c, a novel semi-synthetic taxane, exerts antitumor activity against prostate cancer cells in vitro and in vivo. *Acta Pharm Sin B* 7, 52–58. doi: 10.1016/j.apsb.2016.06.005
- Mediavilla-Varela, M., Pacheco, F. J., Almaguel, F., Perez, J., Sahakian, E., Daniels, T. R., et al. (2009). Docetaxel-induced prostate cancer cell death involves concomitant activation of caspase and lysosomal pathways and is attenuated by LEDGF/p75. *Mol Cancer* 8, 68. doi: 10.1186/1476-4598-8-68

- Mishra, R., Nathani, S., Varshney, R., Sircar, D., and Roy, P. (2020). Berberine reverses epithelial-mesenchymal transition and modulates histone methylation in osteosarcoma cells. *Mol Biol Rep* 47, 8499–8511. doi: 10.1007/s11033-020-05892-8
- Muhamad, N., Plengsuriyakarn, T., and Na-Bangchang, K. (2018). Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: a systematic review. *Int J Nanomedicine* Volume 13, 3921–3935. doi: 10.2147/IJN.S165210
- Mundekkad, D., and Cho, W. C. (2022). Nanoparticles in Clinical Translation for Cancer Therapy. *Int J Mol Sci* 23, 1685. doi: 10.3390/ijms23031685
- Nakonechnaya, A. O., and Shewchuk, B. M. (2015). Growth hormone enhances LNCaP prostate cancer cell motility. *Endocr Res* 40, 97–105. doi: 10.3109/07435800.2014.966383
- Perche, F., and Torchilin, V. P. (2012). Cancer cell spheroids as a model to evaluate chemotherapy protocols. *Cancer Biol Ther* 13, 1205–1213. doi: 10.4161/cbt.21353
- Raitanen, J., Barta, B., Hacker, M., Georg, D., Balber, T., and Mitterhauser, M. (2023). Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines. *Cells* 12, 360. doi: 10.3390/cells12030360
- Ren, G., Li, Y., Ping, C., Duan, D., Li, N., Tang, J., et al. (2022). Docetaxel prodrug and hematoporphyrin co-assembled nanoparticles for anti-tumor combination of chemotherapy and photodynamic therapy. *Drug Deliv* 29, 3358–3369. doi: 10.1080/10717544.2022.2147280
- Rivero-Buceta, E., Vidaurre-Agut, C., Vera-Donoso, C. D., Benlloch, J. M., Moreno-Manzano, V., and Botella, P. (2019). PSMA-Targeted Mesoporous Silica Nanoparticles for Selective Intracellular Delivery of Docetaxel in Prostate Cancer Cells. *ACS Omega* 4, 1281–1291. doi: 10.1021/acsomega.8b02909
- Rodrigues-Ferreira, S., Nehlig, A., Kacem, M., and Nahmias, C. (2020). ATIP3 deficiency facilitates intracellular accumulation of paclitaxel to reduce cancer cell migration and lymph node metastasis in breast cancer patients. *Sci Rep* 10, 13217. doi: 10.1038/s41598-020-70142-7
- Runge, R., Naumann, A., Miederer, M., Kotzerke, J., and Brogsitter, C. (2023). Up-Regulation of PSMA Expression In Vitro as Potential Application in Prostate Cancer Therapy. *Pharmaceuticals* 16, 538. doi: 10.3390/ph16040538

- Ruzycka-Ayoush, M., Kowalik, P., Kowalczyk, A., Bujak, P., Nowicka, A. M., Wojewodzka, M., et al. (2021). Quantum dots as targeted doxorubicin drug delivery nanosystems in human lung cancer cells. *Cancer Nanotechnol* 12, 8. doi: 10.1186/s12645-021-00077-9
- SenGupta, S., Parent, C. A., and Bear, J. E. (2021). The principles of directed cell migration. *Nat Rev Mol Cell Biol* 22, 529–547. doi: 10.1038/s41580-021-00366-6
- Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., et al. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71, 209–249. doi: 10.3322/caac.21660
- Vargas-Accarino, E., Herrera-Montávez, C., Ramón y Cajal, S., and Aasen, T. (2021). Spontaneous Cell Detachment and Reattachment in Cancer Cell Lines: An In Vitro Model of Metastasis and Malignancy. *Int J Mol Sci* 22, 4929. doi: 10.3390/ijms22094929
- Ventura, E., and Giordano, A. (2019). “Cell Cycle,” in *Reference Module in Life Sciences*, (Elsevier). doi: 10.1016/B978-0-12-809633-8.90189-4
- Vinci, M., Gowan, S., Boxall, F., Patterson, L., Zimmermann, M., Court, W., et al. (2012). Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol* 10, 29. doi: 10.1186/1741-7007-10-29
- Wang, X., Decker, C. C., Zechner, L., Krstin, S., and Wink, M. (2019). In vitro wound healing of tumor cells: inhibition of cell migration by selected cytotoxic alkaloids. *BMC Pharmacol Toxicol* 20, 4. doi: 10.1186/s40360-018-0284-4
- Wang, X., Xuetao, X., Wu, M., Wu, P., Sheng, Z., Liu, W., et al. (2022). Inhibitory effect of roboric acid in combination with docetaxel on human prostate cancer cells. *J Enzyme Inhib Med Chem* 37, 542–553. doi: 10.1080/14756366.2021.2018684
- Wüstemann, T., Haberkorn, U., Babich, J., and Mier, W. (2019). Targeting prostate cancer: Prostate-specific membrane antigen based diagnosis and therapy. *Med Res Rev* 39, 40–69. doi: 10.1002/med.21508
- Yang, C., Zhang, W., Wang, J., Chen, P., and Jin, J. (2019). Effect of docetaxel on the regulation of proliferation and apoptosis of human prostate cancer cells. *Mol Med Rep*. doi: 10.3892/mmr.2019.9998

- Zaidieh, T., Smith, J. R., Ball, K. E., and An, Q. (2019). ROS as a novel indicator to predict anticancer drug efficacy. *BMC Cancer* 19, 1224. doi: 10.1186/s12885-019-6438-y
- Zhang, M., Qu, J., Gao, Z., Qi, Q., Yin, H., Zhu, L., et al. (2021). Timosaponin AIII Induces G2/M Arrest and Apoptosis in Breast Cancer by Activating the ATM/Chk2 and p38 MAPK Signaling Pathways. *Front Pharmacol* 11. doi: 10.3389/fphar.2020.601468
- Autio, K. A., Dreicer, R., Anderson, J., Garcia, J. A., Alva, A., Hart, L. L., et al. (2018). Safety and Efficacy of BIND-014, a Docetaxel Nanoparticle Targeting Prostate-Specific Membrane Antigen for Patients With Metastatic Castration-Resistant Prostate Cancer. *JAMA Oncol* 4, 1344. doi: 10.1001/jamaoncol.2018.2168
- Bäcker, A., Göppert, B., Sturm, S., Abaffy, P., Sollich, T., and Gruhl, F. J. (2016). Impact of adjustable cryogel properties on the performance of prostate cancer cells in 3D. *Springerplus* 5, 902. doi: 10.1186/s40064-016-2629-z
- Bahar, E., and Yoon, H. (2021). Modeling and Predicting the Cell Migration Properties from Scratch Wound Healing Assay on Cisplatin-Resistant Ovarian Cancer Cell Lines Using Artificial Neural Network. *Healthcare* 9, 911. doi: 10.3390/healthcare9070911
- Bossio, S., Perri, A., Gallo, R., De Bartolo, A., Rago, V., La Russa, D., et al. (2023). Alpha-Lipoic Acid Reduces Cell Growth, Inhibits Autophagy, and Counteracts Prostate Cancer Cell Migration and Invasion: Evidence from In Vitro Studies. *Int J Mol Sci* 24, 17111. doi: 10.3390/ijms242317111
- Bouchalova, P., and Bouchal, P. (2022). Current methods for studying metastatic potential of tumor cells. *Cancer Cell Int* 22, 394. doi: 10.1186/s12935-022-02801-w
- Bromma, K., Dos Santos, N., Barta, I., Alexander, A., Beckham, W., Krishnan, S., et al. (2022). Enhancing nanoparticle accumulation in two dimensional, three dimensional, and xenograft mouse cancer cell models in the presence of docetaxel. *Sci Rep* 12, 13508. doi: 10.1038/s41598-022-17752-5
- Brüningk, S. C., Rivens, I., Box, C., Oelfke, U., and ter Haar, G. (2020). 3D tumour spheroids for the prediction of the effects of radiation and hyperthermia treatments. *Sci Rep* 10, 1653. doi: 10.1038/s41598-020-58569-4
- Cancino-Marentes, M. E., Hernández-Flores, G., Ortiz-Lazareno, P. C., Villaseñor-García, M. M., Orozco-Alonso, E., Sierra-Díaz, E., et al. (2021). Sensitizing the cytotoxic action of Docetaxel induced by Pentoxifylline in a PC3 prostate cancer cell line. *BMC Urol* 21, 38. doi: 10.1186/s12894-021-00807-6

- Cao, L.-B., Zeng, S., and Zhao, W. (2016). Highly Stable PEGylated Poly(lactic-co-glycolic acid) (PLGA) Nanoparticles for the Effective Delivery of Docetaxel in Prostate Cancers. *Nanoscale Res Lett* 11, 305. doi: 10.1186/s11671-016-1509-3
- Carneiro, B. A., and El-Deiry, W. S. (2020). Targeting apoptosis in cancer therapy. *Nat Rev Clin Oncol* 17, 395–417. doi: 10.1038/s41571-020-0341-y
- Chaudhry, G.-S., Md Akim, A., Sung, Y. Y., and Sifzizul, T. M. T. (2022). Cancer and apoptosis: The apoptotic activity of plant and marine natural products and their potential as targeted cancer therapeutics. *Front Pharmacol* 13. doi: 10.3389/fphar.2022.842376
- Chen, Z., Tai, Z., Gu, F., Hu, C., Zhu, Q., and Gao, S. (2016). Aptamer-mediated delivery of docetaxel to prostate cancer through polymeric nanoparticles for enhancement of antitumor efficacy. *European Journal of Pharmaceutics and Biopharmaceutics* 107, 130–141. doi: 10.1016/j.ejpb.2016.07.007
- Di Gregorio, E., Romiti, C., Di Lorenzo, A., Cavallo, F., Ferrauto, G., and Conti, L. (2022). RGD_PLGA Nanoparticles with Docetaxel: A Route for Improving Drug Efficiency and Reducing Toxicity in Breast Cancer Treatment. *Cancers (Basel)* 15, 8. doi: 10.3390/cancers15010008
- Dini, S., Binder, B. J., Fischer, S. C., Mattheyer, C., Schmitz, A., Stelzer, E. H. K., et al. (2016). Identifying the necrotic zone boundary in tumour spheroids with pair-correlation functions. *J R Soc Interface* 13, 20160649. doi: 10.1098/rsif.2016.0649
- Eder, T., Weber, A., Neuwirt, H., Grünbacher, G., Ploner, C., Klocker, H., et al. (2016). Cancer-Associated Fibroblasts Modify the Response of Prostate Cancer Cells to Androgen and Anti-Androgens in Three-Dimensional Spheroid Culture. *Int J Mol Sci* 17, 1458. doi: 10.3390/ijms17091458
- Edmondson, R., Adcock, A. F., and Yang, L. (2016). Influence of Matrices on 3D-Cultured Prostate Cancer Cells' Drug Response and Expression of Drug-Action Associated Proteins. *PLoS One* 11, e0158116. doi: 10.1371/journal.pone.0158116
- Ehsan, I., Kumari, L., Sen, R., Al Hoque, A., Mukherjee, B., Mukherjee, A., et al. (2022). J591 functionalized paclitaxel-loaded PLGA nanoparticles successfully inhibited PSMA overexpressing LNCaP cells. *J Drug Deliv Sci Technol* 75, 103689. doi: 10.1016/j.jddst.2022.103689
- El Rassy, E., Ghosn, M., Farhat, F., Bakouny, Z., Assi, T., Chahine, G., et al. (2018). Toxicities Associated with Docetaxel-Based Regimens in the Adjuvant Treatment of Early-Stage Breast

Cancer: A Multicenter Prospective Real-Life Experience. *Breast Care* 13, 119–123. doi: 10.1159/000481339

Filipiak-Duliban, A., Brodaczewska, K., Kajdasz, A., and Kieda, C. (2022). Spheroid Culture Differentially Affects Cancer Cell Sensitivity to Drugs in Melanoma and RCC Models. *Int J Mol Sci* 23, 1166. doi: 10.3390/ijms23031166

Friedrich, J., Seidel, C., Ebner, R., and Kunz-Schughart, L. A. (2009). Spheroid-based drug screen: considerations and practical approach. *Nat Protoc* 4, 309–324. doi: 10.1038/nprot.2008.226

Ghassami, E., Varshosaz, J., Minaiyan, M., Nasirikenari, M., and Hoseini, S. M. (2019). Biodistribution, Safety and Organ Toxicity of Docetaxel-Loaded in HER-2 Aptamer Conjugated Ecoflex® Nanoparticles in a Mouse Xenograft Model of Ovarian Cancer. *Recent Pat Nanotechnol* 13, 49–58. doi: 10.2174/1872210513666181128162403

Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z., and Falanga, V. (2017). Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *Journal of Investigative Dermatology* 137, e11–e16. doi: 10.1016/j.jid.2016.11.020

Hashemi, M., Zandieh, M. A., Talebi, Y., Rahmanian, P., Shafiee, S. S., Nejad, M. M., et al. (2023). Paclitaxel and docetaxel resistance in prostate cancer: Molecular mechanisms and possible therapeutic strategies. *Biomedicine & Pharmacotherapy* 160, 114392. doi: 10.1016/j.biopha.2023.114392

He, T., Li, N.-X., Pan, Z.-J., Zou, Z.-H., Chen, J.-C., Yu, S.-Z., et al. (2024). Serine/threonine kinase 36 induced epithelial-mesenchymal transition promotes docetaxel resistance in prostate cancer. *Sci Rep* 14, 729. doi: 10.1038/s41598-024-51360-9

Hsu, C.-Y., Rheima, A. M., Kadhim, M. M., Ahmed, N. N., Mohammed, S. H., Abbas, F. H., et al. (2023). An overview of nanoparticles in drug delivery: Properties and applications. *S Afr J Chem Eng* 46, 233–270. doi: 10.1016/j.sajce.2023.08.009

Imran, M., Saleem, S., Chaudhuri, A., Ali, J., and Baboota, S. (2020). Docetaxel: An update on its molecular mechanisms, therapeutic trajectory and nanotechnology in the treatment of breast, lung and prostate cancer. *J Drug Deliv Sci Technol* 60, 101959. doi: 10.1016/j.jddst.2020.101959

Jouberton, E., Voissiere, A., Penault-Llorca, F., Cachin, F., and Miot-Noirault, E. (2022). Multicellular tumor spheroids of LNCaP-Luc prostate cancer cells as in vitro screening models for cytotoxic drugs. *Am J Cancer Res* 12, 1116–1128.

- Karandish, F., Haldar, M. K., You, S., Brooks, A. E., Brooks, B. D., Guo, B., et al. (2016). Prostate-Specific Membrane Antigen Targeted Polymersomes for Delivering Mocetinostat and Docetaxel to Prostate Cancer Cell Spheroids. *ACS Omega* 1, 952–962. doi: 10.1021/acsomega.6b00126
- Katerji, M., Filippova, M., and Duerksen-Hughes, P. (2019). Approaches and Methods to Measure Oxidative Stress in Clinical Samples: Research Applications in the Cancer Field. *Oxid Med Cell Longev* 2019, 1–29. doi: 10.1155/2019/1279250
- Kulesza, J., Paluszkiwicz, E., and Augustin, E. (2023). Cellular Effects of Selected Unsymmetrical Bisacridines on the Multicellular Tumor Spheroids of HCT116 Colon and A549 Lung Cancer Cells in Comparison to Monolayer Cultures. *Int J Mol Sci* 24, 15780. doi: 10.3390/ijms242115780
- Lee, S.-H., and Lee, Y.-J. (2021). Synergistic anticancer activity of resveratrol in combination with docetaxel in prostate carcinoma cells. *Nutr Res Pract* 15, 12. doi: 10.4162/nrp.2021.15.1.12
- Li, C., Wang, Z., Wang, Q., Ka Yan Ho, R. L., Huang, Y., Chow, M. S. S., et al. (2018). Enhanced anti-tumor efficacy and mechanisms associated with docetaxel-piperine combination- *in vitro* and *in vivo* investigation using a taxane-resistant prostate cancer model. *Oncotarget* 9, 3338–3352. doi: 10.18632/oncotarget.23235
- Lima, T. S., Iglesias-Gato, D., Souza, L. D. O., Stenvang, J., Lima, D. S., Røder, M. A., et al. (2021). Molecular Profiling of Docetaxel-Resistant Prostate Cancer Cells Identifies Multiple Mechanisms of Therapeutic Resistance. *Cancers (Basel)* 13, 1290. doi: 10.3390/cancers13061290
- Liu, Z., and Jiao, D. (2020). Necroptosis, tumor necrosis and tumorigenesis. *Cell Stress* 4, 1–8. doi: 10.15698/cst2020.01.208
- Lu, X., Yang, F., Chen, D., Zhao, Q., Chen, D., Ping, H., et al. (2020). Quercetin reverses docetaxel resistance in prostate cancer via androgen receptor and PI3K/Akt signaling pathways. *Int J Biol Sci* 16, 1121–1134. doi: 10.7150/ijbs.41686
- Lv, G., Sun, D., Zhang, J., Xie, X., Wu, X., Fang, W., et al. (2017). Lx2-32c, a novel semi-synthetic taxane, exerts antitumor activity against prostate cancer cells in vitro and in vivo. *Acta Pharm Sin B* 7, 52–58. doi: 10.1016/j.apsb.2016.06.005
- Mediavilla-Varela, M., Pacheco, F. J., Almaguel, F., Perez, J., Sahakian, E., Daniels, T. R., et al. (2009). Docetaxel-induced prostate cancer cell death involves concomitant activation of

caspase and lysosomal pathways and is attenuated by LEDGF/p75. *Mol Cancer* 8, 68. doi: 10.1186/1476-4598-8-68

Mishra, R., Nathani, S., Varshney, R., Sircar, D., and Roy, P. (2020). Berberine reverses epithelial-mesenchymal transition and modulates histone methylation in osteosarcoma cells. *Mol Biol Rep* 47, 8499–8511. doi: 10.1007/s11033-020-05892-8

Muhamad, N., Plengsuriyakarn, T., and Na-Bangchang, K. (2018). Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: a systematic review. *Int J Nanomedicine* Volume 13, 3921–3935. doi: 10.2147/IJN.S165210

Mundekkad, D., and Cho, W. C. (2022). Nanoparticles in Clinical Translation for Cancer Therapy. *Int J Mol Sci* 23, 1685. doi: 10.3390/ijms23031685

Nakonechnaya, A. O., and Shewchuk, B. M. (2015). Growth hormone enhances LNCaP prostate cancer cell motility. *Endocr Res* 40, 97–105. doi: 10.3109/07435800.2014.966383

Perche, F., and Torchilin, V. P. (2012). Cancer cell spheroids as a model to evaluate chemotherapy protocols. *Cancer Biol Ther* 13, 1205–1213. doi: 10.4161/cbt.21353

Raitanen, J., Barta, B., Hacker, M., Georg, D., Balber, T., and Mitterhauser, M. (2023). Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines. *Cells* 12, 360. doi: 10.3390/cells12030360

Ren, G., Li, Y., Ping, C., Duan, D., Li, N., Tang, J., et al. (2022). Docetaxel prodrug and hematoporphyrin co-assembled nanoparticles for anti-tumor combination of chemotherapy and photodynamic therapy. *Drug Deliv* 29, 3358–3369. doi: 10.1080/10717544.2022.2147280

Rivero-Buceta, E., Vidaurre-Agut, C., Vera-Donoso, C. D., Benlloch, J. M., Moreno-Manzano, V., and Botella, P. (2019). PSMA-Targeted Mesoporous Silica Nanoparticles for Selective Intracellular Delivery of Docetaxel in Prostate Cancer Cells. *ACS Omega* 4, 1281–1291. doi: 10.1021/acsomega.8b02909

Rodrigues-Ferreira, S., Nehlig, A., Kacem, M., and Nahmias, C. (2020). ATIP3 deficiency facilitates intracellular accumulation of paclitaxel to reduce cancer cell migration and lymph node metastasis in breast cancer patients. *Sci Rep* 10, 13217. doi: 10.1038/s41598-020-70142-7

Runge, R., Naumann, A., Miederer, M., Kotzerke, J., and Brogsitter, C. (2023). Up-Regulation of PSMA Expression In Vitro as Potential Application in Prostate Cancer Therapy. *Pharmaceuticals* 16, 538. doi: 10.3390/ph16040538

- Ruzycka-Ayoush, M., Kowalik, P., Kowalczyk, A., Bujak, P., Nowicka, A. M., Wojewodzka, M., et al. (2021). Quantum dots as targeted doxorubicin drug delivery nanosystems in human lung cancer cells. *Cancer Nanotechnol* 12, 8. doi: 10.1186/s12645-021-00077-9
- SenGupta, S., Parent, C. A., and Bear, J. E. (2021). The principles of directed cell migration. *Nat Rev Mol Cell Biol* 22, 529–547. doi: 10.1038/s41580-021-00366-6
- Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., et al. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71, 209–249. doi: 10.3322/caac.21660
- Vargas-Accarino, E., Herrera-Montávez, C., Ramón y Cajal, S., and Aasen, T. (2021). Spontaneous Cell Detachment and Reattachment in Cancer Cell Lines: An In Vitro Model of Metastasis and Malignancy. *Int J Mol Sci* 22, 4929. doi: 10.3390/ijms22094929
- Ventura, E., and Giordano, A. (2019). “Cell Cycle,” in *Reference Module in Life Sciences*, (Elsevier). doi: 10.1016/B978-0-12-809633-8.90189-4
- Vinci, M., Gowan, S., Boxall, F., Patterson, L., Zimmermann, M., Court, W., et al. (2012). Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol* 10, 29. doi: 10.1186/1741-7007-10-29
- Wang, X., Decker, C. C., Zechner, L., Krstin, S., and Wink, M. (2019). In vitro wound healing of tumor cells: inhibition of cell migration by selected cytotoxic alkaloids. *BMC Pharmacol Toxicol* 20, 4. doi: 10.1186/s40360-018-0284-4
- Wang, X., Xuetao, X., Wu, M., Wu, P., Sheng, Z., Liu, W., et al. (2022). Inhibitory effect of roboric acid in combination with docetaxel on human prostate cancer cells. *J Enzyme Inhib Med Chem* 37, 542–553. doi: 10.1080/14756366.2021.2018684
- Wüstemann, T., Haberkorn, U., Babich, J., and Mier, W. (2019). Targeting prostate cancer: Prostate-specific membrane antigen based diagnosis and therapy. *Med Res Rev* 39, 40–69. doi: 10.1002/med.21508
- Yang, C., Zhang, W., Wang, J., Chen, P., and Jin, J. (2019). Effect of docetaxel on the regulation of proliferation and apoptosis of human prostate cancer cells. *Mol Med Rep*. doi: 10.3892/mmr.2019.9998
- Zaidieh, T., Smith, J. R., Ball, K. E., and An, Q. (2019). ROS as a novel indicator to predict anticancer drug efficacy. *BMC Cancer* 19, 1224. doi: 10.1186/s12885-019-6438-y

Zhang, M., Qu, J., Gao, Z., Qi, Q., Yin, H., Zhu, L., et al. (2021). Timosaponin AIII Induces G2/M Arrest and Apoptosis in Breast Cancer by Activating the ATM/Chk2 and p38 MAPK Signaling Pathways. *Front Pharmacol* 11. doi: 10.3389/fphar.2020.601468

Chapter 7 Conclusions and Future Perspectives

7.1 Conclusions

In this work, the development and evaluation of a targeted nanosystem for the delivery of docetaxel to prostate cancer cells was detailed, with the final system being comprised of docetaxel-entrapped anti-PSMA PLGA-PEG nanoparticles.

Firstly, the use of microfluidics was explored for its benefits over solvent evaporation, and proved to be the more efficient method of nanoparticle preparation. The nanosystems prepared by solvent evaporation and microfluidics were pharmaceutically stable and effectively shielded the drug disulfiram within their cores. However, a more desirable nanosystem was achieved with uniform size and stability ($PSD=179\pm2.5$ nm, $\zeta=+32.3\pm0.1$ mV) with higher total drug release (70% after 12 hours), compared with prototypes developed by solvent evaporation.

Next, the optimization by design of experiments and active targeting in PLGA nanoparticles, using chitosan for controlled release was investigated, with folic acid as a targeting agent for PSMA, a receptor expressed highly on prostate cancer tissue. In an attempt to test the versatility of the nanosystem, quercetin, a hydrophobic botanical therapeutic was explored as it had also previously shown *in vitro* anti-cancer activity. The targeted system provided a sustained, pH dependent release of quercetin, (caused by the chitosan and PLGA components of the nanosystem) and showed a higher percentage release of 78% at a tumour-related pH of 6 compared to 55% at a normal physiological pH of 7.4. It also showed higher uptake and lower effective inhibitory concentration of 100 $\mu\text{g/mL}$ of quercetin compared to 300 $\mu\text{g/mL}$ for the non-targeted system on LnCap (PSMA positive) cells, while the effective concentrations were much higher in PSMA negative PC-3 cells at 300 $\mu\text{g/mL}$ for both systems. This showed how the folic acid ligands increased efficacy of the nanosystem by increasing cellular uptake through active targeting of PSMA on LnCap cells. However, no increase in uptake and toxicity could be attributed to the chitosan component of the system.

Thereafter, the microfluidics method of fabrication and optimization with DOE were combined to prepare anti-PSMA-PLGA-PEG nanoparticles loaded with docetaxel. The optimized system had a production yield of $63.2 \pm 1.1\%$, a drug loading of $11.5 \pm 0.66\%$ and antibody conjugation efficiency of $74.3 \pm 4.6\%$. It showed high stability characteristics and was uniform in size, with a particle size of 160.5 ± 4.2 and a ζ potential of -28.1 ± 1.3 mV, and these results can be attributed to the efficient, microfluidics-based method of preparation. The system displayed a controlled, pH dependent release profile, with higher drug release of 72% at a pH of 6, associated with tumour tissue, and a comparatively low drug release of 26% of docetaxel at a

physiological pH of 7.4. It also provided enhanced selective uptake and cytotoxicity with an effective inhibitory concentration of 8 ng/mL of docetaxel, compared to 13,5 ng/mL of the non-targeted system on PSMA positive LnCap cells. Both systems displayed higher effective concentrations of 20 ng/mL for the targeted and 25 ng/mL for the non-targeted system on PSMA negative cells PC-3 cells and minimal toxicity on non-cancerous cells. This clearly demonstrated the PSMA-specific activity of the targeted system and therefore, this nanosystem warranted further exploration for its *in vitro* and *in vivo* targeting effects on prostate cancer cells.

Hence, it was investigated if the docetaxel-loaded PLGA-PEG-anti-PSMA nano-system would have intra-cellular effects on physiological processes such as cell motility and programmed cell death and if it could inhibit the growth of 3D spheroids cultured from PSMA positive cells. It was found that the targeted system inhibited LnCap cell migration by $89,4 \pm 8,4\%$, compared to $48,1 \pm 2,7\%$ inhibition by the non-targeted system after 72 hours of treatment. Preferential mitotic arrest in the G2/M phase of LnCap cells was also observed, with a $30,3 \pm 3,0\%$ and $15,8 \pm 2,8\%$ arrest by the targeted and non-targeted systems respectively. However, no effects on migration or cell cycle were observed on the PSMA negative PC-3 cell line. This increase in the inhibition of cellular processes by the targeted system on PSMA positive LnCap cells, together with its the lack of comparable inhibition on PSMA negative PC3 cells, distinctly indicated the PSMA-specific influence on the efficacy of the nano-systems. Both nano-systems were also more effective than free docetaxel at the inhibition of *in vitro* 3D LnCap spheroids, with $48,6 \pm 9,3\%$ and $62,9 \pm 8,6\%$ inhibition by the non-targeted and targeted systems, respectively, compared to $17,6 \pm 7,6\%$ inhibition by docetaxel after seven days of spheroid growth. However, the nano-systems did not show long term inhibition on spheroid growth compared to free docetaxel and we speculated that the concentration of docetaxel used could have been too low to exert any lasting effect on the 3D model. The systems were tested for biocompatibility on nude MF1 mice and it was found that all systems, including free docetaxel, did not have significant toxic effects on the major organs of the mice after one dose.

In conclusion, the design of the final system by exploring fabrication methods, statistical optimization, and the inclusion of cell penetrating, stealth and active targeting moieties resulted in a drug delivery system with superior physical and drug release properties and anti-cancer toxicity while retaining *in-vivo* biocompatibility.

7.2 Limitations

A few limitations were encountered during the course of this study. Firstly, the testing of the PLGA-PEG-CHI system prepared by solvent evaporation proved difficult for the formulations with low PEG content, due to the lack of solubility. The preparation of the PLGA-CHI-folic acid

nano-system required that all experiments were conducted in the dark, due to the photosensitivity of folic acid, and this could prove to be challenging in future work involving upscaling of the system. Finally, aspects of the animal study precluded much of the in-vivo work planned for the study. The strain of mice seemed to have very low breeding rates and since they have recessive genes, acquisition of the number and sex of the animals required in this study was difficult.

7.3 Future Recommendations

The nanosystems prototyped in this study showed potential to be developed as a successful cancer nanomedicine. Three different active compounds were separately evaluated to demonstrate the versatility of the nanosystems developed. The PLGA-PEG-anti-PSMA nanosystem was most promising due to its demonstrated targeting ability and its high inhibition of prostate cancer cell growth at a relatively low concentration of drug. This formulation could be further optimized by varying the PLGA, PEG and antibody constituents for maximum cellular uptake, and it could possibly be augmented with a secondary delivery component, such as another drug for enhanced therapeutic efficacy. A promising direction is to further optimize this nano-platform to incorporate DNA or RNA moieties for gene therapy, as the targeted nanosystem would tackle problems such as instability and rapid clearance, which are commonly encountered when using genetic material for treatment. The versatility of the nanoparticles could also be explored by attaching different targeting ligands, such as synthetic small molecule ligands, designed to bind PSMA, or other ligands to target different cancers. The enhanced tumour targeting abilities could be validated by tumour invasion assays and live cell toxicity studies. Viability assays on non-cancerous cells could be conducted to compare the toxicity of the increased effective drug concentration that was required to impact viability and growth of prostate cancer spheroids. The 3D spheroid study could focus on concentration dependent toxicity, fluorescent microscopy and 3D-bioprinting of tissue organoid cultures. Since none of the treatments showed any short-term detrimental effects on the major organs of nude MF1 mice, further testing of the nano-systems *in vivo* should be done using the induction of xenograft tumours in the nude mouse model

**Appendix 1 Supplementary Information for Chapter 3: Comparative
Nanofabrication of PLGA-Chitosan-PEG Systems Employing Microfluidics and
Emulsification Solvent Evaporation Techniques**

Table 1S1: Size and dispersity data for formulations prepared by the solvent evaporation method

<u>Solvent</u>	<u>PVA</u> <u>/%</u>	<u>Formulation</u>	<u>PSD</u> <u>/nm</u>	<u>PDI</u>
Acetone	0	Empty PLGA	373±14	0,098±0,05
		PLGA-DSF	216±10	0,248±0,05
		PLGA-CHI	485±235	0,695±0,1
		PLGA-CHI-PEG	288±109	0,540±0,02
	0.5	Empty PLGA	254±5,9	0,423±0,07
		PLGA-DSF	274±8,9	0,139±0,04
		PLGA-CHI	372±8,1	0,176±0,06
		PLGA-CHI-PEG	503±5	0,074±0,02
	1	Empty PLGA	1043±368	0,736±0,1
		PLGA-DSF	566±26	0,298±0,02
		PLGA-CHI	595±68	0,485±0,1
		PLGA-CHI-PEG	1119±154	0,400±0,03
	2	Empty PLGA	429±117	0,635±0,1
		PLGA-DSF	395±15	0,257±0,1
		PLGA-CHI	931±97	0,827±0,1
		PLGA-CHI-PEG	839±36	0,728±0,04

	0	PLGA-CHI(son)	792±42	0,791±0,02
		PLGA-CHI-PEG(son)	717±21	0,454±0,07
	0.5	PLGA-CHI(son)	271±12	0,116±0,07
		PLGA-CHI-PEG(son)	324±19	0,354±0,006
	1	PLGA-CHI(son)	372±8,0	0,176±0,06
		PLGA-CHI-PEG(son)	502±4,9	0,074±0,02
	2	PLGA-CHI(son)	808±95	0,873±0,1
		PLGA-CHI-PEG(son)	484±23	0,660±0,06
	0.5	Empty PLGA	7914±3812	0,653±0,2
		PLGA-DSF	1369±360	0,977±0,02
		PLGA-CHI	1645±157	0,993±0,007
		PLGA-CHI-PEG	402±26	0,391±0,05
Chloroform	1	Empty PLGA	1300±333	0,985±0,01
		PLGA-DSF	672±16	0,709±0,2
		PLGA-CHI	1294±153	0,523±0,2
		PLGA-CHI-PEG	1194±91	0,252±0,05
	2	Empty PLGA	1585±149	0,246±0,1
		PLGA-DSF	524±114	0,576±0,1
		PLGA-CHI	2059±388	0,916±0,1
		PLGA-CHI-PEG	868±32	0,636±0,3
	1	PLGA-CHI(son)	269±9,8	0,169±0,02
		PLGA-CHI-PEG(son)	257±2,7	0,103±0,02
	2	PLGA-CHI(son)	1097±166	0,806±0,1

		PLGA-CHI-PEG(son)	441±26	0,123±0,1
Dichloromethane	0.5	Empty PLGA	569±42	0,765±0,1
		PLGA-DSF	345±14	0,397±0,01
		PLGA-CHI	523±130	0,407±0,08
		PLGA-CHI-PEG	139±12	0,989±0,01
	1	Empty PLGA	10063±6940	0,778±0,1
		PLGA-DSF	2020±241	0,244±0,1
		PLGA-CHI	25316±25316	1±0
		PLGA-CHI-PEG	685±106	0,656±0,2
	2	Empty PLGA	471±245	0,529±0,2
		PLGA-DSF	376±44	0,416±0,02
		PLGA-CHI	407±20	0,368±0,1
		PLGA-CHI-PEG	1006±25	0,387±0,1
	1	PLGA-CHI(son)	844±39	0,426±0,2
		PLGA-CHI-PEG(son)	221±4,1	0,028±0,02
	2	PLGA-CHI(son)	562±55	0,476±0,07
		PLGA-CHI-PEG(son)	234±31	0,784±0,1

Table 1S2: Mean particle size, dispersity and zeta potential data for formulations with varying flow rate ratios and total flow rates.

<u>FRR</u>	<u>TFR</u> <u>/mLmin⁻¹</u>	<u>PSD</u> <u>/nm</u>	<u>PDI</u>	<u>Zpotential</u> <u>/mV</u>
1:1	8	387±6.3	0.191±0.004	26.3±1.3
1:1	12	238±4.3	0.073±0.004	24.2±2.1
1:1	15	289±2.3	0.066±0.03	22.3±2.2
3:1	8	218±1.0	0.165±0.01	34.5±1.0
3:1	12	342±2.0	0.127±0.02	33.0±1.4
3:1	15	304±2.3	0.258±0.002	28.5±2.0
5:1	8	330±9.8	0.126±0.01	20.6±0.6
5:1	12	248±3.5	0.246±0.007	25.7±0.8
5:1	15	244±6.0	0.246±0.004	39.6±3.8

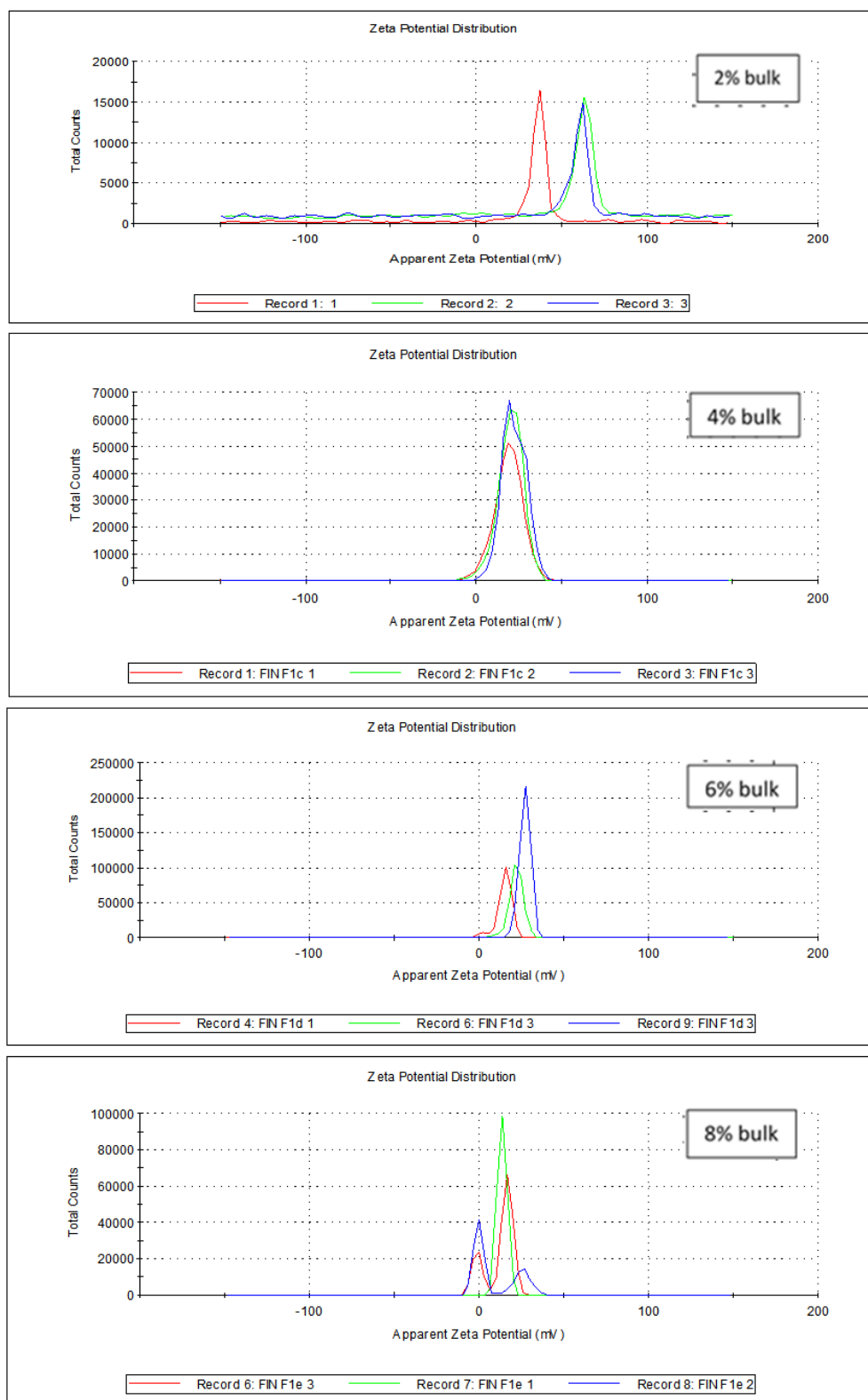


Figure 1S1(A): Representative zeta potential plots showing surface charge variation with increasing % PEG coating density of solvent evaporation (bulk) prepared formulations.

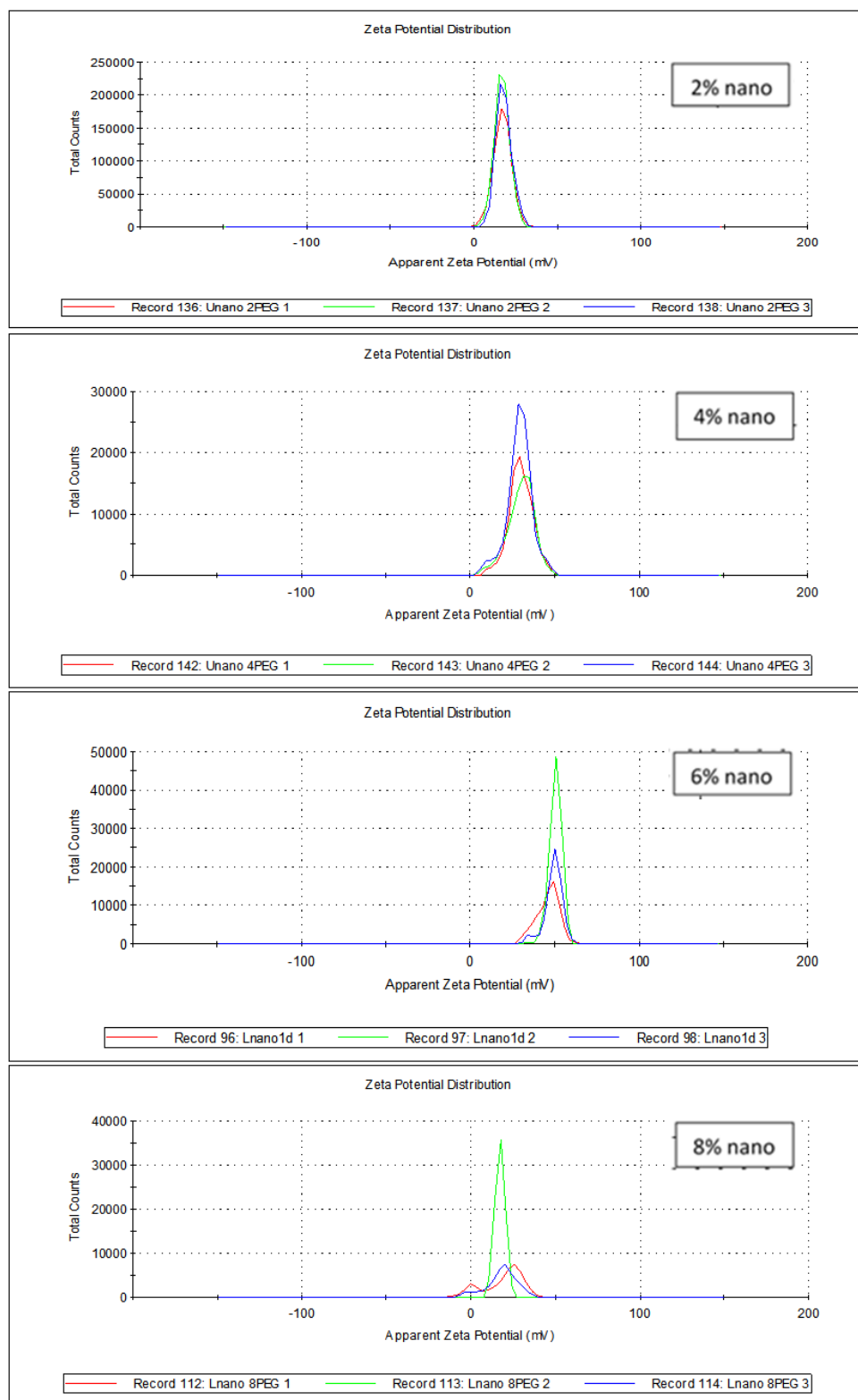


Figure 1S1(B): Representative zeta potential plots showing surface charge variation with increasing % PEG coating density of microfluidics (nano) prepared formulations.

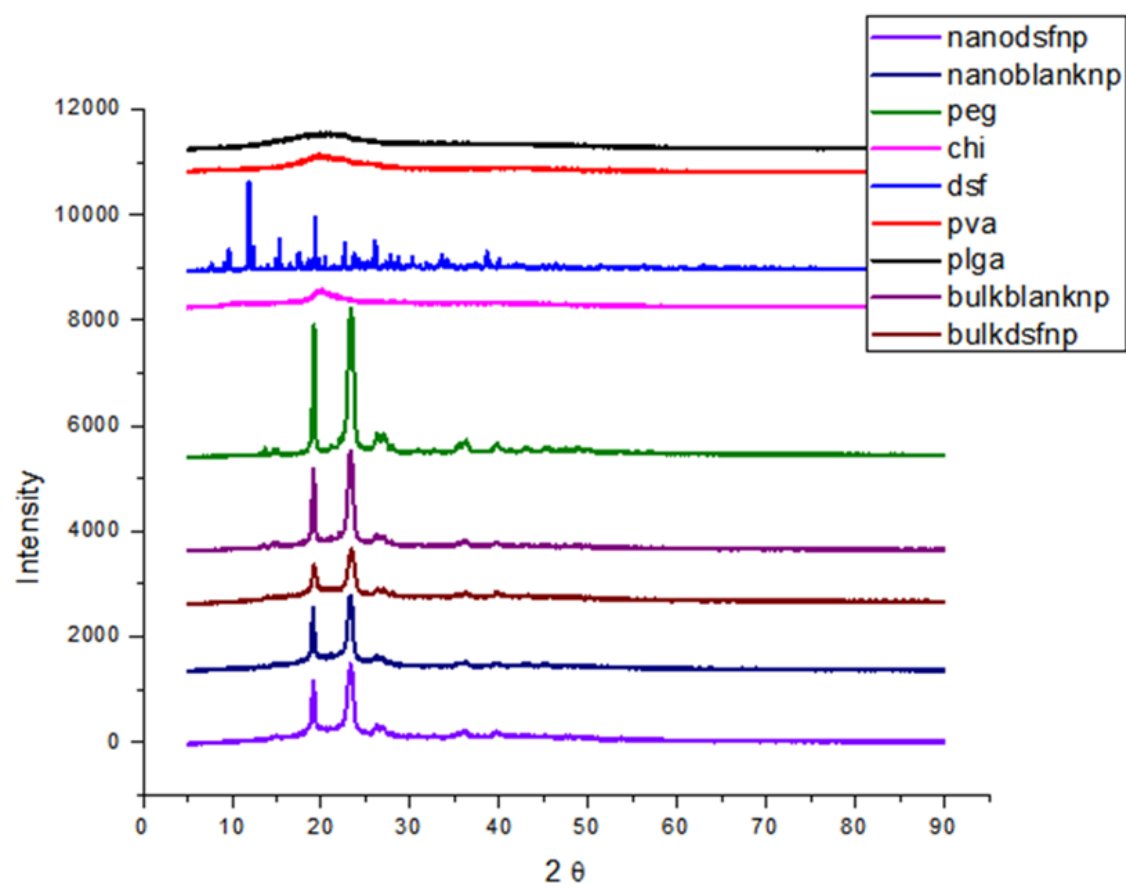


Figure 1S2: XRD spectra of the starting materials, blank and loaded nanoparticles (6% PEG) prepared by microfluidic (nano) and solvent evaporation (bulk) methods. Disulfiram is expressed as DSF.

Appendix 2 Supplementary Information for Chapter 4: Design of Chitosan-Coated, Quercetin-Loaded PLGA Nanoparticles for Enhanced PSMA-Specific Activity on LnCap Prostate Cancer Cells

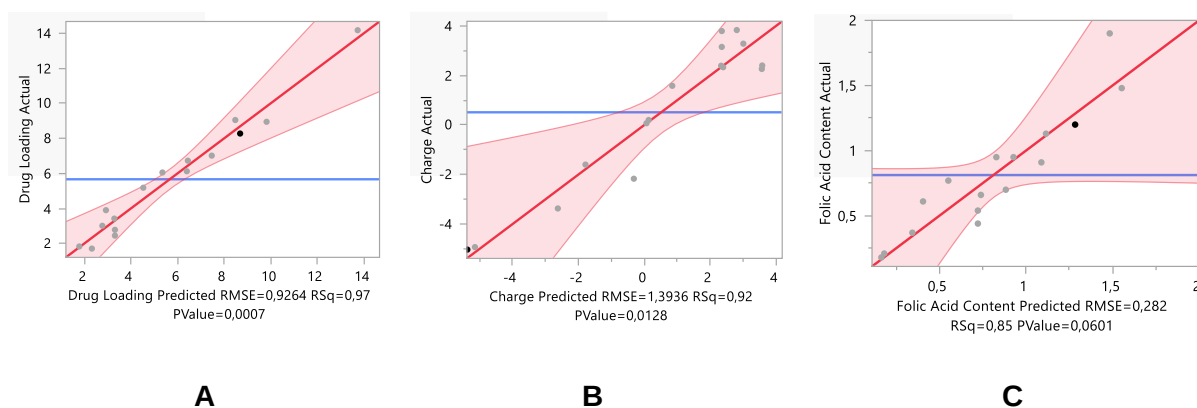


Figure 2S1. Actual vs Predicted plots of quercetin loading (A), charge (B) and folic acid content (C) – from JMP V17

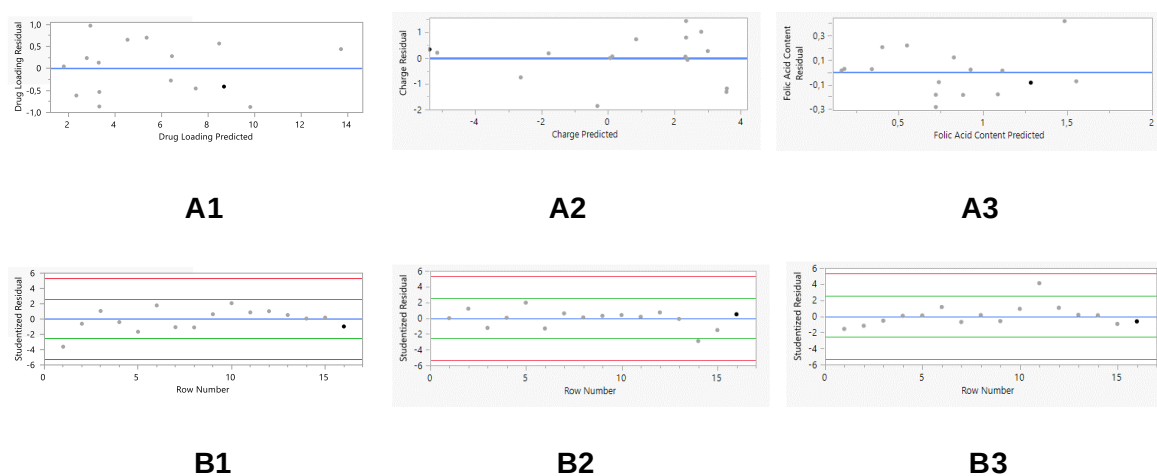
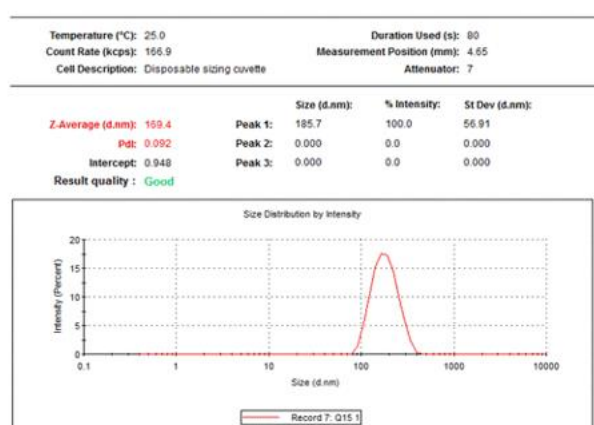


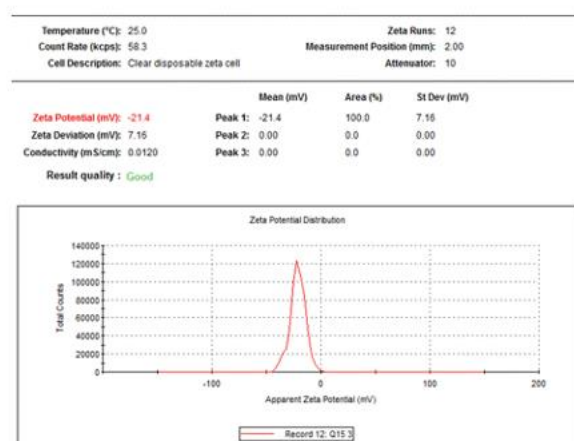
Figure 2S2. Residual Predicted plots (A) and Studentized Residuals (B) of quercetin loading (1), charge (2) and folic acid content (3) - from JMP V17

Source	Logworth		PValue
Quercetin(10,30)	3,937		0,00012
Folic Acid(15,45)	2,983		0,00104
Chitosan(10,60)	2,470		0,00338
Quercetin*Quercetin	2,387		0,00410
Quercetin*Chitosan	1,536		0,02912
Chitosan*Chitosan	1,313		0,04861
Folic Acid*Folic Acid	1,251		0,05614
Chitosan*Folic Acid	0,601		0,25083
Quercetin*Folic Acid	0,243		0,57098

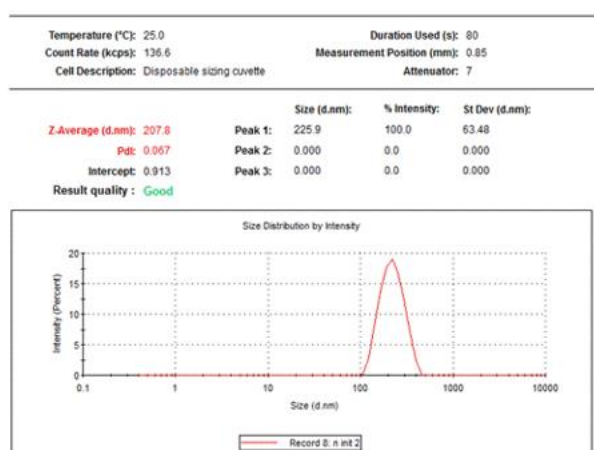
Figure 2S3. Main effects data obtained using a central composite design – from JMP V17



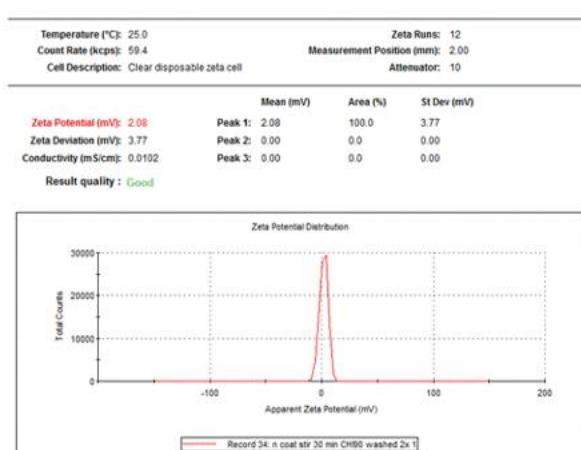
A1



A2



B1



B2

Figure 2S4. 1-2: Representative Graphs of Size and Potential of **A-** un-coated nanoparticles and **B-** coated nanoparticles.

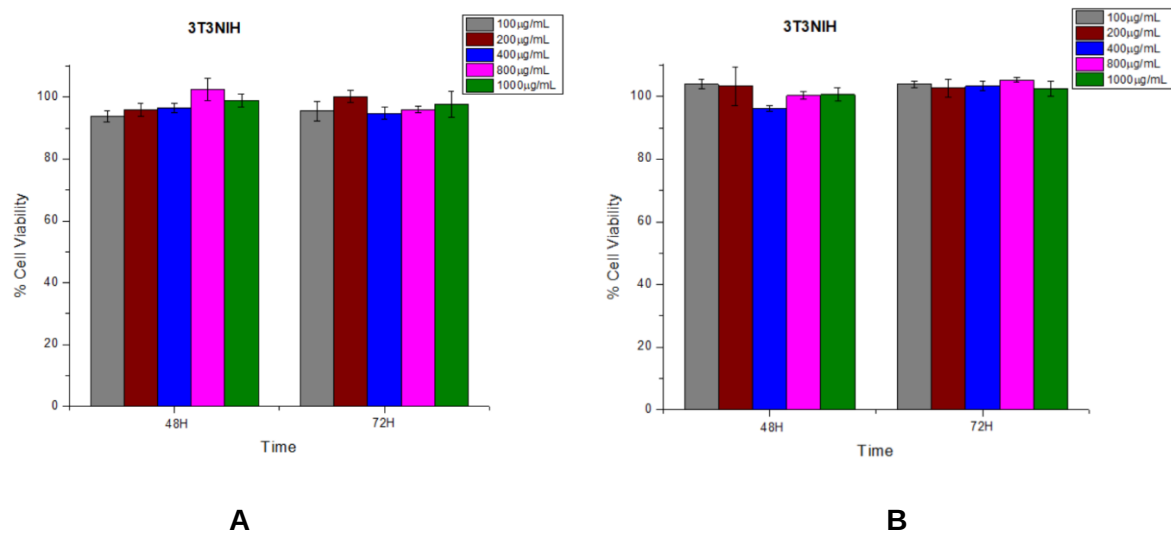


Figure 2S5. Cell viability of 3T3-NIH cells after being treated with unloaded uncoated (**A**) and coated (**B**) nanoparticles for 48 and 72 hours

Appendix 3 Supplementary Information for Chapter 5: Optimized Active Targeted PLGA-PEG Nanoparticles Functionalized with Anti-PSMA Antibody Provide Controlled Release of Docetaxel and Increased Uptake and Cytotoxicity in Prostate Cancer Cells

Effect Summary

Source	Logworth	PValue
Initial AB Amount(5,15)	2,490	0,00324
P:PP ratio*P:PP ratio	2,185	0,00653
Initial Drug Amount(10,20)	1,528	0,02962
Initial AB Amount*Initial AB Amount	1,436	0,03664
P:PP ratio(1,2)	1,421	0,03790 ^
P:PP ratio*Initial Drug Amount	1,135	0,07333
P:PP ratio*Initial AB Amount	0,349	0,44819
Initial Drug Amount*Initial AB Amount	0,291	0,51210
Initial Drug Amount*Initial Drug Amount	0,203	0,62700

Figure 3S1. Main effects data obtained using a central composite design – from JMP V17

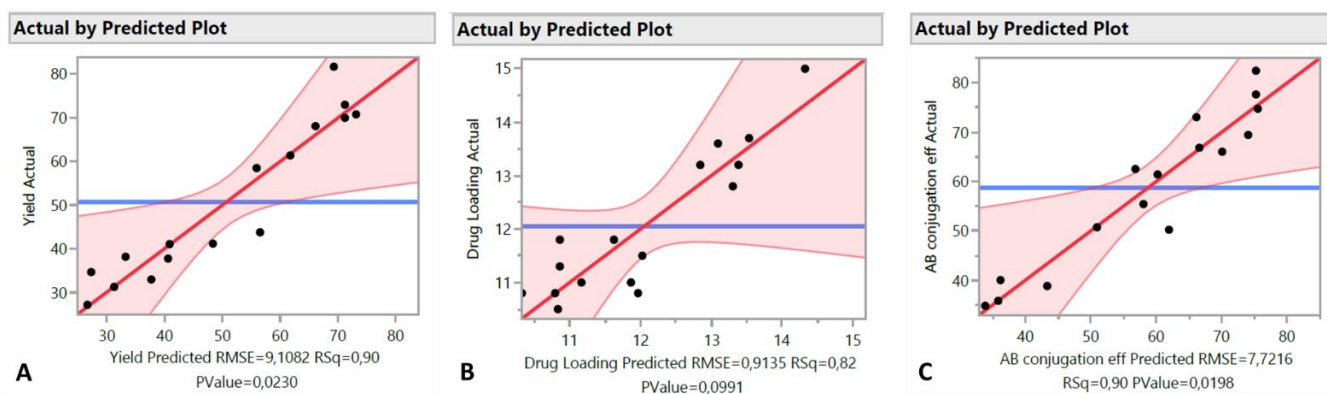


Figure 3S2. Actual vs Predicted plots of yield (A), drug loading (B) and antibody conjugation efficiency (C) – from JMP V17

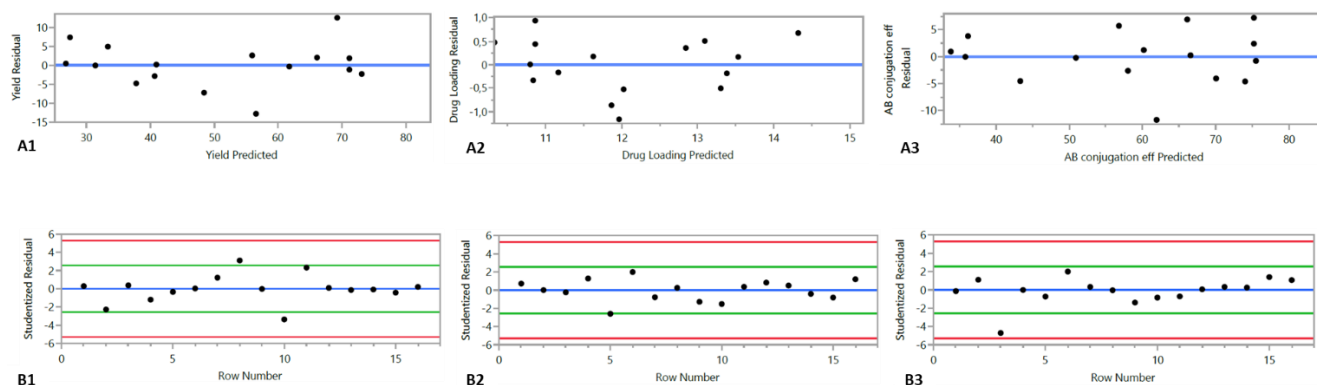


Figure 3S3. Residual Predicted plots (A) and Studentized Residuals (B) of yield (1), drug loading (2) and antibody conjugation efficiency (3) - from JMP V17

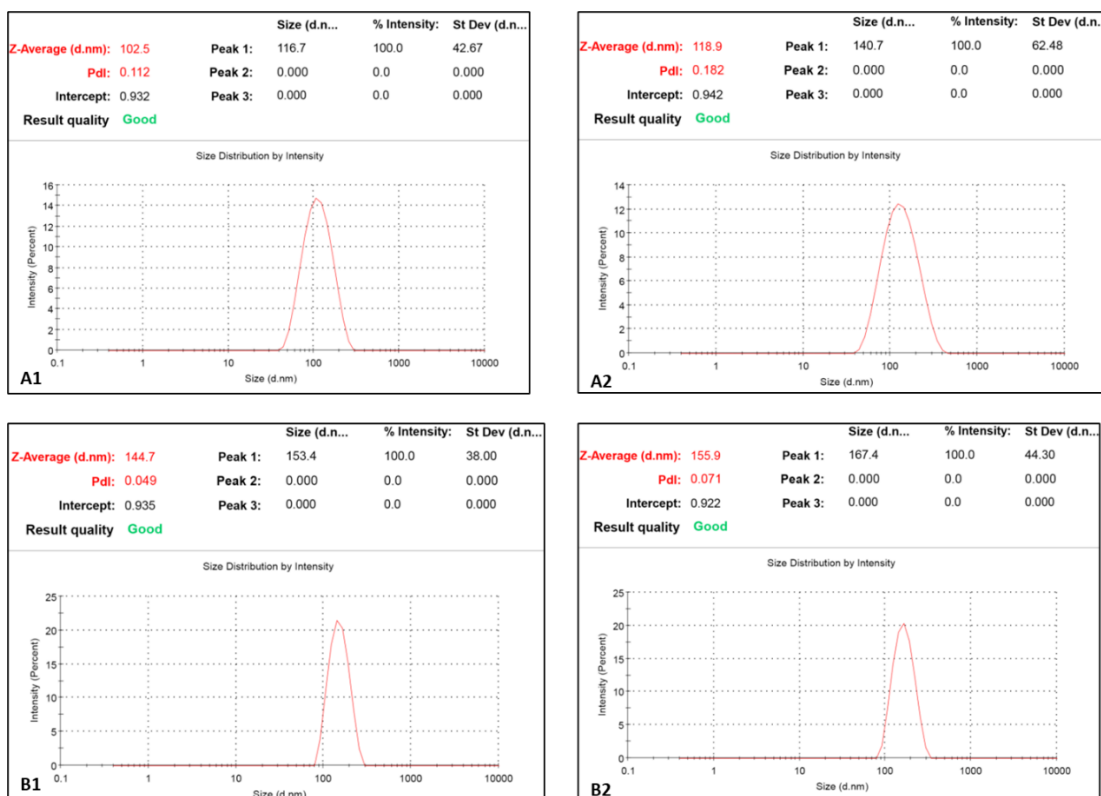


Figure 3S4. 1-2: Representative Graphs showing average size of A1 and A2 - unloaded and loaded non targeted nanoparticles (NTN) and B1 and B2 – unloaded and loaded actively targeted nanoparticles (ATN)

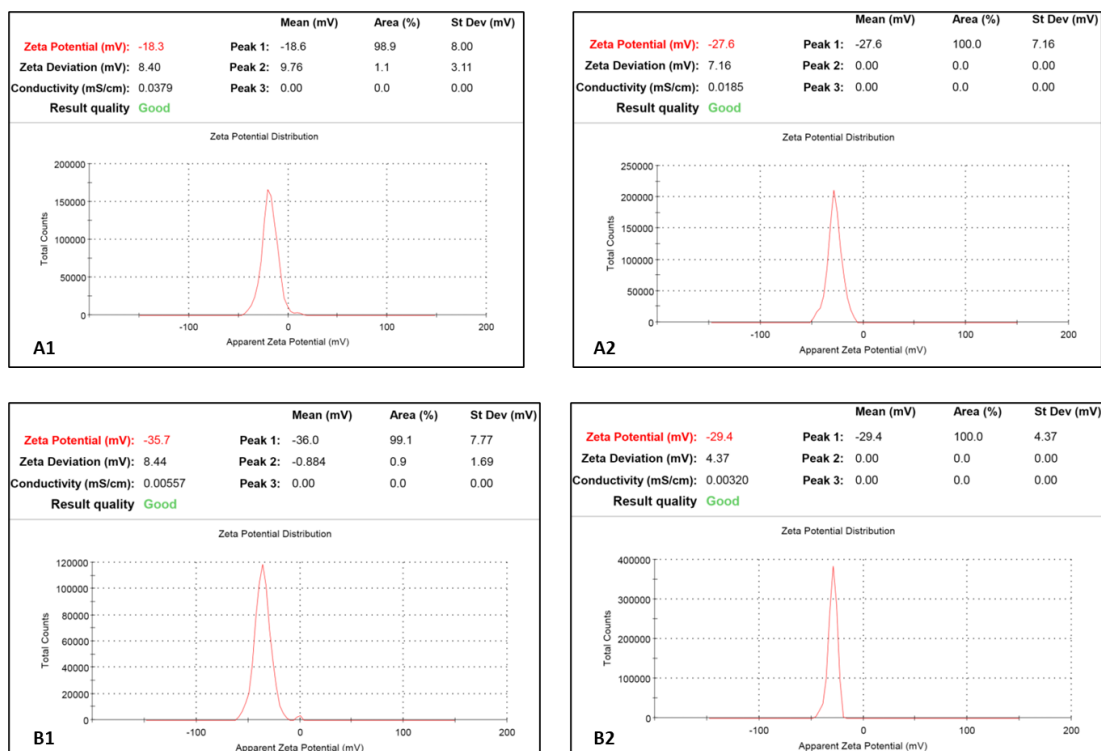


Figure 3S5. 1-2: Representative Graphs showing average Zeta Potential of A1 and A2 - unloaded and loaded non targeted nanoparticles (NTN) and B1 and B2 – unloaded and loaded actively targeted nanoparticles (ATN)

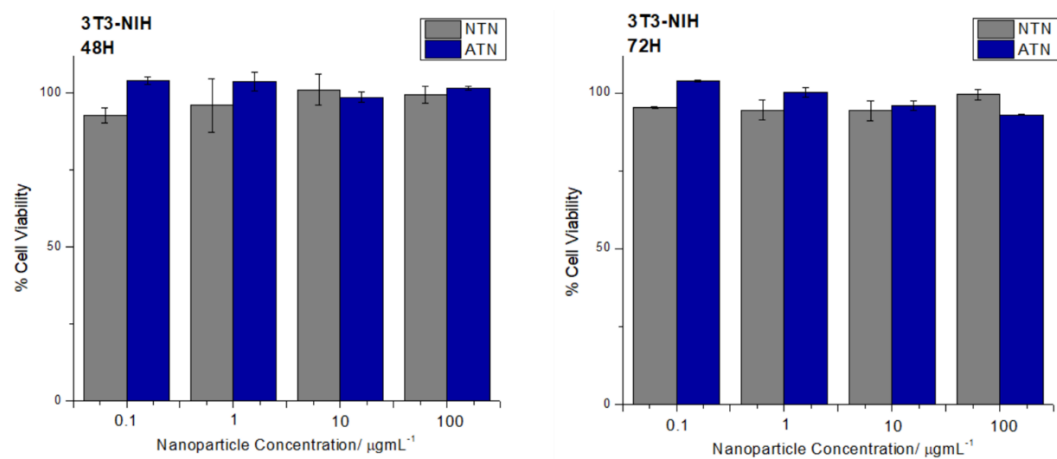


Figure 3S6. Cell viability of 3T3-NIH cells after being treated with unloaded NTN and ATN systems for 48 and 72 hours

Appendix 4 Animal Ethics Clearance Certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)



STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/11/02/C

APPLICANT: Ms D Essa

School: School of Therapeutic Sciences; Department: ; Location: WRAF

PROJECT TITLE: Poly (lactide-co-glycolide) Based Nanoparticles for Prostate Cancer Treatment in a Nude Mouse Model

Category: C; Species and Numbers involved: 48X, 4-6 Weeks old (20g), Male, MF1 - Nude Mice

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 29 Nov 2022. This approval remains valid until 5 Feb 2025 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed:  Date: 06/02/2023
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed:  Date: 7/02/2023

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine