

AN ARCHITECTURALLY-CONFIGURED NANOPARTICULATE SYSTEM FOR TARGETED TREATMENT OF HEPATITIS B VIRUS INFECTION

LATAVIA SINGH

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Pharmacy



Supervisor:

Professor Viness Pillay, Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa

Co-Supervisor:

Associate Professor Yahya Essop Choonara, Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa

Co-Supervisor:

Doctor Lisa Claire du Toit, Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa

Johannesburg, 2015

DECLARATION

I, Latavia Singh, declare that this dissertation is my own work. It is being submitted for the degree of Master of Pharmacy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.



Signature

This ...29th... day of ...April...

ABSTRACT

Viruses boast a highly skilled mode of entry into the cells of all living organisms. This occurs due to the intelligent make-up of these biological systems. A drug delivery system was designed to mimic viruses ordinarily, hence taking advantage of cell entry ease that these viruses parade and ultimately, as a result, ensuring intracellular antiviral transmittal to targeted sites in the body. The Hepatitis B Virus (HBV) was used as a representative virus with representative antiviral drug being Lamivudine (LMV). An architecturally-configured nanoparticulate system (ACNS) was formed by a novel graft copolymerization of hydrophobic epsilon-Caprolactam (ECL) onto the backbone of hydrophilic Hyaluronic acid (HA) (HA-g-ECL). HA-g-ECL showed competence in forming amphiphilic micelles broadening areas of its application in drug delivery. A Box-Behnken experimental design strategy generated formulations thoroughly screened in terms of variables (copolymer, surfactant and solvent) affecting responses (size, drug entrapment efficiency and mean dissolution time). ACNS particles were created with sizes varying from 32.03nm to 132.95 nm holding a negative surface charge. LMV content ranged from 18.52 – 47.77 %. Sustained drug release with an initial burst release was attained. A single optimal formulation was computed by way of statistical variable response optimization. Commendable desirability percentages were achieved for measurement outcomes. A cryoprotectant screening evaluation determined 20 % mannitol to be optimal in maintaining ACNS size. Optimal ACNS was surface-modified with a viral-mimicry targeting ligand for liver concentration. Linoleic acid (LA) was graft copolymerized to chitosan synthesizing chitosan-linolate (C-LA) employed as a coat (C-LA ACNS). Tests to assess response data deviation of C-LA ACNS from optimal ACNS were carried out with positive outcomes. Ex vivo internalization via fluorescent microscopy and imaging studies in liver HepG2 cells showed positive uptake in both optimal ACNS and C-LA ACNS with exemplary findings for C-LA ACNS due to an augmented intracellular receipt. In vivo appraisal proceeded in a rodent animal model dispensing C-LA ACNS intraportally. Ultrasound imaging confirmed echogenic C-LA ACNS robust in the hepatic area. Ultra Performance Liquid Chromatography was executed on blood plasma and hepatic tissue samples for LMV detection and quantification. C-LA ACNS proved an impressive hepato-targeting ability with LMV $C_{max} = 91.723 \mu\text{g/mL}$ in liver tissue against LMV $C_{max} = 8.947 \mu\text{g/mL}$ in blood plasma at equivalent time points. ACNS demonstrated significant liver targeting ability on the grounds that a fatty acid be incorporated into its structure. Evolution of this mechanism will lead to favorably high levels of antiviral drugs within sites in the body that are laden with viral disease and will assist in destruction of the virus. This system will prove particularly beneficial in the treatment of opportunistic infections associated with HIV/AIDS and HBV.

RESEARCH OUTPUTS

1. Review Paper

Cell Portage & Therapeutic Bearers: A Hepatitis B Viral View. Latavia Singh, Yahya E. Choonara, Lomas Kumar Tomar, Charu Tyagi, Lisa C. du Toit, Pradeep Kumar and Viness Pillay (abstract in Appendix A). *To be submitted to Journal of Pharmaceutical Sciences.*

2. Research Papers

Novel synthesis and *in vitro* characterization of an amphiphilic Hyaluronic acid-graft-epsilon Caprolactam copolymer. Latavia Singh, Yahya E. Choonara, Lomas K. Tomar, Charu Tyagi, Pradeep Kumar, Lisa C. du Toit and Viness Pillay (abstract in Appendix B). *To be submitted to Journal.*

Trend reflection in the design and *in vitro* characterization of HA-g-ECL forming Lamivudine-loaded nanoparticles. Latavia Singh, Yahya E. Choonara, Lomas K. Tomar, Charu Tyagi, Pradeep Kumar, Lisa C. du Toit and Viness Pillay (abstract in Appendix C). *To be submitted to Journal of Pharmacy and Pharmaceutical Sciences.*

PATENT APPLICATION

Nanoparticulate system for intracellular drug delivery. 2014. Latavia Singh, Lisa Claire du Toit, Pradeep Kumar, Viness Pillay, Yahya Essop Choonara. South Africa Patents and Trade Mark Office, Patent Journal, Volume 47, Number 6 (Appendix D).

ACCOLADES

Awarded a 'First Time Inventor' certificate for disclosing my first Wits invention that was issued by the Technology Transfer Office, located in Wits Enterprise, and recognizing me as part of the Wits Innovators Forum (Appendix E).

ANIMAL ETHICS CLEARANCE

I, Latavia Singh, hereby confirm that the study entitled “An *in vivo* evaluation of an architecturally-modified nanoparticulate system for intracellular drug delivery in rats” attained approval by the Animal Ethics Screening Committee of the University of the Witwatersrand. Ethics Clearance Number 2014/01/C (Appendix F).

DEDICATION

My dissertation is dedicated to my parents, Prithiraj and Jaiwanthie Singh. Thank you for instilling qualities in me that has sculpted the person I am today. All I have achieved thus far is because of you. Thank you for teaching me the value and importance of education. Your continual support and sacrifices throughout my academic career has been most humbling. Eternally grateful.

ACKNOWLEDGEMENTS

I acknowledge my gratitude to a number of people whose invaluable contribution resulted in this research study:

- To my supervisor - Prof. Viness Pillay, and co-supervisors - Ass Prof. Yahya Essop Choonara and Dr. Lisa Claire du Toit, thank you for allowing me the opportunity to undertake cutting edge research under your much-in-demand supervision and enabling me to reach an academic level of such magnitude.
- Thank you to Mr. Pradeep Kumar. Your awe-inspiring knowledge and wisdom has been exceptionally helpful. Thank you to Dr. Lomas Tomar and Dr. Charu Tyagi for your extensive help and effort with my review and research papers. Thanks to Dr. Divya Bijukumar for her expertise and assistance with the cell component of my study.
- Thank you to the head of Department of Pharmacy and Pharmacology and the department lecturers for their support, encouraging and motivating words. My gratitude goes out to Prof. Sandy Van Vuuren for allowing me use of her equipment. Thank you to the staff of the department for their help and assistance wherever and whenever necessary. To my friends and colleagues of WADDP, warm thanks for all the help and support.
- To the Animal Ethics Screening Committee and the Central Animal Services staff and technicians, many thanks for your aid and advice with animal procedures. Thank you to Prof. Kennedy Erlwanger for his ingenuity as a veterinarian accomplishing an unpracticed and complex surgical procedure with such ease.
- To my family, I thank them for their tremendous support throughout my years of study. Thanks go to my sisters - Sasha Singh and Natasha Singh, for all their unconditional care and help, financially and emotionally during my years of study at Wits. The immense gratitude I harbor- you could never imagine.
- Funders without which this research project would have been impossible are the National Research Foundation of South Africa and Department of Education of Gauteng. Thank you for the sponsorship allowing me to complete this research study.

TABLE OF CONTENTS

	Page
DECLARATION	ii
ABSTRACT	iii
RESEARCH OUTPUTS	iv
PATENT APPLICATION	v
ACCOLADES	vi
ANIMAL ETHICS CLEARANCE	vii
DEDICATION	viii
ACKNOWLEDGEMENTS	ix
LIST OF FIGURES	xviii
LIST OF TABLES	xxv
LIST OF EQUATIONS	xxvii

CHAPTER 1 INTRODUCTION

1.1. Background to this study	1
1.2. Rational and Motivation for this study	3
1.3. Novelty of this study	5
1.4. Aim and Objectives	6
1.5. Overview of this dissertation	6

CHAPTER 2 A LITERATURE REVIEW ON HEPATO-TARGETING: AN OVERVIEW OF DRUG DELIVERY AND TREATMENT STRATEGIES FOR HEPATITIS B VIRUS INFECTION

2.1. Introduction	9
2.2. Intracellular drug delivery and hepatitis B viral entry	10
2.2.1. Endocytic uptake mechanisms	11
2.2.2. Intracellular drug delivery aids	14
2.2.2.1. Emulsifiers as facilitators of intracellular drug delivery	14
2.2.2.2. pH-sensitive carriers in intracellular drug delivery	14
2.2.2.3. Peptide based strategy for intracellular drug delivery	15

2.2.3. Nuclear Targeting	18
2.3. Conventional anti-HBV therapies and drugs	21
2.3.1. Cytokines and Nucleot(s)ide analogues	21
2.3.2. Thiazolide anti-infectives	25
2.3.3. Small interfering ribonucleic acids	25
2.3.4. Heteroarylpyrimidines	27
2.4. Hepato targeting: A delve into attempts at earlier performed research studies	29
2.5. Novel and recent advances in drug delivery developments for anti-HBV therapeutics	32
2.5.1. Woodchuck Apolipoprotein A-1	32
2.5.2. Bionanocapsules	33
2.5.3. Bilosomes	33
2.5.4. Recombinant Viral Vectors	33
2.5.5. Polymeric Hydrogels	34
2.5.6. Polymeric Microparticles	34
2.5.7. Nanoparticles	34
2.5.7.1. Inorganic Nanoparticles	34
2.5.7.2. Polymeric Nanoparticles	35
2.5.8. Lipids	37
2.5.8.1. Ionizable Lipid Nanoparticles	37
2.5.8.2. Cationic Lipids	37
2.5.8.3. Liposomes	37
2.5.8.4. Lipoplexes	38
2.5.8.5. High Density Lipoprotein	38
2.5.8.6. Solid Lipid Nanoparticles	38
2.5.9. Cell Penetrating Peptides	39
2.6. Concluding Remarks	39

CHAPTER 3

SYNTHESIS AND CHARACTERIZATION OF A NOVEL AMPHIPHILIC HYALURONIC ACID- GRAFT-EPSILON CAPROLACTAM COPOLYMER

3.1. Introduction	41
3.2. Materials and Methods	43
3.2.1. Materials	43
3.2.2. Desalting process of hyaluronic acid	43

3.2.3. PEG-assisted solubilization of HA in DMSO	44
3.2.4. Anionic graft (co)polymerization of HA and ECL (HA- <i>g</i> -ECL)	45
3.2.5. Graft copolymer batches and parameters	47
3.2.6. Fourier Transform Infrared spectroscopy analysis of copolymer synthesis	48
3.2.7. Differential Scanning Calorimetry	48
3.2.8. Thermogravimetric Analysis	48
3.2.9. Approximate Molecular Weight measurement of HA- <i>g</i> -ECL	48
3.2.10. Visualization of HA- <i>g</i> -ECL using Scanning Electron Microscopy	49
3.2.11. Micelle characterization	49
3.2.11.1. Determination of the Critical Micelle Concentration of HA- <i>g</i> -ECL	49
3.2.11.2. Micelle formation from amphiphilic HA- <i>g</i> -ECL	49
3.2.11.3. Morphology and size determination of HA- <i>g</i> -ECL micelles utilizing Transmission Electron Microscopy	50
3.3. Results and Discussion	50
3.3.1. Grafting Efficiencies and Graft Ratios of novel copolymer	50
3.3.2. Evidence of grafting occurrence through infrared spectra observations	52
3.3.3. Differential Scanning Calorimetry thermal profiles	54
3.3.4. Thermogravimetric Analysis thermal profiles	56
3.3.5. Approximate Molecular Weight determination of HA- <i>g</i> -ECL	58
3.3.6. Visualization of HA- <i>g</i> -ECL via Scanning Electron Microscopy	60
3.3.7. HA- <i>g</i> -ECL micellization ability	62
3.4. Concluding Remarks	63

CHAPTER 4

TREND REFLECTION IN THE DESIGN AND *IN VITRO* CHARACTERIZATION OF HA-G-ECL FORMING LAMIVUDINE-LOADED NANOPARTICLES

4.1. Introduction	65
4.2. Materials and Methods	68
4.2.1. Materials	68
4.2.2. Synthesis of the amphiphilic graft copolymer	68
4.2.3. Construction of a Box-Behnken design for the preparation/formulation of copolymeric nanoparticles	68
4.2.4. Preparation of LMV-loaded HA- <i>g</i> -ECL copolymeric nanoparticles	70
4.2.5. Transmission Electron Microscopy of the formed HA- <i>g</i> -ECL copolymeric	

nanoparticles	71
4.2.6. Size and Zeta Potential determination via Dynamic Light Scattering	71
4.2.7. Thermal analysis of HA- <i>g</i> -ECL copolymeric nanoparticles	71
4.2.7.1. Differential Scanning Calorimetry	71
4.2.7.2. Thermogravimetric Analysis	71
4.2.8. Construction of a calibration curve to quantify LMV by its ultraviolet spectrophotometric absorptivity	72
4.2.9. Drug Encapsulation Efficiency of HA- <i>g</i> -ECL copolymeric nanoparticles	72
4.2.10. <i>In vitro</i> dissolution studies of LMV-loaded HA- <i>g</i> -ECL copolymeric nanoparticles	72
4.3. Results and Discussion	73
4.3.1. Morphological characterization of HA- <i>g</i> -ECL copolymeric nanoparticles	73
4.3.2. Size and Zeta Potential determination of HA- <i>g</i> -ECL copolymeric nanoparticles by Dynamic Light Scattering	74
4.3.3. Thermal characterization of HA- <i>g</i> -ECL copolymeric nanoparticles	78
4.3.3.1. Differential Scanning Calorimetry	78
4.3.3.2. Thermogravimetric Analysis	79
4.3.4. A calibration curve for LMV for evaluation of its entrapment and release	82
4.3.5. Drug Entrapment Efficiencies of HA- <i>g</i> -ECL copolymeric nanoparticles	83
4.3.6. <i>In vitro</i> drug release profile of HA- <i>g</i> -ECL copolymeric nanoparticle design formulations	85
4.3.7. Analysis of the Box-Behnken design	88
4.3.7.1. Residual error plot analysis for Drug Entrapment Efficiency, Size and Mean Dissolution Time	90
4.3.7.2. Response surface and contour plot analysis for Drug Entrapment Efficiency, Size and Mean Dissolution Time	90
4.4. Concluding Remarks	95

CHAPTER 5

STATISTICAL OPTIMIZATION, *IN VITRO* CHARACTERIZATION AND ELUCIDATION OF A CHITOSAN-GRAFT-LINOLEIC ACID COATED HA-G-ECL LAMIVUDINE-LOADED COPOLYMERIC ACNS FOR TARGETED HEPATO DELIVERY

5.1. Introduction	96
5.2. Materials and Method	98
5.2.1. Materials	98

5.2.2. Preparation of an optimized LMV-loaded ACNS by the Box-Behnken design strategy	98
5.2.2.1. Response Optimization	98
5.2.2.2. Generation of the optimum ACNS	99
5.2.3. HA- <i>g</i> -ECL amphiphilic graft copolymer synthesis	100
5.2.4. Drug-loaded HA- <i>g</i> -ECL copolymeric nanoparticle preparation	100
5.2.5. Chemical modification of chitosan through graft synthesis with linoleic acid producing Chitosan-Linolate	100
5.2.6. Selection process for a suitable cryoprotectant in favor of HA- <i>g</i> -ECL nanoparticle size maintenance	102
5.2.7. Preparation of C-LA coating and coated optimized HA- <i>g</i> -ECL ACNS	103
5.2.8. Determination of molecular vibrational bands utilizing Fourier Transform Infrared spectroscopy	103
5.2.9. Transmission Electron Microscopy imaging of the optimized C-LA coated ACNS	104
5.2.10. Thermal analysis employing Differential Scanning Calorimetry	104
5.2.11. Thermal analysis employing Thermogravimetric Analysis	104
5.2.12. Dynamic Light Scattering size and zeta potential determination of the optimized ACNS	104
5.2.13. Drug Entrapment Efficiency of the optimized ACNS	104
5.2.14. <i>In vitro</i> drug release profile of the optimized ACNS	105
5.3. Results and Discussion	105
5.3.1. Predicted and Observed values for the optimized ACNS	105
5.3.2. Selection of a suitable cryoprotectant to limit the increase of ACNS size	106
5.3.3. Structural variation investigations via Fourier Transform Infrared spectroscopy	107
5.3.4. Morphological investigations of optimized C-LA coated ACNS utilizing Transmission Electron Microscopy	111
5.3.5. Differential Scanning Calorimetry thermogram analysis of C-LA and the optimized ACNS	111
5.3.6. Thermogram analysis of C-LA and the optimized ACNS thermal degradation	115
5.3.7. Size and Zeta Potential evaluations exploiting Dynamic Light Scattering	117
5.3.8. <i>In vitro</i> drug release profile of the C-LA coated and uncoated ACNS	119
5.4. Concluding Remarks	121

CHAPTER 6

EX VIVO INVESTIGATIONS: VISUALIZATION OF CELLULAR INTERNALIZATION OF C-LA COATED ACNS INTO A HEPATIC CELL LINE

6.1. Introduction	122
6.2. Materials and Methods	125
6.2.1. Materials	125
6.2.2. Employment of Aseptic technique for cell procedures	125
6.2.3. HepG2 cell culturing and cell maintenance protocols	126
6.2.3.1. Cell Revival	126
6.2.3.2. Cell Subculturing	127
6.2.3.3. Cell Counting	127
6.2.3.4. Cell Freezing	129
6.2.5. Preparation of the optimized ACNS samples and controls	129
6.2.6. Visualization of intracellular uptake of the fluorescent ACNS	130
6.2.6.1. Confocal fluorescence microscopy imaging	130
6.2.6.2. High speed fiber-optic fluorescence microscopy live imaging	130
6.3. Results and Discussion	131
6.3.1. HepG2 cell culturing	131
6.3.2. Visualization of intracellular uptake of fluorescent ACNS by means of confocal fluorescence microscopy imaging and high speed fiber-optic fluorescence microscopy live imaging	131
6.4. Concluding Remarks	136

CHAPTER 7

IN VIVO ASSESSMENT OF THE ACNS VIA INTRAPORTAL IV ADMINISTRATION IN THE RAT MODEL: A PILOT STUDY

7.1. Introduction	137
7.2. Materials and Methods	139
7.2.1. Materials	139
7.2.2. Animal Ethics Clearance	139
7.2.3. Animal Housing Conditions and Husbandry	139
7.2.4. <i>In vivo</i> studies on the rat model	140

7.2.4.1. Need for a Pilot study	140
7.2.4.1.1. Logistic time points	140
7.2.4.1.2. Surgical approach	140
7.2.4.2. Experimental Design	141
7.2.5. Intricate intraportal injection surgical procedure	144
7.2.6. Animal welfare and humane end-points	146
7.2.7. High-frequency ultrasound imaging	147
7.2.8. Blood and liver sampling	148
7.2.9. Liver removal and treatment	149
7.2.10. Applying Ultra Performance Liquid Chromatography to ascertain LMV content in blood and tissue samples	150
7.2.10.1. Ultra Performance Liquid Chromatography analysis conditions: Washes, mobile phases and separation parameters	150
7.2.10.2. Preparation of stock and standard solutions	151
7.2.10.3. Verifying of optimal solvent/s to be used for liquid-liquid drug extraction procedure	151
7.2.10.4. Precision and accuracy validation	152
7.2.10.5. Construction of a calibration curve to gauge drug quantities in blood plasma and liver treated samples	152
7.3. Results and Discussion	152
7.3.1. Conclusive findings from the Pilot study for the commencement of the main study	152
7.3.1.1. Day 1 – Practice Surgery	153
7.3.1.2. Day 2 – Intraportal vein injections on comparison groups C and D	153
7.3.1.3. Day 3 – Intraportal vein injections on placebo and test groups A and B	153
7.3.1.4. Summary and Resolutions	154
7.3.2. High-frequency ultrasound preclinical imaging	155
7.3.3. Elution times of LMV and the internal standard	156
7.3.4. Substantiation of most optimal solvent/s for liquid-liquid extraction in drug detection clarity and recovery	158
7.3.5. Validation of precision and accuracy	160
7.3.6. Construction of a calibration curve for LMV quantification in blood plasma	160
7.3.7. Quantification of LMV concentrations in blood plasma and livers of rats	161
7.4. Concluding Remarks	164

CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions	165
8.2. Recommendations	166
References	169
Appendices	198
Appendix A	198
Appendix B	200
Appendix C	201
Appendix D	202
Appendix E	205
Appendix F	206

LIST OF FIGURES

	Page
Figure 1.1: Schematic representation of a) a typical virus and b) the ACNS	4
Figure 1.2: Schematic representation of the uptake of the ACNS: a) cell attachment; b) endocytosis; c) ACNS within endosome; d) fusion; e) endolysosomal escape; f) free drug release and g) carrier degradation	5
Figure 2.1: The HBV virion (Modified from Chen et al., 2008)	9
Figure 2.2: Basic steps involved in the intracellular delivery of therapeutic agents (TAs) (Modified from Faraji and Wipf, 2009)	12
Figure 2.3: Entry processes involved, vesicular formation and breakdown of intracellular components (Modified from Li et al., 2012)	13
Figure 2.4: pH sensitive carriers for intracellular targeting (Modified from Koren and Torchilin, 2012)	15
Figure 2.5: A schematic depicting the applications of CPPs as a mode of intracellular delivery of therapeutics (Modified from Koren and Torchilin, 2012)	17
Figure 2.6: Maturation-dependent nuclear entry of HBV capsids: a) Localization of hepatitis B capsids in HuH-7cells. Capsids (green, <i>Left</i>) and NPCs (red, <i>Center</i>) were stained by indirect immunofluorescence. Merged images are shown in the last column (<i>Right</i>). Recombinant capsids, devoid of viral polymerase (P-C) do not bind to cellular structures, immature capsids (ImmatC) show nuclear binding, whereas mature capsids (MatC) generate intranuclear capsids. Scale bar: 10 μ m; and b) Views of nuclear envelope cross sections with adjacent cytoplasm (c) and nucleoplasm (n) from a <i>Xenopus laevis</i> oocyte that has been microinjected with ImmatC. Arrowheads point to capsids associated with the nuclear face of the	

NPC. Scale bar: 100 nm (Adapted from Köser et al., 2012)	20
Figure 2.7: A schematic depicting the receptor-mediated endocytosis by the ASGP-R on hepatocytes (Modified from Lai et al., 2010)	29
Figure 2.8: Liver targeting by nanoparticle (NP) therapeutics: a) Nanosized particles of less than 200 nm with specific functionalities aid in the evasion of premature Kupffer cell clearance; b) Nanosized particles extravasate into the space of Disse through sinusoidal fenestrations in basal lamina absence; c) A high local concentration of NP therapeutics diffusing across the loosely organized extracellular matrix in the space of Disse; d) Non-specific endocytic uptake; and e) Receptor-mediated uptake by the hepatocyte (Modified from Li et al., 2010)	31
Figure 3.1: Chemical structure of a) HA and b) ECL	42
Figure 3.2: Illustration of HA desalting using dialysis tubing (<i>Mw 12044 Da</i>)	44
Figure 3.3: Digital images displaying the set-up for anionic graft copolymerization; a – N ₂ line, b – plugs and stoppers, c – polymers in DMSO, d – pot of heated oil, e – heated temperature-controlled magnetic stirrer, f – 3-necked round bottom flask, g – magnet, h – chemicals for copolymer synthesis, i – N ₂ tank pressure gauge/dial, j – N ₂ tank	46
Figure 3.4: Synthetic graft (co)polymerization of HA- <i>g</i> -ECL	47
Figure 3.5: Digital images of physical appearances of lyophilized A) HAmPEG complex and B) HA- <i>g</i> -ECL copolymer	51
Figure 3.6: Comparative FTIR spectra of HAmPEG, ECL and HA- <i>g</i> -ECL	53
Figure 3.7: Ring opening polymerization of ECL (Adapted from Sepe, 2008)	54
Figure 3.8: Comparative differential scanning calorimetry thermograms of a) HA sodium salt, b) mPEG, c) HAmPEG, d) ECL and e) HA- <i>g</i> -ECL	55

Figure 3.9: Thermogravimetric analytical and derivative thermogravimetry curves of A) HAmPEG, B) ECL and C) HA- <i>g</i> -ECL	57
Figure 3.10: Graphs of viscosity for copolymer concentrations of a) 0.01 %, b) 0.001 % and c) 0.0001 % ^{w/v}	58
Figure 3.11: Scanning electron micrographs of a) ECL at 5000X magnification, b) HA sodium salt at 5250X magnification, c) HAmPEG at 5150X magnification and d) HA- <i>g</i> -ECL at 5050X magnification	61
Figure 3.12: The determination of the CMC value for HA- <i>g</i> -ECL micelles	62
Figure 3.13: Transmission electron micrographs of formed HA- <i>g</i> -ECL micelles	63
Figure 4.1: Graphic representation of a three-factor Box-Behnken design (Adapted from Lawson, 2010)	67
Figure 4.2: Transmission electron micrograph of HA- <i>g</i> -ECL LMV-loaded nanoparticles at 50000X magnification. a) F14, b) F10, c) F1	74
Figure 4.3: Comparison of the effect of variables on nanoparticle sizes. A) Copolymer amount, B) Surfactant amount, C) Solvent volume	77
Figure 4.4: Zeta size and potential distribution profiles of Formulation 6 from the Box-Behnken design showing a) size of 74.60±6.76 nm and b) zeta potential of -16.8±6.55 mv	77
Figure 4.5: Comparison of the effect of variables on DSC. A) Copolymer amount, B) Surfactant amount, C) Solvent volume	79

Figure 4.6: Comparison of the effect of variables on thermal degradation. A) Copolymer amount, B) Surfactant amount, C) Solvent volume	81
Figure 4.7: Calibration curve of LMV in PBS (0.1 M, pH 7.4) at 277 nm	83
Figure 4.8: <i>In vitro</i> LMV release profiles from HA-g-ECL copolymeric nanoparticles. a) Formulations 1-5, b) Formulations 6-10 and c) Formulations 11-15, (SD≤2.5 in all cases, n=3)	87
Figure 4.9: Residual plots for the design responses of size, DEE and MDT	92
Figure 4.10: Response surface plots showcasing the effects of HA-g-ECL, Pluronic F-127 and DCM on DEE (%), Size (nm) and MDT (hours)	93
Figure 4.11: Response contour plots showcasing the effects of HA-g-ECL, Pluronic F-127 and DCM on DEE (%), Size (nm) and MDT (hours)	94
Figure 5.1: Desirability plots demonstrating variable values required for optimized formulation preparation and desirability values expected for responses	99
Figure 5.2: Digital image of A) synthesized lyophilized C-LA in physical form	101
Figure 5.3: Schematic of the synthesis of C-LA (Adapted from Kim et al., 2011)	102
Figure 5.4: Bar graph illustrating the comparative assessment of observed measurement responses between optimized coated and uncoated ACNS	106
Figure 5.5: FTIR spectra of a) native chitosan, b) native linoleic acid and c) C-LA	109
Figure 5.6: FTIR spectra of a) C-LA coated LMV-loaded ACNS, b) C-LA, c) LMV and d) uncoated LMV-loaded ACNS	110
Figure 5.7: Transmission electron micrograph of optimized C-LA coated ACNS	111

Figure 5.8: Differential scanning calorimetry curves; a. Native low molecular weight chitosan, b. Native LA and c. C-LA	112
Figure 5.9: Differential scanning calorimetry curves; a. Pure LMV, b. Mannitol, c. Optimized non-coated LMV-loaded ACNS, d. HA- <i>g</i> -ECL, e. Optimized C-LA coated LMV-loaded ACNS and f. C-LA	114
Figure 5.10: Thermogravimetric analytical and derivative thermogravimetry curves of A) Chitosan, B) LA and C) C-LA	116
Figure 5.11: Thermogravimetric analytical and derivative thermogravimetry curves of A) Pure LMV, B) Mannitol, C) Optimized C-LA coated LMV-loaded ACNS and D) Optimized uncoated LMV-loaded ACNS	117
Figure 5.12: DLS profiles of the optimized ACNS before C-LA coating; a) size and b) zeta potential	118
Figure 5.13: DLS profiles of the optimized ACNS after C-LA coating; a) size and b) zeta potential	119
Figure 5.14: Fractional drug release profiles of the uncoated and C-LA coated optimized ACNS formulations	120
Figure 6.1: Fluorophore chemical structures. A) Cy3, B) Cy5, C) Fluorescein and D) Tetramethylrhodamine (Adapted from Renberg, 2006)	123
Figure 6.2: Illustration of the Improved Neubauer Hemacytometer scrutinizing the grid layout, adapted from http://homepages.gac.edu/~cellab/chpts/chpt1/figure8.html , [Accessed August 5 th , 2014]	128
Figure 6.3: Confocal microscopy images of 1. Plain HepG2 cells at magnifications of A - 10X, B - 20X and C - 40X; 2. Uncoated optimized ACNS uptake (40X magnification) under A i) & ii) - Brightfield light and B i) & ii) - Fluorescent light and 3. C-LA Coated optimized ACNS uptake (40X magnification) under A i) & ii) –	

Brightfield light and B i) & ii) - Fluorescent light 133

Figure 6.4: High speed fiber-optic fluorescent live images of a. fluorescent optimized ACNS, b. fluorescent uncoated optimized ACNS and HepG2 cells (>30 minutes incubation), c. fluorescent uncoated optimized ACNS and HepG2 cells (>24 hours incubation), d. fluorescent C-LA coated optimized ACNS and HepG2 cells (>30 minutes incubation), e. fluorescent C-LA coated optimized ACNS and HepG2 cells (>24 hours incubation) 135

Figure 7.1: Ultrasound images of livers in the rodent animal model following injection administrations of microbubbles in A) tail vein, B) portal vein and C) hepatic artery. Enhanced microbubble contrast (green) is achieved with hepatic artery or portal vein with minimal contrast observed in tail vein (Adapted from Bagi and Andresen, 2010) 138

Figure 7.2: Digital image showing Sprague Dawley rats housed in single in cages at the CAS animal experimental room 140

Figure 7.3: Schematic representation of *in vivo* animal studies to be conducted on the rat model with depiction of assigned experimental and control groups 143

Figure 7.4: Successive digital images depicting the entire portal vein injection surgical procedure 145

Figure 7.5: Digital images of recovery stage post-surgery; a) Rat 1 from group A recovering from anaesthesia, b) A recovery room with blankets and heater 147

Figure 7.6: Digital image of anaesthetized Rat 3A undergoing an ultrasound imaging procedure 148

Figure 7.7: Digital aerial view image of liver removed from Rat 1 Group C in 10% ν formaldehyde solution 149

Figure 7.8: 96 hour time point ultrasound liver images of rat administered with A) Optimized C-LA coated LMV-loaded ACNS, B) Optimized C-LA coated non

drug-loaded ACNS, C) Oral LMV and D) IV LMV 156

Figure 7.9: Elution depictions on 2D and PDA 3D plot for A. Spiked LMV (Retention time = 0.905 minutes) and AZT (Retention time = 1.756 minutes) in double deionized water and B. Spiked LMV (Retention time = 0.888 minutes) and AZT (Retention time = 1.732 minutes) in blank plasma sample 157

Figure 7.10: Calibration curve of LMV in blank plasma at 277nm 161

Figure 7.11: *In vivo* LMV concentrations measured in, a) IV administration in plasma, b) IV administration in liver, c) Oral administration in plasma, d) Oral administration in liver, e) C-LA ACNS intraportal administration in plasma, f) C-LA ACNS intraportal administration in liver 164

LIST OF TABLES

	Page
Table 2.1. Summary of anti-HBV cytokine and nucleot(s)ide analogue therapeutic agents as a treatment plan	23
Table 2.2. Summary of anti-HBV thiazolide, siRNA and HAP therapeutic agents as a treatment plan	28
Table 3.1. Quantities of hydrophilic to hydrophobic polymer used for optimal grafting efficacy	45
Table 3.2. Grafting parameters of the various copolymeric formulations synthesized	52
Table 3.3. Depiction of last 5 runs of HA- <i>g</i> -ECL concentrations to determine viscosity average value	59
Table 4.1. Variables for the Box-Behnken factorial design parameters	69
Table 4.2. Formulation (F) compositions obtained from the Box-Behnken experimental design	70
Table 4.3. Comparative thermal events: Three step thermal degradation and glass transition temperatures of copolymeric nanoparticles	82
Table 4.4. Drug entrapment efficiency and particle sizes of formulations	84
Table 4.5. MDT values obtained for formulations generated from the Box-Behnken design	88
Table 4.6. Variable effects with estimated <i>p</i> -values on outcomes	89

Table 4.7. Regression and residual errors ANOVA	90
Table 5.1. Constraints employed for optimized formulation	99
Table 5.2. Optimized LMV-loaded HA-g-ECL copolymeric nanoparticle formulation developed by the response surface technique	100
Table 5.3. Cryoprotectants used for HA-g-ECL nanoparticle size maintenance assessment	103
Table 5.4. Predicted experimental and desirability measurements of the optimized HA-g-ECL based nanoparticle formulation	105
Table 5.5. Estimation of cryoprotectants flowability, redispersibility and size maintenance functional ability for optimized ACNS	107
Table 7.1. UPLC gradient method for the elution of LMV and AZT (internal standard)	151
Table 7.2. Solvent selection for liquid-liquid extraction procedure	158
Table 7.3. Recovery of LMV drug in spiked blank plasma from selected deproteinating agents (n=3)	159
Table 7.4. Recovery of LMV drug in spiked blank plasma from variable selected deproteinating agent ratios (n=3)	159
Table 7.5. Inter- and Intra- day variability testing precision and accuracy of assay	160

LIST OF EQUATIONS

	Page
Equation 3.1. GE (%)	47
Equation 3.2. GR (%)	47
Equation 3.3. Mark-Houwink	48
Equation 4.1. Box-Behnken quadratic model	68
Equation 4.2. DEE (%)	72
Equation 4.3. MDT	73
Equation 4.4. Polynomial regression: DEE	89
Equation 4.5. Polynomial regression: Size	89
Equation 4.6. Polynomial regression: MDT	89
Equation 6.1. Cell Counting	128
Equation 7.1. RSD (%)	152

CHAPTER 1

INTRODUCTION

1.1. Background to this study

Amongst all of the disease causing agents in humans, viruses are the most notorious, active and important. Viruses which are obligate parasites have DNA or RNA as their genetic material with mutation rates that are markedly different and quantifiable. Some show genetic stability and others show varying rates of mutation. Numerous new viruses have been reported worldwide causing major outbreaks eg. Human Herpesvirus-8 (HHV-8) in 1995, Avian Influenza A (H5N1) Virus in 1997, Nipah virus in 1999, Severe Acute Respiratory Syndrome (SARS) Virus in 2002 and Chikungunya Virus (CHIKV) in 2005 [WHO Report, http://www.searo.who.int/entity/emerging_diseases/documents/b0005.pdf, Accessed 21st April, 2015]. The perpetual viral emergence and re-emergence poses a serious public health concern. Viral latency and resistance, improper diagnosis, toxicity and immunosuppression caused by antivirals may be due to this. Thus, there is a struggle against viruses and foolproof antiviral therapies are lacking against them. To make matters worse, drugs are failing in human clinical trials (Saxena et al., 2009).

This study aims to utilize a pair of polymers that have not been previously conjoined and surface modified, and architecturally resembles the structural outline of viruses in general. The Hepatitis B Virus (HBV) will be used as a model virus with the model antiviral drug being lamivudine (LMV). A vast majority of individuals worldwide are chronically infected with HBV and it is considered one of the most lethal human pathogens. The virus consists of a partially double stranded circular DNA genome that is encapsulated within the viral capsid and envelope and it undergoes reverse transcription during its replication cycle (Xia et al., 2006). The main danger of patients suffering from a chronic HBV infection lies in the emergence of cirrhosis leading to morbidity and mortality from hepatocellular cancer or drawbacks arising from the cirrhosis such as portal hypertension and liver failure (Lebray et al., 2003). The principal aims that need to be addressed to attain a favourable therapeutic approach in a hepatitis B infection should include a reduction in the viral load to imperceptible levels, a decrease in the rate of progression of the disease and a reduced rate of evolution of drug-resistant HBV (Bartholomeusz et al., 2003).

Many significantly effective antiviral agents are active intrinsically but they are shown to be associated with unacceptable systemic toxicity. It may be possible to achieve the following when using novel controlled delivery systems: prolonged efficacy, optimum therapeutic responses and a reduced toxicity by, reproducibly and predictably transporting and promoting the release of the drug to the specific target environment. The site of infection such as the liver in this case clearly makes it useful to architecturally design therapeutic agents with their carriers targeted at the level of the organ. However, targeting agents at the cellular level is not very easy because the life cycle of the virus is limited to the inside of the host's cells, with a reduced selective toxicity of antiviral agents.

Compared to interferon-alpha (IFN), these agents selectively target the viral reverse transcriptase inhibitor to cause inhibition of the HBV genome replication and are considered to be superior with respect to the extent and time it takes to drop HBV DNA levels. Likewise, numerous challenges and drawbacks still remain with the use of oral anti-HBV agents. These are the high rate of relapse with short term therapy, the emergence of drug resistant genotypic mutations of HBV in long term therapy and drug resistance (Ghany and Liang, 2007; Li et al., 2010; Liaw et al., 2004; Palumbo, 2008). An attempt to enhance efficacy and diminish viral resistance occurring has been the recently emerged concept of combination therapy. Still, only minimal data is available and no combination therapy proved to be beneficial as compared to monotherapy (Asselah et al., 2005; Nash and Alexander, 2008).

Hepatocyte drug delivery with specificity to their receptors such as the asialoglycoprotein receptor (ASGP-R) has made possible the selectivity and targeting of drugs and foreign genes to these cells to combat dreaded diseases such as the HBV. Several attempts have been made to label carriers (polymers, human serum albumin, recombinant high density lipoproteins (HDL) with ASGP-R specific ligands (galactose, lactose, acetylgalactosamine, asialofetuin) to design specific carriers for drug and gene delivery to hepatocytes. Several notable studies have also been conducted concerning small interfering ribonucleic acids (siRNAs) as drug delivery treatments for HBV.

With that said this study suggests a novel architecturally-configured nanosized system (ACNS) encompassing the union of polymers, encapsulating therapeutic molecules that will be effective in targeted intracellular therapeutic drug delivery, as an example, an antiviral to the liver, selectively targeting hepatocytes, and site-specifically delivering the therapeutic molecule to render the formidable HBV to imperceptible levels.

1.2. Rationale and motivation for this study

Drawbacks arising from the conventional antiviral delivery systems include those of poor oral absorption or bioavailability, accumulation in tissues leading to toxicity and a very rapid elimination from the body. Other significant problems that may be encountered are severe and unwanted side effects, large interindividual variations in bioavailability as well as an impractical administration frequency (Meijer et al., 1992). A drug delivery system to propose targeted controlled delivery of LMV is necessitated in view of its harmful and toxic side-effects. The most significant warning and precaution constantly highlighted to patients using the oral form of the drug is the great risk of developing lactic acidosis and a severe form of hepatotoxicity that may include hepatomegaly and steatosis which could be fatal. In addition to this and according to the U.S. Food and Drug Administration (FDA) [http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/021003s015,021004s015lbl.pdf, Accessed January 7th, 2014] there is evidence of exacerbation of the viral disease once treatment is halted. Arduous dosing frequencies could be the reason for discontinuation of treatment embracing a system capable of controlled and sustained delivery of the drug. There is also the risk of emergence of resistant HIV-1 and HBV infection when complete and required treatment doses are not attained. Formulating an intracellular targeted virus-like nanoparticulate system, as depicted in Figure 1.1, should prove to be operative and overcome a number of the challenges linked with conventional antiviral drug delivery systems.

Targeted drug delivery comprises a therapeutic agent, a targeting moiety and a carrier system where the drug is incorporated by passive absorption or chemical conjugation. The choice of carrier is extremely important as it determines the fate of the drug (Park et al., 2010). The virus-like nanoparticle system will be administered via intravenous injection for targeted drug delivery, owing to their small size (Park et al., 2010). When nanoparticles are injected into the bloodstream, they are rapidly cleared by the reticuloendothelial system (RES), thus leading to a decreased pharmacological action (Harush-Frenkel et al., 2007). Surface coating of the system with a substance that is able to alter the immune response and clearance may overcome this problem (Zahr et al., 2006). This can be achieved by simply employing a polymer in the outer make-up of the carrier system that possesses the function in avoidance of macrophage uptake and increasing the circulation to efficiently target diseased cells. The constituents in the formulation of the ACNS will only have an appearance of a viral entity in instances where the hydrophobic polymer mimics the envelope, the hydrophilic core with antiviral mimics its DNA

and the chemically adsorbed targeting ligand are ordered in the same manner of arrangement as the glycoprotein spikes posing the similar relevant functions in terms of entry into a cell.

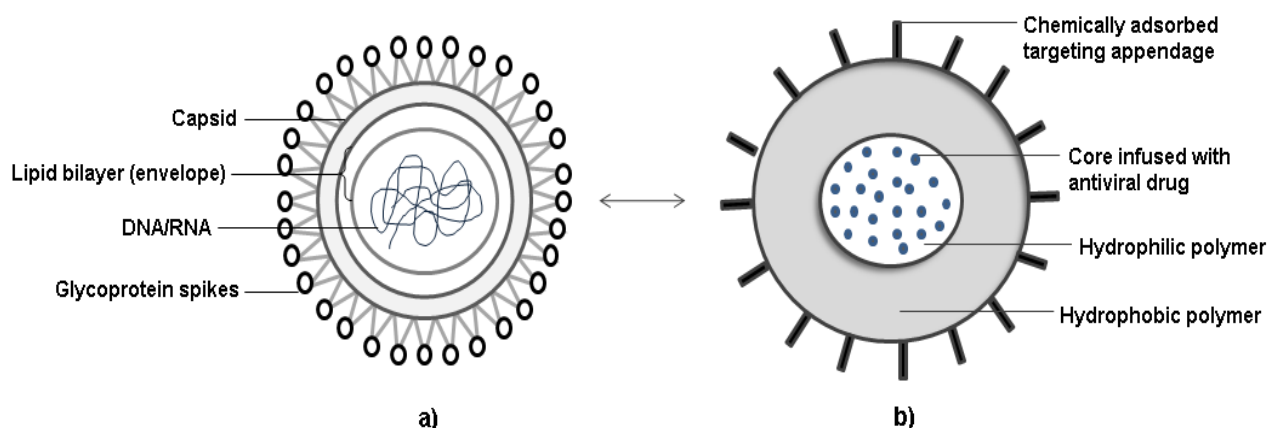


Figure 1.1: Schematic representation of a) a typical virus and b) the ACNS.

The mechanism of intracellular uptake of the ACNS is depicted in Figure 1.2. On approach to a cell, the ACNS is endocytosed following the action of surface attachment of the targeting ligand. The drug which is still intact within the hydrophilic core, together with the carrier, end up in small vesicles (endosomes) within the cell. These endosomes mature and fuse with lysosomes. In a normal situation of uptake, during this process, there is a drop in pH within these vesicles and together with enzymatic degradation within the lysosomes, inactivation of the drug and its carrier occurs before it is released into the cytosol to allow for its therapeutic action (Breunig et al., 2008). Therefore, at this point there should be endolysosomal escape of the drug-carrier system owing to the properties of the bulk hydrophobic polymer, followed by drug unpacking and degradation of the biodegradable delivery system to eliminate toxicity (Panyam et al., 2002).

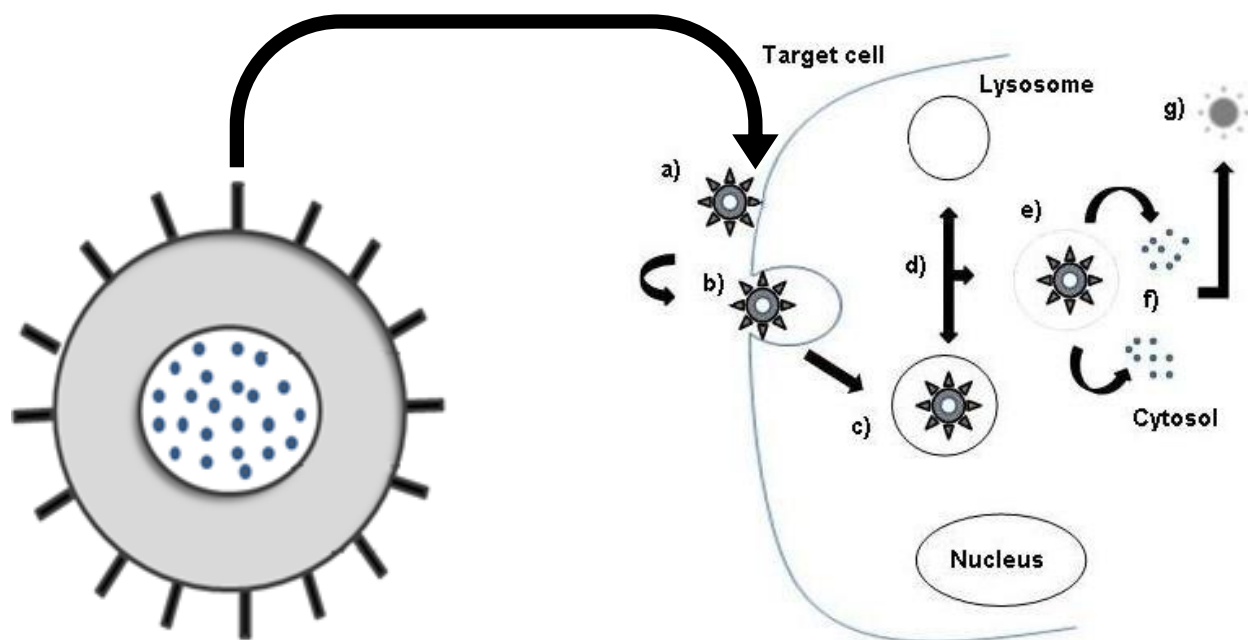


Figure 1.2: Schematic representation of the uptake of the ACNS: a) cell attachment; b) endocytosis; c) ACNS within endosome; d) fusion; e) endolysosomal escape; f) free drug release and g) carrier degradation.

1.3. Novelty of this study

1. The design of a novel, nano-sized architecturally-configured system is achieved by virtue of copolymerization of a hydrophobic and hydrophilic polymer in an original manner, with a double emulsion solvent-evaporation method for the amphiphilic copolymer to be made into nanoparticles encapsulating an antiviral in the ACNS core. Targeting nano-appendages on the nanoparticle surface will be carried out by a method of chemical adsorption onto the newly copolymerized nanoparticles.
2. The design of a nanosized system: The blood-brain barrier (BBB) acts to hinder the transmission of several important essential and therapeutic agents. Thus in the design of a system that is nanoscopic, encapsulating therapeutic molecules and genes that may be effective in diagnosis and therapy, delivery across the BBB will prove to be successful.
3. The ACNS will be formulated to specifically target the formidable HBV. An inordinate advantage arises by the addition of targeting appendages, made from target-specific ligands, (Figure 1.1) adsorbed onto the surface of the polymer foundation to be directed to

infected hepatocytes harboring the virus and thus directing and delivering the therapeutic specifically to this site.

1.4. Aim and objectives

The aim of this study is to design a nanoparticulate system in order to gain targeted intracellular delivery and subsequently attain release of free drug into the cytosol specifically of hepatic cells. The above mentioned aim can be achieved by an outline of the following objectives:

1. To select a suitable combination of a hydrophilic and hydrophobic polymers to formulate the nanoparticulate system for entry into cells.
2. To utilize the method of double emulsion solvent-evaporation in the development of the nanosized drug-carrier complex.
3. To elucidate the physicochemical parameters of the nanoparticulate system such as size, surface chemistry and morphological analysis including their shape and ionic charge, structure of the system and its adhesion to the cell surface.
4. To execute *in vitro* studies in order to assess the drug release profile of the ACNS investigating whether it is capable of providing sustained drug release over time.
5. To execute *ex vivo* studies utilizing a specific cell line in order to visualize uptake of the ACNS.
6. To execute *in vivo* studies on an appropriate animal model to evaluate the bioavailability of the ACNS and thus its therapeutic efficacy.

1.5. Overview of this dissertation

Chapter One is an introductory chapter in which the infamous virus as a driving force for disease and its pathophysiology is discussed. The dreadful HBV is more specifically examined and challenges faced with conventional antiviral agents are also addressed. This chapter highlights the rationale behind the configuration of the ACNS and outlines the novelty, aim and objectives of the study.

Chapter Two is a literature review that discusses aspects of intracellular targeting and therapeutic delivery. It also reviews drug delivery strategies against HBV previously attempted

by researchers as well as focusing on the more recent and novel approaches employed for HBV eradication.

Chapter Three has its complete focal point on the synthesis and development of a novel amphiphilic graft copolymer that is to be the foundation of the ACNS for the study. The method by which the copolymer is fabricated is evaluated by conducting tests to measure its yield as well as proving the occurrence of graft copolymerization in addition to several tests being conducted to fully characterize the ACNS.

Chapter Four aims to fully elucidate the trends seen with selected variables and their effect on response outcomes for the design and development of an optimal ACNS generated from an experimental model. The selected variables were amount of copolymer, amount of surfactant and volume of solvent. The responses for optimization were drug entrapment efficiency (DEE), size and mean dissolution time (MDT).

Chapter Five encompasses the optimization of ACNS. The optimized formulation is fully characterized by testing size, DEE, MDT and assessing its predicted and observed measurements from the model. Structural variations in the make up of the copolymeric nanosystem are explored, thermal profiles using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) are evaluated and all of these are contrasted against a coated optimized ACNS for targeted drug delivery to the hepatic region

Chapter Six is an *ex vivo* component of the study and describes the ability of the ACNS to be internalized intracellularly by HepG2 cells. The imaging microscopy tests employed to visualize the uptake of un/coated ACNS were confocal fluorescence microscopy and a high speed fiber-optic fluorescence live imaging system.

Chapter Seven outlines the *in vivo* study that was undertaken to evaluate the efficacy of ACNS on a rat model. This was a pilot study. A first-time tried intraportal injection procedure was applied to ensure targeting of ACNS to the liver. Ultrasound imaging was used to detect ACNS on live rats. Rats were euthanized, blood samples were taken and livers were removed to assess drug release from ACNS. Livers were homogenized and treated for detection of drug. Ultra Performance Liquid Chromatography (UPLC) was utilized to quantify and detect the drug in biological samples.

Chapter Eight is a conclusion to the dissertation and provides an insight into future outlooks and recommendations in which the study can be enhanced.

CHAPTER 2

A LITERATURE REVIEW ON HEPATO-TARGETING: AN OVERVIEW OF DRUG DELIVERY AND TREATMENT STRATEGIES FOR HEPATITIS B VIRUS INFECTION

2.1. Introduction

Hepatitis B Virus (HBV), a formidable DNA virus, is transmitted percutaneously, sexually and perinatally resulting in worldwide mortality from cirrhosis, liver failure and hepatocellular carcinoma. This virus replicates its genome through an RNA intermediate utilizing reverse transcription. Figure 2.1 is a schematic depicting the HBV virion. The virus, with a diameter of 40-42 nm, consists of a partially double-stranded, circular DNA genome that is encapsulated within the viral capsid and envelope and it undergoes reverse transcription during its replication cycle (Chen et al., 2008). Even though perinatal HBV infection can lead to high levels of the virus replicating and lacking substantial injury to the liver in the earlier years of life, eventually the risk of cirrhosis and hepatocellular carcinoma (HCC) progression will be directly proportional to the HBV DNA level maintained over time consistently and persistently. Liver histologic and clinical improvements are associated with reduced HBV replication. Thus, interventions that decrease HBV replication are expected to put a halt on the progression of liver disease and display an improvement in the natural history of chronic HBV infection (Dienstag, 2008).

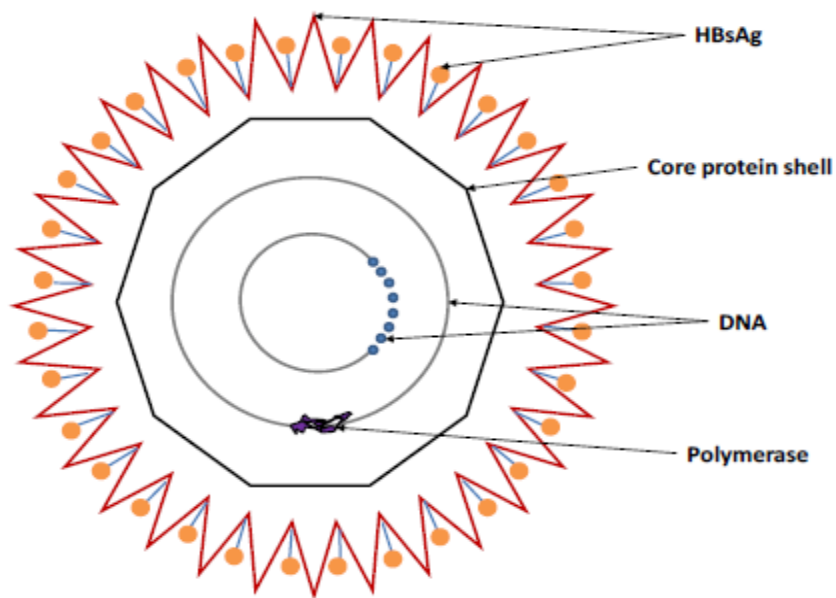


Figure 2.1: The HBV virion (Modified from Chen et al., 2008).

A significant threat to patients suffering from a chronic HBV infection lies in the emergence of cirrhosis leading to morbidity and mortality from hepatocellular cancer or drawbacks arising from the cirrhosis such as portal hypertension and liver failure. Anti-HBV therapy, over the last few years, promised an enhanced effectiveness against HBV. Drugs such as interferon- α_{2B} , pegylated interferon- α_{2A} , lamivudine, adefovir, entecavir and telbivudine have all been recognized and acknowledged for their treatment and direct antiviral activity against chronic HBV (Ruiz-Sancho et al., 2007). The principal aims that need to be addressed to attain a favourable therapeutic approach in an HBV infection should include a reduction in the viral load to imperceptible levels, a decrease in the rate of progression of the disease and a reduced rate of evolution of drug-resistant HBV (Bartholomeusz et al., 2003). The mechanics for targeted drug delivery are foremost in consideration for parenteral administration in addition to shielding therapeutics from degradation and untimely elimination. These mechanics are drug delivery vehicles consisting of soluble carriers eg. synthetic polymers, particulate carriers eg. micro- and nano- particles and target-specific recognition moieties eg. monoclonal antibodies. Dosage forms exist in sizes from the molecular level to macrodevices. Nanoparticles (usually less than 200 nm) are solid colloidal particles utilized in parenteral drug delivery and targeting.

Classes of drug targeting fall into two groups namely active and passive targeting. Active targeting utilizes certain interactions at the target site such as those of ligand-receptor and antigen-antibody binding, or in other cases, signals such as temperature or magnetic fields that can be applied externally. Passive targeting involves adjusting carrier systems' physicochemical properties to that of the physiological and histological features of the target site. At the target site of action, in this case being the liver, a drug is transported by its carrier. For this seemingly straight-forward and simple approach to work, multiple fundamental requirements (nonspecific interactions, access to the target site, drug release and suitability) must be satisfied.

2.2. Intracellular drug delivery and hepatitis B viral entry

A broad range of drugs require an intracellular mode of delivery so as to exert their therapeutic action in any of the affected organelles found therein. Thus, the importance lies not only in delivery of the therapeutic agent to a specified tissue but also to the specified cellular compartment. Transporting a diverse range of biologically active molecules and agents into cells remains a general crucial problem in drug delivery. A biological membrane's lipophilic nature poses restriction to the direct entry of these compounds (Torchilin, 2006). As a result of the 'curiosity' of certain cells, the ingestion of drug delivery systems/substances ensues. The

endocytosis process embraces a notion of membrane manipulation in which substances are encased and absorbed. Along with the above concept of cellular internalization of therapeutic agents and their carriers, exist reports that show that endocytosis is conjointly the putative uptake mechanism for the HBV particles. A study suggests entry of HBV into human hepatocytes occurs by attachment to the asialoglycoprotein-receptor (ASGP-R) molecules that are found on these liver cells. This manifests via the viral preS1-related envelope binding site for the ASGP-R receptor. Building on it, a study demonstrated that a bio-nanocapsule (BNC) containing a liposome with hepatitis B virus surface antigen (HBsAg) L protein embedded as a transmembrane protein and within it containing several drugs and genes were to be delivered with specificity to human hepatic cells in the same manner as and according to the HBV's mechanism of infection of these cells in the early stage (Yamada et al., 2012).

2.2.1. Endocytic uptake mechanisms

Phagocytosis, pinocytosis and receptor-mediated endocytosis (depending on either the clathrin and/or caveolae-mediated processes) are three subtypes of endocytosis. Neutrophils, macrophages and dendritic cells form the RES and such cells can be grouped under the phagocytosis subtype, achieving the internalization of materials of up to 10 μm in diameter (Kohane, 2007). Sub-micron material and substances in solution are taken up by the pinocytosis mechanism and this internalization can be carried out by basically all cell types. Receptor-mediated endocytosis via the caveolin- and clathrin-coated pits involves a triggered signal that is sent to the intracellular space. This occurs upon binding of ligands to the innumerable receptors found on the cellular membrane. The signal generates an assembly of biochemical pathways and may also cause the ingestion of the ligand with its appended drug loaded delivery device (Faraji and Wipf, 2009). The means of HBV recombinant capsid-like core particle uptake and its predetermined course intracellularly was explored once it had been bound to heparan sulfate proteoglycans (Cooper and Shaul, 2006). Results reflected internalization of these particles through clathrin-mediated endocytosis. Figure 2.2. depicts the steps involved in the intracellular delivery of therapeutic agents.

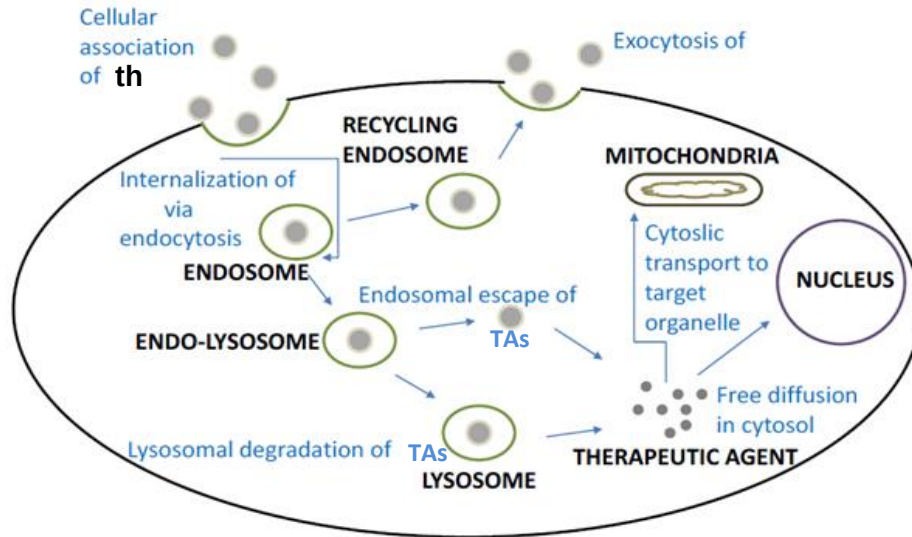


Figure 2.2: Basic steps involved in the intracellular delivery of therapeutic agents (TAs) (Modified from Faraji and Wipf, 2009).

However, there is a problem with the endocytic pathway in terms of particles entering the cell. Molecules and particles that have entered the cell become entrapped in small vesicles within the cell, namely endosomes. These endosomes mature and fuse with lysosomes. In a normal situation of uptake, during this process, there is a drop in pH within these vesicles together with enzymatic degradation within the lysosomes owing to the action of the lysosomal enzymes found therein (Figure 2.3.).

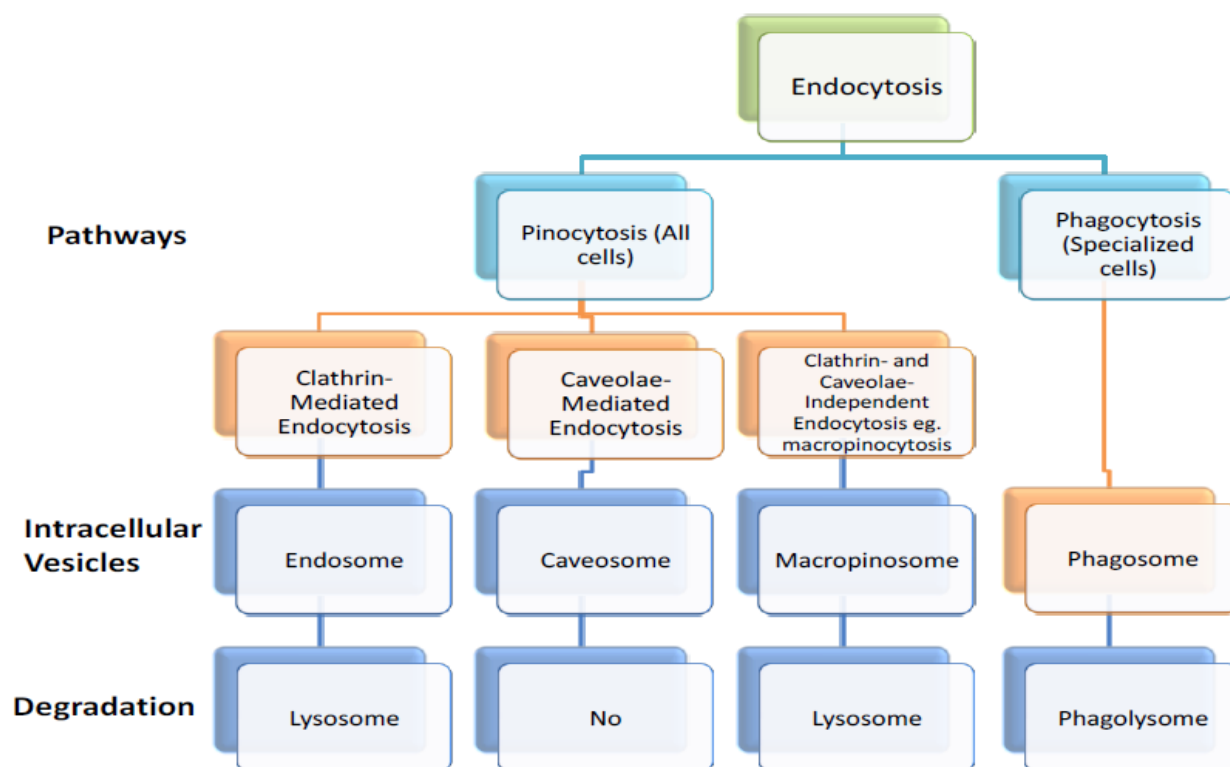


Figure 2.3: Entry processes involved, vesicular formation and breakdown of intracellular components (Modified from Li et al., 2012).

Inactivation of the drug and its carrier occurs before it is released into the cytosol to allow for its therapeutic action. Therefore, only a small amount of unaffected substance appears in the cytoplasm of the cell causing the major problem of bioavailability. Thus far, attempts have been sought to bring assorted macromolecular drugs and drug-loaded carriers directly into the cytoplasm, protecting the therapeutic agents and DNA from the lysosomal degradation and ultimately enhancing the efficiency and therapeutic potential of the drug or DNA incorporation into the genome of the cell (Vasir and Labhasetwar, 2007). These attempts although collective and diversified have been only somewhat successful. A method of destabilizing the endosomal membrane can prove to substantially enhance the escape of the drug delivery system from the endosome. However, this destabilization can also be toxic to the cells and be discordant for some therapeutic agents. The means of escape as for PLGA nanoparticles which do not open the endolysosomes comprises a direct interaction of the nanoparticles with the endo-lysosomal membrane as a result of the surface charge reversal from anionic to cationic that occurs under acidic conditions within the vesicle (Stepensky, 2010). Translocation motif (TLM) is a cell permeable peptide found in the surface protein of human HBV. Intracellularly, their activation by

endosomal proteases sources their disclosure on the surface of the virus. This unveiling of the TLM is crucial in the mediation of viral particle translocation across the endosomal membrane and effectively into the cytosol (Lepère et al., 2007).

2.2.2. Intracellular drug delivery aids

2.2.2.1. Emulsifiers as facilitators of intracellular drug delivery

Polyvinyl alcohol, an emulsifier in varying concentrations, has been shown to alter surface charges of drug loaded delivery devices and results have also shown its influence on intracellular uptake and cellular distribution/endolysosomal escape (Sahoo et al., 2002). Therefore, types of emulsifiers and corresponding concentrations may also modulate intracellular delivery and distribution. The potential of another polymer, polyethylene glycol (PEG), is marveling. PEG chains can be grafted onto the surface of polymeric nanoparticles or be covalently attached to a different polymer through the co-polymer synthesis process, to achieve surface modifications. It may also be surface adsorbed or entrapped into the matrix of the drug loaded delivery device. PEG modification prevents the aggregation of surface modified liposomes and greatly delays particle content leakage of the therapeutic load in comparison to unmodified nanoparticles. PEG protects a therapeutic load from carrier uptake by the RES, from metabolism and from excretion and has therefore been employed in promoting prolonged circulation. Functionalized PEG chains can have trans-activating transcriptional activator (TAT) peptide covalently attached to it. Inclusion of a TAT peptide in a PEG-nanoplex compared to TAT in poly(ethyleneimine) (PEI) particles without the PEG, clarified an enhanced transfection efficiency *in vivo*, suggesting that PEG is necessary for site-specific localization (Vasir and Labhasetwar, 2007). PEG conjugated to interferon- α (IFN- α), the main treatment for chronic HBV, have been shown to augment the pharmacokinetics of standard IFN (Asselah et al., 2007). HBsAg clearance is also established with PEG IFN (Sonneveld and Janssen, 2011).

2.2.2.2. pH-sensitive carriers in intracellular drug delivery

The use of pH-sensitive carriers such as liposomes and cell penetrating molecules, mentioned later, are the most efficient and thus favored noninvasive methods. Liposomes with its pH sensitive components, coalesces with the endovacuolar membrane as a result of the action of the lowered endosomal pH. This promotes destabilization and content release into the cytosol (Torchilin, 2006). It should also be noted that certain diseased tissues, tumours, infections and inflammations illustrate an acidic environment in comparison to their surrounding non-diseased

tissues; therefore stable pH sensitive carriers at physiological pH 7.4 will dispense their contents within these acidic conditions and subsequently into the intracellular compartment (Lembo and Cavalli, 2010). pH-sensitive liposomes delivered the iminosugar N-butyldeoxynojirimycin (NB-DNJ) into the endoplasmic reticulum of mammalian cells (Costin et al., 2002). Researchers stated that NB-DNJ had also exhibited efficiency in hindering the duplication of the HBV by their restraint within hepatic cells. An illustration of a pH-sensitive carrier for intracellular drug delivery is depicted in Figure 2.4.

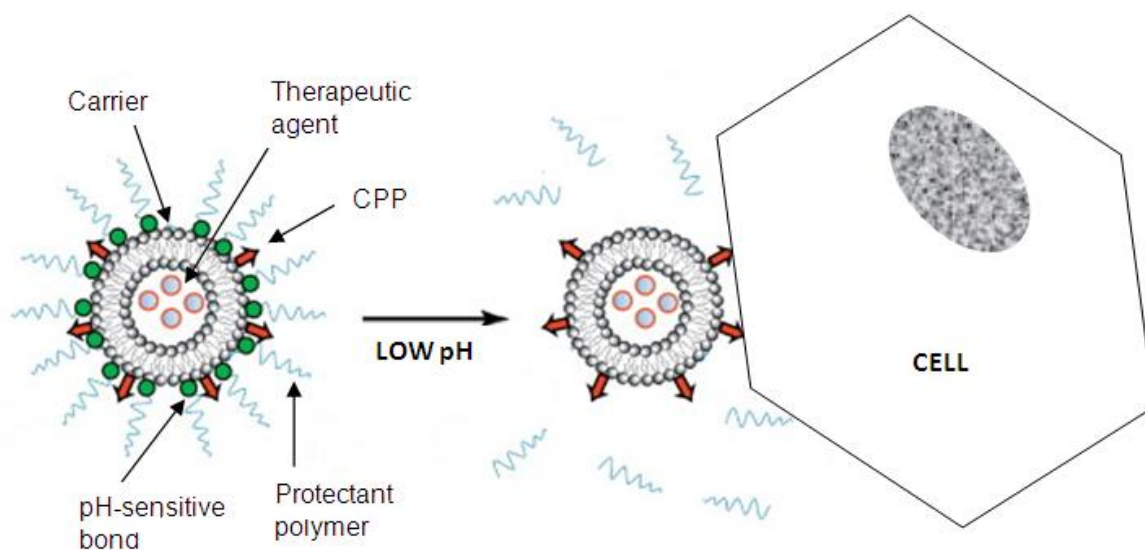


Figure 2.4: pH sensitive carriers for intracellular targeting (Modified from Koren and Torchilin, 2012).

2.2.2.3. Peptide based strategy for intracellular drug delivery

To help internalizing the nanocarrier systems, cell penetrating peptides (CPPs) such as the TAT peptide, amongst the many, have been coupled to the surface of liposomes and this coupling led to their adept delivery intracellularly via a non-endocytic route. Molecules that are modified with CPPs are enhanced to traverse cell membranes and deliver their cargo into the cytosol or the nucleus. CPPs contain a little under 30 amino acids which for the most part are amphipathic maintaining a net positive charge. For instance, the CPP HBV TLM mentioned earlier has the amino acid sequence PLSSIFSRIGDP (Fonseca et al., 2009). Cargoes with molecular weights several times their own weight have been shown to be intracellularly delivered by CPPs. Several advantages over conventional modes exist since CPPs delivery mode is efficient for a variety of cell types and has a potential therapeutic function in terms of cellular delivery

(Torchilin, 2006). Synthetic recombinant CPPs and nucleocapsid subunits have been contrived to incommode the HBVs encasing as a result receding the viral yield. CPPs have also been considered for delivering antibodies intracellularly (Gupta et al., 2005) and a single-chain variable fragment antibody that works against the HBVs core protein has also been delivered intracellularly via a CPP (Xun et al., 2013). A schematic showing various strategies employed in intracellular delivery of therapeutics, including CPPs delivery mode, is depicted in Figure 2.5.

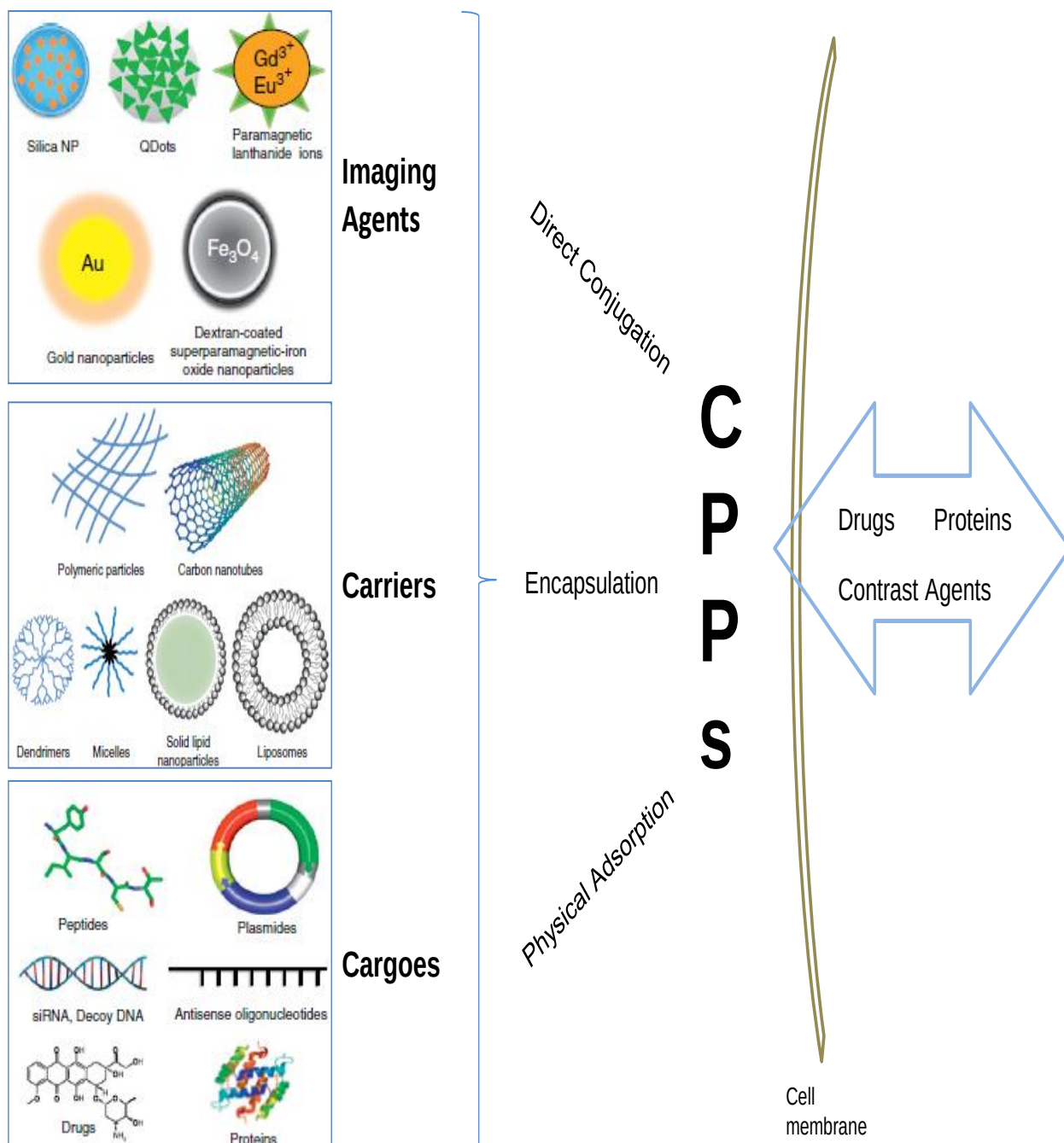


Figure 2.5: A schematic depicting the applications of CPPs as a mode of intracellular delivery of therapeutics (Modified from Koren and Torchilin, 2012).

Ovalbumin was used as a model antigen that was integrated to the CPP TLM acquired by the HBV and this was applied as a vaccine (Bleifuss et al., 2006). The authors reported a prevailing

efficacy of TLM attached ovalbumin compared to ovalbumin alone concerning cellular and humoral immune system responses. Adequate transfection has also been observed both *in vitro* and *in vivo* with a lowered toxicity thus successful delivery to the cytosol makes the CPP approach useful for intracellular targeting (Mäe and Langel, 2006).

Targeting residues conjugated to a peptide-based drug can be sufficient for its desired accumulation in the targeting organelle following its delivery to the cytoplasm. An example is the ER signal peptide that was conjugated to the antigenic peptide by a 1:1 drug: targeting residue ratio. This has been reported to have resulted into an enhanced localization in the endoplasmic reticulum of the target cells following its release from the nanoparticle or liposome-based drug delivery system (Stepensky, 2010). Viral vectors are efficient for gene delivery however, their use has shown to be limited following safety concerns. Furthermore, the alternative non viral vectors have problems with nuclear translocation and endolysosomal escape (Gupta, 2005). In addition, fusogenic peptides function with an end result being endolysosomal escape of the drug delivery system. An interaction with phospholipid membranes follows a resultant pore formation or the induction of membrane fusion and/or lysis (Lochmann et al., 2004). It has been noted that the incorporation of synthetic membrane-active peptides into a delivery system housing drugs such as oligodeoxynucleotides (ODNs), peptides, or plasmid DNA for its intracellular delivery has been possible due to its fusogenic property (Vasir and Labhasetwar, 2007). A study demonstrated a distinct inhibition of the HBV's gene expression and replication by compounding an ODN specific to the polyadenylation signal for HBV, to a vesiculate system for DNA (Wu and Wu, 1992). This targeted the ASGP-R on hepatocytes.

2.2.3. Nuclear Targeting

In order to achieve the desired therapeutic and pharmacological effects at the intracellular level, an evolution of specialized drug delivery systems ought to have specificity and targeting ability in a sustained manner aimed at a cell organelle. Eukaryotic cells encase the nucleus, a significant subcellular structure and a place where DNA replication and transcription occur. Polymeric nanoparticles are interesting and intelligent tools to achieve successful and efficient delivery of DNA and/or drugs to the nucleus. One of the ways in which drug loaded delivery devices could be targeted to the nucleus itself is through the chemical coupling of ligands to their surface, a frequently seen and noted targeting mechanism. Organelle-specific localization signals such as the nuclear localization signal (NLS) could be critical in the development of a drug loaded device as a gene delivery system for nuclear targeting. A study concluded that to

promote an antiviral activity that is detectable by ODNs, a minimum NLS is needed to be conjugated to the polymer in use so as to be delivered in a more efficient way to the nucleus. Another study showed that hepatitis delta antigen (HDag), a hepatitis delta virus (HDV) encrypted protein, is conveyed through to the nucleus as a protein complex (Xia et al., 1992). Authors had identified an NLS within the HDag which ensured the nuclear transport, as well as remnants of the protein that did not incorporate the NLS but were still transferred to the nuclear region via a leucine zipper sequence that must be present on the HDag. A nuclear pore complex (NPC) which is present in the nuclear membrane allows all passive and active transport into and out of the nucleus. It comprises a pore-like, molecular sieve function where molecules that are smaller than 40-45k Da in size have the ability to diffuse freely. Larger proteins that need to pass through require NLS in order to be targeted to the nucleus specifically. Detection of nuclear targeting was brought about by gold nanoparticles and quantum dots usage. In addition to being easily synthesized in several size distributions, these two can be easily detected by transmission electron microscopy or fluorescence-based techniques and therefore have been used as a kind of model nanoparticle for nuclear direction. Researchers reported on the precore protein (P22) of the HBV and its transportation into a cells nucleus following discharge (Ou et al., 1989). This occurred by virtue of signal peptide cleavage and ensues a cytosolic process. A form of nuclear transport mechanism involves the endosome trafficking in a cell which causes a resultant early release of nanoparticles into the cytosol and the shielding of the nanoparticles from the consecutive degradation by cytoplasmic nucleases. Endosomes carry the nanoparticle away from the plasma membrane of the cell to perinuclear locations (Li et al., 2012). A prototype example of a maturation-dependent nuclear entry of HBV capsids is depicted in Figure 2.6.

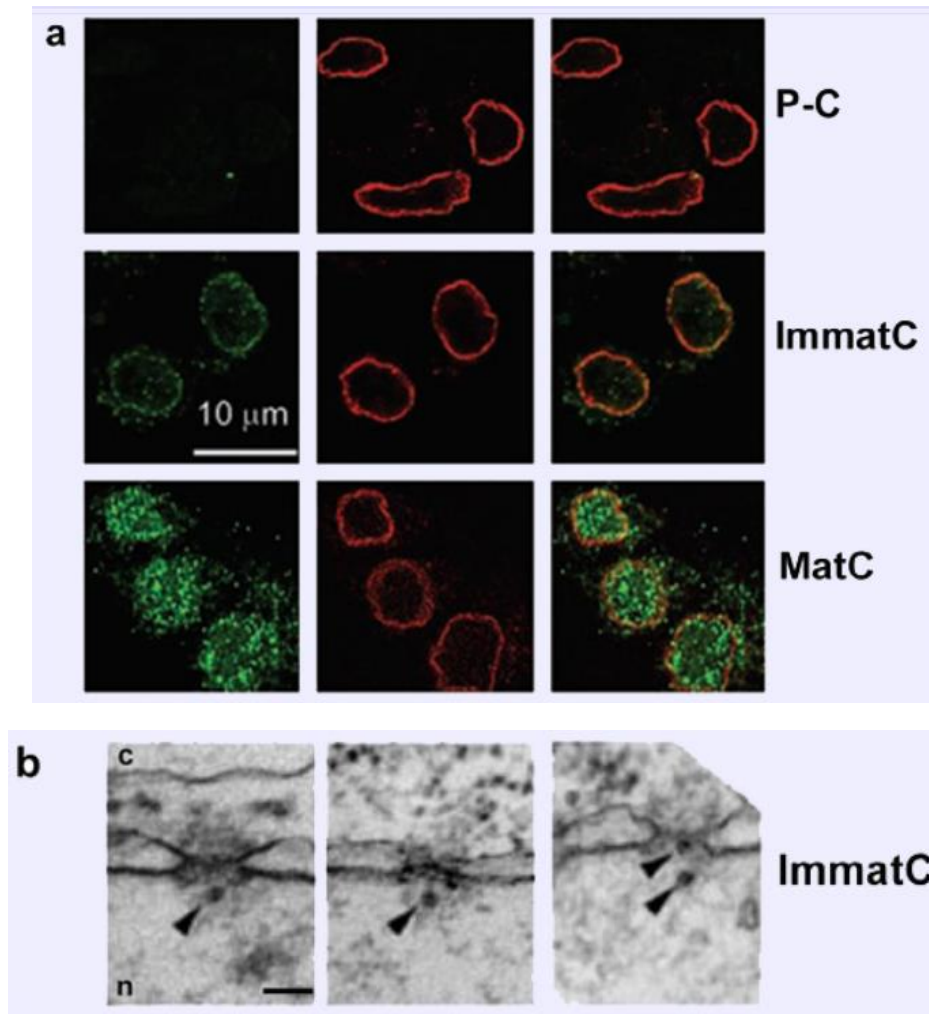


Figure 2.6: Maturation-dependent nuclear entry of HBV capsids: a) Localization of hepatitis B capsids in HuH-7 cells. Capsids (green, *Left*) and NPCs (red, *Center*) were stained by indirect immunofluorescence. Merged images are shown in the last column (*Right*). Recombinant capsids, devoid of viral polymerase (P-C) do not bind to cellular structures, immature capsids (ImmatC) show nuclear binding, whereas mature capsids (MatC) generate intranuclear capsids. Scale bar: 10 μ m; and b) Views of nuclear envelope cross sections with adjacent cytoplasm (c) and nucleoplasm (n) from a *Xenopus laevis* oocyte that has been microinjected with ImmatC. Arrowheads point to capsids associated with the nuclear face of the NPC. Scale bar: 100 nm (Adapted from Köser et al., 2012).

2.3. Conventional anti-HBV therapies and drugs

2.3.1. Cytokines and Nucleot(s)ide analogues

Immune responses and inflammation involving the restore of tissue, the presenting of antigens, actuating cells and differentiation of bone marrow are all functions that engage small-scale superfluous proteins released from the cells of the body and immune system and partake in the accountability for developmental regularization. These are known as cytokines. As mentioned earlier, the IFN- α cytokine has displayed supportive findings toward HBV abatement. IFNs suppress HBV viral copying. IFN- α provides for the restitution of T-helper lymphocyte responses as a result of its antiviral activities and immunoregulatory processes, thus it remains the mainstay cytokine presently utilized in the intervention of chronic HBV. Solely, it allows for anti-hepatitis B e antigen (HBeAg) seroconversion in 25 % of chronic HBV diseased cases (Tassopoulos et al., 1997; Larrubia et al., 2009). Researchers examined the impression that IFN left on patients with anti-HBeAg positive chronic HBV in terms of disease progression by pursuing reexamination after an average of 6 years (Brunetto et al., 2002). Conclusive results were a 2.5 fold decline in the progression of HBV. Perennial pursue of its effects were validated in other studies declaring that IFN treatment stimulates an enlarged survival rate, a diminished possible growth of HCC and consequently amelioration of liver histology. Future follow-ups showed a sustained clinical subsidence and HBsAg seroconversion (Janssen et al., 1999; van Zonneveld et al., 2004). A study pointed out that lambda-IFN has the potential to be useful for therapeutic purposes in the alleviation of chronic HBV or hepatitis C virus (HCV) (Robek et al., 2005). Lambda-IFN is similar to IFN- α and IFN- β in its antiviral behaviour to inhibit HBV when it conveys its effects by means of a distinguishable receptor composite in a modified murine heaptocyte cell line. It also lowered HCV replication in Huh7 cells. Data in a study assessing the antiviral impact of IFN- α_{2a} or IFN- α_{2b} with attached PEG demonstrated close to 30 % and 3-5 % HbeAg seroconversion and HbsAg seroconversion rates respectively, and HBsAg was seen in around 7 % of patients after a 6 month follow-up (Zoulim, 2007).

Nucleot(s)ide analogues can be perceived as prodrugs as their role as inhibitor of polymerase is generated by phosphorylation process of their nucleoside triphosphates or diphosphate (Zhou and Littler, 2006). Lamivudine (LMV) is a cytidine analogue that when compared to IFN- α , does not have a targeted immunoregulatory outcome but does show the response restoration of T-helper lymphocytes suited to HBV for the initial months of its therapy. The other nucleotide

analogues could also feature the same function (Ishikawa, 2012). LMV is a generally well-endured antiviral and its treatment conduces a fall in HBV DNA level by 3 to 5 log₁₀ copies/mL following 12 months of treatment equated to starting line measurements, an accompanied prompt removal of HBeAg and standardized/decreased levels of serum alanine aminotransferase (ALT). Protracted LMV therapy may result in a drop in the peril of developing liver cirrhosis and HCC (de Clercq et al., 2010). Patients in America harboring untreated chronic HBV exhibited well-disposed results on HBVs effects involving chemical processes in the body with reference to its histologic characteristics. HBeAg responses were generally kept up after LMV administration (Dienstag et al., 1999), however, it was shown in another examination that HBeAg responses were really not kept up after LMV administration surcease (Chan et al., 2007). LMV therapy is suggested in people who do not show any seroconversion. The vast problem of drug resistance is seen immensely with LMV and its resistance moderates a higher HB viral load (Zoulim, 2007).

One of the nucleotide analogues, Adefovir dipivoxil (ADV), proved its ability in decreasing the HBV DNA levels as well as showing an improved liver histology in serum. It is also an option for treatment of LMV-resistant patients with a case of chronic HBV (Hadziyannis and Papatheodoridis, 2004; Dai et al., 2007). Patients with chronic HBV were randomly assigned to receive specific dosages of ADV everyday for the duration of 48 weeks of a study (Marcellin et al., 2003). Results suggested a histologic liver progression, a receded amount of HBV DNA and ALT and a strengthened rate of HBeAg seroconversion. A study demonstrated that solid lipid nanoparticles (SLNs) are novel drug delivery systems for ADV for anti-HBV activity (Zhang et al., 2008). SLNs provide non-biototoxicity, drug targeting and sustained drug release as well as harbouring the incorporated compound in a protective fashion and it is also chemically degradable. A fluorescence marker (octadecylamine-fluorescein isothiocyanate) showed the uptake of SLN by HepG2.2.15 cells. Compared to the free ADV, a significant drop in levels of HBsAg, HBeAg and HBV DNA levels *in vitro* was observed. ADV was reviewed in its treatment of chronic HBV and propounded that it was also found to perpetuate its therapeutic efficiency after 3 years of its endured treatment (Hadziyannis and Papatheodoridis, 2004).

Entecavir (ETV) is a powerful inhibitor of HBV replication and was permitted in 2005, for its use against active HBV and liver disease including affirmation of a continuous rise in serum ALT. It is also in concord with an amicable side effect profile and a lesser chance of drug resistance progression (Zoulim, 2007; Basu and Brown Jr., 2012). The long-term productiveness of ETV monotherapy in patients with perceptible HBV DNA levels was explored (Zoutendijk et al., 2011). Conclusive findings suggested ETV could be extended after 48 weeks particularly

distinctly in patients with less of HBV DNA as it precipitates a virological reaction in most of these patients.

Tenofovir disoproxil fumarate (TDF) has potent antiviral activity against HBV but has been investigated more in patients who are HIV positive and have been co-infected with HBV. Many examinations have implied that TDF notably reduced HBV viral load and is probable that it is more persuasive in therapy compared to ADV (Zoulim, 2007). ETV and TDF were reviewed in the clinical setting where it was shown that ETV produced an average of 86 % virologic responses in patients until 4 years of treatment and TDF produced an average of 82 % virologic responses until 1 year 9 months of treatment. There were laudatory reports on tolerability, commendable safety profiles and lesser occurrences of drug resistance for both ETV and TDF (Pol and Lampertico, 2012). Researchers proffered that 5 years of treatment with TDF was effectively impervious and may lead to the reversion of liver cirrhosis through endured abolition of HBV (Marcellin et al., 2013).

The nucleot(s)ide telbivudine is known to be affiliated with the largest HBeAg seroconversion rates and the property of immunomodulation. This makes it reciprocal in character to PEGylated IFN (Wang et al., 2013b). Serum HBsAg levels, after 36 months of telbivudine treatment, were evaluated in 162 cases that were positive for HBeAg and who upheld inconspicuous HBV DNA serum levels in a persistent manner. The speedy fall in serum HBsAg levels in the first 12 months of telbivudine therapy pinpoints patients who are more inclined to acquiring the compete removal of HBsAg (Wursthorn et al., 2010). Table 2.1 summarizes the impression that cytokines and nucleot(s)ide analogues have on HBV.

Table 2.1: Summary of anti-HBV cytokine and nucleot(s)ide analogue therapeutic agents as a treatment plan

Therapeutic agent	Effectiveness as anti-HBV therapy	Reference
IFN- α^a /-PEG ^b /-lamda ^c	Suppressed HBV viral copying, restored T-helper lymphocyte responses/ immunoregulatory, allows for anti-HbeAg ^d seroconversion, prolonged decline in the progression of HBV, prolonged diminished growth of HCC ^e , sustained clinical subsidence and HBsAG ^f seroconversion, subdued HCV ^g replication	Tassopoulos et al., 1997; Janssen et al., 1999; Brunetto et al., 2002; van Zonneveld et al., 2004; Larrubia et al., 2009

Therapeutic agent	Effectiveness as anti-HBV therapy	Reference
LMV ^h	T-helper lymphocyte response restored, HBV DNA level decline, rapid HBeAg removal, decreased levels of serum ALT ⁱ , minimal risk of developing liver cirrhosis and HCC, improved liver histology, sustained HBeAg responses after treatment	Dienstag et al., 1999; Chan et al., 2007; Zoulim, 2007; de Clercq et al., 2010; Ishikawa, 2012
ADV ^j	Decreased HBV DNA levels, improved serum liver histology, treatment in LMV-resistance, decreased ALT, increased HBeAg seroconversion, decreased HBsAg and HBeAg levels, prolonged therapeutic efficacy	Marcellin et al., 2003; Hadziyannis and Papatheodoridis, 2004; Dai et al., 2007; Zhang et al., 2008
ETV ^k	Potent HBV inhibition, minimal drug resistance progression, effective long-term, precipitates virological responses, favorable side effect profile	Zoulim, 2007; Zoutendijk et al., 2011; Basu and Brown Jr., 2012
TDF ^l	Potent HBV inhibition, decreased HBV viral load, more persuasive therapy compared to ADV, sustained virologic responses, favorable safety profile, prolonged liver cirrhosis reversal	Zoulim, 2007; Pol and Lampertico, 2012; Marcellin et al., 2013
Telbivudine	Highest HBeAg seroconversion rate, immunomodulation, rapid decrease in HBsAg levels, possible complete removal of HBsAg	Wurstthorn et al., 2010; Wang et al., 2013b

^aInterferon- α ; ^bPegylated-Interferon; ^cLamda-Interferon; ^danti-Hepatitis B e antigen; ^eHepatocellular carcinoma; ^fHepatitis B surface antigen; ^gHepatitis C virus; ^hLamivudine; ⁱAlanine aminotransferase; ^jAdefovir dipivoxil; ^kEntecavir; ^lTenofovir disoproxil fumarate

2.3.2 Thiazolide anti-infectives

Thiazolides have recently displayed promise as antiviral therapeutic agents that may amplify existing or forthcoming treatments against hepatitis. Nitazoxanide (NTZ) is a thiazolide anti-infective that has activity against a range of viruses in cell culture models, anaerobic bacteria, protozoa and helminthes. NTZ antiviral exertion was first stumbled upon when patients with AIDS (co-infected with HBV or HCV) were being treated for cryptosporidial diarrhea as it was the first thiazolide that was primarily transpired for the treatment of the protozoan *Cryptosporidium parvum*. In studies of patients with chronic HBV, NTZ administration evoked the seroconversion of HBeAg and HBsAg with the overall abolishment of HBV DNA levels. In other studies of patients with chronic HCV, the administration of NTZ together with PEGylated IFN- α_{2a} , with or without the addition of ribavirin, manifested efficiency and acceptable safety of the thiazolide (Korba et al., 2008a; Rossignol and Keeffe, 2008; Keeffe and Rossignol, 2009). Together with the metabolite of NTZ, Tizoxanide, and other thiazolides, reports have indicated the effective inhibition of both HBV and HCV replication in standard antiviral assays conducted. NTZ showed activity against both genotypes 1a and 1b HCV, the frequent LMV and ADV-resistant HBV mutants as well as certain HCV mutants. It induced a reduction in the Hep2.2.15 cell producing HBV proteins; however it did not have an effect on HBV RNA transcription (Korba et al., 2008b). Researchers performed a QSAR examination on a variety of thiazolides and they assessed its conclusion on HBV replication and activity (Stachulski et al., 2011). Briefly, the broad-spectrum NTZ presented efficacy against viruses amongst other things, the novel 2-hydroxybenzoyl-N-(5-chlorothiazol-2-yl) amide displayed robust and selective HBV replication hindrance and in comparison to some analogous salicyloylanilides, favorable activity against HBV was shown with numerous 4'-and 5'-substituted thiazolides. It has been delineated that the action of removal with substitution of the nitro group found on NTZ with a group that is not reducible will allow for novel thiazolides to be formed that sustain a wide-spectrum of viral activity only, which would play a valuable role in HBV abatement (Rossignol, 2009).

2.3.3 Small interfering ribonucleic acids

Small interfering ribonucleic acids (siRNAs) with an efficient delivery system can overcome barriers, and cause the inhibition of gene expression of specific proteins. The mechanism by which this is achieved is known as RNA interference (RNAi). It is a natural procedure that protects the genome by targeting a particular messenger RNA (mRNA) for degradation and in

the event inhibiting protein synthesis and therefore, viral gene transcription, expression and ultimately replication. There are several notable studies that have been conducted concerning siRNA. Merely to achieve siRNA hepatocyte targeting, in one such study, a vehicle: siRNA Dynamic PolyConjugates was developed (Rozema et al., 2007). This technology involves a membrane-active polymer that confers its activity only until it reaches the endosome acidic environment and acquires the ability to drop off its siRNA cargo precisely to hepatocytes after a simple intravenous injection. Novel hepatotropic nontoxic lipid-based vector systems were generated to deliver chemically unmodified siRNAs to the liver (Carmona et al., 2008). Triggered PEGylated siRNA-nanoparticles were formulated. Due to the PEG coupling, the resulting pH sensitive oxime linkage causes the release of nucleic acids from the endosomes. Suppression of markers of HBV replication on account of triggered PEGylated siRNA-nanoparticles go up to 3-fold relative to controls. Results from another study show that siRNA delivery follows a surface charge and size dependent manner (Wang et al., 2010). In another study, the aim was to optimally design siRNAs targeting the aberrant HBV and this was authenticated via quantitative structure-activity relationship (QSAR) analysis methods (Fu et al., 2008). Cocktails of siRNAs could also be problem solving as combinations of siRNAs would cleave multiple sights on the mRNA target, making it difficult for restoration thereof (Chen et al., 2008). Researchers demonstrated a study that showed the effects of an HBV specific 21-bp siRNA that is directed to the HBsAg region, a site where three prominent viral mRNAs project over one another, has on HBV replication in a cell culture system and a mouse model (Giladi et al., 2003). Results point out the marked inhibition of viral antigens, their transcripts and DNA and thus HBV replication as a whole.

A study demonstrated an evaluation of synthetic siRNA combinations targeting various sites of HBV transcripts on its replication and antigen expression *in vitro* (Chen et al., 2005). Results showed that the siRNAs targeting the polymerase and pre-core region specifically inhibited virus replication and antigen expression in a dose-dependent manner. This was done efficiently compared to the use of single siRNAs at the same final concentration. In addition, no apoptotic change was observed in the cells after the combination siRNA treatment. A specialized delivery system known as a SNALP (stable nucleic-acid-lipid particle) contained chemically modified siRNA in its liposome form (Morrissey et al., 2002). These were administered intravenously into HBV replicating mice. HBV DNA reductions specifically lasted for days and weeks with this particular dosing. 3 mg/kg/day intravenous injections taken 3 times a day reduced serum HBV DNA $>1.0 \log_{10}$. A mouse model carrying replicating HBV was injected intravenously with

synthetic siRNA/apo A-I/1,2-dioleoyl-3-trimethylammonium-propane complexes (Kim et al., 2007). The nanoparticles displayed liver specificity with low doses of less than or equal to 2mg/kg still rendering effectiveness in only a single treatment with persistence of the therapeutic effect for 8 days. Observably, administration of these nanoparticles significantly diminished viral protein expression by receptor-mediated endocytosis. Researchers in another study coinjected a compounded N-acetylgalactosamine-melittin-like peptide with a siRNA compounded to cholesterol that is directed to coagulation factor 7 and validated the oppression of HBV's RNA, DNA and proteins with a lengthy effective continuity (Wooddell et al., 2013). Data proposed this system of RNAi-based therapeutics as procuring strong potential for chronic HBV infections. Another recent study added to the illustration of RNAi allaying HBV by proclaiming viral clearance of HBV from the liver of transgenic mice by recombinant adenoviruses expressing HBV-directed short hairpin RNAs (Uprichard et al., 2005).

2.3.4 Heteroarylpyrimidines

Heteroarylpyrimidines (HAPs) were identified as powerful inhibitors of the HBV capsid maturation step. The interaction site of the core protein-HAP and the exact point in the replication cycle where the HAP allows its principal function is unknown however the mode of action is the attachment to and degradation of the core protein. Cellbased HBV replication assays portrayed a greater potency of HAPs than LMV in the therapeutic treatment of HBV (Cuestas et al., 2010). Researchers tested the effect that an HAP [methyl 4-(2-chloro-4-fluorophenyl)-6-methyl-2-(pyridin-2-yl)-1,4-dihydropyrimidine-5-carboxylate] had on HBV capsid protein congregation (Stray et al., 2005). Results depict the HAP commanding the detachment of HBV capsids and consequently being capable of relaying diversified effects emanating from the incongruous assembling of HBV's capsid proteins. Therefore, actuating and decontrolling the assemblage of a virus could lead to prevailing antiviral-based treatments. A range of HAPs built on a crystallized arrangement of a capsid-HAP conjugate was formulated to attempt to better understand the HBV capsid assembly and replication in HepG2.2.15 cells *in vitro* (Bourne et al., 2008). The kinetics of assembly *in vitro* corresponded sufficiently with the prohibition of HBV in the cell culture. Results also alluded to contention between suitable and unsuitable assembly because of the interrelation of assembly kinetics and virus restriction. BAY 41-4109 is an HAP that was evaluated for its antiviral activity in transgenic rodents carrying HBV at variance of dosages (3-30 mg/kg, b.i.d/ t.i.d for 28 days). In relation to LMV, BAY 41-4109 was just as competent in lowering HBV DNA in a dose-dependent fashion. Results also suggested

lowered HBcAg in excised hepatic sections in disparity with LMV treated rodents indicating BAY 41-4109 bears no resemblance in the mechanism of action of LMV as an antiviral. BAY 41-4109 pharmacokinetics revealed swift absorption and a 30% bioavailability (Pan et al., 2008). BAY 41-4109 was also studied for its causative effect on the intracellular EGFP-Core fusion proteins into HepG₂ cells. It is conclusive that BAY 41-4109 is a powerful inhibitor of HBV having myriad effects on the arrangement of HBV's capsid to achieve this antiviral result (Haryanto et al., 2007). Pyrazole and pyrimidine derivatives were also formulated to assess their antiviral exertion on HBV. Facts presented with controlled to high inhibitory activities (El-Sayed et al., 2009). Table 2.2 reflects a compendary of thiazolides, siRNAs and HAPs efficiency as HBV eradicators.

Table 2.2: Summary of anti-HBV thiazolide, siRNA and HAP therapeutic agents as a treatment plan

Therapeutic agent	Effectiveness as anti-HBV therapy	Reference
Thiazolides	Potent and selective HBV replication inhibition, HBeAg and HBsAg seroconversion, decreased HBV DNA levels, treatment in LMV and ADV-resistance, decreased Hep2.2.15 cell HBV proteins, no HBV RNA transcription effect	Rossignol and Keeffe, 2008; Korba et al., 2008a; Korba et al., 2008b; Keeffe and Rossignol, 2009; Rossignol, 2009; Stachulski, 2011
siRNAs ^a	Gene expression inhibited, viral antigens inhibition, HBV transcripts inhibition, decreased serum HBV DNA and RNA ^b levels, suppressed HBV replication markers, mRNA ^c cleavage, polymerase and pre-core region targeting, effective continuance of treatment	Carmona et al., 2008; Chen et al., 2008; Uprichard et al., 2005; Rozema et al., 2007
HAPs ^d	Potent HBV capsid maturing inhibition, HBV core protein degradation, increased potency than LMV, potent antigens inhibition effect, misassembly of HBV's capsid proteins, dose-dependent decrease in HBV DNA levels, decreased HBcAg ^e	Stray et al., 2005; Bourne et al., 2008; Pan et al., 2008; Haryanto et al., 2007; El-Sayed et al., 2009; Cuestas et al., 2010

^aSmall interfering ribonucleic acids; ^bRibonucleic acid; ^cMessenger RNA; ^dHeteroarylpyrimidines;

^eHepatitis B core antigen

2.4. Hepato targeting: A delve into attempts at earlier performed research studies

Hepato or liver targeting does not necessarily reflect hepatocyte targeting as other cells in the liver such as the Kupffer cells, possess the ability to phagocytose. Therefore, carriers need to be modified chemically to achieve the ability of encompassing liver cell type specificity (Cuestas et al., 2010).

The ASGP-R has been amongst the most distinguished and investigated of all receptors and was first corroborated by Ashwell and Morell (1974). The ASGP-R has provided for a location to accommodate cell-cell intercommunication that is membrane bound. It allows for targeting specificity of various therapeutic agents and has been the locus of incrimination for HBV uptake (Stockert, 1995). Figure 2.7 illustrates the ASGP-R mediated endocytosis (Lai et al., 2010). Several attempts have been made to label carriers (polymers, human serum albumin, and recombinant HDLs) with ASGP-R specific ligands (galactose, lactose, acetylgalactosamine, asialofetuin) to design specific carriers for drug and gene delivery to hepatocytes.

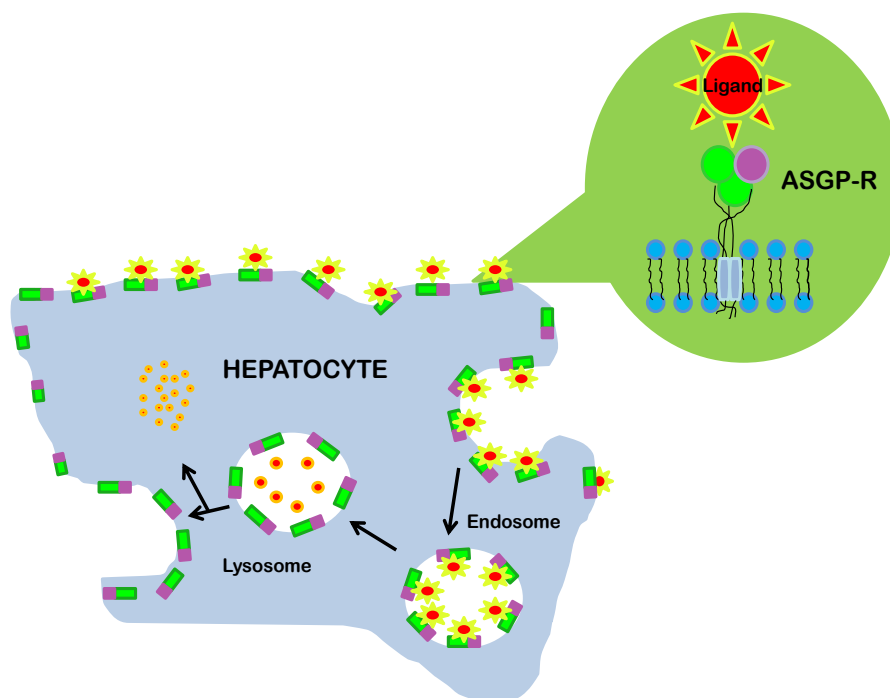


Figure 2.7: A schematic depicting the receptor-mediated endocytosis by the ASGP-R on hepatocytes (Modified from Lai et al., 2010).

Organ-specific targeted delivery has been achieved using linear aminopolysaccharide, chitosan, its derivatives and the likes. Studies have demonstrated liver targeting ability. Systems that target the liver employ passive trapping of the drug loaded delivery device by the RES or the active targeting established through recognition between hepatic receptor and ligand-bearing drug loaded delivery devices. In one such study, lactosaminated N-succinyl-chitosan was synthesized and its potential assessed as a liver specific drug carrier (Kato et al., 2001). It was found that this carrier accumulated in the liver and distributed throughout the liver as a result of interaction with the ASGP-R.

Lactose conjugated to polyion complex micelles that consisted of PEG grafted to chitosan also indicated promising potential as liver specific nanocarriers to deliver the drug, diammonium glycyrrhizinate (Yang et al., 2009). Authors of one study modified the surface of chitosan nanoparticles by conjugating it with glycyrrhizin. The conjugation occurred through a process of oxidation of the glycyrrhizin with sodium periodate (Lin et al., 2008). *In vitro* studies showed localization of the glycyrrhizin conjugated chitosan nanoparticles in hepatocytes and the intracellular uptake amounted to 4.9 times than that in hepatic nonparenchymal cells. The nanoparticle dose as well as the incubation time were significant markers to the cellular uptake process. Liver targeting was achieved by a specific interaction between the glycyrrhizin and the hepatocytes, a ligand-receptor interaction.

High density lipoproteins (HDL) present another assuring hepatic targeting carrier. They take up phospholipids and cholesterol from the body's peripheral tissues and delivers them to the liver hepatocytes via apolipoprotein (apo) A-I, the major lipoprotein component in HDL. Researchers of one study designed divalent and trivalent cluster glycosides attached to the antiviral nucleoside 9-(2-phosphonylmethoxyethyl) adenine (PMEA). This conjugation promoted ASGP-R affinity (Biessen et al., 2000). Data from this work indicated more than 90 % of the therapeutic agent to be taken up by liver parenchyma cells thereby reducing its localization in other tissues significantly. Dextran conjugation is also seen favourable light. Synergistically, modification of dextran with galactose and mannose results in its selectivity for hepatocytes and Kupffer cells respectively (Cuestas et al., 2010). Liver-specific treatment and HCC targeting of doxorubicin is possible when the polymers used are functionalized with galactosamine (Seymour et al., 2002). Figure 2.8. depicts and follows the movement of a therapeutic drug loaded to liver hepatocyte.

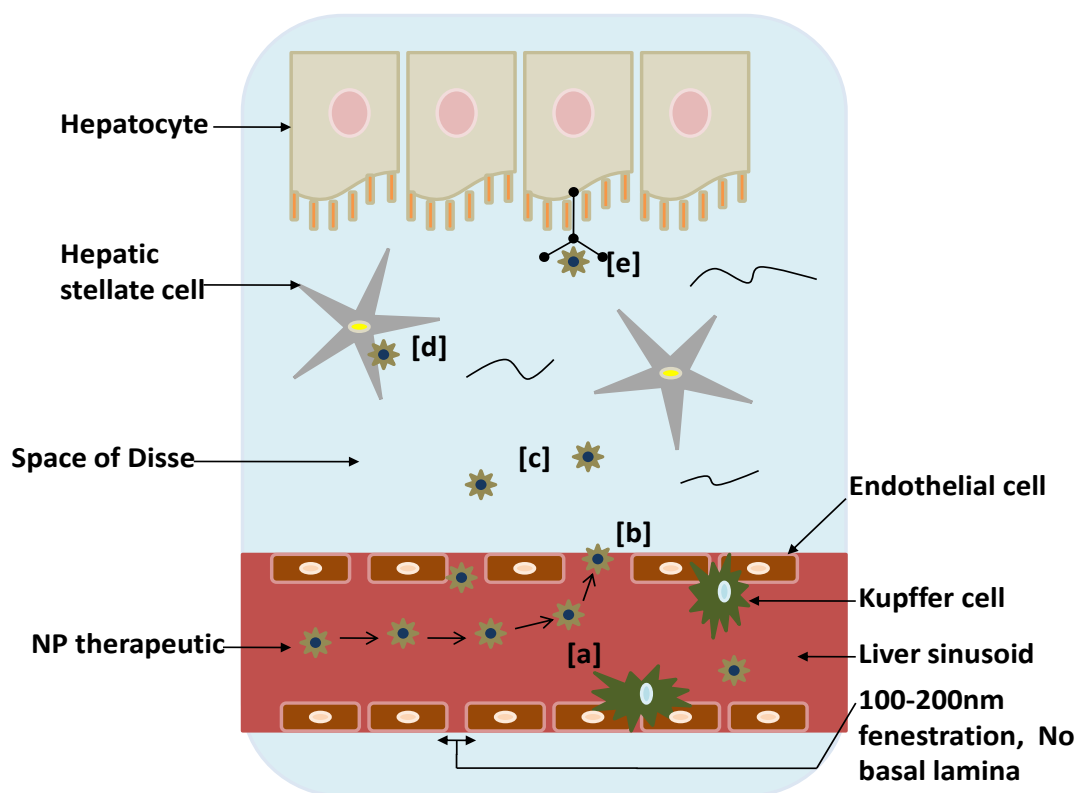


Figure 2.8: Liver targeting by nanoparticle (NP) therapeutics: a) Nanosized particles of less than 200nm with specific functionalities aid in the evasion of premature Kupffer cell clearance; b) Nanosized particles extravasate into the space of Disse through sinusoidal fenestrations in basal lamina absence; c) A high local concentration of NP therapeutics diffusing across the loosely organized extracellular matrix in the space of Disse; d) Non-specific endocytic uptake; and e) Receptor-mediated uptake by the hepatocyte (Modified from Li et al., 2010).

Various drug carriers often fail in delivering drugs to extravascular sites. A strategic plan would be to use low molecular weight prodrugs that have the ability to distribute themselves throughout the body but cleave intracellularly to the active form of the drug by an organ-specific enzyme. HepDirect prodrugs are a series of phosphate and phosphonate prodrugs that pursue a cytochrome P450-catalyzed oxidative cleavage reaction inside the hepatocytes. This certainly classifies a liver-targeting drug delivery approach. These prodrugs are cyclic 1, 3-propanyl esters that contain a ring substrate that presents sensitivity to oxidative cleavage by the cytochrome P450 with specificity to CYP3A4 (Erion et al., 2004).

Studies were also performed to test the potential of bile acids to deliver drugs distinctively to the liver since these bile acids are transported across the plasma membrane in the portal domain (Kramer et al., 1992). The liver-specific drug chlorambucil was used and covalently linked to 7 alpha, 12 alpha, -dihydroxy-3 beta-(omega-aminoalkoxy)-5-beta-cholan-24-oic acid to form chlorambucil-bile acid conjugates and studies revealed success of bile acid molecules to behave as carriers for drug molecules and their specificity for the liver. Researchers designed a nanoparticle-based model delivery system that simulated nonviral gene delivery particles with respect to their surface properties in an attempt to identify design constraints to aid next generation gene delivery to the liver (Popielarski et al., 2005a; Popielarski et al., 2005b). Four nanoparticles were formulated from polystyrene beads viz. Gal-50 and Gal-140 which are galactosylated; and MeO-50 and MeO-140 which are methoxy terminated. The 50 and 140 denotes the mean diameter of the nanoparticles in nanometers. Galactose is incorporated as a targeting ligand and provision of serum stability is attained with PEGylation of the nanoparticles. Results show that a slight anionic, galactose- PEGylated nanoparticle should size up to around 50 nm and 140 nm in diameter in order to selectively target hepatocytes and Kupffer cells, respectively. Overall, these studies and findings support the concept that targeted delivery of antivirals to the liver increases the productiveness of these therapeutic agents in the treatment of viral liver infections and reducing their toxicity profiles in other bodily tissues and organs as a result of systemic exposure (Cuestas et al., 2010).

2.5. Novel and recent advances in drug delivery developments for anti-HBV therapeutics

2.5.1. Woodchuck Apolipoprotein A-1

A significant protein component of high density lipoprotein is apolipoprotein A-1 and functions to passage cholesterol from the body's tissues to the liver where it is then excreted. Authors therefore studied woodchucks infected with the woodchuck hepatitis virus as a model to investigate chronic HBV and HCC in patients (Fioravanti et al., 2011). They proposed that the therapeutic effectiveness of the woodchuck animal model may be interpreted into a successful therapeutic plan for patients that are HBV diseased. Their study recounts the characterizing and cloning crafts of woodchuck Apo-A1 and the means of augmentation that allow it to be applied as a novel drug delivery strategy for HBV.

2.5.2. Bionanocapsules

Bionanocapsules were linked with a lipoplex that contained HBsAg L protein entrenched as a transmembrane protein and was formulated and further complexed with PEI and DNA to produce stable complexes that was an efficient gene carrier for targeting to the human liver. The particles were delivered to the cells in the same manner of HBV infection in the primal phases (Somiya et al., 2012; Yamada et al., 2012).

2.5.3. Bilosomes

Researchers improving on an earlier study of bile salt stabilized vesicles (bilosomes) development have complexed the bilosomal system with cholera toxin B subunit. They loaded the system with HBsAg to elevate M-cell specificity and its transmucosal uptake. Conclusive results displayed significant anti-HBsAg IgG antibody titre response for this specific bilosomal system propounding assuring potential for target specific oral immunization against HBV (Shukla et al., 2008; Shukla et al., 2010).

2.5.4. Recombinant Viral Vectors

A novel vector established on an HBV mutant that has great ability to replicate and holds a 207-bp deletion in the preS1/polymerase spacer region was constructed by researchers. They applied a new insertion approach that maintains the progression of the polymerase open reading frame and as a result recombinant HBV carrying protein/siRNA genes were attained and showed replication. They were confined with efficiency in hepatic cells. Hereon followed favourable wild type HBV (WT-HBV) antigen expression inhibition (Hong et al., 2013). Another study utilized a recombinant adenovirus vector for recombinant HBV delivery. The vector granted adept transduction of HepG2 cells generating WT-HBV in addition to transferred recombinant HBV yielding dominant viral replication. Viral clearance and successive HBV seroconversion was obtained due to the foreign-antigen-specific T-cell response that was prompted by the recombinant adenovirus/recombinant HBV immunotherapy. The study represented a profitable plan of action to do away with immune tolerance and thus ridding chronic HBV (Wang et al., 2014).

2.5.5. Polymeric Hydrogels

Authors developed a novel scheme to lessen the amount of HBV vaccine doses by allowing it to be released from a bodily depot that would respond to oral stimuli. This was carried out by merging the HBV vaccine into a biohybrid hydrogel depot with a PEG foundation. In mice, the depot was receptive to the oral intake of novobiocin, the stimulus molecule. This led to proper immunization in the mice (Gübeli et al., 2013). A diblock copolymeric hydrogel constituting (monomethoxy poly(ethylene glycol)-co-poly(lactic-co-glycolic acid)) was also developed by authors. They used this to deliver both HBsAg and GM-CSF, an immune modulator and a haematopoietic growth factor. Their results displayed a valid and easy technique for actuating anti-hepatitis B surface antibodies in nonresponsive patients (Chou et al., 2010).

2.5.6. Polymeric Microparticles

PLGA and Dextran polymers were melded using a solvent evaporating method to formulate microspheres. Recombinant HBV vaccine was added into the copolymeric complex. *In vivo* investigations concluded PLGA/Dextran microspheres to be able to encourage an acceptable immune response (Moni et al., 2011). Another study that attempted to do away with repetitive injectable doses, designed once off HBsAg adsorbed microspheres composing poly(D,L)-lactide-co-glycolide (PLGA) (L/G 50:50 and 75:25). It was developed to evaluate its ability for provoking the cell mediated immune response against HBsAg. Data exhibited the success of the PLGA microspheres as a vaccine in causing effective immune responses against HBV (Saini et al., 2011).

2.5.7. Nanoparticles

2.5.7.1. Inorganic Nanoparticles

A study concerned with imaging in liver cancer placed emphasis on a proteoglycan known as Glypican-3 (GPC-3) which is involved with enabling cell growth that is found to be overexpressed in HCC. Superparamagnetic iron oxide novel multifunctional nanoparticles were developed. Particles were conjugated to streptavidin and Alexa Fluor 647. It was found with confocal fluorescence microscopy (CFM) that biotin-conjugated GPC-3 monoclonal antibody was confined only to the cellular surface of HepG2 cells expressing GPC-3. The GPC-3 nanoparticle system proved that it can be utilized in imaging for HCC visualization as well as

capability to be used as a vehicle for delivery of therapeutics targeting tumors (Park et al., 2011). Researchers also used magnetic nanoparticles' surface in another study to prepare multifunctional HCC targeting agents by blending bis-*N*-hydroxysuccinimide ester and OSu-activated fluorescent dye Cy3. A mono-antennary and tri-antennary galactosyl ligands were each fixed onto the fluorescent magnetic nanoparticles and their uptake into HepG2 and HeLa cells were evaluated by CFM. Results show that this system is a good ligand transporter and its multivalent ligand assembly improves on the cell interaction with HepG2 receptors. The galactosyl Cy3 magnetic nanoparticles were also found to be non-cytotoxic (Lai et al., 2010). SiO₂ nanoparticles were also used for attachment to HBV-like particles in order to transit immune response-regulating agents for targeted treatment (Dekhtyar et al., 2012).

2.5.7.2. Polymeric Nanoparticles

Poly(vinylbenzyl-O-β-D-galactopyranosyl-D-gluconamide) (PVLA) is known to be site specifically taken up into hepatocytes via ASGPR. Therefore, researchers synthesized a copolymer, poly(N-p-vinylbenzyl-[O-β-D-galactopyranosyl-(1→4)-D-gluconamide]-co-N-p-vinylbenzyl-6-[2-(4-dimethylamino)benzaldehydehydrazono]nicotinate) (P(VLA-co-VNI)), and this was tagged with (99m)Tc for liver imaging. Results revealed promising potential for more of its application in the evaluation of liver cell function (Yang et al., 2011). Authors achieved site specificity to hepatocytes by synthesizing a galactosylated PEG-graft-PEI derivative via the modification of a biscarbamate cross-linked PEI by-product with PEG and lactobionic acid. The complex held a galactose moiety for hepatocyte targeting. It could also efficiently incorporate plasmid DNA into nanoparticles. Data displayed its successful targeting to the hepatocytes (Wang et al., 2013c). In a different study, nanoprecipitation and solvent evaporation techniques were used to prepare cationic poly(lactide) (PLA)-based nanoparticles together with PEI and chitosan as surface coating components. mPEG-PLA-PEI nanoparticles showed best ability to impede HBV surface antigen and thus allowing for transfection of siRNA (Wang et al., 2010). Researchers used a double-emulsification technique to prepare HBsAg passively adsorbed onto the surface of cationic PLGA nanoparticles to deliver IFN-α to site-specific hepatocytes. HbsAg coated (99m)Tc-labeled PLGA nanoparticles results presented notable liver recovery in contrast to normal PLGA nanoparticles (Giri et al., 2011). The particle size and hydrophobicity effects of porous PLA and PLGA nanoparticles were assessed on cell mediated and mucosal immune responses. The nanoparticles accommodated a set amount of HBsAg and administration occurred via pulmonary delivery. Data revealed that hydrophobic particles that were larger in size, greater than 500 nm, derived a potent increase in secretory IgA, IFN-γ and interleukin-2

levels as opposed to hydrophilic particles that were smaller than 500 nms. The larger sized hydrophobic particles also showed that they could be more easily taken up into rat alveolar macrophages. The study demonstrated the competence of the nanoparticles to induce augmented immune responses (Thomas et al., 2011). Authors also developed polymeric nanoparticles formulated for HBV gene silencing making use of the common biodegradable polymer PLGA again, but in this study, the cationic polymer chitosan is embodied into its matrix. The idea is to advance plasmid DNAs loading efficiency and cellular internalization. Conclusive data revealed better HBV silencing with the chitosan-PLGA system compared to plain plasmid DNA alone or simple PLGA nanoparticles. A successful plasmid DNA carrier system was formulated (Zeng et al., 2011). Micelles were developed in one study for incorporating Lamivudines (LMVs) prodrug, LMV stearate. LMV stearate was synthesized to increase LMVs lipophilicity being a very hydrophilic drug. Stearic acid-graft-chitosan oligosaccharide micelles were prepared and loaded with LMV stearate. Results showed that the micelles had constrained effects on HBVs antigen expression and DNA replication and this was more conspicuous when comparing with LMV or its prodrug alone (Li et al., 2010). Acyclovir was also complexed to chitosan-*g*-stearate through use of a succinate linker for anti HBV activity. The acyclovir-chitosan-*g*-stearate was able to self assemble in aqueous solution constructing micelles. According to data there was a significant escalation in its inhibitory effect of HbsAg compared to plain acyclovir alone. An all round success of cellular internalization and anti HBV effects was observed with acyclovirs complexation to chitosan-*g*-stearate (Huang et al., 2011). Authors also revealed an assuring hepatic targeted siRNA delivery system for gene expression silencing formulated from N-acetylgalactosamine functionalized mixed micellar nanoparticles. The N-acetylgalactosamine micellar nanoparticles were self aggregated in aqueous solution from N-acetylgalactosamine functionalized PEG-*b*-poly(ϵ -caprolactone) and cationic poly(ϵ -caprolactone)-*b*-poly(2-aminoethyl ethylene phosphate) (PCL-*b*-PPEEA). The targeting effect to the hepatocytes was displayed by noteworthy enhanced fluorescent siRNA found in primary hepatocytes implying positive anti HBV therapy in liver disease (Wang et al., 2013d). A drug carrier was synthesized from glycyrrhetic acid-modified sulfated chitosan with glycyrrhetic acid being the targeting ligand to HepG2 cells. The micelles formed from this complex revealed swift and eminent ability for *in vivo* liver targeting. Moreover, the formed micelles showed specificity for liver cancer cells in contrast to normal liver cells (Tian et al., 2012).

2.5.8. Lipids

2.5.8.1. Ionizable Lipid Nanoparticles

Researchers of a study found that apolipoprotein E, amongst its other roles, also had the ability to pose as an endogenous targeting ligand for ionizable lipid nanoparticles excluding cationic lipid nanoparticles. And the second means of targeting via an exogenous ligand was formed from an N-acetylgalactosamine cluster. Both target specific systems seemed profoundly adequate in carrying ionizable lipid nanoparticles to the hepatic environment (Akinc et al., 2010).

2.5.8.2. Cationic Lipids

Novel cationic lipids were synthesized from *N*-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) that is conjugated to a dialkylglycylamide moiety to form *N,N'*-dioctadecyl-*N*-4,8-diaza-10-aminodecanoylglycine amide (DODAG). This was used to form lipoplex nanoparticles containing siRNA and these efficiently led to the *in vivo* delivery of siRNAs to the liver of transgenic mice mediating the suppression of HBV replication that showed a greater comparison to LMV with minimal observable liver toxicity (Mével et al., 2010). In other studies, a cationic lipid-DNA-complex (CLDC) was assessed for its capacity to aid HBsAg in extracting immune responses and to cause a decline in HBV DNA levels in transgenic mice. It was triumphant in accomplishing both of these functions (Morrey et al., 2011). Researchers evaluated the use of a synthetic HBV preS-derived lipopeptide, HBVpreS/2-48(my) (HBVP), known to be equipped with a coercive ability for liver tropism, in site specificity for liver cell delivery. The lipopeptide was conjugated to PEGylated liposomes (HBVP-Lip). Data pointed out the capability of HBVP-Lip to deliver cargo to hepatocytes with definitive target specificity both *in vitro* and *in vivo* (Zhang et al., 2014).

2.5.8.3. Liposomes

Cationic liposomes have been considered as novel adjuvant systems because they themselves are not adequate enough as immunostimulating agents. Therefore ligands that do acquire this function are coalesced with the liposomes acknowledging them as adjuvants to the functional division of the complex and thus bringing about potential application for enhanced HBV vaccine delivery (Christensen et al., 2011). In one study, asialofetuin was affixed to cationic liposomes for hepatocyte selectivity and conjoined to it were diverse cyclodextrins and plasmid DNA for gene transfer. From the cyclodextrins, γ -cyclodextrin complexed with the DNA-asialofetuin liposome showed the most dominant transfection efficiency with zero cytotoxicity, highest

entrapment ratio of the DNA and the ability to stabilize the membrane of the asialofetuin-liposome. γ -cyclodextrin was noted as an amplifier of gene transfer efficiency in liposomes appended with asialofetuin (Motoyama et al., 2011).

2.5.8.4. Lipoplexes

Authors evaluated the practicality of hindering HBV replication *in vivo* utilizing the recently reported altitrol-containing class of synthetic siRNAs. They were executed as lipoplexes and assessed *in vivo* using an HBV transgenic mouse model. Observations and findings revealed success in the silencing of HBV replication with no toxicity. Results correlated with conceding future application of altitrol-containing siRNA therapeutic lipoplexes (Hean et al., 2010).

2.5.8.5. High Density Lipoprotein

A study illustrating HDL's capability of transporting microRNAs (miRNA) was performed. miRNAs being a novel group of ailing biomarkers, warranted an efficient targeting potential, therefore its export to HDL was achieved by sphingomyelinase. Additional findings revealed that the delivery by means of HDL was reliant on scavenger receptor class B type I. Healthy patients and patients with high cholesterol levels exhibited dissimilar HDL-miRNA effects and this inspection specified HDL's means of cell-cell intercommunication allowing for miRNA transfer (Vickers et al., 2011). In other studies, contrast agents for MR imaging in the liver were developed using reconstituted HDL enclosing gadolinium-labeled-cholesterol as nanoparticles. This would be advantageous to determine anatomical variations in the liver. The contrast agent nanoparticles bound to HDL receptors on the HepG2 cells consenting uptake. Researchers also investigated whether the popular drug doxorubicin hydrochloride could be efficiently encompassed into reconstituted HDL for liver targeting. A doxorubicin-HDL compound was formed and assessments showed that the compound was successfully taken up into liver cells laden with the HDL-specific scavenger receptor class B type 1, had heightened and effective cytotoxicity results against many cell lines and has the ability to lessen cancer progression to a greater degree than the drug in liposomes. In this case the reconstituted HDL was fecund yet again in drug deliverance targeting the diseased liver [Rui et al., 2012; Yuan et al., 2013).

2.5.8.6. Solid Lipid Nanoparticles

Cationic and mannosylated solid lipid nanoparticles (SLNs) were prepared to show potential as a vehicle for HBV vaccine delivery via the subcutaneous route. The mannosylated SLNs displayed superior cellular internalization, a reduced amount of cytotoxicity as well as causing a

larger TH1 immune response type (Mishra et al., 2010). Cationic SLNs were also reconstructed from native low density lipoproteins and were formed to have application as specific target systemic delivery of connective tissue growth factor siRNA (siCTGF). The system was developed for the liver fibrosis treatment in HBV. Fluorescence imaging and single-photon emission computed tomography (SPECT) allowed for biodistribution studies which rooted the specific targeting, delivery and build-up of cationic solid lipid nanoparticles/siCTGF nanocomplexes in the liver (Kong et al., 2013).

2.5.9. Cell Penetrating Peptides

Mentioned earlier, a study took advantage of the complimentary execution of cytoplasmic transduction peptide (CTP) in delivering its payloads to hepatocytes. This was carried out by researchers purifying an anti-HBV core single-chain variable fragment welded to CTP and followed by assessment of its potential in HBV inhibition. It markedly reduced HBV DNA levels (Xun et al., 2013). Authors developed a series of artificial recombinant peptides along with cell penetrating sequence R7 and various nucleocapsid binding subunits. Conclusive data showed that the synthetic recombinant CPPs holding nucleocapsid binding subunits can enter into cells readily, cause the obstruction of nucleocapsid assembly and arrest HBV release (Pan et al., 2011). A most recent novel drug delivery platform with its groundwork on a CPP motif called X-Pep, derived from the extreme N-terminal region of the X-protein of HBV, is being said to retain application in having drugs delivered straight to cells specifically (Montrose et al., 2014).

Researchers in another study also co-injected an N-acetylgalactosamine-melittin-like peptide with a siRNA compounded to cholesterol that is directed to coagulation factor 7 and validated the oppression of HBV's RNA, DNA and proteins with a lengthy effective continuity. Data proposed this system of RNAi-based therapeutics as procuring strong potential as a novel therapeutic for chronic HBV infections (Wooddell., 2013).

2.6. Concluding Remarks

Much needs to be done in the development of novel liver-specific drug delivery strategies to do away with the high morbidity and mortality that is related to diseases caused by the HBV infection. There should be a demand for carriers that reach the height of optimization with intracellular targeting, protection of its contents and efficient delivery thereof. Adequate information has been sought out with respect to receptors that dwell on the specific liver cell

types, therefore, with this knowledge and more, receptor-specific ligands can be embodied in the design of nanocarrier systems to achieve heightened efficiency of therapy. A desired characteristic of nanocarriers and an added advantage to anti-HBV therapy is the capacity to incorporate agents together with the required drug for liver imaging. The discovery of novel drug candidates together with relevant carriers should also prove to be promising in HBV eradication, or at least significant reduction. However, the objective of these drug candidates for treatments of a diseased liver owing to HBV is to attain clinical recovery and reparation in the least amount of time achievable to be devoid of the virus generating a resistant strain. It should be expected for future anti-HBV therapies to embrace a medley of agents such as the nucleot(s)ide analogs together with immunostimulants and curative vaccines. Once these ideal goals have been reached and achieved, quality of life will be greatly improved for patients suffering from this debilitating disease.

CHAPTER 3

SYNTHESIS AND CHARACTERIZATION OF A NOVEL AMPHIPHILIC HYALURONIC ACID-GRAFT-EPSILON CAPROLACTAM COPOLYMER

3.1. Introduction

Hyaluronic Acid (HA), allied to a category of substances referred to as glycosaminoglycans, is a naturally occurring linear polysaccharide that comprises of interchanging links of D-glucuronic and *N*-acetyl-D-glucosamine residues (Oh et al., 2010; Kogan et al., 2007; Kuo et al., 1991) [Figure 3.1.a]. The reason for its natural biocompatibility arises from the fact that HA is sourced from various origins such as those of animal tissue as well as microbial fermentations (Oh et al., 2010). HA is employed as a scaffold constituent in tissue engineering and has many uses in the preparation of polymeric drug delivery systems (Palumbo et al., 2006). Synthetic functionalization of HA through grafting has been reviewed in a favorable light and derived sizeable deliberation in biomedical relevance (Radhakumary et al., 2011). Polymer grafting encompasses surface modification of polymers, to enhance the performance of these polymeric materials (Kato et al., 2003). Block and graft copolymers and polysaccharides of a lipophilic nature are amphiphilic macromolecules that have been vastly examined for utilization as a targeted drug delivery system (Jeong et al., 2012). In the case of poly(vinyl) alcohol, the graft copolymerization method has been reported to generate a higher graft yield compared to other methods of chemical modification (Zheng et al., 2005). Conjugation and crosslinking allows for chemical modification of HA by an amalgam grafted onto the backbone of HA via a single bond and assorted HA chains are affiliated by two or more links, respectively (Schanté et al., 2011). These chemical modifications cause the HA's remedial properties to be adjusted via targeting its many sites and applying these functionalizations in diverse solvents to attain an effect that preferably correspond to the product traits being sought (Schanté et al., 2011). In one study, preparation of graft copolymers were accomplished by the linking of mono amine-terminated poloxamer with HA employing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxysuccinimide to conjugate the two polymers (Cho et al., 2003). HA was also modified to determine its stability and thus resistance to enzymatic degradation by grafting numerous amino acids onto the carboxylic functional group of HA. The results proved successful with achievement of degradation resistance likened to native HA (Schanté et al., 2012).

A hydrophobic polymer, epsilon-Caprolactam (ECL) [Figure 3.1.b)], bears favorable properties for structural integrity and stability in a solvent. These comprise durability, hardness and an ability to extend (Grahowski and Borg, 1964). The synthetic fiber NYLON-6

is a constitution of ECL monomers. A graft copolymerization process was carried out between ECL and a Kevlar-49 fiber (Kim et al., 1997). The resultant copolymer achieved enhanced thermal and mechanical properties in comparison to the original Kevlar-49 fiber alone. ECL is also a salient precursor to the malleable material, plastic (Maheswari et al., 2003; Vilas and Tojo, 2010; Witjkamp et al., 1999). Anionic polymerization is the approach applied to elicit the linkage of polyesteramides from the cyclic monomers ECL and ϵ -caprolactone and these have been characterized (Chromcová et al., 2008).

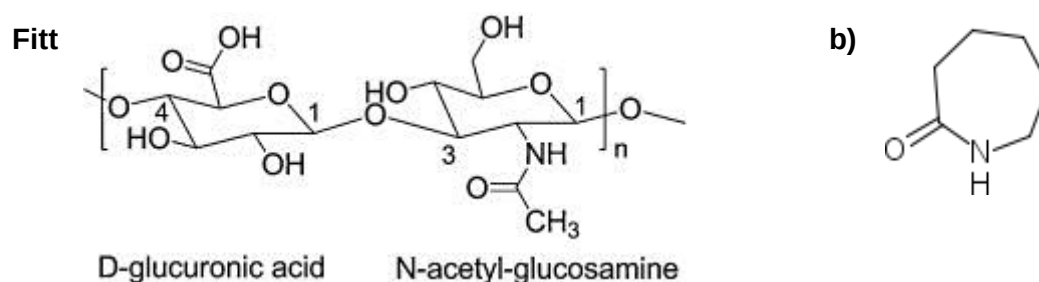


Figure 3.1: Chemical structure of a) HA and b) ECL.

Thus anionic polymerization is a preferred method of choice to graft the ECL onto HA. But HA is unsatisfactorily soluble in essential organic solvents and this limitation restricts its operation in drug delivery systems when there is need for its chemical constitution with hydrophobic polymers for an effective specific outcome (Lee et al., 2009b). Poly (ethylene glycol) (PEG) is most frequently employed in polymer drug delivery and is covalently affixed by means of one or more of its chains to a peptide/non-peptide or protein molecule. This polymer is widely used because of its robust characteristics of nontoxicity, increasing of half-life, being well soluble in water and having no antigenic or immunogenic response (Knop et al., 2010; Veronese and Pasut, 2005). PEGylation also allows for tumor targeting by way of an improved permeability and retention effect. The most attractive function of PEG is its ability to augment the solubility of hydrophobic compounds in an aqueous or organic environment (Banerjee et al., 2012; Roberts et al., 2002). Comparable to HA, many carbohydrates, proteins and DNA need to be dissolved in a separate organic phase to reach a steady state release of the drug delivery formulation. PEG may form complexes with these biomacromolecules to assist in solubilization of these compounds (Mok et al., 2008).

Therefore the aim of this chapter is to synthesize a novel copolymer by an anionic graft copolymerization process for linking hydrophobic ECL chains onto the backbone of hydrophilic HA for use in drug delivery. To attain this, the hydrophilic HA requires modification for anhydrous dimethylsulfoxide (DMSO) solubilization by the additive

methoxyPEG. The PEG nanocomplex formed with HA (HAmPEG) will enable graft copolymerization of the polymer with ECL. Overall characterization tests and investigations into micellar formation ability of the amphiphilic copolymer will determine whether there may be potential for application in targeted drug delivery and more specifically, against HBV, in this study.

3.2. Materials and Methods

3.2.1. Materials

Hyaluronic acid sodium salt from *Streptococcus equi*, epsilon-Caprolactam ($\geq 99\%$), Poly(ethylene glycol) methyl ether (mPEG) (average $M_w \sim 2000$ Da), Dialysis tubing cellulose membrane ($M_w 12044$ Da), *N*-acetylcaprolactam (99 %) (*N*-ACL), Sodium hydride (NaH) (powder moistened with oil, 55-65 % gas volumetric), Fluorescein isothiocyanate (FITC) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulphoxide (DMSO) and Dichloromethane (DCM) were purchased from Merck Chemicals (Pty) Ltd. (Germiston, Gauteng, South Africa). All other chemicals and reagents were of analytical grade.

3.2.2. Desalting process of hyaluronic acid

HA was converted to its acidic form by removal of its salt (Lee et al., 2008). Dry HA (1 g) was added to 100 mL of deionized water and left to stir until all HA was dissolved forming a viscous solution. This was dialyzed ($M_w 12044$ Da) for 24 hours against 3-5 L of deionized water to convert the sodium salt to the acid form (Figure 3.2.). The process can be hastened by having the beaker on a magnetic stirrer, disrupting the equilibrium between the 2 compartments continuously. The acid form of HA was recovered by lyophilization with a freeze-dryer (Labconco Freeze-Dry Systems, Labconco Corp., Kansas City, MO, USA).

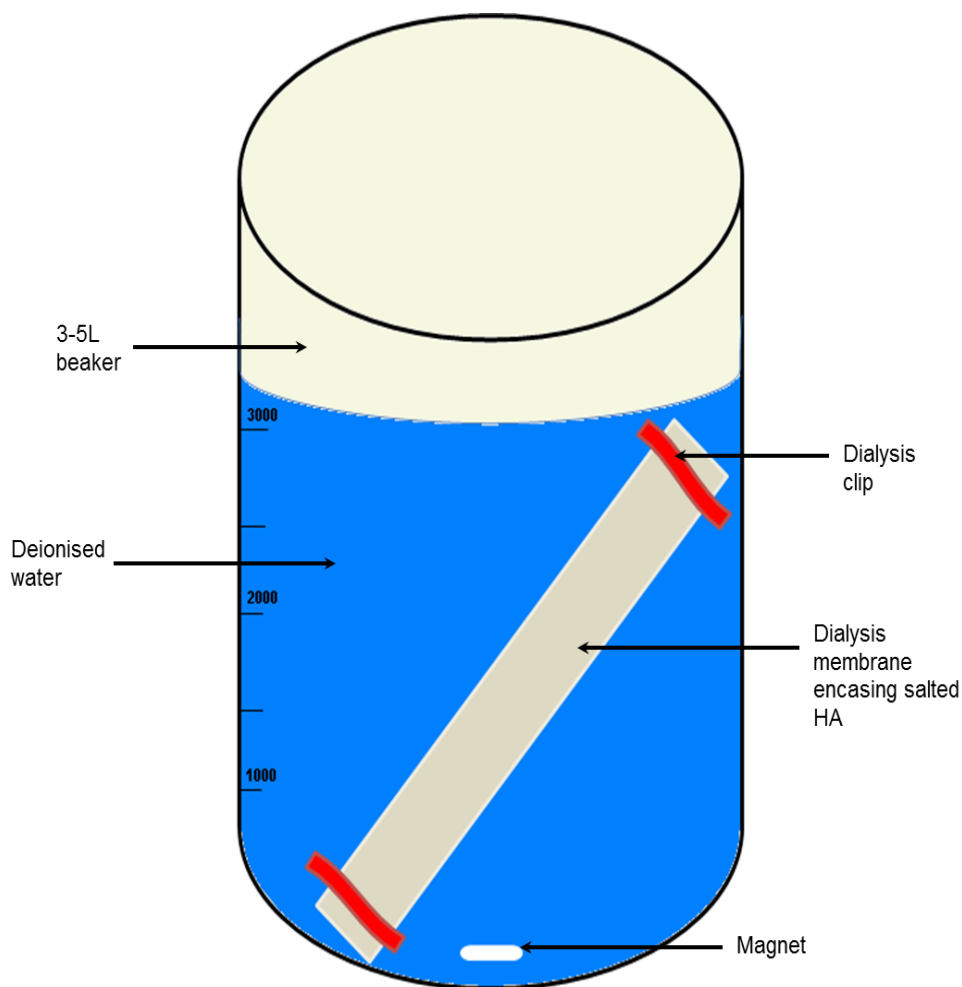


Figure 3.2: Illustration of HA desalting using dialysis tubing (M_w 12044 Da).

3.2.3. PEG-assisted solubilization of HA in DMSO

Desalted HA and methoxyPEG (mPEG) at an mPEG/HA weight ratio of 5:1 was added in 25 mL deionized water to form a mixture. The solution was well-stirred for 3 days and lyophilized to obtain dry HAmPEG complex powder. A total of 100 mg of lyophilized HAmPEG complexes was added in 1 mL of anhydrous DMSO under dry N_2 , vigorously stirred at 80 °C for 2 hours to solubilize the mPEG-attached HA (Lee et al., 2008).

3.2.4. Anionic graft (co)polymerization of HA and ECL (HA-g-ECL)

A set of five batches in alternating quantities of HA to ECL was prepared (Refer to Table 3.1.). The polymers were added together and plugs and stoppers ensured a sealed system that was purged with N₂ gas for 30 minutes. *N*-acetylcaprolactam (1mL) was added to the system through a syringe after which the tube was placed into an oil bath at a temperature of 140 °C. After 15 minutes of stirring at this temperature, 0.153 g of NaH was carefully added to the flask. The polymerization was allowed to proceed for 3 hours under N₂ gas (Tarkin-Tas and Mathias, 2010) (Refer to Figure 3.3). After cooling down to room temperature, the mixture was dialyzed (*Mw* 12044 Da) against deionized water for 48 hours and put to freeze for 24 hours at -70 °C. The proposed graft copolymer was lyophilized. An illustration of the graft copolymerization of HA and ECL is depicted in Figure 3.4.

Table 3.1: Quantities of hydrophilic to hydrophobic polymer used for optimal grafting efficacy.

Batch No.	HA* ¹ (mg/mL)	ECL* ² (mg)
1	100	900
2	300	700
3	500	500
4	700	300
5	900	100

*Volume and weight of *N*-ACL and NaH kept constant

¹HA- Hyaluronic acid

²ECL- epsilon Caprolactam

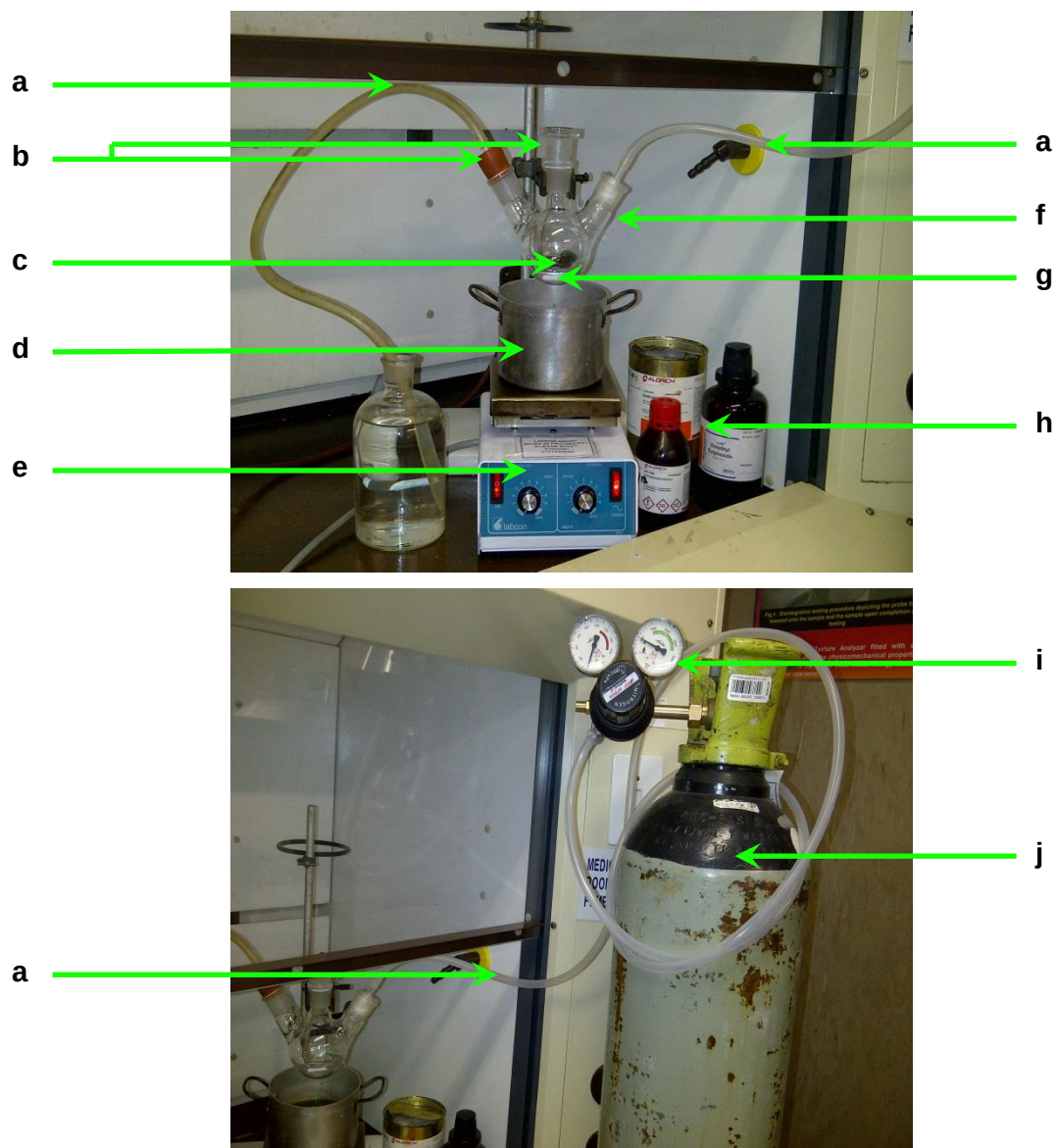


Figure 3.3: Digital images displaying the set-up for anionic graft copolymerization; a – N_2 line, b – plugs and stoppers, c – polymers in DMSO, d – pot of heated oil, e – heated temperature-controlled magnetic stirrer, f – 3-necked round bottom flask, g – magnet, h – chemicals for copolymer synthesis, i – N_2 tank pressure gauge/dial, j – N_2 tank.

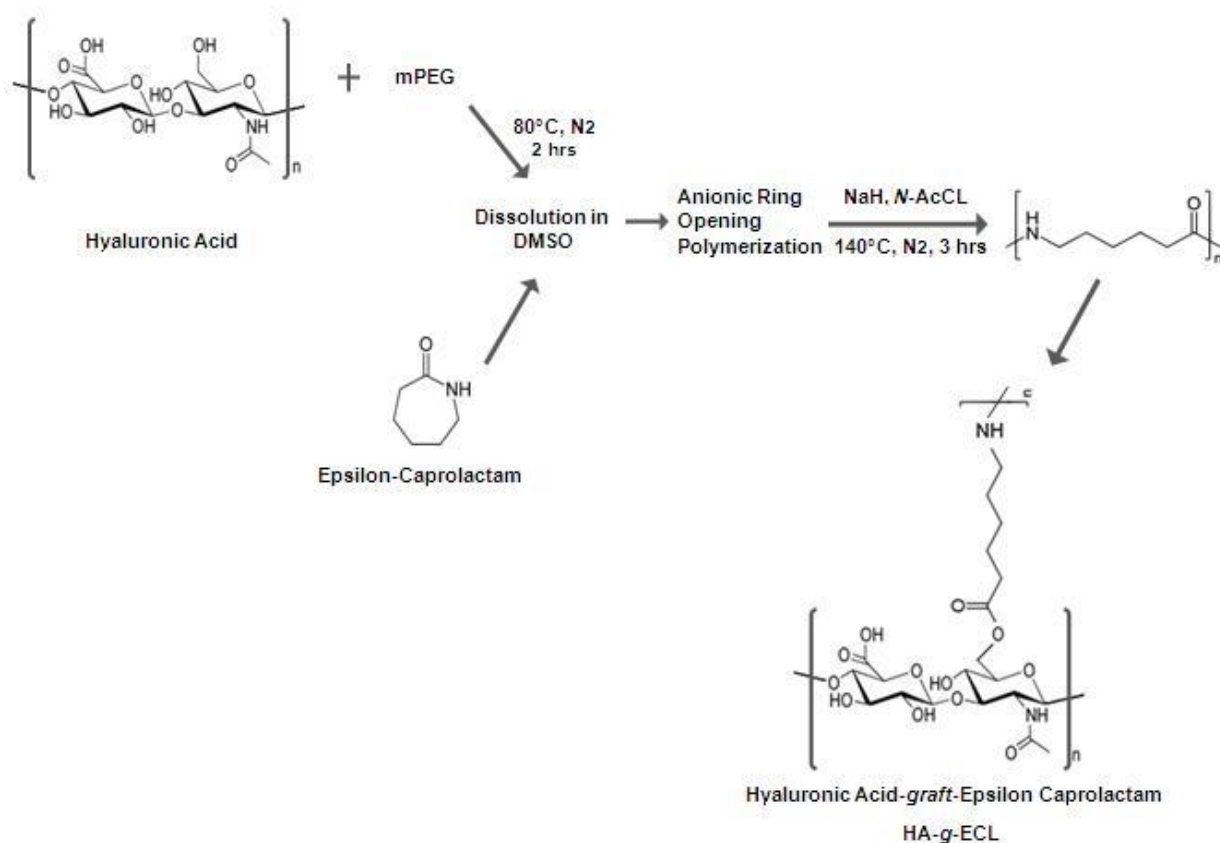


Figure 3.4: Synthetic graft (co)polymerization of HA-g-ECL.

3.2.5. Graft copolymer batches and parameters

Anionic graft copolymerization was performed on 5 batches consisting of varying mass ratios of HA:ECL (1:9 – 9:1). Percentage of grafting efficiency (GE) and percentage graft ratio (GR) were calculated according to Equation 1 & 2, respectively (Kumar et al., 2012):

$$GE (\%) = \frac{\text{Mass of graft copolymer (g)} - \text{Mass of HAmPEG (g)}}{\text{Mass of graft copolymer (g)}} \times 100 \quad \text{Equation 3.1.}$$

$$GR (\%) = \frac{\text{Mass of graft copolymer (g)} - \text{Mass of HAmPEG (g)}}{\text{Mass of HAmPEG (g)}} \times 100 \quad \text{Equation 3.2.}$$

3.2.6. Fourier Transform Infrared Spectroscopy analysis of copolymer synthesis

Fourier transform infrared (FTIR) spectroscopy analysis was performed to ascertain the copolymerization between HA and ECL and the non covalent interaction between HA and mPEG. FTIR allow for structural changes and variations to be assessed between compounds as a result of molecular fingerprint spectra that are derived for each. Spectra were obtained utilizing a Perkin Elmer Spectrum 2000 FTIR spectrometer with a MIRTGS detector (PerkinElmer Spectrum 100, Llantrisant, Wales, UK).

3.2.7. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) thermal analysis was carried out on mPEG, HA, HAmPEG, ECL and HA-*g*-ECL samples to characterize their structural states and thermophysical properties, utilizing a DSC 1 with updated STARe Thermal Analysis software (Mettler Toledo, Switzerland). Accurately weighed samples of dry formulations were put into aluminium crucibles, sealed, and heated in the temperature range 25 °C – 300 °C at a heating rate of 10 °C/min under constant purging of nitrogen. The reference standard used was an empty aluminium crucible.

3.2.8. Thermogravimetric Analysis

Thermal decomposition characteristics of HA-*g*-ECL copolymer and its constituents were carried out on a Themogravimetric Analyzer TGA 4000 (PerkinElmer Life And Analytical Sciences Inc., Shelton, CT USA). Weighed out samples within a range of 8-20 mg were placed in specialized crucibles. TGA analysis was conducted over a heating range of 30 °C – 900 °C at a rate of 10 °C/min under a nitrogen stream.

3.2.9. Approximate Molecular Weight measurement of HA-*g*-ECL

Viscosity (η) of different concentrations of solutions of HA-*g*-ECL dissolved in 0.2 M NaCl was measured using a modular advanced rheometer (HAAKE MARS Modular Advanced Rheometer, Thermo electron Corporation, Karlsruhe, Germany) with a C35/1° Ti sensor. This was used to determine molecular weight of the graft copolymer using the Mark-Houwink equation (Equation 3.3.) as described by Lapčák, Jr. L and authors (1998):

$$[\eta]=K.M^{\alpha}$$

Equation 3.3.

where $[\eta]$ is the intrinsic viscosity, K and α are constant parameters specific to a polymer and solvent system and M is the molecular weight. K and α constants specific for HA were 0.0339 mL/g and 0.778, respectively (Caspersen et al., 2014).

3.2.10. Visualization of HA-*g*-ECL using Scanning Electron Microscopy

The surface morphology of HAMPEG, ECL and HA-*g*-ECL samples were observed by use of scanning electron microscopy (FEI Phenom™, Hillsboro, Oregon, USA). Powders were mounted on stubs and gold coated by use of a sputter-coater (SPI Supplies, STRUCTURE PROBE INC, West Chester, Pennsylvania, USA). Comparisons in surface structural variations were then evaluated at a $\approx 5000\times$ magnification.

3.2.11. Micelle characterization

3.2.11.1. Determination of the Critical Micelle Concentration of HA-*g*-ECL

A test to quantitatively elucidate the amphiphilic character of HA-*g*-ECL in its ability to form micelles was carried out. The critical micelle concentration (CMC) of HA-*g*-ECL copolymers was measured by a FITC UV measurement method, as described by Lin et al (2013). Micelles were prepared at a concentration of 1mg/mL by sonicating it in deionized water for 3 minutes (Ultrasonic processor VCX 130 Watt (Sonics & Materials Inc., Newtown, CT, USA). A volume of 50 μ L FITC solution at a concentration of 5×10^{-4} M was added into HA-*g*-ECL solutions ranging in concentration from $10 - 1 \times 10^{-7}$ mg/mL. The relative ratio of absorbance between the wavelength of 455 and 475 nm (A_{455nm}/A_{475nm}) was determined using a nanophotometer (NanoPhotometer™, Implen GmbH, Munchen, Germany) by obtaining UV absorbance values at each concentration. A graph of absorption ratio 455 nm/475 nm against HA-*g*-ECL micellar log concentrations were plotted to determine the CMC value.

3.2.11.2. Micelle formation from Amphiphilic HA-*g*-ECL

An emulsion solvent evaporation method was used to prepare micelles. A copolymer concentration of 10 mg/mL was dissolved in dichloromethane. This was added dropwise into 10 mL deionized water with ultrasonication for 5 minutes [power on/off (10s/2s)], without any added surfactants in an attempt to investigate their ability of solely forming copolymeric

micelles. DCM was removed under vacuum in an oven (Vacuum Drying Oven “VACUTERM” EV-50, Raypa, Barcelona, Spain).

3.2.11.3. Morphology and size determination of HA-*g*-ECL micelles utilizing Transmission Electron Microscopy

The surface characteristics and shape of micelles formed were investigated with transmission electron microscopy (TEM) (JEOL 1200 EX, Tokyo, Japan, 120 keV). A drop from a micelle aqueous dispersion of 10mg/mL was placed on a copper grid composing perforated carbon film for viewing. The sizes of micelles were clearly visualized from the scale bar.

3.3. Results and Discussion

3.3.1. Grafting Efficiencies and Graft Ratios of novel copolymer

Figure 3.5 show the physical appearance of synthesized HAmPEG and HA-*g*-ECL. Table 3.2 reflects the impact that alternating quantities of the hydrophilic and hydrophobic polymers have on the efficiency of grafting and its ratio. The optimal values obtained for GE and GR is achieved with a 1:9 ratio of HA to ECL (100 mg/mL HA and 900 mg ECL). It was noted that an increase in the HA:ECL ratio causes a decrease in both GE and GR. The larger quantity of HA over ECL suggests that steric hindrance is most likely the reason for poor GE and GR. Molecules as large as HA wields considerable steric hindrance. Their molecular domains are bulky especially in the case of HA holding the ability of hydration and extension (Forrester and Lackie, 1981). Thus it can be deemed that steric hindrance hinders graft copolymerization at greater HA:ECL ratios accordingly reflecting the supreme GE and GR of 117.8 % and 54.1 %, respectively, at the lowest 1:9 ratio of HA:ECL. The 1:9 ratio of HA:ECL was therefore used in subsequent experiments and chapters.

A



B



Figure 3.5: Digital images of physical appearances of lyophilized A) HAMPEG complex and B) HA-*g*-ECL copolymer.

Table 3.2: Grafting parameters of the various copolymeric formulations synthesized.

Sample Batch	HA/ECL ¹ ratio	Yield (%)	GE ² (%)	GR ³ (%)
1	1:9	4.59	117.80	54.10
2	3:7	15.29	96.20	49.00
3	5:5	26.94	85.60	46.12
4	7:3	39.86	75.61	43.05
5	9:1	52.78	70.52	41.36

¹HA/ECL- Hyaluronic acid to epsilon Caprolactam

²GE- Grafting efficiency

³GR- Graft ratio

3.3.2. Evidence of grafting occurrence through infrared spectra observations

FTIR spectra were observed and analyzed to compare individual constituents to the synthesized graft copolymer in an attempt to visualize the occurrence of actual graft copolymerization. Figure 3.6 illustrates the comparison of individual polymers participating in the graft copolymerization process and the final polymer HA-*g*-ECL. As evidenced in Figure 3.6, HAmPEG exhibited its strong broad band at 3399 cm⁻¹ which is characteristic of O-H and N-H stretching vibrations. The band at 2880 cm⁻¹ is characteristic of C-H stretching vibrations. The 1614 cm⁻¹ and 1412 cm⁻¹ bands can be assigned to the asymmetric (C=O) and symmetric (C-O) stretching modes of the HA carboxyl groups. The bands that extend from 1500 cm⁻¹ to 1700 cm⁻¹ are characteristic of amide I and II bands (Yue, 2012). An intense band ranging from 945 cm⁻¹ to 1203 cm⁻¹ is consistent with COO⁻ stretching in alcohols. The ECL spectrum demonstrates its characteristic peaks. The two bands 3199 cm⁻¹ and 3292 cm⁻¹ represent the N-H stretching vibration and the bands of 2856 cm⁻¹ and 2927 cm⁻¹ show the C-H stretches from its alkyl part. The strong 1654 cm⁻¹ band can be attributed to C=O stretching (Rusu et al., 2009). In HA-*g*-ECL infrared spectra, bands show a new peak at 1735 cm⁻¹ which is indicative of an ester formation unseen in the parent compounds and accompanying characteristic C-O stretches at 1240 and 1104 cm⁻¹.

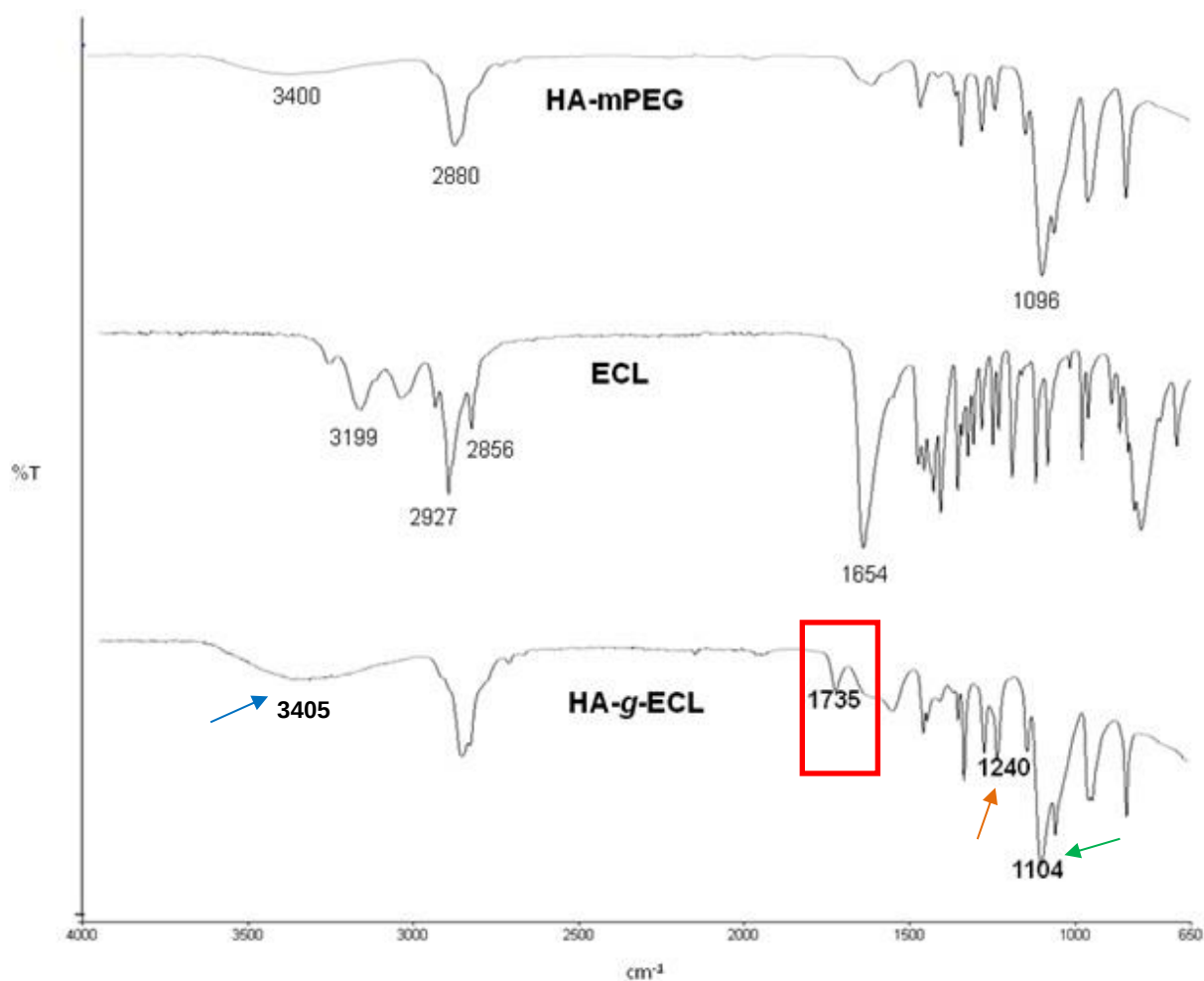


Figure 3.6: Comparative FTIR spectra of HAmPEG, ECL and HA-g-ECL.

The analysis of FTIR spectra revealed that the essential chemical structure of native compounds is retained in the prepared copolymer. The new peak that arises at 1735 cm^{-1} in HA-g-ECL is the point of conjugation due to a covalent chemical bond formation. As a result of the ring-opening of ECL, an acyl group is left exposed (Havlice et al., 1999; Sepe, 2008) (Figure 3.7). Esterification of HA occurs at the hydroxyl group via the acyl group giving rise to the new ester peak. The peaks at 1204 and 1104 cm^{-1} are indicative of C-O stretches due to the ester formation (Zhang and James, 2004). The increase in intensity of band around 3405 cm^{-1} is due to additional N-H stretching on the copolymer as a whole as a result of conjugation with the ECL derivative.

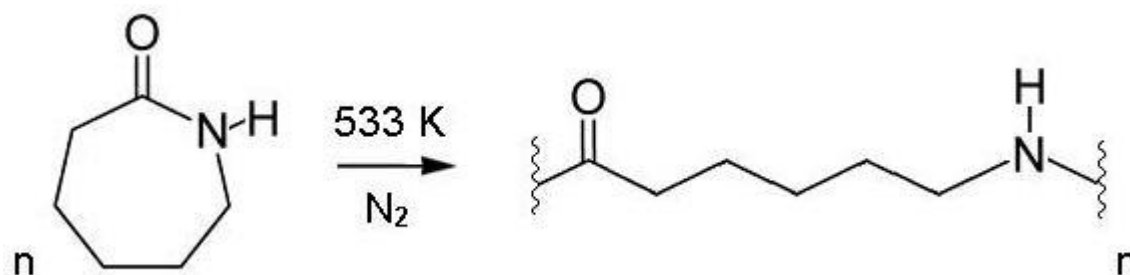


Figure 3.7: Ring opening polymerization of ECL (Adapted from Sepe, 2008).

3.3.3. Differential Scanning Calorimetry thermal profiles

Figure 3.8 exhibits the DSC curves of native polymers HA sodium salt, mPEG and ECL with their derivatives HAmPEG and HA-*g*-ECL in contrast. In Figure 3.8a HA sodium salt displays its characteristic broad endothermic transition at 113.21 °C which correlates with moisture loss from the HA molecule (Collins and Birkinshaw, 2008). The endotherm resembles a dehydration process as a result of evaporation. Its exothermic degradation peak is also seen at 238.18 °C. The ordered mPEG shows its maximum melting peak at 55.72 °C and thermostability as far as 300 °C can be seen. Figure 3.8c shows the HAmPEG complex attaining a lowered decomposition temperature (218.95°C) compared to HA sodium salt. This could be due to the less ordered state of HA in view of mPEG's attachment to the molecule. The peak melting point of ECL can be seen at 75.78 °C from Figure 3.8d as the first endothermic transition. The second endothermic transition could be attributed to the degradation point of ECL at a temperature of 258.72 °C. Figure 3.8e presents the thermal profile of the copolymer. Only one endothermic transition is seen at 52.69 °C followed by remarkable thermostability greater than either of its parent polymers alone. The shift to a lower temperature in the melting endotherm from 56.30 °C to 52.69 °C indicates that the graft copolymerization of ECL monomers onto the backbone of HAmPEG changed the order, arrangement and thus the crystal structure of the molecule chain. There was destabilization of the molecules structure with the formation of new bonds to give a less ordered structure (Gill et al., 2010). An additional observation is the disappearance of the endothermal peak corresponding to the degradation of ECL. This indicates grafting onto HA.

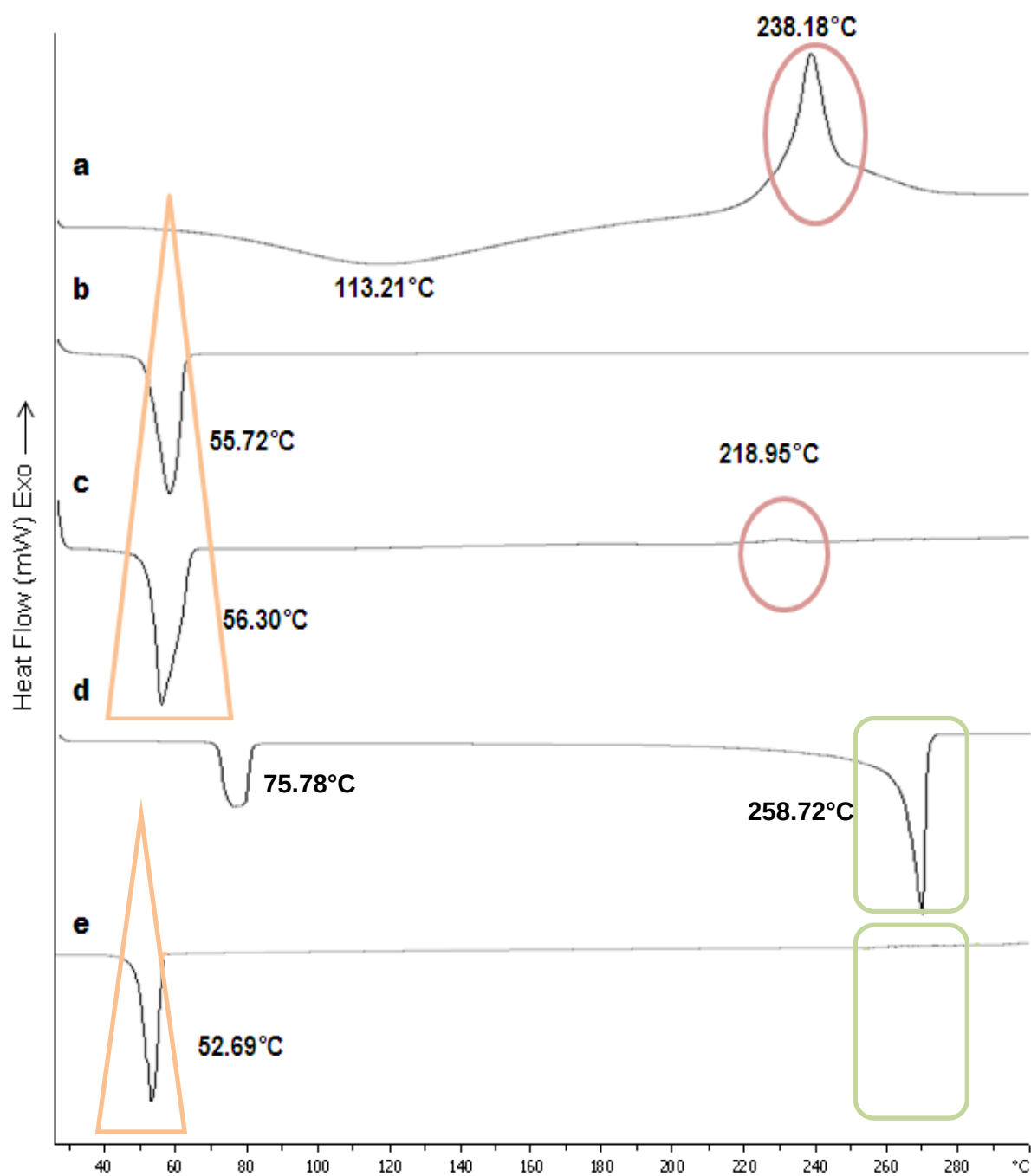
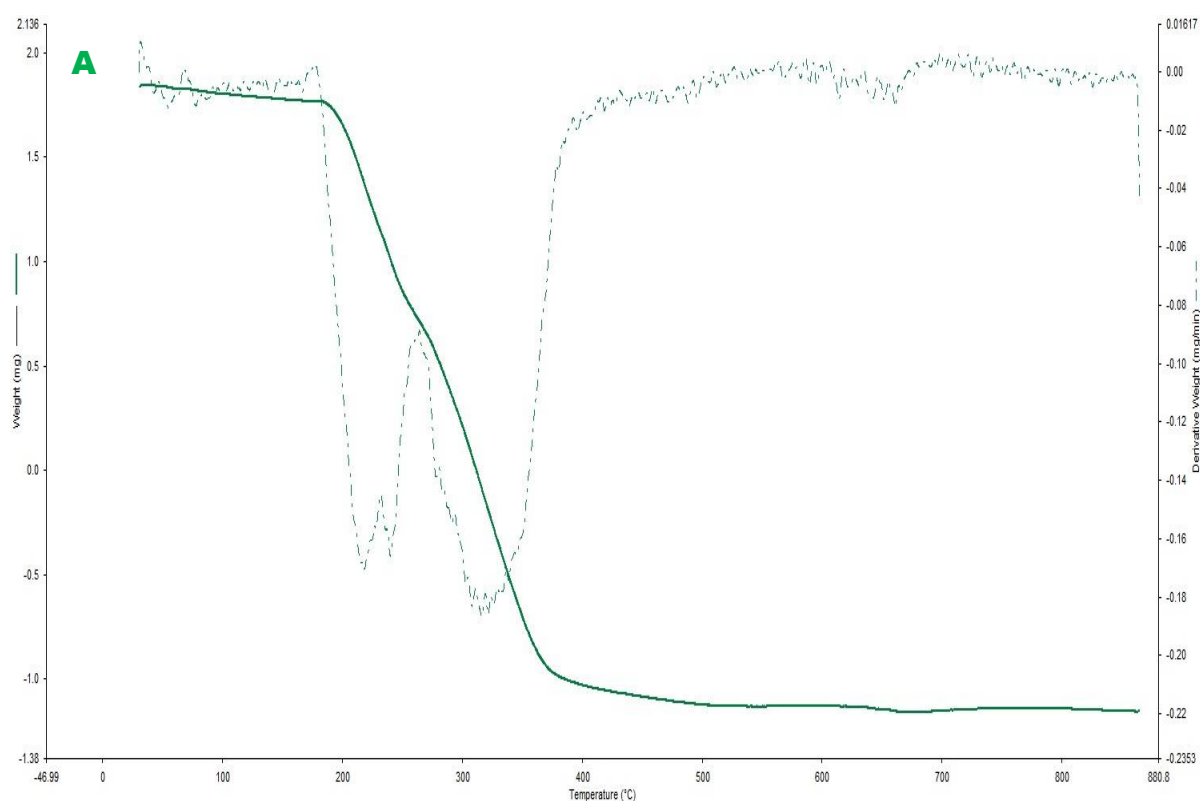


Figure 3.8: Comparative differential scanning calorimetry thermograms of a) HA sodium salt, b) mPEG, c) HAMPEG, d) ECL and e) HA-*g*-ECL.

3.3.4. Thermogravimetric Analysis thermal profiles

Figure 3.9 depicts the thermograms of degradation obtained together with their first derivative curves for HAmPEG (A), ECL (B) and HA-g-ECL (C). The solid curves denote thermogravimetric analysis curves and the dash-dot curves denote the derivative thermogravimetric analysis curves for each sample. ECL, HA-g-ECL and HAmPEG proceed with 1, 2 and 3 degradation steps, respectively. The onset temperature of degradation (T_{onset}) for ECL is shown to occur at 143.56 °C and its complete degradation is attained at 252.77 °C. These results correlate well with that of the DSC thermal profile of ECL depicted in Figure 3.8 d). The peak of greatest rate of weight loss change i.e. the point of inflection (T_{peak}) at 218.84 °C for HAmPEG correlates well with the DSC thermal profile for HAmPEG [Figure 3.8 c)] which has been established as the degradation of HA. The initial 4.52 % weight loss observed in HAmPEG can be attributed to the loss of water molecules at this stage. It was noted that the T_{onset} for HA-g-ECL copolymer occurred at 211.63 °C and that for the native polymer constituents were 143.56 °C and 191.85 °C for ECL and HAmPEG, respectively. This demonstrated the thermal stability of the newly synthesized HA-g-ECL over its parent polymers alone.



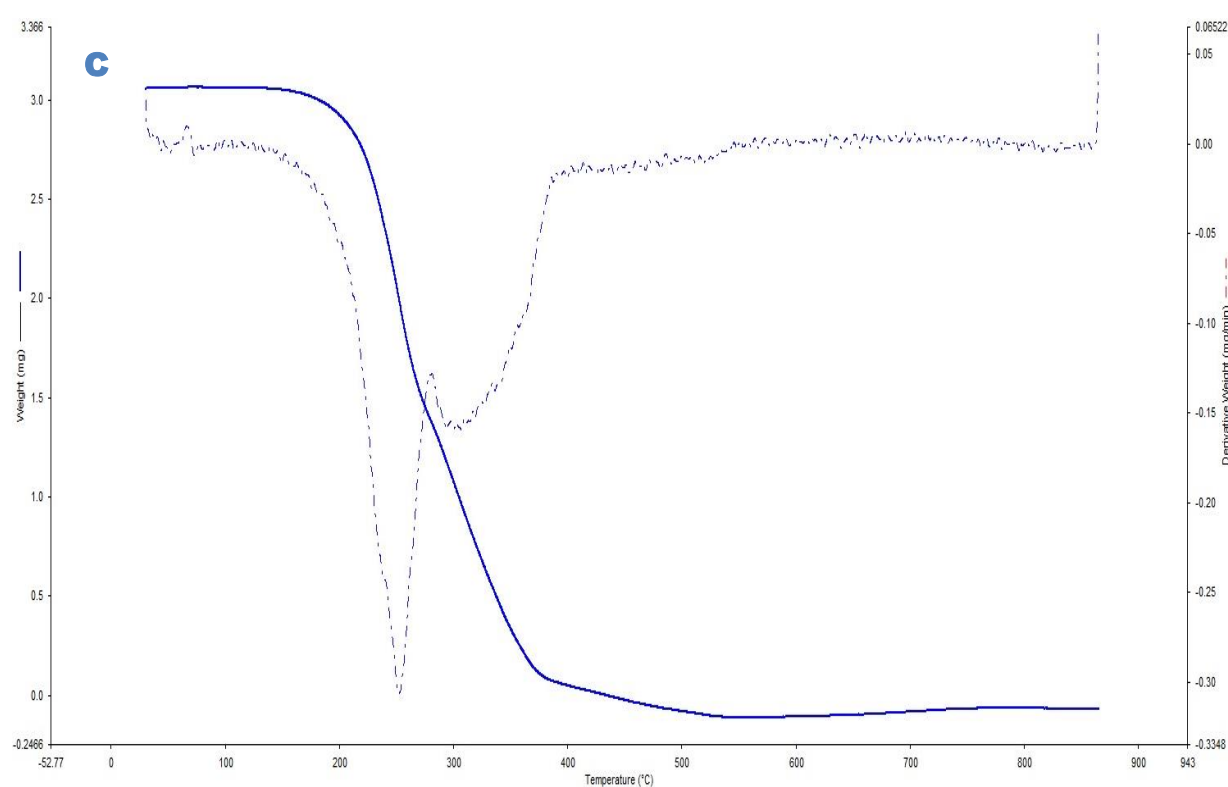
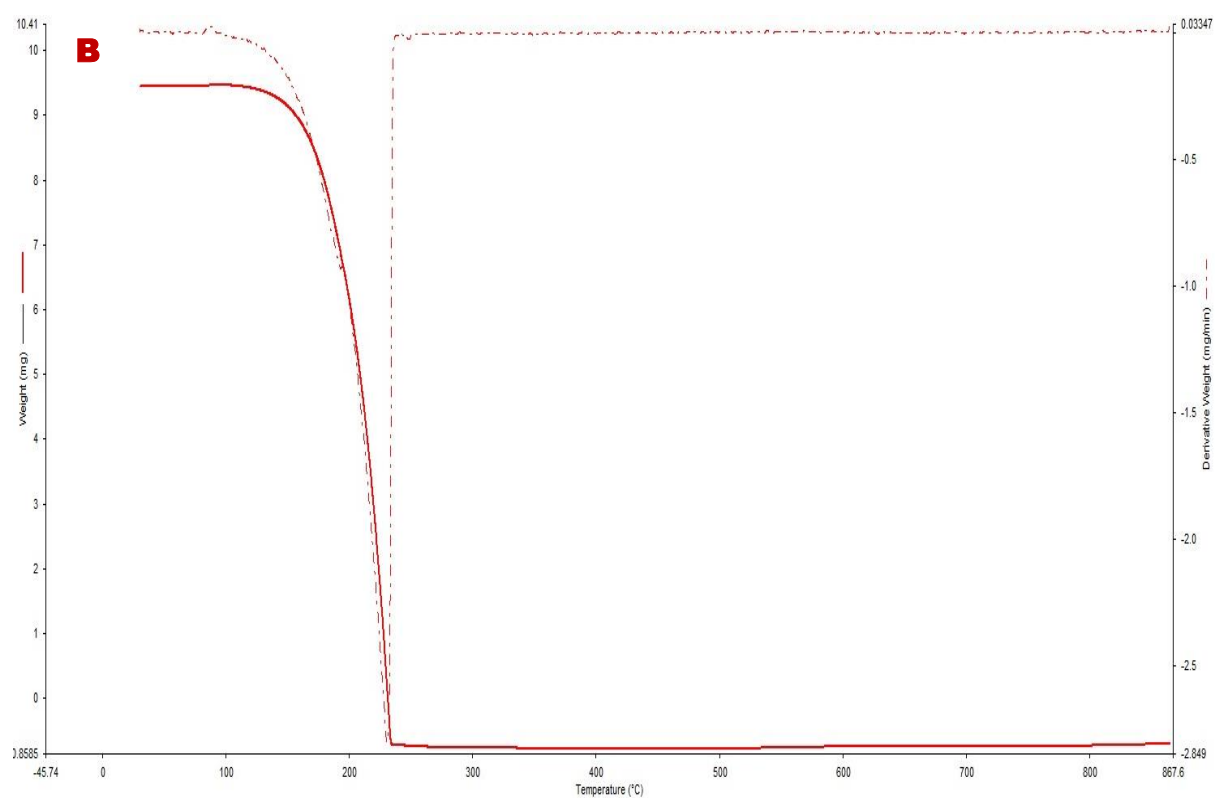


Figure 3.9: Thermogravimetric analytical and derivative thermogravimetry curves of A) HAmPEG, B) ECL and C) HA-g-ECL.

3.3.5. Approximate Molecular Weight determination of HA-g-ECL

The average molecular weight of the novel copolymer was determined adopting intrinsic viscosity testing and measurements made on variable concentrations of HA-g-ECL in deionized water (0.01-0.0001 %). Figure 3.10 presents the graphs of viscosity against time for each HA-g-ECL concentration. Table 3.3 shows the last 6 runs of viscosity estimations for each copolymer solution.

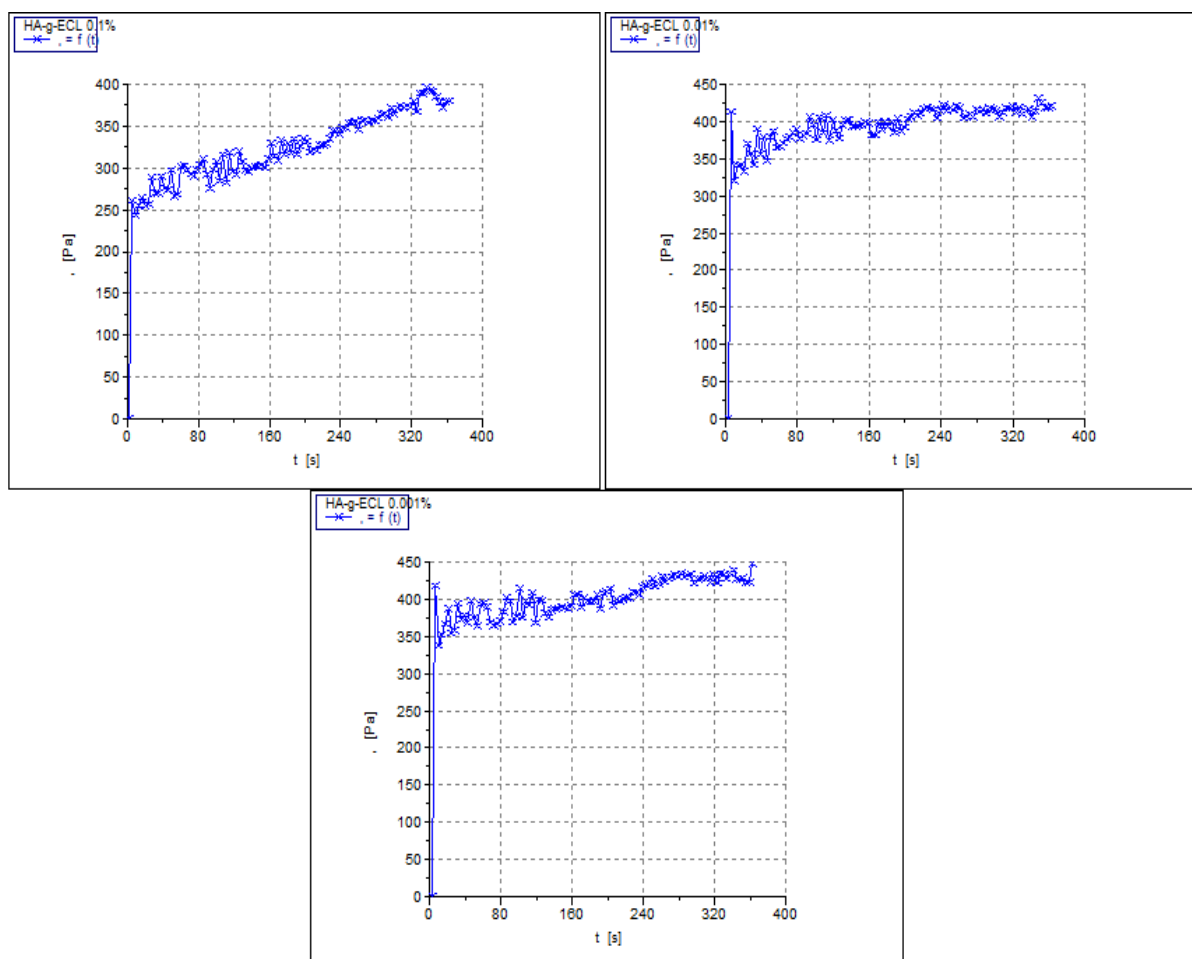


Figure 3.10: Graphs of viscosity for copolymer concentrations of a) 0.01 %, b) 0.001 % and c) 0.0001 % w/v .

Determining the average intrinsic viscosity value of the last 4 runs at each concentration allowed its substitution into the Mark-Houwink formula (Equation 3.3) with the already determined K and α parameters which are known constants that are dependant on a particular polymer, its solvent used and the temperature at which the measurement is performed. Some of the determinants affecting the value obtained for molecular weight comprise polydispersity, temperature, chain stiffness, topology and type of solvent

(Bhattarai, 2012). The specified HA K and α constants were 0.0339 mL/g and 0.778 in a 0.2 M NaCl solvent system at temperature 25 °C. These were inserted into the equation together with the obtained average intrinsic viscosity value of 864.55 mPas. The approximate molecular weight calculated for the synthesized HA-g-ECL copolymer was 3.398×10^7 Da. The molecular weight of the starting Hyaluronic Acid Sodium Salt from *Streptococcus Equi* was a high range of $1.5 - 1.8 \times 10^6$ Da. Yet again, this demonstrates that the anionic graft copolymerization procedure for covalently attaching ECL to the backbone of HA was successful causing an increase in the molecular weight of the compound as a result of its linkage. ECL bestowed a lipophilic attribute onto the water soluble polymer conferring an amphiphilic derivative that could have potential in a variety of biomedical applications

Table 3.3: Depiction of last 5 runs of HA-g-ECL concentrations to determine viscosity average value.

Run	t [s]	t seq [s]	η [mPas]	T [°C]
0.01%^w/v				
95	344.0	341.8	826.7	24.01
96	347.7	345.5	803.5	23.98
97	351.3	349.1	779.8	23.98
98	354.9	352.7	759.7	23.99
99	358.6	356.4	766.7	23.99
100	362.2	360.0	767.1	23.99
0.001%^w/v				
95	344.8	341.8	871.2	24.01
96	348.4	345.5	900.9	24.01
97	352.0	349.1	875.7	24.00
98	355.7	352.7	853.4	24.01
99	359.3	356.4	846.9	23.97
100	363.0	360.0	843.4	23.99
0.0001%^w/v				
95	344.8	341.8	900.0	23.99
96	348.5	345.5	886.5	23.99
97	352.1	349.1	883.4	24.02
98	355.7	352.7	862.4	24.01
99	359.4	356.4	853.0	24.01
100	363.0	360.0	898.4	23.99

3.3.6. Visualization of HA-*g*-ECL via Scanning Electron Microscopy

Figure 3.11 are scanning electron micrographs that display the external surface and morphologies of freeze-dried novel synthesized copolymer against its source homopolymers at a 5000 – 5250 X magnification. Forms of HA with its attached sodium salt as well as the free acid with attached mPEG are visualized (Figure 3.11b & c). The porous nature of the hydrophilic polymer is evident in the micrographs and this is undeniably expected being the most hygroscopic molecule naturally present and having a significant characteristic of water retention (Wang et al., 2013a). Figure 3.11a shows the micrograph of ECL with its crystal-like appearance and having a smooth surface. HA-*g*-ECL in Figure 3.11d demonstrates areas of smoothness as well as a somewhat rough surface. This could be understood as a consequence of bringing together two polymers that are not physically similar in their outward features. The portions of irregular surface on the copolymer is plausible by way of the vast porosity of HA. Embedded particles are visible on the surface of HA-*g*-ECL copolymer in Figure 3.11d.

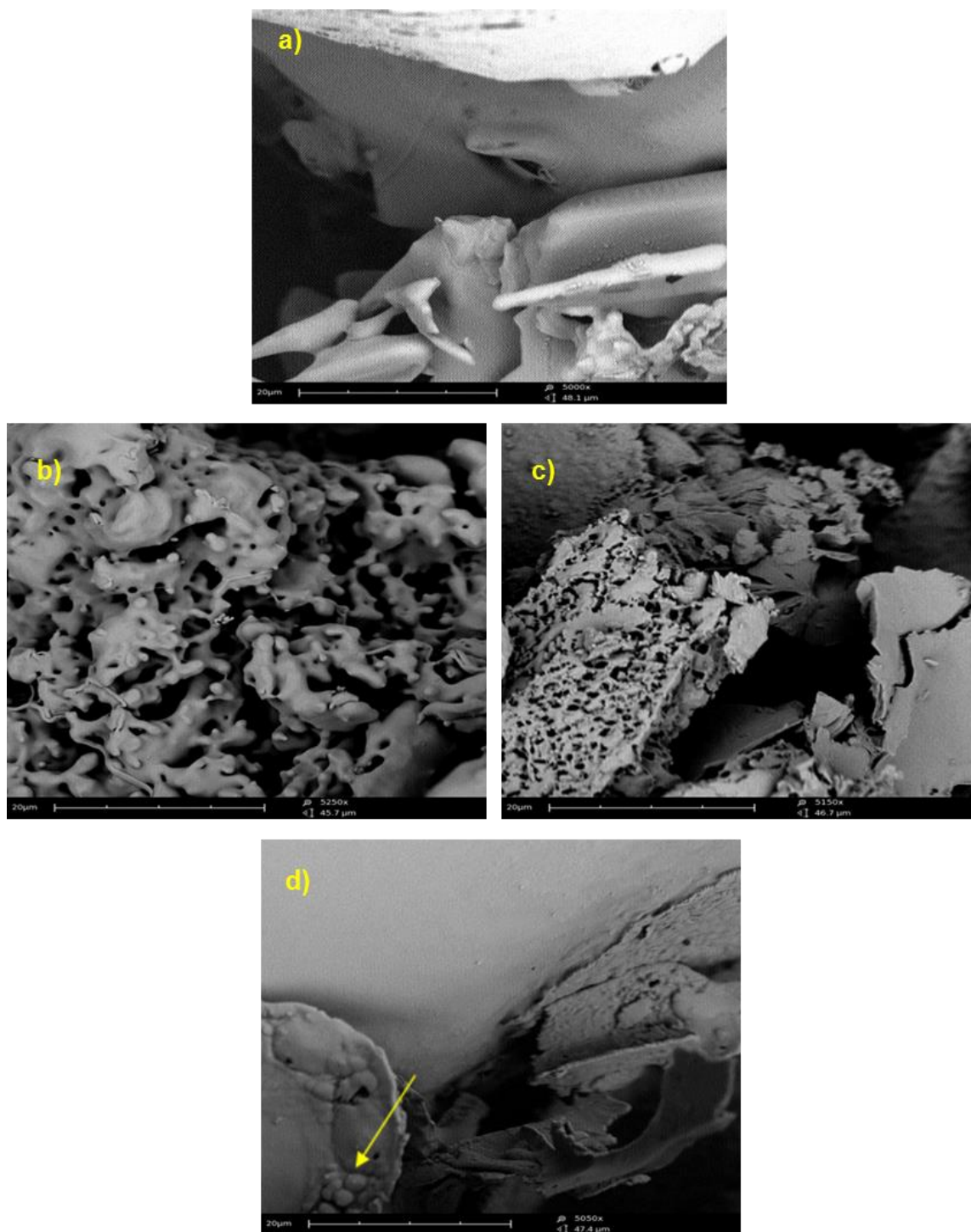


Figure 3.11: Scanning electron micrographs of a) ECL at 5000X magnification, b) HA sodium salt at 5250X magnification, c) HAMPEG at 5150X magnification and d) HA-g-ECL at 5050X magnification.

3.3.7. HA-g-ECL micellization ability

It is conventional for amphiphilic copolymers possessing a satisfactory hydrophilic/hydrophobic balance to produce micellar networks when added to distinct solvents (Wu et al., 2005). Thus, the CMC of HA-g-ECL was determined by the use of FITC as a fluorescent probe. UV absorbance measurements as a ratio were made and tested on a $10 - 1 \times 10^{-7}$ mg/mL HA-g-ECL concentration range. A plot of the absorbance ratio 455nm/475nm against the log concentrations of HA-g-ECL was made to determine the point at which the copolymer formed micelles (Figure 3.12). The point of intersection of the two tangent lines drawn is the point at which to calculate the CMC value on the graph (Lin et al., 2013). At the lower concentrations of HA-g-ECL, there is only a small difference in the absorbance ratio. A sudden increase in the absorption ratio is noted as the copolymer concentration heightened. This signifies the partitioning of the fluorophore into the hydrophobic core of the micelles (Shen et al., 2009). The point at which this occurs, 0.0063 mg/mL, is depicted on the graph, this being the CMC. Length ratio of polymers, total molecular weight and the chain architecture of the copolymer are factors that affect micellization. Depending on an aqueous or non-aqueous solvent, CMC decreases with increasing length ratio which favors the insoluble polymer and CMC increases with increasing length of the soluble polymer (Alexandridis and Lindman, 2000).

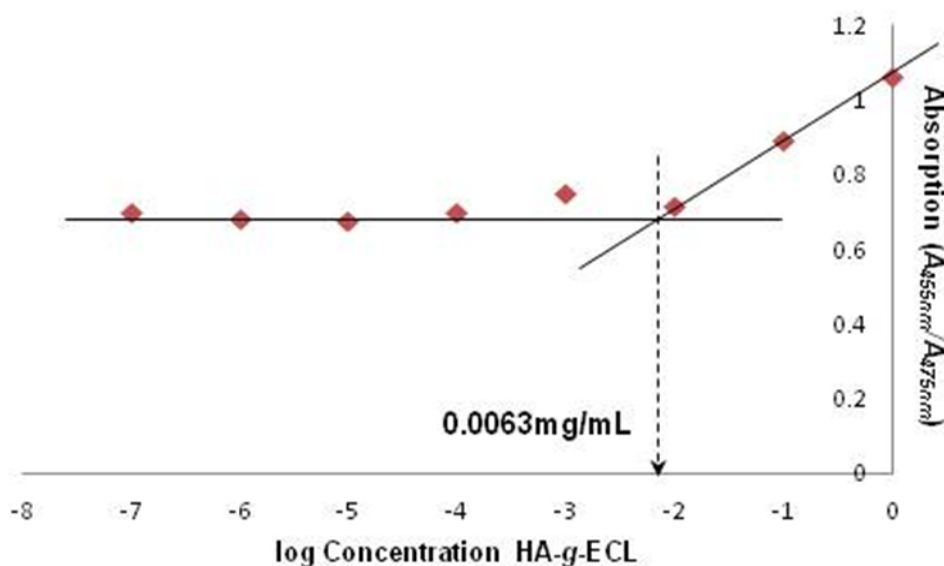


Figure 3.12: The determination of the CMC value for HA-g-ECL micelles.

To investigate the capability of HA-*g*-ECL to efficiently form copolymeric micelles in aqueous solution without the aid of a surfactant, a solvent evaporation method was utilized. Investigations into their morphology and size was carried out with TEM. Figure 3.13 reveals the images obtained at a magnification of 50000X of HA-*g*-ECL micelles formed. It can be seen that the morphology of formed micelles were that of an intact structure and well-defined shape. Micelles were spherically discernible and it is known that due to the amphiphilicity of HA-*g*-ECL copolymer forming micelles in an aqueous solution; the hydrophobic ECL portions form the core of the micelle and the hydrophilic HA chains establishes the micelle corona.

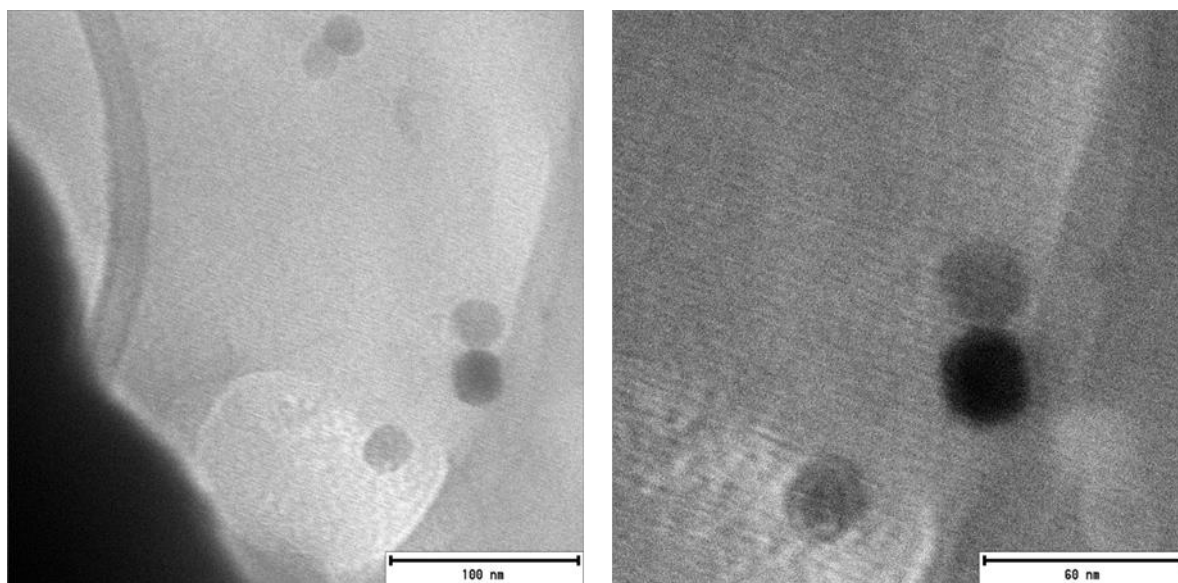


Figure 3.13: Transmission electron micrographs of formed HA-*g*-ECL micelles.

The size of HA-*g*-ECL micelles can easily be clarified from the TEM images. Sizes of about 25-30 nm could be calculated from the scale bar of images (Figure 3.13).

3.4. Concluding Remarks

Chapter 2 described several drug delivery polymer-based strategies formulated to make an impact on HBV. In this chapter, a novel copolymer was described and synthesized for its use as the foundation to a drug targeting HBV system in following chapters. This chapter put forth an anionic ring opening graft copolymerization mechanism that successfully allowed the grafting of ECL monomeric branches onto HA chains forming a novel amphiphilic HA-*g*-ECL copolymer that acquired the ability to form copolymeric micelles. An optimal ratio of hydrophile to hydrophobe was selected for efficient grafting followed by a full characterization

of the novel graft copolymer. Micelles prepared were of a consistent size range and shape which can be exploited as potential candidates for important applications in nanoscale drug delivery. The core of the micelle is lipophilic and can be loaded with hydrophobic drugs acting as an assuring carrier to impart sustained drug release. However, the aim of this study is to encapsulate a model drug that is hydrophilic into this novel copolymeric system utilizing a method that is construed in the ensuing chapter. Because of this counter orientation of hydrophilic and hydrophobic polymers, the ACNS particles are in actual fact 'reverse micelles' (Trivedi and Kompella, 2010).

CHAPTER 4

TREND REFLECTION IN THE DESIGN AND *IN VITRO* CHARACTERIZATION OF HA-G-ECL FORMING LAMIVUDINE-LOADED NANOPARTICLES

4.1. Introduction

The 21st century has welcomed nanotechnology as a looming and empowering technology due to the merging of science in various fields occurring at the nanoscale. The production of nanoparticles is a significant part of nanotechnology as definitive characteristics are recognized with nanoscale particles, crystals and layers. These nanoelements capitalize on the following outcomes:

- Advanced biological, chemical and physical features as a consequence of size scaling,
- The development of new phenomena as a result of the reduced size,
- Utilizing various routes engendering different atomic and molecular material networks,
- A pronounced hike in particle system processing speeds and magnitude of complexity (Roco, 1999).

The attractiveness of biodegradable polymeric nanoparticles is derived by the polymers ability of acting as drug delivery agents achieving controlled release of drugs, target organ specificity, serving as gene therapy carriers as well as delivering therapeutics via a peroral administration route (Soppimath et al., 2001).

HA has been employed as a coat or usually via conjugation with another chemical entity to be utilized in target specific intracellular delivery of anticancer therapeutics or genes. This polymer is known to be receptor-mediated endocytosed by way of CD44 and RHAMM receptors found on cells (Jin et al., 2010). The preference for HA in nanoparticle anticancer drug delivery is derived from the fact that it is biodegraded by hyaluronidase which is found to be abundant in malignancies and cancers. Many researchers exploit this reality to attain rapid drug release at the tumour site (Yeo, 2013). The diversity in chemical modification approaches with HA, via cross-linking or conjugation, gives way for many choices in synthesizing new HA derivatives possessing diversified characteristics (Schantè et al., 2011). Copolymers synthesized from grafting HA with PEG have been investigated for their various characteristics that were additionally developed (Moriyama et al., 1999). PLGA chains were grafted onto the backbone of HA forming targeting micelles carrying doxorubicin (Lee et al., 2009b). Copolymerizing polylactic acid onto HA backbone produced a hydrophobic compound that had the ability to assemble in aqueous media (Palumbo et al.,

2006). HA and poly(ϵ -caprolactone) (PCL) were graft copolymerized to produce micelles in aqueous media that entrapped fibroblast growth factor 1 (Lin et al., 2013). Another study reported preparation of a hydrogel by grafting of poloxamer and HA. The hydrogel successfully entrapped ciprofloxacin which could be released in a sustained manner (Cho et al., 2003). Oxaliplatin was also attached to a chemically modified HA-chitosan entity and this entire compound was contained in Eudragit S100 coated pellets for efficient targeting specifically to colon tumours (Jain et al., 2010). HA was used to coat paclitaxel containing poly(butyl cyanoacrylate) nanoparticles by a radical polymerization method (He et al., 2009). Wu and coworkers (2012) reported on conjugates of histidine and HA forming nanoparticles containing doxorubicin.

The cytosine analogue LMV has proved to be quite effective in treating human HBV by inhibiting HBV reverse transcriptase terminating DNA synthesis (Cuestas et al., 2010). Due to its unfavorable and serious side effects such as that of lactic acidosis, and the inconvenience of daily dosing, the drug should ideally be released in a controlled manner from a biodegradable and biocompatible system avoiding undesired effects. In one study, LMV was loaded into PLGA nanoparticles that were coated with bovine serum albumin for intracellular HBV delivery in one study (Wang et al., 2012). The PLGA particles were successfully internalized and showed sustained release of the drug. In another study, polymethacrylic acid nanoparticles encapsulated LMV and the release mechanism was that of zero order and was sustained over a duration of 24 hours (Tamizhrasi et al., 2009). LMV was also encapsulated in nanoparticles synthesized from glycyrrhizin linked to chitosan for liver specificity. An initial burst release of the drug was noted followed by a sustained release (Mishra et al., 2014).

Design of experiments (DOE) is an approach that is selected to achieve the most data and information from an experimental model by fabricating additional experiments that will produce maximum informational results statistically. This can then be utilized in limit evaluations and validating the experimental model. Prior to data retrieval, the experiment is designed by addressing factors such as conditions under which experiments are performed, the choice of input variables and their modifications and determinants addressed by experimenter upon which they will observe phenomena being examined (Franceschini and Macchietto, 2008). The three-factor three-level Box Behnken design (Figure 4.1.) was chosen for this study to optimize the conditions for formulating the nanostructured system.

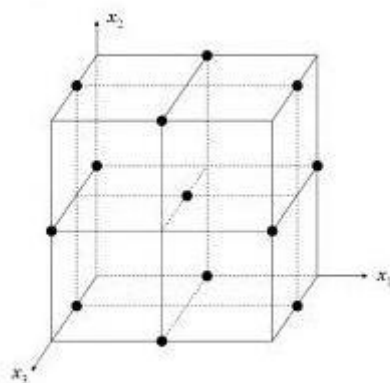


Figure 4.1: Graphic representation of a three-factor Box-Behnken design (Adapted from Lawson, 2010).

The advantages of Box-Behnken designs over Central Composite designs are that a varying of factors over three levels is merely necessary possibly leading to less cost on experimenting and they usually need less total runs except for cases in five-factors (Lawson, 2010). A study aimed to optimize PLGA nanoparticles loaded with lorazepam by assessing the effects of processing variables of polymer, surfactant, drug and aqueous/organic ratio on the desired responses of Z-average and drug entrapment efficiency utilizing the Box-Behnken design. All data collected led to the successful estimation of optimized nanoparticles safety in Sprague-Dawley rats in the establishment of a nose-to-brain pathway (Sharma et al., 2014). One other study using the Box-Behnken design unfolded and optimized a method for obtaining PCL nanoparticles laden with *Uncaria tomentosa* extract. PCL concentration, polyvinyl alcohol concentration and aqueous/organic volume ratio were the processing variables studied with drug entrapment efficiency, particle mean diameter, polydispersity, and zeta potential as the desired responses. Conclusive results suggested a successful Box-Behnken design approach by the development of an optimized nanoparticle formulation with desired features (Ribeiro et al., 2013).

In the following study, formulation variables- copolymer, surfactant and solvent quantities, were selected to be optimized to elicit the desired responses- maximum drug entrapment efficiency, minimized particle size and minimized mean dissolution time. Trends in the consequences of variable quantity combinations are examined and discussed in this chapter to begin developing a physicochemical understanding of the novel synthesized copolymer in terms of the chosen parameters.

4.2. Materials and Methods

4.2.1. Materials

Hyaluronic acid sodium salt from *Streptococcus equi*, epsilon-Caprolactam ($\geq 99\%$), Poly(ethylene glycol) methyl ether (mPEG) (average $M_w \sim 2000$ Da), Dialysis tubing cellulose membrane (M_w 12044 Da), N-acetylcaprolactam (99 %), Sodium hydride (NaH) (powder moistened with oil, 55-65 % gas volumetric) and Pluronic F-127 were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Lamivudine (LMV) was received as an *ex-gratia* sample from Adcock Ingram Ltd. (Germiston, Gauteng, South Africa). Dimethyl sulphoxide (DMSO) and Dichloromethane (DCM) were purchased from Merck Chemicals (Pty) Ltd. (Germiston, Gauteng, South Africa). All other chemicals and reagents were of analytical grade.

4.2.2. Synthesis of the amphiphilic graft copolymer

The copolymerization process was carried out as outlined in *Chapter 3, Sections 3.2.2. Desalting process of hyaluronic acid, 3.2.3. PEG-assisted solubilization of HA in DMSO and 3.2.4. Anionic graft (co)polymerization of HA and ECL (HA-g-ECL).*

4.2.3. Construction of a Box-Behnken design for the preparation/formulation of copolymeric nanoparticles

A 3-factor, 3-centre points Box-Behnken quadratic design was ascribed for statistical analysis carried out by use of a Minitab Statistical Software, Version 14 (Minitab Inc., State College, PA, USA) to optimize the formulation limitations and gauge their effects according to % DEE, size and mean dissolution time (MDT) from drug release curves. Experimental runs (15) are engineered and the nonlinear computer-generated quadratic model is expressed as Equation 4.1.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad \text{Equation 4.1.}$$

where, Y is the measured response associated with each factor level combination; b_0 is an intercept; b_1 to b_{33} are regression coefficients computed from the observed experimental values of Y ; and X_1 , X_2 and X_3 are the coded levels of independent variables. The terms X_1X_2 and X_i^2 ($i = 1, 2$ or 3) represent the interaction and quadratic terms, respectively (Chopra et al., 2007; Motwani et al., 2008). The independent factors and dependent variables along with their limits are depicted in Table 4.1. These values were ordained proceeding from preformulation experimentation. The quantities of each chemical ingredient

employed in all 15 formulations received from the Box-Behnken quadratic design are listed in Table 4.2.

Table 4.1: Variables for the Box-Behnken factorial design parameters.

Independent Variables	Limits	
	Lower	Upper
Amount of HA-g-ECL ¹ copolymer (mg)	150	350
Amount of Pluronic F-127 surfactant (g)	1	2
Volume of DCM ² solvent (mL)	5	20
Variable Response		
Particle size (nm)	Minimize	
DEE ³ (%)	Maximize	
MDT ⁴ (hrs)	Minimize	

¹HA-g-ECL- Hyaluronic acid-graft-epsilon Caprolactam

²DCM- Dichloromethane

³DEE- Drug Entrapment Efficiency

⁴MDT- Mean Dissolution Time

Table 4.2: Formulation (F) compositions obtained from the Box-Behnken experimental design.

Formulation Run	Copolymer (HA-g-ECL) (mg)	Surfactant (Pluronic F-127) (g)	Solvent (DCM) (mL)
F1	300	1	12.5
F2	300	1.5	20
F3	225	2	20
F4	225	1.5	12.5
F5	150	2	12.5
F6	300	2	12.5
F7	150	1.5	5
F8	225	1.5	12.5
F9	225	1	20
F10	150	1.5	20
F11	150	1	12.5
F12	225	1.5	12.5
F13	300	1.5	5
F14	225	1	5
F15	225	2	5

4.2.4. Preparation of LMV-loaded HA-g-ECL copolymeric nanoparticles

The copolymeric nanoparticles were prepared by a double emulsion (w/o/w) solvent-evaporation method. Drug (60 mg/mL) was dissolved in deionized water. Varying amounts of copolymer (150 mg, 225 mg & 300 mg) were dissolved in varying amounts of DCM (5 mL, 12.5 mL & 20 mL). The primary emulsion was prepared by adding the aqueous drug solution to the organic copolymeric solution with sonication [pulse on(10 s)/off(2 s)] for 20 minutes using an ultrasonic processor VCX 130 Watt (Sonics & Materials Inc., Newtown, CT, USA). Variable amounts of Pluronic F-127 surfactant (1 g, 1.5 g & 2 g) were dissolved in 100 mL of distilled water and the external aqueous phase solution was placed under a propeller stirrer and the primary emulsion added into this with a needle and syringe in a dropwise manner to form nanoparticles. The resultant secondary emulsion was put in a vacuum oven to remove the organic solvent (Trade Raypa® Digital drying oven, Barcelona, Spain maintained at 40 °C, -0.6 bar).

4.2.5. Transmission Electron Microscopy of the formed HA-g-ECL copolymeric nanoparticles

The surface morphological and structural transitions of HA-g-ECL nanoparticles were determined by TEM (JEOL 1200EX, Tokyo, Japan) at a 50000X magnification. A concentrated drop of the nanoparticle suspension was placed on the TEM copper grid ahead of ultrasonication of the suspension. Staining of the grid was not a necessary requirement as the nanoparticles were clearly visible to the eye under microscope view. The copper grid was dried at room temperature for approximately 2 hours before analysis with the microscope.

4.2.6. Size and Zeta Potential determination via Dynamic Light Scattering (DLS)

The size and zeta potential of prepared nanoparticles was estimated by a Zetasizer NanoZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The nanoparticle formulation suspension (1 mL) was diluted to 10 mL with deionized water and filtered through a 0.22 μm filter (Millipore Co., Massachusetts, USA). The filtrate in the sterilized cuvette (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was made to reach the mark of maximum limit for dynamic light scattering testing. A particle's Brownian motion in a suspended liquid form impels laser light to be scattered at varying intensities. Essentially, Brownian motion velocity and thus the particle sizes can be measured when these intensity variations are analyzed. Tests were performed in triplicate.

4.2.7. Thermal analysis of HA-g-ECL copolymeric nanoparticles

4.2.7.1. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) thermal analysis was carried out on design nanoparticle samples utilizing a DSC 1 with updated STARe Thermal Analysis software (Mettler Toledo, Switzerland). Accurately weighed 2 mg samples (Murnane et al., 2008) of dry formulations were put into aluminium crucibles, sealed, and heated in the temperature range 25 $^{\circ}\text{C}$ – 200 $^{\circ}\text{C}$ at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ under constant purging of nitrogen. The reference standard used was an empty aluminium crucible.

4.2.7.2. Thermogravimetric Analysis

Thermal decomposition characteristics of design nanoparticle samples were carried out on a Thermogravimetric Analyzer TGA 4000 (PerkinElmer Life And Analytical Sciences Inc.,

Shelton, CT USA). TGA analysis was conducted over a heating range of 30 °C – 900 °C at a rate of 10 °C/min under a nitrogen stream.

4.2.8. Construction of a calibration curve to quantify LMV by its ultraviolet spectrophotometric absorptivity

A calibration curve was constructed by preparing a stock solution of LMV in phosphate buffered saline (PBS) (0.1 M, pH 7.4) at a concentration of 0.3 mg/mL. Dilutions from stock were prepared at a range of concentrations (0.003-0.015 mg/mL). UV absorbance values at the various concentrations were recorded on a nanophotometer (NanoPhotometer™ IMPLEN, Munich, Germany) at a wavelength of 277 nm. A linear curve was plotted and its R² value calculated with the assays being evaluated in triplicate for reproducibility.

4.2.9. Drug encapsulation efficiency of HA-g-ECL copolymeric nanoparticles

Lyophilized LMV-loaded HA-g-ECL nanoparticles were left to stir vigorously for 96 hours in PBS (0.1 M, pH 7.4) to attain complete drug leaching from the nanoparticles. The formulation was centrifuged at 20000 rpm for 10 minutes to separate free drug from the HA-g-ECL copolymer. A volume of 4 µL supernatant was used to determine the amount of free drug therein employing ultraviolet spectroscopy (NanoPhotometer™ IMPLEN, Munich, Germany) at λ_{max} 277 nm. The amount of drug in the nanoparticles was determined by Equation 4.2.

$$\text{Drug Encapsulation Efficiency (\%)} = \frac{\text{Actual drug amount in nanoparticles}}{\text{Theoretical drug amount of nanoparticles}} \times 100 \quad \text{Equation 4.2.}$$

4.2.10. *In vitro* dissolution studies of LMV-loaded HA-g-ECL copolymeric nanoparticles

In vitro LMV release studies were carried out by placing HA-g-ECL nanoparticles in dialysis tubing (*Mw* 12044 Da) with 2 mL of normal saline. The membrane bag was inserted into a jar with a tightly sealed lid containing 50 mL PBS buffer (0.1 M, pH 7.4). The complete system was placed in an orbital shaker bath (Labex, Stuart SBS40®, Gauteng, South Africa) kept constant at 37 °C and shaking at 25 rpm. At predetermined time intervals, 100 µL of the release medium was sampled and replaced by 100 µL of PBS buffer. The amount of LMV released was detected and calculated using UV spectroscopy at λ_{max} 277 nm and enumerated from a construction of a standard curve of linearity, respectively. Values of the

mean dissolution time (MDT) were estimated for each formulation with exertion of Equation 4.3. where M_t is the fraction of dose released in time $t_i = (t_i + t_{i-1})/2$ and M_∞ corresponds to the loading dose (Mufamadi et al., 2013). Each test was carried out in triplicate.

$$\text{MDT} = \sum_{i=1}^n t_i \left(\frac{M_t}{M_\infty} \right) \quad \text{Equation 4.3.}$$

4.3. Results and Discussion

4.3.1. Morphological characterization of HA-g-ECL copolymeric nanoparticles

Transmission electron microscopy (TEM) was used to study the size and structure of the prepared copolymeric nanoparticles. TEM imaging reflected HA-g-ECL drug loaded nanoparticles in the nanometer range of consistent near-spherical morphology following preparation procedures. The surfactant served to reduce the surface tension contributing to the particles spherical shape and low aggregation tendencies. Figure 4.2 shows nanoparticles prepared with 1 g of Pluronic F-127, 225 mg HA-g-ECL and 5 mL DCM (F14); 1.5 g of Pluronic F-127, 150 mg HA-g-ECL and 20 mL DCM (F10) and 1 g of Pluronic F-127, 300 mg HA-g-ECL and 12.5 mL DCM (F1). Most nanoparticles were of an unvarying spherical shape with minimal aggregation. The slight aggregation of copolymeric nanoparticles seen in Figure 4.2b for F10 is due to the formation of smaller sized particles (discussed in *Chapter 4, Section 4.3. Results and Discussion, Subsection 4.3.2. Size and Zeta Potential determination of HA-g-ECL nanoparticles by DLS*). The smaller the size of particles, the more susceptible they are to agglomeration (Elzey and Grassian, 2010). The formation of spheres, in terms of structural integrity of particles, can be attributed to the curing conditions in formulating (Ruiz-Pérez et al., 2010). From the TEM image it will be seen that the average diameter of nanoparticles corresponds to the measurements made by DLS later (Figure 4.3A and 4.3C).

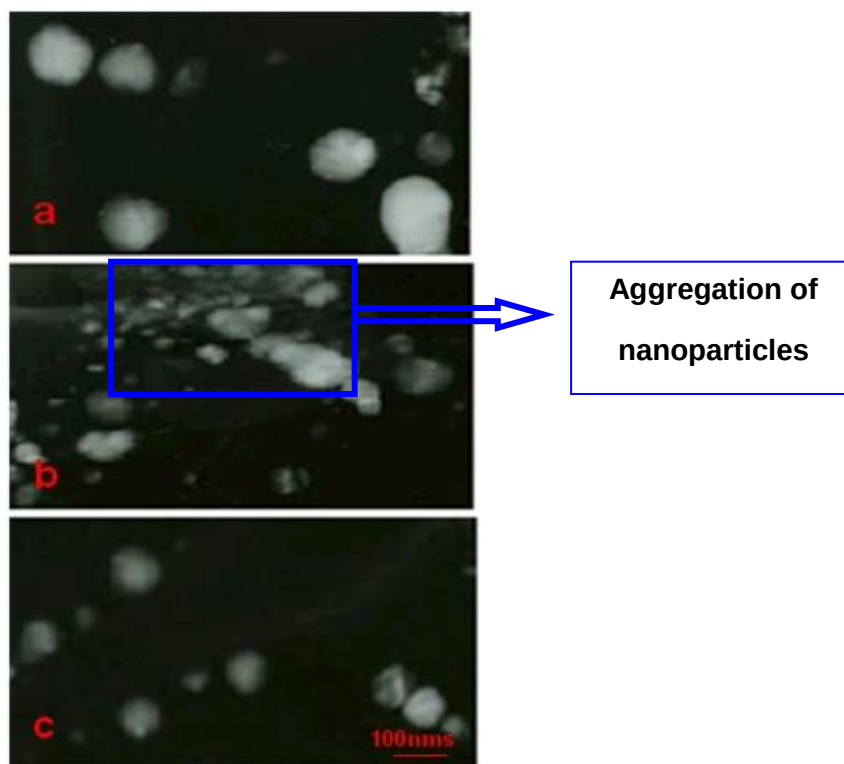


Figure 4.2: Transmission electron micrograph of HA-g-ECL LMV-loaded nanoparticles at 50000X magnification. a) F14, b) F10, c) F1.

4.3.2. Size and Zeta Potential determination of HA-g-ECL copolymeric nanoparticles by Dynamic Light Scattering

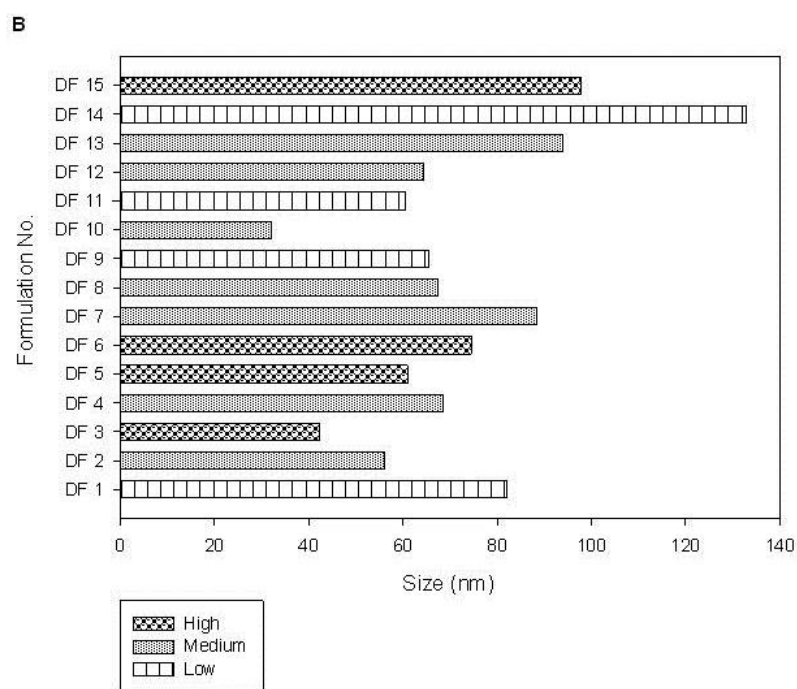
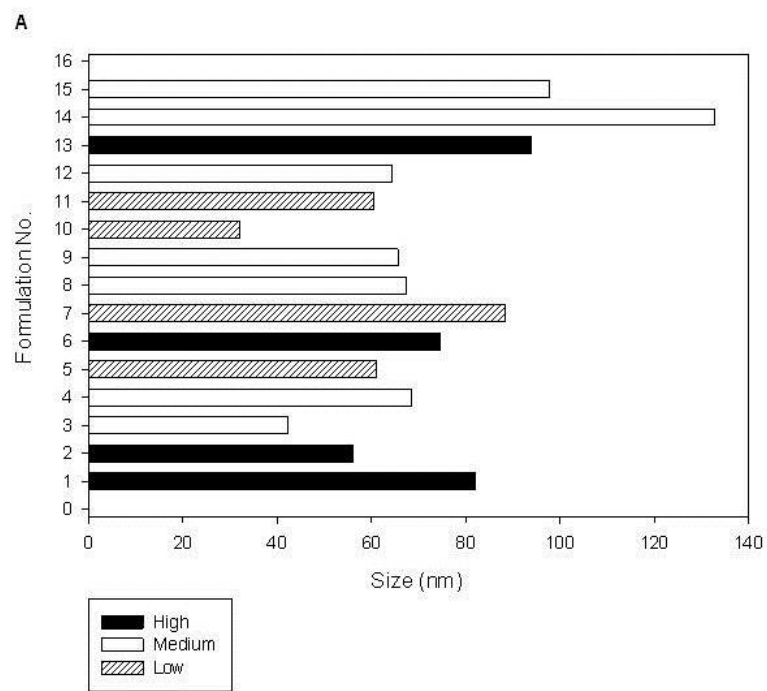
Particle size is significant as its measurement begets a direct pertinence to formulation aspects such as durability, drug entrapment, release, intracellular delivery and biodistribution (Sahana et al., 2008). The trends in terms of size comparisons of nanoparticles show that higher amount of copolymer used in formulation results in larger particle sizes (Figure 4.3). An increase in mean particle diameter can be seen from F11 to F1, F5 to F6 and F7 to F13 when the HA-g-ECL copolymer amount is increased from 150 mg to 300 mg whilst the other variables are kept constant. F10 comprised of copolymeric nanoparticles of the smallest size of 32.03 nm with 150 mg copolymer used in its formulation. It may be presupposed that the higher amount of HA-g-ECL conducted an elevation in the frequency of the collisions between the molecules which led to the union of particles in the formulation process thus raising their mean diameter. An increase in sizes was also brought about by the raise in viscosity of the organic phase which affected the interplay between the internal and external phases of the formulation. Elevating copolymer amounts or concentration increases the viscosity of the two phases causing an increase in the particle size diameter on account of

the unequal distribution of shear energy (Chakravarthi, 2008). The size of nanoparticles depends on the stability of the emulsion when it is dropped into the dispersion medium with the occurrence of collision and coalescence developing and following.

Smaller amounts of Pluronic F-127 produced nanoparticles of larger sizes. Results reflected a modest decrease in nanoparticle size when the surfactant amount increased. Comparisons between F1 and F6, F9 and F3 and F14 and F15 demonstrate this. The Pluronic F-127 is an amphiphilic non-ionic surfactant with ethylene oxide as the hydrophilic block and a central propylene oxide as the hydrophobic block (Yapar and İnal, 2012). The surfactants amphiphilic character allows it to adsorb onto the hydrophobic portion of the copolymer (ECL) while its hydrophilic property dispenses steric stabilization as a result of it possibly being solvated by the water (Sarkari et al., 2002).

The change in the volume of the solvent, DCM, used in design formulations had an inverse effect on the prepared HA-*g*-ECL nanoparticles, with lower volume giving larger size particles and vice versa. There showed considerable size increases of 37.92 nm, 55.56 nm, 56.46 nm and 67.37 nm between F13 & F2, F15 & F3, F7 & F10 and F14 & F9, respectively, when HA-*g*-ECL and Pluronic F-127 amounts were held constant. The formulation containing a small amount of DCM, being the dispersed phase, has a high viscosity and is added dropwise into the continuous phase. A higher energy magnitude is presumably necessary to partition the droplets in the emulsion into smaller ones. Thus due to the higher internal phase viscosity, mean nanosizes were increased. Formation of the smaller nanosizes with a higher DCM volume occurs because droplet coalescence is not permitted due to the spacious solvent volume readily available for diffusion into the external aqueous phase.

In summary, the size of the nanoparticles were directly proportional to HA-*g*-ECL copolymer and inversely proportional to both surfactant and solvent. Distribution profiles of Formulation 6 from the experimental design is shown in Figure 4.4. Zeta potential measurements of all formulations in the design displayed a negative surface charge in the range of -11.7 to -24.8 mv.



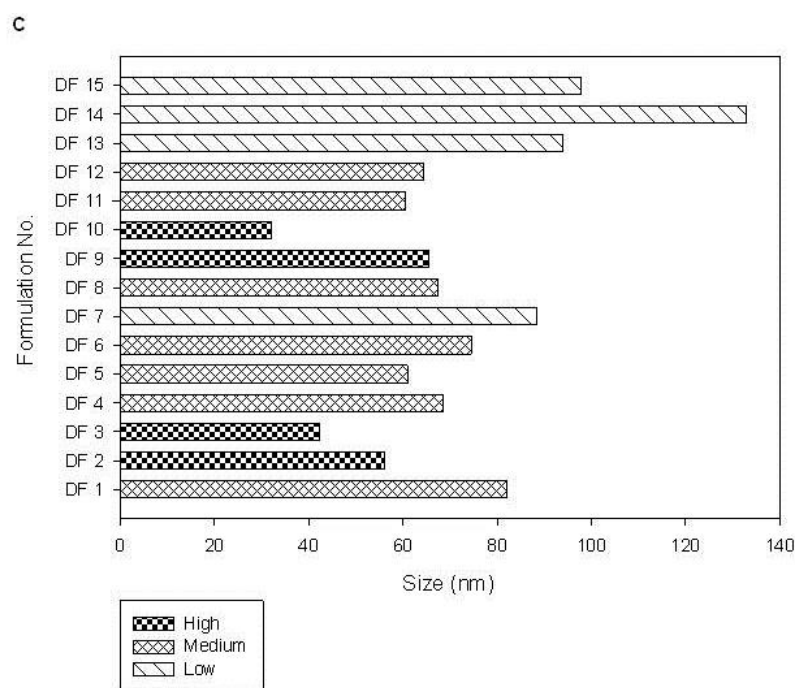


Figure 4.3: Comparison of the effect of variables on nanoparticle sizes. A) Copolymer amount, B) Surfactant amount, C) Solvent volume.

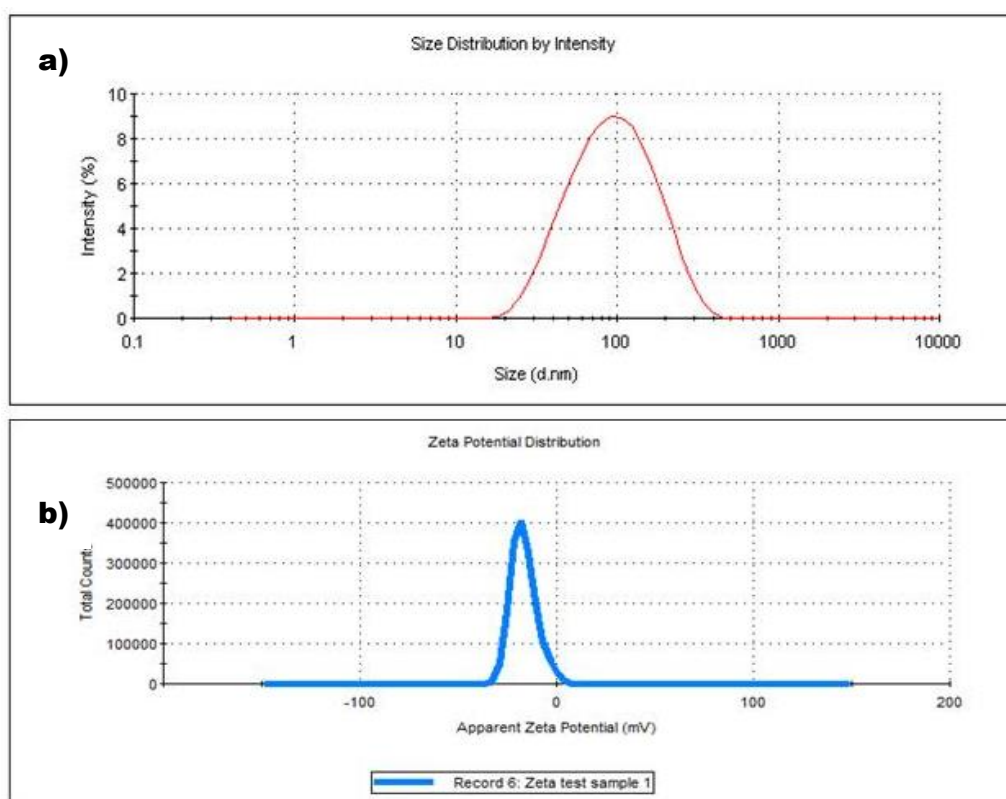


Figure 4.4: Zeta size and potential distribution profiles of Formulation 6 from the Box-Behnken design showing a) size of 74.60 ± 6.76 nm and b) zeta potential of -16.8 ± 6.55 mv.

4.3.3. Thermal characterization of HA-g-ECL copolymeric nanoparticles

4.3.3.1. Differential Scanning Calorimetry

DSC thermograms revealed slight yet fairly noticeable differences comparing maximum and minimum limits of process variables for particle design formulations. HA-g-ECL copolymer amount of 150 mg and 300 mg has been compared in F5 and F6 respectively (Figure 4.5A); surfactant amount of 1 g and 2 g has been compared in F1 and F6 respectively (Figure 4.5B) and solvent volume of 5 mL and 20 mL has been compared in F13 and F2 respectively (Figure 4.5C). These were evaluated in terms of phase transitions on each thermal curve as shown in Figure 4.5. Melting point peaks were noted to be similar at $\sim 54.50^{\circ}\text{C}$ for all formulations. Differences were seen with the glass transition thermal event. Glass transition temperature (T_g) is strongly dependent on water moisture content. With increasing water content, the T_g decreases to a certain extent (Lapčák Jr. et al., 2010). Therefore an increased copolymer amount would equal an added amount of water molecules and thus increased moisture content in the HA structure causing the T_g to decrease from around 41.48°C (F5) to 31.57°C (F6) (Figure 4.5A & Table 4.3). It is also known that T_g is reduced when dispersants such as plasticizers or surfactants are added as a result of improved flow of particles (Jadhav et al., 2009). This can be seen with F6 containing the higher surfactant amount of 2 g showing a T_g of 31.57°C as opposed to 39.21°C with the lower 1 g surfactant in F1 (Figure 4.5B & Table 4.3). Formulations comparing the effects of solvent volume reveal T_g values larger for F2 than for F13 as depicted in Figure 4.5C and Table 4.3. In essence, a greater solvent volume would cause greater particle dispersion in the aqueous phase as it is being dropped through the needle and syringe and thus leading to a higher diffusion rate. A smaller solvent volume in the internal phase would yield a higher copolymer concentration and thus increased viscosity resulting in greater tendency of the copolymeric particles to coalesce and agglomerate. The latter would eventually result in larger particles. Consequently, the rationale links to that for greater copolymer amounts compared to smaller amounts as discussed above. Larger particles would indicate greater copolymer content and thus a smaller T_g for F13 with the larger solvent volume compared to F2 (Table 4.3).

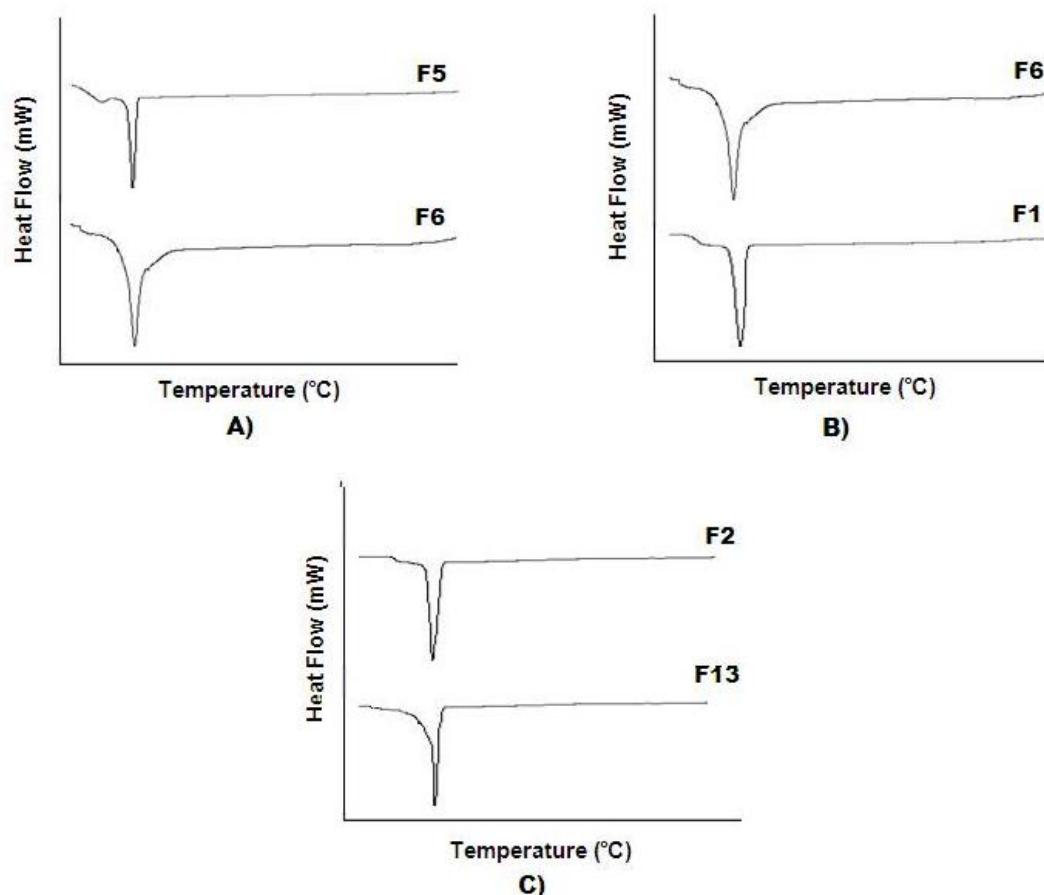


Figure 4.5: Comparison of the effect of variables on DSC. A) Copolymer amount, B) Surfactant amount, C) Solvent volume.

4.3.3.2. Thermogravimetric Analysis

Thermal decomposition curves are depicted in Figure 4.6. In all of the TGA curves it can be noted that an initial increase in weight occurs before melting and decomposition processes commence. The initial weight increase that occurs could possibly be a result of carbon impurities in the formulations undergoing chemical reactions causing the formation of non-volatile or volatile products (Gabbott, 2008). Thermal stability of formulation variables are revealed- an increase in the amount of copolymer does not significantly improve its thermal stability. A slight change of about 2.7 °C is observed for one fold increase in copolymer content (Figure 4.6A). However the onset of melting occurs first with F6. A lower surfactant amount also increases thermal stability by just approximately 2 °C (Figure 4.6B). Although there is no considerable difference noted in each design formulation due to variable parameters, all formulations did display a 3-step degradation procession. Table 4.3. summarizes the temperature ranges of each degradation step for design formulations

representing upper and lower limits of the design variables. The first gradual step of each degradation (the initial mass change) can be attributed to the evaporation of water i.e. the moisture loss on melting because HA has a high water-sorption and water retention magnitude and low molecular weight products. The main thermal event with maximum loss of sample weight occurs at the second stage. This is the apt chemical breakdown of the copolymer. The final degradation moment corresponded to the decomposing of sample material. However, a greater residual mass is seen with F1 and F6. The melting that begins with F6 compared to F5 can be attributed to the increased amount of moisture due to the higher copolymeric content of F6 and thus more H₂O molecules. This allows for evaporation first. Carbon black pigment is unable to decompose in nitrogen and is left behind. This is the residual mass. F6 has a higher copolymeric content compared to F5 resulting in greater carbon pigment and thus greater residual mass. F1 produces larger sized particles compared to F6 therefore it would also generate more residual mass. A slight poor thermal stability is seen with a formulation utilizing a greater solvent volume with polymer degradation occurring approximately 2 °C earlier compared with a smaller volume of solvent (Figure 4.6C). The reason lies in the already established explanation of the inverse relationship between particle size and solvent volume. Due to the expansive surface area of smaller compared to larger sized particles, consequently, thermal degradation would occur at a faster rate. The residual mass of either formulation were almost the same.

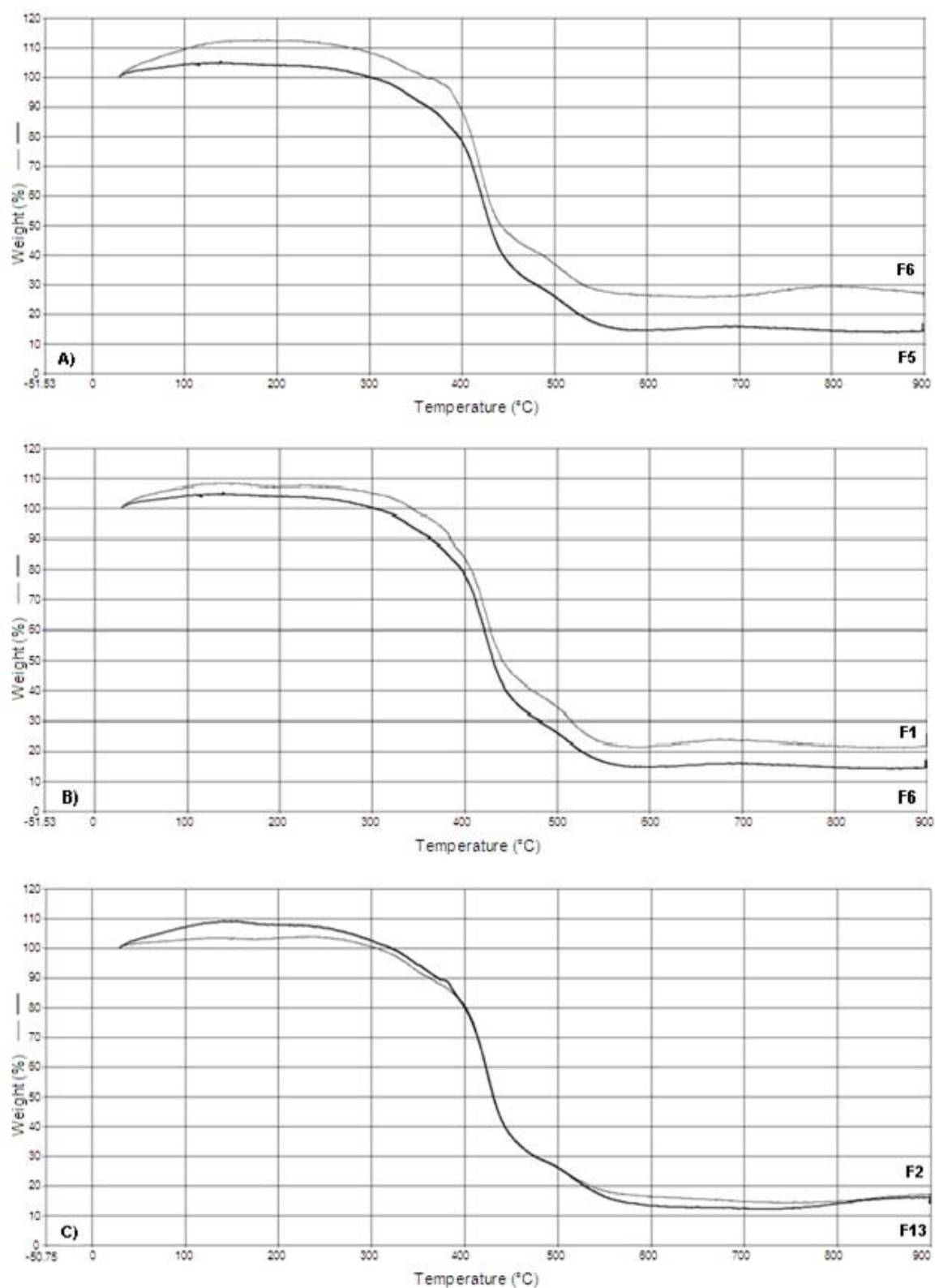


Figure 4.6: Comparison of the effect of variables on thermal degradation. A) Copolymer amount, B) Surfactant amount, C) Solvent volume.

Table 4.3: Comparative thermal events: Three step thermal degradation and glass transition temperatures of copolymeric nanoparticles.

	Degradation temperature range (°C)			¹ T _g (°C)
	1 st	2 nd	3 rd	
F1	30-220	270-480	490-600	39.21
F2	30-360	380-495	495-605	34.10
F3	30-350	360-490	495-600	35.15
F4	30-340	360-480	490-600	38.38
F5	30-340	370-460	490-550	41.48
F6	30-355	370-480	490-610	31.57
F7	30-330	340-460	480-600	42.07
F8	30-340	360-480	490-600	38.57
F9	30-340	350-480	490-600	36.92
F10	30-350	370-460	490-600	37.22
F11	30-340	370-480	495-605	37.98
F12	30-340	360-480	490-600	38.21
F13	30-385	390-490	495-600	32.56
F14	30-340	350-480	490-610	34.03
F15	30-340	350-480	495-600	33.73

¹T_g - Glass transition temperature

4.3.4. A calibration curve for LMV for evaluation of its entrapment and release

A calibration curve was plotted as depicted in Figure 4.7. This was used to ascertain the amount of LMV entrapped in HA-*g*-ECL nanoparticles. Concentrations of drug solution in PBS (0.1 M, pH 7.4) ranged from 0.003 – 0.015 µg/mL and was measured at a wavelength of 277 nm.

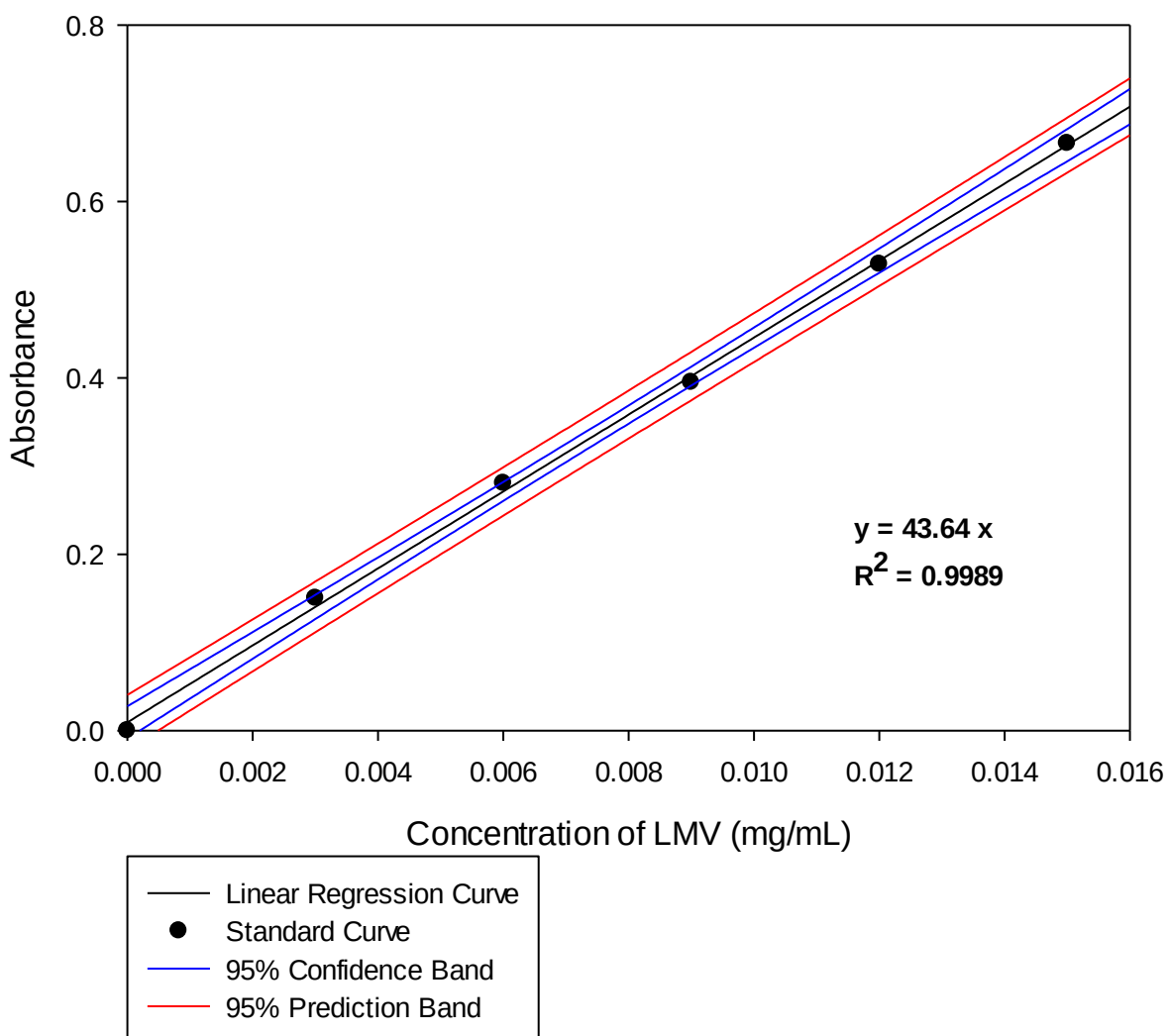


Figure 4.7: Calibration curve of LMV in PBS (0.1 M, pH 7.4) at 277 nm.

4.3.5. Drug Entrapment Efficiencies of HA-g-ECL copolymeric nanoparticles

Before trend analysis, it is evident that drug entrapment efficiency results reveal that LMV was successfully incorporated in the HA-g-ECL nanoparticles. The efficiency of entrapment of LMV varied from 18.52 ± 1.23 % to 47.77 ± 0.10 % reflected in Table 4.4. F2, 6 & 13 comprising of 300 mg of copolymer depicted highest percentage of drug entrapment (44.93 ± 1.07 %, 46.96 ± 0.94 % and 47.77 ± 0.10 %, respectively). It is evident that entrapment efficiency somewhat decreases when the amount of surfactant Pluronic F-127 is increased from 1 g to 2 g (Refer to Figure 4.10 DEE response surface plots) while other variables are fixed. When the volume of DCM increases from 5 mL to 20 mL (with additional variables

sustained) drug entrapment efficiency is virtually the same with only a very weak decrease in its percentage (Figure 4.10).

Table 4.4: Drug entrapment efficiency and particle sizes of formulations.

Formulation Code	Entrapment efficiency (%) $\pm$ SD	Particle size (nm)	PDI
F1	27.17 $\pm$ 0.55	82.13	0.260
F2	44.93 $\pm$ 1.07	56.03	0.330
F3	23.52 $\pm$ 0.72	42.28	0.381
F4	38.19 $\pm$ 0.34	68.46	0.278
F5	18.52 $\pm$ 1.23	61.07	0.463
F6	46.96 $\pm$ 0.94	74.60	0.364
F7	27.25 $\pm$ 0.67	88.49	0.261
F8	37.58 $\pm$ 0.88	67.43	0.296
F9	35.10 $\pm$ 0.14	65.58	0.436
F10	26.47 $\pm$ 0.11	32.03	0.234
F11	20.26 $\pm$ 0.59	60.59	0.431
F12	38.15 $\pm$ 0.32	64.44	0.300
F13	47.77 $\pm$ 0.10	93.95	0.234
F14	31.57 $\pm$ 0.42	132.95	0.359
F15	30.35 $\pm$ 0.91	97.85	0.356

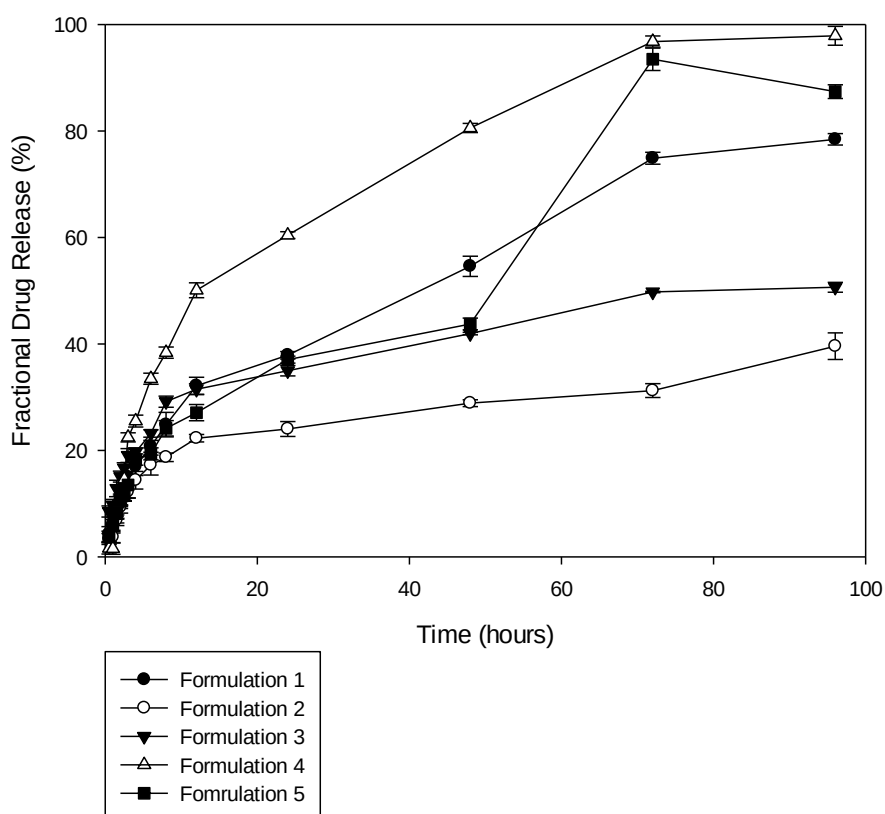
Drug entrapment efficiency results showed that elevated copolymer amounts in the emulsion formulation led to a heightening of the effectiveness in LMV encapsulation. It is plausible that the steepened viscosity level of the dispersed organic phase led to LMV being contained from leaching to the external water phase and this led to the improvement of drug entrapment efficiency (Jadhav et al., 2009). The chemical interaction between LMV and the copolymer may also have contributed to the raised entrapment efficiency percentage. If a drug has more solubility for the continuous phase it will be more inclined to this phase and migrate leading to more of the drug leaching through diffusion (Dhakar et al., 2012). The enlarged copolymer load could also grant more room to entrap LMV. Smaller particles carry a greater surface area-to-volume ratio which would increase drug adsorption on the particles surface precipitating lowered encapsulation ability and loading efficiency (Jana et al., 2014) and consequently a faster release rate, interpreted in a later discussion. Affinity of the hydrophilic drug to the large external aqueous phase is great and the shorter distance that a smaller particle provides from its center to its surface allows for rapid escape and thus low encapsulation efficiency. An elevated surfactant concentration beyond its critical micellar

concentration will diminish its effective lowering of the surface tension as well as particle size (this would therefore lower the entrapment efficiency) hence the reason for the inverse relationship of entrapment efficiency to surfactant (Ekambaram and Sathali, 2011; Sathali and Rajalakshmi, 2010). The almost constant entrapment efficiency with an increased solvent volume may correspond with the small amount of LMV (drug dose/amount is constant) in a greater unmatched volume of DCM solvent. The combined effects of variables on DEE display regions of response surface maxima in all plots and demonstrate that low to intermediate amounts of surfactant, higher amounts of copolymer and a moderate volume of solvent lead to greatest entrapment efficiency.

4.3.6. *In vitro* drug release profile of HA-g-ECL copolymeric nanoparticle design formulations

The cumulative release of LMV drug from all the 15 HA-g-ECL nanoparticles is shown as a percentage in Figure 4.8. The release of LMV eventuated in two phases: a stage of initial burst release ensued by a stage of slow and steady release. The HA-g-ECL copolymer nanoparticles manifested an initial drug release in PBS solution with a full release of drug from 6 out of the 15 design formulations (F4, 7, 8, 10, 12 and 15) occurring over a period of 4 days (96 hours). The burst effects were noted to a lesser degree (inaugural bursts of 20-30 %) in formulations 1, 2, 6 and 13 accommodating a higher amount of copolymer in correlation with formulations 5, 7, 10 and 11 (inaugural bursts of 50-65 %) harbouring the least amount of copolymer. Drug release from smallest size nanoparticle formulation F10 (32.03 nm) was observed to occur considerably rapidly in comparison to the larger sized F14 (132.95 nm) which released only 76.90 % of drug over 96 hours. The *in vitro* drug release profiles show a lagging sustained release of LMV especially with F2. The possible drug release mechanism as described by Sinha and Trehan (2003) include i) desorption either from the surface or through pores on the particle; ii) depending on the characteristics of the polymer concerned, via diffusion either by way of the unscathed polymer wall or a water-surged wall; iii) bulk degradation and the rate of polymer eroding depending on polymer properties. The prompt initial release of drug is ascribed to drug that is adsorbed to the surface of the nanoparticles. Subsequently, a diffusion-controlled delayed release phase proceeds. It is understood that the increased amount of HA-g-ECL preceded a compact and compressed polymeric state and thus burdened the ease of drug diffusion constraining the burst effect. A relatively dense structure would also prompt reduced release of the surface bound drug. A smaller amount of copolymer produces a less compact polymer phase which prompts drug migration towards the surface of the nanoparticles during the drying phase where large pores would have formed and diffusion would be independent of copolymer

control (Huang and Brazel, 2007). An accelerated release of drug was obtained with the smaller particles as these would possess a larger surface area for advanced drug migration. This lagged drug release at physiological pH 7.4 may be salutary because a prolonged release of LMV will diminish the side effects that are related to a copious uptake of LMV drug in an unspecified manner. Table 4.5 reveals the MDT values for Box-Behnken design formulations. The range spans from 14.01 – 34.28 hours with these extremes being those of Formulation 7 and Formulation 5, respectively. Formulation 7 produced particles of a larger size (88.49 nm) compared to Formulation 5 producing particles of size 61.07 nm. This falls in line with the explanation that smaller sized particles would lead to a faster release of LMV and hence a greater MDT value due to enhanced drug migration as a result of the larger surface area. The larger particles of Formulation 7 prolonged LMV release (smaller MDT value) because of the compact copolymeric structure retarding LMV release in comparison.



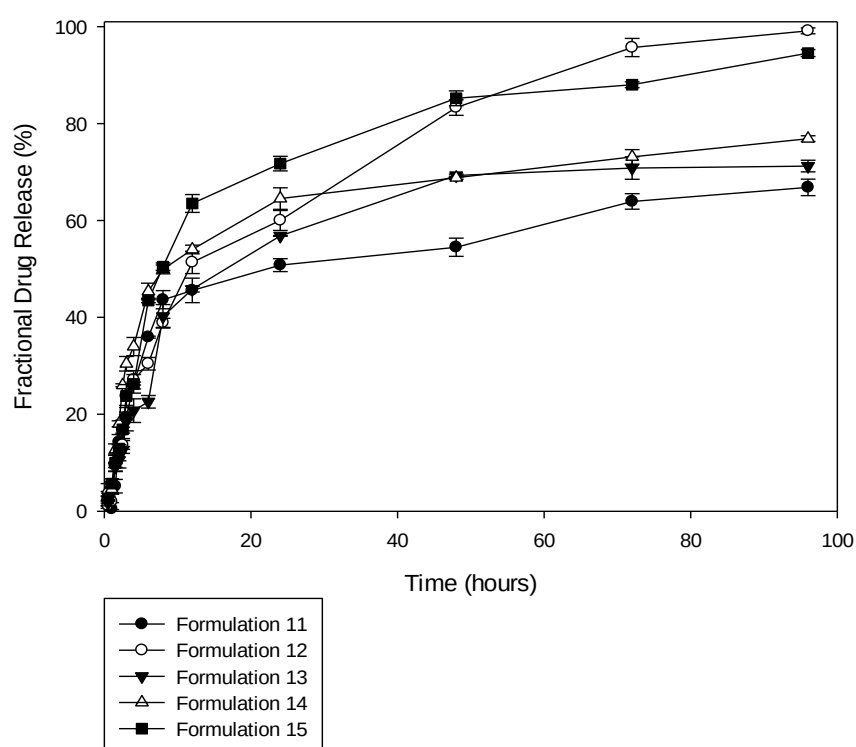
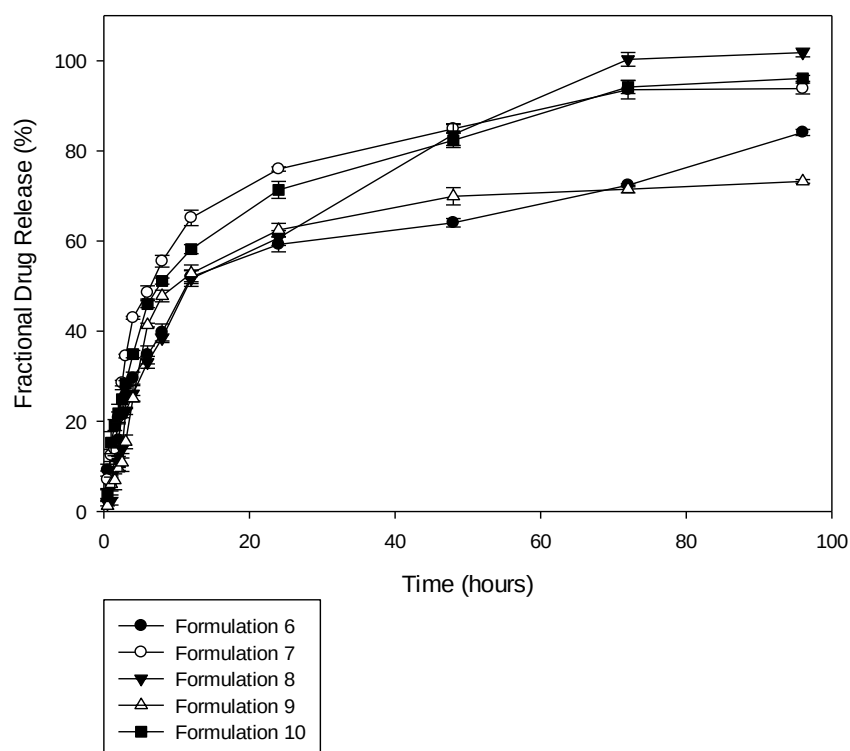


Figure 4.8: *In vitro* LMV release profiles from HA-*g*-ECL copolymeric nanoparticles. a) Formulations 1-5, b) Formulations 6-10 and c) Formulations 11-15, (SD $\leq$ 2.5 in all cases, n=3).

Table 4.5: MDT values obtained for formulations generated from the Box-Behnken design.

Formulation No.	MDT value (hrs)
1	30.22
2	28.61
3	26.08
4	22.69
5	34.28
6	24.05
7	14.01
8	23.34
9	12.53
10	17.94
11	18.20
12	23.04
13	15.66
14	14.44
15	17.60

4.3.7. Analysis of the Box-Behnken Design

Linear regression was employed to optimize the ACNS by incorporating the responses from each of the given variables into an experimental design utilizing the MINITAB® software to yield a possible optimized formulation. A prediction of variable confidence intervals were determined and their effects with the estimated *p*-values are reflected in Table 4.6. A *p*-value of a variable ≤ 0.05 is treated as significant and the response is to be noted.

Table 4.6: Variable effects with estimated *p*-values on outcomes.

Term	<i>p</i> -value		
	DEE	Size	MDT
HA-g-ECL	0.836	0.206	0.961
Plu F-127	0.087	0.105	0.381
DCM	0.602	0.032	0.217
HA-g-ECL ²	0.550	0.207	0.118
Plu F-127 ²	0.025	0.099	0.435
DCM ²	0.920	0.166	0.005
HA-g-ECL*Plu F-127	0.080	0.689	0.008
HA-g-ECL*DCM	0.842	0.371	0.141
Plu F-127*DCM	0.340	0.559	0.100

The full linear polynomial regression equations that were generated by the MINITAB® software for drug entrapment efficiency, size and mean dissolution time are as described by Equation 4.4, 4.5 and 4.6:

$$\begin{aligned} \text{Drug Entrapment Efficiency} = & -44.4737 + 0.0511[\text{HA-g-ECL}] + 74.9458[\text{Plu F-127}] + \\ & 1.0069[\text{DCM}] - 0.0003[\text{HA-g-ECL}^2] - 32.4317[\text{Plu F-127}^2] + 0.0048[\text{DCM}^2] + 0.1435[\text{HA-g-} \\ & \text{ECL*Plu F-127}] - 0.0009[\text{HA-g-ECL*DCM}] - 0.6907[\text{Plu F-127*DCM}] \end{aligned} \quad \text{Equation 4.4}$$

$$\begin{aligned} \text{Size} = & 179.972 + 0.653[\text{HA-g-ECL}] - 133.346[\text{Plu F-127}] - 10.193[\text{DCM}] - 0.001[\text{HA-g-ECL}^2] \\ & + 39.722[\text{Plu F-127}^2] + 0.141[\text{DCM}^2] - 0.053[\text{HA-g-ECL*Plu F-127}] + 0.008[\text{HA-g-ECL*DCM}] \\ & + 0.787[\text{Plu F-127*DCM}] \end{aligned} \quad \text{Equation 4.5}$$

$$\begin{aligned} \text{Mean Dissolution Time} = & -8.0034 - 0.0063[\text{HA-g-ECL}] + 17.7193[\text{Plu F-127}] + 1.3384[\text{DCM}] \\ & + 0.0004[\text{HA-g-ECL}^2] + 4.5483[\text{Plu F-127}^2] - 0.1155[\text{DCM}^2] - 0.1483[\text{HA-g-ECL*Plu F-127}] + \\ & 0.0040[\text{HA-g-ECL*DCM}] + 0.6927[\text{Plu F-127*DCM}] \end{aligned} \quad \text{Equation 4.6}$$

Additionally, Analysis of Variance on the regression and residual error from each response was carried out and is emulated in Table 4.7. The adjusted sum of squares for regression over that for residual error was large in each case of response. This implies that regression justifies the data generated. The *p*-values in all cases of responses were shown to be significant (≤ 0.05) with size and MDT displaying the most significance.

Table 4.7: Regression and residual errors ANOVA.

	DF	Adj SS	Adj MS	F	P
DEE					
Regression	9	1093.857	121.5396	5.03	0.045
Residual Error	5	120.858	24.1716		
Total	14				
Size					
Regression	9	7915.23	879.470	9.90	0.011
Residual Error	5	444.22	88.843		
Total	14				
MDT					
Regression	9	550.629	61.1810	9.22	0.012
Residual Error	5	33.173	6.6347		
Total	14				

DF- Degrees of freedom

Adj SS- Adjusted sum of squares

Adj MS- Adjusted mean squares

4.3.7.1. Residual error plot analysis for Drug Entrapment Efficiency, Size and Mean Dissolution Time

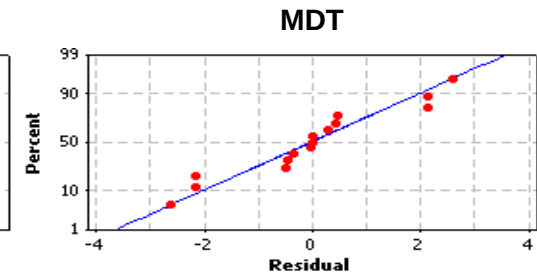
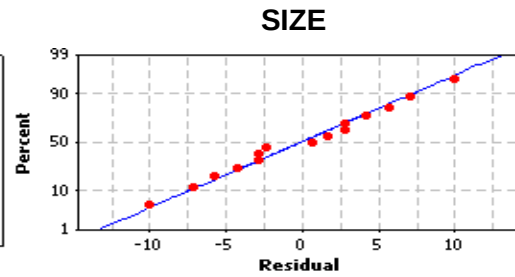
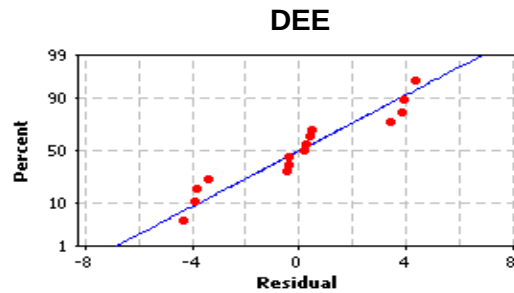
The residual plots for DEE, size and MDT design responses are shown in Figure 4.9 and evaluates the residual error of the design. The normal probability plots for all three responses displayed the scatter points all along and around the straight line which indicated that there was satisfactory distribution of residual data. The residual versus the fitted values showed random scatter around the line of 0 which meant that the error variances were uniform. Ideally, the histogram plots should be bell-shaped graph layouts and because this was not observed in all responses suggested the presence of large residual data points. The residual versus the order of the data plots reflect a non-random error and shows serial correlation that is positive for all responses.

4.3.7.2. Response surface and contour plot analysis for Drug Entrapment Efficiency, Size and Mean Dissolution Time

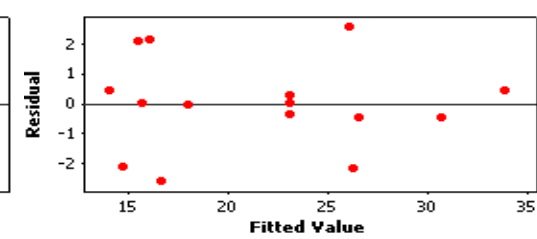
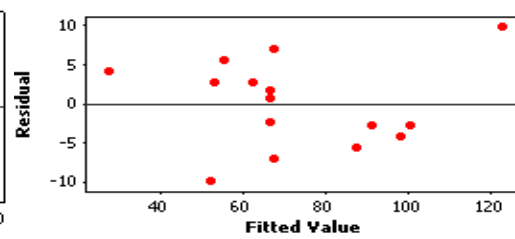
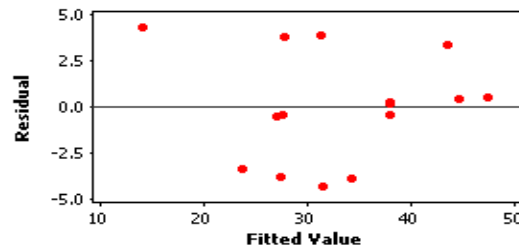
Figure 4.10 and 4.11 showcase the surface and contour plots for respective responses and their synergy with variables established by the Box-Behnken experimental design. From the plots for DEE, a significant p -value of 0.025 (Table 4.6) was obtained for the effect of

surfactant quantity on DEE (Refer to *Chapter 4, Section 4.3. Results and Discussion, Subsection 4.3.5. Drug entrapment efficiencies of HA-g-ECL nanoparticles* for discussion). A significant p -value of 0.032 was generated for the outcome that volume of solvent had on the size response and this is evident by the response and contour plots for depicted for size. A trend is noted where the volume of solvent on increasing causes a reduction in the size value and vice versa. As mentioned earlier in *Chapter 4, Section 4.3. Results and Discussion, Subsection 4.3.2. Size and Zeta Potential determination of HA-g-ECL nanoparticles by DLS*, a larger solvent volume adds space for droplets of the emulsion to be readily dispersed while dropped into the external aqueous phase, not being forced to conjoin, where larger sizes would result. The model also computed considerably significant p -values of 0.005 and 0.008 for effects on MDT by solvent volume and copolymer/surfactant quantities, respectively. Figure 4.10 and 4.11 plots clearly show a lowering of the MDT value as DCM levels lower. The link between these two lie in the explanation that lower DCM volumes cause larger particle sizes to be produced as discussed above. Larger particle sizes would lead to a more controlled release of drug and thus a smaller MDT value because of the packed and condensed copolymeric make-up of the larger particle. Similarly attested on the plots for copolymer quantity effects on MDT response, the substantiation abides.

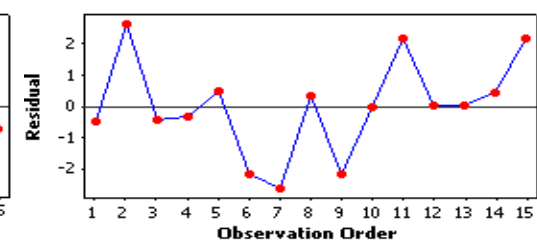
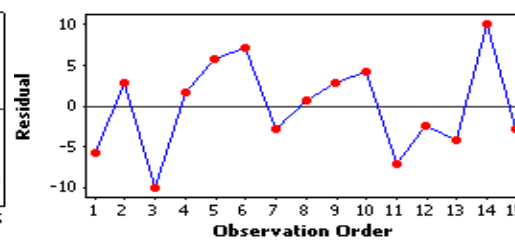
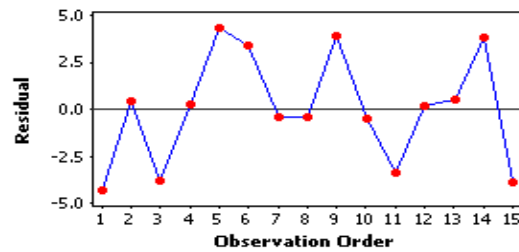
Normal
Probability Plot
of the Residuals



Residuals
Versus the



Residuals
Versus the
Order of the
Data



Histogram of

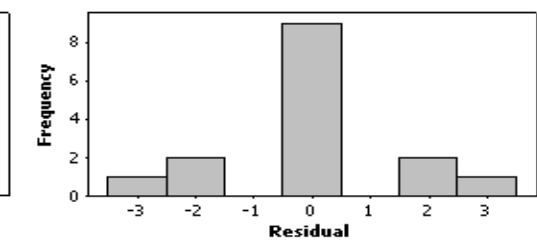
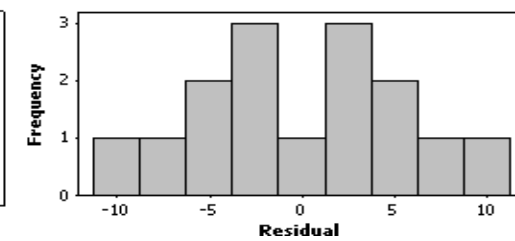
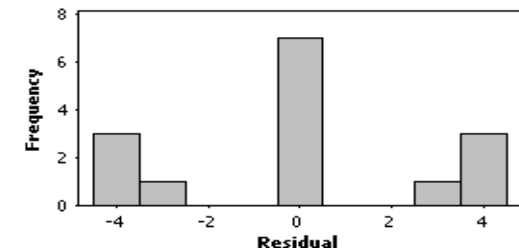


Figure 4.9: Residual plots for the design responses of size, DEE and MDT.

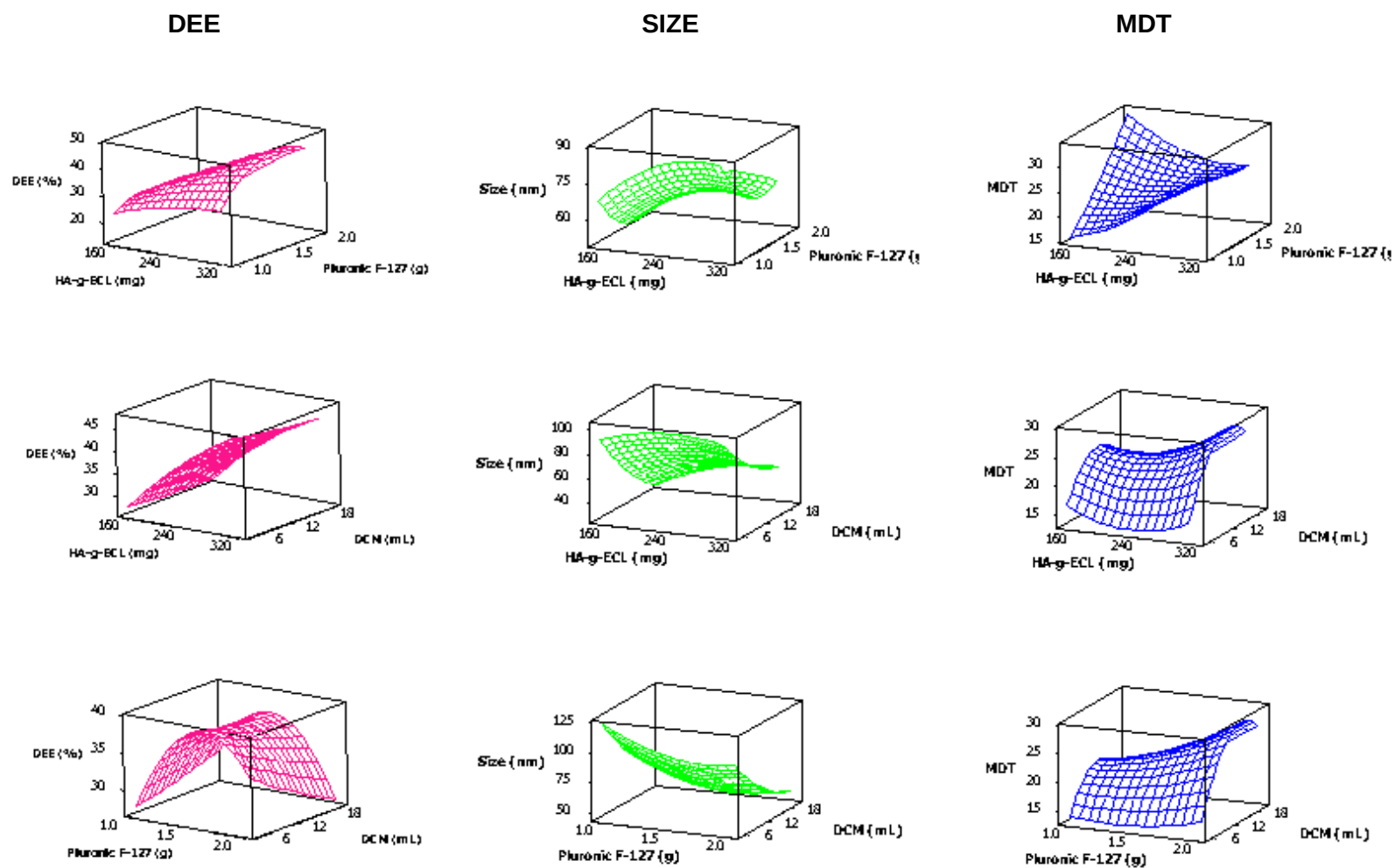


Figure 4.10: Response surface plots showcasing the effects of HA-g-ECL, Pluronic F-127 and DCM on DEE (%), Size (nm) and MDT (hrs).

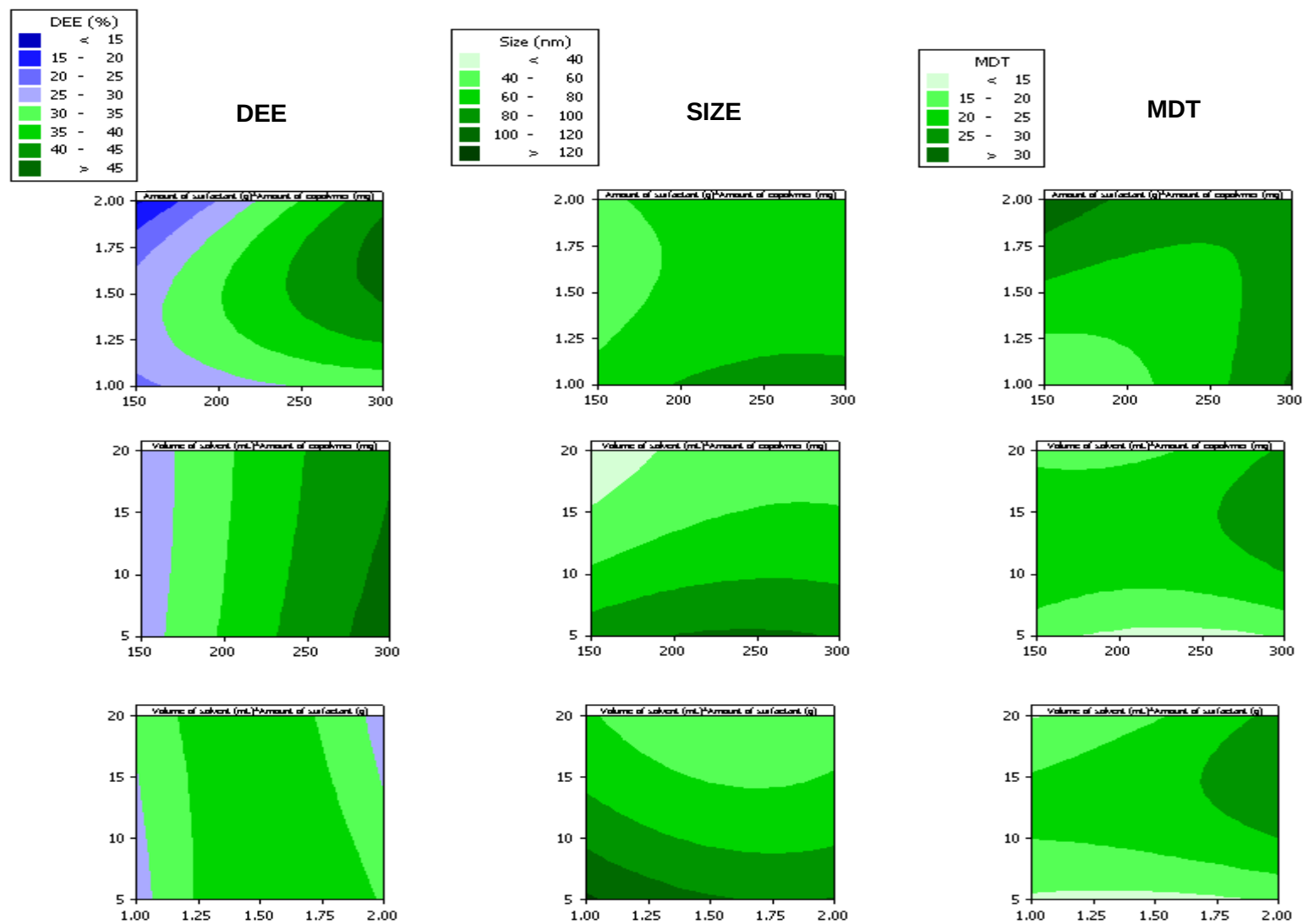


Figure 4.11: Response contour plots showcasing the effects of HA-g-ECL, Pluronic F-127 and DCM on DEE (%), Size (nm) and MDT (hrs).

4.4. Concluding Remarks

The information procured from this chapter indicated trends seen when two polymers were grafted together configuring an amphiphilic copolymer. A Box-Behnken experimental design strategy was used to generate formulations to assess these trends in terms of copolymer, surfactant and solvent quantity (variables) effects on nanoparticle size, drug entrapment efficiency and mean dissolution time (responses). The prepared nanoparticles displayed definite characteristics as a result of its constituents that were utilized in the formulation procedure. When intensifying the concentration of the element making up a particle, the average diameter of the particle will also be increased and this may be due to the rise in viscosity of the internal phase. An increase in viscosity will naturally create a retardation of movement of any substance. This would therefore affect drug entrapment and release. Pluronic F-127 enabled non-agglomeration of particles observed on TEM as well as acceptable nanometer size ranges reflected by DLS that would be ideal for intracellular delivery of the system. DCM, furthermore, accorded viscosity changes to the internal phase having a conclusive effect on nanoparticle sizes, drug entrapment and release. Surfactant and solvent quantities are crucial in the development of a drug delivery system as these impart and mend to different degrees, depending on the substances concerned in formulation: stability, solubility and consequently bioavailability of the actives. Thus, it is indeed possible to utilize the novel HA-g-ECL copolymeric nanoparticles for encapsulation and sustained release delivery of therapeutic molecules. The copolymer may have potential for various other drug delivery vehicle forms with restriction not being limited to a nanoparticle form. Surface modifiers may also allow for site-specific targeting of the HA-g-ECL based drug delivery system for directed effects. The following chapter unveils the optimal conditions for ACNS production that were determined by the Box-Behnken design.

CHAPTER 5

STATISTICAL OPTIMIZATION, *IN VITRO* CHARACTERIZATION AND ELUCIDATION OF A CHITOSAN-GRAFT-LINOLEIC ACID COATED HA-G-ECL LAMIVUDINE-LOADED COPOLYMERIC ACNS FOR TARGETED HEPATO DELIVERY

5.1. Introduction

Globally, liver contagion caused by HBV is colossal. Approximately two billion people are affected and infected by the virus with 350 million people categorized as chronic carriers. A supposed number of 1.2 million people die annually from disease caused by HBV (Lavanchy, 2004; van Tong et al., 2014). Furthermore, co-infection with HIV-1 and the hepatitis viruses has the tendency to lead to more severe consequences of chronic liver disease. The human HIV retroviruses utilize the same infection route employed by the hepatitis viruses and thus co-infection by these viruses is a frequent occurrence (Moreira et al., 2013). Currently, antiviral treatment is insufficient due to poor effectiveness of therapy and the major problem of drug resistance. In addition, when antiviral treatment is interrupted, viral cccDNA is still prevalent in hepatocytes thus viral rebound is often noticed (Cuestas et al., 2010). Therefore, there remains a need for drug delivery systems to efficiently target infected liver parenchyma.

Chitosan - a derivative of chitin, is an α (1 $\rightarrow$ 4)-linked 2-amino-2-deoxy- β -D-glucopyranose that has undergone *N*-deacetylation. The degree to which it undergoes deacetylation is varied and as a result is ultimately a copolymer product of *N*-acetylglucosamine and glucosamine. Both chitin and chitosan are polymers found in copious amounts naturally and they possess attractive properties such as those of biocompatibility, biodegradability, non-toxicity and have the capacity to adsorb onto cells in a combative manner (Dutta et al., 2004).

Essential fatty acids fall into the category of polyunsaturated fatty acids which are strictly obtained from diet. Polyunsaturated fatty acids are 18, 20 and 22 carbon chain lengths. One type of polyunsaturated fatty acid, linoleic acid (LA), contains an 18 carbon chain with 9 and 12 positioned double bonds in *cis* arrangement along its length (Du et al., 2009). These polyunsaturated fatty acids have approvingly presented anticarcinogen effects and repressed effects on atherosclerotic contusions as a result of heightened immunity effects, reduced body

fat and increase in lean body mass observed in many animal models (Aydin, 2005; Du et al., 2009).

Liver plays a central role in body metabolism. LA in the body first concentrates in the cells of the liver and thus plays a pivotal role in the hepatic environment (Thomas et al., 1988). Accordingly, LA is sought as a trigger ligand to arbitrate internalization into hepatic cells (Cheong et al., 2009). The manner in which this occurs is via recognition pathways utilizing the ASGP-R found on hepatocytes and a biochemical lipid metabolism pathway involving cholesterol and triglyceride synthesis once LA is concentrated in hepatocytes (Cheong et al., 2009; Lee et al., 2009a). LA has also been shown to increase transfection efficiency of lipid nanoparticles when incorporated into the system for hepatic delivery (Yu et al., 2012).

Chitosan and LA have been paired in numerous studies as a result of communal positive properties in diverse areas of biomedical applications. A study demonstrated the effect of LA grafted to chitosan oligosaccharide prepared as micelles for delivery of doxorubicin into tumor cells. Favorable results were obtained with doxorubicin contained in these micelles compared to a solution of doxorubicin hydrochloride alone (Du et al., 2009; Du et al., 2011). Another study needed an efficient topical delivery system for dermal regeneration and repair of tissue. Chitosan was hydrophobically altered with LA and oleic linkage to demonstrate a positive tissue repairing capability (Bonferoni et al., 2014). Hydrogel nanoparticles can also be made up by LA chemically modified with chitosan for reliable encapsulation of trypsin (Liu et al., 2005). Principally and with relevance to this study, the polymer pair have been implicated conjointly for a hepato-targeting ability. A study that has utilized these polymers in similar manner is one that developed a hepatocyte targeting magnetic resonance imaging contrast agent utilizing water soluble chitosan conjugated to LA that was developed into superparamagnetic iron oxide nanocrystals and nanoparticles (Cheong et al., 2009; Lee et al., 2009a).

A scaling down of the vast experiment numbers as a result of vast variable parameters is resolved by the DOE approach. Thus, implementing DOE is advantageous in refining experiments for designing an optimal formulation especially due to complicated glitches that occur as well as the costly procedures that are necessary for experimenting (Kincl et al., 2005). In this chapter chitosan and LA will be graft polymerized and utilized as a chemically adsorbed coating on an optimized ACNS, to capitalize on the physicochemical properties of these compounds in favor of liver and thus HBV targeting.

5.2. Materials and Methods

5.2.1. Materials

Hyaluronic acid sodium salt from *Streptococcus equi*, epsilon-Caprolactam ($\geq 99\%$), Poly(ethylene glycol) methyl ether (mPEG) (average $M_w \sim 2000$ Da), Dialysis tubing cellulose membrane ($M_w 12044$ Da), *N*-acetylcaprolactam (99%), Sodium hydride (NaH) (powder moistened with oil, 55-65 % gas volumetric), Pluronic F-127, Chitosan low molecular weight (75-85 % deacetylated), Linoleic acid ($\geq 99\%$), Acetic acid (glacial, $\geq 99.85\%$), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, crystalline (EDC) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Lamivudine (LMV) was received as an *ex-gratia* sample from Adcock Ingram Ltd. (Germiston, Gauteng, South Africa). Dimethyl sulphoxide (DMSO), D-Mannitol ($\geq 99\%$) ($M_w 182.17$ g/mol) and Dichloromethane (DCM) were purchased from Merck Chemicals (Pty) Ltd. (Germiston, Gauteng, South Africa). All other chemicals and reagents were of analytical grade.

5.2.2. Preparation of an optimized LMV-loaded ACNS by the Box-Behnken design strategy

5.2.2.1. Response Optimization

An optimized formulation encompassing constraints that ensured the smallest nanometer sized particles (≤ 100 nm), the greatest drug entrapment efficiency and a sustained LMV release was acquired by use of statistical software (Minitab[®], V14, Minitab Inc[®], PA, USA). These constraint settings are displayed in Table 5.1. Figure 5.1 portrays desirability plots of the single optimal formulation generated as a result of constraint optimization. The optimal desirability value was shown to be 0.959.

Table 5.1: Constraints employed for optimized formulation.

Variable Response	Level
Size (nm)	Minimize
DEE (%)	Maximize
MDT (hrs)	Minimize

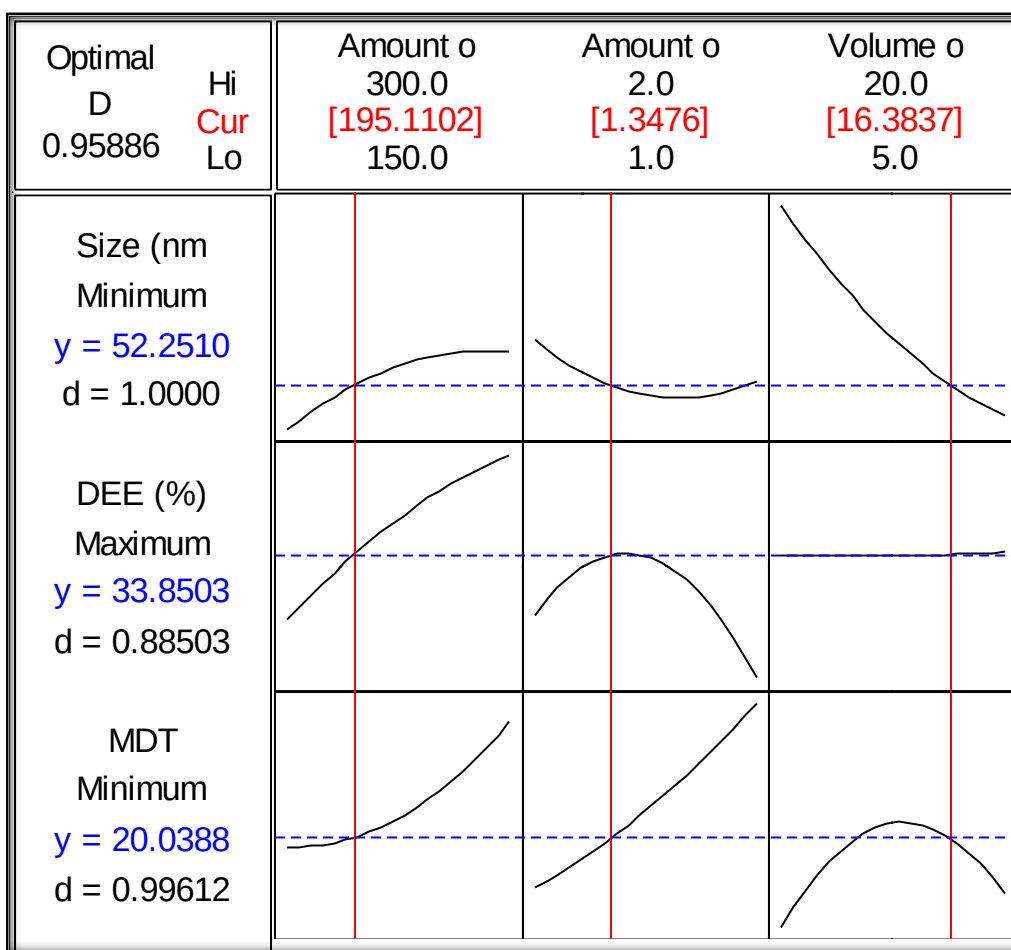


Figure 5.1: Desirability plots demonstrating variable values required for optimized formulation preparation and desirability values expected for responses.

5.2.2.2. Generation of the optimum ACNS

A representation of the quantities derived from response prediction for the optimal copolymeric nanoparticle formulation is shown in Table 5.2.

Table 5.2: Optimized LMV-loaded HA-*g*-ECL copolymeric nanoparticle formulation developed by the response surface technique.

Formulation Components	Optimized formulation
HA- <i>g</i> -ECL copolymer (mg)	195.11
Pluronic F-127 surfactant (g)	1.35
DCM solvent (mL)	16.38

5.2.3. HA-*g*-ECL amphiphilic graft copolymer synthesis

The copolymerization process was carried out as outlined in *Chapter 3, Sections 3.2.2. Desalting process of hyaluronic acid, 3.2.3. PEG-assisted solubilization of HA in DMSO and 3.2.4. Anionic graft (co)polymerization of HA and ECL (HA-*g*-ECL).*

5.2.4. Drug-loaded HA-*g*-ECL copolymeric nanoparticle preparation

The preparation of nanoparticles was carried out as described in *Chapter 4, Section 4.2.4. Preparation of LMV-loaded HA-*g*-ECL copolymeric nanoparticles*, utilizing the optimized variable quantities according to Table 5.2.

5.2.5. Chemical modification of chitosan through graft synthesis with linoleic acid producing Chitosan-Linolate

The grafting of linoleic acid to the polymer, chitosan was carried out according to the method by Kim and co-workers (2011) with slight modification. Low molecular weight chitosan (1 %^w/_v) was dissolved in 1 %^v/_v dilute aqueous acetic acid. A volume of 20mL chitosan solution was used with 0.77 mL linoleic acid for the graft procedure. The linoleic acid was dissolved in 36.80 mL DMSO. The chemical link, EDC, was added into the chitosan solution at a weight of 0.394 g and was made to dissolve. The two solutions were added together and grafting of linoleic acid to chitosan was performed through EDC chemistry at pH 4.75 through vigorous stirring for 24 hours. C-LA was received after its precipitation in 200 mL organic solvent (32 % Ammonia solution). The product was centrifuged at 5000 rpm (Table Top High Speed Centrifuge (TG16-WS), Hunan Xiangyi Laboratory Instrument Development Co., Ltd, Hunan, China) for 10 minutes three times washing with deionized water at each interval to remove any trace of

organic solvent. C-LA was then lyophilized to obtain a semi-stiff cotton wool-like textured product (Figure 5.2). An illustration of the grafting synthesis of C-LA is depicted in Figure 5.3.



Figure 5.2: Digital image of A) synthesized lyophilized C-LA in physical form.

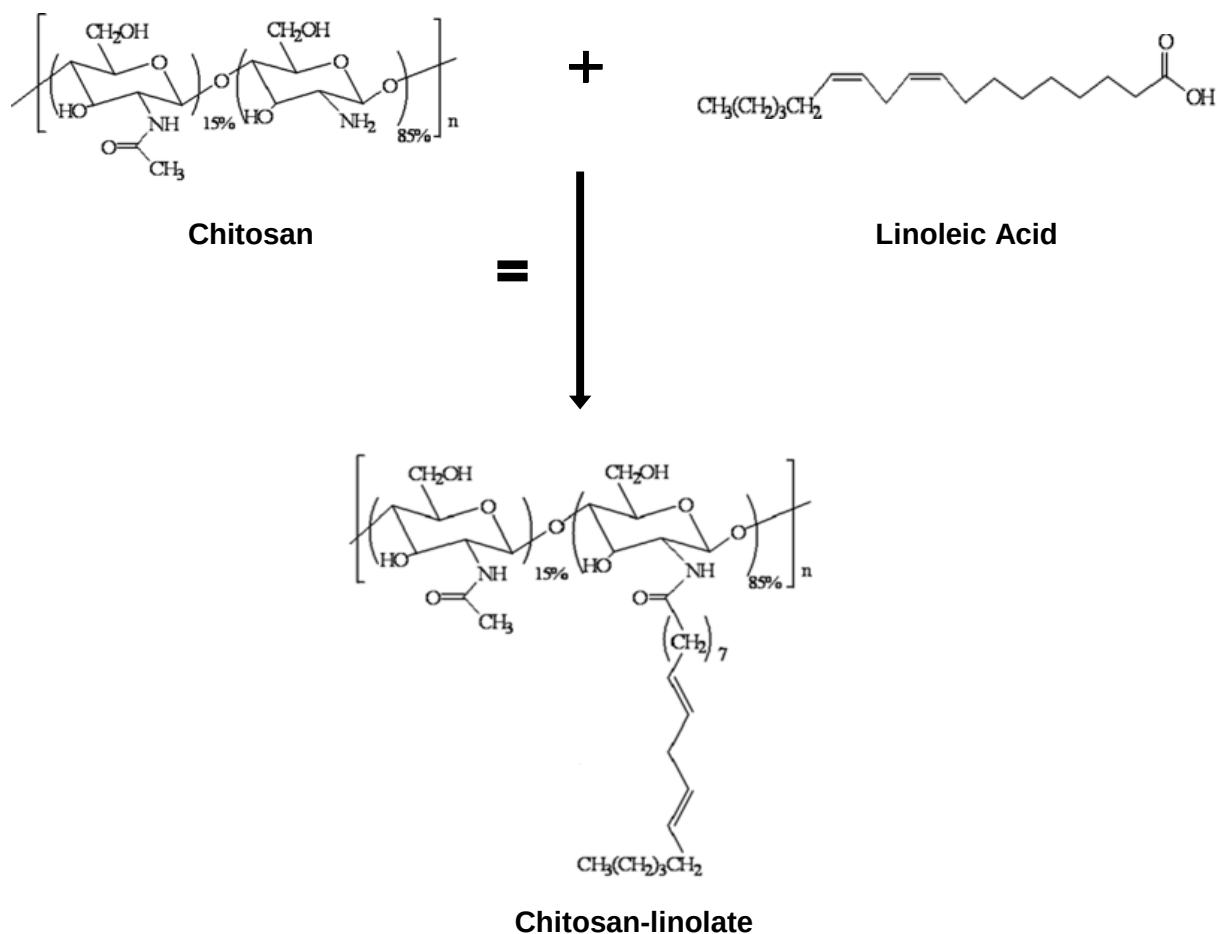


Figure 5.3: Schematic of the synthesis of C-LA (Adapted from Kim et al., 2011).

5.2.6. Selection process for a suitable cryoprotectant in favor of HA-g-ECL nanoparticles size maintenance

A range of cryoprotectants at a range of different concentrations were tried and tested to maintain the size of formed HA-g-ECL nanoparticles as this is one of the significant variable responses of the three that must be kept constant for targeted hepatic cell delivery. Table 5.3 shows various cryoprotectants at their respective concentrations that were added to Formulation 7 of the Box-Behnken design acquiring a size of 88.49 nm. These were added into the formulation before freezing at -80 °C for 24 hours and lyophilizing. Thereafter, powdered cryoprotected HA-g-ECL nanoparticles were redispersed in the same volume of liquid initially

held in before freezing. Cryoprotectants were evaluated by their effect on nanoparticles in terms of their flowability, redispersibility and size maintenance.

Table 5.3: Cryoprotectants used for HA-*g*-ECL nanoparticle size maintenance assessment.

Cryoprotectant	Concentration (%^w/_v)
Sucrose	3
	16
	30
Glucose	3
	16
	30
Lactose	1
	3
	8
Mannitol	3
	10
	20
Pluronic F-127	1

5.2.7. Preparation of C-LA coating and coated optimized HA-*g*-ECL ACNS

Lyophilized 2 %^w/_v C-LA was made to dissolve in 1 %^v/_v dilute acetic acid with magnetic stirring for 3 hours. Powdered HA-*g*-ECL nanoparticles (10.5 mg) with cryoprotectant was added to and redispersed in the C-LA coating solution. The solution was taken off the magnetic stirrer and left in a dark cupboard for 24 hours to allow for chemical adsorption of C-LA onto HA-*g*-ECL nanoparticles to occur. The coating-nanoparticle solution was then centrifuged on high (15000 rpm, 99 minutes) (Universal 320R, Hettich Zentrifugen, Germany) to remove excess coating from the nanoparticles.

5.2.8. Determination of molecular vibrational bands utilizing Fourier Transform Infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) analysis was employed to obtain infrared spectra of native chitosan, native linoleic acid and synthesized C-LA as well as the optimized ACNS, coated optimized ACNS, LMV and non LMV-loaded optimized ACNS. The vibrational characteristics of the samples were detected in response to infrared radiation allowing for interpretation of the quality and consistency of the sample compounds. Infrared spectra were created on a Spectrum 100 FTIR Spectrometer (PerkinElmer Life And Analytical Sciences Inc., Shelton, CT USA).

5.2.9. Transmission Electron Microscopy imaging of the optimized C-LA coated ACNS

TEM imaging of the coated optimized ACNS was carried out as outlined in *Chapter 4, Section 4.2.5. Transmission Electron Microscopy (TEM) of the formed copolymeric nanoparticles.*

5.2.10. Thermal analysis employing Differential Scanning Calorimetry

Optimized samples were thermally analyzed according to the method described in *Chapter 4, Section 4.2.7, Subsection 4.2.7.1. Differential Scanning Calorimetry.*

5.2.11. Thermal analysis employing Thermogravimetric Analysis

Optimized samples together with their individual constituents were thermogravimetrically analyzed as described in the method under *Chapter 3, Section 3.2.8 Thermogravimetric analysis.*

5.2.12. Dynamic Light Scattering size and zeta potential determination of the optimized ACNS

DLS measurements of optimized samples before and after coating were performed as described in *Chapter 4, Section 4.2.6. Size and Zeta Potential determination via Dynamic Light Scattering (DLS).*

5.2.13. Drug Entrapment Efficiency of the optimized ACNS

According to the method outlined in *Chapter 4, Section 4.2.9. Drug encapsulation efficiency (DEE) of HA-g-ECL nanoparticles*, the optimized coated and uncoated ACNS formulations were tested for their DEE.

5.2.14. *In vitro* drug release profile of the optimized ACNS

Optimized coated and uncoated ACNS formulations were subjected to drug release profiling according to the process described in *Chapter 4, Section 4.2.10. In vitro dissolution studies of LMV-loaded HA-g-ECL nanoparticles*.

5.3. Results and Discussion

5.3.1. Predicted and Observed values for the optimized ACNS

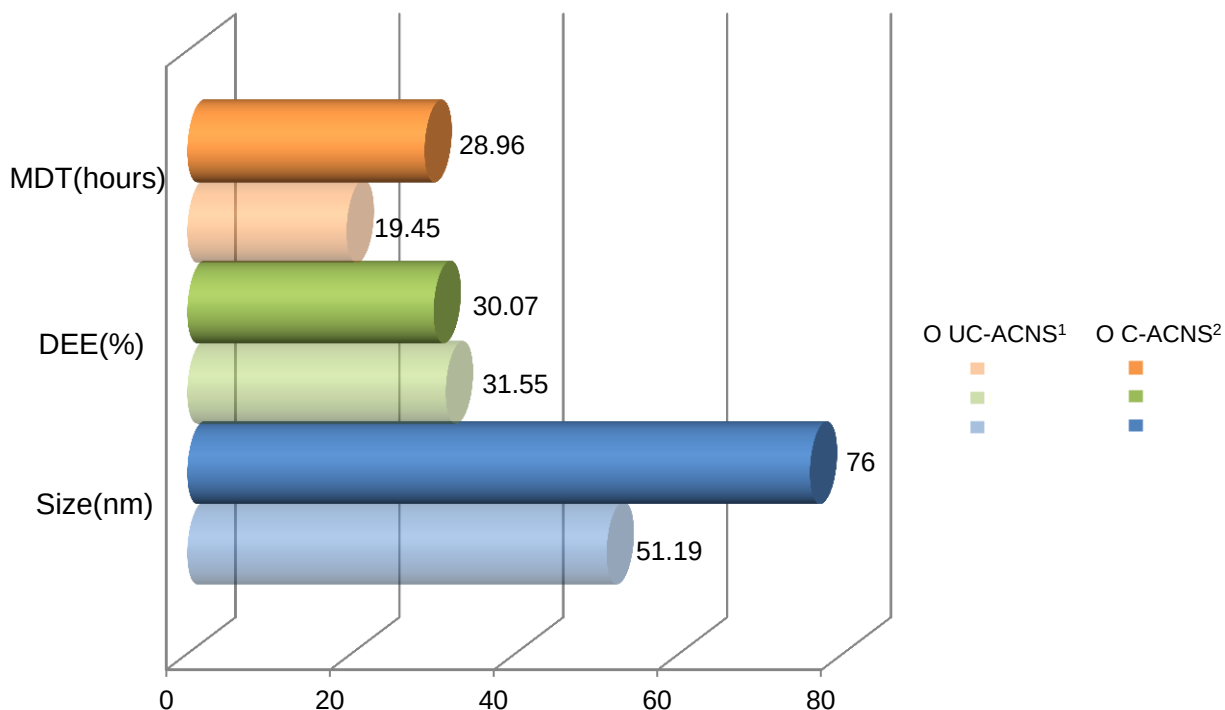
An ACNS formulation was optimized by way of variable response optimization utilizing the Minitab® statistical software (Minitab®, V14, Minitab Inc®, PA, USA). The optimal formulation computed from the programme constituted quantity use of 195.1102 mg HA-g-ECL, 1.3476 g Pluronic F-127 and 16.3837 mL DCM. Table 5.4 portrays the predicted and observed measurements achieved with their corresponding desirability value. All of the results were good with the best desirability of 100 % achieved for size response and the least desirable (yet acceptable) of 93.21 % obtained for DEE.

Table 5.4: Predicted experimental and desirability measurements of the optimized HA-g-ECL based nanoparticle formulation.

Measured Variable Response	Predicted Measurement	Observed Measurement	Desirability (%)
Size (nm)	52.25	51.19±4.37	100
DEE (%)	33.85	31.55±3.10	93.21
MDT (hrs)	20.04	19.45±1.59	97.01

Bearing in mind that the targeting for the drug to the liver for HBV eradication involves the coating of the ACNS which occurs after optimization of the plain uncoated ACNS, a comparison of the C-LA coated ACNS was made to the optimized plain ACNS which is shown in Figure 5.4. Variations in results were noted with an expected increase in size of approximately 25 nm to the

ACNS due to the addition of diameter to the structure as a result of the chemical adsorption of the C-LA coat. This was still acceptable being below 100 nm for hepatocyte internalization. DEE reflected an approximate 1.5 % reduction which could be overlooked and MDT showed a considerable increase in hours of approximately 9.5 which was most desired as the response optimization goal for the ACNS initially was a maximized MDT value. This was also expected due to the coat serving as a barricade to LMV and sustaining its release from the ACNS structure.



¹O C-ACNS- Optimized coated architecturally configured nanoparticulate system

²O UC-ACNS- Optimized uncoated architecturally configured nanoparticulate system

Figure 5.4: Bar graph illustrating the comparative assessment of observed measurement responses between optimized coated and uncoated ACNS.

5.3.2. Selection of a suitable cryoprotectant to limit the increase of ACNS size

A variety of cryoprotectants were evaluated for their ability to sustain the size of formed ACNS nanoparticles due to freeze-drying processes that alter its properties causing agglomeration in aqueous redispersion and thus a defective size increase. Sucrose, glucose, lactose and

mannitol sugars as well as 1 % Pluronic F-127 solution, were prepared in concentrations depicted in Table 5.5. The cryoprotectants were assessed on their capacity to efficiently cause the freeze-dried ACNS to flow, redisperse and maintain its initial size (Formulation 7 of 88.49 nm from the experimental design) before freezing at -80 °C and freeze-drying. Results indicate the optimal cryoprotectant to be 20 % mannitol which caused only a slight increase in size of the ACNS by 0.97 nm in addition to the ACNS demonstrating good flowability and redispersibility. Mannitol at this concentration was thus employed in preparation of the ACNS for the subsequent stage of formulation with C-LA coating.

Table 5.5: Estimation of cryoprotectants flowability, redispersibility and size maintenance functional ability for optimized ACNS.

Cryoprotectant	%w/v	Flowability	Redispersibility	Size (nm)	PDI
Sucrose	3	++	+	97.24	0.322
	16	+	-/+	106.5	0.247
	30	-/+	-/+	117.56	0.235
Glucose	3	-	++	99.58	0.610
	16	-/+	+	122.6	0.286
	30	-/+	+	118.3	0.236
Lactose	1	-/+	+	95.19	0.410
	3	+	++	164.83	0.257
	8	++	++	173.06	0.263
Mannitol	3	-/+	+	108.40	0.288
	10	+	+	99.15	0.352
	20	+	+	89.46	0.257
Pluronic F-127	1	-	-/+	143.1	0.781

---Lump formation; --Very poor; -Poor; -/+Moderately good; +Good; ++Very good

5.3.3. Structural variation investigations via Fourier Transform Infrared spectroscopy analysis

The synthesized C-LA was analyzed with FTIR to ascertain the graft polymerization occurrence between chitosan and LA. Figure 5.5 depicts the spectra obtained for plain chitosan (a), plain LA (b) and the formed C-LA (c). Figure 5.5a demonstrated the characteristic chitosan spectrum displaying the OH vibration at 3286 cm⁻¹, CH stretching vibration at 2868 cm⁻¹, C=O stretching

vibration at 1624 cm^{-1} and the NH of amide stretching vibration at 1558 cm^{-1} (Kim et al., 2011). Figure 5.5b also shows that LA displayed characteristic peaks at 1708 cm^{-1} which resembled the carboxylic acid group, 2854 cm^{-1} and 2923 cm^{-1} frequencies which correlated with the CH stretching vibration and 3008 cm^{-1} which was the alkene peak on the molecule (Kim et al., 2011). Proof that LA grafted onto the structural backbone of chitosan was sought by the disappearance of the carboxylic acid peak at 1708 cm^{-1} suggesting the attachment of the molecule at this point on the chitosan chain. There was also evidence of an increased peak intensity at 1648 cm^{-1} which corresponded to a stronger carbonyl peak as a result of the interaction of chitosan and LA as well as the appearance of an acrylate double bond at 900 cm^{-1} . These FTIR findings indicated successful graft copolymerization between chitosan and LA to form C-LA. Figure 5.6 displays the FTIR spectra of optimized coated and uncoated formulations comparatively with C-LA and LMV. The difference in spectra between the coated and uncoated drug loaded formulations (Figure 5.6a & d) is the intensity of the peak in a) at 1637 cm^{-1} that is larger than the same peak seen in d). This peak in either formulation corresponds to the drug peak at 1631 cm^{-1} which is rightly so as both uncoated and coated ACNS were LMV-loaded. However, the intensity variation occurs because of the C-LA peak at 1648 cm^{-1} which is being detected by infrared radiation at the same point on the coated ACNS resulting in the heightened intensity. Moreover, the broad OH vibration of C-LA is also seen at the same frequency range in the coated ACNS formulation suggesting again that C-LA did indeed exist on the ACNS structure. These raised intensities are unseen on the spectra of the non coated LMV-loaded ACNS.

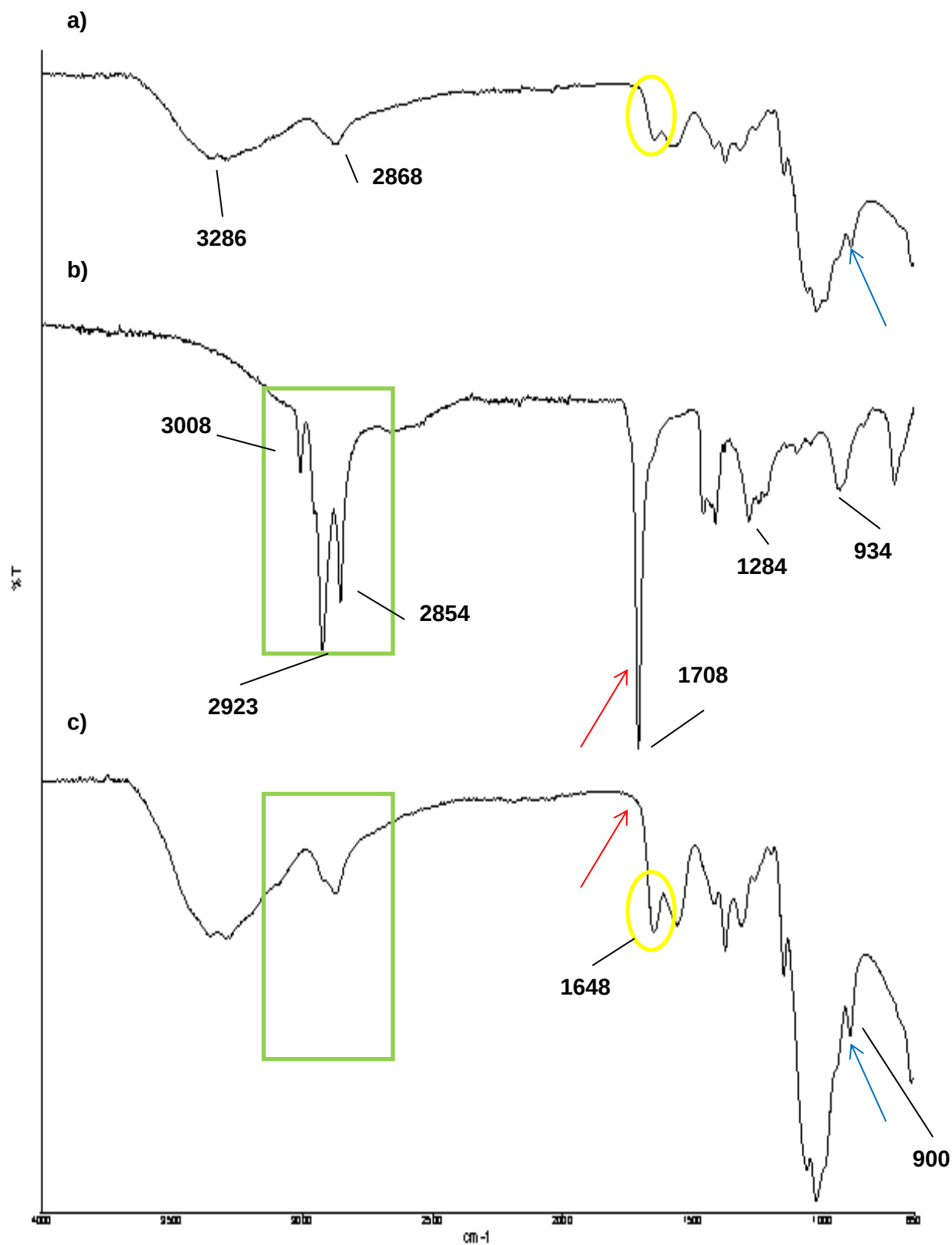


Figure 5.5: FTIR spectra of a) native chitosan, b) native linoleic acid and c) C-LA.

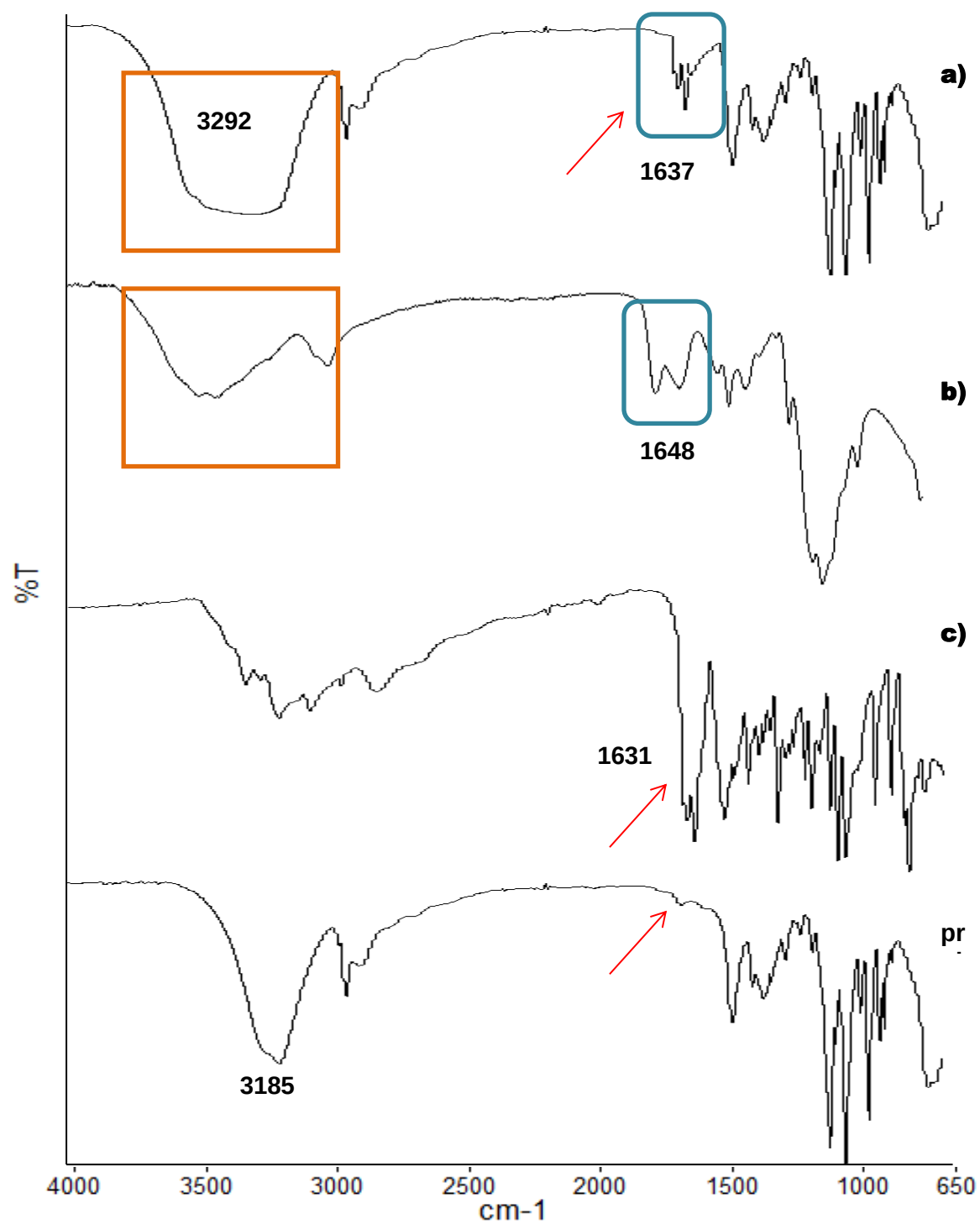


Figure 5.6: FTIR spectra of a) C-LA coated LMV-loaded ACNS, b) C-LA, c) LMV and d) uncoated LMV-loaded ACNS.

5.3.4. Morphological investigations of optimized C-LA coated ACNS utilizing Transmission Electron Microscopy

The TEM image depicted in Figure 5.7 revealed mostly spherically-shaped C-LA coated ACNS particles which were favorably non-agglomerated and properly individualized relaying clarity of ACNS morphology. The C-LA coat can be clearly seen as it imparts the darkened color onto nanoparticles implying and confirming its chemical adsorption onto plain HA-*g*-ECL ACNS. Figure 5.7 also discloses the size of the C-LA coated ACNS later seen to correlate with DLS measurements of size distribution and zeta potential profiling. The transmission electron micrograph was taken at 42000X magnification and it can be estimated by the scale bar that sizes of C-LA coated ACNS particles were in the range of 70-80 nm.

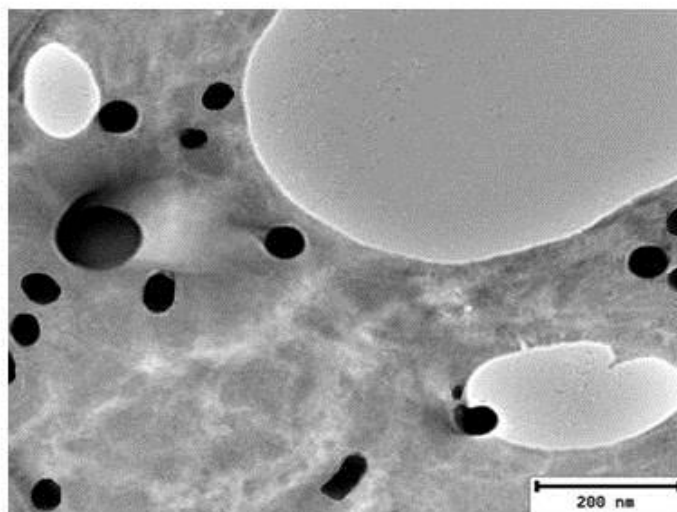


Figure 5.7: Transmission electron micrograph of optimized C-LA coated ACNS.

5.3.5. Differential Scanning Calorimetry thermogram analysis of C-LA and the optimized ACNS

The thermal profiling from DSC is disclosed in Figure 5.8 and Figure 5.9. By comparison, they depict the curves of indigenous polymers to their derivative graft products as well as the LMV-loaded optimized ACNS in its C-LA coated and uncoated form. Figure 5.8 distinguishes between the low molecular weight chitosan and the amorphous LA from its graft product C-LA.

Figure 5.8a displays the broad endotherm of chitosan at 103.76 °C that would correspond to the dehydration of the water molecules on the compound. Its exotherm at the onset temperature of 283.78 °C conforms to decomposition of the molecule. Linoleic acid in Figure 5.8b shows no thermal event until around 260 °C which may correlate to the boiling of LA and/or a fraction of thermal decomposition of the fatty acid followed by a broad exothermic transition at around 280 °C which may correspond to the decomposition products (Kapusniak and Siemion, 2007). The graft copolymerization of LA onto chitosan can be deduced from the new endothermic transition that occurs at a peak maxima of 167.80 °C. This is most probably representative of a melting endotherm as the first broad endothermic transition observed at a ~100 °C can be labelled the T_g as a result of LA's amorphous nature contributing to it. The melting endothermic peak occurs because of the solid nature imparted on the C-LA product and thus an existing but very low extent of crystallinity.

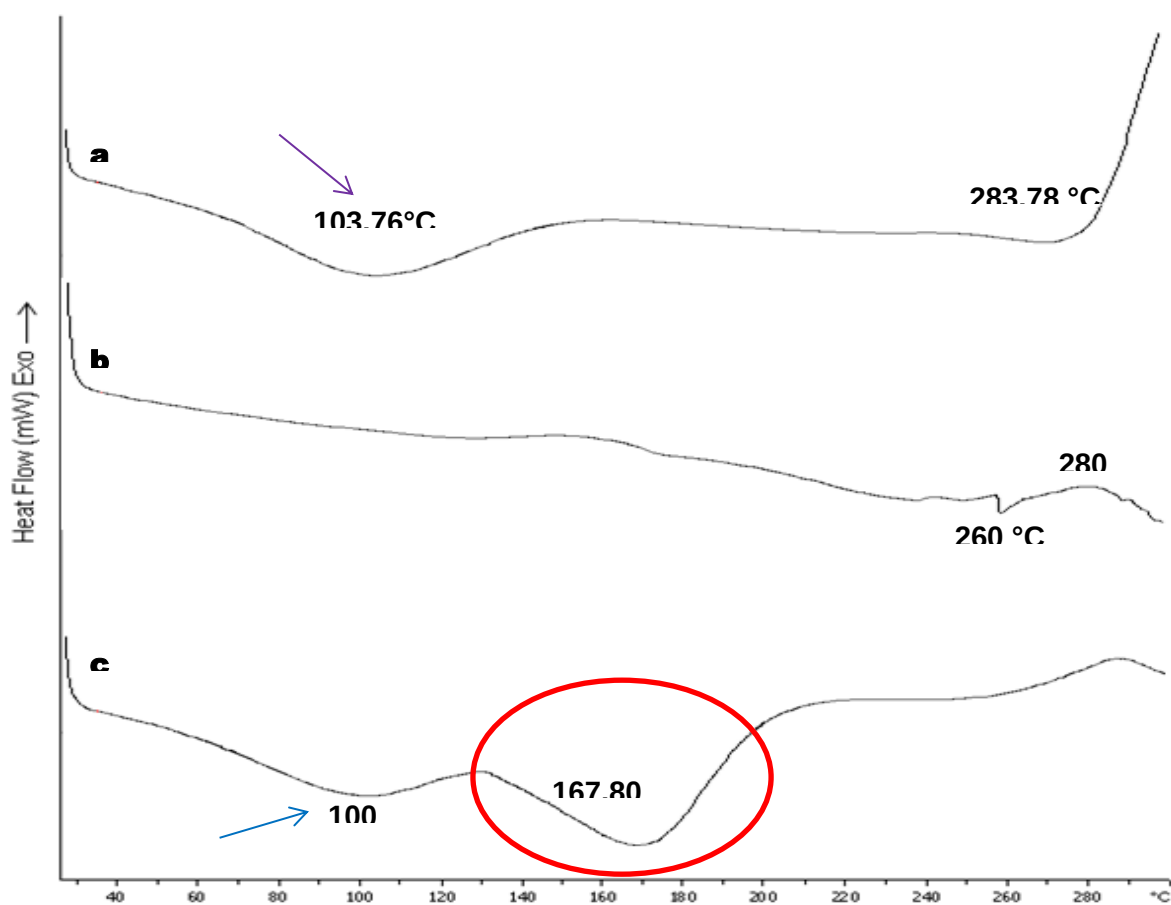


Figure 5.8: Differential scanning calorimetry curves; a. Native low molecular weight chitosan, b. Native LA and c. C-LA.

The drug LMV in its pure form is shown in Figure 5.9a. The sharp endothermic peak at 182.91 °C is representative of its melting thermal transition (Singh and Nath, 2012). The fact that this significant endotherm disappears in the curves of the LMV-loaded optimized formulations (Figure 5.9c & e) implies that the drug has been successfully encapsulated into the copolymeric ACNS structure (Park et al., 2014) within the hydrophilic part of amphiphilicity. The exothermic transition identified at around 270 °C is most probably associated with the degradation of the drug at this point. Figure 5.9b demonstrates the melting endotherm of mannitol at its peak temperature 162.16 °C. Being in its physical form and basically blended in optimized formulations merely to manifest its cryoprotectant effect, its endothermic peak can be seen in both coated and uncoated optimized formulations at 163.05 °C and 162.44 °C respectively with the enthalpy value being greater for the uncoated optimized formulation. In Figure 5.9c the first endothermic peak of 53.08 °C resembles the melting endotherm transition of HA-*g*-ECL (Figure 5.9d). Yet, this endothermic transition is absent in the C-LA coated optimized formulation, providing evidence that the coating indeed exists as it acts as a barrier restricting HA-*g*-ECL from melting at the temperature that it does. In addition, C-LA illustrates its broad endothermic peak at 167.80 °C in Figure 5.9f and the optimized C-LA coated ACNS shows an analogous peak at a slightly lower 162.36 °C (possibly due to disruption from attachment) implying the existence of the coat.

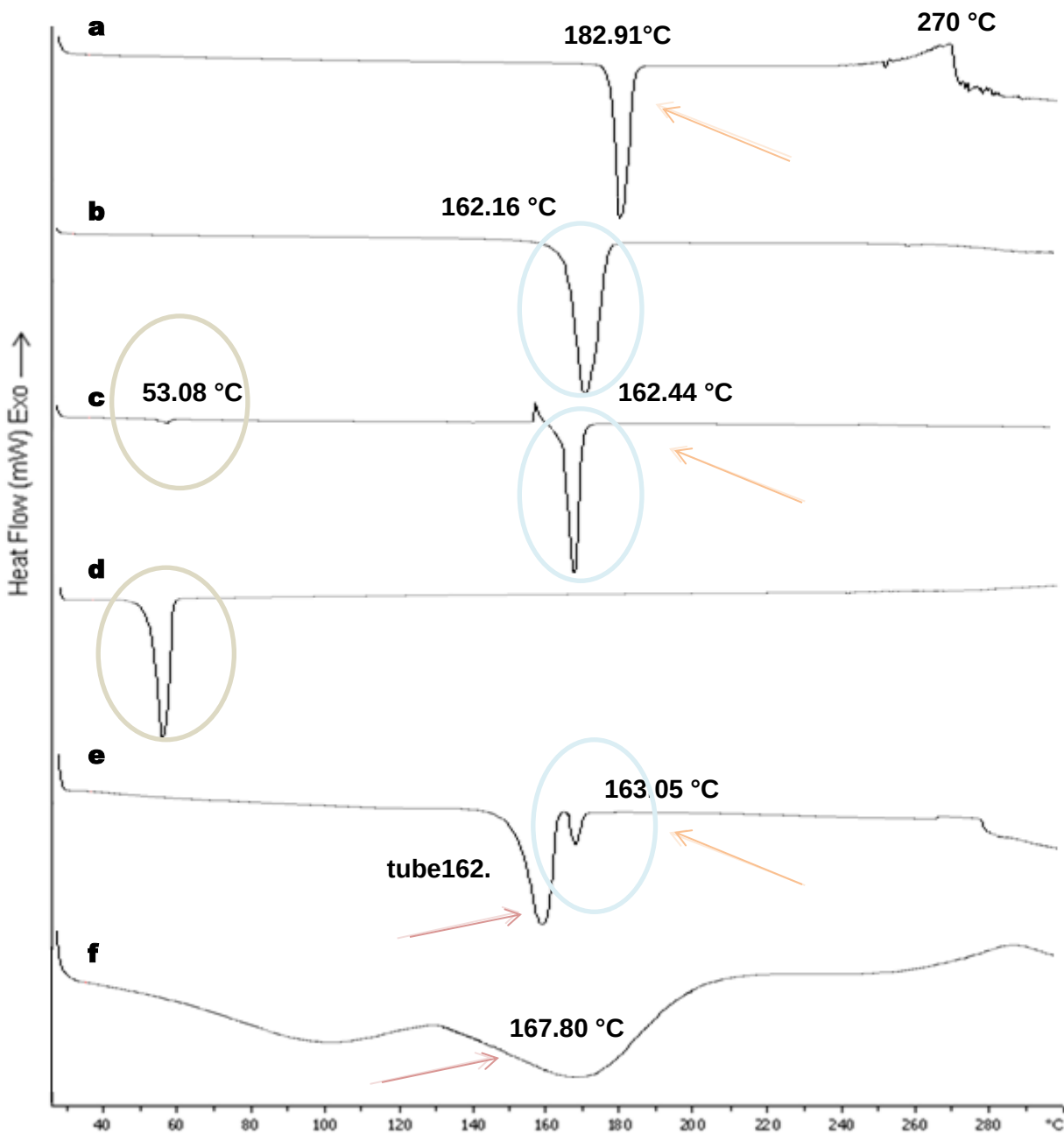


Figure 5.9: Differential scanning calorimetry curves; a. Pure LMV, b. Mannitol, c. Optimized non-coated LMV-loaded ACNS, d. HA-g-ECL, e. Optimized C-LA coated LMV-loaded ACNS and f. C-LA.

5.3.6. Thermogram analysis of C-LA and the optimized ACNS thermal degradation

TGA analysis was carried out to observe the degradation mechanism for samples revealed in Figures 5.10 & 5.11. The thermogravimetric curves (solid) are depicted with their respective first derivative curves (dash-dot). Both polymer and copolymer samples in Figure 5.10 displayed 3 steps of degradation. In the first steps for chitosan (A) and C-LA (C), the T_{peaks} are 81.61 °C and 58.43 °C, respectively. These can be attributed to the loss of water molecules taking place. The following two decomposition steps occurring for C-LA at 277.65 °C and 522.47 °C are as a result of the breakdown of chitosan backbone and the linoleic acid graft, respectively (Hülya et al., 2006). However, due to the graft procedure that caused disruption of the bonds on the chitosan backbone, a decrease in thermal stability was observed with C-LA with T_{onset} occurring at 207.40 °C when compared to the later occurrences of degradation in chitosan and LA alone at 291.32 °C and 265.62 °C, respectively, suggesting greater stability in parent compounds.

Figure 5.11 reveals the thermal degradation profiles for the optimized drug-loaded C-LA coated (C) and non-coated ACNS (D) in addition to LMV pure drug (A) and the cryoprotectant used in formulation, mannitol (B). A prominent thermal stability difference can be noted between the drug-loaded optimized C-LA coated and uncoated ACNS. A T_{onset} difference of 5.73 °C is obtained between the two suggesting that C-LA coat confers greater degradation stability in an optimized ACNS ($T_{\text{onset}} = 280.81$ °C) compared to the poor uncoated optimized ACNS ($T_{\text{onset}} = 275.08$ °C). Another notable feature calculated from the degradation thermograms of the comparative optimized formulations were the residual mass percentages. The uncoated ACNS had a residual mass of 1.748 % and the coated ACNS had a residual mass of 4.325 %. This confirmed evidence of successful C-LA chemically adsorbed coating onto plain HA-*g*-ECL ACNS by indicating that the extra mass was due to extra components on the chemical structure of plain HA-*g*-ECL ACNS. The degradation thermogram of the pure drug proceeded in two stages (Figure 5.11 A). The major weight loss that is noted is at $T_{\text{peak}} = 295.73$ °C and this is attributed to the evolving water molecules that are present in the chemical structure of LMV. The second decomposition occurs at $T_{\text{peak}} = 599.73$ °C and this is attributed to the breakage of the N6-C12 bond in the drug molecule (Ramkumaar et al., 2012). Mannitol has the ability to contain thermal energy avoiding weight loss stably below a $T_{\text{peak}} = 300.15$ °C (Kumaresan et al., 2011). The T_{onset} of Mannitol was extrapolated to be 315.35 °C from Figure 5.11 B and the complete

weight loss of the sample was completed in one step with the major portion of thermal degradation occurring at $T_{\text{peak}} = 381.83\text{ }^{\circ}\text{C}$.

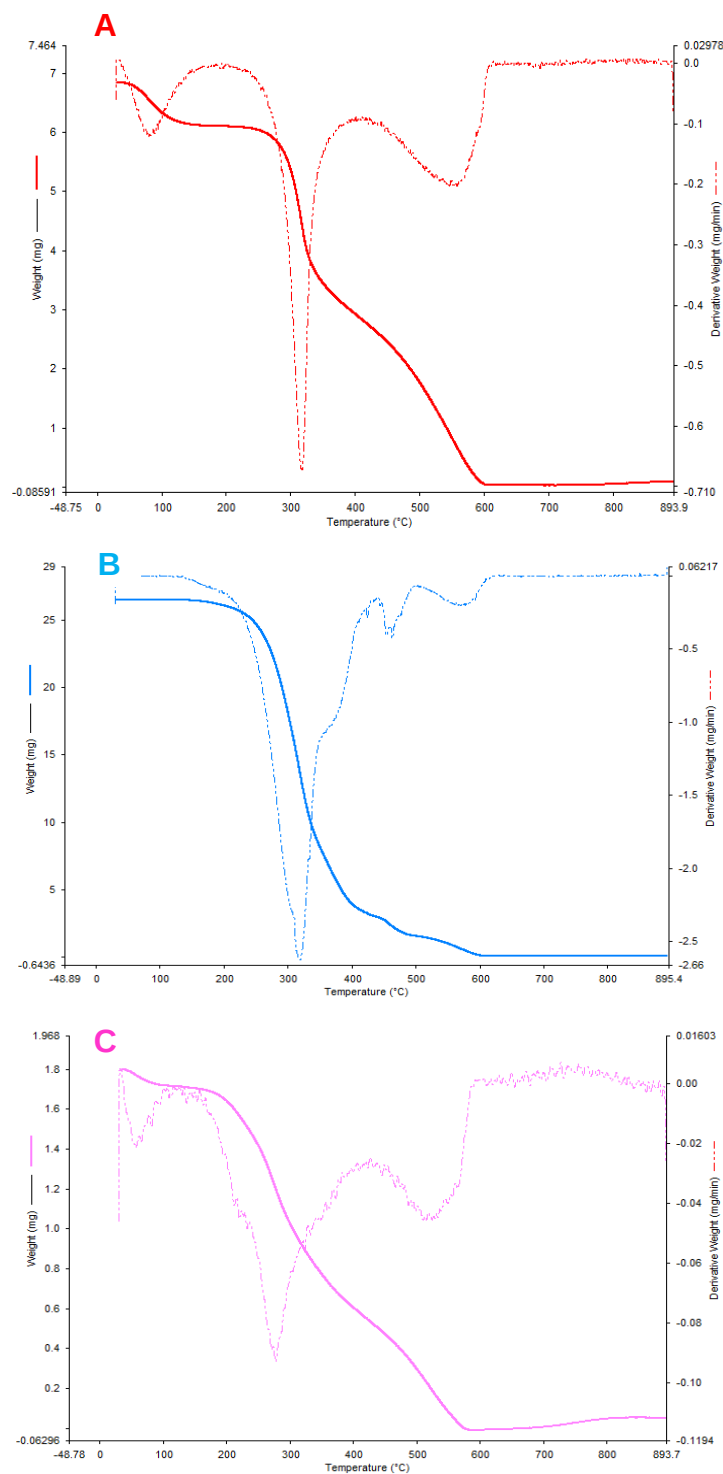


Figure 5.10: Thermogravimetric analytical and derivative thermogravimetry curves of A) Chitosan, B) LA and C) C-LA.

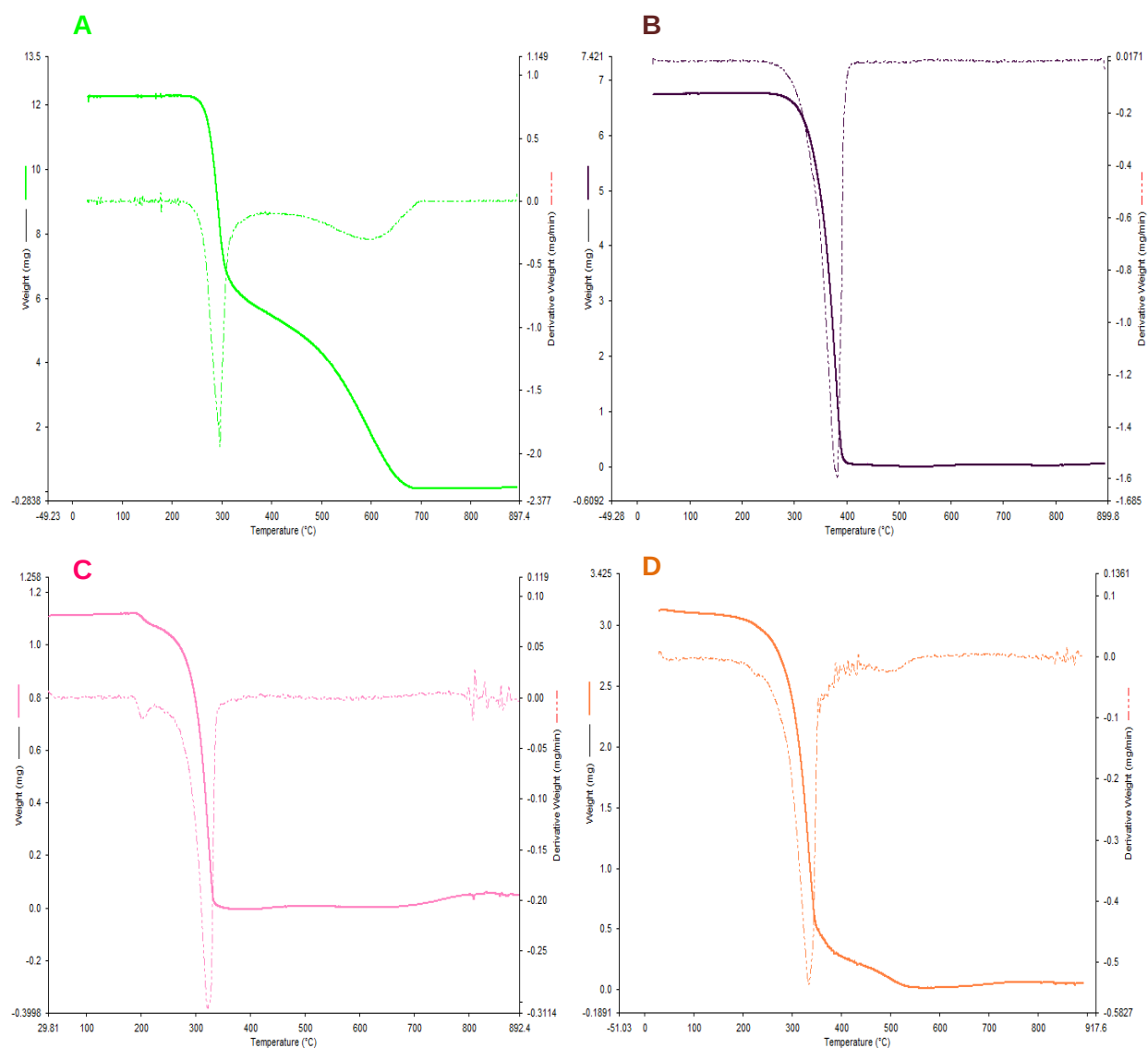


Figure 5.11: Thermogravimetric analytical and derivative thermogravimetry curves of A) Pure LMV, B) Mannitol, C) Optimized C-LA coated LMV-loaded ACNS and D) Optimized uncoated LMV-loaded ACNS.

5.3.7. Size and zeta potential evaluations exploiting Dynamic Light Scattering

Dynamic light scattering was employed to assess the size and surface charge distribution of the optimized ACNS formulation before and after it was subjected to C-LA coating. Figure 5.12 displays the DLS size and zeta potential graphs obtained for the optimized ACNS before C-LA

was chemically adsorbed onto it. The size distribution by intensity displayed a peak well below 100 nm. This corresponded to the measurement of 51.19 ± 4.37 nm that was recorded. Zeta potential distribution displayed a negative charge of -19.6 ± 3.19 mV on the surface of the ACNS copolymeric structure. Figure 5.13 depicts the DLS distributions of size and zeta potential of the optimized ACNS following C-LA chemical adsorption. The DLS graph of size reveals proof of successful coating of the targeting ligand by the observed shift in peak size closer to the 100 nm mark corresponding to 76.00 ± 14.98 nm. Furthermore, the zeta potential distribution showed a complete opposite positive surface charge of $+25.7 \pm 2.87$ mV on the ACNS structure which evidently reflects the charge of chitosan on the nanosystems surface.

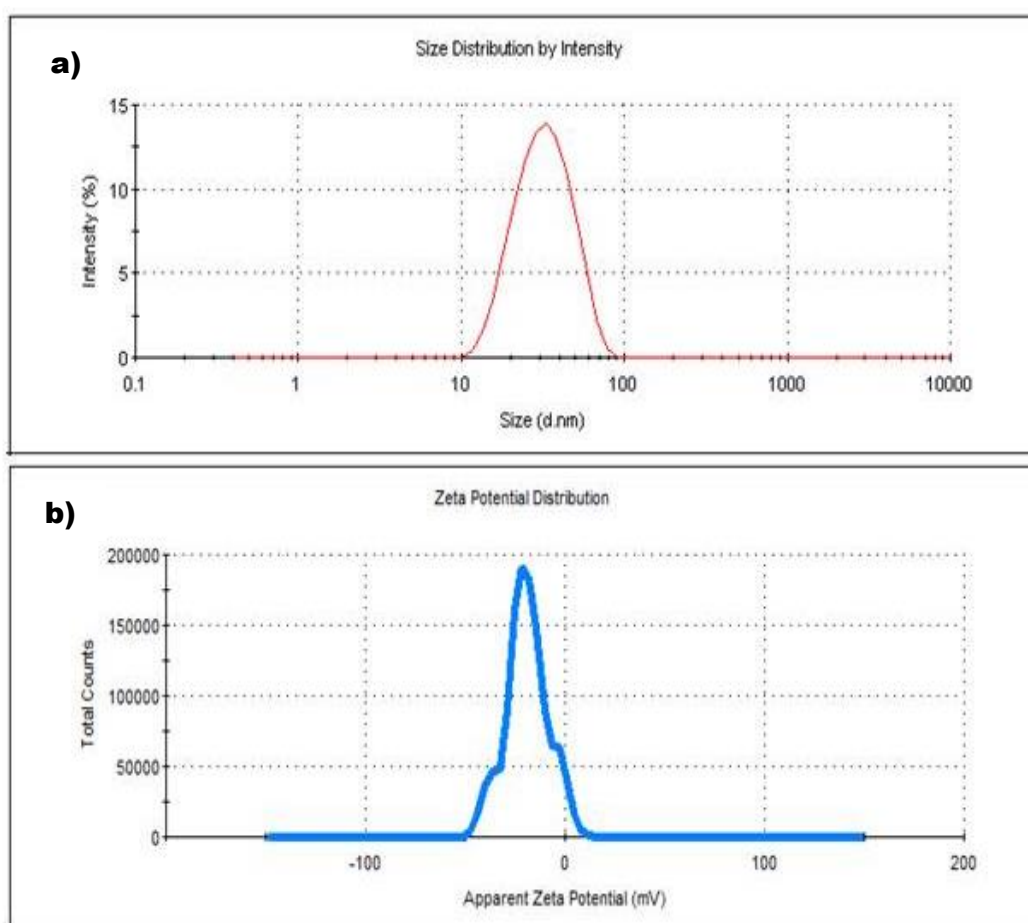


Figure 5.12: DLS profiles of the optimized ACNS before C-LA coating; a) size and b) zeta potential.

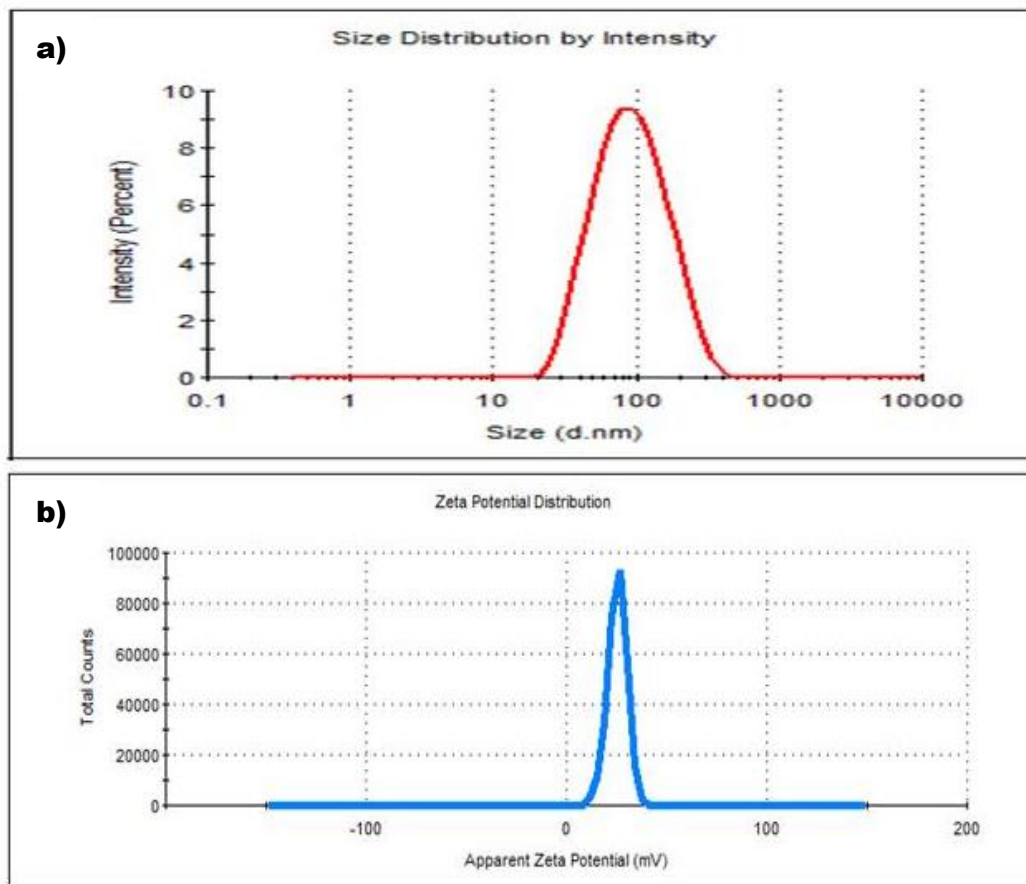


Figure 5.13: DLS profiles of the optimized ACNS after C-LA coating; a) size and b) zeta potential.

5.3.8. *In vitro* drug release profiles of the C-LA coated and uncoated ACNS

In vitro drug release profiles of optimized C-LA coated and uncoated LMV-loaded ACNS formulations are illustrated in Figure 5.14. Both formulations displayed sustained release profiles however the ACNS lacking coating showed an initial burst release of 40.78 % within 8 hours compared to the C-LA coated ACNS which was efficiently sustained due to a layer surrounding the nanosystem that drug must pass through and ultimately constraining the burst effect. The copolymer of the ACNS uncoated nanosystem holds drug that is adsorbed on its surface which is exposed to the diffusion medium resulting in prompt initial burst release thereof. The size difference between the coated and uncoated ACNS is also vast which leads to the much smaller sized uncoated ACNS of approximately 50nm dispelling drug rapidly due to the relatively shorter distance to the nanoparticle surface for migration. The coated C-LA ACNS

showed a lagged release of drug with merely 41.89 % released after 4 days. Comparatively, 66.93 % was released after 4 days in uncoated ACNS. A further consequence to exposed copolymeric ACNS is the open pores readily in contact with diffusion media that provides a direct channel for the hydrophilic LMV to move through. Coating adsorbed to the surface would act as a sealant preventing this passage of drug. LA comprises a lipid property. Lipids are infamous for demonstrating difficulty in being passively impermeable providing a relatively condensed blockade for the hydrophilic LMV. MDT values of 28.96 ± 2.20 and 19.45 ± 1.59 hours was achieved with C-LA coated ACNS and uncoated ACNS, respectively. Thus, the chitosan-linolate adsorbed onto the outer make-up of ACNS displayed the more favorable near zero-order drug release of LMV.

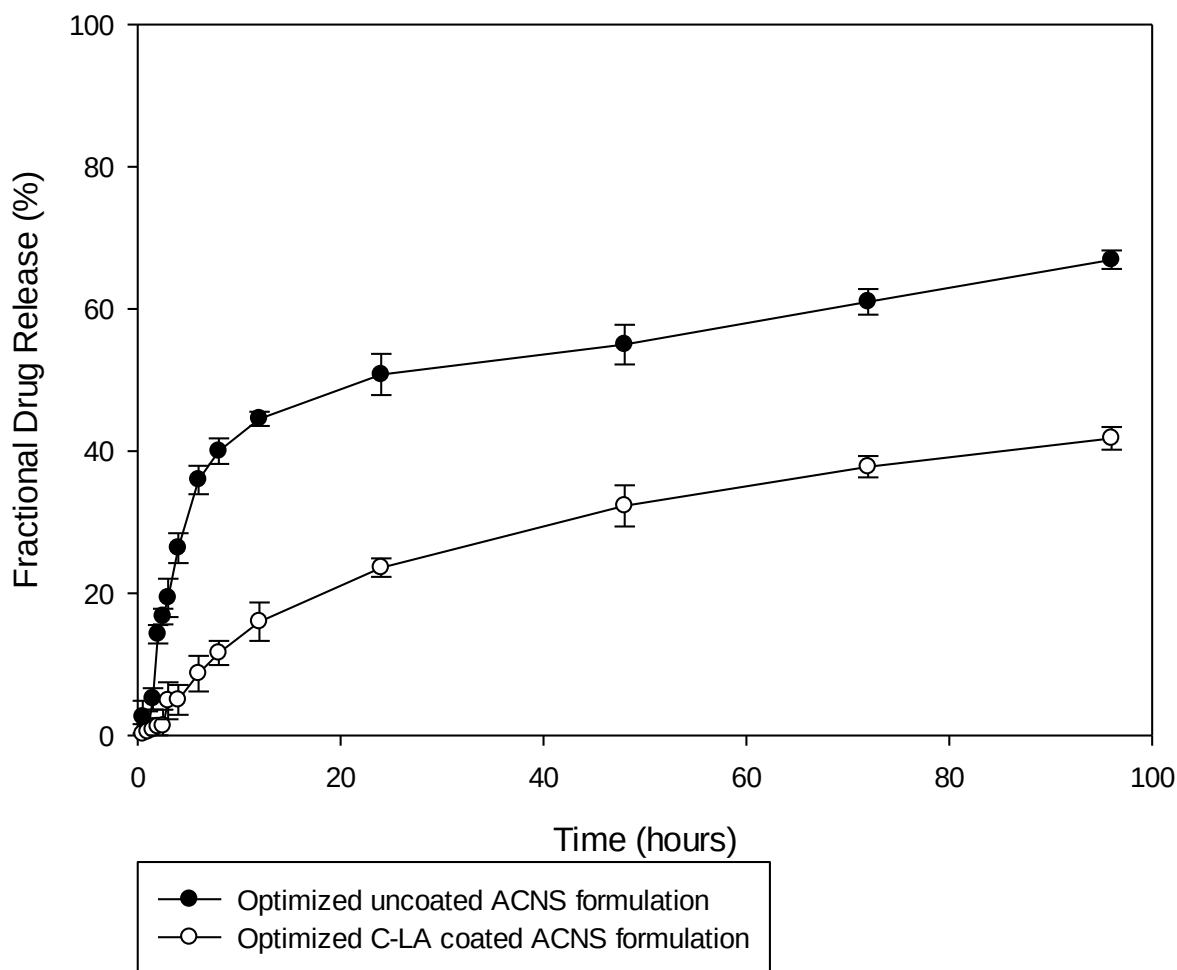


Figure 5.14: Fractional drug release profiles of the uncoated and C-LA coated optimized ACNS formulations.

5.4. Concluding Remarks

The present chapters objective was to elucidate and characterize the processed parameters previously statistically determined using the Box-Behnken model, to have a considerable effect on the three responses (DEE, size and MDT) to obtain their optimized values. The optimization process was carried out by use of polynomial mathematical equations and response surface plots which allowed for the determination of the compound independent variables with predictable profile of experimental responses. An optimized ACNS was developed and fully characterized to evaluate its physicochemical characteristics as well as compare predicted and observed response measurements attained by the model. Responses measurements showed good correlation. In addition, a graft copolymerization between chitosan and LA occurred successfully synthesizing C-LA. Preparation of a C-LA coating into a chemically adsorbed copolymeric liver targeting ligand also developed successfully as discerned by DLS size and surface charge characterization tests as well as an enhanced sustained drug release observation. The *in vitro* characterization and elucidation of the optimized C-LA coated ACNS was most rewarding, however the most critical investigation lay in the actual performance of the nanosystem as a hepatocyte targeting therapeutic complex which is divulged in the following chapter as cellular internalization of the ACNS is explored.

CHAPTER 6

EX VIVO INVESTIGATIONS: VISUALIZATION OF CELLULAR INTERNALIZATION OF C-LA COATED ACNS INTO A HEPATIC CELL LINE

6.1. Introduction

Nanoparticulate carrier systems have attained significance in nanomedicine through conferring appealing pharmacokinetic feats for various drug treatments. Apt drug delivery is achieved by augmented solubility and stability of drug provided by these nanoparticulate carrier systems including a lagged systemic circulation and target specificity leading to accumulation of therapeutic at the target area. Moreover, proper efficacious cellular passage is achievable by these delivery systems (Song et al., 2013). Nanoparticulate carrier systems display physical and chemical traits that determine its fate at the level of biological system such as its transit across the cell surface. Some of these traits constitute the makeup of the nanoparticulate system, its surface chemistry, charge and size (Bannunah et al., 2014). From the time inorganic nanoparticles have been successfully internalized into cells, they have become a long-established means of fluorescent imaging in live cells (Serdiuk et al., 2014). Nanomachinery are readily taken up into a cell and localized herein at varying intensities significantly dependent on the chemical makeup of the surface of the nanomaterial along with their shape and size which notably impacts cellular internalization. A particular cell will internalize a nanoparticle size suited to the distinctiveness of the cell. In terms of shape, it was found that spherically shaped nanostructures have been taken up more readily than rod-shaped nanostructures. In terms of surface charge, positively charged nanostructures have been most competent in passing over a cells membrane and reaching internal locations (Verma and Stellacci, 2010).

Phagocytotic and non-phagocytotic processes such as that of clathrin- and caveolin-mediated endocytosis and macropinocytosis, are mechanisms by which nanoparticles are cellularly internalized. Phagocytosis is an inadmissible mechanism because of macrophages identifying the nanostructures as foreign bodies and thus eliminating them resulting in failed therapy. Phagocytosis should be avoided by surface modification of nanostructures with ligands, exposing a hydrophilic surface and preparing extremely nanosized particles. Target site delivery necessitates efficiency in decisiveness about a nanoparticle's surface modification and ligand attachments. Receptor-mediated endocytosis occurs through the high-affinity receptors found

on the surface of most cells that seize nanoparticles. Surface caveolin is a dimeric protein that is triggered by the nanostructures that facilitates their internalization. Surface clathrin coated pits cause the transit of nanoparticles into cells leading to the formation of endosomes once inside. Macropinocytosis is an internalization route that is limited to larger particles (Gad, 2008; Plajnšek et al., 2012).

Fluorescent chemical substances that are within the scope of re-emitting light are termed fluorophores. They emit fluorescence by way of the electron excitation and its subsequent falling back into the orbit of an electron. These chemical compounds have the ability to perform sensitive detection simultaneously evading concerns with safety and special execution of the experiment. They have long been implemented in ligand assays for their function (Renberg, 2006). Figure 6.1 show some of the frequently ordained fluorophores being fluorescein, tetramethylrhodamine and the organic Cy3 and Cy5 dyes used in DNA and protein microarrays.

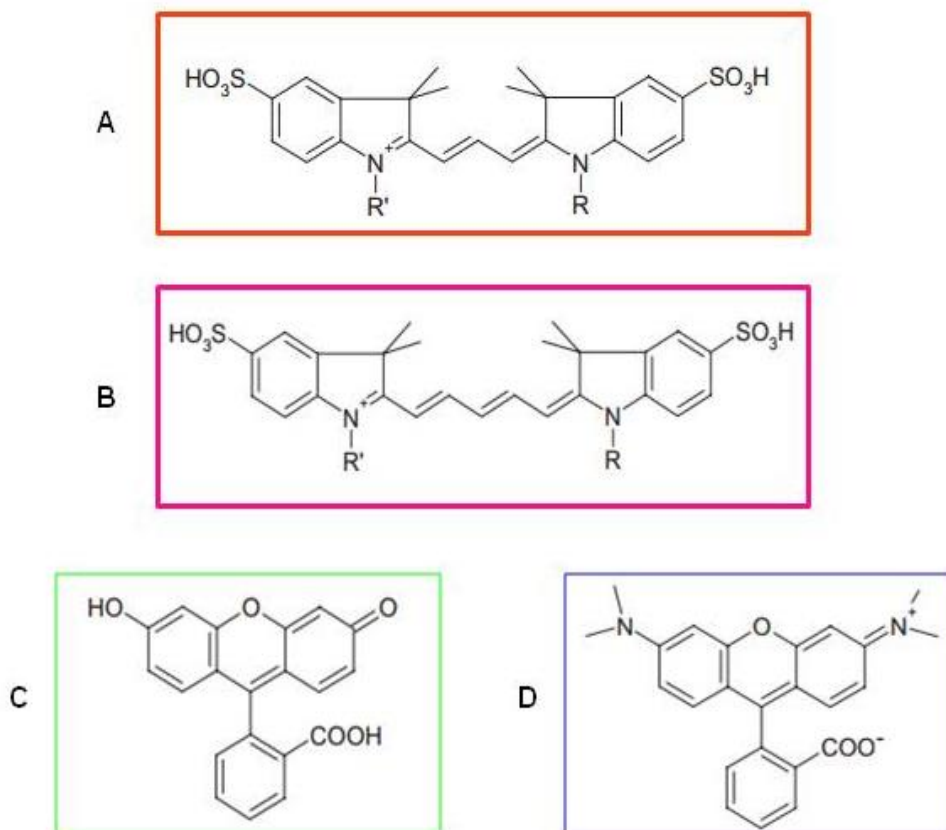


Figure 6.1: Fluorophore chemical structures. A) Cy3, B) Cy5, C) Fluorescein and D) Tetramethylrhodamine (Adapted from Renberg, 2006).

The mechanism by which fluorescent dyes impart their reactivity is through direct labeling of proteins and their blends. Reactive derivatives that are prominent are those of isothiocyanates and *N*-hydroxysuccinimide esters that are readily clasped covalently to lysine side chain amino groups and amine terminal groups (Renberg, 2006). Fluorescence microscopy is routinely availed to detect cellular uptake of a compound upon ligand binding and supplementing the chemical entity that is of interest with the fluorescent dye. After the attachment process, polymers in particular command a legion of fluorescent dye washing cycles because of the extra background fluorescence that is imperative to be removed. Thereupon, polymeric macromolecules holding a fluorescent label can illuminate the pathway upon cellular endocytosis tracking the movement of the entire nanostructure (Mangold et al., 2008).

Many cell lines of liver source are utilized extensively in biomedical research. The organotypic HepG2 cell line was chosen for the *ex vivo* component of this study as it is usually utilized as an *in vitro* surrogate for the specified organ of importance – the human liver; as is the case for Caco-2 and Caki-1 for human intestine and human kidney resembling, respectively (Hilgendorf et al., 2007). This most resourceful HepG2 cell line was formerly sequestered by Aden et al. in the year 1972 from an 11 year old Argentine male with a primary hepatoblastoma. HepG2 cells are able to preserve particular actions lacking in most cultured primary hepatocytes such as the extrusion of major plasma proteins. Accordingly, HepG2 cells are sought when it comes to liver disease research, therapeutic actions and mechanisms as well as gene transcription and gene expression (Mersch-Sundermann et al., 2004). Studies that employed fluorescein isothiocyanate (FITC) as a fluorophore together with use of the HepG2 cell line reported valuable and favorable data. A study that simultaneously used rhodamine B isothiocyanate with FITC on silica nanoparticles demonstrated in flow cytometry that the cellular internalization of the particles was reliant on size, concentration and culturing time. Results reflected nanoparticles to be concentrated in cytoplasm, endosomes and HepG2 cell surfaces (Hu et al., 2011). Jiang et al (2013) reported on HepG2 cells subjected to cholesterol-modified pullulan nanoparticles that were covalently appended with FITC. Fluorometry showed nanoparticle uptake reliance on concentration and temperature. Results also implicated clathrin-mediated endocytosis and macropinocytosis in the cellular internalization. One more study illustrated fluorescence on a novel chitosan derivative that was linked with many galactose residues in an antennary manner. Additionally, a galactosylated chitosan derivative was also prepared. Data displayed poor fluorescence with plain FITC conjugated chitosan nanoparticles, greater

fluorescence with FITC conjugated galactosylated chitosan nanoparticles and the best fluorescence with FITC conjugated multiple galactose-chitosan nanoparticles in HepG2 cells (Mi et al., 2007).

The objective of the following *ex vivo* study is to visibly ascertain the HepG2 internalization of the C-LA coated verse the uncoated ACNS formulations by way of fluorescence labeling, also applying FITC as the fluorophore. Fluorescent microscopy was used to establish the extent of internalization of each.

6.2. Materials and Methods

6.2.1. Materials

HepG2 cells were obtained from ATCC (American Type Culture Collection, Manassas, USA). Flourescein 5(6)-isothiocyanate (FITC) (Bioreagent, mixture of 2 components, ≥ 90 % (HPLC)), 0.25 % Trypsin-EDTA Solution (2.5 g porcine trypsin and 0.2 g EDTA, 4 Na/L HBSS), Fetal Bovine Serum (FBS) (cell-culture tested, USA origin, sterile-filtered, hybridoma tested), Penicillin-Streptomycin Solution (Pen-Strep) (stabilized with 10 000 units penicillin and 10 mg streptomycin/mL, sterile-filtered) and Trypan Blue Solution (0.4 %) were all purchased from Sigma Aldrich (St. Louise, MO, USA). RPMI 1640 w: L-Glutamine, w: 2.0 g/L NaHCO_3 , sterile filtered, was purchased from Pan Biotech (PAN-Biotech GmbH, Aidenbach, Germany). A Bright-Line Hemacytometer (supplied with two cover slips) was purchased from Sigma Aldrich (St. Louise, MO, USA). Tubes used for centrifuging were purchased from The Scientific Group, Randburg, South Africa. Tissue cell flasks and other cell culture equipment was purchased from Lasec (South Africa). The coated and uncoated optimized formulations were prepared as discussed in Chapters 3, 4 and 5. All other chemicals used were of analytical grade and employed as purchased.

6.2.2. Employment of Aseptic technique for cell procedures

Sterility and avoidance of cell contamination was ensured by aseptic method procedures put into place. Aseptic techniques provide a barrier against viruses, fungi and bacteria laden naturally in the air from reaching cell culture. The designated laboratory used for cell culturing and procedures is a sterile environment exposed to UV radiation when not in use. Before the commencement of any procedures as well as entering the sterile work area, good and proper

personal hygiene was made certain of and hands were washed. Gloves were worn to cover up skin, a sterile laboratory coat was used to cover up clothes that held dust and dirt and a mask was used to cordon off contamination from breathing or talking. The sterile work area was a laminar flow unit (Labotec, Midrand, South Africa) and its surface was wiped with 70% ethanol solution before placing in it cell culture and/or media and reagents that were used in cell culturing. All bottles, flasks and pipettes were sprayed with the alcohol before taking into the unit. Gloved hands were periodically rubbed with alcohol before and after leaving the laminar flow unit. A lit bunsen burner was used in the laminar flow unit with handling and work performed around the flame. All container mouths and closures were flamed upon opening and closing. Media and reagents for reuse were film sealed at their capped lids. All cell culture procedures were performed as rapidly as possible to minimize contamination.

6.2.3. HepG2 cell culturing and cell maintenance protocols

The HepG2 cell line used for the uptake study was cultured according to a standard protocol utilizing the RPMI 1640 media (with L-Glutamine and 2.0g/L NaHCO_3). Media was supplemented with 10 % v/v FBS and 1 % v/v Pen-Strep. Media and supplements were warmed to 37 °C at each preparation time before adding to cell culture flasks. Frozen solutions were placed in warm water to thaw 45 minutes before preparation.

6.2.3.1. Cell revival

The first step in the culturing process was the revival of the cell line from frozen in storage. During preparation of media, cell revival required a doubling in the quantities of FBS and Pen-Strep (20 % v/v and 2 % v/v , respectively) due to the compromised state of the cells as a result of the environment it had been subjected to. It was crucial that this step be carried out swiftly to prevent death of cells. The frozen cells were left to thaw around the bunsen burner flame. The cells were immediately transferred to a sterile centrifuge tube and dispersed in a matched volume of media. They were then transferred to a T-25 cm^3 cell culture flask containing 6-10mL media. The flask was gently tilted back and forth to swirl the contents and ensure uniform dispersion of the cells when undergoing cell attachment. The flask with cell culture was incubated at 37 °C in humidified air with 5 % CO_2 . The media was changed 24 hours later with standard undoubled quantities of media.

6.2.3.2. Cell subculturing

Cell culture was viewed daily for their attachment and normal growth. A yellow-orange like discolouration of the media observed in the flask was a sign for a media change. When cells reached confluency, subculturing was necessary. HepG2 cells were allowed to grow to 75 % confluency. Thereafter, the removal of media from the flask was followed by rinsing of the cells using sterile PBS (0.1 M, pH7.4, 37 °C). Trypsin-EDTA (0.25 %) was added to the flask, swirled and left in for a few minutes for cells to change shape and complete the trypsinization process. The duration of this process varies among different cell lines. Trypsinization of HepG2 was relatively quick (± 5 minutes). A confirmation of detachment was obtained through light microscope (Olympus CKS microscope, Olympus, Japan) viewing to observe cells floating completely. The culturing flask may also be tapped thrice gently on the side of cell attachment to dislodge cells, if necessary. A matched volume of media was added to the flask to dilute the Trypsin-EDTA as cell culture should not be exposed to pure Trypsin-EDTA longer than necessary. The Trypsin-EDTA-media contents were then transferred to a sterile tube and centrifuged at 5000 rpm for 3 minutes. A pellet of cells was noted at the bottom of the tube. The Trypsin-EDTA-media was discarded and 1 mL fresh media was added to the pellet with redispersion. Cells were then passaged to two T-75 cm³ holding 20 mL fresh media. Cells were viewed under the microscope for proper dispersion in each flask.

6.2.3.3. Cell counting

Cell counting was carried out on the day cell splitting/ passaging was required. A sterile hemacytometer was the instrument used for counting and this was done using the trypan blue exclusion assay. Trypan blue is a dye that stains the nuclei of non-viable or dead cells isolating them from live viable ones and allowing this distinction to be observed under the light microscope. A 0.4 %^{w/v} trypan blue solution in sterile PBS (0.1 M, pH 7.4) was prepared for cell counting. A cell volume of 20 μ L and a trypan blue volume of 60 μ L were mixed together. This was a 1:3 ratio which produced a dilution factor of 4. Figure 6.2 depicts a hemacytometer with grid on which stained and unstained cells were displayed and visualized under the light microscope. The four quadrants of the hemacytometer grid (numbers depicted in Figure 6.2) that held cells not stained were counted and inserted into Equation 6.1 to obtain the total number of HepG2 cells.

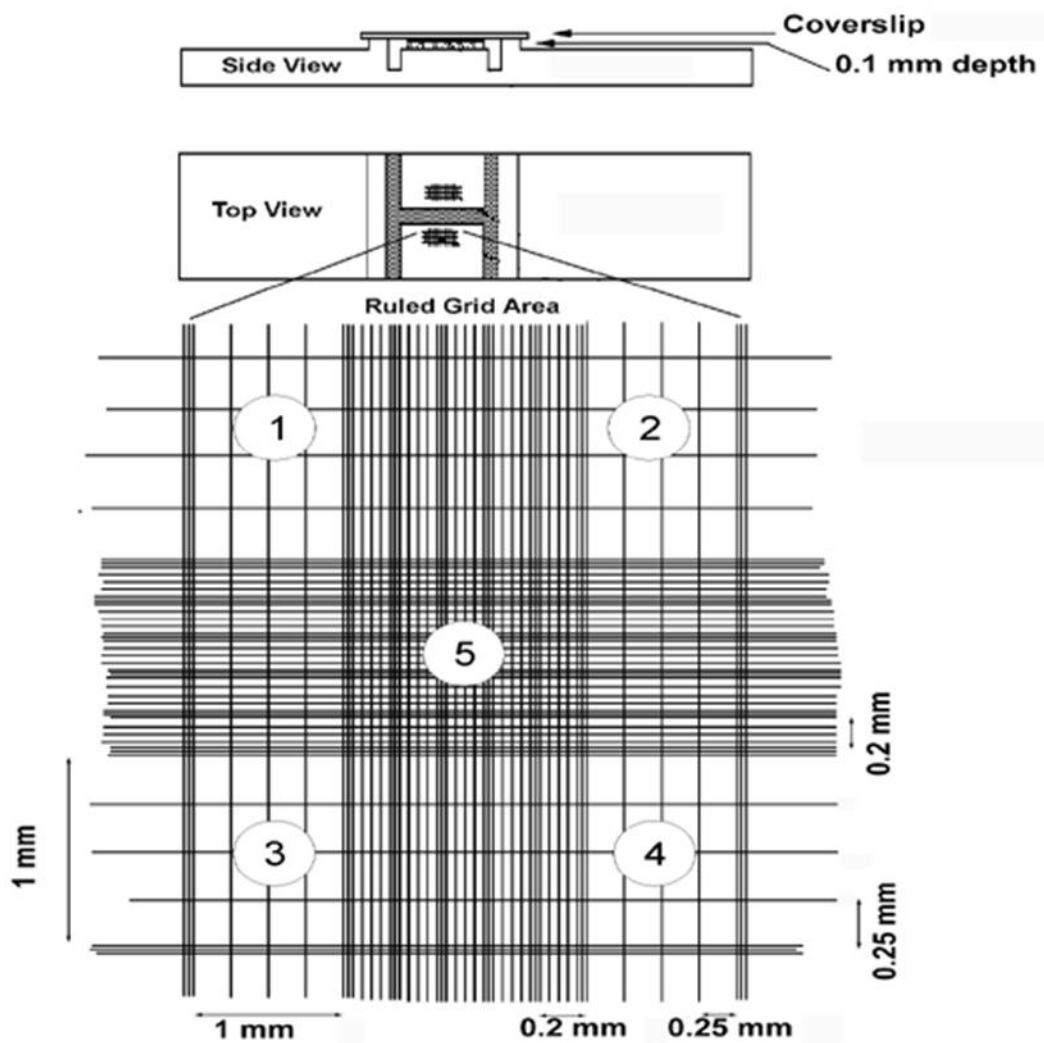


Figure 6.2: Illustration of the Improved Neubauer Hemacytometer scrutinizing the grid layout, adapted from <http://homepages.gac.edu/~cellab/chpts/chpt1/figure8.html>, [Accessed August 5th, 2014].

$$\frac{\text{Number of cells counted}}{\text{Number of quadrants counted}} \times \text{DF} \times 10^4 \text{ cells/mL} \quad \text{Equation 6.1.}$$

where DF is the dilution factor used and 10^4 is a constant. Cells of 95 % viability were employed in ensuing uptake image tests.

6.2.3.4. Cell freezing

Freezing of HepG2 cells was necessary for preserving them in storage encountering probable use in later stages of experimentation. The media in which they were frozen was constituted with a solution of 0.5 %_{v/v} DMSO in FBS. Cells were counted before placing in the freezer and a number of $10^5 - 2 \times 10^5$ cells were allocated to 1 mL of freezing solution. However, cells had to be passaged at least twice before they could be used further for experimentation purposes to be sure of an optimal state.

6.2.5. Preparation of the optimized ACNS samples and controls

Internalization uptake studies necessitated at least 4 samples per measurement to ensure a thorough understanding of observations on microscopy images. This constituted:

1. HepG2 cells;
2. Optimized non-/ coated ACNS
3. Optimized C-LA coated ACNS with HepG2 cells and
4. Optimized non-coated ACNS with HepG2 cells.

Sample numbers 1 and 2 served as the controls. FITC was used as the fluorophore on ACNS to detect their movement and receipt by cells in the subsequent fluorescent imaging evaluations. Coated and uncoated samples (2 mg/mL) were combined with FITC (1 mg/mL) in sterile PBS (0.1 M, pH 7.4). For conjugation to occur, this solution was stirred for 40 minutes at 37 °C whilst protecting the light-sensitive FITC from light. These samples containing both FITC conjugated to ACNS (FITC-ACNS) and free FITC were then dialyzed against 3 L deionized water for 24 hours to remove the free FITC. The dialyzing beaker was covered with foil to protect the FITC-ACNS from light.

6.2.6. Visualization of intracellular uptake of the fluorescent ACNS

6.2.6.1. Confocal fluorescence microscopy imaging

Confocal microscopy was utilized as the first method to visualize possible uptake of the ACNS systems through fluorescence detection. A quantity of 200 μL HepG2 cells (2×10^5 cells/mL) were dropped on cover slips that were placed in disposable petri dishes (BRAND®, 55 mm x 16 mm) and carefully spread out to completely fill the cover slip. This was incubated at 37 °C and 5 % CO_2 to allow for the cells to attach to the slips and grow overnight. Care was taken by periodically monitoring the slips every hour to ensure the media had not fully dried out due to the warm temperature of the incubator. Media was carefully replenished if this was the case. FITC-ACNS coated and uncoated formulations were then added with 0.20 μm filter-sterilization (Millipore Co., Massachusetts, USA) together with media to cover slips in a ratio of 1:4. Controls received complete plain media (cells) and complete formulation. These were left for another 24 hours to allow internalization to occur. Thereafter, FITC-ACNS-media were removed and the cover slips were washed thrice with sterile PBS (0.1 M, pH 7.4). A thin layer of PBS was left on and the slips were taken to a fume hood outside the sterile laboratory to fix the cells with 3 % paraformaldehyde. Paraformaldehyde is highly toxic and required the use of protective wear at the fumehood. Fixative solution was added to each cover slip at a volume of 2 mL. Fixing occurred for 15-30 minutes after which it was removed and each slip was washed with PBS (0.1 M, pH 7.4) three to four times. They were left for 24 hours at 4 °C. Finally, the cover slips were mounted on slides with a drop of glycerol. They were left to dry for 3 hours before viewing for fluorescent uptake via microscopy (Zeiss LSM 780, Carl Zeiss Microscopy GmbH, Goettingen, Germany).

6.2.6.2. High speed fiber-optic fluorescence microscopy live imaging

The second method to detect fluorescent uptake of nanosystems was via a high speed fiber-optic fluorescence microscope (Cellvizio® LAB, coupled with Microprobes and ImageCell™ Software; Visualsonics and Mauna Kea Technologies, USA). The cells with FITC-ACNS were visualized as floating entities on images thereby eliminating the step for cell attachment and growth. Media and FITC-formulations were mixed in a ratio of 1:4 and added with 0.20 μm filter-sterilization to 2 mL eppendorf tubes containing 500 μL HepG2 cell culture (5×10^5 cells/mL). Tubes were incubated at 37 °C and 5 % CO_2 for internalization to occur and images were taken

at 30 minutes and 24 hour post incubation time points to evaluate the rate of internalization into HepG2 cells.

6.3. Results and Discussion

6.3.1. HepG2 cell culturing

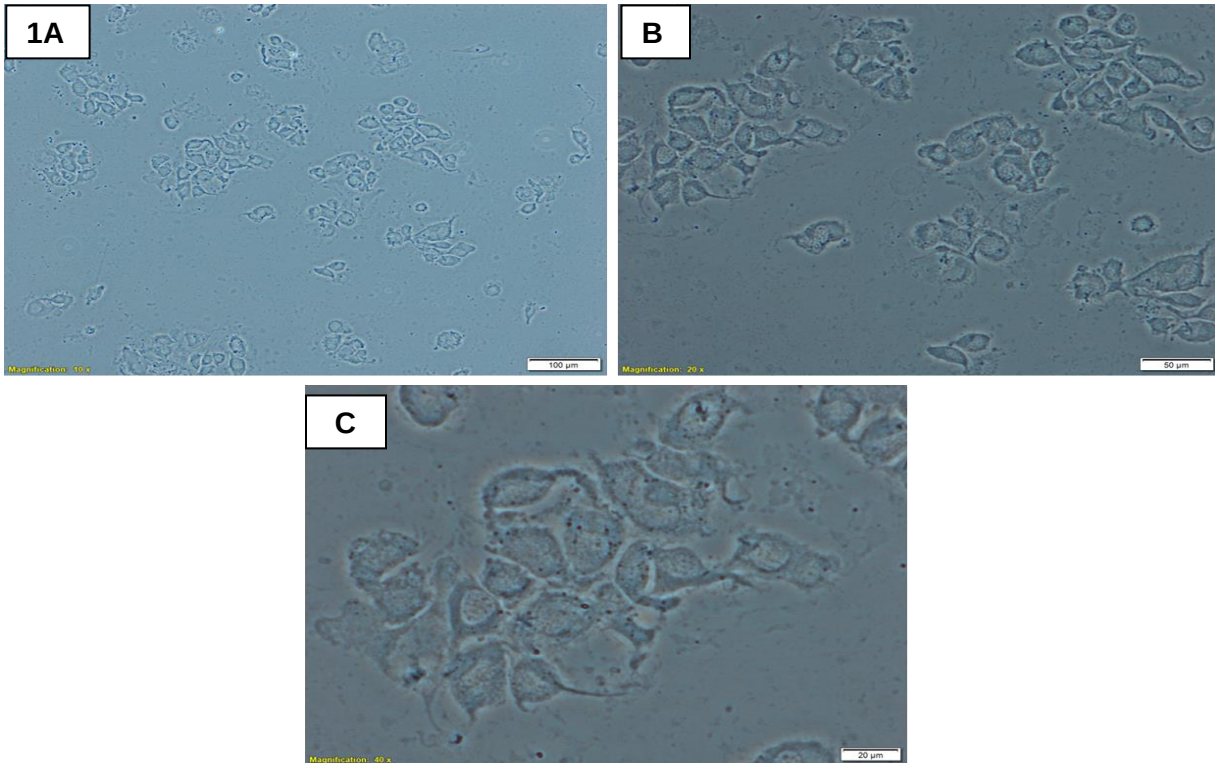
HepG2 cells are human liver carcinoma cells which were cultured according to a standard protocol with Figure 6.2.1A, B and C depicting the morphology of the cells at different magnifications under Brightfield light. HepG2 are epithelial cells and cell growth of this particular cell line was relatively rapid, occurred in a monolayer format and required passaging every 2-3 days. They also displayed a trend of aggregate growth in the flask. Cell death would occur if confluency approached 100 %. The changing of media was essential as media discolouration was a resultant feature of cells depleting the nutrients obtained from the supplements (FBS, L-Glutamine and NaHCO_3) for their growth found therein. The colour change to yellow was due to the acid production during cellular metabolism.

6.3.2. Visualization of intracellular uptake of fluorescent ACNS by means of confocal fluorescence microscopy imaging and high speed fiber-optic fluorescence microscopy live imaging

The investigations made on cellular uptake called for samples of plain cells and plain nanosystems together with the custom test nanosystems as this allowed for a resolute comparison of the efficacy of ACNS coated with C-LA against that lacking coating on the uptake by HepG2 cells. Visualization of the ACNS inside a cell is made possible through fluorescence emanating off FITC. FITC bears the highly amine reactive isothiocyanate entity that is the main conjugator to amine functional groups on molecules (Schmitt et al., 2007). Free FITC removal was a prerequisite to prevent erroneous misleading fluorescent images.

ACNS internalization observations through confocal fluorescent imaging are demonstrated in Figure 6.3 2A i) & ii) and 2B i) & ii) and 3A i) & ii) and 3B i) & ii) 24 hours after incubation, for the uncoated optimized FITC-ACNS and C-LA coated optimized FITC-ACNS, respectively. The snapshots are taken in Brightfield light initially followed by a comparison against their respective fluorescent images in the unchanged position and magnification (40X) observed. It is quite clear

that a distinct enhanced fluorescence is found in cells incubated with the FITC-ACNS system that was coated with C-LA [Figure 6.3 3A i) & ii) and 3B i) & ii)] while a dimmer fluorescence is noted within cells with the uncoated FITC-ACNS system [Figure 6.3 2A i) & ii) and 2B i) & ii)].



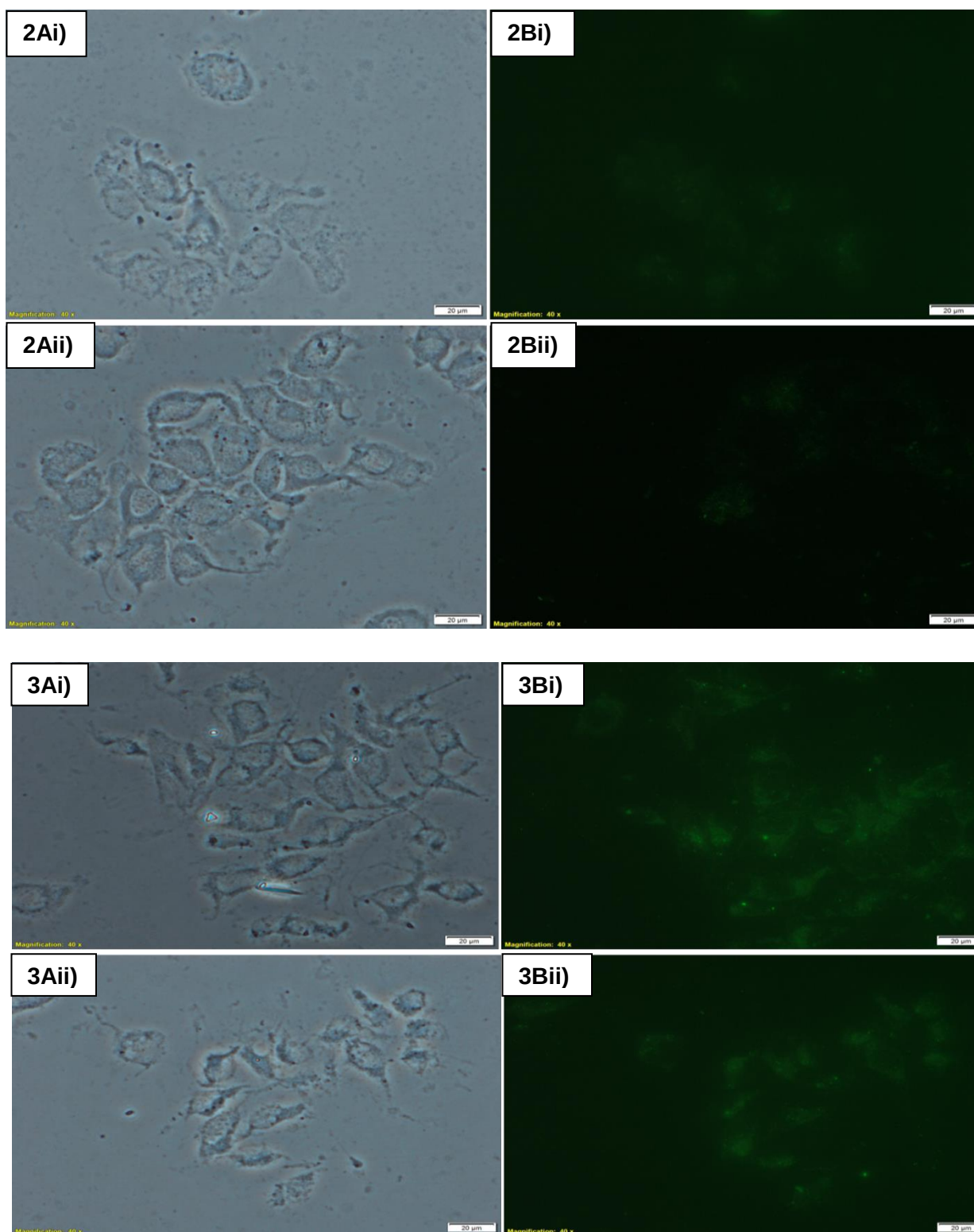


Figure 6.3: Confocal microscopy images of 1. Plain HepG2 cells at magnifications of A - 10X, B - 20X and C - 40X; 2. Uncoated optimized ACNS uptake (40X magnification) under A i) & ii) –

Brightfield light and B i) & ii) - Fluorescent light and 3. C-LA Coated optimized ACNS uptake (40X magnification) under A i) & ii) - Brightfield light and B i) & ii) - Fluorescent light (*Refer to attached CD for electronic image of Figure 6.3 depicting fluorescence clarity*).

Similarly, the live fluorescent images of fiber-optic imaging (Figure 6.4) display an augmented uptake of C-LA coated FITC-ACNS after both 30 minutes (d) and 24 hours (e) of incubation compared to uncoated FITC-ACNS at 30 minutes (b) and 24 hours (c) of incubation. An augmented uptake is designated by a brighter fluorescent cluster. These are the floating HepG2 cells harvesting ACNS fluorescent particles. Figure 6.4a shows an image of the appearance of plain FITC-ACNS in solution. The yellow arrows indicate mild aggregation of FITC-ACNS to differentiate between HepG2 cell internalized ACNS shown as the larger clusters in Figure 6.4b, c, d & e. Also quite clear-cut is the observance that the longer the incubation time of the ACNS systems with cells, the greater the internalization into the cell (30 minutes → 24 hours).

The reason for both cases of imaging reflecting coated C-LA ACNS as hepato-preferrable lies with the prominent liver targeting ligand, linoleic acid, employed in the make-up of the system. Linoleic acid is one of the fundamental polyunsaturated fatty acids that exist and is often used as a hepatic trigger entity by virtue of its pivotal role occurring in the liver (Cheong et al., 2009), because the liver is an organ that performs a cardinal role in lipid metabolism and via the metabolic pathway- fatty acid oxidation, linoleic acid is markedly implicated (Macarulla et al., 2005). The link resides here.

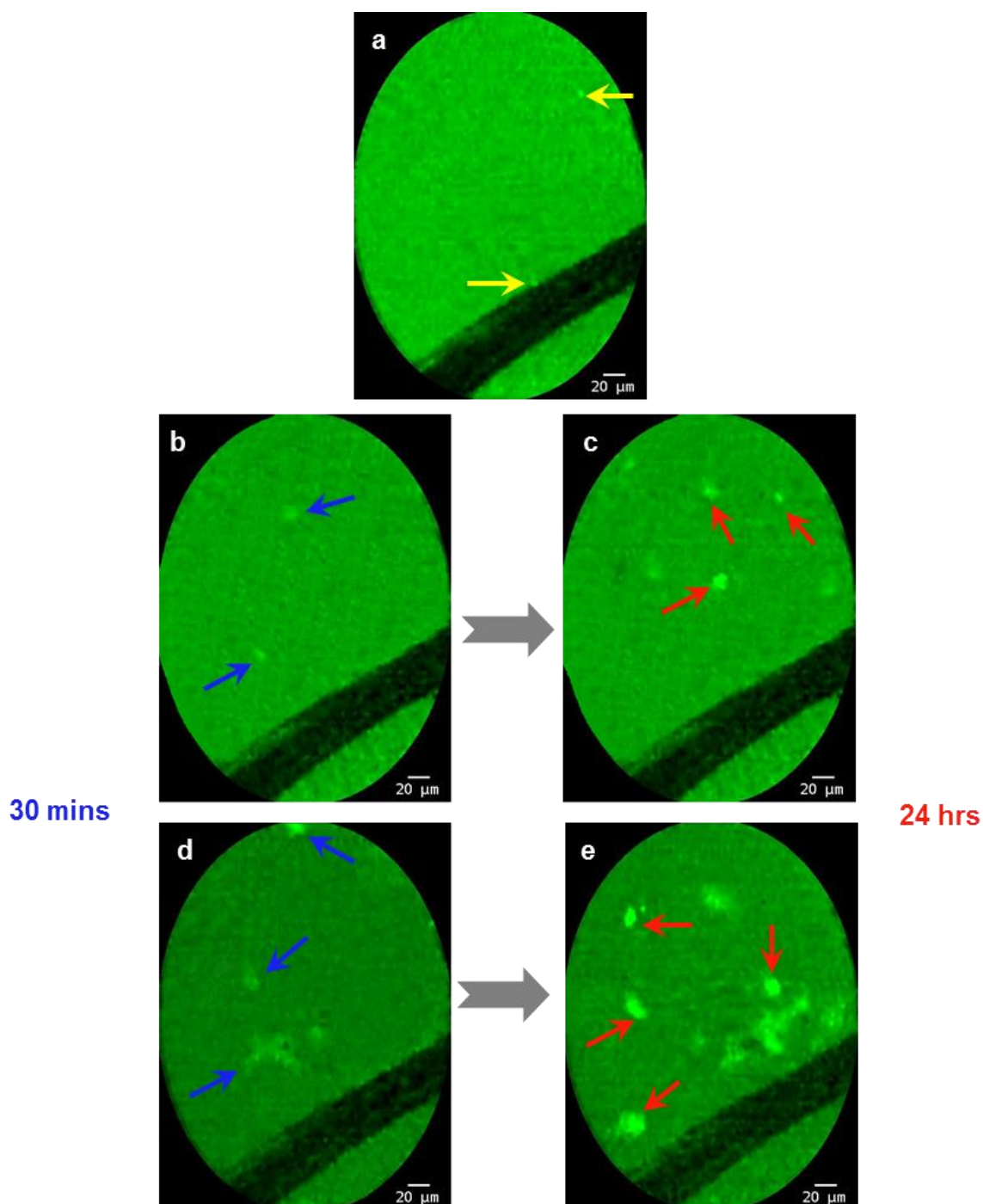


Figure 6.4: High speed fiber-optic fluorescent live images of a. fluorescent optimized ACNS, b. fluorescent uncoated optimized ACNS and HepG2 cells (>30 minutes incubation), c. fluorescent uncoated optimized ACNS and HepG2 cells (>24 hours incubation), d. fluorescent C-LA coated optimized ACNS and HepG2 cells (>30 minutes incubation), e. fluorescent C-LA coated optimized ACNS and HepG2 cells (>24 hours incubation).

6.4. Concluding Remarks

Effectual binding of FITC molecule onto ACNS surface both coated and uncoated was accomplished. Two types of fluorescence microscopy techniques were employed to investigate the nanoparticle distribution within HepG2 cells. It was quite apparent to see that in large quantities, the rapid uptake of ACNS formulation by HepG2 cells was that which was coated with chitosan-linolate. This resembled active targeting. The content of ACNS surface therefore stipulated the kinetics of cellular internalization. The presence of chitosan relaying enhanced binding, a liver cells attraction toward linoleic acid as a consequence of lipid metabolism, and a favorable <100 nm particle size all owed to the enhanced preferential ACNS binding at the cell surface and subsequent internalization.

CHAPTER 7

***IN VIVO* ASSESSMENT OF THE ACNS VIA INTRAPORTAL IV ADMINISTRATION IN THE RAT MODEL: A PILOT STUDY**

7.1. Introduction

The information acquired thus far from previous chapters on *in vitro* work performed is beneficial only when the conclusive results are equated to *in vivo* data. The reason for this is because of the possible and real threat to mankind or even an environmental threat that could be perilous as a result of the compound being surveilled (Han et al., 2012).

Animals are crucial to comprehending and exploring therapies that are essential for a variety of both human and animal ailments. As models, they are able to aid in deciding whether medicinal products and many other compounds are practical, adequate and safe (Stokes and Marsman, 2014). The introduction of a chemical substance into an animal will lead to interactions within its body at different sites where information on the unforeseen side effects or safety of the substance being studied can be acquired. The intricacy of pharmacokinetic and pharmacodynamic data cannot be replicated solely on cell cultures or laboratory procedures (*in vitro* testing) simulating a living physiological environment (Bertau et al., 2008). However, animal welfare is equally pivotal and should not be neglected. When necessity calls for *in vivo* examinations, the 3Rs ideology for animal welfare should never be lost sight of:

- Reduction entails using a minimal amount of animals concurrently achieving the pertinent information necessary to develop valuable conclusive data.
- Refinement entails a change that is realized during the study that may possibly require a reduction in the harshness of the inhumane operations the animals are to be subjected to.
- Replacement entails applying scientific methods that are feasible on non sentient matter instead of sentient animals (Madden et al., 2012).

Chronic liver disease encompasses a liberal loss of liver function and transformation of the liver parenchyma into fibrotic and cirrhotic tissue. Pathogenesis of this disease normally engenders portal hypertension and ultimately complete hepatic failure. Causative agents of chronic liver disease are hepatitis B and C viral infections, both nonalcoholic and alcoholic fatty liver disease, schistosomiasis infections and biliary fibrosis. Again, complete and thorough comprehension of liver disease pathogenesis is necessary on a dependable

experimental animal model. Thus far, an appropriate animal model has not been identified as one suitable enough to emulate hepatic disease. Only certain attributes of the disease are currently possible to assume in an animal model. Rodents are noted by investigators as most relevant animal models due to their small size, are short-lived and as a result of these, are particularly easy maintenance. Moreover, they have a considerably noteworthy genetic resemblance to humans allowing them to be most suitable a choice of model for human liver disease studies (Liu et al., 2013).

The liver acquires approximately 75 % of its blood supply from the portal vein therefore an additional and enhanced liver targeting approach would be administration of ACNS via an intraportal injection. In the study of portal haemodynamics for instance, a procedure allowing for an accessible reproducible administration of liquid solutions into the portal circulation of an animal model would be favored. This expedites inquiry into a drugs metabolism, possible toxicity of certain drugs on the liver, as well as examining and evaluating the functioning of a drug targeted in the treatment of hepatic diseases (Blumgart et al., 1971). There are a lot of complications related to injections into the hepatic circulatory system and as such, injections into the tail veins of rodents are merely implemented in *in vivo* studies. The problem with this is the small analogy with that of a clinical setting (Bagi and Andresen, 2010). Figure 7.1 expresses the differences in accumulation of microbubbles in the liver that are seen when the injection administrations occur via A) tail vein, B) portal vein and C) hepatic artery.

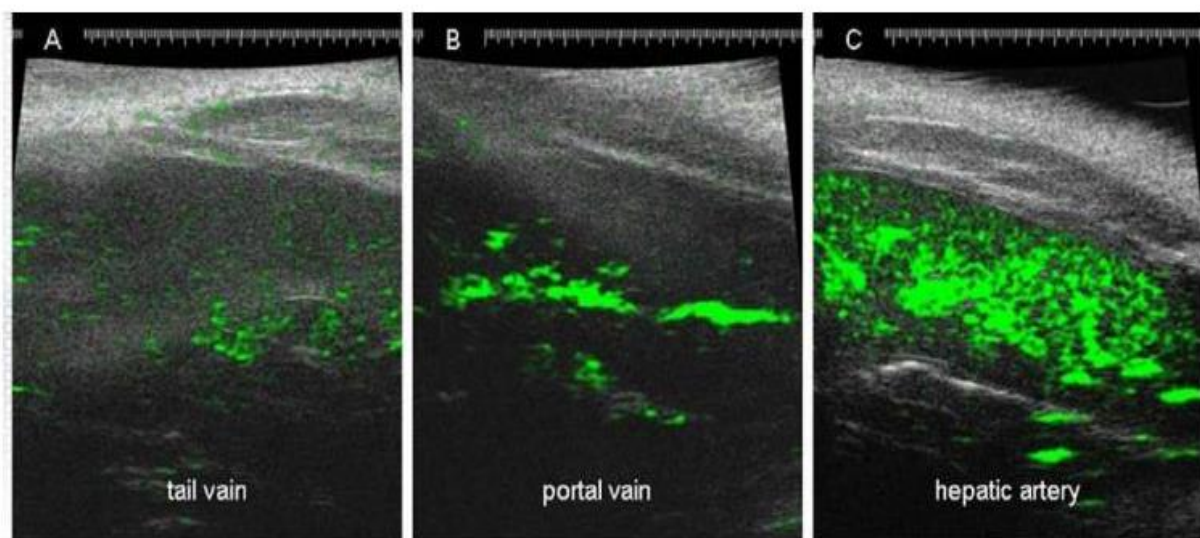


Figure 7.1: Ultrasound images of livers in the rodent animal model following injection administrations of microbubbles in A) tail vein, B) portal vein and C) hepatic artery. Enhanced microbubble contrast (green) is achieved with hepatic artery or portal vein with minimal contrast observed in tail vein (Adapted from Bagi and Andresen, 2010).

The *in vivo* determination of drug released within the liver and evaluating an efficient liver targeting ability of the novel drug delivery system compared to the conventional systems are one of the significant outcome measurements in this chapter. This was achieved through incorporation of a targeting appendage polymer design in the outer make-up of the drug delivery system for attachment, a liver targeting fatty acid incorporated in these appendages and an administration route (intraportal) that would reach the liver first.

7.2. Materials and Methods

7.2.1. Materials

Sprague Dawley rats employed in this study were received as per the protocols of the Central Animal Services (CAS) of the University of the Witwatersrand. Lamivudine (LMV) was received as an *ex-gratia* sample from Adcock Ingram Ltd. (Germiston, Gauteng, South Africa). Zidovudine (AZT) was purchased from GlaxoSmithKline (Middlesex, UK). Double de-ionized water was obtained from a Milli-Q system, (Milli-Q, Millipore, Johannesburg). All solvents utilized for UPLC-UV detection were of UPLC grade whereas all other reagents were analytical grade.

7.2.2. Animal Ethics Clearance

The Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand provided an animal ethics clearance for this research study (No. 2014/01/C) (Appendix F).

7.2.3. Animal Housing Conditions and Husbandry

Housing conditions were maintained according to the CAS Standard Operating Procedures (guidelines set by the South African National Standard for the care and use of animals for scientific purposes). In both pilot and main study, the Sprague Dawley rats were housed in single in cages at the CAS which were monitored on a 12 hour light/ 12 hour dark cycle (Figure 7.2). They were provided with water and rat feed *ad libitum*. Rats were also observed daily for one hour periods to detect behaviour changes. They were weighed on a weekly basis and a complete fill-out of a score sheet was made with observations as a result of the state of the animals noted.



Figure 7.2: Digital image showing Sprague Dawley rats housed in single in cages at the CAS animal experimental room.

7.2.4. *In vivo* studies on the rat model

7.2.4.1. Need for a Pilot Study

7.2.4.1.1. Logistic time points

A pilot study was conducted to evaluate a definite need for a 12 hour time point as it clashed with and disrupted CAS units operating times. Information acquired would evaluate a definite need for a 12 hour time point. CAS staff may not be available at this time point to conduct necessary surgical procedures and drug administrations in view of the CAS units operating times. Findings from blood samples with reference to fate of drug at 0, 3, 5, 8 and 24 hours may provide an insight into definite time points for use in main study that could allow for the exclusion of a 12 hour time point.

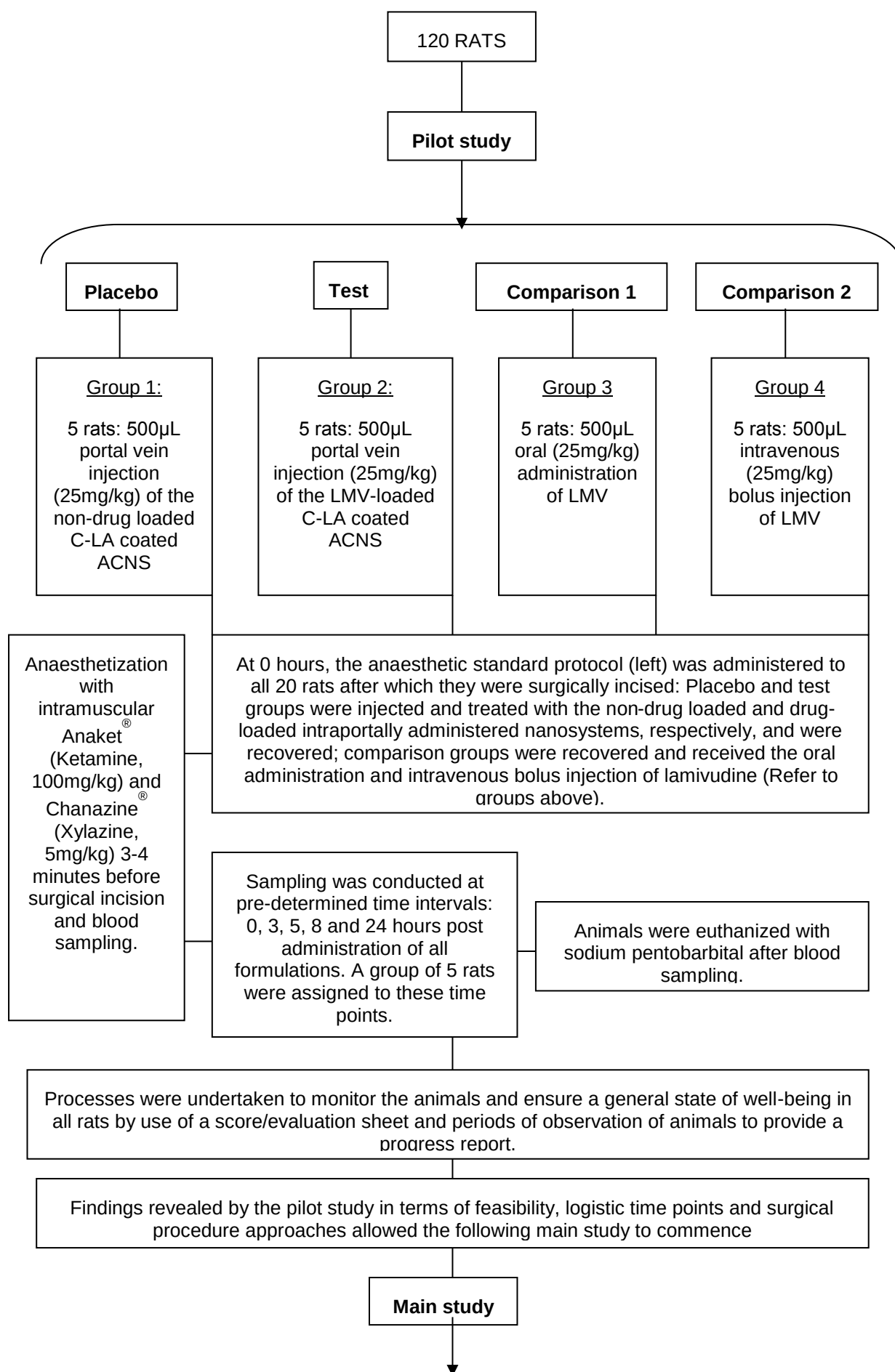
7.2.4.1.2. Surgical approach

The procedure described for the intraportal vein injection (Jacob et al., 2004) was one untried by the CAS veterinarians. A test of this method on a small number of animals would allow an

assessment of feasibility of the method by observing the animal and its state supposing death occurred due to possible excessive bleeding in the process and/or embolism in the liver and lungs. A main study could not commence if this had developed on all rats.

7.2.4.2. Experimental Design

Twenty rats with an initial weight of ~225 g were assigned to 4 groups (5 rats per group) for the pilot study. The main study comprised one hundred rats with an initial weight of ~225g which were assigned to 4 groups (25 rats per group) (Refer to Flow diagram, Figure 7.3). Group 1 was a placebo group of healthy rats which received an injection of the non-drug loaded C-LA coated ACNS. The group was administered with the nanosystem intravenously via the portal vein. The test group (Group 2) was administered with the LMV-loaded C-LA coated ACNS via intraportal injection. Group 3 and Group 4 were comparison groups. Group 3 involved the model drug, LMV being administered orally in healthy rats. Healthy rats of Group 4 received LMV administered through an intravenous bolus injection via the tail vein. The control non-drug loaded delivery system (placebo group) and the drug-loaded delivery system (test group) were the only groups that received an intraportal injection. All rats were caged singly post-surgery. For each group in the main study, a biodistribution imaging procedure was done 15 minutes before blood sampling analysis and livers were removed immediately after blood sampling and euthanasia.



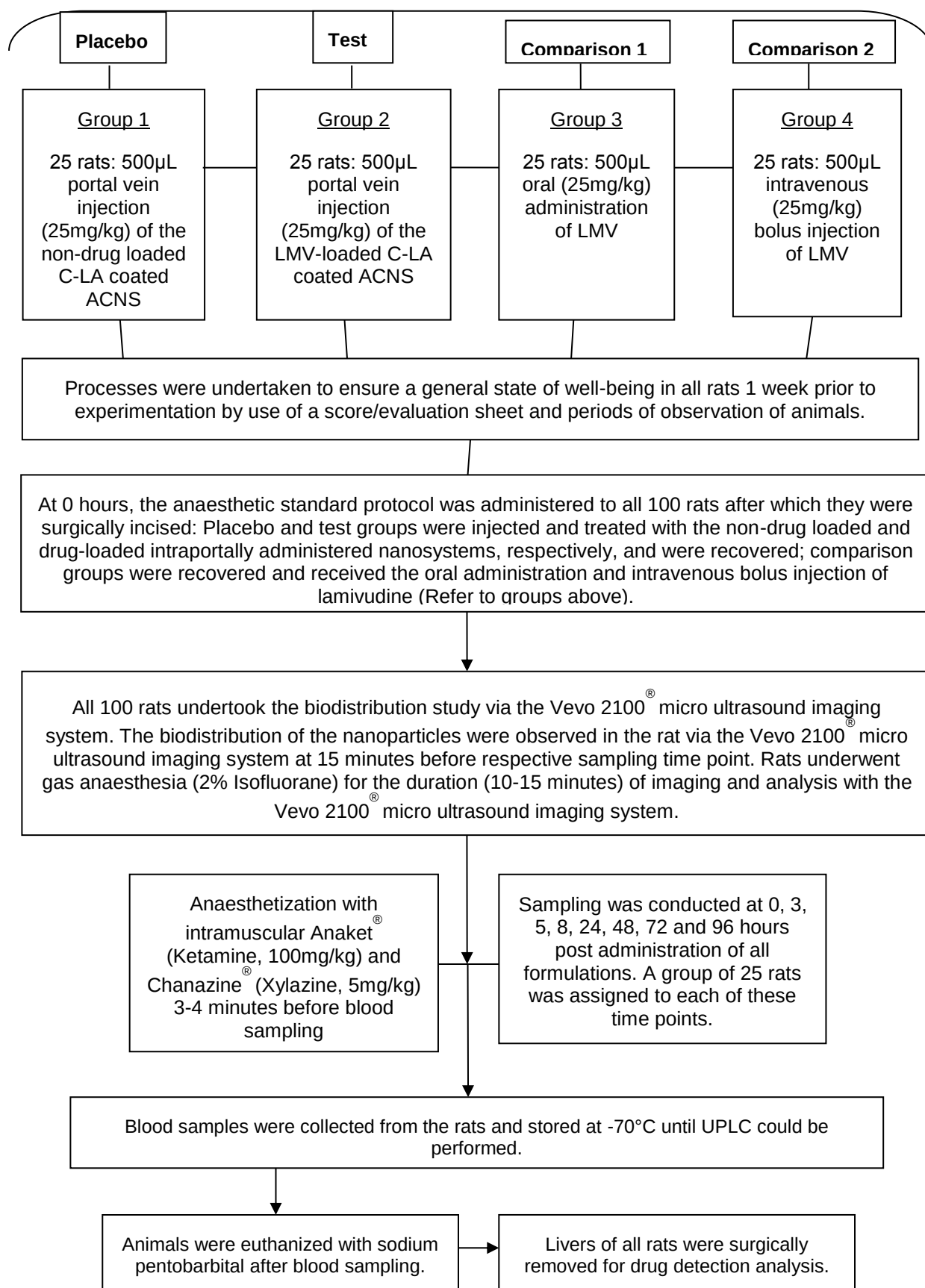


Figure 7.3: Schematic representation of *in vivo* animal studies to be conducted on the rat model with depiction of assigned experimental and control groups.

7.2.5. Intricate intraportal injection surgical procedure

A surgical procedure was performed on all groups of rats initially, although not necessary for respective treatments and administrations, but to maintain animals in a standard state due to the surgery that is necessary for the intraportal injection in Groups 1 and 2. It took 10-15 minutes to anaesthetize, open, inject, close and recover each rat. In Groups 1 and 2 rats, a transverse incision was made directly beneath the xyphoid process towards the animals left side. The portal vein was located, isolated and an intraportal injection was performed slowly without excessive pressure to avoid complications and the death of the animals (Jacob et al., 2004). A volume of 500 μ L was injected into the portal vein. Rats of Groups 3 and 4 were anaesthetized and the same incision was performed after which they were closed and recovered. All of the above applied to the pilot and the main study. The registered veterinarians did not have experience with the technique of intraportal injection therefore the pilot study provided insight into the feasibility of the surgical approach. Figure 7.4 outlines the intricate intraportal injection surgical procedure. A description of the procedure is as follows:

- a) Anaesthetized rat shaven, prepared and ready for surgery.
- b.i – b.iii) Transverse incision being made above liver.
- c.i – c.vi) Locating the portal vein.
- d) Slow and controlled injection into the portal vein.
- e) Careful placement of organ back into abdominal cavity.
- f.i – f.iii) Suturing of incision.

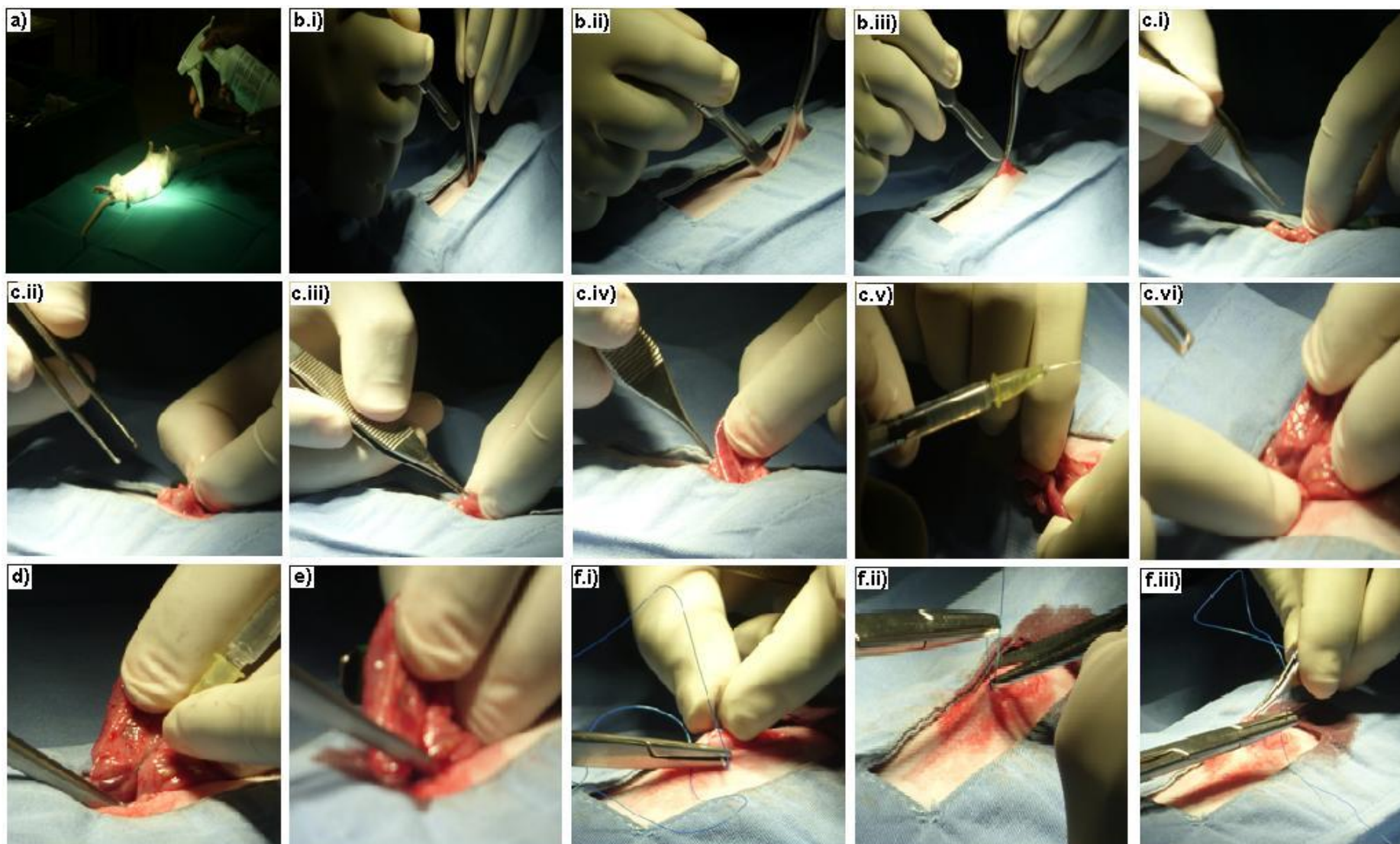


Figure 7.4: Successive digital images depicting the entire portal vein injection surgical procedure.

7.2.6. Animal welfare and humane end-points

The recovery period post intraportal surgery was a critical stage to ensure scrutinized observation and monitoring of animals. Figure 7.5 depicts the contribution to animal welfare in providing an environment most suitable to the specific needs of the animals by provision of heat to ease the discomfort experienced in recovery. Analgesia was employed when needed especially for rats of time points ≥ 24 hours, immediately, to relieve pain experienced by the rats as a result of the complex surgery. When any altered or disparate behaviour of the rats were recognized, they would have immediately been removed from the study. Use of score sheets and twice daily observation periods ensured the welfare of the animal and avoidance of any suffering. The following were clinical signs that would have required the rat to be euthanized:

- Signs of internal bleeding through stitches;
- Rats that lost 15 % of their body weight when compared to controls;
- Rats that lost 10 % of their body weight in 24 hours;
- Self-mutilation of the feet and
- Rats that developed seizures or convulsions (Schoefield, 2012).

Any of the above wayward findings would have allowed for pertinent data in evaluating the ACNSs biosafety.



Figure 7.5: Digital images of recovery stage post-surgery; a) Rat 1 from group A recovering from anaesthesia, b) A recovery room with blankets and heater.

7.2.7. High-frequency ultrasound preclinical imaging

Ultrasound imaging was used to observe the biodistribution of the ACNS within the rat using the Vevo 2100® Micro Imaging Platform enhanced with the Cellvizio® Lab Module (VisualSonics (Pty) Ltd, Toronto, Ontario, Canada). This was readily observed due to the impedance mismatch between the polymeric nanoparticles and body tissues/fluids. Imaging was conducted on anaesthetized rats following drug and drug delivery system administrations to the relevant animal groups 15 minutes

before a sampling time point. The rat was anaesthetized with xylazine and ketamine (1:4 mixture, 0.1 mL/100g) at CAS before being taken to the Dept. of Pharmacy and Pharmacology laboratory to perform the ultrasound imaging. The anaesthetized animal was placed on the stage fitted with a heated pad and its snout placed in the opening of the tube delivering 2 % Isoflurane gas anaesthesia and oxygen (Figure 7.6). A warmed gel specific to ultrasound imaging was gently smeared on the shaved area of the rat exposing the surgical stitches in the vicinity of the liver. The probe was placed on the area of interest and imaging was conducted.

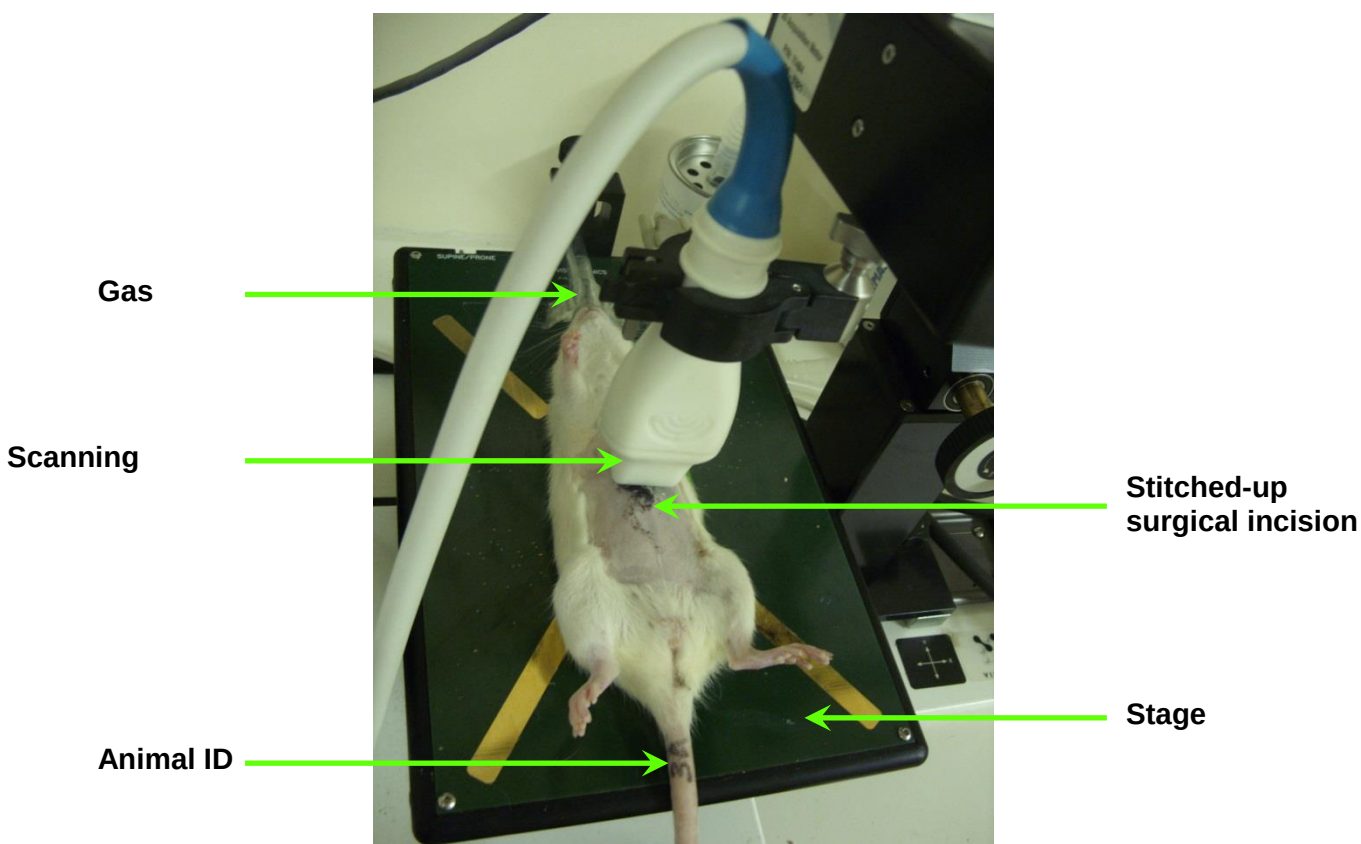


Figure 7.6: Digital image of anaesthetized Rat 3A undergoing an ultrasound imaging procedure.

7.2.8. Blood and liver sampling

Blood samples and liver tissue samples were obtained from rats for drug detection in Ultra Performance Liquid Chromatography (UPLC) analysis. These were terminal procedures for each individual rat to allow for sufficient blood collection and organ removal (Beeton et al., 2007). Blood sampling was carried out to determine that minimal to no drug was released from the delivery system in the circulatory system due to release in the liver (hepatic system). The sampling was taken via

intracardiac puncture in rats. Blood samples of 5 mL were acquired with a sterile syringe while the animals were under anaesthesia with IM Anaket® (Ketamine, 100 mg/kg) and Chanazine® (Xylazine, 5 mg/kg). Sampling was conducted at fixed time intervals (Refer to Figure 7.2) and blood samples were collected in vacutainer tubes containing clotting factor to draw out serum. Animals were then euthanized with sodium pentobarbital. After euthanasia, the livers of the rats were extracted for UPLC analysis. The blood tubes were left to stand overnight at 4 °C for collection of plasma the next day. The supernatant, containing the plasma, was carefully aspirated and transferred into clean 2 mL eppendorf tubes and were put to freeze at -80 °C immediately till further UPLC analysis.

7.2.9. Liver removal and treatment

The livers of euthanized rats were surgically removed to be homogenized and evaluated through UPLC for any drug release in the liver. The liver removal surgery took 1-2 minutes for each rat. The rat was opened up with a medial incision and the liver was revealed. It was carefully cut, removed and transferred immediately to a beaker of 100mL ice-cold 0.9 % NaCl solution to reduce its temperature. It was further placed in 10 % v/v formalin solution and stored in liquid nitrogen until homogenization and analysis of the organ could commence. Figure 7.7 shows a liver that was removed at the 8 hour time point of rat from Group C.



Figure 7.7: Digital aerial view image of liver removed from Rat 1 Group C in 10 % v/v formaldehyde solution.

For the homogenization process, 100 mg/mL of liver tissue was ground to a pulp with sterile saline using a pestle and mortar in a fumehood. The liver pulp was then transferred to ice and underwent further homogenization by non-pulsed ultra-sonication for 10 minutes at an amplitude of 80 (Vibra-Cell Ultrasonicator, Sonics, USA). The thoroughly homogenized liver was then transferred to clean eppendorf tubes to be centrifuged on high at 15000 rpm for 15 minutes (Universal 320R, Hettich Zentrifugen, Germany). Supernatant was aspirated with a pipette and transferred further to clean tubes for storage at -80 °C for UPLC analysis.

7.2.10. Applying Ultra Performance Liquid Chromatography to ascertain LMV content in blood and tissue samples

Sensitive analytical methods for quantification are required in order to improve the understanding toward the relationship between pharmacokinetics, pharmacology and the bioavailability of LMV within a system (Wang et al., 2009). UPLC is an analytical method used to quantify and improve the analysis of samples encountered during pharmaceutical development (Wren et al., 2006). The blood and liver samples were analyzed to gather information about the drug molecule through UPLC to determine drug concentration against time. The concentration of LMV from blood and livers sampled during the study was used to determine the ability of the drug delivery system to be superior than the comparators by achieving better drug release profiles.

7.2.10.1. Ultra Performance Liquid Chromatography analysis conditions: Washes, mobile phases and separation parameters

Chromatographic detection and quantification of LMV was performed making use of a Waters Acuity™ Ultra Performance Liquid Chromatographic (UPLC) system (Waters Corporation, Milford, Massachusetts, USA) which comprised a binary solvent manager, a sample manager and was fitted with a photodiode array detector (PDA). An Acuity UPLC® HSS T3 1.8 µm; 2.1x150 mm analytical column (Waters Corporation, Dublin, Ireland), fitted to a VanGuard™ Pre-column 3/Pk 2.1x5 mm column (Waters Corporation, Dublin, Ireland), was employed for drug detection at a temperature of 40 °C. Before proceeding with runs, the machine had to be primed with a standard set of solvents that consisted of 100 % methanol, 100 % acetonitrile (ACN), a strong wash made up of 90/10 ACN/water and a weak wash made up of 10/90 ACN/water. Table 7.1 shows the gradient method that was employed for detection and quantification of LMV with Zidovudine (AZT) being employed as the internal standard, and mobile phases of 0.2 % Formic acid in water (A) and 100 % ACN utilized.

Table 7.1: UPLC gradient method for the elution of LMV and AZT (internal standard).

Time (min)	Flow Rate (mL/min)	0.2% Formic Acid	% Acetonitrile
0	0.5	90	10
1.0	0.5	70	30
1.5	0.5	90	10

The photoiodide UV/vis detection occurred at 277 nm. The sample injection volume was 10 μ L and the runtime spanned 3 minutes. All solvents and solutions were filtered with 0.22 μ m membrane filters (GHP Acrodisc® 13 mm Syringe Filters, Pall Corporation, New York, USA).

7.2.10.2. Preparation of stock and standard solutions

A stock solution of LMV was prepared to be used for the working standard solutions in the concentration range 8-40 μ g/mL (n=5). This was achieved by dissolving 10 mg of LMV in 100 mL ultra-pure double de-ionized water (Milli-Q Gradient, Millipore, MA, USA, electrical conductivity 18.2 M Ω .cm at 25 °C) and serial dilutions made thereof. The internal standard, AZT, was prepared and used at an unvarying concentration of 500 μ g/mL.

7.2.10.3. Verifying of optimal solvent/s to be used for liquid-liquid drug extraction procedure

A liquid-liquid extraction procedure was selected because of LMVs approving low protein binding feat. The chosen solvent/s were pipetted at a volume of 1500 μ L into plasma and liver tissue homogenate aliquots of 100 μ L in eppendorf microtubes (Eppendorf AG, Hamburg, Germany), followed by vortexing (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for 1 minute to extract the proteins that would be found therein. The solvent-plasma/solvent-liver tissue homogenate samples were then centrifuged on high at a speed of 12000 rpm for 10 minutes at 4 °C (Universal 320R, Hettich Zentrifugen, Germany). The supernatant was decanted into clean eppendorf tubes and samples were dried completely directing a gentle stream of N₂ into the tubes in a fumehood. The residue was reconstituted with 500 μ L of mobile phase A and spiked with the uniform internal standard concentration, before being filtered into the UPLC Waters glass vials and placing in the sample compartment.

7.2.10.4. Precision and accuracy validation

Inter- and intra- day accuracy and precision elucidation is a between-day and within-day reproducibility test of the assay and involved the preparation of three different spiked concentrations of plasma samples processed as three replicates and treated by the extraction procedure defined in *Subsection 7.2.10.3* on three discontinuous days (Inter-day precision) as well as repeatability in one day (Intra-day). Drug concentrations were prepared in the range of 1, 2.5 and 5 µg/mL. The validation was computed by means of percentage relative standard deviation (%RSD) in Equation 7.1.

$$\%RSD = \frac{SD}{Mean} \times 100 \quad \text{Equation 7.1}$$

where SD is standard deviation.

7.2.10.5. Construction of a calibration curve to gauge drug quantities in blood plasma and liver treated samples

A calibration curve was constructed to quantify the levels of LMV found in blood plasma compared to the liver of rats. Frozen plasma and liver treated samples were thawed and 100 µL of sample were transferred to clean 2 mL eppendorf tubes. Added to this were the extraction solvents mentioned in *Subsection 7.2.10.3* and profiled in imminent subsections. Tubes with sample were subjected to the extraction procedure portrayed in *Subsection 7.2.10.3*. Following spiking with the internal standard, samples were filtered and analyzed.

7.3. Results and Discussion

7.3.1. Conclusive findings from the Pilot study for the commencement of the Main study

A pilot study (Figure 7.3) was conducted to:

- Evaluate a definite need for a 12 hour time point as it clashes with and disrupts CAS units operating times;
- Assess the feasibility of the surgical approach which involves an intraportal vein injection procedure previously untried by CAS veterinarians by observing the animal and its state

supposing death occurs due to possible excessive bleeding and/or embolism in the liver and lungs.

7.3.1.1. Day 1 – Practice Surgery

Three CAS unit rats were used to perform a mock surgical procedure of portal vein injection. A 26 gauge needle was used for the intraportal vein injection of sterile water. Outcomes: The entire surgical procedure proved successful, in terms of the mid-line incision and location of the portal vein, up until the injection with the 26 gauge needle. It was found that the needle gauge was slightly large for the tiny vessel and caused the rat to bleed incessantly. It was gathered that a smaller gauge needle (27-30 gauge) was needed to cause minimal to no vein damage for intraportal administration.

7.3.1.2. Day 2 - Intraportal vein injections on comparison groups C and D

Five rats from group C and five rats from group D were treated with oral and IV LMV, respectively. Portal vein injections were not necessary for these groups; however a sham surgery was conducted on all experimental animals to ensure a standard state for accurate results. The blood sampling and euthanasia time points to be assessed for the pilot study were 0, 3, 5, 8 and 24 hours. This occurred in a random, alternating fashion with rats assigned to the 8 hour time point undergoing the experimental procedures first. Outcomes: A procedure was undertaken for accommodating the 8 hour time point to occur within CAS operating times (08:00-16:30). Therefore, rats for both groups belonging to the 8 hour time point were worked on first which brought the end of the day's experimental procedure to 16:35 the latest. The preparations before the procedures commence needed to occur 15 minutes in advance (07:45) to save up on unnecessary lost time.

7.3.1.3. Day 3 – Intraportal vein injections on placebo and test groups A and B

Five rats from group A and five rats from group B were treated with the C-LA coated LMV-loaded ACNS and C-LA coated non-drug loaded ACNS, respectively, via the intraportal injection administration route. The time points of 0, 3, 5, 8 and 24 hours were assessed in the same manner as demonstrated with groups C and D in *Subsection 7.3.1.2*. Outcomes: Day 3 involved the complicated intraportal injections which required thorough concentration and careful animal

handling. For this reason, the last sampling and intracardiac puncture 8 hour time point was expected to shift slightly later than anticipated. Therefore compensation for this would be an earlier start to the days procedures (>15 minutes in advance). Main study sampling points would proceed for more days after the 24 hour time point therefore a lack of a 12 hour time point would certainly not misconstrue drug level readings in the least bit. A thinner 27 gauge needle was used for intraportal injections of Day 3 to evaluate its efficacy. Amongst ten rats, two displayed problems. Rat 1 A had lost a considerable amount of blood during the procedure and had to be treated with Ringer's Lactate solution to replace the lost volume. Rat 5 A had to be terminated as it showed continuous blood loss from the wound, whilst in its cage throughout the day. The 27 gauge needle was clearly not thin enough. As a result, the main study had to make use of a much thinner 29 or 30 gauge needle to avoid damage to the vein and ensure bleeding did not occur at all at the site of injection.

7.3.1.4. Summary and Resolutions

The exclusion of a 12 hour time point was carried through and an intraportal vein injection on the rat model was feasible but with use of an extremely thin needle. Thirty gauge needles had been sought, ordered and received for its use in the main study.

7.3.2. High-frequency ultrasound preclinical imaging

High-frequency ultrasound imaging is a non-invasive procedure that encompasses a process of sound wave generation from transducers that pass into a living entity. When this occurs, the waves are reflected back and the transducer then converts them into 2D and 3D images. Anaesthetized rats, 15 minutes before a sampling and termination point, were imaged in the supine position (Figure 7.6) with the transducer being positioned over the rats liver which is suspended just beneath the diaphragm anatomically. Images obtained were to show the presence of C-LA coated ACNS (as well as its movement through the vein through the instruments capability of 3D tracking) due to the acoustic mismatch between the materials of the ACNS from that of body tissue (Yang et al., 2008). Figure 7.8 is an ultrasound image acquired at the last time point of 96 hours displaying the median/cystic lobe of rat livers administered with the C-LA coated LMV-loaded ACNS (A), C-LA coated non drug-loaded ACNS (B), oral LMV (C) and IV LMV (D). The red circled parts denote the homogenous liver parenchyma. A healthy rat liver would show medium level echogenicity on an ultrasound image as normal distinguishing features together with a regular hepatic surface (Resende et al., 2011). This is also denoted by the red circles. However, an enhanced echogenic portion on the liver lobe is evident in Figure 7.8 A & B of rats that were administered with the C-LA coated ACNS systems that is absent in Figure 7.8 C & D. The first reason for this lies in the high concentration of cryoprotectant, mannitol, which was used in the formulation of the C-LA ACNS in its initial stages of preparation for size maintenance. Mannitol is known to have an acoustic property that causes ultrasound reflectivity and produces echogenic systems in which it is incorporated greater than any other sugar (Huang et al., 2001). The other reason for the high contrast liver lies in the targeting ligand used in the make-up of the coating of the ACNS, linoleic acid. It is well-known that linoleic acid is a fatty acid that is classified as a lipid. Materials such as lipids are also highly echogenic because they acquire the potential of acoustic backscattering as a result of the gas/air bubble they are capable of capturing causing the echogenic effect (Son et al., 2014). This proves in Figure 7.8 A & B images that the C-LA coated ACNS is still present in the liver 4 days later compared to the conventionals (Figure 7.8 C & D) which show minimal to no echogenicity.

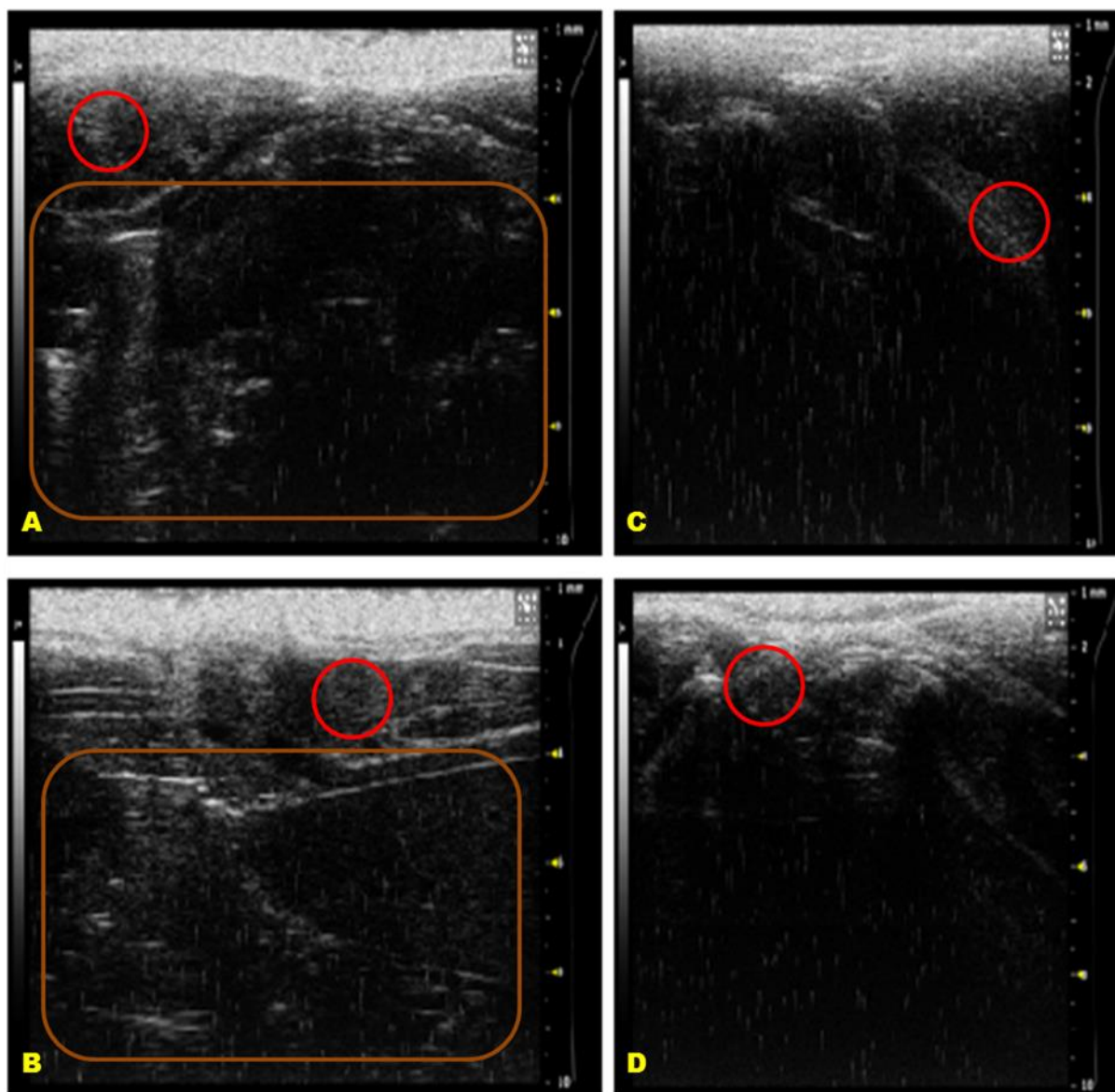


Figure 7.8: 96 hour time point ultrasound liver images of rat administered with A) Optimized C-LA coated LMV-loaded ACNS, B) Optimized C-LA coated non drug-loaded ACNS, C) Oral LMV and D) IV LMV.

7.3.3. Elution times of LMV and the internal standard

The chromatographic conditions employed for quantification and detection of LMV and its internal standard, AZT, proved to be successful as chromatographic data showed peaks that corresponded to their elution times. Figure 7.9 reflects the 2D chromatogram plot and its interrelated 3D PDA plot of LMV and internal standard spiked in ACN:double deionized water (1:1) (A) and LMV and internal

standard spiked in blank plasma (B). LMV was the first to elute at a retention time of 0.905 minutes and internal standard at 1.756 minutes in a 1:1 ratio of ACN and double deionized water. However, a slight difference in elution times was noted with both drug and internal standard when spiked in a blank plasma sample. A difference of 0.017 minutes was seen when LMV separated in the blank plasma sample with elution occurring earlier at 0.888 minutes. Likewise, with AZT a slightly higher difference of 0.024 minutes was eminent in its elution with the blank plasma sample occurring at 1.732 minutes.

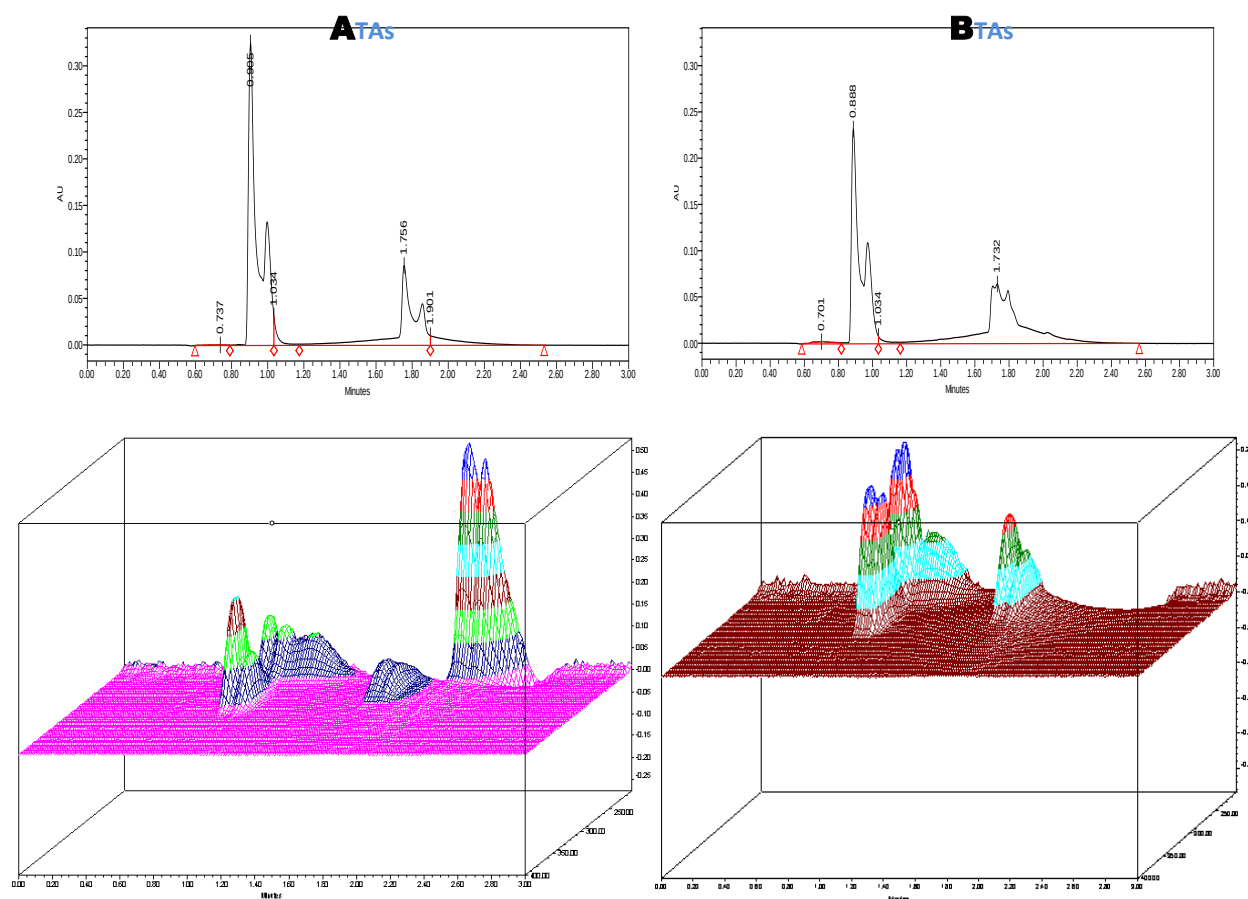


Figure 7.9: Elution depictions on 2D and PDA 3D plot for A. Spiked LMV (Retention time = 0.905 minutes) and AZT (Retention time = 1.756 minutes) in double deionized water and B. Spiked LMV (Retention time = 0.888 minutes) and AZT (Retention time = 1.732 minutes) in blank plasma sample.

7.3.4. Substantiation of most optimal solvent/s for liquid-liquid extraction in drug detection clarity and recovery

The blood plasma samples and liver tissue treated samples from rats must be 'clean' before injection into the UPLC column as protein makes up the bulk of the constituents which produce interferences on a chromatogram leading to flawed data. It is therefore crucial to employ the most favourable solvent/s that manages to achieve almost total cleanliness of *in vivo* samples. A wide range of solvents were tested to achieve minimal interference through stray peaks originating from blank plasma samples. Solvents were utilized in solitary as well as ratios. Table 7.2 displays the solvents tried and tested with success or fail.

Table 7.2: Solvent selection for liquid-liquid extraction procedure.

Deproteinizing agent	Baseline Result
Acetonitrile	↕↕
Ethyl Acetate	↕
Perchloric Acid (6 %)	↕↕
Trichloroacetic acid (10 %)	↕↕↕
Ethanol	↕↕
Methanol	↕↕
*Acetonitrile/Ethyl Acetate	↕↕
*Dichloromethane/Ethyl Acetate	↕
*Trichloroacetic Acid (10 %)/Ethanol	↕↕↕
*Formic Acid (1 %)/Acetonitrile	↕↕
#Acetonitrile/Dichloromethane/Ethyl Acetate	↕
#Ammonium Sulphate/Acetonitrile/Ethanol	↕↕
*Zinc Sulphate/Acetonitrile	↕↕

↕ - Closest to baseline; ↕↕- Further from baseline; ↕↕↕- Furthest from baseline

*1:1

#1:1:1

The table shows solvents that resulted in a peak that was either small enough (closest to the baseline) that could be ignored for erroneous results and utilized for extraction, slightly heightened

which could interfere with drug detection elution (further from the baseline) or large enough to completely dispel any useful data (furthest from the baseline). Ideally, a solvent resulting in peaks formed closest to the baseline would be preferable. This was seen with Ethyl Acetate (EA), a 1:1 ratio of Dichloromethane/Ethyl Acetate (DCM/EA) and a 1:1:1 ratio of ACN/DCM/EA. The solvent and solvent combinations were further tested to evaluate which generated the best recovery of LMV. This is depicted in Table 7.3.

Table 7.3: Recovery of LMV drug in spiked blank plasma from selected deproteinating agents (n=3).

	¹ EA	^{2,*} DCM/EA	^{3,#} ACN/DCM/EA
Recovery (%)	71.253	41.378	93.179
⁴RSD (%)	2.586	19.221	5.422

¹Ethyl Acetate

^{2,*}Dichloromethane/Ethyl Acetate, 1:1

^{3,#}Acetonitrile/Dichloromethane/Ethyl Acetate, 1:1:1

⁴Percent Relative Standard Deviation

The solvent blend of ACN/DCM/EA showed the best drug recovery percentage of 93.179 % with an RSD percentage value of 5.422. These were acceptable results and the combinations of these solvents were thus chosen for liquid-liquid extraction in cleaning the blood plasma and hepatic tissue treated samples. However, paramount emphasis is on the actual recovery of LMV from the extraction procedure and consequently the solvent blend was tried and tested on spiked LMV blank plasma samples (10 µg/mL) at ratios of 3:2:1 and 5:1:2.5 to estimate the optimal recovery of LMV against the 1:1:1 ratio already demonstrated. Table 7.4 reflects the data obtained with the varying ratios of the solvent blend.

Table 7.4: Recovery of LMV drug in spiked blank plasma from variable selected deproteinating agent ratios (n=3).

	¹ ACN/DCM/EA		
	1:1:1	3:2:1	5:1:2.5
Recovery (%)	93.179	83.844	91.720
²RSD (%)	5.422	3.875	0.893

¹Acetonitrile/Dichloromethane/Ethyl Acetate

²Percent Relative Standard Deviation

A 1:1:1 ratio of ACN/DCM/EA yielded the highest amount of drug recovery with a 5:1:2.5 ratio coming in second with 91.720 % of recovered drug. A 3:2:1 ratio holding EA in the least amount proved to be unsatisfactory with the lowest recovery of 83.844 %. Accordingly, a 1:1:1 ratio of ACN/DCM/EA was used in the liquid-liquid drug extraction procedure for all blood plasma and hepatic tissue treated samples.

7.3.5. Validation of precision and accuracy

Assessments of inter- and intra- day variability were performed for precision and accuracy validation and results are reflected in Table 7.5. Concentrations of drug solutions used were 1, 2.5 and 5 µg/mL. The RSD value for intra- and inter- day variability with concentrations 1 (1 µg/mL), 2 (2.5 µg/mL) and 3 (5 µg/mL) ranged from 1.023-3.834 %, 0.246-3.565 % and 1.095-3.085 %, respectively. These results were admissible (Karnes and March, 1993) and validated precision and accuracy of the sample treatment procedure as well as method.

Table 7.5: Inter- and Intra- day variability testing precision and accuracy of assay.

	Intra-day			Inter-day		
	1	2	3	1	2	3
%RSD Concentration 1	3.620	2.048	1.023	3.834	1.405	2.836
%RSD Concentration 2	1.275	1.604	1.152	3.565	1.076	0.246
%RSD Concentration 3	1.570	3.085	1.915	2.627	2.290	1.095

7.3.6. Construction of a calibration curve for LMV quantification in blood plasma

A calibration curve was plotted to be able to quantify LMV in the biological fluid samples and this is depicted in Figure 7.10. A drug concentration range of 1-5 µg/mL (n=5) was used and the ratio of the area under the curve of LMV to that of AZT was calculated and plotted against concentration (µg/mL). The calibration curve showed good linearity with a correlation coefficient (R^2) of 0.989.

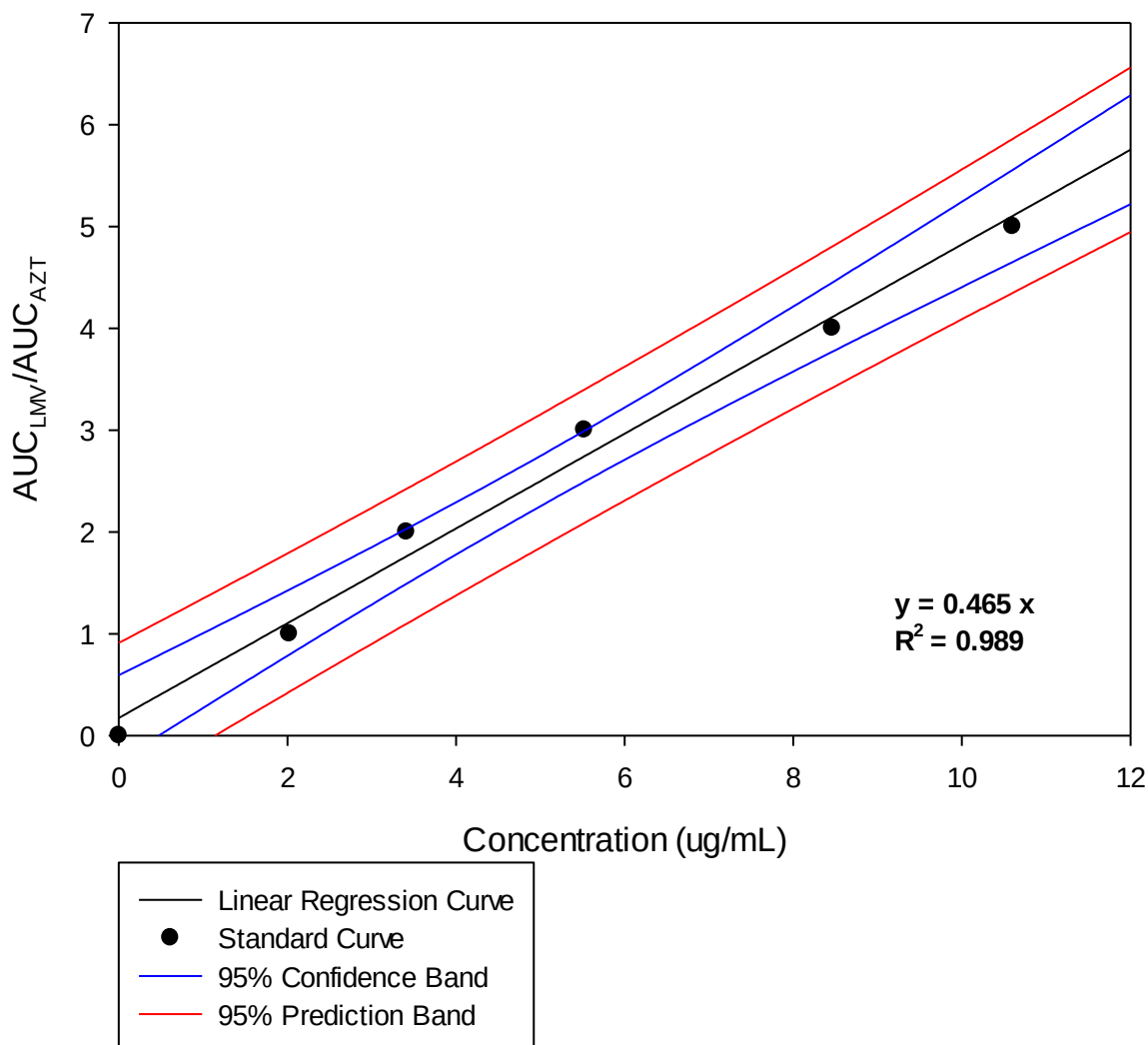


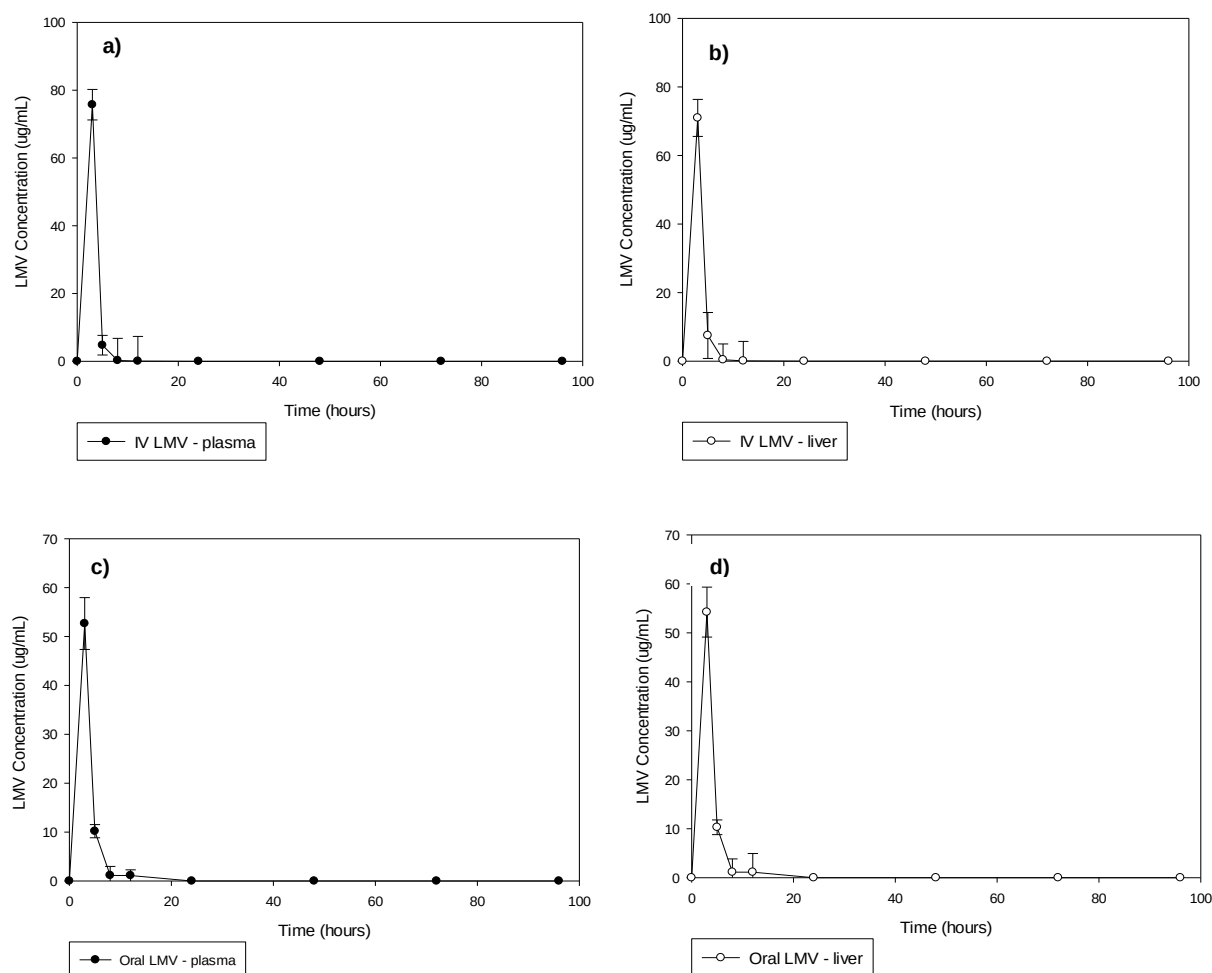
Figure 7.10: Calibration curve of LMV in blank plasma at 277nm.

7.3.7. Quantification of LMV concentrations in blood plasma and livers of rats

An imperative final assessment to be made in this *in vivo* study was the actual quantification of LMV detected in the blood plasma of rats treated against their levels in the hepatic region. Figure 7.11 discloses contrasting LMV concentrations in plasma and liver of plain drug that was administered intravenously (a & b), orally (c & d) and via the optimized C-LA coated LMV-loaded ACNS (e & f). With reference to Figure 7.11 a & b, following tail vein injection of LMV,

the drug reaches its peak concentration ($C_{\max}$) of 75.736 $\mu\text{g/mL}$ in the first measured time point of 3 hours in plasma and that of the liver is $C_{\max} = 70.983 \mu\text{g/mL}$. These are, without reservation, high peak levels and understandably so as the drug possesses a low plasma protein binding ability ($\sim <36\%$). LMV levels achieved immediately after IV injection in both the plasma and liver were fairly high, but had dropped rapidly by the 5th sampling point. The slightly higher level of concentration in the plasma compared to the liver at this time can be attributed to the apparent fact that the drug was injected directly into the circulatory system leading to immediate soaring concentrations detected therein. The slightly lower LMV concentration in the liver is due to the fact that post administration of most drug compounds via a tail vein injection in the rat model distributes to the liver first (Meibohm, 2006). The lowest level of 0.0368 $\mu\text{g/mL}$ and 0.0582 $\mu\text{g/mL}$ is detected after 12 hours in the blood and liver, respectively. The drug release profiles of the conventional orally administered LMV depicted in Figure 7.11 c & d demonstrate peak serum levels of drug reaching 52.675 $\mu\text{g/mL}$ 3 hours post administration. LMV concentration levels decline rapidly over the next 9-10 hours. The levels of LMV in the liver show a slightly higher $C_{\max} = 54.241 \mu\text{g/mL}$ 3 hours after administration of oral LMV. These results are fair because LMV has been known to have a satisfactory pharmacokinetic profile with bioavailability after oral administration being fairly high ($\sim 80\%$). Despite this favorable reality of the oral conventional LMV, one should not lose sight of the undesirable side effects it avidly holds such as that of lactic acidosis and hepatotoxicity being the more serious forms. Figure 7.11 e & f presents LMV release profiles in the plasma and liver from rats administered with the C-LA coated ACNS. LMV $C_{\max}$ in the liver tissue was achieved 8 hours after the intraportal injection of the C-LA coated ACNS at a remarkable concentration of 91.723 $\mu\text{g/mL}$ compared to LMV $C_{\max}$ of 8.947 $\mu\text{g/mL}$ in plasma at the same time point suggesting desired and successful C-LA ACNS hepato-targeting ability. LMV, after 4 days, reflects a concentration of 73.888 $\mu\text{g/mL}$ in the liver indicating that the drug is being released in a controlled manner overcoming peak-to-trough fluctuations. Fluctuations in LMV being released were not observed. The drug loaded C-LA coated system distinctly surpassed the IV and orally administered LMV. Minimal LMV is detected in the plasma, thus potentially minimizing the systemic side effects of LMV significantly i.e. the oral system does not differentiate between plasma vs. liver delivery and high levels of LMV are detected in the plasma that would lead to the undesirable systemic side effects. The levels of LMV attained in the liver are within therapeutic range as LMV is in fact not a narrow therapeutic range drug and spans across a wide window. Additionally, research studies that fixated on overdosing did not reveal any organ toxicity from the high levels of the drug (Strauch et al., 2011). However, one study did confirm the chromosome damaging effect of

LMV at a concentration dose of 125 µg/mL (48 hour time period) and 150 µg/mL (24 hour time period) in the blood (Bayram and Topaktaş, 2008). Figure 7.11 a) depicting $C_{\max} = 75.736$ µg/mL with the pooling of LMV in blood plasma after intravenous administration is significantly the closest to this toxic concentration of the drug compared to an insignificant $C_{\max} = 8.947$ µg/mL [Figure 7.11 e)] achieved with the ACNS.



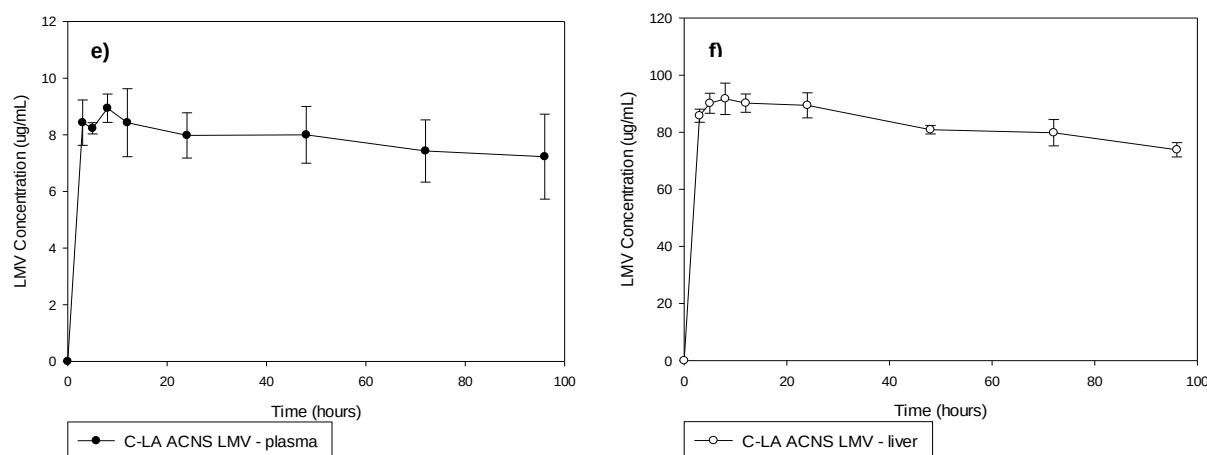


Figure 7.11: *In vivo* LMV concentrations measured in, a) IV administration in plasma, b) IV administration in liver, c) Oral administration in plasma, d) Oral administration in liver, e) C-LA ACNS intraportal administration in plasma, f) C-LA ACNS intraportal administration in liver.

7.4. Concluding Remarks

This *in vivo* chapter demonstrated that the ACNS is a suitable carrier for a hydrophilic antiviral and further demonstrated an exceptional ability of targeting when coated or chemically modified on its surface. The absent model disease, HBV, and drug, LMV, were applied for affirmation of the ACNS functionality. A coat consisting of chitosan linked LA on its surface was employed to target the hepatic environment wholly. Lucrative data were obtained with majority of LMV found pooled in ACNS and pseudo-released in the liver as compared to the circulatory system. A pilot study was carried out to assess the feasibility of an intraportal administration in the rodent model and this turned out successful as well as successful in providing the augmented hepato-targeting. Biodistribution images proved coated ACNS targeting ability and positive lagged drug release in the liver for days with potential of sustenance for more hours to days. This study opened up areas for ACNS to reveal its potential in diverse applications as a drug/gene carrier and targeting system.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusion

Numerous pathogenic viruses exist which cause infectious viral diseases in humans when specific cells are attacked and virus replication occurs due to immunity of the host failing. A renowned trait of these microorganisms is their rapid spreading ability. In consequence, viral pandemics are not unusual with pointed fingers at HIV/AIDS currently rife globally. The most alarming and recent outbreak occurrence is the Ebola Virus where numbers of Ebola cases and infected persons are soaring at present, with fear of this virus also spanning to a 'pandemic' proportion.

Duly, there is dire concern and desperation to control and curb a viral infection because being tricky organisms, they maintain a capacity of adjusting, conforming to be resistant to antivirals as well as incorporating themselves into host DNA at which point tumors are then developed. For this reason, they reside in the nucleus and cytoplasm of cells which necessitates therapeutic action to be directed to these areas. With that said, a novel virus-resembling antiviral intracellular delivery system is put forward in this research study with Hepatitis B Virus (HBV) selected as the model viral disease and Lamivudine (LMV) as the antiviral therapeutic to be delivered into the cytosol of a targeted hepatic cell where HBV would ordinarily reside.

A hydrophile - Hyaluronic acid (HA), and a hydrophobe - epsilon-Caprolactam (ECL), were successfully graft copolymerized in a novel fashion to synthesize an amphiphile (HA-g-ECL) capable of gaining intracellular entry when formulated into suitably sized nanoparticles. Intracellular entry was possible and distinct for cells within hepatic radius due to the surface chemical modification of the architecturally configured nanoparticulate system (ACNS) with a liver targeting ligand coat of Chitosan and the fatty acid, Linoleic acid (LA). The polymer and fatty acid were equally successful in being copolymerized, and in similar manner as HA-g-ECL, with synthesis of Chitosan-Linolate (C-LA). Affixing C-LA as a coat for ACNS was fruitful in conveying a liver targeting ability as evinced in *in vivo* investigations. *Ex vivo* investigations approvingly manifested HepG2 cellular internalization proficiency of C-LA ACNS particles due to a required and achieved nanoscale size. C-LA ACNS was able to deliver acceptably entrapped

LMV to hepatic cells aided by intraportal administration in the rat model. *In vivo* ultrasound imaging over the hepatic region confirmed this. The system provided sustained release of the antiviral over a measured period of 96 hours. Concurrently, results revealed favorably mediocre LMV levels in the blood circulatory system further demonstrating a positive hepato-targeting inclination of C-LA ACNS.

Due to the novel HA-*g*-ECL displaying a valuable capacity that relays to micelle structuring, parallel to most amphiphiles, this proposed amphiphile can be extended to a vast array of therapeutic molecules, not limited to LMV, an antiviral or even a hydrophile. In any event, the basic intent purpose for a micelle is the encapsulation of hydrophobic drugs due to the unembellished primary oil in water emulsion preparatory method that leads to it. Therefore HA-*g*-ECL poses as a platform for sustained hydrophilic or poorly soluble drug release promising a feasible therapeutic carrier. Moreover, HA-*g*-ECL is able to afford intracellular direction of these contained drugs due to its capability of forming extremely nanosized particles and being open to surface decorating with chemical compounds specialized for a targeting requirement. The aim and all of the objectives unfolded at the outset of this dissertation were achieved. This new type of amphiphilic copolymer exhibited worthy intracellular targeted drug delivery potentiality with multifunctional characteristics and therefore could be expanded to a multitude of applications in biomedical areas.

8.2. Recommendations

The rodent animal model employed in this study was not induced with the representative human disease being addressed. HBV was absent in the animal model. A more accurate data presentation is likely to be generated following induction of the actual disease being targeted. Such available animal models that have been studied are HBV-transfected mice and human liver chimeric mouse models for HBV (which consist of the Trimera mouse model and SCID Alb-uPA mice for studying HBV) that could be utilized to determine precise C-LA ACNS therapeutic potential.

An essential aspect of the *in vivo* study entailed immediate -80°C freezing of removed liver tissue following euthanasia of rats for homogenization to establish drug quantities therein. Due to this disparate manner of immediate liver treatment following removal that is not conducive to immunohistochemistry and pathology storage conditions for examination, these tests were

overlooked at this time. This, in addition to a cytocompatibility analysis, should be carried out to further confirm safety or toxicity of ACNS.

Expansion to this study can accommodate another veracity measure optimizing the C-LA coating by using an experimental model mediating variable quantities of chitosan to LA. This should also be considered because LA in excess has been shown to promote prostate cancer risk by enhancing growth of prostate tumor cells in animals.

A portal vein administration was sought and utilized to further enhance the hepatic targeting ability of C-LA ACNS. An alternative to this injectable route is an intrahepatic arterial injection being equally if not more beneficial as a liver targeting route (Illustrated in *Chapter 7, Section 7.1 Introduction, Figure 7.1*).

The HA-g-ECL amphiphile and formed ACNS can be indispensable to a wide range of infectious diseases and ailments. It can be exploited in applications of anticancer mitochondrial therapeutic delivery, targeted nuclear gene delivery, lysosomal targeting of a possible enzyme deficiency and cytoplasmic therapeutic delivery. Need for sophisticated HIV/AIDS treatment is currently paramount. Application of novel ACNS to various intracellular operations can be valuable to eliminate disease. Its performance may be scrutinized by selecting other model diseases (or possible inducing actual disease in animal models) and therapeutic treatments to determine the extent of ACNS as a functional intracellular drug delivery system.

The ACNS resembles a virus with C-LA targeting nano-appendages representing viral glycoprotein spikes for entry into cells. Modification of the 'glycoprotein spikes' to better suit delivery of ACNS with encapsulated therapeutic across the Blood-Brain-Barrier (BBB) can be attained. This study adopted an uninvolved and uncomplicated method of chemical adsorption of targeting nano-appendages on ACNS surface, however, innovative methods and steps such as electrospinning may be undertaken for adsorbing target-specific ligand nano-appendages for slightly arduous biomedical applications such as the above mentioned drug delivery across BBB.

Bearing in mind the points proffered, additional experimental design studies can be executed based on possible predictions able to be derived from this study. This research may be reflected on by scientists utilizing it as an information-rife foundation building on, integrating existing and

acquiring new knowledge for impending pharmaceutical based applications in advanced drug delivery.

REFERENCES

Akinc, A., Querbes, W., De, S., Qin, J., Frank-Kamenetsky, M., Jayaprakash, K.N., Jayaraman, M., Rajeev, K.G., Cantley, W.L., Dorkin, J.R., Butler, J.S., Qin, L., Racie, T., Sprague, A., Fava, E., Zeigerer, A., Hope, M.J., Zerial, M., Sah, D.W., Fitzgerald, K., Tracy, M.A., Manoharan, M., Koteliensky, V., de Fougères, A., Maier, M.A., 2010, 'Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms', *Molecular Therapy* 18, 1357–1364.

Alexandridis, P., Lindman, B., 2000, 'Amphiphilic Block Copolymers: Self-Assembly and Applications Studies in Surface Science & Catalysis', Elsevier, 2000.

Ashwell, G., Morell, A.G., 1974, 'The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins', *Advances in Enzymology and Related Areas of Molecular Biology* 41, 99–128.

Asselah, T., Lada, O., Moucari, R., Martinot, M., Boyer, N., Marcellin, P., 2007, 'Interferon therapy for chronic hepatitis B', *Clinics in Liver Disease* 11, 839-849.

Asselah, T., Riepault, M., Castelnau, C., Giuily, N., Boyer, N., Marcellin, P., 2005, 'The current status of antiviral therapy of chronic hepatitis B', *Journal of Clinical Virology* 34, S115-S124.

Aydin, R., 2005, 'Conjugated linoleic acid: Chemical structure, sources and biological properties', *Turkish journal of Veterinary and Animal Sciences* 29, 189-195.

Bagi, C.M., Andresen, C.J., 2010, 'Models of hepatocellular carcinoma and biomarker strategy', *Cancers* 2, 1441-1452.

Banerjee, S.S., Aher, N., Patil, R., Khandare, J., 2012, 'Poly(ethylene glycol)-prodrug conjugates: concept, design and applications', *Journal of Drug Delivery*, 103973, <http://dx.doi.org/10.1155/2012/103973>.

Bannunah, A.M., Vllasaliu, D., Lord, J., Stolnik, S., 2014, 'Mechanisms of nanoparticle internalization and transport across an intestinal epithelial cell model: effect of size and surface charge', *Molecular Pharmaceutics* 11, 4363-4373.

Bartholomeusz, A., Furman, P., Locarnini, S., 2003, 'Novel approaches in the management of chronic HBV infection', in: Schinazi, R.F., Sommadossi, J-P., Rice, C.M. (Eds.), *Frontiers in Viral Hepatitis* [electronic resource], The Netherlands: Elsevier BV, pp. 225-243.

Basu, P., Brown ,Jr., R., 2012, 'Entecavir for treatment of chronic hepatitis B: A clinical update for the treatment of patients with decompensated cirrhosis', *Open Journal of Internal Medicine* 2, 53-61, <http://www.dx.doi:10.4236/ojim.2012.22012>.

Bayram, S., Topaktaş, M., 2008, 'Confirmation of the chromosome damaging effects of lamivudine in in vitro human peripheral blood lymphocytes', *Environmental and Molecular Mutagenesis* 49, 328-333.

Beeton, C., Garcia, A., Chandy, K.G., 2007, 'Drawing blood from rats through the saphenous vein and by cardian puncture', *Journal of Visualized Experiments* 7, 266.

Bertau, M., Mosekilde, E., Westerhoff, H.V., 2008, *Biosimulation in Drug Development*, Wiley-VCH, Weinheim.

Bhattarai, A., 2012, 'Determination of the average molecular weight of sodium polystyrenesulphonate from viscosity measurement', *Scientific World* 10, 17-19.

Biessen, E.A.L., Valentijn, A.R.P.M., De Vreuh, R.L.A., Van de Bilt, E., Sliedregt, L.A.J.M., Prince, P., Bijsterbosch, M.K., Van Boom, J.H., Van Der Marel, G.A., Abrahams, P.J., Van Berkel, T.J., 2000, 'Novel hepatotropic prodrugs of the antiviral nucleoside 9-(2-phosphonylmethoxyethyl) adenine with improved pharmacokinetics and antiviral activity', *FASEB Journal* 14, 1784-1792.

Bleifuss, E., Kammertoens, T., Hutloff, A., Quarcoo, D., Dorner, M., Straub, P., Uckert, W., Hildt, T., 2006, 'The translocation motif of hepatitis B virus improves protein vaccination', *Cellular and Molecular Life Sciences* 63, 627-635.

Blumgart, L.H., Leach, K.G., McLachlan, M.S.F., Seager, S., Ryan, C.J., 1971, 'Portal venous injection in the rat', *Gut* 12, 585-591.

Bonferoni, M.C., Sandri, G., Dellera, E., Rossi, S., Ferrari, F., Mori, M., Caramella, C., 2014, 'Ionic polymeric micelles based on chitosan and fatty acids and intended for wound healing.

Comparison of linoleic and oleic acid', *European Journal of Pharmaceutics and Biopharmaceutics* 87, 101-106.

Bourne, C., Lee, S., Venkataiah, B., Lee, A., Korba, B., Finn, M.G., Zlotnick, A., 2008, 'Small-molecule effectors of hepatitis B virus capsid assembly give insight into virus life cycle', *Journal of Virology* 82, 10262-10270.

Breunig, M., Bauer, S., Goepferich, A., 2008, 'Polymers and nanoparticles: Intelligent tools for intracellular targeting?' *European Journal of Pharmaceutics and Biopharmaceutics* 68, 112-128.

Brunetto, M.R., Oliveri, F., Coco, B., Leandro, G., Colombatto, P., Gorin, J.M., Bonino, F., 2002, 'Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study', *Journal of Hepatology* 36, 263-270.

Carmona, S., Jorgensen, M.R., Kolli, S., Crowther, C., Salazar, F.H., Marion, P.L., Fujino, M., Natori, Y., Thanou, M., Arbuthnot, P., Miller, A.D., 2008, 'Controlling HBV replication in vivo by intravenous administration of triggered PEGylated siRNA-nanoparticles', *Molecular Pharmaceutics* 6, 706-717.

Caspersen, M.B., Roubroeks, J.P., Qun, L., Shan, H., Fogh, J., RuiDong, Z., Tømmerraas, K., 2014, 'Thermal degradation and stability of sodium hyaluronate in solid state', *Carbohydrate Polymers* 107, 25-30.

Chakravarthi, S.S., 2008, 'Effect of size of PLGA and chitosan-PLGA particles on the cellular association, cytotoxicity, and anti-tumor efficacy of paclitaxel, PhD Thesis, The Faculty of The Graduate College, University of Nebraska, Omaha, Nebraska.

Chan, H.L., Wang, H., Niu, J., Chim, A.M., Sung, J.J., 2007, 'Two-year lamivudine treatment for hepatitis B e antigen-negative chronic hepatitis B: a double-blind, placebo-controlled trial', *Antiviral Therapy* 12, 345-353.

Chen, Y., Cheng, G., Mahato, R.I., 2008, 'RNAi for treating hepatitis B viral infection', *Pharmaceutical Research* 25, 72-86.

Chen, Z., Xu, Z., Ye, J., Yao, H., Zheng, S., Ding, J., 2005, 'Combination of small interfering RNAs mediates greater inhibition of human hepatitis B virus replication and antigen expression', *Journal of Zhejiang University. Science. B.* 6, 236-241.

Cheong, S.J., Lee, C.M., Kim, S.L., Jeong, H.J., Kim, E.M., Park, E.H., Kim, D.W., Lim, S.T., Sohn, M.H., 2009, 'Superparamagnetic iron oxide nanoparticles-loaded chitosan-linoleic acid nanoparticles as an effective hepatocyte-targeted gene delivery system', *International Journal of Pharmaceutics* 372, 169-176.

Cho, K.Y., Chung, T.W., Kim, B.C., Kim, M.K., Lee, J.H., Wee, W.R., Cho, C.S., 2003, 'Release of ciprofloxacin from poloxamer-graft-hyaluronic acid hydrogels in vitro', *International Journal of Pharmaceutics* 260, 83-91.

Chopra, S., Patil, G.V., Motwani, S.K., 2007, 'Release modulating hydrophilic matrix systems of losartan potassium: Optimization of formulation using statistical experimental design', *European Journal of Pharmaceutics and Biopharmaceutics* 66, 73-82.

Chou, H.Y., Lin, X.Z., Pan, W.Y., Wu, P.Y., Chang, C.M., Lin, T.Y., Shen, H.H., Tao, M.H., 2010, 'Hydrogel-delivered GM-CSF overcomes nonresponsiveness to hepatitis B vaccine through the recruitment and activation of dendritic cells', *Journal of Immunology* 185, 5468-5475.

Christensen, D., Korsholm, K.S., Andersen, P., Agger, E.M., 2011, 'Cationic liposomes as vaccine adjuvants', *Expert Review of Vaccines* 10, 513-521.

Chromcová, D., Baslerová, L., Roda, J., Brožek, J., 2008, 'Polymerization of lactams. 99 Preparation of polyesteramides by the anionic copolymerization of ϵ -caprolactam and ϵ -caprolactone', *European Polymer Journal* 44, 1733-1742.

Collins, M.N., Birkinshaw, C., 2008, 'Physical properties of crosslinked hyaluronic acid hydrogels', *Journal of Materials Science. Materials in Medicine* 19, 3335-3343.

Cooper, A., Shaul, Y., 2006, 'Clathrin-mediated endocytosis and lysosomal cleavage of hepatitis B virus capsid-like core particles', *The Journal of Biochemistry* 281, 16563-16569.

Costin, G.E., Trif, M., Nichita, N., Dwek, R.A., Petrescu, S.M., 2002, 'pH-sensitive liposomes are efficient carriers for endoplasmic reticulum-targeted drugs in mouse melanoma cells', *Biochemical and biophysical research communications* 293, 918-923.

Cuestas, M.L., Mathet, V.L., Oubiña, J.R., Sosnik, A., 2010, 'Drug delivery systems and liver targeting for improved pharmacotherapy of the hepatitis B virus (HBV) infection', *Pharmaceutical Research* 27, 1184-1202.

Dai, C., Chuang, W., Hsieh, M., Lee, L., Huang, J., Hou, N., Lin, Z., Chen, S., Hsieh, M., Wang, L., Tsai, J., Chang, W., Yu, M., 2007, 'Adefovir dipivoxil treatment of lamivudine-resistant chronic hepatitis B', *Antiviral Research* 75, 146-151.

de Clercq, E., Férir, G., Kaptein, S., Neyts, J., 2010, 'Antiviral treatment of chronic hepatitis B virus (HBV) infections', *Viruses* 2, 1279–1305.

Dekhtyar, Y., Romanova, M., Kachanovska, A., Skrastina, D., Reinhofa, R., Pumpens, P., Patmalnieks, A., 2012, 'Inorganic nanoparticle as a carrier for hepatitis b viral capsids', in: Vaseashta, A., Braman, E., Susmann, P. (Eds.), 2012, 'Technological innovations in sensing and detection of chemical, biological, radiological, nuclear threats and ecological terrorism', *Netherlands: Springer*, pp. 221-225.

Dhakar, R.C., Maurya, S.D., Saluja, V., 2012, 'From formulation variables to drug entrapment efficiency of microsphere: A technical review', *Journal of Drug Delivery and Therapeutics* 2, 128-133

Dienstag, J.L., 2008, 'Hepatitis B virus infection', *The New England Journal of Medicine* 359, 1486-1500.

Dienstag, J.L., Schiff, E.R., Wright, T.L., Perrillo, R.P., Hann, H.W., Goodman, Z., Crowther, L., Condeay, L.D., Woessner, M., Rubin, M., Brown, N.A., 1999, 'Lamivudine as initial treatment for chronic hepatitis B in the United States', *The New England Journal of Medicine* 341, 1256-1263.

Du, Y.Z., Wang, L., Yuan, H., Hu, F.Q., 2011, 'Linoleic acid-grafted chitosan oligosaccharide micelles for intracellular drug delivery and reverse drug resistance of tumor cells', *International Journal of Biological Macromolecules* 48, 215-222.

Du, Y.Z., Wang, L., Yuan, H., Wei, X.H., Hu, F.Q., 2009, 'Preparation and characteristics of linoleic acid-grafted chitosan oligosaccharide micelles as a carrier for doxorubicin', *Colloids and Surfaces. B, Biointerfaces* 69, 257-263.

Dutta, P.K., Dutta, J., Tripathi, V.S., 2004, 'Chitin and chitosan: Chemistry, properties and applications', *Journal of Scientific and Industrial Research* 63, 20-31.

Ekambaram, P., Sathali, H.A.A., 2011, 'Formulation and evaluation of solid lipid nanoparticles on ramipril', *Journal of Young Pharmacists* 3, 216-220.

El-Sayed, W.A., Ramiz, M.M., Abdel-Rahman, A.A., 2009, 'Anti-hepatitis B virus activity of new N4-beta-D-glycoside Pyrazolo [3,4-d]pyrimidine derivatives', *Zeitschrift für Naturforschung. C, Journal of Biosciences* 64, 323-328.

Elzey, S., Grassian, V.H., 2010, 'Agglomeration, isolation and dissolution of commercially manufactured silver nanoparticles in aqueous environments', *Journal of Nanoparticle Research* 12, 1945-1958.

Erion, M.D., Reddy, K.R., Boyer, S.H., Matelich, M.C., Gomez-Galeno, J., Lemus, R.H., Ugarkar, B.G., Colby, T.J., Schanzer, J., Van Poelje, P.D., 2004, 'Design, synthesis, and characterization of a series of cytochrome P450 3A-activated prodrugs (hepDirect prodrugs) useful for targeting phosph(on)ate-based drugs to the liver', *Journal of the American Chemical Society* 126, 5154-5163.

Faraji, A.H., Wipf, P., 2009, 'Nanoparticles in cellular drug delivery', *Bioorganic and Medicinal Chemistry* 17, 2950-2962.

Fioravanti, J., Gomar, C., Medina-Echeverez, J., Otano, I., Benito, A., Prieto, J., González-Aseguinolaza, G., Berraondo, P., 2011, 'Characterization of woodchuck apolipoprotein A-I: a new tool for drug delivery and identification of altered isoforms in the woodchuck chronic hepatitis model', *Journal of Medical Virology* 83, 1221-1229.

Fonseca, S.B., Pereira, M.P., Kelley, S.O., 2009, 'Recent advances in the use of cell-penetrating peptides for medical and biological applications', *Advanced Drug Delivery Reviews* 61, 953-964.

Forrester, J.V., Lackie, J.M., 1981, 'Effect of hyaluronic acid on neutrophil adhesion', *Journal of Cell Science* 50, 329-344.

Franceschini, G., Macchietto, S., 2008, 'Model-based design of experiments for parameter precision: State of the art', *Chemical engineering Science* 63, 4846-4872.

Fu, J., Tang, Z., Gao, X., Zhao, F., Zhong, H., Wen, M.R., Sun, X., Song, H.F., Qian, X.H., 2008, 'Optimal design and validation of antiviral siRNA for targeting hepatitis B virus', *Acta Pharmacologica Sinica* 29, 1522-1528.

Gabbott, P., 2008, 'A Practical Introduction to Differential Scanning Calorimetry', in 'Principles and Applications of Thermal Analysis', Blackwell Publishing Ltd, Oxford, UK, 2008.

Gad, S.C., 2008, *Pharmaceutical Manufacturing Handbook: Production and Processes*, John Wiley & Sons, Hoboken, New Jersey.

Ghany, M., Liang, T.J., 2007, 'Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B', *Gastroenterology* 132, 1574-1585.

Giladi, H., Ketzinel-Gilad, M., Rivkin, L., Felig, Y., Nussbaum, O., Galun, E., 2003, 'Small interfering RNA inhibits hepatitis B virus replication in mice', *Molecular Therapy* 8, 769-776.

Gill, P., Moghadam, T.T., Ranjbar, B., 2010, 'Differential scanning calorimetry techniques: Applications in biology and nanoscience', *Journal of Biomolecular Techniques* 21:167–193.

Giri, N., Tomar, P., Karwasara, V.S., Pandey, R.S., Dixit, V.K., 2011, 'Targeted novel surface-modified nanoparticles for interferon delivery for the treatment of hepatitis B', *Acta Biochimica et Biophysica Sinica (Shanghai)* 43, 877-883.

Grahowski, T.S., Borg, W., 1964, 'Blends of epsilon caprolactam polymer and graft copolymer alkenyl cyanide and alkenyl substituted aromatic hydrocarbon on polybutadiene', *United States patent 3134746*.

Gübeli, R.J., Schöneweis, K., Huzly, D., Ehrbar, M., Hamri, G.C., El-Baba, M.D., Urban, S., Weber, W., 2013, 'Pharmacologically triggered hydrogel for scheduling hepatitis B vaccine administration', *Scientific Reports* 3, 2610, doi:10.1038/srep02610.

Gupta, B., Levchenko, T.S., Torchilin, V.P., 2005, 'Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides', *Advanced Drug Delivery Reviews* 57, 637–651.

Hadziyannis, S.J., Papatheodoridis, G.V., 2004, 'Adefovir dipivoxil in the treatment of chronic hepatitis B virus infection', *Expert Review of Anti-Infective Therapy* 2, 475-83.

Han, X., Corson, N., Wade-Mercer, P., Gelein, R., Jiang, J., Sahu, M., Biswas, P., Finkelstein, J.N., Elder, A., Oberdörster, G., 2012, 'Assessing the relevance of in vitro studies in nanotoxicology by examining correlations between in vitro and in vivo data', *Toxicology* 297, 1-9.

Harush-Frenkel, O., Debotten, N., Benita, S., Altschuler, Y., 2007, 'Targeting of nanoparticles to the clathrin-mediated endocytic pathway', *Biochemical and Biophysical Research Communications* 353, 26-32.

Haryanto, A., Wijayanti, N., Kann, M., 2007, 'Effect of the HBV capsid assembly inhibitor Bayer 41-4109 on the intracellular localization of EGFP-core fusion proteins', *Indonesian Journal of Biotechnology* 12, 998-1004.

Havlice, J., Brozek, J., Šáchová, M., Nováková, V., Roda, J., 1999, 'Polymerization of lactams, 92. Non-activated anionic polymerization of ϵ -caprolactam initiated with the sodium salt of ϵ -caprolactam', *Macromolecular Chemistry and Physics* 200, 1200-1207.

He, M., Zhao, Z., Yin, L., Tang, C., Yin, C., 2009, 'Hyaluronic acid coated poly(butyl cyanoacrylate) nanoparticles as anticancer drug carriers', *International Journal of Pharmaceutics* 373, 165-173.

Hean, J., Crowther, C., Ely, A., Ul Islam, R., Barichievy, S., Bloom, K., Weinberg, M.S., van Otterlo, W.A., de Koning, C.B., Salazar, F., Marion, P., Roesch, E.B., Lemaitre, M., Herdewijn, P., Arbuthnot, P., 2010, 'Inhibition of hepatitis B virus replication in vivo using lipoplexes containing altritol-modified antiviral siRNAs', *Artificial DNA, PNA and XNA* 1, 17-26.

Hilgendorf, C., Ahlin, G., Seithel, A., Artursson, P., Ungell, A.L., Karlsson, J., 2007, 'Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and

organotypic cell lines', *Drug Metabolism and Disposition: The Biological Fate of Chemicals* 35, 1333-1340.

Hong, R., Bai, W., Zhai, J., Liu, W., Li, X., Zhang, J., Cui, X., Zhao, X., Ye, X., Deng, Q., Tiollais, P., Wen, Y., Liu, J., Xie, Y., 2013, 'Novel recombinant hepatitis B virus vectors efficiently deliver protein and RNA encoding genes into primary hepatocytes', *Journal of Virology* 87, 6615-6624.

Hu, L., Mao, Z., Zhang, Y., Gao, C., 2011, 'Influences of size of silica particles on the cellular endocytosis, exocytosis and cell activity of HepG2 cells', *Journal of Nanoscience Letters* 1, 1-16.

Huang, S.T., Du, Y.Z., Yuan, H., Zhang, X.G., Miao, J., Cui, F.D., Hu, F.Q., 2011, 'Synthesis and anti-hepatitis B virus activity of acyclovir conjugated stearic acid-g-chitosan oligosaccharide micelle', *Carbohydrate Polymers* 83, 1715-1722.

Huang, X., Brazel, C.S., 2007, 'On the importance and mechanisms of burst release in matrix-controlled drug delivery systems', *Journal of Controlled Release* 73, 121-136.

Huang, S.L., Hamilton, A.J., Nagaraj, A., Tiukinhoy, S.D., Klegerman, M.E., McPherson, D.D., Macdonald, R.C., 2001, 'Improving ultrasound reflectivity and stability of echogenic liposomal dispersions for use as targeted ultrasound contrast agents', *Journal of Pharmaceutical Sciences* 90, 1917-1926.

Hülya, A., Baki, H., Sung, C.Y., 2006, 'Grafting of poly(3-hydroxyalkanoate) and linoleic acid onto chitosan', *Journal of Applied Polymer Science* 103, 81-89.

Ishikawa, T., 2012, 'Immunoregulation of hepatitis B virus infection--rationale and clinical application', *Nagoya Journal of Medical Science* 74, 217-232.

Jacob, D., Davis, J., Fang, B., 2004, 'Xenograftic tumor models in mice for cancer research, a technical review', *Gene Therapy and Molecular Biology* 8, 213-219.

Jadhav, N.R., Gaikwad, V.L., Nair, K.J., Kadam, H.M., 2009, 'Glass transition temperature: Basics and application in pharmaceutical sector', *Asian Journal of Pharmaceutics* 3, 82-89.

Jain, A., Jain, S.K., Ganesh, N., Barve, J., Beg, A.M., 2010, 'Design and development of ligand-appended polysaccharidic nanoparticles for the delivery of oxaliplatin in colorectal cancer', *Nanomedicine: Nanotechnology, Biology and Medicine* 6, 179-190.

Jana, U., Mohanty, A.K., Pal, S.L., Manna, P.K., Mohanta, G.P., 2014, '*International Journal of Pharmacy and Pharmaceutical Sciences* 6, 564-567.

Janssen, H.L., Gerken, G., Carreño, V., Marcellin, P., Naoumov, N.V., Craxi, A., Ring-Larsen, H., Kitis, G., van Hattum, J., de Vries, R.A., Michielsen, P.P., ten Kate, F.J., Hop, W.C., Heijtkink, R.A., Honkoop, P., Schalm, S.W., 1999, 'Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment', The European Concerted Action on Viral Hepatitis (EUROHEP), *Hepatology* 30, 238-243.

Jeong, Y.I., Kim, D.H., Chung, C.W., Yoo, J.J., Choi, K.H., Kim, C.H., Ha, S.H., Kang, D.H., 2012, 'Self-assembled nanoparticles of hyaluronic acid/poly(DL-lactide-co-glycolide) block copolymer', *Colloids and Surfaces. B, Biointerfaces* 90, 28-35.

Jiang, L., Li, X., Liu, L., Zhang, Q., 2013, 'Cellular uptake mechanism and intracellular fate of hydrophobically modified pullulan nanoparticles', *International Journal of Nanomedicine* 8, 1825-1834.

Jin, Y-J., Ubonvan, T., Kim, D-D., 2010, 'Hyaluronic acid in drug delivery systems', *Journal of Pharmaceutical Investigation* 40, 33-43.

Kapusniak, J., Siemion, P., 2007, 'Thermal reactions of starch with long-chain unsaturated fatty acids. Part 2. Linoleic acid' *Journal of Food Engineering* 78, 323–332.

Karnes, H.T., March, C., 1993, 'Precision, Accuracy, and Data Acceptance Criteria in Biopharmaceutical Analysis', *Pharmaceutical Research* 10, 1420-1426.

Kato, K., Uchida, E., Kang, E.T., Uyama, Y., Ikada, Y., 2003, 'Polymer surface with graft chains', *Progress in Polymer Science* 28, 209-259.

Kato, Y., Onishi, H., Machida, Y., 2001, 'Biological characteristics of lactosaminated *N*-succinyl-chitosan as a liver-specific drug carrier in mice', *Journal of Controlled Release* 70, 295-307.

Keeffe, E.B., Rossignol, J.F., 2009, 'Treatment of chronic viral hepatitis with nitazoxanide and second generation thiazolides', *World Journal of Gastroenterology* 15, 1805–1808.

Kim, D., Kim, S., Jo, S., Woo, J., Noh, I., 2011, 'Physicochemical properties of chitosan-poly(ethylene oxide) hydrogel modified through linoleic acid', *Macromolecular Research* 19, 396-402.

Kim, E.Y., An, S.K., Kim, H.D., 1997, 'Graft copolymerization of ϵ -Caprolactam onto Kevlar-49 fiber surface and properties of grafted Kevlar fiber reinforced composite', *Journal of Applied Polymer Science* 65, 99-107.

Kim, S.I., Shin, D., Choi, T.H., Lee, J.C., Cheon, G.J., Kim, K.Y., Park, M., Kim, M., 2007, Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I', *Molecular Therapy* 15, 1145-1152.

Kincl, M., Turk, S., Vrečer, F., 2005, 'Application of experimental design methodology in development and optimization of drug release method', *International Journal of Pharmaceutics* 291, 39-49.

Knop, K., Hoogenboom, R., Fischer, D., Schubert, U.S., 2010, 'Poly(ethylene glycol) in drug delivery: Pros and cons as well as potential alternatives', *Angewandte Chemie (International ed. in English)* 49, 6288-6308.

Kogan, G., Šoltés, L., Stern, R., Gemeiner, P., 2007, 'Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications', *Biotechnology Letters* 29, 17-25.

Kohane, D.S., 2007, 'Microparticles and nanoparticles for drug delivery', *Biotechnology and Bioengineering* 96, 203-209.

Kong, W.H., Park, K., Lee, M.Y., Lee, H., Sung, D.K., Hahn, S.K., 2013, 'Cationic solid lipid nanoparticles derived from apolipoprotein-free LDLs for target specific systemic treatment of liver fibrosis', *Biomaterials* 34, 542-551.

Korba, B.E., Elazar, M., Lui, P., Rossignol, J.F., Glenn, J.S., 2008a, 'Potential for hepatitis C virus resistance to nitazoxanide or tizoxanide', *Antimicrobial Agents and Chemotherapy* 52, 4069-4071.

Korba, B.E., Montero, A.B., Farrar, K., Gaye, K., Mukerjee, S., Ayers, M.S., Rossignol, J.F., 2008b, 'Nitazoxanide, tizoxanide and other thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication', *Antiviral Research* 77, 56-63.

Koren, E., Torchilin, V.P., 2012, 'Cell-penetrating peptides: breaking through to the other side', *Trends in Molecular Medicine* 18, 385-393.

Köser, J., Maco, B., Aebi, U., Fahrenkrog, B., 2012, 'The nuclear pore complex becomes alive: new insights into its dynamics and involvement in different cellular processes', *Atlas of Genetics and Cytogenetics in Oncology and Haematology*. Available from: <http://atlasgeneticsoncology.org/Deep/NuclearPoreComplexID20048.html>.

Kramer, W., Wess, G., Schubert, G., Bickel, M., Girbig, F., Gutjahr, U., Kowalewski, S., Baringhaus, K.H., Enhnen, A., Glombik, H., Mullner, S., Neckermann, G., Schulz, S., Petzinger, E., 1992, 'Liver-specific drug targeting by coupling to bile acids', *The Journal of Biological Chemistry* 267, 18598-18604.

Kumar, P., Choonara, Y.E., du Toit, L.C., Modi, G., Naidoo, D., Pillay, V., 2012, 'Novel high-viscosity polyacrylamidated chitosan for neural tissue engineering: Fabrication of anisotropic neurodurable scaffold via molecular disposition of persulfate-mediated polymer slicing and complexation', *International Journal of Molecular Sciences*, 13, 13966-13984.

Kumaresan, G., Velraj, R., Iniyan, S., 2011, 'Thermal analysis of D-mannitol for use as phase change material for latent heat storage', *Journal of Applied Sciences* 11, 3044-3048.

Kuo, J.W., Swann, D.A., Prestwich, G.D., 1991, 'Chemical modification of hyaluronic acid by carbodiimides', *Bioconjugate Chemistry* 2, 232-241.

Lai, C.H., Lin, C.Y., Wu, H.T., Chan, H.S., Chuang, Y.J., Chen, C.T., Lin, C.C., 2010, 'Galactose encapsulated multifunctional nanoparticle for HepG2 cell internalization', *Advanced Functional Materials* 20, 3948-3958.

Lapčík Jr., L., Benešová, K., Lapčík, L., De Smedt, S., Lapčíková, B., 2010, 'Chemical modification of hyaluronic Acid: Alkylation', *International Journal of Pure and Applied Chemistry* 15, 486-496.

Lapčík, Jr. L., Lapčík, L., De Smedt, S., Demeester, J., Chabreček, P., 1998, 'Hyaluronan: preparation, structure, properties, and applications', *Chemical Reviews* 98, 2663-2684.

Larrubia, J.R., Benito-Martínez, S., Miquel-Plaza, J., Sanz-de-Villalobos, E., González-Mateos, F., Parra, T., 2009, 'Cytokines - their pathogenic and therapeutic role in chronic viral hepatitis', *Revista Española de Enfermedades Digestivas* 101, 343-51.

Lavanchy, D., 2004, 'Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures', *Journal of Viral Hepatology* 11, 97-107.

Lawson, J., 2010, *Design and Analysis of Experiments with SAS*, CRC Press, Boca Raton, Florida.

Lee, C.M., Jeong, H.J., Kim, S.L., Kim, E.M., Kim, D.W., Lim, S.T., Jang, K.Y., Jeong, Y.Y., Nah, J.W., Sohn, M.H., 2009a, 'SPION-loaded chitosan-linoleic acid nanoparticles to target hepatocytes', *International Journal of Pharmaceutics* 371, 163-169.

Lee, H., Ahn, C.H., Park, T.G., 2009b, 'Poly[lactic-co-(glycolic acid)]-grafted hyaluronic acid copolymer micelle nanoparticles for target-specific delivery of doxorubicin', *Macromolecular Bioscience* 9, 336-342.

Lee, H., Lee, K., Park, T.G., 2008, 'Hyaluronic acid- paclitaxel conjugate micelles: synthesis, characterization, and antitumour activity', *Bioconjugate Chemistry* 19, 1319-1325.

Lebray, P., Vallet-Pichard, A., Michel, M., Fontaine, H., Sobesky, R., Brèchot, C., Pol, S., 2003, 'Immunomodulatory drugs and therapeutic vaccine in chronic hepatitis B infection', *Journal of Hepatology* 39, S151-S159.

Lembo, D., Cavalli, R., 2010, 'Nanoparticulate delivery systems for antiviral drugs', *Antiviral Chemistry and Chemotherapy* 21, 53-70.

Lepère, C., Régeard, M., Le Seyec, J., Gripon, P., 2007, 'The translocation motif of hepatitis B virus envelope proteins is dispensable for infectivity', *Journal of Virology* 81, 7816-7818.

Li, L., Wang, H., Ong, Z.Y., Xu, K., Ee, P.L.R., Zheng, S., Hedrick, J.L., Yang, Y., 2010, 'Polymer- and lipid-based nanoparticle therapeutics for the treatment of liver diseases', *Nano Today* 5, 296-312.

Li, Q., Du, Y.Z., Yuan, H., Zhang, X.G., Miao, J., Cui, F.D., Hu, F.Q., 2010, 'Synthesis of lamivudine stearate and antiviral activity of stearic acid-g-chitosan oligosaccharide polymeric micelles delivery system', *European Journal of Pharmaceutical Sciences* 41, 498-507.

Li, Y., Wang, J., Wientjes, M.G., Au, J.L., 2012, 'Delivery of nanomedicines to extracellular and intracellular compartments of a solid tumor', *Advanced Drug Delivery Reviews* 64, 29-39.

Liaw, Y., Sung, J.J.Y., Chow, W.C., Farrell, G., Lee, C., Yuen, H., Tanwandee, T., Tao, Q., Shue, K., Keene, O.N., Dixon, J.S., Gray, D.F., Sabbat, J., 2004, 'Lamivudine for patients with chronic hepatitis B and advanced liver disease', *The New England Journal of Medicine* 351, 1521-1531.

Lin, A., Liu, Y., Huang, Y., Sun, J., Wu, Z., Zhang, X., Ping, Q., 2008, 'Glycyrrhizin surface-modified chitosan nanoparticles for hepatocyte-targeted delivery', *International Journal of Pharmaceutics* 359, 247-253.

Lin, T-C., Chen, J-H., Chen, Y-H., Teng, T-M., Su, C-H., Hsu, S-H., 2013, 'Biodegradable micelles from a hyaluronan-poly-(ϵ -caprolactone) graft copolymer as nanocarriers for fibroblast growth factor 1', *Journal of Materials Chemistry B* 1, 5977-5987.

Liu, C-G., Chen, X-G., Park, H-J., 2005, 'Self-assembled nanoparticles based on linoleic-acid modified chitosan: Stability and adsorption of trypsin', *Carbohydrate Polymers* 62, 293-298.

Liu, Y., Meyer, C., Xu, C., Weng, H., Ellerbrand, C.H., ten Dijke, P., Dooley, S., 2013, 'Animal models of chronic liver diseases', *American Journal of Physiology - Gastrointestinal and Liver Physiology* 304, G449-G468.

Lochmann, D., Jauk, E., Zimmer, A., 2004, 'Drug delivery of oligonucleotides by peptides', *European Journal of Pharmaceutics and Biopharmaceutics* 58, 237-251.

Macarulla, M.T., Fernández-Quintela, A., Zabala, A., Navarro, V., Echevarría, E., Churrua, I., Rodríguez, V.M., Portillo, M.P., 2005, 'Effects of conjugated linoleic acid on liver composition and fatty acid oxidation are isomer-dependent in hamster', *Nutrition* 21, 512-519.

Madden, J.C., Hewitt, M., Przybylak, K., Vandebriel, R.J., Piersma, A.H., Cronin, M.T., 2012, 'Strategies for the optimisation of in vivo experiments in accordance with the 3Rs philosophy', *Regulatory Toxicology and Pharmacology* 63, 140-154.

Mäe, M., Langel, U., 2006, 'Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery', *Current Opinion in Pharmacology* 6, 509-514.

Maheswari, R., Shanthi, K., Sivakumar, T., Narayanan, S., 2003, 'Beckmann rearrangement over phosphotungstic acid/SiMCM-41 cyclohexanone oxime to ϵ -caprolactam', *Applied Catalysis. A, General* 248, 291-301.

Mangold, S.L., Carpenter, R.T., Kiessling, L.L., 2008, 'Synthesis of fluorogenic polymers for visualizing cellular internalization', *Organic Letters* 10, 2997-3000.

Marcellin, P., Chang, T.T., Lim, S.G., Tong, M.J., Sievert, W., Shiffman, M.L., Jeffers, L., Goodman, Z., Wulfsohn, M.S., Xiong, S., Fry, J., Brosgart, C.L., 2003, 'Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B', *The New England Journal of Medicine* 348, 808-816.

Marcellin, P., Gane, E., Buti, M., Afdhal, N., Sievert, W., Jacobson, I.M., Washington, M.K., Germanidis, G., Flaherty, J.F., Schall, R.A., Bornstein, J.D., Kitrinis, K.M., Subramanian, G.M., McHutchison, J.G., Heathcote, E.J., 2013, 'Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study', *Lancet* 381, 468-475.

Meibohm, B., 2006, 'Title Pharmacokinetics and Pharmacodynamics of Biotech Drugs: Principles and Case Studies in Drug Development', John Wiley & Sons, 137.

Meijer, D.K.F., Jansen, R.W., Molema, G., 1992, 'Drug targeting systems for antiviral agents: options and limitations', *Antiviral Research* 18, 215-258.

Mersch-Sundermann, V., Knasmüller, S., Wu, X.J., Darroudi, F., Kassie, F., 2004, 'Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents', *Toxicology* 198, 329-340.

Mével, M., Kamaly, N., Carmona, S., Oliver, M.H., Jorgensen, M.R., Crowther, C., Salazar, F.H., Marion, P.L., Fujino, M., Natori, Y., Thanou, M., Arbuthnot, P., Yaouanc, J.J., Jaffrès, P.A., Miller, A.D., 2010, 'DODAG; a versatile new cationic lipid that mediates efficient delivery of pDNA and siRNA', *Journal of Controlled Release* 143, 222-232.

Mi, F.L., Wu, Y.Y., Chiu, Y.L., Chen, M.C., Sung, H.W., Yu, S.H., Shyu, S.S., Huang, M.F., 2007, 'Synthesis of a novel glycoconjugated chitosan and preparation of its derived nanoparticles for targeting HepG2 cells', *Biomacromolecules* 8, 892-898.

Mishra, D., Jain, N., Rajoriya, V., Jain, A.K., 2014, 'Glycyrrhizin conjugated chitosan nanoparticles for hepatocyte-targeted delivery of lamivudine', *Journal of Pharmacy and Pharmacology* 66, 1082-1093.

Mishra, H., Mishra, D., Mishra, P.K., Nahar, M., Dubey, V., Jain, N.K., 2010, 'Evaluation of solid lipid nanoparticles as carriers for delivery of hepatitis B surface antigen for vaccination using subcutaneous route', *Journal of Pharmacy and Pharmaceutical Sciences* 13, 495-509.

Mok, H., Kim, H.J., Park, T.G., 2008, 'Dissolution of biomacromolecules in organic solvents by nano-complexing with poly(ethylene glycol)', *International Journal of Pharmaceutics* 356, 306-313.

Moni, S.S., Natarajapillai, S., Safhi, M.M., 2011, 'Immune augmentation of injectable PLGA—Dextran (PLDEX) a double polymeric microspheres as an adjuvant for hepatitis B vaccine', *World Journal of Vaccines* 1, 104-108.

Montrose, K., Yang, Y., Krissansen, G.W., 2014, 'X-pep, a novel cell-penetrating peptide motif derived from the hepatitis B virus', *Biochemical and Biophysical Research Communications*, <http://dx.doi.org/10.1016/j.bbrc.2014.09.057>.

Moreira, M., Ramos, A., Netto, E.M., Brites, C., 2013, 'Characteristics of co-infections by HCV and HBV among Brazilian patients infected by HIV-1 and/or HTLV-1', *The Brazilian Journal of Infectious Diseases* 17, 661-666.

Moriyama, K., Ooya, T., Yui, N., 1999, 'Hyaluronic acid grafted with poly(ethylene glycol) as a novel peptide formulation', *Journal of Controlled Release* 59, 77-86.

Morrey, J.D., Motter, N.E., Chang, S., Fairman, J., 2011, 'Breaking B and T cell tolerance using cationic lipid-DNA complexes (CLDC) as a vaccine adjuvant with hepatitis B virus (HBV) surface antigen in transgenic mice expressing HBV', *Antiviral Research* 90, 227-230.

Morrissey, D.V., Lee, P.A., Johnson, D.A., Overly, S.L., McSwiggen, J.A., Beigelman, L., Mokler, V.R., Maloney, L., Vargeese, C., Bowman, K., O'Brien, J.T., Shaffer, C.S., Conrad, A., Schmid, P., Morrey, J.D., Macejak, D.G., Pavco, P.A., Blatt, L.M., 2002, 'Characterization of nuclease-resistant ribozymes directed against hepatitis B virus RNA', *Journal of Viral Hepatitis* 9, 411-418.

Motoyama, K., Nakashima, Y., Aramaki, Y., Hirayama, F., Uekama, K., Arima, H., 2011, 'In vitro gene delivery mediated by asialofetuin-appended cationic liposomes associated with γ -cyclodextrin into hepatocytes', *Journal of Drug Delivery*, 476137, <http://www.dx.doi:10.1155/2011/476137>.

Motwani, S.K., Chopra, S., Talegaonkar, S., Kohli, K., Ahmad, F.J., Khar, R.K., 2008, 'Chitosan-sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: Formulation, optimisation and in vitro characterization', *European Journal of Pharmaceutics and Biopharmaceutics* 68, 513-525.

Mufamadi, M.S., Choonara, Y.E., Kumar, P., Modi, G., Naidoo, D., van Vuuren, S., Ndesendo, V.M.K., du Toit, L.C., Iyuke, S.E., Pillay, V., 2013, 'Ligand-functionalized nanoliposomes for targeted delivery of galantamine', *International Journal of Pharmaceutics* 448, 267-281.

Murnane, D., Marriott, C., Martin, G.P., 2008, 'Polymorphic control of inhalation microparticles prepared by crystallization', *International Journal of Pharmaceutics* 361, 141-149.

Nash, K.L., Alexander, G.J.M., 2008, 'The case for combination antiviral therapy for chronic hepatitis B virus infection', *Lancet Infectious Diseases* 8, 444-448.

Oh, E.J., Park, K., Kim, K.S., Kim, J., Yang, J.A., Kong, J.H., Lee, M.Y., Hoffman, A.S., Hahn, S.K., 2010, 'Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives', *Journal of Controlled Release* 141, 2-12.

Ou, J.H., Yeh, C.T., Yen, T.S., 1989, 'Transport of hepatitis B virus precore protein into the nucleus after cleavage of its signal peptide', *Journal of Virology* 63 (1989) 5238-5243.

Palumbo, E., 2008, 'New drugs for chronic hepatitis B: A review', *American Journal of Therapeutics* 15, 167-172.

Palumbo, F.S., Pitarresi, G., Mandracchia, D., Tripodo, G., Giammona, G., 2006, 'New graft copolymers of hyaluronic acid and polylactic acid: synthesis and characterization', *Carbohydrate Polymers* 66, 379-385.

Pan, X.B., Han, J.C., Wei, L., Peng, D.D., Gao, Y., 2008, 'Subcellular distribution and translocation of hepatitis B virus core protein in HepG2.2.15 cells', *Chinese Journal of Hepatology* 16, 29-32.

Pan, X.B., Wei, L., Han, J.C., Ma, H., Deng, K., Cong, X., 2011, 'Artificial recombinant cell-penetrating peptides interfere with envelopment of hepatitis B virus nucleocapsid and viral production', *Antiviral Research* 89, 109-114.

Panyam, J., Zhou, W., Prabha, S., Sahoo, S.K., Labhasetwar, V., 2002, 'Rapid endo-lysosomal escape of poly (DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery', *FASEB Journal* 16, 1217-1226.

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, 426-433.

Park, J.H., Saravanakumar, G., Kim, K., Kwon, I.C., 2010, 'Targeted delivery of low molecular drugs using chitosan and its derivatives', *Advanced Drug Delivery Reviews* 62, 28-41.

Park, J.O., Stephen, Z., Sun, C., Veiseh, O., Kievit, F.M., Fang, C., Leung, M., Mok, H., Zhang, M., 2011, 'Glypican-3 targeting of liver cancer cells using multifunctional nanoparticles', *Molecular Imaging* 10, 69-77.

Plajnšek, K.T., Kocbek, P., Kreft, M.E., Kristl, J., 2012, 'Mechanisms of cellular uptake of nanoparticles and their effect on drug delivery', *Slovenian Medical Journal* 81, 225-235.

Pol, S., Lampertico, P., 2012, 'First-line treatment of chronic hepatitis B with entecavir or tenofovir in 'real-life' settings: from clinical trials to clinical practice', *Journal of Viral Hepatitis* 19, 377-386.

Popielarski, S.R., Pun, S.H., Davis, M.E., 2005a, 'A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 1. Synthesis and characterization', *Bioconjugate Chemistry* 16, 1063-1070.

Popielarski, S.R., Hu-Lieskovan, S., French, S.W., Triche, T.J., Davis, M.E., 2005b, 'A nanoparticle-based model delivery system to guide the rational design of gene delivery to the liver. 2. In vitro and in vivo uptake results', *Bioconjugate Chemistry* 16, 1071-1080.

Radhakumary, C., Nandkumar, A.M., Nair, P.D., 2011, 'Hyaluronic acid-g-poly(HEMA) copolymer with potential implications for lung tissue engineering', *Carbohydrate Polymers* 85, 439-445.

Ramkumaar, G.R., Srinivasan, S., Bhoopathy, T.J., Gunasekaran, S., 2012, 'Quantum chemical and experimental studies on polymorphism of antiviral drug lamivudine', *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy* 98, 265-270.

Renberg, B., 2006, *Fluorescence-based ligand assays for protein detection using affibody affinity proteins*, Stockholm:KTH.

Resende, C., Lessa, A., Goldenberg, R.C.S., 2011, 'Ultrasonic Imaging in Liver Disease: From Bench to Bedside, Ultrasound Imaging - Medical Applications, Minin, O., (Ed.), ISBN: 978-953-307-279-1, InTech, DOI: 10.5772/16878. Available from: <http://www.intechopen.com/books/ultrasound-imaging-medical-applications/ultrasonic-imaging-in-liver-disease-from-bench-to-bedside>

Ribeiro, A.F., de Oliveira Rezende, R.L., Cabral, L.M., de Sousa, V.P., 2013, 'Poly ϵ -caprolactone nanoparticles loaded with *Uncaria tomentosa* extract: preparation, characterization, and optimization using the Box–Behnken design', *International Journal of Nanomedicine* 8, 431-442.

Robek, M.D., Boyd, B.S., Chisari, F.V., 2005, 'Lambda interferon inhibits hepatitis B and C virus replication', *Journal of Virology* 79, 3851-3854.

Roberts, M.J., Bentley, M.D., Harris, J.M., 2002, 'Chemistry for peptide and protein PEGylation', *Advanced Drug Delivery Reviews* 54, 459-476.

Roco, M.C., 1999, 'Nanoparticles and nanotechnology research', *Journal of Nanoparticle Research* 1, 1-6.

Rossignol, J.F., 2009, 'Thiazolides: a new class of antiviral drugs', *Expert Opinion on Drug Metabolism and Toxicology* 5, 667-674.

Rossignol, J.F., Keeffe, E.B., 2008, 'Thiazolides: a new class of drugs for the treatment of chronic hepatitis B and C', *Future Microbiology* 3, 539-545.

Rozema, D.B., Lewis, D.L., Wakefield, D.H., Wong, S.C., Klein, J.J., Roesch, P.L., Bertin, S.L., Reppen, T.W., Chu, Q., Blokhin, A.V., Hagstrom, J.E., Wolff, J.A., 2007, 'Dynamic polyConjugates for targeted in vivo delivery of siRNA to hepatocytes', *Proceedings of the National Academy of Sciences of the United States of America* 104, 12982-12987.

Rui, M., Guo, W., Ding, Q., Wei, X., Xu, J., Xu, Y., 2012, 'Recombinant high-density lipoprotein nanoparticles containing gadolinium-labeled cholesterol for morphologic and functional magnetic resonance imaging of the liver', *International Journal of Nanomedicine* 7, 3751-3768.

Ruiz-Pérez, L., Royston, G.J., Fairclough, J.P.A., Ryan, A.J., 2008, 'Toughening by nanostructure', *Polymer* 49, 4475-4488

Ruiz-Sancho, A., Sheldon, J., Soriano, V., 2007, 'Telbivudine: a new option for the treatment of chronic hepatitis B', *Expert Opinion on Biological Therapy* 7, 751-761.

Rusu, E., Rusu, G., Dorohoi, D., 2009, 'Influence of temperature on structures of polymers with ϵ -caprolactam units studied by FT-IR spectroscopy', *Polimery* 54, 347-352.

Sahana, D.K., Mittal, G., Bhardwaj, V., Ravi Kumar, M.N.V., 2008, 'PLGA nanoparticles for oral delivery of hydrophobic drugs: Influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as a model drug', *Journal of Pharmaceutical Sciences* 97, 1539-1542.

Sahoo, S.K., Panyam, J., Prabha, S., Labhasetwar, V., 2002, 'Residual polyvinyl alcohol associated with poly(D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake', *Journal of Controlled Release* 82, 105-114.

Saini, V., Jain, V., Sudheesh, M.S., Jaganathan, K.S., Murthy, P.K., Kohli, D.V., 2011, 'Comparison of humoral and cell-mediated immune responses to cationic PLGA microspheres containing recombinant hepatitis B antigen', *International Journal of Pharmaceutics* 408, 50-57.

Sarkari, M., Brown, J., Chen, X., Swinnea, S., Williams III, R.O., 2002, 'Johnston KP. Enhanced drug dissolution using evaporative precipitation into aqueous solution', *International Journal of Pharmaceutics* 243, 17-31.

Sathali, H.A.A., Rajalakshmi, G., 2010, 'Evaluation of transdermal targeted niosomal drug delivery of terbinafine hydrochloride', *International Journal of PharmTech Research* 2, 2081-2089.

Saxena, S.K., Mishra, N., Saxena, R., 2009, 'Advances in antiviral drug discovery and development: Part 1: Advancements in antiviral drug discovery', *Future Virology* 4, 101-107.

Schanté, C.E., Zuber, G., Herlin, C., Vandamme, T.F., 2011, 'Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications', *Carbohydrate Polymers* 85, 469-489.

Schanté, C.E., Zuber, G., Herlin, C., Vandamme, T.F., 2012, 'Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids', *Carbohydrate Polymers* 87, 2211-2216.

Schmitt, M., Wagner, J., Jung, G., Hempelmann, R., 2007, 'Functionalized polymer colloids bearing primary amino groups', *Journal of Colloid and Interface Science* 311, 425-429.

Schoefield, J., 2012, 'Salvaging the conscience: The biomedical research dilemma', *VetScript*, 36-40.

Sepe, M., 2008, 'The Materials Analyst, Part 98: A tour of the world of nylon - Part 1', *Plastics Today*.

Serdiuk, T., Alekseev, S., Lysenko, V., Skryshevsky, V., Gèloën, A., 2014, 'Trypsinization-dependent cell labeling with fluorescent nanoparticles', *Nanoscale Research Letters* 9, 1-5.

Seymour, L.W., Ferry, D.R., Anderson, D., Hesslewood, S., Julyan, P.J., Poyner, R., Doran, J., Young, A.M., Burtles, S., Kerr, D.J., 2002, 'Hepatic drug targeting: Phase I evaluation of polymer-bound doxorubicin', *Journal of Clinical Oncology* 20, 1668-1676.

Sharma, D., Maheshwari, D., Philip, G., Rana, R., Bhatia, S., Singh, M., Gabrani, R., Sharma, S.K., Ali, J., Sharma, R.K., Dang, S., 2014, 'Formulation and Optimization of Polymeric Nanoparticles for Intranasal Delivery of Lorazepam Using Box-Behnken Design: In Vitro and In Vivo Evaluation', *BioMed Research International*, 156010, <http://dx.doi.org/10.1155/2014/156010>

Shen, Y., Li, Q., Tu, J., Zhu, J., 2009, 'Synthesis and characterization of low molecular weight hyaluronic acid-based cationic micelles for efficient siRNA delivery', *Carbohydrate Polymers* 77, 95-104.

Shukla, A., Katare, O.P., Singh, B., Vyas, S.P., 2010, 'M-cell targeted delivery of recombinant hepatitis B surface antigen using cholera toxin B subunit conjugated bilosomes', *International Journal of Pharmaceutics* 385, 47-52.

Shukla, A., Khatri, K., Gupta, P.N., Goyal, A.K., Mehta, A., Vyas, S.P., 2008, 'Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes)', *Journal of Pharmacy and Pharmaceutical Sciences* 11, 59-66.

Sinha, V.R., Trehan, A., 2003, 'Biodegradable microspheres for protein delivery', *Journal of Controlled Release* 90, 261-280.

Singh, A.V., Nath, L.K., 2012, 'Evaluation of compatibility of tablet excipients and novel synthesized polymer with lamivudine', *Journal of Thermal Analysis and Calorimetry* 108, 263-267.

Somiya, M., Yoshimoto, N., Iijima, M., Niimi, T., Dewa, T., Jung, J., Kuroda, S., 2012, 'Targeting of polyplex to human hepatic cells by bio-nanocapsules, hepatitis B virus surface antigen L protein particles', *Bioorganic and Medicinal Chemistry* 20, 3873-3879.

Son, S., Min, H.S., You, D.G., Kim, B.S., Kwon, I.C., 2014, 'Echogenic nanoparticles for ultrasound technologies: Evolution from diagnostic imaging modality to multimodal theranostic agent', *Nano Today* 9, 525-540.

Song, Q., Wang, X., Hu, Q., Huang, M., Yao, L., Qi, H., Qiu, Y., Jiang, X., Chen, J., Chen, H., Gao, X., 2013, 'Cellular internalization pathway and transcellular transport of pegylated polyester nanoparticles in Caco-2 cells', *International Journal of Pharmaceutics* 445, 58-68.

Sonneveld, M.J., Janssen, H.L., 2011, 'Chronic hepatitis B: peginterferon or nucleos(t)ide analogues?', *Liver International: Official Journal of the International Association for the Study of the Liver* 31, 78-84.

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Rudzinski, W.E., 2001, 'Biodegradable polymeric nanoparticles as drug delivery devices', *Journal of Controlled Release* 70, 1-20.

Stachulski, A.V., Pidathala, C., Row, E.C., Sharma, R., Berry, N.G., Iqbal, M., Bentley, J., Allman, S.A., Edwards, G., Helm, A., Hellier, J., Korba, B.E., Semple, J.E., Rossignol, J.F., 2011, 'Thiazolides as novel antiviral agents. 1. Inhibition of hepatitis B virus replication', *Journal of Medicinal Chemistry* 54, 4119-4132.

Stepensky, D., 2010, 'Quantitative aspects of intracellularly-targeted drug delivery', *Pharmaceutical Research* 27, 2776-2780.

Stockert, R.J., 1995, 'The asialoglycoprotein receptor: Relationships between structure, function, and expression', *Physiological Reviews* 75, 591-609.

Stokes, W.S., Marsman, D.S., 2014, Chapter 9 - Animal Welfare Considerations in Biomedical Research and Testing, in: Bayne, K., Turner, P.V. (Eds), *Laboratory Animal Welfare*, Academic Press, Boston.

Strauch, S., Jantratid, E., Dressman, J.B., Junginger, H.E., Kopp, S., Midha, K.K., Shah, V.P., Stavchansky, S., Barends, D.M., 2011, 'Biowaiver monographs for immediate release solid oral dosage forms: lamivudine', *Journal of Pharmaceutical Sciences* 100, 2054-2063.

Stray, S.J., Bourne, C.R., Punna, S., Lewis, W.G., Finn, M.G., Zlotnick, A., 2005, 'A heteroaryldihydropyrimidine activates and can misdirect hepatitis B virus capsid assembly', *Proceedings of the National Academy of Sciences of the United States of America* 102, 8138-8143.

Tamizhrasi, S., Shukla, A., Shivkumar, T., Rathi, V., Rathi, J.C., 2009, 'Formulation and evaluation of lamivudine loaded polymethacrylic acid nanoparticles', *International Journal of PharmTech Research* 1, 411-415.

Tarkin-Tas, E., Mathias, L.J., 2010, 'Synthesis and ring-opening polymerization of 5-azepane-2-one ethylene ketal: a new route to functional aliphatic polyamides', *Macromolecules* 43, 968-974.

Tassopoulos, N.C., Koutelou, M.G., Polychronaki, H., Paraloglou-Ioannides, M., Hadziyannis, S.J., 1997, 'Recombinant interferon-alpha therapy for acute hepatitis B: a randomized, double-blind, placebo-controlled trial', *Journal of Viral Hepatitis* 4, 387-394.

Thomas, C., Rawat, A., Hope-Weeks, L., Ahsan, F., 2011, 'Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine', *Molecular Pharmaceutics* 8, 405-415.

Thomas, G., Lorient, C., Pepin, D., Chambaz, J., Bereziat, G., 1988, 'Selective channelling of arachidonic and linoleic acids into glycerolipids of rat hepatocytes in primary culture', *Biochemical Journal* 256, 641-647.

Tian, Q., Wang, X.H., Wang, W., Zhang, C.N., Wang, P., Yuan, Z., 2012, 'Self-assembly and liver targeting of sulfated chitosan nanoparticles functionalized with glycyrrhetic acid', *Nanomedicine* 8, 870-879.

Torchilin, V.P., 2006, 'Recent approaches to intracellular delivery of drugs and DNA and organelle targeting', *Annual Review of Biomedical Engineering* 8, 343-375.

Trivedi, R., Kompella, U.B., 2010, 'Nanomicellar formulations for sustained drug delivery: Strategies and underlying principles', *Nanomedicine* 5, 485-505.

Uprichard, S.L., Boyd, B., Althage, A., Chisari, F.V., 2005, 'Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs', *Proceedings of the National Academy of Sciences of the United States of America* 102, 773-778.

van Zonneveld, M., Honkoop, P., Hansen, B.E., Niesters, H.G., Darwish Murad, S., de Man, R.A., Schalm, S.W., Janssen, H.L., 2004, 'Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B', *Hepatology* 39, 804-810.

van Tong, H., Bock, C.T., Velavan, T.P., 2014, 'Genetic insights on host and hepatitis B virus in liver diseases', *Mutation Research/ Reviews in Mutation Research* 762, 65-75.

Vasir, J.K., Labhasetwar, V., 2007, 'Biodegradable nanoparticles for cytosolic delivery of therapeutics', *Advanced Drug Delivery Reviews* 59, 718-728.

Verma, A., Stellacci, F., 2010, 'Effect of surface properties on nanoparticle-cell interactions', *Small* 6, 12-21.

Veronese, F.M., Pasut, G., 2005, 'PEGylation, successful approach to drug delivery', *Drug Discovery Today* 10, 1451-1458.

Vickers, K.C., Palmisano, B.T., Shoucri, B.M., Shamburek, R.D., Remaley, A.T., 2011, 'MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins', *Nature Cell Biology* 13, 423-433.

Vilas, M., Tojo, E., 2010, 'A mild and efficient way to prepare ϵ -caprolactam by using a novel salt related with ionic liquids', *Tetrahedron Letters* 51, 4125-4128.

Wang, H-M., Chou, Y-T., Wen, Z-H., Wang, Z-R., Chen, C-H., Ho, M-L., 2013a, 'Novel biodegradable porous scaffold applied to skin regeneration', *PLoS ONE* 8, e56330, doi:10.1371/journal.pone.0056330.

Wang, B., Chen, G., Mao, Z., Zhang, Y., Yu, D., Gao, C., 2012, 'Preparation and cellular uptake of PLGA particles loaded with lamivudine', *Chinese Science Bulletin* 57, 3985-3993.

Wang, G.Q., Ding, Y.P., Dong, Y.H., 2013b, 'Telbivudine treatment is associated with high hepatitis B e antigen seroconversion and immune modulatory effects in chronic hepatitis B patients', *Journal of Viral Hepatitis* 20, 9–17.

Wang, J., Feng, S., Wang, S., Chen, Z., 2010, 'Evaluation of cationic nanoparticles of biodegradable copolymers as siRNA delivery system for hepatitis b treatment', *International Journal of Pharmaceutics* 400, 194-200.

Wang, Y., Su, J., Cai, W., Lu, P., Yuan, L., Jin, T., Chen, S., Sheng, J., 2013c, 'Hepatocyte-targeting gene transfer mediated by galactosylated poly(ethylene glycol)-graft-polyethylenimine derivative', *Drug Design, Development and Therapy* 7, 211-221.

Wang, H.X., Xiong, M.H., Wang, Y.C., Zhu, J., Wang, J., 2013d, 'N-acetylgalactosamine functionalized mixed micellar nanoparticles for targeted delivery of siRNA to liver', *Journal of Controlled Release* 166, 106-114.

Wang, Y., Yao, Y., An, R., You, L., Wang, X., 2009, 'Simultaneous determination of puerarin, daidzein, baicalin, wogonoside and liquiritin of GegenQinlian decoction in rat plasma by ultra-performance liquid chromatography–mass spectrometry', *Journal of Chromatography B* 877, 1820–1826.

Wang, Z., Zhu, K., Bai, W., Jia, B., Hu, H., Zhou, D., Zhang, X., Zhang, X., Xie, Y., Bourguine, M.M., Michel, M.L., Lan, K., Deng, Q., 2014, 'Adenoviral delivery of recombinant hepatitis B virus expressing foreign antigenic epitopes for immunotherapy of persistent viral infection', *Journal of Virology* 88, 3004-3015.

Wooddell, C.I., Rozema, D.B., Hossbach, M., John, M., Hamilton, H.L., Chu, Q., Hegge, J.O., Klein, J.J., Wakefield, D.H., Oropeza, C.E., Deckert, J., Roehl, I., Jahn-Hofmann, K., Hadwiger, P., Vornlocher, H., McLachlan, A., Lewis, D.L., 2013, 'Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection', *Molecular Therapy: The Journal of the American Society of Gene Therapy* 21, 973–985.

Wren, S.A., Tchelitcheff, P., 2006, 'Use of ultra-performance liquid chromatography in pharmaceutical development', *Journal of Chromatography A* 1119, 140–146.

Wu, G.Y., Wu, C.H., 1992, 'Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides', *The Journal of Biological Chemistry* 267, 12436-12439.

Wu, J-L., Liu, C-G., Wang, X-L., Huang, Z-H., 2012, 'Preparation and characterization of nanoparticles based on histidine–hyaluronic acid conjugates as doxorubicin carriers', *Journal of Materials Science: Materials in Medicine* 23, 1921-1929.

Wu, Y., Zheng, Y., Yang, W., Wang, C., Hu, J., Fu, S., 2005, 'Synthesis and characterization of a novel amphiphilic chitosan–polylactide graft copolymer', *Carbohydrate Polymers* 59, 165-171.

Wursthorn, K., Jung, M., Riva, A., Goodman, Z.D., Lopez, P., Bao, W., Manns, M.P., Wedemeyer, H., Naoumov, N.V., 2010, 'Kinetics of hepatitis B surface antigen decline during 3 years of telbivudine treatment in hepatitis B e antigen-positive patients', *Hepatology* 52, 1611-1620.

Xia, W., Shen, Y., Xie, H., Zheng, S., 2006, 'Involvement of endoplasmic reticulum in hepatitis B virus replication', *Virus Research* 121, 116-121.

Xia, Y.P., Yeh, C.T., Ou, J.H., Lai, M.M., 1992, 'Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex', *Journal of Virology* 66, 914–921.

Xun, Y., Pan, Q., Tang, Z., Chen, X., Yu, Y., Xi, M., Zang, G., 2013, 'Intracellular-delivery of a single-chain antibody against hepatitis B core protein via cell-penetrating peptide inhibits hepatitis B virus replication in vitro', *International Journal of Molecular Medicine* 31, 369-376.

Yamada, M., Oeda, A., Jung, J., Iijima, M., Yoshimoto, N., Niimi, T., Jeong, S.Y., Choi, E.K., Tanizawa, K., Kuroda, S., 2012, 'Hepatitis B virus envelope L protein-derived bio-nanocapsules: mechanisms of cellular attachment and entry into human hepatic cells', *Journal of Controlled Release* 160, 322-329.

Yang, F., Gu, A., Chen, Z., Gu, N., Ji, M., 2008, 'Multiple emulsion microbubbles for ultrasound imaging', *Materials Letters* 62, 121-124.

Yang, K.W., Li, X.R., Yang, Z.L., Li, P.Z., Wang, F., Liu, Y., 2009, 'Novel polyion complex micelles for liver-targeted delivery of diammonium glycyrrhizinate: in vitro and in vivo characterization', *Journal of Biomedical Materials Research. Part A* 88, 140-148.

Yang, W., Mou, T., Shao, G., Wang, F., Zhang, X., Liu, B., 2011, 'Copolymer-based hepatocyte asialoglycoprotein receptor targeting agent for SPECT', *Journal of Nuclear Medicine* 52, 978-985.

Yapar, E.A., İnal, O., 2012, 'Poly(ethylene oxide)–Poly(propylene oxide)-based copolymers for transdermal drug delivery: an overview', *Tropical Journal of Pharmaceutical Research* 11, 855-866.

Yeo, Y., 2013, *Nanoparticulate Drug Delivery Systems: Strategies, Technologies, and Applications*, John Wiley & Sons, Hoboken, New Jersey.

Yu, B., Hsu, S.H., Zhou, C., Wang, X., Terp, M.C., Wu, Y., Teng, L., Mao, Y., Wang, F., Xue, W., Jacob, S.T., Ghoshal, K., Lee, R.J., Lee, L.J., 2012, 'Lipid nanoparticles for hepatic delivery of small interfering RNA', *Biomaterials* 33, 5924-5934.

Yuan, Y., Wang, W., Wang, B., Zhu, H., Zhang, B., Feng, M., 2013, 'Delivery of hydrophilic drug doxorubicin hydrochloride-targeted liver using apoA1 as carrier', *Journal of Drug Targeting* 21, 367-374.

Yue, W., 2012, 'Preparation of low-molecular-weight hyaluronic acid by ozone treatment', *Carbohydrate Polymers* 89, 709–712.

Zahr, A.S., Davis, C.A., Pishko, M.V., 2006, 'Macrophage uptake of core-shell nanoparticles surface modified with poly (ethylene) glycol', *Langmuir* 22, 8178-8185.

Zeng, P., Xu, Y., Zeng, C., Ren, H., Peng, M., 2011, 'Chitosan-modified poly(D,L-lactide-co-glycolide) nanospheres for plasmid DNA delivery and HBV gene-silencing', *International Journal of Pharmaceutics* 415, 259-266.

Zhang, M., James, S.P., 2004, 'Novel hyaluronan esters for biomedical applications', *Biomedical Sciences Instrumentation* 40, 238-242.

Zhang, X., Miao, J., Li, M., Jiang, S., Hu, F., Du, Y., 2008, 'Solid lipid nanoparticles loading adefovir dipivoxil for antiviral therapy', *Journal of Zhejiang University. Science. B.* 9, 506-510.

Zhang, X., Zhang, Q., Peng, Q., Zhou, J., Liao, L., Sun, X., Zhang, L., Gong, T., 2014, 'Hepatitis B virus preS1-derived lipopeptide functionalized liposomes for targeting of hepatic cells', *Biomaterials* 35, 6130-6141.

Zheng, S.Y., Chen, Z.C., Lu, D.S., Wu, Q., Lin, X.F., 2005, 'Graft copolymerization of water-soluble monomers containing quaternary ammonium group on poly(vinyl alcohol) using ceric ions', *Journal of Applied Polymer Science* 97, 2186-2191.

Zhou, X.X., Littler, E., 2006, 'Nucleoside analogs as anti-HBV agents', *Current Topics in Medicinal Chemistry* 6, 851-865.

Zoulim, F., 2007, 'Emerging drugs for hepatitis B', *Expert Opinion on Emerging Drugs* 12, 199-21.

Zoutendijk, R., Reijnders, J.G., Brown, A., Zoulim, F., Mutimer, D., Deterding, K., Petersen, J., Hofmann, W.P., Buti, M., Santantonio, T., van Bömmel, F., Pradat, P., Oo, Y., Luetgehetmann, M., Berg, T., Hansen, B.E., Wedemeyer, H., Janssen, H.L., 2011, 'Entecavir treatment for chronic hepatitis B: adaptation is not needed for the majority of naïve patients with a partial virological response', *Hepatology* 54, 443-451.

APPENDICES

APPENDIX A

Cell Portage & Therapeutic Bearers: A Hepatitis B Viral View

Latavia Singh^a, Yahya E. Choonara^a, Lomas Kumar Tomar^a, Charu Tyagi^a, Valence M.K. Ndesendo^b, Lisa C. du Toit^a, Pradeep Kumar^a and Viness Pillay^{a,*}

^aWits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown 2193, Johannesburg, Gauteng, South Africa

^bSchool of Pharmacy & Pharmaceutical Sciences, St. John's University of Tanzania, Dodoma, Tanzania

*Corresponding Author:

Professor Viness Pillay

Tel: +27-11-717-2274

Fax: +27-11-642-4355

Fax2Email: +27-86-553-4733

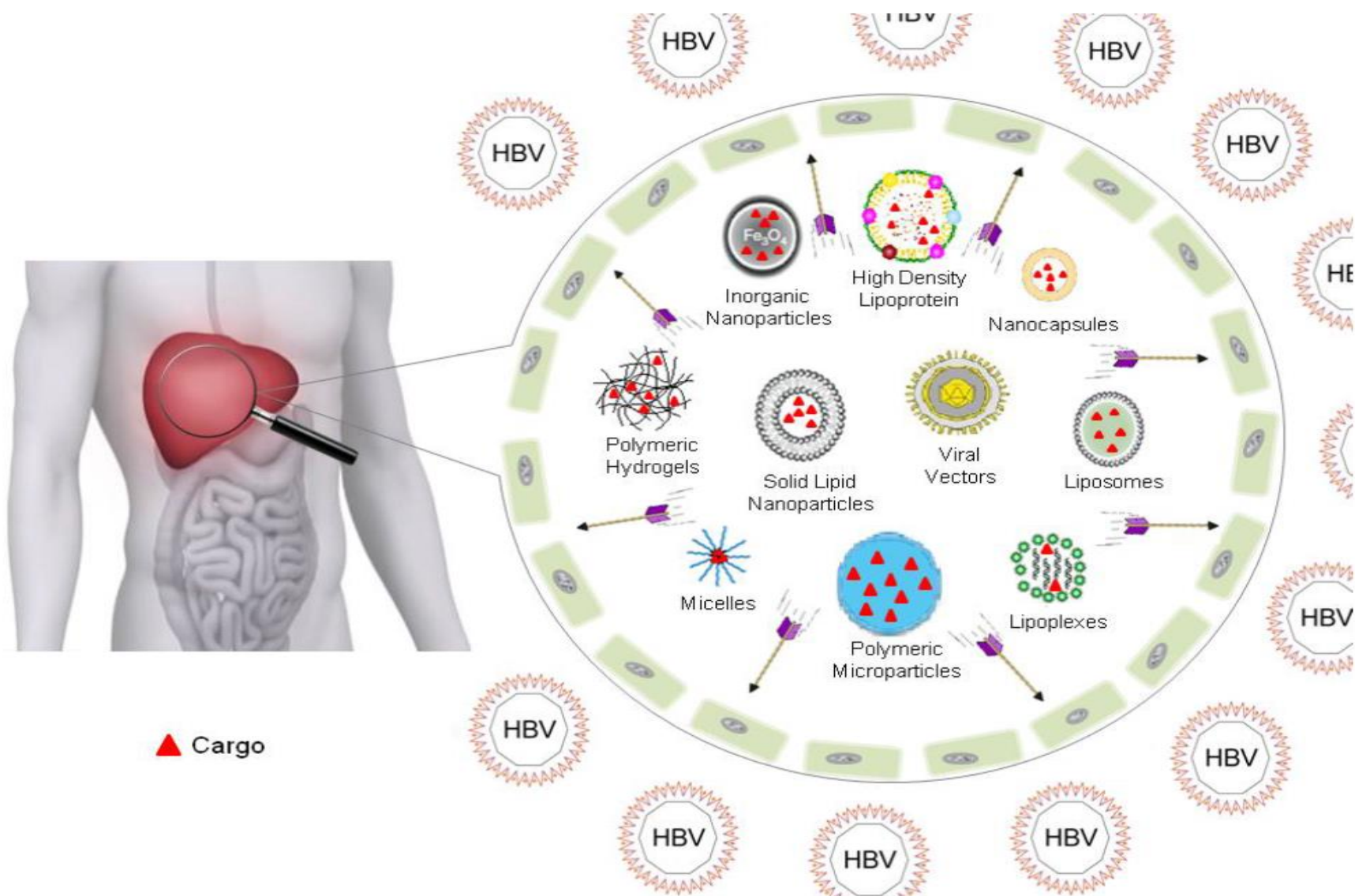
Email: viness.pillay@wits.ac.za

ABSTRACT

A chronic hepatitis B virus (HBV) poses a significant health challenge due to associated morbidity and mortality from cirrhosis and hepatocellular cancer that eventually results in breakdown of liver functionality. Nanotechnology plays a considerable role in the therapeutic approach to reducing viral load levels and drug resistant HBV through drug targeting and thus the rate of evolution of the disease. Apart from tissue targeting, intracellular mode of delivery of a wide range of drugs is dire to wield a therapeutic action in affected organelles. This review encompasses strategies and techniques that have been utilized to target the HBV infected nucleus in liver hepatocytes additionally covering some HBV treatment measures that have previously been sought and challenged in an attempt to expunge the dreadful virus with a significant look at new insights and most recent advances in drug carriers and their role in anti-HBV therapy.

Keywords: hepatitis B virus (HBV); intracellular drug delivery; liver targeting; hepatocyte; asialoglycoprotein receptor (ASGP-R); nanoparticles; cell penetrating peptides (CPPs).

GRAPHICAL ABSTRACT



APPENDIX B

Novel synthesis and *in vitro* characterization of an amphiphilic Hyaluronic acid-graft-epsilon-Caprolactam copolymer

Latavia Singh¹, Yahya E. Choonara¹, Lomas K. Tomar¹, Charu Tyagi¹, Pradeep Kumar¹, Lisa C. du Toit¹, Vines Pillay^{1*}

University of the Witwatersrand, Department of Pharmacy & Pharmacology, Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa

*Correspondence: vines.pillay@wits.ac.za

A novel amphiphile was synthesized employing the hydrophilic Hyaluronic acid (HA) and hydrophobic epsilon-Caprolactam (ECL) and subjecting these to an anionic ring opening graft copolymerization technique to allow for grafting of ECL monomeric branches onto the backbone of the HA chain. HA-g-ECL was formed by evidence of grafting obtained via fourier transform infrared spectroscopy (FTIR), x-ray diffraction (XRD) analyses and differential scanning calorimetry (DSC). A thermal degradation profile of the copolymer was attained using thermogravimetric (TGA) analysis. The novel HA-g-ECL displayed a grafting efficiency (GE) and graft ratio (GR) in ranges of 70.52 - 117.80% and 41.36 – 54.10%, respectively. The most optimal GE and GR was achieved with a ratio of 1:9 of HA:ECL. The average molecular weight of novel HA-g-ECL was determined by viscosity measurements to be 3.398×10^7 Da. Scanning electron microscopy (SEM) demonstrated areas of smoothness and roughness on the external surface of the copolymer. HA-g-ECL acquired the ability to form copolymeric micelles and the critical micelle concentration (CMC) determined by fluorescein isothiocyanate (FITC) as a fluorescent probe, was found to occur at a concentration of 0.0063mg/mL. Micelles prepared were visualized with transmission electron microscopy (TEM) revealing a consistent 25-30nm size range and distinct spherical shape which can be exploited as potential candidates for important applications in nanoscale drug delivery.

Trend reflection in the design and *in vitro* characterization of HA-g-ECL forming Lamivudine-loaded nanoparticles

Latavia Singh¹, Lomas K. Tomar¹, Charu Tyagi¹, Pradeep Kumar¹, Lisa C. du Toit¹, Yahya E. Choonara¹, Viness Pillay^{1*}

University of the Witwatersrand, Department of Pharmacy and Pharmacology, Medical School, 7 York Road, Parktown, 2193, Johannesburg, South Africa

*Correspondence: viness.pillay@wits.ac.za

ABSTRACT - PURPOSE. The impetus for this study was to engineer a novel Lamivudine (LMV, model drug) loaded nanoformulation by conjugating, Hyaluronic Acid (HA) and epsilon-Caprolactam (ECL) (HA-g-ECL), and determining their inclination and tendencies in affecting desired responses. **METHODS.** HA-g-ECL copolymer and subsequently its nanoparticles were prepared by anionic graft copolymerization and a double emulsion solvent-evaporation method respectively. A Box Behnken experimental design was used to generate 15 formulations to be assessed and characterized *in vitro*. Verification of actual graft transpiring was carried out by a fourier transform infrared spectroscopy (FTIR) report. The nanoparticle formulations were evaluated for their physicochemical properties by various standard techniques - the investigation of mean particle size diameter by virtue of dynamic light scattering (DLS), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), transmission electron microscopy (TEM), drug entrapment efficiency (DEE) and drug release. **RESULTS.** Particle size of the formulations was observed to be inversely proportional to the amounts of both surfactant and solvent and directly proportional to copolymer mass. Lower quantities of HA-g-ECL copolymer in the formulation as compared to higher quantities resulted in higher encapsulation efficiencies of HA-g-ECL nanoparticles. A lower amount of Pluronic F-127 surfactant and a greater volume of dichloromethane (DCM) solvent also contributed to the high encapsulation efficiency. Approximately 65% LMV was released in the burst effect in the first 15 hrs with design formulations comprising least amount of HA-g-ECL while ~20% LMV with highest level of HA-g-ECL. **CONCLUSIONS.** Grafting of ECL onto HA successfully produced an amphiphilic copolymer. Varying the quantities of copolymer, surfactant and solvent proved effective for nanoparticle preparation and had implications in controlling the drug's entrapment efficiency and release from the copolymeric system.

APPENDIX D

JUNE 2014		PATENT JOURNAL	
PATENT JOURNAL INCLUDING TRADE MARKS, DESIGNS AND COPYRIGHT IN CINEMATOGRAPH FILMS			
JUNE 2014		VOL 47 • No.06	
 Companies and Intellectual Property Commission a member of the dti group			
Part II of II			
ISSUED MONTHLY		DATE OF ISSUE: 25 JUNE 2014	
ISSN 2223-4837			
		Page 1	

PATENT JOURNAL

INCLUDING TRADE MARKS, DESIGNS AND
COPYRIGHT IN CINEMATOGRAPH FILMS

VOL. 47 No. 06

Date of Issue: 25 JUNE 2014

PATENTS, TRADE MARKS, DESIGNS AND COPYRIGHT OFFICE

Official notices of proceedings under:

The Patents Act, 1978

The Designs Act, 1993

The Trade Marks Act, 1963

The Trade Marks Act, 1993

The Registration of Copyright in Cinematograph Films Act, 1977

Registrar of Patents, Trade Marks, Designs and Copyright

Note: CIPC acting on behalf of the Government of the Republic of South Africa, cannot guarantee the accuracy of its publications or undertake any responsibility for errors or omissions or their consequences.

~72: DAVIS, Gary;DIMITRAKIS, Georgios;DODDS, Christopher;HAMILTON, Erin;KINGMAN, Samuel~ 33:AU
~31:2011904632 ~32:08/11/2011

2014/04092 ~ Complete ~54:PROCESS FOR PRODUCING METHANATION CATALYST AND PROCESS FOR
METHANATION OF SYNTHESIS GAS ~71:BASF SE, D-67056 LUDWIGSHAFEN, GERMANY, Germany ~72:
MILANOV, Andrian;QUERNER, Claudia;ROUSSE, Thomas;SCHUNK, Stephan;STRASSER,
Andreas;WASSERSCHAFF, Guido~ 33:EP ~31:11188238.7 ~32:08/11/2011

2014/04090 ~ Complete ~54:PROCESS FOR PRODUCING REFORMING CATALYST AND REFORMING OF
METHANE ~71:BASF SE, D-67056 LUDWIGSHAFEN, GERMANY, Germany ~72: MILANOV,
Andrian;ROUSSE, Thomas;SCHUNK, Stephan;SCHWAB, Ekkehard;STRASSER,
Andreas;WASSERSCHAFF, Guido~ 33:EP ~31:11188237.9 ~32:08/11/2011

2014/04091 ~ Complete ~54:ORAL SPRAY FORMULATIONS AND METHODS FOR ADMINISTRATION OF
SILDENAFIL ~71:SUDA LIMITED, LEVEL 1, UNIT 12, 55 HOWE STREET, OSBORNE PARK 6017 WESTERN
AUSTRALIA, AUSTRALIA, Australia ~72: BERGSTROM, David;OPAWALE, Foye~ 33:US ~31:61/566,879
~32:05/12/2011

2014/04088 ~ Complete ~54:ACCURATE DOSE CONTROL MECHANISMS AND DRUG DELIVERY SYRINGES
~71:UNITRACT SYRINGE PTY LTD, SUITE 3, LEVEL 11, 1 CHIFLEY SQUARE, SYDNEY, NEW SOUTH
WALES 2000, AUSTRALIA, Australia ~72: CASTAGNA, Lou;SHETTY, Gautam, N.~ 33:US ~31:61/568,509
~32:08/12/2011

2014/04093 ~ Complete ~54:METHOD AND DEVICE FOR ROLLING ROLLING STOCK AND USE OF A
COOLING LUBRICANT ~71:SMS SIEMAG AG, Eduard-Schloemann-Strasse 4, Germany ~72: Arnt
KOHLRAUSCH;Dr. Hartmut PAWELSKI;Dr. Olaf Norman JEPSEN~

2014/04081 ~ Provisional ~54:A COOLING TOWER WITH EFFICIENCY ENHANCING ACCESSORY
~71:STELLENBOSCH UNIVERSITY, Admin B, Victoria Street, Stellenbosch, South Africa ~72: GAUCHÉ,
Paul;VAN NIEKERK, Johannes Lodewikus~

2014/04110 ~ Provisional ~54:WHEEL BAROW TANKER ~71:FELIX FRANCOIS DE VILLIERS , 53 Badger Lane
Aqua Vista Mountain Estate Bronkhorstspuit , South Africa ~72: FELIX FRANCOIS DE VILLIERS~

2014/04111 ~ Provisional ~54:LADDER EXTENSION ~71:ALBERT NORMAN WESSON , 25 North Rand Road
Beyers Park Boksburg , South Africa ~72: ALBERT NORMAN WESSON~

2014/04117 ~ Provisional ~54:AN IMPLANTABLE SENSOR FOR DISEASE DETECTION AND TREATMENT
~71:UNIVERSITY OF THE WITWATERSRAND ,JOHANNESBURG , 1 Jan Smuts Avenue , South Africa ~72: LISA
CLAIRE DU TOIT;MPHO PHEHELLO NGOEPE;PRADEEP KUMAR;VINESS PILLAY;YAHYA ESSOP
CHOONARA~

2014/04119 ~ Provisional ~54:NANOPARTICULATE SYSTEM FOR INTRACELLULAR DRUG DELIVERY
~71:UNIVERSITY OF THE WITWATERSRAND ,JOHANNESBURG , 1 Jan Smuts Avenue , South Africa ~72:
LATAVIA SINGH;LISA CLAIRE DU TOIT;PRADEEP KUMAR;VINESS PILLAY;YAHYA ESSOP CHOONARA~

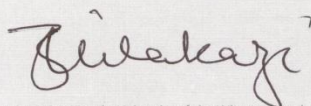
2014/04127 ~ Provisional ~54:WEAPON RECOIL MANAGEMENT ASSEMBLY ~71:Emerging World
Technologies South Africa (Pty) Limited, No. 54 De Havilland Crescent, Perseus Technopark, South Africa ~72:
LOOCK, JOHAN HENDRIK;SAVIDES, CRAIG ANTHONY~

First Time Inventor

Certificate awarded to

Latavia Singh

for having disclosed their first Wits invention



Prof Zebion Vilakazi, DVC Research, University of the Witwatersrand, Johannesburg

22 July 2014

This award is issued by the Technology Transfer Office of the University, located in Wits Enterprise. As a recipient of this award, the Technology Transfer Office welcomes you to the Wits Innovator's Forum, an elite group of Wits researchers who translate their research into innovations that have potential for use in society and industry for the benefit of South Africans.



Wits Innovators Forum

APPENDIX F



STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/01/C

APPLICANT: L Singh

SCHOOL: Pharmacy & Pharmacology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *An in vivo evaluation of an architecture-modified nanoparticulate system for intracellular drug delivery in rats*

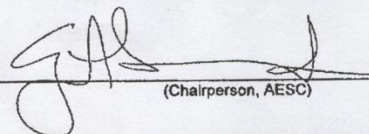
Number and Species

120 Rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 28 JANUARY 2014. This approval remains valid until 27 JANUARY 2016.

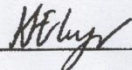
The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: 
(Chairperson, AESC)

Date: 10/2/2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: 
(Registered Veterinarian)

Date: 10/2/2014

cc: Supervisor: Prof V Pillay
Director: CAS

Works 2000/ain0015/AESCcert.wps