

A PROLONGED RELEASE *IN SITU* RECONFIGURING DEVICE FOR THE DELIVERY OF DRUGS TO SOLID TUMORS

AMEENA WADEE



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, South Africa

Co-Supervisors:

Professor Yahya E. Choonara

Department of Pharmacy and Pharmacology, University of the Witwatersrand, South Africa

Ms Lisa C. du Toit

Department of Pharmacy and Pharmacology, University of the Witwatersrand, South Africa

and

Doctor Clem Penny

Department of Medical Oncology, University of the Witwatersrand, South Africa

Johannesburg, 2012

DECLARATION

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

.....

This day of October 2012

RESEARCH OUTPUTS

1. Publications

a. Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit, Clement Penny, Valence MK Ndesendo, Pradeep Kumar and Caragh S Murphy. 2011. Recent advances in the design of drug-loaded polymeric implants for the treatment of solid tumors. *Expert Opin. Drug Deliv.* 8:1323-40. (Abstract in appendix A (I))

b. Determination of the effect of release of methotrexate from an in situ forming implant on colon carcinoma cells and in vivo studies in the rat model. 2011.

Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit, Clement Penny, Pradeep Kumar and Leith Meyer. *South African Pharmaceutical Journal.* 78:49-50. (Abstract in appendix A (II))

2. Conference Outputs : Poster Presentations

a. Farhana Ravat, Ameena Wadee, Viness Pillay, Yahya E. Choonara and Lisa C. du Toit. Formulation approaches for interactive pH-responsive nanoparticles. 29th Annual Conference of the Academy of Pharmaceutical Sciences, 22-26 September 2008, Rustenburg, South Africa. (Abstract in Appendix B (I))

b. Ameena Wadee, Palesa Sekano, Viness Pillay, Yahya E. Choonara and Lisa C. du Toit. The effect of gum arabic on the physicochemical properties and drug release behaviour of thermoresponsive poly(methylvinyl ether) gels. 5th ICPPS conference, 23-26 September 2009, Potchefstroom, South Africa. (Abstract in appendix B (II))

c. Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit, Clement Penny and Valence MK Ndesendo. An injectable in situ forming implant for the treatment of solid tumors utilizing a central composite design. International Symposium on Stimuli Responsive Materials, 26 and 27th October 2010. Mississippi, USA. (Abstract in appendix B (III))

3. Conference Outputs : Podium Presentations

a. Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit and Clement Penny. The development of an in situ forming implant for the treatment of osteosarcoma: Autoclaving as a sterilization method. Postgraduate Cross-Symposium. September 2010, University of the Witwatersrand, Johannesburg, South Africa. (Abstract in appendix B (IV))

b. Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit and Clement Penny. The development of an *in situ* forming implant for the treatment of solid tumors: Determination of the effect of methotrexate release on colon carcinoma cells and in vivo studies in the rat model. 6th ICPPS Conference, Adcock Ingram Young Scientist Competition, 25th- 28th September 2011, Umhlanga, Kwa-Zulu Natal, South Africa. (Abstract in appendix B (V))

4. Patent Application

Ameena Wadee, Viness Pillay, Yahya E. Choonara, Lisa C. du Toit and Clement Penny. An implant for the controlled release of drugs. *SA Patent Application No: 2010/03745*, 02-05-2008 (Preliminary filing).

5. Accolades

Postgraduate Merit Award, University of the Witwatersrand, 2008 and 2009

TATA Africa Scholarship, 2010, 2011

Winner of the Adcock Ingram Young Scientist Award: The development of an *in situ* forming implant for the treatment of solid tumors: Determination of the effect of methotrexate release on colon carcinoma cells and in vivo studies in the rat model. 6th ICPPS Conference, 25th- 28th September 2011, Umhlanga, Kwa-Zulu Natal, South Africa.

ABSTRACT

The poor response of patients to high-dose chemotherapy commonly associated with the treatment of solid tumors has led to research efforts in the development of implants for the delivery of drug directly to solid tumors. In order to prevent surgical complications associated with the placement of an implant at the site of the tumor, efforts have been made to develop implants which form at the site of the tumor.

This study aimed to develop an *In Situ* Forming Implant (ISFI) which was responsive to temperature and able to form an implant when injected into the body and to release drug over a period exceeding one month. To this end, a thermoresponsive polymer, poly(methyl vinyl ether) was selected and following preformulation studies to assess release and gelation temperature, a Design of Experiments approach was utilised to formulate an optimal formulation.

Fourteen formulations were prepared according to a Face-Centred Central Composite Design selected and were assessed for gelation temperature, ease of injectability and Mean Dissolution Time. Utilising the experimental values obtained, regression models for each of the outcomes were generated. The optimal formulation was then determined by selecting the appropriate targets for each of the responses in the design. The optimal formulation was able to gel at body temperature, could be injected into the body and showed release of entrapped chemotherapeutic, methotrexate for a period exceeding one month.

pH-responsive microparticles were also formulated and optimized using a Face-Centred Central Composite Design. Optimized particles were then loaded into the optimized ISFI and displayed faster release of the entrapped drug than with the dispersed drug.

In vitro testing of the ISFI was conducted against solid tumor forming cells and *in vivo* testing was conducted in healthy Sprague-Dawley rats. A few rats developed toxicity to methotrexate after 6 days, however, low quantities of drug were found in the plasma. In addition, drug was present in the surrounding tissue in a high concentration even after 10 days.

ACKNOWLEDGEMENTS

Most importantly, I would like to thank the Creator of all things, Most Beneficial and Most Merciful for allowing me this opportunity to learn so much through this very humbling experience of masters. If I did not have You to turn to in the many failures I endured, I would not have made it this far. To You are due all praises.

It is impossible to quantify the help given by various people that led to the completion of this work. It is also impossible to name every person that has helped me in whatever small way. Please note that the list that follows is by no means complete or as detailed as it should be. The acts of kindness and compassion that were showed to me in this challenging 5 year journey, gave me hope. The gratitude I have expressed and am trying to express here can in no way compensate.

Thank you to my parents for giving me the numerous academic opportunities I know I am extremely privileged to have. Thank you also for allowing me the indulgence of completing my masters. To my aunt, Gorifoi, thank you for all the work you have put into my education and for your endless words of encouragement. To my sister, Safiyya and my brothers Ahmed and Abdurrahman, thank you for the laughter which kept me sane (sort-of) and for not making light of my failure and bad choices but instead helping me through it. To my aunts, uncles, cousins and extended family members, thank you for always reminding me of the importance of perseverance and for your encouragement through the worst parts of the years.

To Prof Viness Pillay, thank you for the opportunity given to me in allowing me to complete my masters under your supervision. Your dedication to excellence and innovative thinking has resulted in me learning life lessons as much as I have learnt scientific ones. Thank you also for ensuring that students at the Wits Advanced Drug Delivery Platform (WADDP) have access to cutting-edge equipment and an excellent facility in which to conduct research.

To my co-supervisors: Prof Yahya Choonara, Ms Lisa du Toit and Dr Clem Penny, my sincerest gratitude for your valuable input. My sincere appreciation for the work all of you have put into my research.

To my uncle, Prof AA Wadee, the Dean of the Faculty of Health Sciences, thank you for your encouragement to pursue my masters and for being a source of inspiration to me.

Thank you to all my friends and colleagues at WADDP and the Dept of Pharmacy and Pharmacology: Kovanya, Clare, Rubina, Yusuf, Zaheeda, Femi, Yasien, Sajida, Shivaan, Deshika, Caragh, Priya, Bongani, Derusha, Thiresen, Thomas, Latavia, Tasneem, Ahmed, Mpho, Teboho, Martina, Felix, Angus, Raeesa, Kealeboga Mokolobate, Mershen, Khadija, Nonhlanhla, Zamanzima, Jonathan and all other masters and PhD students for all your encouragement through my numerous setbacks, assistance in the lab and for the generous sharing of your vastly superior knowledge.

A special mention is required for the PhD students at WAADP, whose research work and publications have been a constant source of awe and motivation to me. To Pradeep Kumar, thank you for your enthusiasm and for all the effort and time you have put into my work. To Ndidi Ngwuluka, thank you for helping me see the bigger picture and for the numerous gems of advice you have shared. You are an inspiration to me of what a strong woman is. To Maluta Mufamadi, thank you for all the encouragement you have given me and for being my “role model”.

To Dr Valence Ndesendo, thank you for your assistance with my review publication and your words of wisdom.

To Safiyyah Laher, thank you for being a friend. Thank you for the cathartic laughter and thank you for helping me through this journey. May Allah (SWT) grant you everything of the best, always.

To Deshnee Naidoo and Natanya Moodley, who are not part of WADDP but without whose assistance this work would not have been possible. Thank you for your expertise and help.

To the lab, administrative and academic staff of the Dept of Pharmacy and Pharmacology: Mr Kleinbooi Mohlabi, Mr Bafana Themba, Mr Sello Ramarumo, Ms Nompumelelo Damane, Mrs Deanne Johnston, Mr David Bayever, Ms Shirona Naidoo, Prof Paul Danckwerts and others, thank you for your willingness to assist me whenever necessary, even when you didn't have to.

To Dr Leith Meyer and the staff of the CAS: thank you for all your time and assistance and for understanding my initial difficulty with handling the animals.

I am also appreciative to the following people who were kind enough to assist me despite having no obligation to do so: Mr Mathipa of the Dept of Surgery for his help with the flow cytometer and for staying late to clean the flow cytometer, Prof Sandy van Vuuren for her advice, the use of her laminar flow unit and autoclave as well as various

other pieces of equipment, Prof Robyn van Zyl for the use of equipment and Mr Chienteng Chen for his assistance with MTT assays. I am extremely grateful.

On a lighter note, I would also like to thank PhD comics (www.phdcomics.com). The comic strips gave me perspective and a sense of camaraderie. I was grateful to know that the suffering of post-graduate students is universal.

Funders that made this research possible were the National Research Foundation of South Africa, TATA Africa and the Department of Pharmacy and Pharmacology.

Finally, to every teacher I have had; formal teachers through primary and high school, religious school and university and informal ones that have taught me lessons of life, thank you for your contribution to my education.

The following quotes gave me strength during my masters and it seems fitting that they are mentioned here:

“Consider the bright morning hours and the night when it grows still and dark. Your Sustainer has not forsaken you, nor does he scorn you: for indeed the life to come will be better for you than this earlier part. And indeed, your Sustainer will grant you (what your heart desires) and you shall be most pleased.” - Al-Quraan (93:1-5)

“Let the beauty of what you love be what you do.” - Rumi

“The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood, who strives valiantly, who errs and comes up short again and again, because there is no effort without error or shortcoming, but who knows the great enthusiasms, the great devotions, who spends himself for a worthy cause; who at the best, knows in the end, the triumph of high achievement and who, at the worst, if he fails, at least he fails while daring greatly, so that his place shall never be with those cold and timid souls who knew neither victory nor defeat.” - Theodore Roosevelt

TABLE OF CONTENTS

CHAPTER 1	1
1.1. The Burden of Cancer.....	1
1.1.1. The Pathology of solid tumors, barriers to treatment and current therapy	1
1.1.2. Current methods proposed for overcoming barriers to effective solid tumor therapy	3
1.1.3. The cost of cancer.....	4
1.2. Rationale and Motivation for the Study	7
1.2.1. Rationale for the chemotherapeutic drug utilised in this study	10
1.3. Aims and Objectives of the Study	10
1.4. Novelty of the ISFI	11
1.5. Overview of this Dissertation	11
CHAPTER 2	13
2.1. Introduction	13
2.2. <i>In situ</i> Forming Implants	15
2.3. Implants Based on Natural Polymeric Sources	17
2.3.1. Chitosan	17
2.3.1.1. <i>In situ</i> forming implants	17
2.3.1.2. Pre-formed chitosan implants.	18
2.3.2. Alginate	19
2.3.2.1. <i>In situ</i> forming alginate implants	19
2.3.2.2. Pre-formed alginate implants	20
2.3.3. Gelatin.....	22
2.3.3.1. <i>In situ</i> forming gelatin implants	22
2.3.3.2. Pre-formed gelatin implants.	22
2.3.4. Albumin	23
2.3.4.1. <i>In situ</i> forming albumin implants	23
2.3.5. Collagen	24
2.3.5.1. <i>In situ</i> forming collagen implants.....	24
2.4. Synthetic, Biodegradable Mono-polymer and Co-polymers as Implant Sources	24

2.4.1. Poly(N-isopropylacrylamide) (pNIPAAm).....	24
2.4.2. <i>In situ</i> forming Atrigel® implants	25
2.4.3. <i>In situ</i> forming poly(organophosphazenes) implants	26
2.4.4. <i>In situ</i> forming poly(ε-caprolactone fumarate) implants	26
2.4.5. Polyethylene glycol (PEG)	26
2.4.5.1. <i>In situ</i> forming PEG implants	26
2.4.5.2. Pre-formed PEG implants	27
2.4.6. Pre-formed implants composed of poly(D,L-lactide-co-glycolide)	27
2.4.7. Pre-formed polyanhydride polymeric implants.....	28
2.5. Synthetic, biodegradable tri-block polymers used for implants.....	31
2.5.1. Tri-block polymer from poly(lactic-co-glycolide and polyethylene glycol (PLGA- PEG-PLGA).....	31
2.5.1.1. Poloxamers/Pluronics®.....	32
2.6. Synthetic non-biodegradable polymers	32
2.6.1. Silicone.....	32
2.7. Surgical pastes.....	33
2.8. Other implants	33
2.9. The ideal implantable system	34
2.10. Concluding Remarks	35
CHAPTER 3	36
3.1. Introduction	36
3.2. Materials and Methods	39
3.2.1. Materials.....	39
3.2.2. Preparation of PMVE solutions	40
3.2.2.1. Preparation of formulations to minimise the burst release of drug	40
3.2.3. Determination of the gelation temperature of the polymeric formulations utilising oscillatory rheology	42
3.2.3.1. Viscoelastic solids	42
3.2.3.2. Determination of the viscoelastic region	45
3.2.3.3. Determination of the gelation temperature of the polymer.....	45
3.2.4. Construction of a standard curve for the quantification of folic acid release from ISFI using ultraviolet spectroscopy	46
3.2.5. Release of folic acid from the formulations under conditions mimicking those at the tumor site	46

3.3. Results and Discussion.....	47
3.3.1. Construction of a standard curve for the quantification of folic acid released from the ISFI.....	47
3.3.2. Determination of the viscoelastic region of PMVE and gelation temperatures.....	48
3.3.3. The effect of increasing polymer concentration on the release of folic acid from the ISFI.....	50
3.3.3.1. The effect of carrageenan, gum arabic, PAAm and other polymers on the release profile of the ISFI formulation	51
3.3.3.2. The effect of gum arabic and CaCl ₂ on drug release from the ISFI formulation.....	53
3.3.3.3. The effect of CaCl ₂ on the release profile of the ISFI formulation and on the gelation temperatures of the ISFI.....	54
3.4. Concluding Remarks	57
CHAPTER 4	59
4.1. Introduction	59
4.2. Materials and Methods	61
4.2.1. Materials.....	61
4.2.2. Design and optimization of the formulations using a Central Composite Design	61
4.2.3. Preparation of <i>in situ</i> forming implant (ISFI) solutions	62
4.2.4. Determination of the viscoelastic behavior and the gelation temperature utilizing oscillatory rheology	63
4.2.5. Determination of the force of injectability of the ISFI	64
4.2.6. Determination of drug release from the ISFI at a simulated tumor site	64
4.2.7. Computational modelling to determine the effect of the addition of a salt to PMVE	65
4.2.8. Determination of an effective method of sterilization for the ISFI	65
4.2.8.1. Materials and methods.....	66
4.2.8.2. Preparation of formulations.....	66
4.2.8.3. Determination of polymeric structural variations.....	66
4.2.8.4. Determination of the gelation temperature of the ISFI.....	67
4.2.8.5. Determination of the release of folic acid from the formulation	67
4.2.8.6. Determination of the sterility of the formulations.....	67
4.2.9. The Effect of Different Temperatures on the Release Profile of the Implant	68
4.2.9.1. Materials and Methods.....	68
4.2.9.2. Determination of the release of drug from ISFI formulations under different temperature conditions.....	68
4.3. Results and Discussion.....	69

4.3.1. Analysis of the Face-Centred Central Composite Design	69
4.3.1.1. Analysis of Variance (ANOVA)	71
4.3.1.2. Examination of the residual error in the design	72
4.3.2. The use of oscillatory rheology in the determination of the thermal gelation temperatures of the ISFI formulations.....	74
4.3.3. Determination of the force of injectability: The force required to inject the ISFI formulations.....	76
4.3.4. Release of folic acid from the <i>ISFIs</i> over 32 days	78
4.3.5. Computational modeling of the method of gelation and the effect of the addition of CaCl ₂	80
4.4. Optimization of ISFI Formulation Responses	82
4.4.1. The release of MTX from the optimized formulation.....	83
4.4.2. Further rheological characterisation of the optimized ISFI.	85
4.4.3. Modelling of the release of drug from the implant.....	86
4.4.4. The effect of autoclaving on the properties of the ISFI	87
4.4.4.1. Vibrational transition analysis	87
4.4.4.2. Rheological determination of the gelation temperature of the autoclaved formulation	88
4.4.4.3. Release of folic acid from autoclaved formulations	88
4.4.4.4. Sterility of the formulations by examination of agar plates	89
4.4.5. The effect of different temperatures on the release profile of folic acid from ISFI formulations.....	89
4.5. Concluding Remarks	91
CHAPTER 5	92
5.1. Introduction	92
5.2. Materials and Methods	93
5.2.1. Materials.....	93
5.2.2. Preliminary investigations into the formulation of microparticles	93
5.2.2.1. Preparation of microparticles	93
5.2.3. Statistical optimization of the microparticles	94
5.2.3.1. Design and optimization of the formulations using a central composite design	94
5.2.4. Determination of drug entrapment and microparticle yield	96
5.2.5. Determination of the release of drug from the microparticles at various pHs	96
5.2.6. Examination of the morphology of the particles.....	97
5.2.7. Determination of the zeta potential of the optimised particles	97
5.3. Results and Discussion.....	97

5.3.1. Calibration curve of folic acid conducted to ascertain concentrations of folic acid entrapped in particles	97
5.3.2. Drug entrapment efficacy of preformulation microparticle formulations	98
5.3.3. Regression equations and examination of the FCCCD	99
5.3.3.1. Residual error in the design	100
5.3.4. Entrapment of drug within the design microparticles	102
5.3.5. Release of folic acid from the optimised microparticle formulation	103
5.3.6. Determination of the morphology of the microparticles using SEM	106
5.4. Response optimization.....	107
5.4.1. Loading of the optimised microparticle formulation with methotrexate	107
5.4.1.1. Determination of the drug entrapment efficacy of the microparticle formulation	108
5.4.1.2. Release of methotrexate from optimized microparticle formulations	108
5.4.1.3. Determination of the zeta potential of the particles.....	109
5.5. Investigation of the effect of microparticles in the ISFI	110
5.5.1. Determination of the release of MTX from the combination of MTX-loaded particles and the ISFI	110
5.5.2. Rheological investigation of the formulation	112
5.6. Investigation of the effect of bicarbonate loaded microparticles	112
5.6.1. Preparation of the formulation.....	112
5.6.2. Determination of the effect of bicarbonate particles on the release of drug entrapped within the implant	113
5.7. Concluding Remarks	114
CHAPTER 6	115
6.1. Introduction	115
6.2. Materials and Methods	118
6.2.1. Materials.....	118
6.2.2. Methods	119
6.2.2.1. Aseptic technique.....	119
6.2.2.2. Culturing and counting of cells.....	119
6.2.2.3. Cell counting using the trypan blue exclusion assay and a haemocytometer	120
6.2.2.4. The inclusion of controls into test runs.....	120
6.2.2.5. MTT assay	120
6.2.2.6. Determination of the effects of the drug release elutes and microparticles on HT29 cells, MCF7 cells and DLD1 cells utilising flow cytometry	123

6.3. Results and Discussion.....	124
6.3.1. The effects of methotrexate, polymer and microparticles on HT29 cells using the MTT assay	124
6.3.2. The effect of drug elutes, placebo elutes and microparticles on HT29, MCF7 and DLD1 cells determined using flow cytometry	127
6.4. Conclusion.....	129
 CHAPTER 7	 130
7.1. Introduction	130
7.1.1. Biocompatibility of implanted materials.....	130
 7.2. Materials and Methods	 131
7.2.1. Materials.....	131
7.2.2. Receipt, marking and treatment of animals	132
7.2.3. Biocompatibility studies in rats	132
7.2.4. <i>In vivo</i> studies on the rat model	132
7.2.4.1. Sterile preparation of formulations	133
7.2.4.2. Determination of thermally responsive drug release from the implant into the bloodstream and monitoring of body temperature of animals: pilot study	133
7.2.4.3. Determination of drug release from the ISFI over 10 days and the effect of the application of ice on the release of MTX from the ISFI	134
7.2.5. Evaluation of the toxicity of the drug <i>in vivo</i>	138
7.2.5.1. Weighing of animals and cage activity.....	138
7.2.5.2. Analysis of blood obtained from treated animals	138
7.2.6. Visualisation of the ISFI utilising ultrasound imaging.....	139
7.2.7. Utilization of Ultra Performance Liquid Chromatography in the determination of the methotrexate concentration in blood and tissue samples obtained from rats ..	139
7.2.7.1. UPLC analysis.....	141
7.2.7.2. Preparation of weak and strong washes and mobile phases	142
7.2.7.3. Drug content in the blood	143
7.2.7.4. Determination of the residual drug in ISFIs	145
7.2.7.5. Determination of the drug content in surrounding tissue	146
 7.3. Results and Discussion.....	 147
7.3.1. Biocompatibility of the ISFI in the rat model	147
7.3.1.1. Implantation site and tissue necrosis	147
7.3.1.2. Tissue mineralization	148
7.3.1.3. Granulomatous inflammation and fibroplasia	148
7.3.1.4. Focal skin ulceration	148

7.3.2. <i>In vivo</i> testing of the ISFI	149
7.3.2.1. Sterile preparation of ISFI	149
7.3.2.2. Construction of a calibration curve in order to quantify the amount of methotrexate in blood and tissue samples and precision and accuracy of the UPLC method.....	150
7.3.2.3. Observations and findings of the pilot study	151
7.3.2.4. Main study	153
7.4. Concluding Remarks	161
CHAPTER 8	162
8.1. Conclusions.....	162
8.2. Recommendations	163
REFERENCES	164
APPENDICES	183

LIST OF FIGURES

	Page
Figure 1.1: Simplified schematic showing the barriers to effective therapy of solid tumors (venous blood drainage not shown).	2
Figure 1.2: Schematic showing 4 factors affecting the costs associated with intravenous chemotherapy.	6
Figure 1.3: Proposed mechanism of the ISFI formation and drug release when loaded with microparticles	9
Figure 2.1: Schematic depicting some of the mechanisms of formation of implants <i>in situ</i> .	15
Figure 2.2: Schematic showing drug loading and release of the drugs from the modified alginate hydrogel (Adapted from data in Bouhadir et al., 2001).	21
Figure 2.3: Schematic depicting a longitudinal section through the disc shaped implant with the loading and release patterns of two drugs clearly illustrated (Adapted from data in Göpferich,1997).	29
Figure 2.4: Schematic depicting a cross-section through the implant (Adapted from data in Vogelhuber et al., 2001).	30
Figure 3.1: Digital images showing the transition of PMVE from a) a solution to b) a gel following heating to 37°C.	37
Figure 3.2: Chemical structure of PMVE showing the hydrophilic oxygen that is responsible for the solvation of the polymer at room temperature.	38
Figure 3.3: Schematic showing a model to aid in the understanding of viscoelastic materials (Adapted from Marriott, 2004).	42
Figure 3.4: Phase shifts in different materials following application of a sinusoidal strain (Adapted from Wyss et al. 2007).	44
Figure 3.5: a) Schematic showing the drug release method used to determine the release of folic acid from the ISFI, b) digital image showing the actual drug release vessel with the suspended implant within the dialysis tubing clearly shown.	47
Figure 3.6: Calibration curve of the absorbance of folic acid in PBS (pH 6.75) using UV spectroscopy at 280nm (in all cases n=3 and SD<0.025).	48
Figure 3.7: Rheological plot of deformation vs. stress showing the yield stress of PMVE.	49
Figure 3.8: Stress sweeps obtained for PMVE at various frequencies.	49
Figure 3.9: Gelation temperatures of different concentrations of PMVE.	50
Figure 3.10: Drug release profiles of formulations with increasing amounts of PMVE (n=2, SD< 0.09).	51

Figure 3.11: a) Drug release profiles obtained from the carrageenan containing ISFI formulations (n=2, SD< 0.1) b) The effect of gum arabic on the drug release profile of PMVE (25 hour profile). (37°C, pH 6.75, n=2, SD< 0.069).	53
Figure 3.12: PMVE-gum arabic and CaCl ₂ (0.1M) compared to a combination of PMVE-CaCl ₂ (0.1M) on the drug release profile (37.5°C, pH 6.75). (37°C, pH 6.75, n=2, SD< 0.04).	54
Figure 3.13: The effect of increasing the quantity of CaCl ₂ on the release of drug from ISFI formulations (37°C, pH 6.75, n=2, SD< 0.051).	55
Figure 3.14: The effect of CaCl ₂ on PMVE with regards to gelation temperature.	56
Figure 3.15: Release of folic acid from the sodium bicarbonate containing implant (37°C, pH 6.75, n=2, SD<0.5).	57
Figure 4.1: Some advantages of utilizing a design of experiments in the development of an optimal formulation.	60
Figure 4.2: Diagrammatic representation of a Face-Centred Central Composite Design with two factors (X ₁ and X ₂) showing the centre point, 4 factorial points and four axial points (Adapted from Verseput, 2000).	61
Figure 4.3: Textural analysis instrument set-up used to determine Force of Injectability of the ISFI through syringe fitted with a 18G needle.	64
Figure 4.4: Schematic representation of agar plate and the streaking of the autoclaved formulation.	68
Figure 4.5: Figure showing the method used for drug release during experimental determination of the effect of ice on the implant.	69
Figure 4.6: Residual plots of the data for gelation temperature, force of injectability and MDT for the ISFI formulation.	73
Figure 4.7: Typical profile obtained during rheological testing of samples. The black circle demarcates the crossover between the storage modulus (G') and the loss modulus (G'') and hence the value that was used as the gelation temperature.	74
Figure 4.8: The effect of CaCl ₂ on the gelation temperature of formulations (n=3, SD< 0.35).	75
Figure 4.9: a) Bar chart for gelation temperature showing the experimental versus the predicted values (R ² =0.9971) and b) Response surface plot correlating the Gelation temperature with the concentration of PMVE and CaCl ₂ present in the formulation.	76
Figure 4.10: Typical profile obtained for a formulation filled syringe versus that of an air-filled syringe.	77
Figure 4.11: a) Graph showing the experimental values obtained for force of injectability compared to the predicted or "fitted" values (R ² =0.9164) and b) Response surface plot correlating the force of injectability with the concentration of PMVE and CaCl ₂ present in the formulation.	78

Figure 4.12: Drug release profiles from formulations over a period of 40 days where a) represents formulations 1-5 b) represents formulations 6-10 and c) represents formulations 11-14 (37°C, pH 6.75, n= 3, SD< 0.1).	79
Figure 4.13: Response surface plot showing the relationship between MDT and the PMVE and CaCl ₂ concentration in the ISFI formulations.	80
Figure 4.14: Computational modeling showing a) The PMVE Chain with R-representing chain elongation on both ends, the R is represented by large balls b) Water associated PMVE chains in the aqueous medium, only part shown, chain elongation R is shown in olive green. The -OCH ₃ are in water association, red dots represent water molecules and oxygen atoms in the polymer chain as well. In c) CaCl ₂ (the slate-green balls) in the medium, with water and PMVE chain as associated with water molecules, and around the polymer chain. Figure denotes size relationship of the salt with the polymer chain atoms (and not chain length and size). d) CaCl ₂ in association with water molecules which are itself in association with the PMVE polymer methoxyl's oxygen and hereby giving a compacted matrix where in all the entities are in close proximity as well as in the limits of inter-atomic distances required for the physicochemical interaction. The salt, CaCl ₂ is also in layered state between the two polymer strands and could not be inter-wined/embed into the polymer chain itself.	82
Figure 4.15: a) Calibration curve obtained for MTX and b) drug release profile from the optimized formulation containing MTX over a period of 50 days. (pH=6.75, n=3, SD< 0.024).	84
Figure 4.16: Rheological profile of the optimized ISFI at different stress values.	85
Figure 4.17: FTIR spectra comparing the autoclaved and unautoclaved formulations.	87
Figure 4.18: Rheological determination of gelation showed no apparent change between autoclaved and un-autoclaved formulations.	88
Figure 4.19: Release profiles showing the release of folic acid from autoclaved and un-autoclaved formulations (n=3, SD<0.067).	89
Figure 4.20: a) The effect of increased temperature on the release profile of folic acid from the ISFI over 10 days (pH 6.75, n=2, SD< 0.039) and b) the effect of ice application on the release profile from the ISFI over 10 days (pH 6.75, n=2, SD< 0.06).	90
Figure 5.1: Chemical structure of a) chitosan and b) poly(methacrylic acid-co-methyl methacrylate).	93
Figure 5.2: Calibration curve of folic acid in NaOH	98
Figure 5.3: Residual plots obtained for the responses examined in the design.	101
Figure 5.4: a) Bar graph showing the DEE: experimental and predicted values and b) response surface plot showing the relationship between the polymer concentration and DEE.	102

Figure 5.5: a) Calibration curve of folic acid in PBS, pH 7.4 and b) typical release profile obtained from a microparticle formulation.	104
Figure 5.6: Response surface plots generated by the FCCCD analyzing the effect of CHT/PMMA concentrations and sonication time on the responses a) mean dissolution time at pH 6.75 and b) mean dissolution time at pH 7.4.	106
Figure 5.7: Electron micrographs showing the spindle like morphology of the microparticles.	107
Figure 5.8: Calibration curve of MTX in hydrochloric acid.	108
Figure 5.9: Release of MTX from the optimized formulation at three different pH values (37°C, n=3, SD<0.03).	109
Figure 5.10: Zeta potential distribution obtained for the optimized formulation.	110
Figure 5.11: Release of MTX from drug loaded particles in the ISFI (37°C, pH 6.75, n=2, SD< 0.04)	111
Figure 5.12: Rheological profile of the optimized implant containing MTX loaded particles.	112
Figure 5.13: Release of folic acid from the ISFI loaded with sodium bicarbonate particles (37°C, pH 6.75, n=2, SD< 0.09).	113
Figure 6.1: Yellow MTT is reduced to purple formazan crystals in the mitochondria of living cells.	116
Figure 6.2: Schematic showing the principle of hydrodynamic focusing utilised by the flow cytometer within the flow cell to allow the flow of single cells past the detector.	117
Figure 6.3: Figure showing a simplification of the fluidics and optic systems of the FACSCalibur (BD Biosciences, New Jersey, USA), the flow cytometer used in this study (adapted from: http://www.bdbiosciences.com/support/training/index.jsp).	118
Figure 6.4: MTT calibration curve obtained for HT29 cells	121
Figure 6.5: Bar graph illustrating the reduction in viability of the HT29 cells when treated with the drug release elutes of the optimized ISFI	125
Figure 6.6: Digital image showing an MTT plate following addition of DMSO as the solubilising agent for formazan crystals. Note the highlighted area indicating the presence of undispersable polymer deposits and the final four wells showing no deposit.	126
Figure 6.7: Plot showing the decrease in the viability of the cells following treatment with the particles versus treatment with the elutes prepared (n=3, SD< 0.1).	127
Figure 6.8: a) Typical dot plot obtained showing the viable cells (region R2), and cells which have taken up the 7AAD stain (region R4) b) counting beads are shown in region R3 and c) toxicity of elutes on HT29, MCF7 and DLD1 cells.	128

Figure 7.1 : Flow diagram showing overall design of <i>in vivo</i> studies (*Sampling procedure is elaborated on in Figure 7.2).	135
Figure 7.2: Flow diagram depicting the treatment each of the experimental groups (i.e. Both Group1 and Group 2) were to undergo.	136
Figure 7.3: a) Sketch showing the proposed area of implantation of the ISFI in the flank of the animal b) Digital image showing the actual site of implantation and c) digital image showing the injection of the ISFI into the flank of the rat.	137
Figure 7.4: PDA plot showing the absorbance of MTX (retention time= 1.5mins) and PYZ (retention time= 1.1.mins).	142
Figure 7.5: Digital images showing the resected tissue surrounding the implant with a) showing the implant <i>in situ</i> as indicated by the black arrow and b) shows the resected implant with some of the implant still remaining as indicated by the black arrow. This area was then scraped using a scalpel to collect the remaining implant.	146
Figure 7.6: Digital image showing the resected implant with surrounding tissue.	147
Figure 7.7: Histopathological analysis of local tissue surrounding the area of implantation of the implant in a) non-treated animal b) after 2 days of implantation c) after 6 days of implantation and d) after 10 days of implantation.	149
Figure 7.8: Typical chromatogram obtained for methotrexate (retention time=1.408min) and internal standard, pyrazinamide (retention time=0.932min)	150
Figure 7.9: Calibration curve obtained of Methotrexate in blank plasma	151
Figure 7.10: MTX levels in the blood of the pilot study animals clearly showing the peak of drug at 1 hour following the application of ice to the area	152
Figure 7.11: Flow chart demonstrating the numbering of the experimental animals.	153
Figure 7.12: Blood methotrexate concentrations in baseline animals (SD< 0.034)	155
Figure 7.13: The effect of application of ice to the region of implantation (SD<0.06)	157
Figure 7.14: a) Calibration curve of MTX in PBS b) calibration of MTX in methanol c) remaining drug in the implant over 4 or 10 days and after the application of ice to the region d) drug in the surrounding tissue after 4 or 10 days and after the application of ice e) weight loss in rats carrying the MTX loaded implants for 10 days and f) weight loss in in placebo receiving rats.	159
Figure 7.15: Ultrasound imaging showing the absence of the implant in a) placebo rat and the presence of the ISFI in b)	160
Figure 7.16: a) Image obtained using 2 dimensional ultrasound b) three-dimensional image obtained from scan of implant area showing the medial and sagittal planes and c) and d) three-dimensional plotting of the area of the implant. Note the tapering of the implant indicating the point of withdrawal of the needle from the flank leaving a “trail” of the implant.	161

LIST OF TABLES

	Page
Table 2.1: Currently available implant systems for the treatment of cancer.	14
Table 2.2: Drug, type of tumor and the polymers employed for <i>in situ</i> forming implants.	17
Table 2.3: Comparison of the advantages and disadvantages of <i>in situ</i> forming implants.	35
Table 3.1: Composition of ISFI formulations formulated for investigation	41
Table 4.1: Formulation variables and responses applied in design.	62
Table 4.2: Compositions of ISFI solutions formulated in accordance with FCCCD design template.	63
Table 4.3: Table summarizing the standard error of the model (S) and the coefficient of determination (R^2) values obtained for the regression equations.	70
Table 4.4: Probabilities of the effects of the variables on the outcomes.	71
Table 4.5: Analysis of variance for regression and residual errors.	71
Table 4.6: Summary of the obtained values for each of the responses and the predicted values and desirability.	83
Table 4.7: Value of R^2 for different models of mechanisms of drug release	86
Table 5.1: Investigation of variables for the formation of microparticles	94
Table 5.2: Table listing formulations as well as the drug entrapment efficacy obtained for each formulation	98
Table 5.3: Formulation variables and responses applied in design	95
Table 5.4: Composition of microparticle formulations in accordance with the FCCCD	95
Table 5.5: MDT values obtained for the formulation generated by the FCCCD	105
Table 5.6: Values obtained for each response as well as the model predicted values and the desirability.	107
Table 6.1: Summary of the controls used in the study.	120
Table 6.2: Responses to a foreign body that may occur (Taken from Williams, 2008)	131
Table 6.3: UPLC method for the elution of methotrexate and internal standard pyrazinamide.	142
Table 6.4: Inter- and intra- day precision and accuracy of the method were determined (only one set of interday data is shown).	151
Table 6.5: Table showing the effect of the application of ice on the rats on the local and systemic temperatures.	152

LIST OF EQUATIONS

	Page
Equation 3.1	40
Equation 3.2	43
Equation 3.3	43
Equation 3.4	45
Equation 3.5	45
Equation 3.6	45
Equation 3.7	47
Equation 4.1	65
Equation 4.2	70
Equation 4.3	70
Equation 4.4	70
Equation 5.1	96
Equation 5.2	99
Equation 5.3	99
Equation 5.4	100
Equation 6.1	123
Equation 6.2	143

CHAPTER 1

INTRODUCTION

1.1. The Burden of Cancer

Cancer is the broad classification for a number of diseases characterised by the uncontrollable growth and spread of cells. It is a leading cause of death worldwide and it is estimated that deaths from cancer will continue to increase and cancer will be responsible for 7.9 million deaths by 2030 (Globocan, 2008, IARC, 2010). Cancer is estimated to be the cause of 1 in 8 deaths worldwide, more than HIV/AIDS, malaria and tuberculosis combined (American Cancer Society, 2010). Of all the deaths estimated to be caused by cancer in the United States of America for 2010, solid tumors account for approximately 90% (American Cancer Society, 2010).

1.1.1. The Pathology of solid tumors, barriers to treatment and current therapy

Solid tumors are characterised by masses of abnormal cells often in sheets or nests surrounded by supporting stroma. This stroma contains connective tissue cells and other cells but is composed mainly of blood vessels (Murray and Carmichael, 1995). Solid tumors develop their own blood supply and it has been reported that a tumor cannot grow more than 1-2mm in diameter without causing angiogenesis (Folkman, 1990). A highly perfused tissue would imply excellent drug delivery to the area. However, the problem lies in the structure of the vasculature. Angiogenesis is often erratic in solid tumors and tumors often consist of a well perfused outer region and a poorly perfused necrotic centre. Hence, systemically administered drugs may reach the exterior layers but rarely reach the central area (Murray and Carmichael, 1995). This poor perfusion is mainly due to the low movement of drugs from the blood vessels into the stroma of the tumor and hence to the cancerous cells. Although the endothelium of the blood vessels in the area of the tumor does appear to “leak” more than normal cells due to large spaces between the endothelial cells, abnormally formed basement membrane and fenestration (Munn, 2003), drugs are not taken up well into the stroma. This is thought to be due to the high interstitial hydrostatic pressure in the stroma or a diminished vascular pressure in the tumor region (Makrilia et al., 2009). The problem of erratic vasculature architecture is compounded by variable blood flow rates in the tumor region due in part to variable vessel sizes as shown in Figure 1.1. As Figure 1.1 also shows, blood flow may be high in some regions and low in others and

this pattern is not constant and can vary in minutes to hours (Patan et al., 1996). Further, Munn (2003) proposes that much of the systemically delivered drug which does diffuse into the stroma is shunted through the tumor and is reabsorbed by other blood vessels or enters the lymphatics of the tumor which are found mostly on the periphery of the tumor. Hence very little of the systemically delivered drug is actually delivered to the cells.

Another barrier to treatment is the 3-dimensional structure of the solid tumors, in that drug penetration into solid tumors is 5-10 fold less when compared to penetration into a single layer of cells (Au et al., 2001).

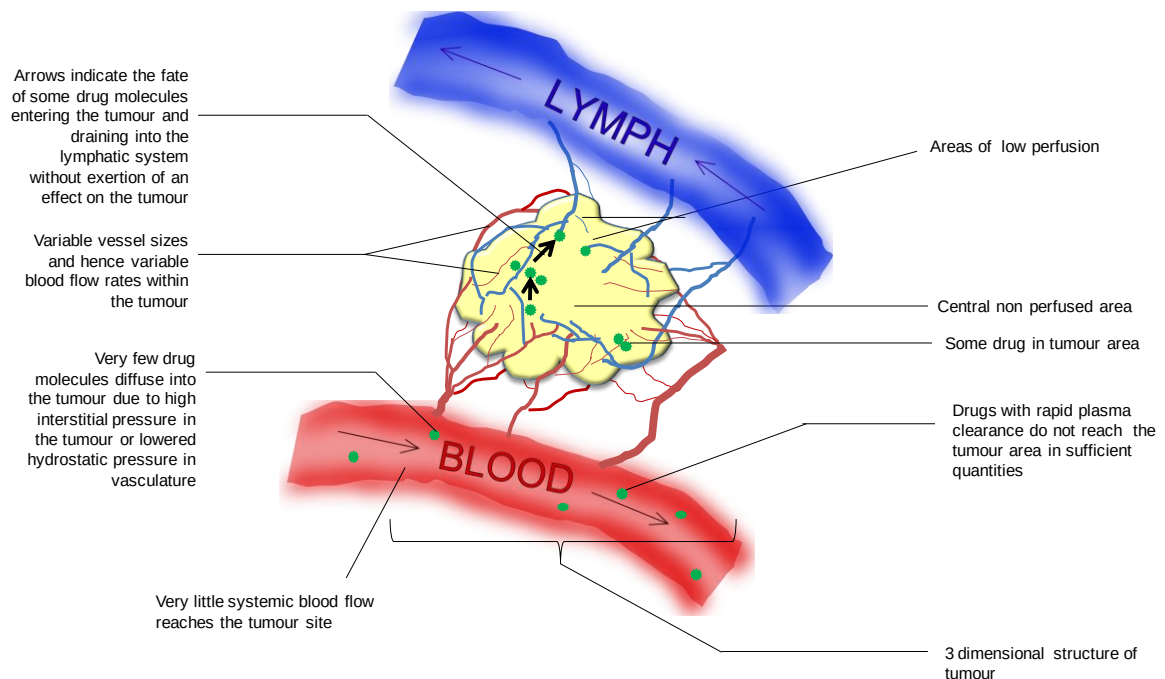


Figure 1.1: Simplified schematic showing the barriers to effective therapy of solid tumors (venous blood drainage not shown).

Current therapy of tumors involves surgical resection, the delivery of drugs mostly via the intravenous route (Wong et al., 2007) and the use of adjunctive radiotherapy. Oral therapy is often used following surgical resection or in hormone sensitive tumors. However, as discussed, oral and even intravenous drug delivery cannot successfully treat the majority of solid tumors. In order to overcome the problem of delivery of drugs to solid tumors as highlighted, high doses of drug are given to patients via the

intravenous route. However, this worsens the problem as only a small portion of systemic blood flow reaches the tumor, and hence only a small fraction of the delivered dose is available at the site of the tumor (Weinberg et al., 2007). Instead most of the drug reaches healthy tissue and exerts its action resulting in numerous side effects. Hence the host (i.e. the patient) tolerability to the high doses of chemotherapy that are often used must also be considered (Gaze et al., 1995 and Schiller, 2008). Further, some drugs pose an additional barrier as they have rapid plasma clearance and as a result short exposure times to the tumor (Weinberg et al., 2007).

1.1.2. Current methods proposed for overcoming barriers to effective solid tumor therapy

One solution to the problems associated with the delivery of drugs to solid tumors is the development of novel drugs or other therapeutics which only target cancer cells. However, the time taken for a novel compound from development stage to reach the patient is a long one and the expense of such development is considerable (Adams and Brantner, 2010). Another simpler solution to the treatment of solid tumors is to find ways to deliver existing drugs such that the current problems faced are overcome. Hence focus has shifted to more targeted drug delivery systems (Hughes, 2005). Numerous methods of delivering drugs to solid tumors have been proposed and this includes the use of implants at the site of the tumor (Seong et al., 2003; Ruel-Gariépy et al., 2004 and Han et al., 2008).

Carmustine loaded poly-lactic-co-glycolic-acid (PLGA) microparticles in an implantable wafer have shown exceptional results in the treatment of malignant glioma, a solid tumor of the brain (Seong et al., 2003). An implant of paclitaxel intended for injection at the tumor site has also been developed and shown to prolong the life of the tumor-bearing rats considerably (Ruel-Gariépy et al, 2004). Han and co-workers (2008) synthesised a chitosan hydrogel which combined an anti-cancer drug (chemotherapy) and a vaccinia virus vaccine (immunotherapy). This device prolonged the lives of tumor bearing rats by approximately 60 days. An implanted drug delivery system at the site of the tumor offers solutions at many levels. The drug is not as systemically distributed as an intravenously administered drug and subsequently a lower dose can be administered and the chances of systemic side-effects are significantly reduced. Further some of the diffusion barriers are reduced as the drug is now at high

concentrations at the site of the tumor and hence would be able to diffuse more rapidly and penetrate the solid tumor effectively. Further, the implant can be formulated to release drug over a number of weeks or even months decreasing the need for the patient to return to the hospital for systemic treatment, or eliminating the need to take medication daily and hence improving patient acceptability and compliance (Vassileva et al., 2007 and Pulsipher et al., 2009).

Drug delivery systems which are micro- or nano- enabled have also been designed for the treatment of solid tumors. Microparticles such as those formulated by Lin and co-workers (2005), consisted of PLGA microparticles loaded with doxorubicin, a potent anti-cancer drug. PLGA was also utilized to formulate paclitaxel loaded microparticles for the treatment of brain cancer by Elkharraz et al. (2006). As mentioned previously, PLGA microparticles were also formulated by Seong et al. (2003) and incorporated into wafers for the treatment of malignant glioma. In terms of nanoparticles, Cegnar and co-workers (2004) have used poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing a novel chemotherapeutic, cystatin and successfully showed the efficacy of nanoparticulate drug delivery to tumors. The advantages of nano-enabled devices are numerous and include the ability of these small particles to penetrate biological barriers (Hughes, 2005), and to be retained at the tumor site as per the enhanced permeation and retention effect (EPR) (Matsumura and Maeda, 1986). However, the problem with particles on the micro and nano level is the difficulties experienced in the scale up process and ultimate wide spread use.

These systems are by no means the entirety of the research that has been done on solid tumor treatment. Chapter 2 of this dissertation expands on the numerous other systems that have been researched.

1.1.3. The cost of cancer

The costs of cancer are considerable. It is estimated by the US National Institute of Health that the overall cost of cancer in 2010 is \$263.8 billion: \$102.8 billion for direct medical costs, \$20.9 billion for indirect morbidity costs such as the lost of productivity due to illness and \$140.1 billion for indirect mortality costs such as lost of productivity

due to premature death (American Cancer Society, 2010). While exact South African statistics are not available, a similar pattern is expected.

Considering medical costs and intravenous delivery of drugs, the following must be considered. Chemotherapeutics, especially newer modalities, are costly to manufacture and a further problem with intravenously delivered drugs is the costs involved: a pharmacist is often required to extemporaneously prepare the intravenous dose and nurses are required for administration. In South Africa, where public health care provides for many patients with various disease states, who cannot afford medical insurance, the healthcare system is overburdened. This may further complicate effective treatment of solid tumors as often patients may not be able to be treated appropriately due to lack of resources. It should also be noted that older drugs often have greater side-effect profiles. A study of drug sales in 25 countries was undertaken by Jönsson and Wilking, (2007) with 19 European countries and 6 countries outside of Europe including South Africa. The study showed the slow uptake of new drugs in South Africa (Jönsson and Wilking, 2007).

Chemotherapeutics are often given weekly to patients and a factor cited by American patients (American Cancer Society, 2010) is the transport costs. In South Africa, the situation is expected to be the same with a further complication of rural living. Patients in rural areas must be expected to travel to urban areas to receive treatment at secondary or tertiary healthcare institutions. Naturally this provides a significant challenge. Patients failing to come in to the hospital run the risk of complications such as resistance of cancer cells to therapy thought to be influenced by subtherapeutic levels of drug (Weinberg et al., 2007).



Figure 1.2: Schematic showing 4 factors affecting the costs associated with intravenous chemotherapy.

Another South African situation is that the overburdened health care system and the exorbitant cost associated with private health care means that some patients may not be diagnosed until later stages of cancer and treatment at that stage may be more extensive and costly. Considering the burden of other diseases in SA such as HIV, tuberculosis and malaria, cancer is neglected compared to these infectious diseases.

Lastly, considering the side effects associated with cancer chemotherapy, treatment of these may add to costs as well. Complications such as neutropenic pneumonia which may arise in patients receiving chemotherapy must also be treated. In cases of severe neutropenia or anaemia or other complications, patients may require hospitalisation which has even more costs associated with it and may be associated with further complications such as nosocomial infections. Factors affecting the cost of chemotherapy are schematically represented in Figure 1.2.

1.2. Rationale and Motivation for the Study

Considering the challenges facing drug delivery to solid tumors such as poor blood flow and perfusion as outlined above as well as the costs involved in the treatment of solid tumors, the development of an effective method of drug delivery will prove beneficial.

This study aimed to develop a novel stimuli responsive biodegradable *In Situ* Forming Implant (hereafter referred to as the ISFI). As mentioned previously, tumor sites are unique in their biochemistry and hence the microenvironment of a solid tumor varies from the environment surrounding healthy cells. Two of the microenvironmental characteristics selected for study in this dissertation were temperature and pH. The pH of interstitial fluid surrounding a tumor is approximately 6.75 which differs significantly from the interstitial fluid of normal body cells of approximately 7.23 (Martin and Jain, 1994). An *in situ* forming implant would overcome the problems of poor systemic drug delivery and the reduction in side effects and complications would reduce costs.

The ISFI was to be injected at the site of the tumor and would form an implant in this position due to its response to an inherent stimulus present at the tumor site. The ISFI was intended to treat solid tumors in general, by using colon cancer as a model for solid tumors.

The ISFI as described in this study would exist as a sol at room temperature. Upon administration via an injection through an 18G needle to the site of the tumor, the sol would interconvert to a gel-state. The ISFI was hence expected to be responsive to temperature of the body. The ISFI would be injected into the tissue using conventional means to prevent further costs. The drug would be dispersed in the formulation and release from the implant would occur slowly, ideally over a period exceeding one month. The delivery over a month would reduce visits to the hospital hence decreasing costs.

Should the ISFI release the drug too quickly, the use of particles (either micro- or nano-meters in size), as described in Figure 1.3 in the interests of improving the ISFI, was to be considered. These particles were to be composed of a stimuli-responsive polymer

and were to serve to improve the release of drug from the implant. Firstly the particles would contain the drug and the response of the polymer, that forms the micro- or nano-particle, to the stimulus in the tumor environment would result in the release of the drug. Alternatively the particles would contain a substance, experimentally determined, hereafter referred to as the erosion controlling substance (ECS), which would influence the erosion of the polymer forming the hydrogel matrix by either increasing or decreasing the rate of erosion of the hydrogel. The stimulus for the particles was envisaged to be pH, and the micro- or nano- particles composed of a pH responsive polymer would respond to the pH at the tumor and release either the drug or the ECS (contained in the particles).

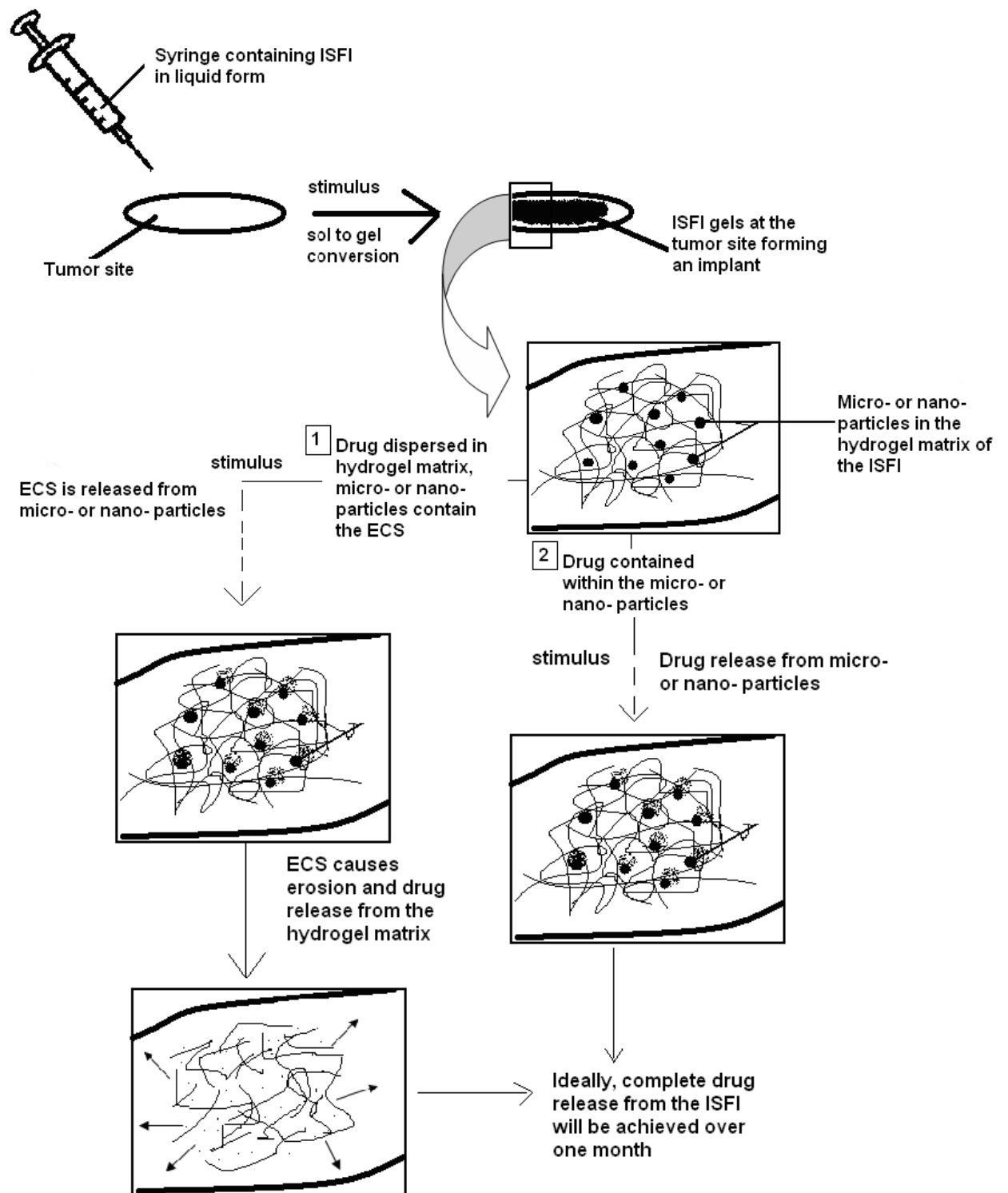


Figure 1.3: Proposed mechanism of the ISFI formation and drug release when loaded with microparticles

1.2.1. Rationale for the chemotherapeutic drug utilised in this study

Folic acid acts as a coenzyme for a number of biochemical reactions *in vivo*. Dihydrofolate reductase is an enzyme requiring the presence of folic acid especially in the de novo synthesis of thymidylic acid and purine nucleotides and hence is important in the division of cells. Methotrexate inhibits the activity of this enzyme due to its structural similarity to folic acid (Chu and Sartorelli, 2004). As cancer cells are actively dividing, the drug inhibits the rapid division of the cells, leading to a decrease in the size of the tumor. It is widely used in the treatment of neoplastic diseases especially in combination treatment regimens (Avendaño and Menéndez, 2008). Due to the high cost of chemotherapeutic drugs, methotrexate provides the ideal model due to its similarity to folic acid. Folic acid is a much cheaper and more readily available drug and as a result the majority of preliminary work conducted in this study was with folic acid. Methotrexate was only used in the optimized formulation and during *in vitro* cell line work and *in vivo* animal work conducted.

1.3. Aims and Objectives of the Study

The aim of this study was to formulate an ISFI for the treatment of solid tumors using colon cancer as a suitable model. Polymers used in the formulation of this implant would be stimuli-responsive and respond to specific stimuli around tumor site. The ISFI would be injected at the site of the tumor and form an implant *in vivo*. In this way the ISFI, containing a suitable drug, would release drug in a controlled manner- either from the micro- or nano- particles or directly from the implant at the site of the cancer lesion. In order to achieve this, the following objectives needed to be achieved:

1. To select a suitable stimuli-responsive polymer in order to formulate an implant that will form *in situ* and to modify these polymers with the addition of excipients as necessary.
2. To develop an implant that could release the model drug, folic acid over a period exceeding one month.
3. To optimise the ISFI formulation using a Design of Experiments approach.
4. To investigate the possibilities of a micro-enabled system. The microparticles would contain the drug or would serve as a way to enhance the biodegradation of the polymer and would also be optimized using an experimental design.
5. To determine the physicochemical properties of the various components of the optimized ISFI and microparticles, including the rheological properties of the ISFI and the morphology of the microparticles.

6. To load the implant with the cytotoxic drug, methotrexate and investigate the *in vitro* drug release.
7. To determine an effective sterilisation method for the ISFI such that would not be detrimental to the either release or the formation of the implant *in vivo*.
8. To investigate the effect of the release fractions on solid tumor cell lines.
9. To perform *in vivo* studies in the Sprague-Dawley rat model, in order to determine the drug release behaviour from the ISFI and the biocompatibility of the implant.

1.4. Novelty of the ISFI

The novelty of the ISFI lies mostly in the use of a polymer which has not been used for such purposes before. In addition the following objectives were to be achieved in order to formulate a novel ISFI:

1. Drug release would be over a prolonged period, ideally for at least one month.
2. Micro- or nano- particles would be incorporated into the ISFI, if necessary. These particles would contain the drug or an erosion controlling substance (ECS).

1.5. Overview of this Dissertation

Chapter One serves as the introduction of the dissertation and describes the pathophysiology of solid tumors in order to highlight the problems preventing the successful treatment of solid tumors. The novelty of the work and the aims and objectives of this work are also discussed.

Chapter Two is a literature review focussing on the work that has been conducted by other researchers in the development of polymeric implants for the treatment of solid tumors. It also provides insight into the use of stimuli responsive materials as implant-forming systems.

Chapter Three outlines the preformulation investigations undertaken in the development of the *in situ* forming system. Utilising this information, variables for an experimental design were obtained.

Chapter Four describes the utilization of a Face-Centred Central Composite Design in the determination of an optimum ISFI formulation. The formulations were tested for *in vitro* release behaviour, ease of injectability and gelation temperature. An investigation into the effects of autoclaving as a sterilisation method are also described.

Chapter Five introduces the pH responsive particles, briefly describes preformulation of the particles and subsequent optimization of the particles using a Face-Centred Central Composite Design. The effect of some formulation variables on the response parameters (drug entrapment and drug release) was ascertained and utilised in the development of an optimal formulation. The combination of ISFI and particles was also investigated for rheological properties and drug release and are reported on.

Chapter Six describes the *in vitro* testing of the implant and particles on various cancer cell lines. The use of flow cytometry and MTT assays are described and the results obtained from the two methods of viability testing of cells are compared.

Chapter Seven reports on the *in vivo* work conducted on the Sprague-Dawley rat model. Ultra Performance Liquid Chromatography quantification of the amount of drug in the plasma is described and the results evaluated. The biocompatibility of the implant is also discussed.

Chapter Eight lists shortcomings of the system and provides recommendations and suggestions for future work on this system.

CHAPTER 2

RECENT ADVANCES IN THE DESIGN OF DRUG- LOADED POLYMERIC IMPLANTS FOR THE TREATMENT OF SOLID TUMORS

2.1. Introduction

Implantable systems offer a number of advantages in the delivery of drugs to the human body. From a therapeutic point of view, high levels of drug can be achieved at the site of implantation. Considering conventional treatment methods for solid tumors often involve administration of high doses of chemotherapeutic agents (Gaze et al., 1995 and Schiller et al., 2008) and chemotherapeutic agents such as cytotoxic drugs are limited by toxicity to healthy body cells (Vassileva et al., 2007 and Pulsipher et al., 2009), an implant delivering high doses of drug only (or mostly) to the tumor area offers a clear advantage. It has been shown that employing an implant at the site of the tumor instead of an intravenously delivered chemotherapeutic agent may overcome this problem (Vassileva et al., 2007 and Berrada et al., 2005). The chances of systemic side-effects are much reduced due to the localized therapy exerted by the implant.

From a patient-centered viewpoint, implant systems in general, offer the advantage of compliance such as in the treatment of schizophrenia, where mental incapacitation may affect compliance or in the prevention of pregnancy where forgetting to take a single tablet can result in treatment failure. In the case of cancer where patient compliance is not usually a factor to be considered, implants offer convenience. An implant formulated to release drug over a number of weeks or even months decreases the need for the patient to return to the hospital weekly for systemic treatment, or eliminates the need to take medication daily and hence improves patient acceptability.

A number of implants have been marketed for the treatment of cancer and are registered with Food and Drug Administration (FDA) USA are Zoladex[®], Gliadel[®] and Eligard[®] (Table 2.1). Each of these implants is discussed more fully under the polymer utilised in the formulation of the implant.

Table 2.1: Currently available implant systems for the treatment of cancer.

Product	Condition	Drug incorporated	Year of approval by FDA	Company
Zoladex [®]	Advanced prostate cancer	Goserelin	1996	AstraZeneca PLC
Gliadel [®]	Malignant human glioblastoma	BCNU	1997	Guilford Pharmaceuticals, Inc
Eligard [®]	Advanced prostate cancer	Leuprolide acetate	2002	Atrix Labs, Inc

The use of implanted devices in the treatment of cancer can be divided into two therapeutic applications. Either the implant is intended for the delivery of chemotherapeutics as treatment or the implant is intended for “prophylaxis” following tumor resection (Jackson et al., 2000). The reasoning behind “prophylactic” applications is that there is often recurrence of the tumor within 2cm of the resected site (Jackson et al., 2000). Gliadel[®] is used for the treatment of malignant human glioblastoma and is placed at the site of the tumor. Zoladex[®] and Eligard[®] are used for the treatment of advanced prostate cancer and exert their action centrally by inhibiting central hormone production and are therefore not placed at the site of the tumor.

Implants that are intended for the treatment of tumors are often *in situ* forming implants (Eliaz and Kost, 2001). These are injected into the body and form a solid implant at the tumor site often in response to a local stimulus. While an obvious advantage of such a system is that invasive surgery is not required and the system can be easily injected into the patient using conventional methods, a possible disadvantage is the use of a local stimulus for formation of the implant since patient variability in local tumor conditions may influence the formation of the implant. Implants used for prophylaxis are often pastes or pre-formed implants that are applied to or placed at the site during surgery (Brem, 1996 and Gopferich, 1997). These implants may be formed by a number of methods, including crosslinking and photo-polymerization. An advantage of such a system is that all implants are of the same size and shape while a major disadvantage is that surgical implantation is required. This review provides a critical assessment of the various attempts that have been made in the novel treatment of tumors using polymeric implants that are intended for the delivery of drugs directly to the tumor sites.

2.2. *In situ* Forming Implants

In situ forming implants (ISFIs) exist as solutions (sol) at room conditions and convert into a gel like phase when exposed to a certain stimulus. Such a stimulus may be ionic or chemical crosslinking (Pezron et al., 1988) or photopolymerisation (Bosch et al., 2000 and Wei et al., 2005) or a local environmental stimulus such as pH, ionic strength or temperature (Klouda and Mikos, 2008). There are also a number of *in situ* forming implants which are not based on stimuli-sensitive nature of the implant but rather form as a result of organic solvent diffusion (Pucetti and Leblanc, 1996). Figure 2.1 shows the main mechanisms of implant formation.

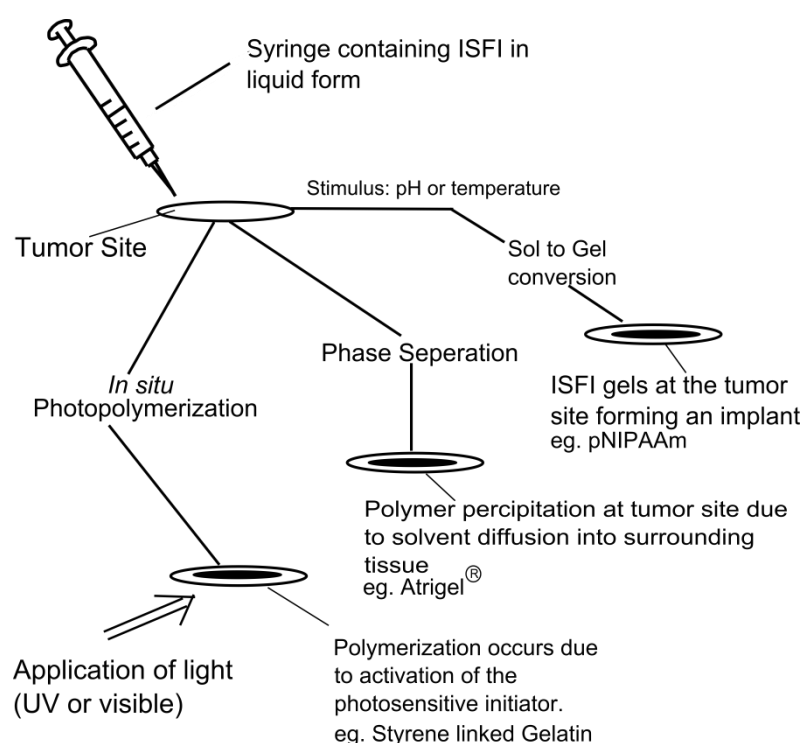


Figure 2.1: Schematic depicting some of the mechanisms of formation of implants *in situ*.

The stimuli-sensitive polymers which have been intensively investigated are those which respond to temperature change (Park and Song, 2006; Zhang and Misra, 2007; Lue et al., 2008 and Cole et al., 2009). This is due to the slightly higher temperature of most tumors (Babich, 2005). These polymers can form gels by two mechanisms. This is either through exhibition of an upper critical solution temperature (UCST) or a lower critical solution temperature (LCST). The UCST is exhibited by polymers which have some critical temperature below which they form a precipitated gel like phase whereas

heating the gel forms a homogenous solution (He et al., 2008). The LCST group of polymers exists as solutions below the LCST but form gels at temperatures exceeding the LCST (ten Brinke and Karasz, 1984; Geever et al., 2006; Zhou et al., 2007; Klouda and Mikos, 2008 and He et al., 2008). An example of such a polymer is poly(N-isopropylacrylamide) (pNIPAAm). The biggest advantage of thermal gelation is that it occurs almost instantaneously following the polymer reaching the LCST while other methods of chemical gelation take a few minutes (Hacker et al., 2008). The lag phase between the formation of a solid from the liquid phase may give rise to an undesirable initial burst effect (Schuetz et al., 2008). The pH of the interstitial fluid that surrounds the tumor cells is approximately 6.75. Thus, it differs quite considerably from the pH of the interstitial fluid that surrounds the normal body cells, which is approximately 7.23 (Martin and Jain, 1994). This presents another stimulus at the site of the tumor that can be utilized to develop stimuli sensitive implants (Schmaljohann, 2006).

As depicted in Figure 2.1, implants that form *in situ* may do so as a result of phase separation. In this case, the implant consists of a blend of a water insoluble polymer and a water-miscible solvent. This is then injected into the tumor area, the solvent diffuses out of the solution causing the polymer to precipitate out and an *in situ* implant is formed (Ravivarapu et al., 2000). The obvious downfall of such a system is the potential toxicity of the implant if the solvent used is toxic or not biocompatible. Eligard[®] is a registered product that utilizes this technology for the delivery of leuprolide acetate. The polymer that has been employed is poly (D,L-lactide-co-glycolide) and the pharmaceutically acceptable solvent is (N-methyl-2-pyrrolidone) (NMP). The PLG copolymer consists of a 75:25 ratio of D,L lactide to glycolide. Table 2.2 summarizes the attempts made by a number of researchers in the treatment of various tumors using *in situ* forming implants.

Table 2.2: Drug, type of tumor and the polymers employed for *in situ* forming implants.

Polymer used	Drug used	Model and tumor line	Reference
Chitosan-GP	Paclitaxel	EMT-6 (mouse mammary tumor)	Ruel-Gariépy et al., 2004
Chitosan-GP	DOX and vaccinia viral vaccine	Murine TC-1 (cervical cancer)	Han et al., 2008
Chitosan-GP	Camptothecin	RIF-1 (mouse fibroblastoma)	Berrada et al., 2005
Photocrosslinkable Chitosan	Paclitaxel	3LL (Lewis lung tumor)	Obara et al., 2005
Acrylated Chitosan-Poloxamer®	Conjugated DOX	A549 (Human Lung adenocarcinoma)	Cho et al., 2009
Albumin-TAD	DOX	WiDr (human colon carcinoma)	Kakinoki and Taguchi, 2007
Collagen-ephidrine	5-FU/ DOX/ cisplatin	BxPC-3 and PANC-1 (human pancreatic cancer)	Smith et al., 1995
pNipaam-co-AA	-	SKOV-3 (human ovarian tumor)	Meyer et al., 2001
PEG	Topotecan	MAT BIII (breast cancer)	Lalloo et al., 2006
Atrigel®	Cisplatin	T4N2aMO(squamous carcinoma of supraglottic larynx)	Chen et al., 2003
Pluronic® F127/Poloxamer 407	Paclitaxel	B16F1 (murine melanoma)	Amiji et al., 2002

GP: glycerophosphate; DOX: Doxorubicin; TAD: Tartaric acid derivative; 5-FU: 5-flourouracil; AA: Acrylic acid; PEG: polyethylene glycol

2.3. Implants Based on Natural Polymeric Sources

2.3.1. Chitosan

2.3.1.1. *In situ* forming implants

Of all natural stimuli-responsive polymers, chitosan has been studied extensively in terms of its use for the treatment of cancer (Berrada et al., 2005; Ho et al., 2005 and Cho et al., 2009). The properties of chitosan, such as thermo- and pH- responsiveness as well as its biocompatibility, biodegradability, and mucoadhesiveness, make chitosan an ideal candidate for implant formation. In addition, chitosan can be used at low concentrations for the purpose of decreasing the chances of toxic effects *in vivo* (Berrada et al., 2005). A further advantage is that the chitosan solutions can be easily sterilized (Ruel-Gariépy and Leroux, 2004). Gelation of chitosan can be achieved through three mechanisms. Firstly, in the presence of salts such as glycerophosphate,

chitosan can form a gel when exposed to body temperature (Chenite et al., 2000). Secondly, the formation of a chitosan hydrogel can be triggered by UV irradiation as already documented by Ono et al., (2000) who used azide-derived chitosan. Thirdly, chitosan is highly soluble at low pHs and poorly soluble at high pHs, thus forming viscous gels at physiological pH (Ganguly and Dash, 2004).

Recently, Cho et al. (2009) conjugated doxorubicin (DOX) to acrylated chitosan and Pluronic[®] in an attempt to obtain sustained release of the chemotherapeutic agent. The hydrogels formed as a result of both photo-crosslinking as well thermoresponsiveness. As a result the formed hydrogels were mechanically robust and when compared to gels consisting of free DOX in a mixture of chitosan and Pluronic[®], the release from the DOX-conjugated-chitosan-Pluronic[®] was significantly slower and over a longer period of time (40% DOX was released after 15 days) and the burst release that was established with the other hydrogel was not observed here. *In vivo* animal studies were conducted in athymic mice bearing human lung adenocarcinomas. The decrease in the tumor size appeared to mimic the obtained *in vitro* results as the size of the tumors decreased rapidly initially in mice receiving hydrogels containing free DOX (due to the burst release of drug) followed by an increase in tumor size indicating re-growth of the tumor due to the lack of further drug release. In contrast, mice receiving the DOX-conjugated-chitosan-Pluronic[®] showed a slow decrease in the size of the tumor initially but the tumor size at the end of 30 days was 22% less than the baseline tumor, indicating significant improvement. However, although the photo-initiator used was FDA approved, the use of photo-crosslinking can be detrimental to the drug and lead to degradation of the drug and must be performed for the minimum amount of time (Cho et al., 2009). This can potentially be a drawback to clinical application of the formulation as alteration of the required time for irradiation may lead to different release profiles.

2.3.1.2. Pre-formed chitosan implants.

Chitosan egg-Phosphatidyl (C-eP) implants were investigated by Ho et al., 2005 as well as Soo et al, 2008. Both implants were intended for the local treatment of ovarian cancer and contained paclitaxel. Ho et al., 2005 implants demonstrated *in vitro* zero order drug release over a period of 5 days. Soo et al., 2008 loaded the C-eP implant with PLA-b-PEG/PLA nanoparticles loaded with paclitaxel. They subsequently investigated the *in vitro* behavior of the implant in ascites fluid, of which a build up often

occurs in ovarian cancer. Furthermore, the effect of the implant in mice was investigated. The *in vitro* and *in vivo* results correlated well and the release of paclitaxel *in vivo* was over a period of 4 weeks (Soo et al., 2008). No local signs of toxicity were observed. Ho et al., 2005 investigated the effects of the implant on multi-drug resistance. The effect of the sustained release of paclitaxel from the implant was compared with the effect of intermittent intraperitoneal dosing with paclitaxel formulated in Cremophor EL[®]. The determination of multi drug resistance was carried out *in vitro* on cancer cell lines and *in vivo* on tumor bearing mice. After 14 days, cells that received paclitaxel- Cremophor EL[®] showed a two-fold increase in mRNA levels, an effect that was not observed with cells receiving the implanted device (Ho et al., 2005). In addition, there was also a significant induction of efflux activity of the drug transporter, PGP by the paclitaxel-Cremophor EL[®] dosing which was not seen with the implanted system. This indicates that the sustained delivery of drugs may be beneficial in reducing the development of multi-drug resistance.

In another study, Vassileva et al., 2007 compared the overall toxicity as well as the anti-tumor effects of a paclitaxel loaded C-eP implant to paclitaxel formulated in Cremophor EL[®]. These studies revealed that the Cremophor EL[®]-paclitaxel formulation was associated with significant toxicity, lethality, abnormal peritoneal organ morphology and hepatic inflammation, whereas mice receiving the implanted device encountered no such problems (Vassileva et al., 2007). In addition, the maximum tolerable dose was much higher for the implant (up to 280 mg/kg/week) when compared to the Cremophor EL[®]-paclitaxel formulation (20 mg/kg/week) (Vassileva et al., 2007). A drug loading of 60 mg/kg in the implant had an enhanced cytotoxic effect compared to the same dose administered intraperitoneally as the Cremophor EL[®]-paclitaxel formulation indicating the potential of this implant system as a drug delivery device.

2.3.2. Alginate

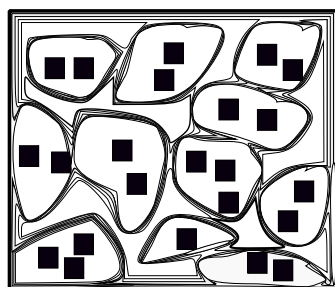
2.3.2.1. *In situ* forming alginate implants

Alginate is a polysaccharide derived from seaweed. It is essentially a block copolymer consisting of β -D-mannuronic and α -L-guluronic acid which gels in the presence of divalent cations such as calcium (van Tomme et al., 2008). The polymer rapidly forms a gel by crosslinking in the presence of calcium ions and this gelation mechanism has been utilized by Hori et al. (2009) to develop an *in situ* forming implant. Calcium ions

were entrapped within alginate microspheres and dispersed in an alginate solution. Upon injecting this formulation into the body, calcium ions were released from the microspheres and assisted by the calcium concentration *in vivo*; the alginate solution took 60 minutes to form a stable gel (Hori et al., 2009). The implant was intended for the delivery of immunomodulators in the treatment of cancer. The soluble factor (IL2) was incorporated into the implant with ease by simple mixing with the alginate solution. Hori et al. (2009) also investigated the feasibility of attaching immunomodulatory oligonucleotides to the microspheres for delivery purposes. The IL2 was 95% bioactive after delivery via the alginic implant indicating the feasibility of this drug delivery system. Approximately 80% of the oligonucleotides were loaded to the microspheres and the release was slow (approximately 20% over one week). This study highlights the possibility of dual-functional microspheres and the potential for the effective treatment of cancer through delivery of immunomodulators to the tumor site. However, the delivery of other smaller chemotherapeutics may not be as effective as the time take for a stable gel to form is quite lengthy and this may lead to an initial burst effect (Hori et al., 2009).

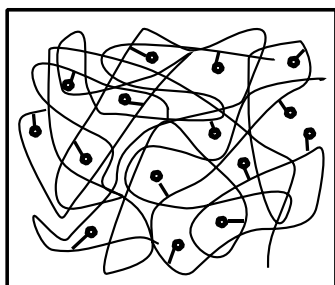
2.3.2.2. Pre-formed alginate implants

Bouhadir et al., 2001 made use of an alternate method of gelation by oxidising the alginate to form lower molecular weight oligomers and then crosslinked them using adipic dihydrazide. The alginate based implant was loaded separately with three drugs in different ways in order to demonstrate the different release profiles that could be obtained from this hydrogel. The three cytotoxics that were utilised were methotrexate (MTX), doxorubicin (DOX) and mitoxantrone (Bouhadir et al., 2001). Figure 2.2 depicts the loading mechanisms that were utilized for each drug as well as the mechanisms of release of the chemotherapeutic agents.



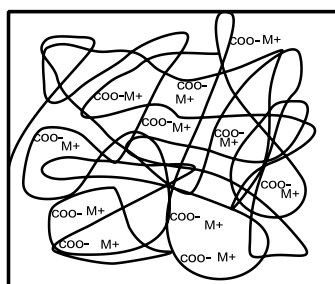
Methotrexate was loaded into the pores of the hydrogel and released via diffusion of the drug from the hydrogel

■ Methotrexate



Doxorubicin was covalently attached to the polymer backbone via azone bond and release was via hydrolysis of this labile bond

● Doxorubicin
— Hydrozone bond



Mitoxantrone formed ionic complexes with the carboxylate groups on the sugar residues of the polymer background and release of the drug was due to dissociation of the ionic complex due to the degradation of the hydrogel

M⁺ Mitoxantrone
COO⁻ Carboxylate group

Figure 2.2: Schematic showing drug loading and release of the drugs from the modified alginate hydrogel (Adapted from data in Bouhadir et al., 2001).

MTX was released over two days but this was extended to seven days with a higher concentration of the covalent crosslinker, adipic dihydrazide (Bouhadir et al., 2001). The reason for the prolonged release was the reduced swelling of the hydrogel that occurred with the higher concentration of the crosslinking agent, resulting in a slower diffusion-based drug release. The release of doxorubicin was over a period of days to weeks depending on the concentration of adipic dihydrazide. Likewise, the release of mitoxantrone was substantially controlled upon increasing the concentration of adipic dihydrazide. At high concentrations of adipic dihydrazide, the release of mitoxantrone was only 10% over a period of 22 days (Bouhadir et al., 2001). Thus, this system exemplifies that a number of drugs may be loaded into the hydrogel depending on the chemical nature of the drug. This allows specific loading for specific drugs and allows the implant to have many uses. Bouhadir et al. (2001) also investigated the release of

the three drugs when loaded simultaneously to determine whether the implant had potential to be used to deliver multiple drugs. *In vitro* release studies showed that MTX was released completely over 9 days, whereas DOX and mitoxantrone were released over 17 days. More than 20% of DOX was released over the first 13 days while majority of the mitoxantrone was released in the last 4 days. This sequential release of drug shows that the implant may be used to deliver multiple drugs which may prove useful in the treatment of drug resistant tumors (Bouhadir et al., 2001).

2.3.3. Gelatin

2.3.3.1. In situ forming gelatin implants

Gelatin is derived from naturally occurring collagen and exhibits thermo-reversible behavior. At temperatures below 25°C the polymer forms a gel while above 30°C it reverts back to solution form (Klouda and Mikos, 2008). This is not favourable behavior for pharmaceutical preparations and therefore various attempts have been made to modify the temperatures of gelation-containing preparations to a more physiologically acceptable temperature range (Van Den Bulcke et al., 2000 and Klouda and Mikos, 2008). Gelatin is also a pH responsive polymer (Gupta et al., 2002). *In situ* forming gelatin hydrogels for postoperative chemotherapy have been formulated by Okino and co workers (2002). Styrene derived gelatin together with the initiator camphorquinone was utilised and polymerisation in the presence of visible light irradiation occurred. This *in situ* forming hydrogel was intended for the delivery of drug to newly resected tumor sites. The formation of the hydrogel was investigated on the surface of rat liver and despite the potential toxicity of the free radicals produced by the initiator, the *in vivo* application of the hydrogel showed no necrotic damage to the hepatocytes (Okino et al., 2002). The mechanism of formation of these hydrogels is due to photopolymerisation and is not dependant on the temperature sensitivity of the gelatin. However, due to the application of UV light that is required; this formulation can only be used during surgical resection of the tumor or for tumors occurring on the skin.

2.3.3.2. Pre-formed gelatin implants.

Liu et al., 2003 attempted to develop gelatin hydrogels for the treatment of cancer using a method previously developed by Tabata and Ikada (1999), Kang et al. (1999) and Hong et al. (2000) for producing gelatin hydrogels meant for purposes other than cancer treatment. The hydrogels contained Interleukin 12 (IL12) and were tested on

colon carcinomal cells implanted subcutaneously into the backs of rats. The effect of these hydrogels on the tumors was evaluated and the cytotoxicity of the IL12 was found to be higher in the subcutaneously administered gelatin hydrogel formulation than in the subcutaneously administered free IL12 (Liu et al., 2003). Konishi et al., (2003) have also studied the release of chemotherapeutic agents namely cisplatin from preformed gelatin hydrogels intended for implantation following tumor resection. They established that the release of the incorporated cisplatin was not via diffusion but rather a function of degradation of the hydrogel (Konishi et al., 2003). In 2005, the same group investigated the release of cisplatin and adriamycin from the hydrogel. Adriamycin was released at a quicker rate than the cisplatin and the combination showed a synergistic inhibition of tumor growth *in vivo* (Konishi et al., 2005). In addition, when adriamycin was injected percutaneously into the mice as a control, scar formation was noted at the injection site. Contrary to this, the adriamycin-loaded hydrogel did not induce such an effect in the mice (Konishi et al., 2005). The authors postulated that the lack of scar formation was due to the slow release of the drug from the hydrogel.

2.3.4. Albumin

2.3.4.1. In situ forming albumin implants

Human serum albumin has been used to produce an *in situ* forming hydrogel which was combined with a tartaric acid derivative (TAD) (Kakinoki et al., 2007). TAD is a tissue adhesive that has the advantage of maintaining the position of the implant in the desired site (Kakinoki et al., 2007). The release of doxorubicin from this hydrogel was investigated and *in vitro* studies showed an initial burst effect due to surface drug followed by release from within the gel for up to 100 hours (4 days). A follow-up *in vivo* study by Kakinoki and Taguchi, 2007 tested the formulation in rats bearing the human colon carcinoma. They found that the size of tumors in rats had decreased to a greater extent in comparison to the tumors treated with free drug or the control. However, the drug was only detectable for 3 days, as expected from the *in vitro* results (Kakinoki and Taguchi, 2007).

2.3.5. Collagen

2.3.5.1. In situ forming collagen implants.

Collagen is a naturally occurring polysaccharide from which gelatin is derived. An injectable formulation containing collagen and ephedrine together with cisplatin as a drug registered as Intrados[®] (Matrix Pharmaceuticals) has provided some promising results in treating hepatocellular carcinoma (Mok et al., 2001; Wenig et al., 2002). Ephedrine serves to constrict blood vessels in the affected area thereby reducing the diffusion and hence loss of drug from the area. The delivery of these chemotherapeutic agents to tumors of the head and neck, trunk and liver for the treatment of hepatocellular carcinoma has been shown to be successful in humans (Mok et al., 2001; Wenig et al., 2002). In addition, the collagen/ephedrine combined with cisplatin has also demonstrated efficacy in obstructive oesophageal cancer in humans (Monga et al., 2000) while the gel in combination with 5-fluorouracil has proven good efficacy in pancreatic cancer of rats (Smith et al., 1995). Collagen-poly(HEMA) hydrogels were synthesized by Jeyanthi and Rao (1990). The drug release from the gels was found to be independent of time and followed zero order kinetics. The maximum amount of drug release from the gels which followed zero order kinetics was 98% over 10 days for 5-Fluorouracil (Jeyanthi and Rao, 1990). The gels containing Mitomycin C and Bleomycin had zero order release of 52% over 12 days and 74% over 11 days respectively. This is longer than the release profiles obtained for collagen and ephedrine indicating that this formulation is superior to Intrados[®]. However, the drug release is still for a short period of time only.

2.4. Synthetic, Biodegradable Mono-polymer and Co-polymers as Implant Sources

2.4.1. Poly(N-isopropylacrylamide) (pNIPAAm)

With the increasing interest in the polymers that are stimuli-sensitive, otherwise referred to as “smart polymers”, a number of studies have focused on pNIPAAm due to its thermosensitivity (Packhaeuser et al. 2004; Han et al, 2009). The polymer exists as a solution below its lower critical solution temperature (LCST) and gels at temperatures above LCST. The LCST of pNIPAAm is 32°C (Winnik, 1990). This temperature can easily be increased to body temperature or the temperature of a tumor by co-polymerisation of pNIPAAm with hydrophilic polymers (Jones et al., 2007). However, the problem with pNIPAAm is its lack of biodegradability (Yu et al., 2007). In an effort to

overcome this problem as well as improve the LCST of the hydrogel, researchers have polymerized pNIPAAm with various other polymers (often hydrophilic in nature) such as hydroxyethylmethacrylate (HEMA) (Jones et al., 2007), acrylic acid (Kim and Healy, 2003; Yu et al., 2007), as well as gelatin and hyaluronic acid (Ohya et al., 2005). Co-polymerization with polymers such as propylacrylic acid and poly(2-dimethyl amino)ethyl methacrylate (DMAEMA) leads to the formation of polymers that are pH- and thermoresponsive (Kim and Healy, 2003; Yu et al., 2007). Although most of these polymeric hydrogels were not synthesized specifically for the treatment of cancer, they have shown promising potential for use in such applications.

2.4.2. *In situ* forming Atrigel® implants

Atrigel® (Atrix Laboratories, Inc) consists of biodegradable polymers such as polylactic acid (PLA) or poly(lactic-co-glycolic acid) (PLGA) together with a biocompatible hydrophilic solvent such as N-methyl-2-pyrrolidone. The solvent renders the polymer soluble and when the system is injected into the body, the solvent dissipates into the surrounding tissue leaving behind a formed implant. This technology was initially designed for the treatment of periodontal disease (Packhauser et al., 2004). In 2000, Ravivarapu and co-workers developed a formulation of Atrigel® loaded with leuprolide acetate and showed the ability of the implant to suppress serum testosterone for a 3 month period. A 6-month formulation has since been developed and Atrigel® is now used to deliver leuprolide acetate for the treatment of advanced prostate cancer in the product known as Eligard®. One of the problems facing the *in situ* forming implants is the quick initial release of drug (Packhauser et al., 2004). In order to overcome this problem, Astaneh et al., 2007 created a zinc-leuprolide complex which was loaded into a PLGA *in situ* forming implant. This implant was compared to Eligard® showing that the use of the zinc complex reduced the burst period slightly and also resulted in a continuous, zero-order release profile following the burst phase. Comparably, Eligard® showed a very rapid initial release of the drug (almost 40% over 24 hours) (Astaneh et al., 2007). Atrigel® has also been loaded with cisplatin for the potential treatment of head and neck cancers (Chen et al., 2003). The efficacy of this system was investigated in a chimeric mouse model carrying the human head and neck squamous cell carcinoma (Chen et al., 2003). Drug release was 80% at the 7th day. The ALZA Corporation, Inc. has also designed *in situ* forming implants that are very similar to the Atrigel® technology (except for the solvents used) but they have not yet been investigated for use as anticancer formulations.

2.4.3. *In situ* forming poly(organophosphazenes) implants

Recently Al-Abd et al., 2010 investigated the release of DOX from a poly(organophosphazene) hydrogel *in vitro* and in rats with human gastric tumor xenografts. Release of the drug was over 5 weeks and the hydrogel was retained for 7 weeks. The concentration of polymer used was also relatively low (10%^{w/v}). This group of polymers has shown a great potential for the development of an ideal implant system for cancer chemotherapy due to the ease with which they can be modified to serve the purpose of the respective drug delivery system (Al-Abd et al., 2010).

2.4.4. *In situ* forming poly(ϵ -caprolactone fumarate) implants

Poly(ϵ -caprolactone fumarate) is a relatively new photo-crosslinkable polymer which possesses both biocompatibility and good fluidity making it an ideal candidate for the development of an injectable *in situ* forming implant (Sharifi et al, 2009). This polymer was used by Sharifi et al. (2009) to develop an *in situ* forming implant for the delivery of tamoxifen citrate to breast cancer patients. *In vitro* studies conducted showed extremely slow release of the drug (less than 5% of drug after 400 hours) and 40-60% of MCF-7 (breast cancer) cells incubated with the formulation were killed (Sharifi et al, 2009). This formulation has potential for further development and *in vivo* work should be completed.

2.4.5. Polyethylene glycol (PEG)

2.4.5.1. *In situ* forming PEG implants

Qiu et al., (2003) synthesized a PEG-based copolymer that was capable of *in situ* gelling. This was achieved as a result of chemical cross linking of the multiple thiol groups that lie on the polymer backbone. However, the polymer as well as the crosslinking agent must be injected into the area (Qiu et al, 2003). Although hydrogel formation was achieved *in vivo*, the loaded hydrophilic anti-cancer drug, topotecan, was released very rapidly, probably due to the large pores in the hydrogel (Qiu et al, 2003). As a result, Laloo et al. (2006) formulated a two phase drug release system. A portion of the drug was entrapped in the PEG hydrogel and some within liposomes which were then loaded into the hydrogel. The drug release profile showed a quick initial release followed by a longer controlled release over several days (Laloo et al.,

2006). Tumor masses (MAT BIII breast cancer cells) in rats were also significantly reduced with the use of this hydrogel (Lalloo et al., 2006).

2.4.5.2. Pre-formed PEG implants

Tauro et al. (2005) proposed a PEG hydrogel system for the treatment of Glioblastoma Multiforme. Highly invasive tumors are often associated with higher amounts of matrix metalloproteases (MMPs) in the area surrounding the tumor. These proteins cause tumor invasion as well as neovascularisation (Tauro et al., 2005). The developed system proposed the use of these proteases to control the release of the drug from the hydrogel matrix. The drug (cisplatin) was linked to the polymer backbone by means of peptide linkages. As the MMPs diffused through the hydrogel, the peptide bonds were cleaved by the MMPs and as the result the drug was released into the area surrounding the tumor. This allowed controlled release of the drug at the site of the tumor. The controlled release effect depended on the amount of MMPs present and hence the dose of drug delivered corresponded to the invasiveness of the tumor (Tauro et al., 2005).

2.4.6. Pre-formed implants composed of poly(D,L-lactide-co-glycolide)

Poly(D,L-lactide-co-glycolide) (PLGA) is a well known polymer, the use of which in the medical field is well-documented. In the treatment of cancer, recent advances have been made into the use of this polymer as a foam or a nanofiber disc intended to be inserted intra- or peritumorally (Lee et al., 2009 and Ranganath et al., 2010). Lee et al., 2009 formulated a micro-porous PLGA foam intended for the delivery of drug to the brain. The foams were loaded with paclitaxel and showed *in vitro* release of up to 8 weeks. The foams were also placed intracranially in healthy mice and assessment after 28 days revealed that therapeutic amounts of paclitaxel were found throughout the mouse brain (Raganath et al., 2010). Nanofiber discs were prepared by Ranganath and workers, (2010) and loaded with paclitaxel and implanted into the brains of mice bearing human glioblastoma. Paclitaxel was also found to have diffused from the disc to penetrate a large area of the brain and that the concentrations of paclitaxel were maintained for 42 days which exceeds the release obtained by previous researchers (Raganath et al., 2010). This formulation seems extremely promising for further development.

2.4.7. Pre-formed polyanhydride polymeric implants

The most successful of the pre-formed implants are the Gliadel[®] implants intended for the treatment of malignant human glioma (Brem, 1996; Perry et al., 2007; Chopko, 2009; Argyriou et al., 2009). These implants have been developed with the intention of delivering anticancer drugs following at least partial resection of the tumor (Jain, 2005). A study that was conducted by Brem et al., 1995 using Gliadel[®] showed that patients who received the implant containing an anticancer drug had a 6 month survival rate 50% greater than those who received a placebo implant. In 1996, the FDA (United States) approved this implant. However, the implant has only been shown to prolong the lives of patients by one year (Perry et al., 2007; Chopko, 2009; Argyriou et al., 2009). Gliadel[®] consists of poly(carboxyphenoxypropane/sebacic acid) anhydride, 20:80 (cPP-SA) (Biodel[®]) and the drug, carmustine (BCNU) co-dissolved in methylene chloride and spray dried into microspheres. The microspheres are then compressed into discs of dimensions 1.4cm in diameter and 1.0 mm in thickness. The discs are sterilized by gamma irradiation.

Following the success of the Gliadel[®] implant, Göpferich (1997) aimed to develop an implant for release of at least two drugs, the release of one drug lasting two weeks and the second drug being released over the next week. This would decrease the resistance of cancer cells as they would be exposed to a number of different drugs over a certain period of time (Göpferich, 1997). A surface-eroding polymer, poly(1,3-bis[*p*-carboxyphenoxy]propane-co-sebacic acid) (p(CPP-SA)) was used and two drugs were loaded into the implant. One drug was in the “core” and the other was in the “mantle”. Drug release from this implant was therefore a function of the erosion of the polymer. However, it was found that the release of the second drug occurred prematurely (Göpferich, 1997). Thus, instead of increasing the thickness of the mantle which would have caused the implant to be too large, a slow-eroding polymer namely poly (D,L-lactic acid) was used. A four layer device was formulated as depicted in Figure 2.3. With this implant release periods of up to four weeks were obtained (Göpferich, 1997).

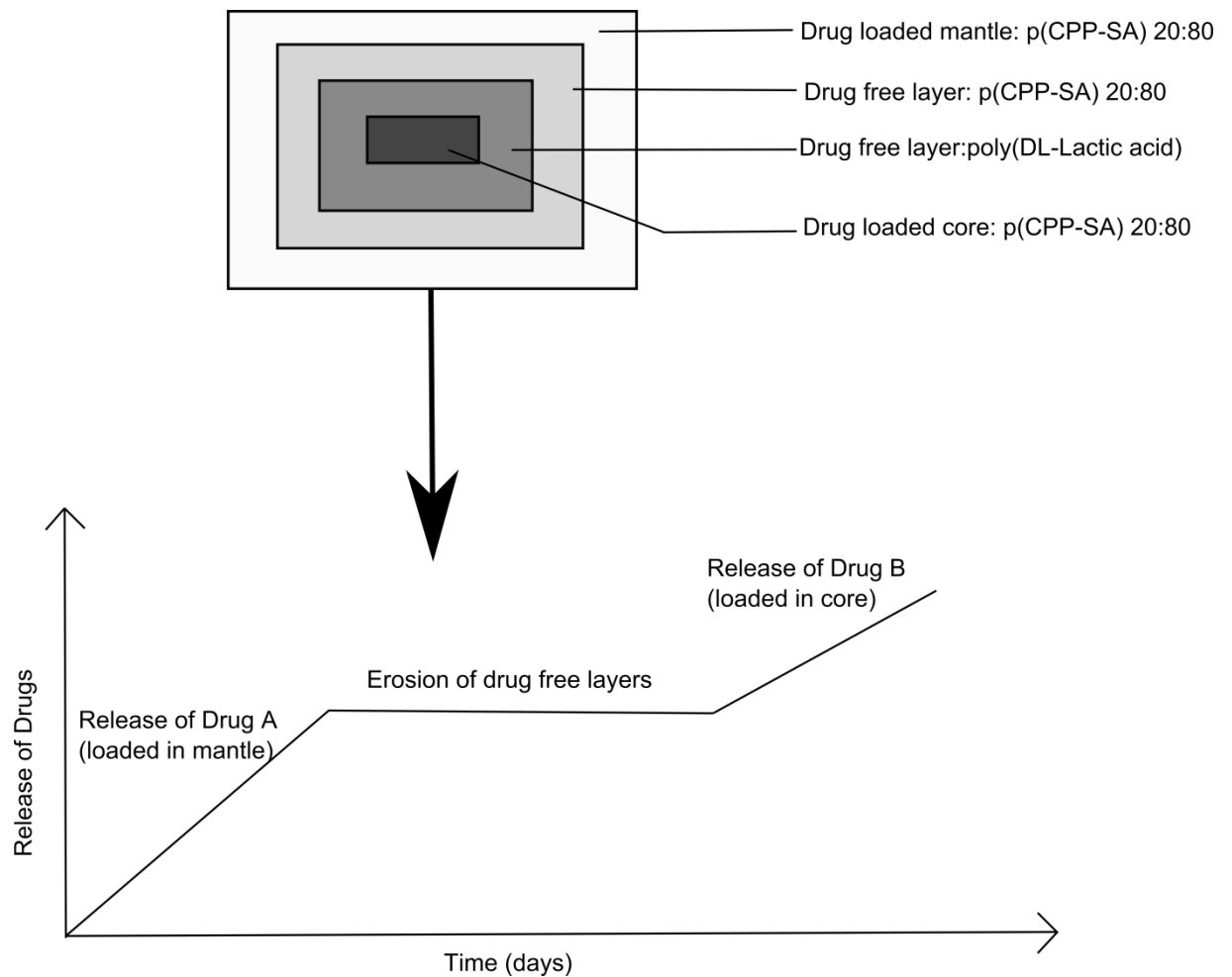


Figure 2.3: Schematic depicting a longitudinal section through the disc shaped implant with the loading and release patterns of two drugs clearly illustrated (Adapted from data in Göpferich,1997).

Although the implant devised by Göpferich (1997) was proven to allow release of drug from the core after two weeks, Vogelhuber et al. (2001) attempted to create an implant that had a greater lag phase before the release of the drug. This was meant to allow the implant to have more applications as a drug delivery system. Furthermore, this implant would also address the problem of the low survival rate of patients with human glioblastoma despite therapy with Gliadel[®], as this implant would be capable of delivering a number of drugs to the tumor region in a controlled manner. This attempt registered some promising results (Vogelhuber et al., 2001).

The greatest challenge that was faced during the attempt to improve the implant designed by Gopferich (1997) was the size of the implant. The implant had dimensions

of 8mm diameter and 4mm height and this made the *in vivo* testing in a rat model impossible (Vogelhuber et al., 2001). As a result Vogelhuber et al. (2001) investigated the drug release from an implant consisting two layers one containing the drug (drug loaded core) and the other containing no drug (drug free mantle) (Figure 2.3). This reduced the size of the implant to a diameter of 2mm and a height of 1.8mm (Vogelhuber et al., 2001).

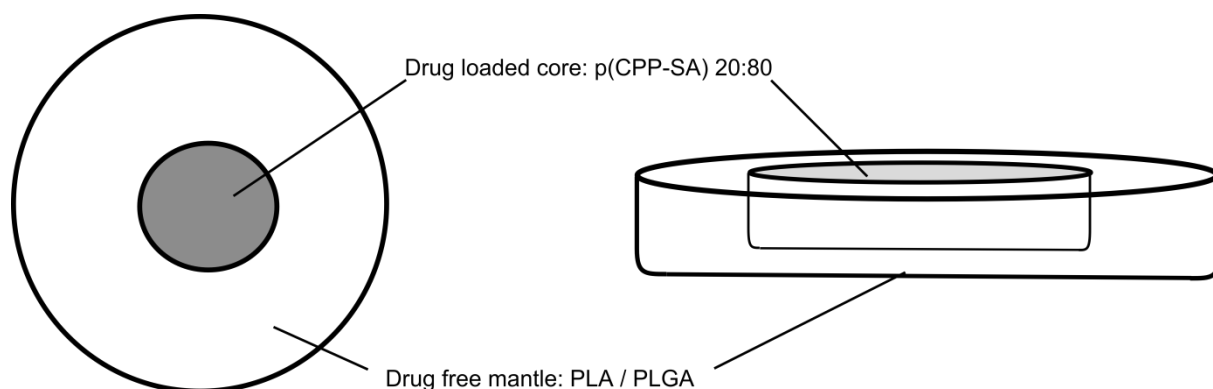


Figure 2.4: Schematic depicting a cross-section through the implant (Adapted from data in Vogelhuber et al., 2001).

The mantle consisted of either PLA or PLGA whilst the core consisted of polyanhydride, p(CPP: SA) 20:80 loaded with the drug. The effect of tempering the implant in silicon oil was also investigated. Delayed onset of drug release with this implant was achieved with the release being delayed by 10 days post-implantation when PLGA50₁₇ was used and by 58 days when PLA50₃₀ formed the mantle layer (Vogelhuber et al., 2001). Furthermore, it was shown that tempering of the implant allowed for erosion and drug release to be controlled by the type of polymer used and the effects of pores and other defects in the polymer mantle affected the release of the drug far more than the polymer erosion (Vogelhuber et al., 2001).

More recently Hiremath et al. (2008) have synthesized cylindrical biodegradable implants intended for the delivery of tamoxifen citrate to breast cancers by the placement of the implant into the affected area. The employed polyanhydride polymer was poly(sebacic acid-co-rinoleic-ester anhydride) in a ratio of 70:30 w/w [poly(SA-RA) 70:30 w/w]. The implants were prepared using the standard melt manufacturing

method and *in vitro* studies revealed that the release of drug from 10% and 20% loaded implants was 42.36 and 62.60% after 30 days indicating prolonged delivery of the drug to the tumor. It was also estimated that drug release would be complete after 72 days for the 10% loaded implant and 100 days for the 20% loaded implant (Hiremath et al., 2008).

2.5. Synthetic, biodegradable tri-block polymers used for implants

2.5.1. Tri-block polymer from poly(lactic-co-glycolide and polyethylene glycol (PLGA-PEG-PLGA)

ReGel[®] (MacroMed) is a tri-block polymer (ABA or BAB) whereby the A block is poly(lactic-co-glycolide) and the B block is poly(ethylene glycol). The tri-block polymer is water soluble at room temperature but insoluble at body temperature (37°C) (Zentner et al., 2001). It has been established that due to its insoluble gel state *in vivo*, the implant can remain intact at the site of application for at least 1 month (Jeong et al., 2004). The implant is also biodegradable. The rate of degradation of the polymer, the permeability of the matrix of the hydrogel, the sol-gel transition temperature as well as the burst release rate depend on the molecular weight of the polymers and the ratio of PLGA to PEG (Jeong et al., 2000). In addition, the above mentioned polymer characteristics are also influenced by the ratio of lactide: glycolide as well as the concentration of these elements and the end group of the PLGA polymer component. Formulations of ReGel[®] containing paclitaxel have been trademarked OncoGel[®]. This tri-block polymer has also been used to develop a drug delivery system for the delivery of testosterone (Chen et al., 2005) and the delivery of interleukin 2 or cancer immunotherapy and this formulation is trademarked Cytoryn[®] (Madan et al., 2009). More recently, the polymer has been used in a formulation containing PEGylated camptothecin (Yu et al., 2007). The purpose of adding a polyethylene glycol (PEG) group to the camptothecin was to enhance the release of the drug in a controlled manner. This was achieved and in addition the solubility of the drug was also enhanced (Yu et al., 2007). Furthermore, the sol-gel transition temperature was decreased and the viscosity of the implant with the PEGylated drug was higher than that of the implant without the PEGylated drug. Ostensibly, this will improve the implants behavior *in situ*.

2.5.1.1. Poloxamers/Pluronics®

These are triblock co-polymers consisting of poly(ethylene oxide)-b-poly(propyleneoxide)-b-poly(ethylene oxide) (PEO-PPO-PEO). Poloxamers have also been investigated as potential implant-forming systems due to their thermoreversible nature (Jeong and Gutowska, 2002). Sol to gel transitions occur at 5-30°C and gel to sol transitions at 35-50°C (Jeong and Gutowska, 2002). Alteration of the molecular weights, composition and concentration of the polymer results in a change in the sol to gel transition (gelation) temperature of the polymer (Klouda and Mikos, 2008). However, the actual formation of the gel phase is poorly understood (Klouda and Mikos, 2008). Pluronic products include Pluronic® F127, P85 and L61. The cytotoxicity of these polymers has been investigated and the study revealed that F127 had no cytotoxic potential while both P85 and L61 decreased the growth of the tumor cells (Exner et al., 2005). Amiji et al., 2002 created a paclitaxel containing implant using Pluronic® F127, which was administered to melanoma bearing mice. The results were promising as 91% of rats survived up to 15 days post-implantation compared to 58% in the control group (Klouda and Mikos, 2008). This study seems to be an exception as other formulations containing Pluronics rarely released drugs for more than a period of a few days (Ruel-Gariépy and Leroux, 2004).

2.6. Synthetic non-biodegradable polymers

2.6.1. Silicone

The use of silicone in the formulation of implants has not been investigated extensively as silicone is not biodegradable (He et al., 2003). He et al., 2003 investigated the effects of silastic implants loaded with 5-Fluorouracil (5FU) - a drug which has an extremely short half life (10 minutes). The dimensions of the implants were 2mm (outer diameter), 0.5mm (wall thickness) and 25mm in length. Each pellet contained 13.2mg of 5FU. These implants were surgically implanted into the livers of Wistar rats bearing Walker-256 carcinosarcoma. When the implant was placed in the left lobe of the liver at the site where the tumor had been implanted, the tumor inhibition was found to be as high as 96.3%, with one of the animals in this group surviving the entire length of the experiment and no viable tumor was detected in this animal (He et al., 2003). Biodistribution results showed a high concentration of 5FU in the tissues adjacent to the area in which the implant was placed but the amount of 5FU found in other tissues was lower, indicating a lower toxicity of this implant than when administered parentally. In addition, 5FU was found in the blood for 1 to 8 weeks, despite its short half life,

indicating that there was a controlled release of the drug from the implant (He et al., 2003). Pathological studies were also carried out on the tissue surrounding the implant and no fibrous capsule was found around the implant, indicating the lack of immunological attack on the implant (He et al., 2003). While these results seem to indicate the feasibility of the system for cancer treatment, the lack of biodegradability potential in silicone cannot be ignored. Non-biodegradation necessitates the removal of the implant following the drug release. This is not likely to have high patient acceptability and also multiple operations may lead to further complications.

2.7. Surgical pastes

Most of the pastes developed for use in cancer have been formulated prior to 2005 and include those developed by Dordunoo et al. (1997), Winternitz et al. (1996) and Jackson et al. (2000, 2004). Dordunoo et al. (1997) investigated the impact of a paclitaxel-containing surgical paste composed of poly(caprolactone). This polymer has a biodegradation lifetime of between 6-9 months, making it an ideal polymer for sustained drug release. The paste was to be delivered by heating of the paste to temperatures of between 50-55°C and delivering it via injection or application to the tumor area following the resection of the tumor. This paste showed tumor regression in 63% of the mice treated (Dordunoo et al., 1997). The same polymer and drug was used by Hunter et al. (1997). The application of this paste was carried out on a murine tumor model. The results indicated that the implant delayed the re-growth of tumors that were not resected entirely (Hunter et al., 1997). Blends of PCL and methoxypolyethylene glycol were also investigated by Winternitz et al., 1996, obtaining drug release over a number of days.

2.8. Other implants

Numerous other implants also exist which are intended for the treatment of cancer but cannot be implanted locally. These include: Zoladex[®] (AstraZeneca PLC), which consists of PLGA rods containing goserelin, a LHRH analogue which is injected subcutaneously and is used in the treatment of advanced prostate cancer (Dijkman et al., 1990). Results from clinical studies have revealed that the implant may be effective over at least 14 weeks when injected into the upper arm of patients (Dijkman et al., 1990). An implant of a gonadotropin releasing hormone agonist namely histrelin, (administered subdermally) has also shown efficacy in the treatment of prostate cancer

(Chertin et al., 2000). Implanted devices which carry radiopharmaceuticals to the site of the tumor have also been synthesized (Sofou et al., 2008).

2.9. The ideal implantable system

When considering implants in the treatment of solid tumors, *in situ* forming implants have an advantage over pre-formed implants as they rule out the need for surgical interventions and hence have both the benefit of higher patient acceptability and reduction of a multitude complications which may occur during surgery. These implant systems also allow delivery of the drugs intratumorally rather than peritumorally as is the case with pre-formed implants. The ideal characteristics of an ideal polymer for an implant include responsiveness to a stimuli such as temperature, pH (~6.75) or light (photopolymerizable); biocompatibility, biodegradability, low cytotoxicity, mucoadhesivity, low concentration effectiveness, easily sterilizable, long storage capability, the ability to form mechanically robust hydrogels, easily modifiable and lastly, the polymer should have an LCST around body temperature. An appropriately designed and defined implant should provide a sustained delivery of drugs (beneficial in reducing the development of multi-drug resistance), retention of activity of the bioactives such as IL2, controlled rate of degradation of the polymer, desirable permeability of the hydrogel matrix, desirable sol-gel transition temperature and finally feasibility of the drug delivery system which also depends on the chemical nature of the drug. The most commonly cited problems with the polymer/polymeric implant include: i) quick burst release or release of the bioactive at a lower pH (pH 5-6); ii) negatively charged entities which may interact with bioactive components once in the body; and iii) formation of turbid gels thus necessitating the need of stabilizers during lyophilization. Quite often this results in the formation of hydrogel with very low strength which are also expensive to manufacture. The use of photo-crosslinking can as well be detrimental to the drug e.g. leading to degradation of the drug. Such shortcomings of *in situ* forming implants are described in Table 2.3.

Table 2.3: Comparison of the advantages and disadvantages of *in situ* forming implants.

System	Photocrosslinkable systems	Local stimuli responsive systems	Solvent diffusion systems
Mechanism of operation	Use of an initiator in the implant and an external light source	Responds to local environment e.g. pH or temperature	Contains an organic solvent which diffuses into surrounding body tissue leaving implant <i>in situ</i>
Advantages	Do not require surgical implantation and removal Cost of production (manufacturing is lower than for similar targeted systems e.g. micro- or nano- particles) (Packhauser et al., 2004) Can be used in the delivery of “sensitive ” molecules e.g. proteins (Packhauser et al., 2004)		
Disadvantages	1. Area of injection limited by penetration of the light source (Packhauser et al., 2004) 2. Possibility of toxicity of the initiator (Okino et al., 2002) 3. Drug may be damaged when crosslinking occurs (Cho et al., 2009)	1. Inter-patient variations 2. Differences in injection techniques can result in differences in release kinetics 3. Sterilization technique may pose challenge 4. Initial burst release may occur (Schuetz et al., 2008 and Kakinoki and Taguchi, 2007)	1. Possibility of toxicity of the solvent 2. Initial burst phase release (Schuetz et al., 2008 and Astaneh et al., 2007)

2.10. Concluding Remarks

A few of the systems described in this chapter such as Intrados[®], Atrigel[®], Eligard[®], Gliadel[®], ReGel[®], Cremophor EL[®]-paclitaxel, OncoGel[®], Cytoryn[®] and Zoladex[®] have shown great potential but further development of these systems is warranted for the effective treatment of solid tumors. This chapter served to summarise the various attempts that have been made in the development of an ideal implant system for the treatment of solid tumors. The advantages of an implant system have been highlighted and the opportunities offered by *in situ* forming implants mentioned. Challenges associated with *in situ* forming implants have also been clearly delineated. Subsequent chapters of this dissertation will attempt to overcome the associated challenges and develop an *in situ* forming implant capable of delivery a chemotherapeutic drug to solid tumors over prolonged periods of time.

CHAPTER 3

PREFORMULATION STUDIES CONDUCTED FOR DESIGNING THE *IN SITU* FORMING IMPLANT

3.1. Introduction

Thermoresponsive polymers as described in Chapter 2 of this dissertation are useful for the formation of *in situ* forming implants as these polymers are able to reconfigure from a sol state, at some defined temperature to a gel state, with a change in temperature. Some polymers such as poly(acrylic acid) exhibit an Upper Critical Solution Temperature. At low temperatures the polymer solution exhibits phase separation whereas at higher temperatures, homogeneity is achieved (He et al., 2008). In contrast, polymers exhibiting a Lower Critical Solution Temperature (LCST) undergo a phase separation following heating. Alteration from a homogenous solution to a two phase system consisting of a solid phase and a liquid phase is typically observed (Bokias et al., 2000). The formation of the solid phase in LCST polymers was of interest as this could be used for the formation of an *In Situ* Forming Implant (ISFI).

Poly(methylvinyl ether) (PMVE) is a water soluble, biocompatible polymer which displays thermoresponsiveness and is reported to have a LCST of 32-38°C (Karayanni and Staikos, 2000; Madbouly and Ougizawa, 2005; Nurkeeva et al., 2005). Drug delivery systems using PMVE in combination with maleic acid have been investigated for the delivery of various drugs such as pilocarpine (Urti, 1985) and tetracaine (Woolfson et al., 1998). PMVE-co-maleic anhydride is also used commercially as a denture fixative. However, these systems and products do not utilise the thermoresponsive behaviour of PMVE. Currently there is no literature on the use of PMVE in the formulation of a thermoresponsive drug delivery system for the site specific treatment of solid tumors.

PMVE was selected as it offers two significant advantages for its use as an implantable system for drug delivery. Firstly, the polymer converts from a solution into a gel instantaneously upon heating to its LCST (Figure 3.1) and secondly, the polymer is biocompatible (Nurkeeva et al., 2005). The “sharp” transition from solution phase to the precipitated gel-like phase was considered a distinct advantage as it would stop a

significant burst release of drug as observed in other *in situ* forming implants (Astaneh et al., 2007 and Schuetz et al., 2008).

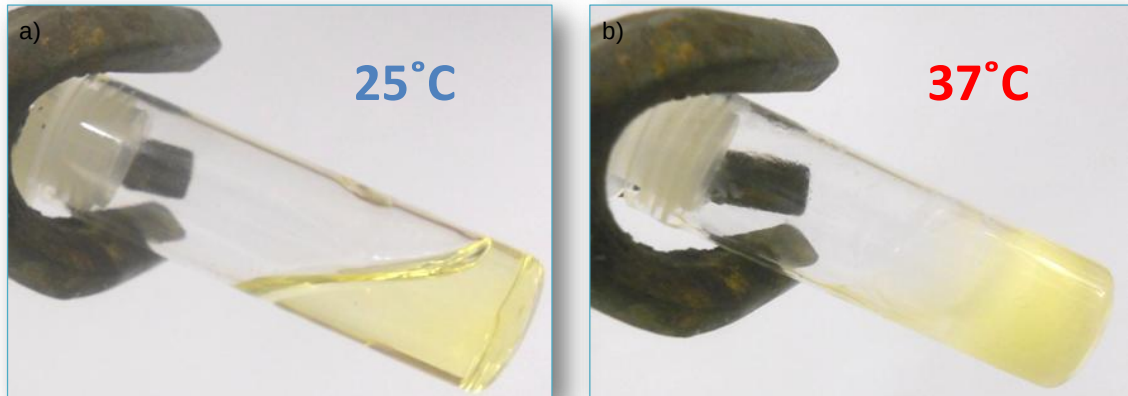


Figure 3.1: Digital images showing the transition of PMVE from a) a solution to b) a gel following heating to 37°C.

Many other *in situ* forming systems take a few minutes to form *in situ* and as a result the drug has sufficient time to be released from the formulation and into the surrounding tissue (Hacker et al., 2008 and Schuetz et al., 2008).

Gelation of PMVE is thought to be as a result of its hydrophilic (ether oxygens) and hydrophobic (vinyl backbone and methyl side groups) regions as shown in Figure 3.2 (Karayanni and Staikos, 2000).

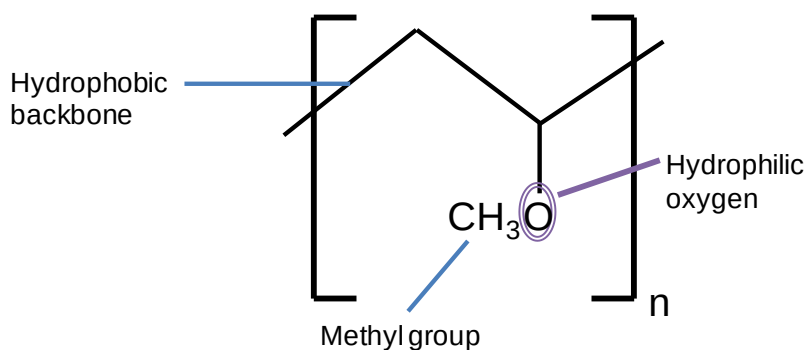


Figure 3.2: Chemical structure of PMVE showing the hydrophilic oxygen that is responsible for the solvation of the polymer at room temperature.

As a result the polymer is solvated at room temperature (surrounded by water molecules) due to the presence of the ether oxygens on the polymer. However, at temperatures exceeding the LCST of the polymer, the hydration sheath surrounding the polymer chains is damaged and the interactions between the hydrophobic areas of the polymer such as the vinyl backbone and methyl groups on the polymer, become more important and as a result the water molecules are forced out, resulting in the “collapse” of the polymeric strands (precipitation of the sol or gel formation) (Karayanni and Staikos, 2000).

Other polymers displaying thermosensitivity, such as hydroxypropylcellulose and methylcellulose in combination with other additives, were also investigated, but are not discussed further in this dissertation, since results obtained with these polymers were not as desirable as those obtained with PMVE.

In order to control the initial burst release from the PMVE formulations, other polymers were added to the formulation. The burst effect was postulated to be related to the syneresis of water on formation of the implant and hence various water absorbable polymers were selected to minimise this effect. Gum arabic, a polysaccharide extracted from the gum of the acacia tree (Phillips et al., 1996), carrageenan, a polysaccharide of seaweed origin (Usov, 1998) and polyacrylamide (PAAm) a polymer able to imbibe large amounts of water (Martínez-Ruvalcaba et al., 2008) were investigated. An additional reason for the selection of carrageenan was due to the research conducted by Tomšič et al., 2008 on the thermogelation properties of κ -carrageenan. As the

polymer available was of mixed composition, i.e. it contained all forms of carrageenan and not solely κ -carrageenan, the results obtained would be of interest.

The effect of a salt, calcium chloride in addition to the polysaccharides was also investigated due to the influence of calcium ions on the gelation characteristics of polysaccharides such as carrageenan (Usov, 1998).

Other polymers investigated included alginate, hydroxypropylcellulose and gellan gum. However these results are not reported on due to undesirable results obtained. Polyacrylic acid (PAA) was also used and the results are reported here. Other salts investigated included potassium chloride and sodium bicarbonate. Sodium bicarbonate showed promising results and is discussed further.

3.2. Materials and Methods

3.2.1. Materials

Poly(methyl vinyl ether) (PMVE) (50%wt in water), folic acid (pteroylglutamic acid, 98% purity), polyacrylamide (PAAm) ($M_w = 5-6 \times 10^6$ g/mol), carrageenan, sodium alginate (with a viscosity of a 2% solution at 25°C reported to be ~3500 cP) and dialysis tubing (MWCO 12kDa, flat width 33mm) were purchased from Sigma-Aldrich (Steinheim, Germany). A sample of hydroxypropylcellulose (Klucel®, Type EF) was obtained from Hercules Incorporated (DE, USA). Sodium hydroxide pellets (NaOH, $M_w = 40$ g/mol), calcium chloride dihydrate (CaCl_2 , $M_w = 147.02$ g/mol) and gum arabic powder were obtained from Rochelle Chemicals (Johannesburg, South Africa). Sodium dihydrogen phosphate (NaH_2PO_4 , $M_w = 141.96$ g/mol), and sodium bicarbonate (NaHCO_3 , $M_w = 84.01$ g/mol) was purchased from Merck, Wadeville, Gauteng, South Africa. Potassium chloride (KCl, $M_w = 74.56$ g/mol), potassium dihydrogen phosphate ($M_w = 136.09$ g/mol) and sodium chloride (NaCl, $M_w = 58.44$ g/mol) were obtained from ACE Chemicals (Southdale, South Africa). Double deionised water (Milli-Q Gradient, Millipore, MA, USA, electrical conductivity 18.2 M Ω .cm at 25°C) was used to prepare all solutions of the polymer and all other buffered solutions.

3.2.2. Preparation of PMVE solutions

Solutions of PMVE were diluted to 10, 20 or 30%^{w/v} using double deionised water. Since the high viscosity of the supplied PMVE (50%^{w/v}) made accurate measuring of the polymer as a liquid challenging, Equation 3.1 was utilised to determine the volume of solutions. Briefly, the polymer was weighed (m) and the density of PMVE ($\rho=1.03$) was used to determine the volume (v) of polymer solution. Using the volume obtained, dilutions were then made using deionised water to yield polymeric solutions of the correct concentration.

$$\rho = \frac{m}{v} \dots\dots\dots \text{Equation 3.1}$$

In order to adjust the pH, a standard quantity of 100 μ L of NaOH (1M) was added to 10mL of each solution prepared. This altered the pH of the solution to ~ 6.75.

3.2.2.1. Preparation of formulations to minimise the burst release of drug

PAAm combinations with PMVE were prepared. Briefly, a solution of PAAm (1% or 5%^{w/v}) was prepared and added to 20% PMVE formulations in ratios of 1:10 or 10:1.

Other formulations were prepared as shown in Table 3.1. Gum arabic, carrageenan and the salts were added as powders with the correct weight to appropriately diluted PMVE solutions to produce formulations with concentrations as listed in Table 3.1. When the effect of a combination of both CaCl₂ and gum arabic was investigated, formulations were prepared as follows. Briefly, to produce the formulation containing 0.1M CaCl₂ and 4%^{w/v} gum arabic: 0.284g of CaCl₂ was added to 20mL of a PMVE solution diluted to the correct concentration. The solution was mixed until the salt had dissolved in the polymer solution. Gum arabic (0.8g) was then added to the solution to yield a formulation as described in Table 3.1. The solution was then physically stirred using a glass rod until a homogenous solution formed. The model drug, folic acid was added to the solutions at a concentration of 5mg/mL per formulation.

Table 3.1: Composition of ISFI formulations formulated for investigation

Formulation	Concentration of PMVE (% ^w /v)	Concentration of Gum Arabic (% ^w /v)	Concentration of Carrageenan (% ^w /v)	Concentration of CaCl ₂ (M)	Concentration of NaHCO ₃ (M)
1	10	0	0	0	0
2	20	0	0	0	0
3	30	0	0	0	0
The effect of gum arabic					
4	20	1	0	0	0
5	20	4	0	0	0
6	20	10	0	0	0
The effect of carrageenan					
7	20	0	1	0	0
8	20	0	5	0	0
9	20	0	10	0	0
The effect of gum arabic and CaCl ₂					
10	20	4	0	0.05	0
11	20	4	0	0.1	0
12	20	4	0	0.2	0
The effect of CaCl ₂					
13	20	0	0	0.05	0
14	20	0	0	0.1	0
15	20	0	0	0.2	0
16	20	0	0	0.3	0
17	10	0	0	0.2	0
The effect of NaHCO ₃					
18	20	0	0	0	0.05
19	20	0	0	0	0.1

3.2.3. Determination of the gelation temperature of the polymeric formulations utilising oscillatory rheology

The study of the flow properties was considered extremely significant to this study as it is central to the mechanisms by which the ISFI functions. At room temperature the implant remains in the liquid state to allow delivery via a 18G needle and at body temperature (37°C) the implant forms a solid-like structure. In order to characterise and analyse the flow behaviour and determine the LCST or gelation temperature of the ISFI formulations, rheology studies were conducted using a Haake Modular Advanced Rheometer System (ThermoFisher Scientific, Germany). As the polymeric material acts as a viscoelastic solid, some background on viscoelastic solids is provided.

3.2.3.1. Viscoelastic solids

Polymer solutions often exhibit viscoelastic properties. The term “viscoelastic” implies that they behave neither like a liquid which flows when a force is applied and do not return to their original shape (as depicted by the dashpot, Figure 3.3(a)) nor like an elastic material which when a force is applied to it, deforms and upon removal of the force returns to its original shape (much like a spring: Figure 3.3(b)). Instead, a viscoelastic solid exhibits properties of both these materials Figure 3.3(c) (Marriott, 2004).

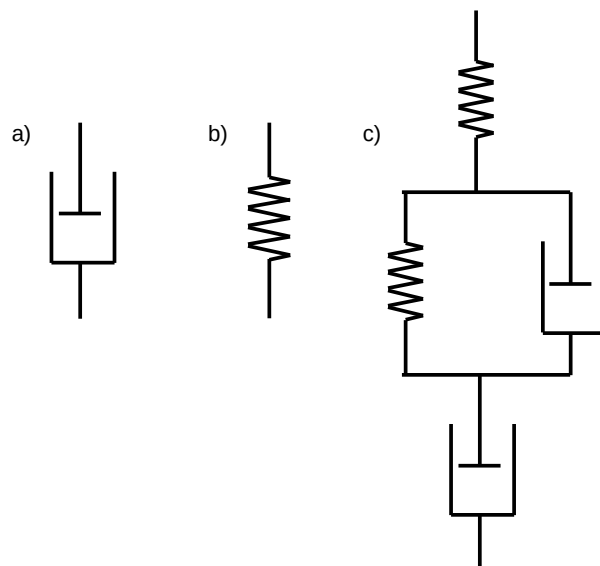


Figure 3.3: Schematic showing a model to aid in the understanding of viscoelastic materials (Adapted from Marriott, 2004).

For elastic materials, Hook's Law of Elasticity is used to characterise the flow properties and the term shear modulus (G) is used to relate the relationship between the shear stress (τ) and the deformation (γ) (Equation 3.2). Liquids are defined by Newton's Law of Viscosity (Equation 3.3) whereby the shear stress (τ) is proportional to the shear rate of the liquid ($\frac{\Delta\gamma}{\Delta t}$).

$$\tau = G\gamma \dots \dots \dots \text{Equation 3.2}$$

$$\tau = \eta \frac{\Delta\gamma}{\Delta t} \dots \dots \dots \text{Equation 3.3}$$

In order to characterise viscoelastic solids, the use of oscillatory rheology is required. The principle of oscillatory rheology can be explained as the induction of a sinusoidal shear deformation in the sample being analysed and the measurement of the resultant stress. As shown in Figure 3.4 the application of a sinusoidal strain results in different resultant stress patterns in elastic solids, viscous fluids and a viscoelastic solid. The viscoelastic solid shows a phase shift that lies between the phase shift of 0 for an elastic solid and $\pi/2$ for a viscous fluid (Wyss et al., 2007).

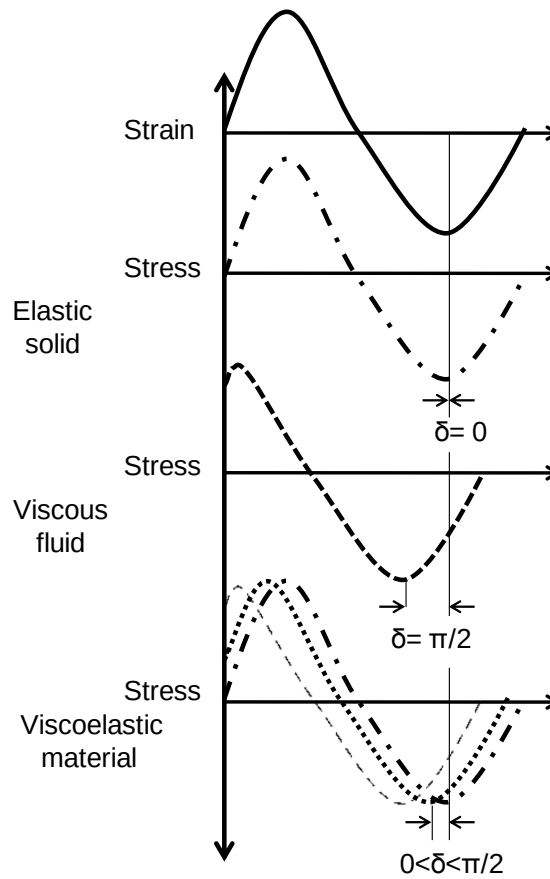


Figure 3.4: Phase shifts in different materials following application of a sinusoidal strain (Adapted from Wyss et al. 2007).

For viscoelastic solids, as the behaviour of the sample is between the extremes of elastic and viscous behaviour, additional terms are required for definition relating the elastic modulus component or storage modulus, G' , and the viscosity modulus or loss modulus, G'' , to the angular frequency, ω . The complex modulus, G represents the total resistance of the substance to oscillatory shear and is determined using Equation 3.4. The complex viscosity (η') is the flow resistance of the sample and is determined using Equation 3.5 and Equation 3.6.

$$G = G' + iG'' = (G'^2 + G''^2)^{\frac{1}{2}} \dots\dots\dots \text{Equation 3.4}$$

$$\tan \delta = \frac{G''}{G'} \dots\dots\dots \text{Equation 3.5}$$

$$\text{And } \eta' = \frac{G}{\omega} \dots\dots\dots \text{Equation 3.6}$$

Where δ is the phase angle, G' is the storage modulus, G'' is the loss modulus, η' is the complex viscosity and ω is the angular frequency.

3.2.3.2. Determination of the viscoelastic region

Viscoelastic solids should be tested for their properties within the viscoelastic region. In order to determine the viscoelastic region of the formulation samples, each sample was placed on the lower plate of the rheometer and the yield stress of the formulation was first determined, as this is the point at which a minimum critical shear stress is exceeded and hence the point at which a viscoelastic substance begins to flow. Stress sweeps at 0.01Hz, 1Hz and 10Hz were then conducted using an applied strain of 0.1-70Pa ensuring that the minimum strain applied was less than the yield stress value obtained. The stress sweep plots were then analysed and the plateau or linear viscoelastic region where both G' (storage modulus) and G'' (loss modulus) were independent of the stress amplitude, was used as the viscoelastic region. The first point of deviation from the plateau and the frequency at which it occurred was used for subsequent temperature ramping tests.

3.2.3.3. Determination of the gelation temperature of the polymer

In order to determine the lower critical solution temperature (LCST) and hence the gelation temperature of the ISFI formulation, the temperature of samples was ramped from 20-50°C at a rate of 0.25°C/min while applying the predetermined stress obtained from the stress sweeps previously described, at the frequency observed in that test. The gelation temperature was determined as the temperature at which the cross-over of G' and G'' occurred i.e., the point at which the formulation was no longer acting as a liquid (G'') but as a solid (G'). In all cases a solvent trap was used to prevent sample evaporation.

3.2.4. Construction of a standard curve for the quantification of folic acid release from ISFI using ultraviolet spectroscopy

A stock solution of folic acid in phosphate buffered saline (PBS) was prepared at a concentration of 2%^{w/v}. Serial dilutions were then made to yield a concentration range of (0.0018-0.15mg/mL). The solutions were then assayed using a UV Spectrophotometer (Specord 40, Analytik Jena, AG, Germany) using a wavelength of 280nm. Assays were conducted in triplicate and on two different days, to determine whether the results obtained were reproducible.

3.2.5. Release of folic acid from the formulations under conditions mimicking those at the tumor site

Drug release studies were conducted in an orbital shaker bath (Orbital Shaker Incubator, LM-530, Yihder, Taiwan) (37°C, 25rpm). A dialysis tubing method similar to that described by Graves et al. (2007) was used. Briefly, the dialysis tubing was thoroughly rinsed to remove preservative fluid and then cut into 10cm pieces. One end of the tubing was tied and the tubing was filled with 5mL of dissolution fluid (PBS, 6.75, 37°C). The formulation being tested (3mL) was injected into the dialysis tubing and the other end of the tubing was also tied. The dialysis bags were then placed into vessels filled with 100mL of PBS (pH 6.75) as shown in Figure 3.5 and the vessels were placed into the shaker bath. Samples of 10mL were drawn at: 1 hour, 4.5 hours and 1, 2, 3, 5, 7, 12 and 17 days. Pre-warmed buffer (10mL) was replaced at each time interval to maintain sink conditions. Samples were analysed using a UV spectrophotometer (Specord 40, Analytik Jena, AG, Germany) at the wavelength for folic acid, 280nm. PBS was prepared in accordance with tissue culture requirements with the following molar quantities: 137mM NaCl, 2.7mM KCl, 10mM NaH₂PO₄, 2mM KH₂PO₄ (Sambrook et al., 1989). The solution was adjusted to pH 6.75 in order to mimic conditions of the tumor site (Martin and Jain, 1994).

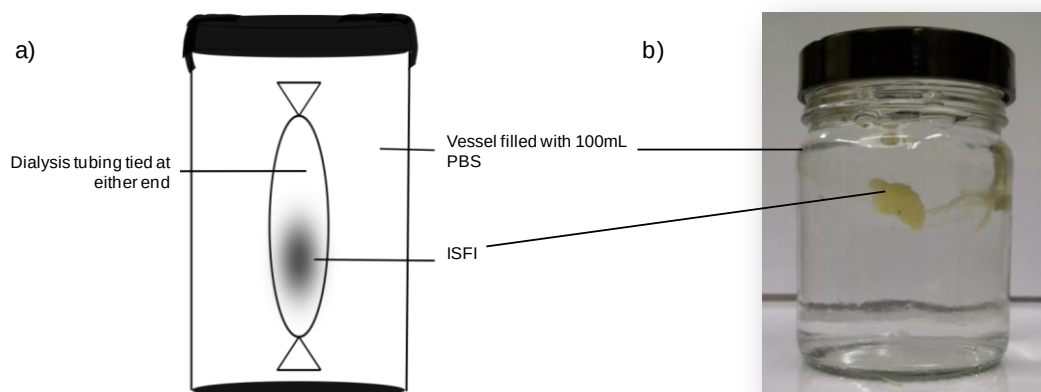


Figure 3.5: a) Schematic showing the drug release method used to determine the release of folic acid from the ISFI, b) digital image showing the actual drug release vessel with the suspended implant within the dialysis tubing clearly shown.

3.3. Results and Discussion

3.3.1. Construction of a standard curve for the quantification of folic acid released from the ISFI

Beer Lamberts Law (Equation 3.7) describes a linear relationship between the absorbance (A) of a dilute substance ($<0.01M$) and its concentration (c), provided that the pathlength of the light (b) through the solution remains constant. It also describes some absorptivity value that is specific for the substance being assayed. This specific absorptivity can be used on the determination of concentration of released drugs utilising the constructed standard curve relating absorbance to concentration.

$$A = \alpha \cdot a \cdot b \cdot c \dots \dots \dots \text{Equation 3.7}$$

As shown in Figure 3.6, a straight line through the origin was obtained. Regression coefficients obtained for the calibration curves conducted on two consecutive days were 0.998 and 0.991 with the absorptivity values only differing slightly from each other. The standard deviations between absorbance values obtained for solutions having the same concentration on the same day was < 0.025 and the standard deviation between absorbances of the same solution on consecutive days was 0.045. These results

indicate the precision of the instrument and allowed the usage of the UV spectrophotometer in subsequent tests.

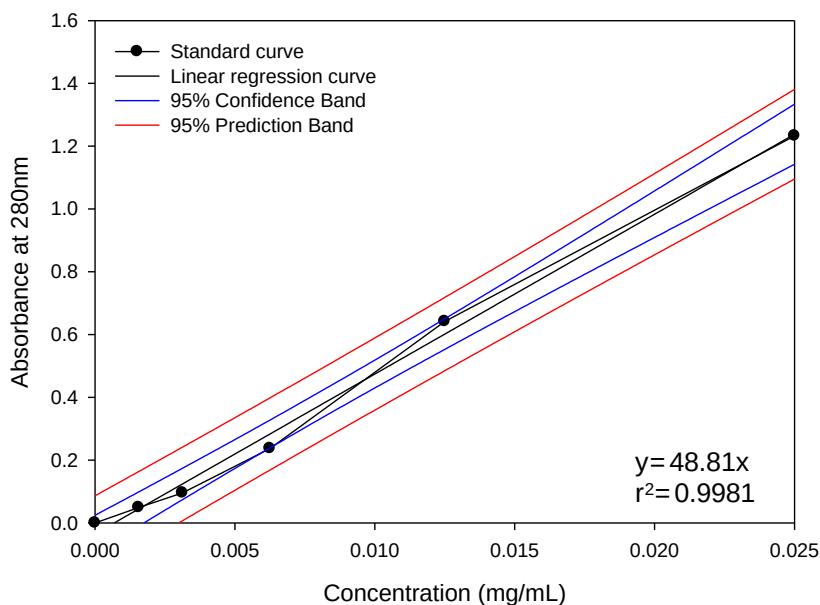


Figure 3.6: Calibration curve of the absorbance of folic acid in PBS (pH 6.75) using UV spectroscopy at 280nm (in all cases $n=3$ and $SD<0.025$).

3.3.2. Determination of the viscoelastic region of PMVE and gelation temperatures

In accordance with the process described in Chapter 3, Section 3.2.3.2 the viscoelastic region of PMVE was first determined. As shown in Figure 3.7, the yield stress of the polymer was determined. Using this data, subsequent tests were run at stress values which did not exceed this value. Consequently stress sweeps were run from 0.1-70Pa, with the yield stress value within the minimum and maximum stresses. Results of the stress sweeps as shown in Figure 3.8 clearly depict the plateau phase associated with the viscoelastic phase and the point of first deviation from the plateau was used for subsequent tests. As the stress sweep at 10Hz showed the most horizontal plateau, it was therefore used for subsequent tests, together with a stress of 13.4Pa.

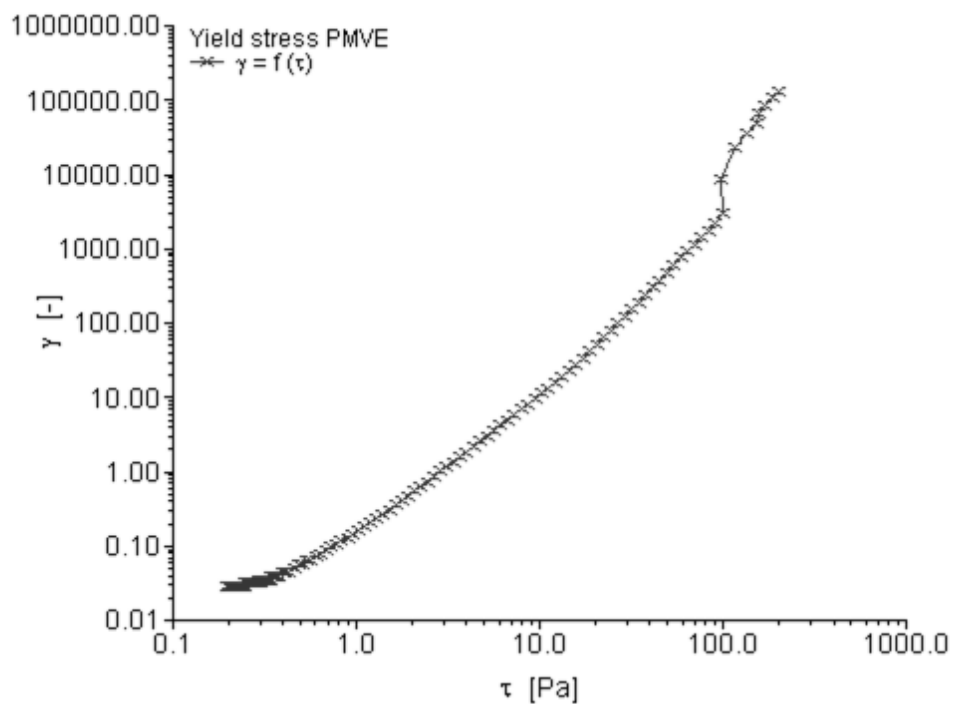


Figure 3.7: Rheological plot of deformation vs. stress showing the yield stress of PMVE.

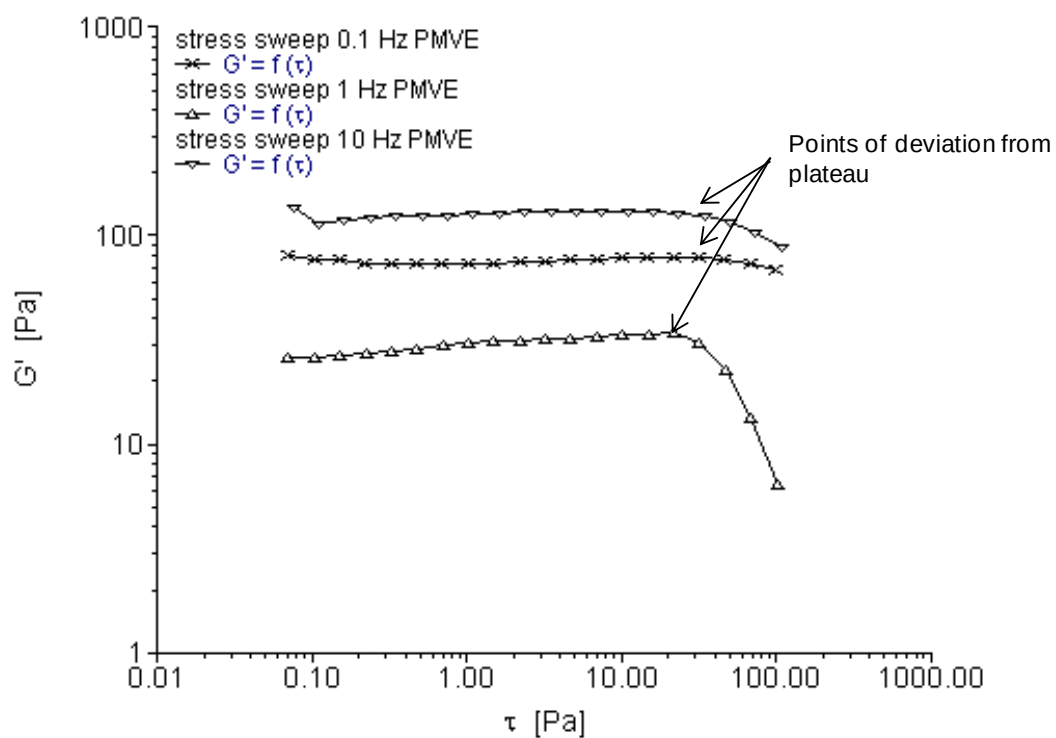


Figure 3.8: Stress sweeps obtained for PMVE at various frequencies.

As shown by the gelation temperatures in Figure 3.9, the gelation temperature of PMVE changes with a change in concentration. However as can be seen the storage modulus varies with the higher concentration of the polymer and the effect is highest when the concentration of polymer is highest.

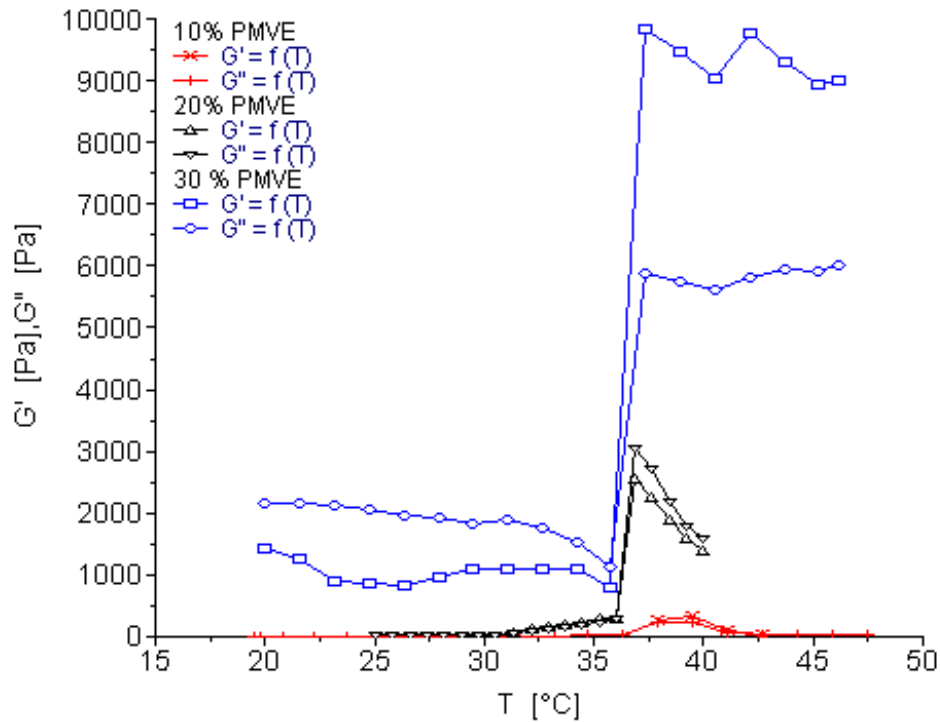


Figure 3.9: Gelation temperatures of different concentrations of PMVE.

3.3.3. The effect of increasing polymer concentration on the release of folic acid from the ISFI

As can be seen in Figure 3.10, the 10%^{w/v} formulation showed a significant burst release over 3 days, whereas the other formulations showed less of an initial burst release.

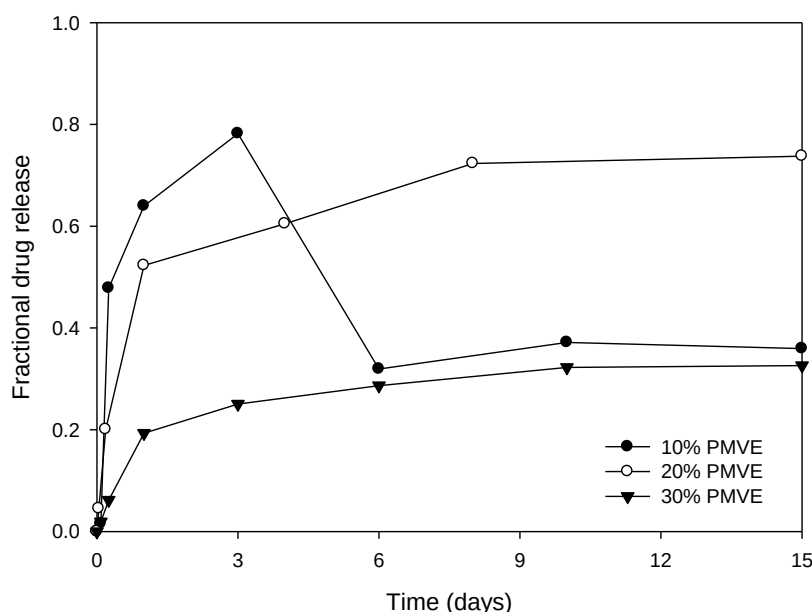


Figure 3.10: Drug release profiles of formulations with increasing amounts of PMVE (n=2, SD< 0.09).

The erratic release of drug from the 10%^{w/v} formulation coupled with the initial burst release excluded it from further analysis. The 20%^{w/v} PMVE formulation showed prolonged release, however, about 50% of the drug was released in 24 hours. The 30%^{w/v} formulations showed prolonged release and a very low burst effect. However, the increase in PMVE in the 30%^{w/v} also increased the viscosity of the formulation considerably. As a result, the 20%^{w/v} PMVE containing formulation was chosen for further modification. Of concern was the burst release from the 20%^{w/v} formulation that occurred over 24 hours and attempts were made to decrease this burst release through the addition of various polymers.

3.3.3.1. The effect of carrageenan, gum arabic, PAAm and other polymers on the release profile of the ISFI formulation

As shown by Figure 3.11a, the addition of carrageenan to the formulation did not change the burst release of the ISFI and actually appeared to have increased the burst effect while the addition of gum arabic (Figure 3.11(b)) also had very little impact on decreasing the burst release of drug from the polymer (24 hour profile shown). Increasing amounts of either gum arabic or carrageenan in the formulation decreased the release (i.e. a 10%^{w/v} carrageenan formulation had lower release than a 1%^{w/v} formulation). However, the increasing quantity of polymer naturally increased the

viscosity, an unwanted effect as this would cause difficulty in administration of the ISFI. The possible lack of effect of these two polymers on the drug release is possibly due to the large size of the polysaccharide molecules which did not interact with the PMVE and hence did not decrease the burst effect from the formulations perhaps indicating that the burst release is not in fact due to the syneresis of water from the formulation or that the gum arabic and carrageenan polymers were unable to absorb this water with the drug. It is a possibility that the burst release of the drug was caused by surface deposition of the drug. Rheological characterization was performed but results were unremarkable.

The addition of PAAm, a non-ionic polymer did not prove fruitful either as upon standing formulations separated into two distinct phases indicating immiscibility. PAAm is classified as a proton acceptor (Koussathana et al., 1997) and as a result the phase separation and lack of interaction is possibly a result of the fact that PMVE is also a proton acceptor rather than a proton donator due to its $-\text{OCH}_3$ group (Nurkeeva et al., 2005). The interaction between a proton donator and a proton acceptor was clearly seen when attempts were made to add PAA to PMVE solutions. The addition of PAA even at low concentrations ($0.05\% \text{w/v}$) caused precipitation of PMVE. This is in accordance with the literature as reported by Cowie et al. (1989), and is a result of hydrogen bond formation between PAA and PMVE due to the proton donating groups of PAA.

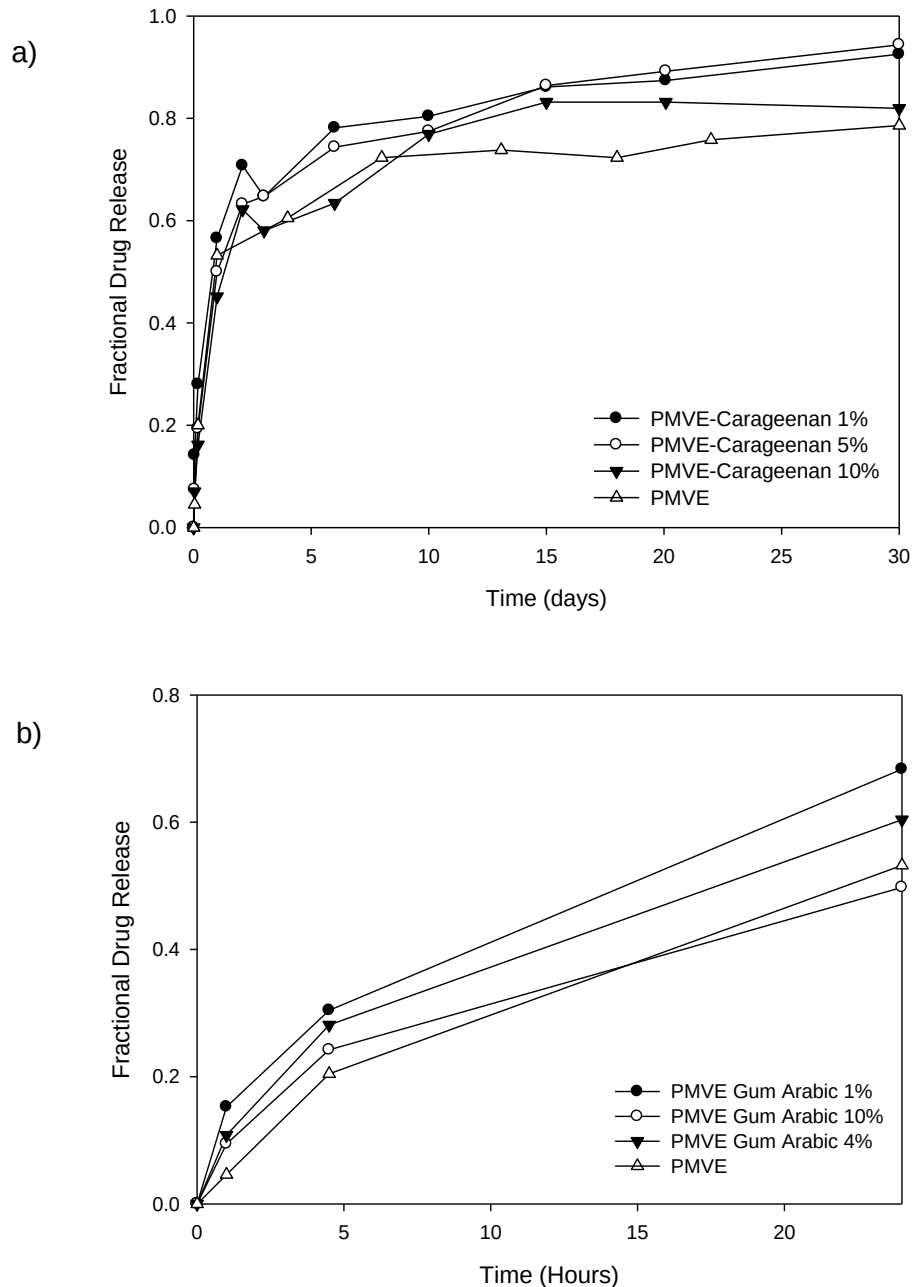


Figure 3.11: a) Drug release profiles obtained from the carrageenan containing ISFI formulations ($n=2$, $SD < 0.1$) b) The effect of gum arabic on the drug release profile of PMVE (25 hour profile). (37°C , $\text{pH } 6.75$, $n=2$, $SD < 0.069$).

3.3.3.2. The effect of gum arabic and CaCl_2 on drug release from the ISFI formulation

Following unsuccessful results with carrageenan and gum arabic, CaCl_2 was then investigated in addition to the gum arabic and carrageenan, in the hope that the salt would increase the viscosity of the polysaccharide polymers (Uosv,1998) and hence decrease the release from the ISFI. However, the addition of CaCl_2 to carrageenan formulations did not appear to affect the release tremendously while the addition of the

salt to gum arabic-containing formulation proved more successful as shown in Figure 3.12.

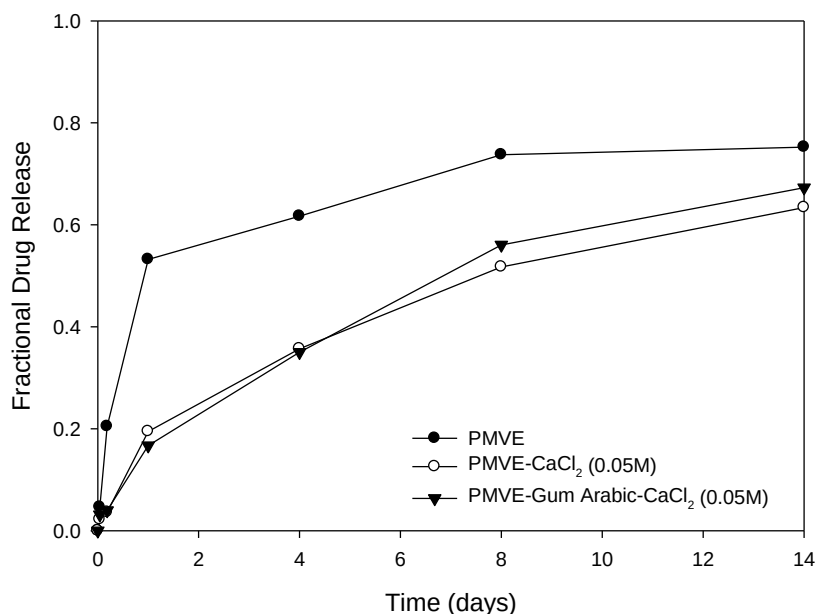


Figure 3.12: PMVE-gum arabic and CaCl₂ (0.1M) compared to a combination of PMVE-CaCl₂ (0.1M) on the drug release profile (37.5°C, pH 6.75). (37°C, pH 6.75, n=2, SD< 0.04).

The addition of both gum arabic and CaCl₂ caused an initial slower release of folic acid. However, as the effect of gum arabic minimal as described previously, the effect of the salt on drug release was then investigated and found to be as effective in reducing the burst effect as the combination of gum arabic and CaCl₂ (Figure 3.12). For this reason, gum arabic was no longer utilised and the effect of differing concentrations of CaCl₂ were investigated.

3.3.3.3. The effect of CaCl₂ on the release profile of the ISFI formulation and on the gelation temperatures of the ISFI

Following the discovery that the salt was responsible for the decrease in the burst release of drug from the polymer, CaCl₂ was added to formulations in different concentrations and drug release and rheological assessment was performed.

As shown in Figure 3.12, the addition of CaCl_2 slowed the release of folic acid from the ISFI and the increasing quantity of CaCl_2 decreased the release further (Figure 3.13). However, increasing the concentration of CaCl_2 to concentrations above 0.3M caused immediate precipitation of PMVE in the formulation. In addition, it was intended that the quantity of salt used would be kept to a minimum due to concerns of local toxicity. A discussion of the possible effect of CaCl_2 on PMVE follows in Chapter 4, Section 4.3.5.

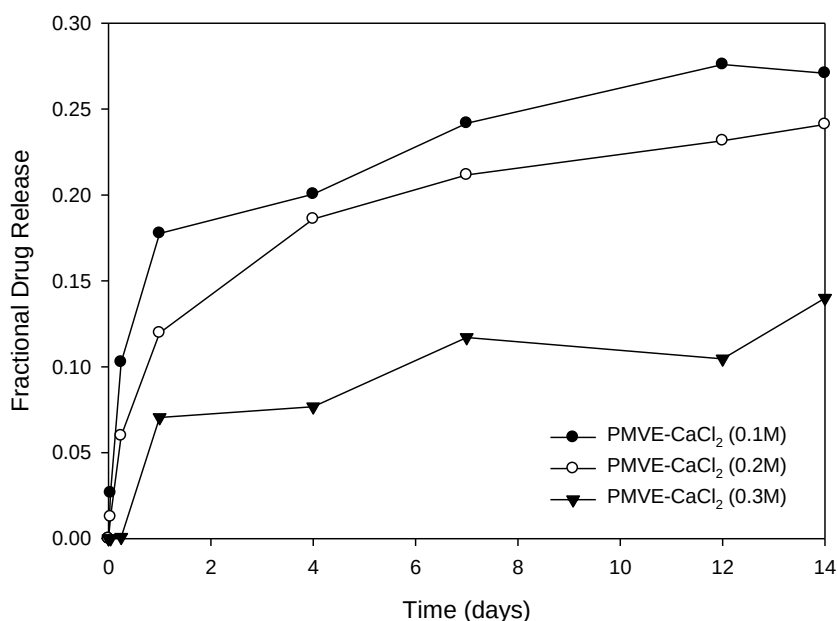


Figure 3.13: The effect of increasing the quantity of CaCl_2 on the release of drug from ISFI formulations (37°C , pH 6.75, $n=2$, $\text{SD} < 0.051$).

The effect of CaCl_2 on formulations containing 10% $^w/v$ PMVE was also investigated and showed a decreased burst release although still present. Unfortunately while decreasing the burst release of drug from the ISFI, CaCl_2 also decreased the gelation temperature of the polymer. Formulations containing 0.3M CaCl_2 had a gelation temperature of 26.0°C ($\pm 0.3^\circ\text{C}$) and this was counter-productive as this temperature was too close to ambient temperatures which could result in gelation of the ISFI prior to administration or while handling. For this reason 0.3M formulations of CaCl_2 were not considered ideal and higher concentrations of PMVE were not investigated. As can be noted in Figure 3.14, the G' values for the formulations also increased with an increase in CaCl_2 concentration.

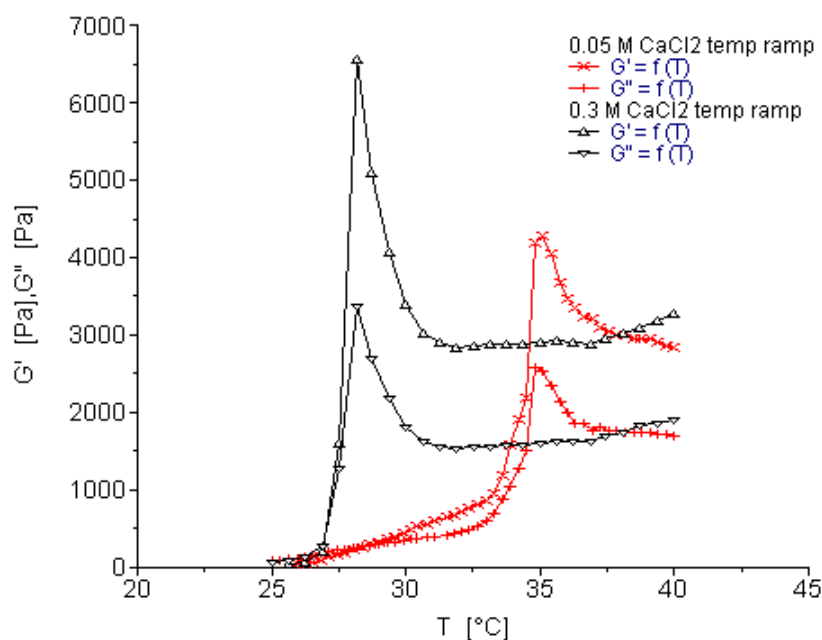


Figure 3.14: The effect of CaCl_2 on PMVE with regards to gelation temperature.

Following the success of CaCl_2 , the effect of other salts were also investigated. Of these NaHCO_3 showed promising results. The addition of NaHCO_3 increased the release of drug from the polymer and on visual inspection of the formulation in the dissolution medium appeared to have increased the breakdown of the polymer formulation (Figure 3.15). Higher concentrations of NaHCO_3 proved detrimental as they caused immediate precipitation of the polymer. This formulation was considered to be of interest as it increased the rate of folic acid release a great deal and hence could be considered for use as the “erosion controlling substance” mentioned in Chapter 1, Section 1.2 of this dissertation. While the mechanism of increased release from the formulations containing NaHCO_3 have not been elucidated, and hence it is unknown whether NaHCO_3 does in fact increase the erosion of the polymer, addition of NaHCO_3 increased the release of the drug. While data on PMVE is not readily available, studies have been performed on the PMVE-maleic anhydride copolymer. Finne et al. (1989) explained that the alteration of the pH in that polymer by the addition of salts caused an increase in the pH of the polymer surface and consequently lead to an increase of the entrapped drug. Without the addition of the basic salts, H^+ ions accumulated on the surface of the polymer inhibiting polymer dissolution (Finne et al., 1989). However, the source of the H^+ ions is the carboxylic esters (maleic anhydride esters) (Heller, 1985) on the polymeric chain and hence this effect is not possible with PMVE.

Regardless of the mechanism of increasing the drug release from the polymer, a faster release indicated that the salt be investigated further. For this reason it was further investigated in microparticles and results obtained are reported in Chapter 5 of this dissertation.

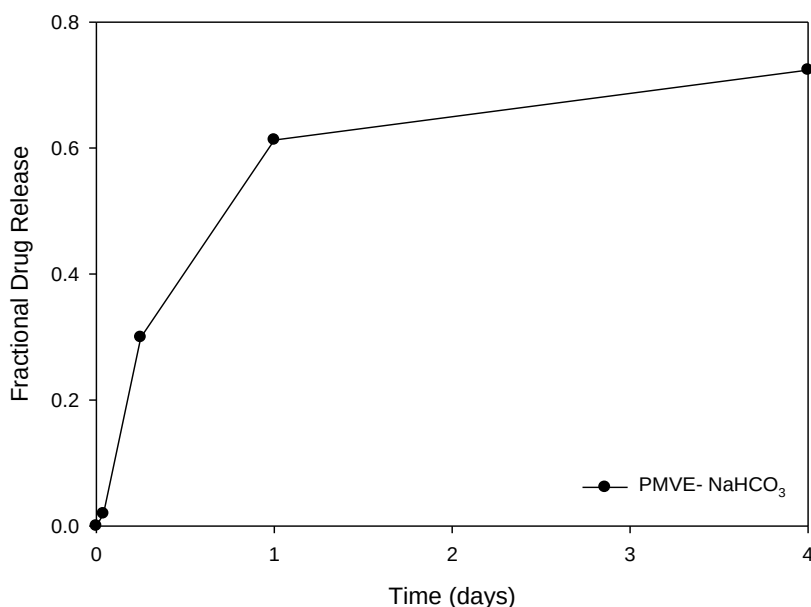


Figure 3.15: Release of folic acid from the sodium bicarbonate containing implant (37°C, pH 6.75, n=2, SD<0.5).

Rheological analysis of formulations containing NaHCO₃ also proved interesting as the gelation temperature of the formulations also decreased exactly as noted with CaCl₂ containing formulations. However the G' value was much lower than both the formulation containing an equivalent amount of PMVE and a CaCl₂ containing formulation. This is an interesting discovery as the addition of CaCl₂ caused a decrease in gelation temperature but an increase in the storage modulus. It is possible that the ionic nature of NaHCO₃ caused a decrease in the gelation temperature and the decrease in the storage modulus was caused by the effect of pH change on the polymer.

3.4. Concluding Remarks

A preliminary ISFI formulation was developed, rheologically tested and drug release from the formulation was determined. It was established that PMVE was a viable

choice of polymer for the formulation of an ISFI. However, there was a slight burst effect observed when the polymer was used in isolation especially at lower concentrations. CaCl_2 was investigated and showed promise in decreasing the release of folic acid from the ISFI. An experimental design was selected for the determination of a statistically optimal ISFI formulation and input formulation variables were therefore as follows: PMVE concentration (10-30% $^{w/v}$) and CaCl_2 concentration (0.05M-0.2M). Chapter 4 describes the Design of Experiments (DoE) strategy selected and the process of determining the optimal ISFI formulation.

CHAPTER 4

STATISTICAL OPTIMIZATION OF THE *IN SITU* FORMING IMPLANT

4.1. Introduction

Statistical optimization has gained popularity due to the numerous advantages that are associated with the use of such a process (Singh et al., 2005). Traditionally, the design of a drug delivery system has been based on trial and error. This method generally involves variation of one variable at a time in the formulation and can be a time consuming process. In addition success depends on characteristics of the formulator such as previous experience, knowledge base, and intuition (Singh et al., 2005). Using this method a satisfactory formulation which satisfies the desired outcomes can be achieved, however the optimal formulation is rarely achieved (Singh et al., 2005). Design of Experiments (DoE) fits experimental data to statistical equations and then utilizes these models in the prediction of formulation performance and in the optimization of the formulation. DoE hence offers a rational method of connecting various experiments and obtaining information from fewer experiments (Singh et al., 2005). Essentially it is the rationality of such an approach that makes it a useful tool in the scientific method. Some advantages of design of experiments (Lewis et al., 1999, Singh et al., 2005 and Furnaletto et al., 2006) are shown in Figure 4.1.

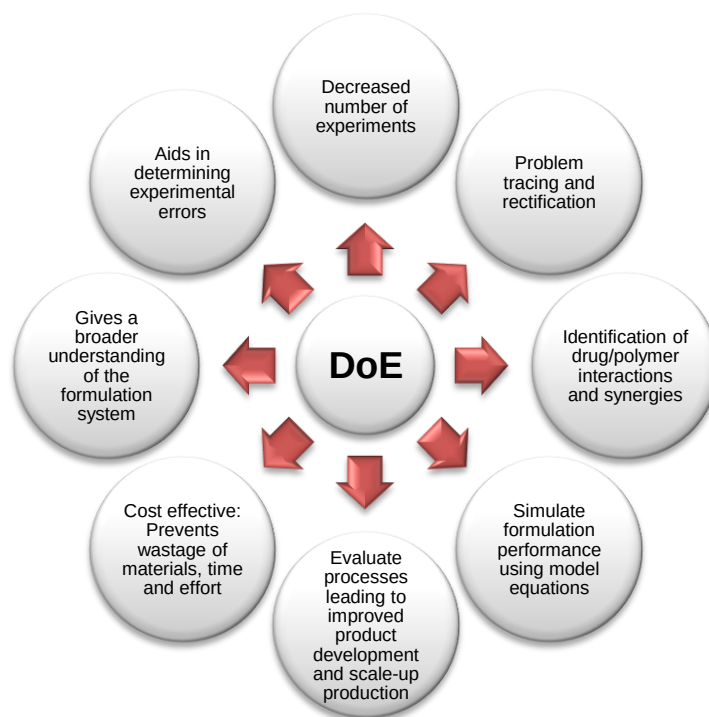


Figure 4.1: Some advantages of utilizing a design of experiments in the development of an optimal formulation.

As concluded in Chapter 3, two variables or factors had been identified to be of interest in the design of an ISFI. The aim of this phase of the study was to employ an experimental design to develop a thermoresponsive implant which would be easily injectable into human tissue, form *in vivo* in response to the temperature of the human body and would be capable of providing release of drug for at least one month. As the response i.e. the effect of the variable on these measured outcomes described was not expected to be linear and response surface plots were required, a Face-Centred Central Composite Design was chosen rather than a factorial design.

Face-Centred Central Composite Designs contain an embedded 2^k (where k = number of factors) factorial design but with the addition of a group of star points and a central point as shown graphically in Figure 4.2. The star points serve to estimate curvature of the response of the variables to the outcome hence providing an idea of the response surface and allowing estimation of interactions. A response surface is a graphical representation of the mathematical data obtained from the design of experiments and a response surface plot shows the relationship of two variables on an outcome. This provides valuable information in understanding the contributions of variables on a

single outcome (Singh et al., 2005). The 9 points as shown in Figure 4.2 constitute the experimental runs. In this study the centre-points were run 6 times, giving a total of 14 formulations.

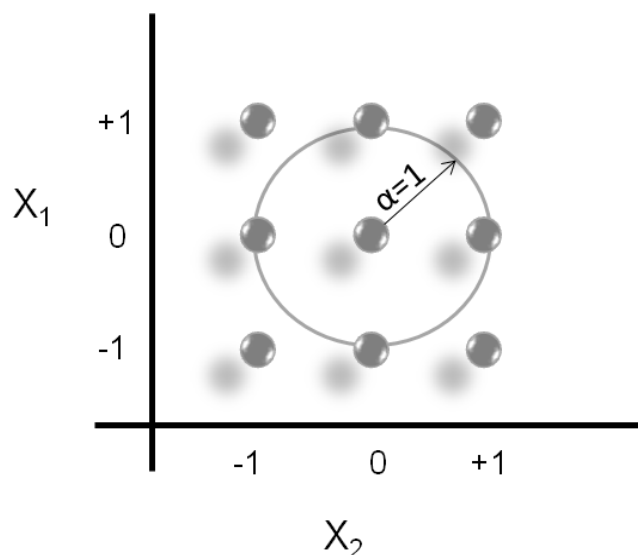


Figure 4.2: Diagrammatic representation of a Face-Centred Central Composite Design with two factors (X_1 and X_2) showing the centre point, 4 factorial points and four axial points (Adapted from Verseput, 2000).

4.2. Materials and Methods

4.2.1. Materials

Materials used were as described in Chapter 3, Section 3.2.1 of this dissertation. Methotrexate ($\geq 95\%$ purity) was purchased from Sigma-Aldrich, Steinheim, Germany.

4.2.2. Design and optimization of the formulations using a Central Composite Design

A two-factor Face-Centred Central Composite Design (FCCCD) was employed for the determination of the effect of the two formulation variables (PMVE concentration and CaCl_2 concentration) on the gelation temperature, force of injectability and Mean Dissolution Time (MDT) of the ISFI.

The ISFI was designed to be easily injectable using standard injection methods and for this reason the force of injectability was minimized to ensure that the optimal formulation would be easily injectable (i.e. less force would be required to inject the formulation *in vivo*). The release of the entrapped chemotherapeutic was to be over a period of one month (> 30 days) which was demonstrated in preliminary results. Finally, the ISFI was intended to form *in vivo* at around the temperature of the body ($37 \pm 0.5^\circ\text{C}$). As preliminary studies revealed that the addition of CaCl_2 contributed to a decrease in the gelation temperature and the ISFI was not to form at temperatures approaching room temperature as this would contribute to difficulty in injecting the polymer solution, the ISFI was optimized within the design by maximizing the gelation temperature. For each of the two parameters selected, two factors were fixed, an upper and a lower level as summarized in Table 4.1 and these values were obtained from preliminary studies undertaken. Also shown in Table 4.1 are the responses and the optimization constraints for each variable. The design was both generated and analysed using Minitab V15 software (Minitab[®] Inc, PA, USA) and for the design 14 experimental formulations were obtained as summarized in Table 4.2.

Table 4.1: Formulation variables and responses applied in design.

	Levels		Objective
	Upper	Lower	
Parameters			
PMVE (% ^w / _v)	30	10	
CaCl ₂ (M)	0.20	0.05	
Responses			
Gelation Temperature (°C)			Maximize
MDT*			Minimize
Force of Injectability (N)			Minimize

*MDT: Mean Dissolution Time

4.2.3. Preparation of *in situ* forming implant (ISFI) solutions

The experimental design template provided by the FCCCD was utilised to prepare 14 formulations containing varying quantities of polymer and salt. Compositions of the formulations prepared are shown in Table 2. All formulations were prepared in duplicate. Briefly, to produce the formulation a 30%^w/_v PMVE formulation containing 0.1M CaCl_2 : 15g of PMVE (50%^w/_v) (density, $\rho=1.03\text{g/mL}$) was diluted with 9.71mL deionised Milli-Q water to give a 30%^w/_v solution. CaCl_2 (0.142g) was then added to

10mL of the 30%^{w/v} PMVE solution. The solution was then placed under magnetic stirring until a homogenous solution formed. NaOH (1M, 150 μ L) was added to each formulation while the pH was measured to ensure that the pH of the implant was approximately 7.2. When drug release studies were conducted, folic acid or methotrexate (MTX) (for the optimized formulation) was added to the solutions in a concentration of 15mg/3mL of formulation.

Table 4.2: Compositions of ISFI solutions formulated in accordance with the FCCCD design template.

Formulation Number	Concentration of PMVE (% ^{w/v})	Concentration of CaCl ₂ (M)
1	30	0.200
2	10	0.200
3	20	0.125
4	20	0.125
5	10	0.050
6	30	0.050
7	20	0.125
8	20	0.200
9	30	0.125
10	20	0.125
11	20	0.125
12	20	0.125
13	10	0.125
14	20	0.050

4.2.4. Determination of the viscoelastic behavior and the gelation temperature utilizing oscillatory rheology

A Haake Modular Advanced Rheometer System (ThermoFisher Scientific, Germany) fitted with a 2° Titanium probe was used for these studies. Stress sweeps at 0.1Hz, 1Hz and 10Hz were conducted on the samples to determine the linear viscoelastic region for the formulations. Using the information obtained, samples were then exposed to a fixed strain and oscillation (18Pa, 10Hz) while the temperature of the sample was increased from 20-40°C in a ramped temperature flow curve (0.042°/min). The LCST was defined as the temperature at which there was a significant increase in the storage modulus (G') and dynamic viscosity (η'). All tests were conducted in duplicate.

4.2.5. Determination of the force of injectability of the ISFI

A Textural Analyser (TA.XTplus Texture Analyser, StableMicroSystems, England) was fitted with a 2mm cylindrical steel probe and a 5kg load-cell and the samples were tested for the ability to be easily injected. The maximum force required to depress the plunger of a syringe filled with the implant formulation was determined and compared with the force required to depress the plunger of a water filled syringe. An 18G needle was fitted at the end of the syringe. Figure 4.3 shows the setup of the equipment used to determine the force of injectability. A typical test involved advancing the probe at a predetermined velocity onto the plunger of the sample-filled syringe in accordance with the following parameters: pre-test and post-test speeds 1mm/s and 3mm/s respectively; test speed 2mm/s; maximum compression force 40N; trigger force 0.001N. Data acquisition was performed at 200 points/sec via Texture Exponent for Windows software, V3.2.

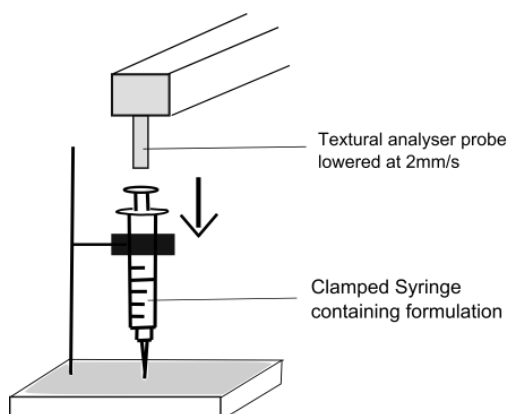


Figure 4.3: Textural analysis instrument set-up used to determine Force of Injectability of the ISFI through syringe fitted with a 18G needle.

4.2.6. Determination of drug release from the ISFI at a simulated tumor site

Drug release studies were conducted in an orbital shaker bath ($37 \pm 0.5^\circ\text{C}$, 25rpm). As described in Chapter 3, Section 3.2.5 of this dissertation a dialysis tubing method similar to that described by Graves et al., (2007) was used. 30mL samples were drawn at the following intervals: 6 hours, 1 day, 3 days, 5 days, 9 days, 13 days and 17 days, 22 days, 27 days, 32 days and 40 days. Pre-warmed buffer (30mL) was replaced at each time interval to maintain sink conditions. Large volumes were extracted to compensate for the poor solubility of the drug. Samples were passed through a $0.22\mu\text{m}$

pore size filter (Cameo Acetate membrane filter, Millipore Co., Bedford, MA, USA) and analysed using a UV spectrophotometer (Specord 40, Analytik Jena, AG, Germany) at the wavelength for methotrexate (MTX), 306nm or for folic acid (FA), 280nm and the amounts of MTX or FA were quantified using a calibration curve for each of the drugs. Each ISFI formulation was tested in triplicate. The Mean Dissolution Time (MDT) as described by Pillay and Fassihi (1998) at 32 days was calculated for each of the formulations using Equation 4.1 and a maximum MDT is the fastest drug release rate achievable (Govender et al., 2005).

$$MDT = \sum_{i=1}^n t_i \frac{M_t}{M_{\infty}} \dots\dots\dots \text{Equation 4.1}$$

Where M_t is the fraction of dose released in time ($t_i = t_i + t_{i-1}$)/2 and M_{∞} corresponds with the loading dose.

4.2.7. Computational modelling to determine the effect of the addition of a salt to PMVE

Computational and structural modeling was performed to determine the effect of the addition of a highly water soluble salt to solutions of PMVE. Predictive models were created using ACD/I-Lab V5.11 (Add-on) software (Advanced Chemical Development Inc., Toronto, Canada, 2000).

4.2.8. Determination of an effective method of sterilization for the ISFI

In order for the implant to be tested in the animal model an effective method of sterilization had to be determined. Two types of sterilization exist: terminal sterilization and aseptic processing. Terminal sterilization is of the greatest benefit as it involves sterilization of the final product only. However, methotrexate is a thermo-labile drug and therefore terminal sterilization could not be considered. It was therefore proposed that the polymer solution be sterilized and the drug added to the formulation aseptically.

Approved methods for sterilization according to the United States Pharmacopoeia (1999) involve the following: steam sterilization, dry heat sterilization, gas sterilization, sterilization by ionizing radiation, sterilization by filtration and aseptic processing. Filtration of the implant would prove difficult due to the viscous nature of the formulation, and irradiation of polymeric materials can lead to the formation of reactive species such as free radicals. Irradiation of polymers can be detrimental in two ways: it can cause an increase in molecular weight (cross linking) or it can cause chain scission and degradation (decrease in molecular weight) (Andriola Silva Brun-Graepi et al., 2009). Nurkeeva et al, 2004 have reported the use of gamma irradiation in the crosslinking of PMVE. Oven sterilization (dry heat) was not considered due to the length of time required for sterilization. Instead autoclaving (steam sterilization) was selected. Autoclaving utilizes high steam pressures to kill viable microorganisms.

4.2.8.1. Materials and methods

Materials utilised were as described in Chapter 3, Section 3.2.1 of this dissertation. Blood agar was purchased from Oxoid, UK, sterilized appropriately and then poured into sterile petri dishes.

4.2.8.2. Preparation of formulations

Formulations were prepared according to the centre point of the design (PMVE 20%^{w/v} and CaCl₂ 0.125M) as this would give 5 previous runs obtained from the design for comparison purposes. Samples were bottled into amber glass bottles with screw tops and placed in a Butterworth HD158 autoclave and allowed to run at 121°C for 15 minutes. This is the minimum time allowed by the British Pharmacopoeia (British Pharmacopoeia, 1993). Samples were removed following autoclaving and allowed to reach room temperature. Samples were then opened aseptically and previously autoclaved double deionised water was added to make up the volume lost during autoclaving.

4.2.8.3. Determination of polymeric structural variations

Fourier Transformed Infrared Spectroscopy (FTIR) was utilized in order to determine if any variations in vibrational frequencies had occurred following autoclaving of the formulations. Differences in vibrational frequencies would be indicative of bond

formation or loss and hence structural variations on the polymeric structure. FTIR was conducted on samples using a Perkin Elmer Spectrophotometer (Perkin Elmer Spectrum 100, Beaconsfield, United Kingdom). Formulations were air dried before testing to remove excess water.

4.2.8.4. Determination of the gelation temperature of the ISFI

The gelation temperature of the autoclaved formulations was determined as per Section 4.2.4 of this chapter.

4.2.8.5. Determination of the release of folic acid from the formulation

Drug release studies were performed as per Chapter 4, Section 4.2.6 of this dissertation. Folic acid was utilized as the model drug and was added to the formulations aseptically.

4.2.8.6. Determination of the sterility of the formulations

Once formulations had been autoclaved and reconstituted to correct volume under aseptic conditions (discussed further in Chapter 6, Section 6.2.2 of this dissertation), formulations were streaked onto blood agar plates using a sterile spatula as depicted in Figure 4.4 and incubated at 25°C and 37°C for 24 hours. As can be noted in Figure 4.4, each time the formulation was streaked onto one half of the plate and the other half was left untreated to serve as a negative control i.e. to ensure that the agar was not contaminated. Incubation at 25°C was to determine the presence of fungal growth whereas incubation of the plate at 37°C was to test for the presence of bacteria. Remaining formulations were capped and placed in a refrigerator (4°C) and retested in the same way after one month.

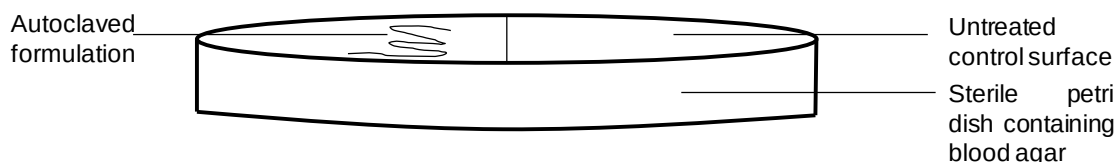


Figure 4.4: Schematic representation of agar plate and the streaking of the autoclaved formulation.

4.2.9. The Effect of Different Temperatures on the Release Profile of the Implant

Considering that the temperature in the tumor region, especially a rapidly growing tumor, is slightly higher than the temperature of normal tissue (Babich, 2005), the release of folic acid from formulations was determined at higher temperatures. While the release of entrapped drug from the implant prove satisfactory, the effect of ice on the release profile of the ISFI was examined in an attempt to increase the release of drug from the implant should that prove necessary.

4.2.9.1. Materials and Methods

Materials utilized were as described in Chapter 4, Section 4.2.1 of this chapter.

4.2.9.2. Determination of the release of drug from ISFI formulations under different temperature conditions

Drug release studies were conducted as per Chapter 4, Section 4.2.6 of this dissertation with some modifications. For studies determining the impact of an increase in temperature studies, vessels prepared as described in Chapter 4, Section 4.2.6, were placed in a rotating water bath (Labex SBS40) maintained at $38.5 \pm 0.5^\circ\text{C}$ and set at a rotation speed 25rpm. For the determination of the impact of ice on the release profile, vessels were placed in a shaker bath as described in Chapter 4, Section 4.2.6. Unlike in Chapter 4, Section 4.2.6, samples drawn were taken directly from the dialysis bag rather than from the exterior fluid as described previously. This made the application of ice for short periods of time possible without having the effect of waiting for the dissolution fluid to heat up following the application of ice. The following procedure was carried out. A 22G needle was attached to the dialysis bag, as shown in Figure 4.5 and the fluid surrounding the implant was drawn directly from this point. Samples were taken before and after the application of ice. At each time point: 6 hours,

1 day, 4 days and 10 days, 3mL of PBS was withdrawn through the needle and replaced with prewarmed buffer. The dialysis tubing was then removed from the vessel and placed in a beaker on a bed of crushed ice. After the given time interval: 2 minutes or 5 minutes, a 3mL sample was withdrawn from the tubing through the needle and replaced with prewarmed buffer. The dialysis tubing containing the implant was then returned to the vessel which was placed in the shaker bath maintained at 37°C. Adjustments for the different volumes of the samples were made.

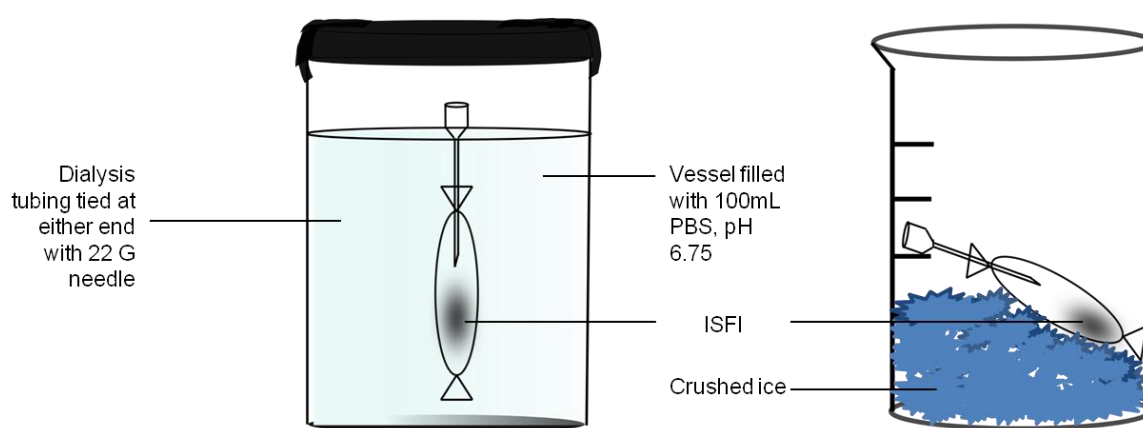


Figure 4.5: Figure showing the method used for drug release during experimental determination of the effect of ice on the implant.

4.3. Results and Discussion

4.3.1. Analysis of the Face-Centred Central Composite Design

Regression (ordinary least squares) was used to fit the experimental data by estimating the effects of each of the variables on an outcome such that the sum of squared differences between predicted and observed responses was minimized. The standard error of the model(S) and the R^2 value of the model indicate the fit of the model and are summarized in Table 4.3. Regression equations generated for gelation, force of injectability and MDT are as described by Equations 4.2, 4.3 and 4.4. Response surface plots describing the effect of each variable on the outcomes are discussed under each of the outcomes (Figure 4.9, Figure 4.11 and Figure 4.13).

$$\text{Gelation} = 33.6410 - 0.02633[\text{PMVE}] - 1.9367[\text{CaCl}_2] - 1.1353[\text{PMVE} \cdot \text{PMVE}] - 0.1353[\text{CaCl}_2 \cdot \text{CaCl}_2] + 0.075[\text{PMVE} \cdot \text{CaCl}_2] \dots \text{Equation 4.2}$$

$$\text{Force of Injectability} = 28.7086 + 9.8433[\text{PMVE}] - 1.0967[\text{CaCl}_2] + 3.8967[\text{PMVE} \cdot \text{PMVE}] - 1.6633[\text{CaCl}_2 \cdot \text{CaCl}_2] + 5.2625[\text{PMVE} \cdot \text{CaCl}_2] \dots \text{Equation 4.3}$$

$$\text{MDT} = 18.4370 + 0.4485[\text{PMVE}] - 1.9718[\text{CaCl}_2] + 2.1155[\text{PMVE} \cdot \text{PMVE}] + 4.2038[\text{CaCl}_2 \cdot \text{CaCl}_2] + 0.6254[\text{PMVE} \cdot \text{CaCl}_2] \dots \text{Equation 4.4}$$

Table 4.3: Table summarizing the standard error of the model (S) and the coefficient of determination (R^2) values obtained for the regression equations.

	Gelation (°C)	Force of Injectability (N)	MDT*
S	0.1501	4.196	2.720
R^2	99.4	85.8	69.4

* MDT= Mean Dissolution Time

The lower the standard error of the model (S) the better the fit of the model. The standard error for gelation is fairly low and the R^2 value also shows the good fit of the model. Force of injectability has a less desirable fit with a slightly lower R^2 value and MDT has a satisfactory fit. The model also predicts the confidence intervals of the variables and a significant value ($p \leq 0.05$) indicates that the predicted effect of the variable can be considered to be of importance (Table 4.4). These p-values together with the response surface plots describing the effect of each variable on the outcomes are discussed under each of the outcomes (Figure 4.9, Figure 4.11 and Figure 4.13).

Table 4.4: Probabilities of the effects of the variables on the outcomes.

Term	p-value		
	Gelation (°C)	Force of Injectability (N)	MDT
PMVE	0.004	0.001	0.698
CaCl ₂	0.000	0.542	0.119
PMVE*PMVE	0.000	0.169	0.240
CaCl ₂ *CaCl ₂	0.180	0.534	0.038
PMVE*CaCl ₂	0.923	0.040	0.660

4.3.1.1. Analysis of Variance (ANOVA)

Variance or how widely the data is distributed can provide valuable information. ANOVA was employed in the further examination of the models and data obtained for regression and the residuals is as summarized in Table 4.5.

Table 4.5: Analysis of variance for regression and residual errors.

Source	DF	Adj SS	Adj MS	F	P
ANOVA for Gelation					
Regression	5	27.3802	5.4760	243.14	0.000
Residual Error	7	0.1577	0.0225		
Total	13	27.9807			
ANOVA for Force of Injectability					
Regression	5	740.941	148.188	8.42	0.007
Residual Error	7	123.229	17.604		
Total	13	865.347			
ANOVA for MDT					
Regression	5	116.033	23.207	3.14	0.084
Residual Error	7	51.802	7.400		
Total	13	169.393			

DF= degrees of freedom, Adj SS= Adjusted Sum of Squares, Adj MS= Adjusted Mean squares.

The sum of squares for regression is the sum of the deviations of the fitted response values from the mean response value whereas the residual sum of squares is the sum of deviations of the observed response values from the fitted values. In all the outcomes, the adjusted sum of squares for regression exceeds that of the adjusted sum of squares for the residual error indicating that regression accounts for most of the data. This is confirmed by the F statistic value which is the ratio of mean squares of

regression to the mean squares of the residual. The p value is significant ($p \leq 0.05$) for gelation and slightly higher for force of injectability and much higher for MDT indicating that at least for gelation the coefficient is not 0.

4.3.1.2. Examination of the residual error in the design

Of important consideration in the determination of the efficacy of an experimental design is the assessment of the residual error. Figure 4.6 presents the various residual plots that require examination. The normal probability plots (a-c) for all the responses showed approximately straight lines indicating a normal distribution of residuals. The residuals versus fitted value scatter plots (d-f) showed random scatter of the residuals around 0 as expected. This plot serves to detect non-constant variance, missing higher order terms and outliers. None of these were observed in the plotted graphs. The plots of the residuals versus the order of the data are used to determine the time dependence of the residuals. The plotted curves (g-i) showed no clear pattern indicating a lack of dependence. Histograms, plotting residuals against frequency (j-l), should be bell shaped curves. However the graphs obtained were not bell shaped and this seemed to indicate the presence of outliers (data points with large residuals). However, as the sample size is very small (< 50) a histogram is not considered the best choice in the examination of normality and the normal probability plot is far more sensitive ([NIST/Sematech](#), accessed 2012-02-06).

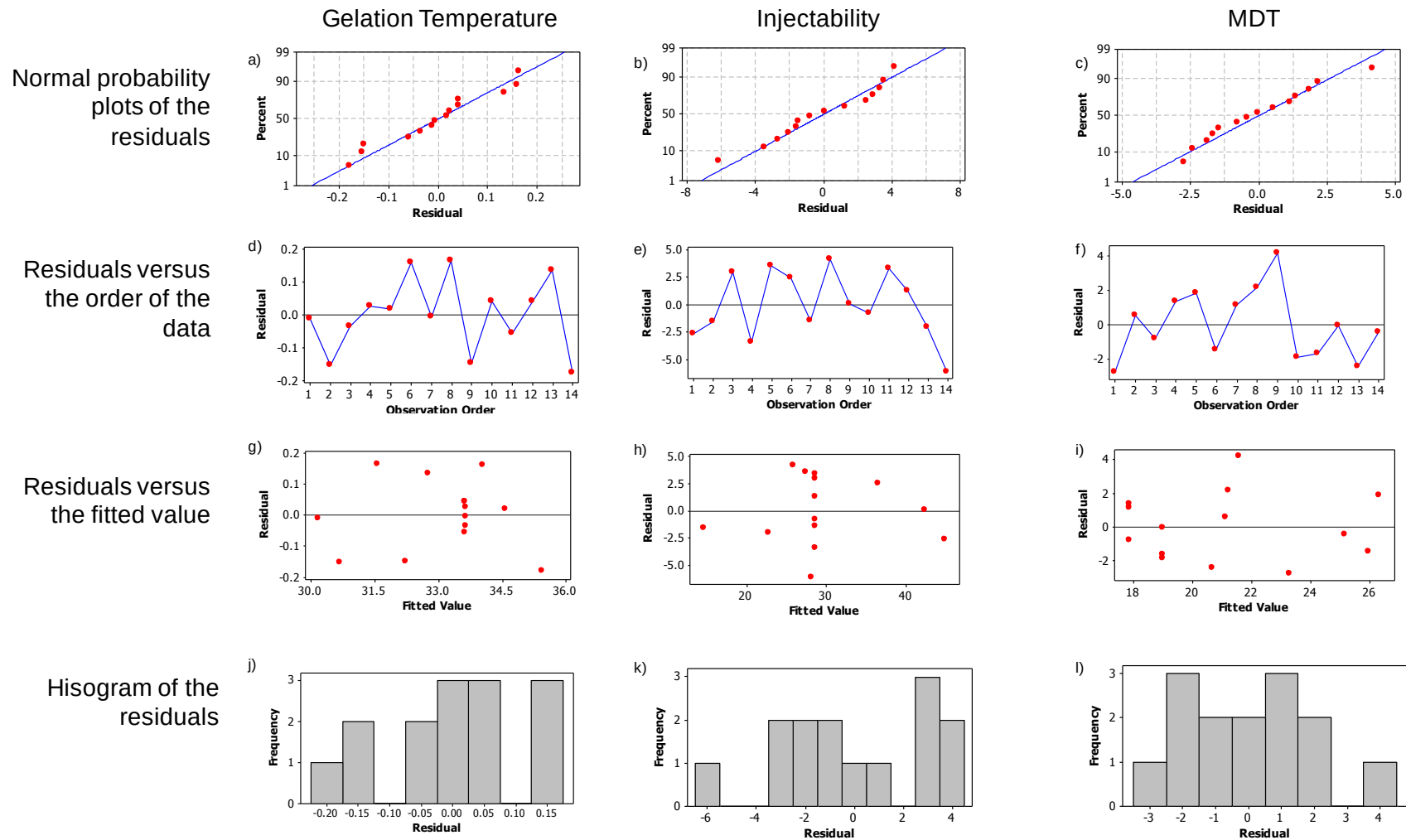


Figure 4.6: Residual plots of the data for gelation temperature, force of injectability and MDT for the ISFI formulation.

4.3.2. The use of oscillatory rheology in the determination of the thermal gelation temperatures of the ISFI formulations

All the formulations tested exhibited the typical pattern expected of a thermoresponsive system displaying a Lower Critical Solution Temperature (LCST) (Figure 4.7). As clearly annotated in Figure 4.7, the crossover of the storage and loss modulus was considered the gelation temperature (T_g°) for the formulation. The storage modulus (G') of a viscoelastic solid is associated with the solid properties or the elastic energy storage properties which indicated that the sample would return to its original state following removal of the deformational energy, while the loss modulus (G'') explains the behavior of the viscoelastic solid when it is acting as a liquid i.e. the viscous properties of the sample representing the dissipation of energy after the application of the deformational stress. Hence at the point at which the storage modulus exceeded the loss modulus, the formulation was behaving more like a solid than a liquid and hence this was used to determine the thermal gelation temperature.

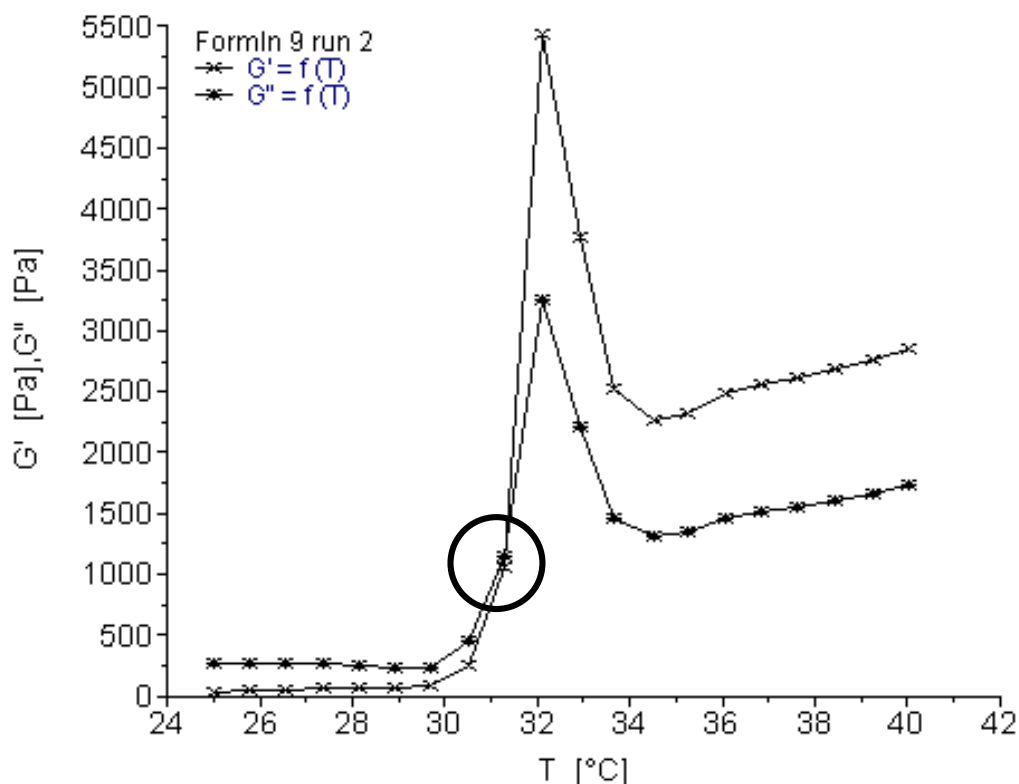


Figure 4.7: Typical profile obtained during rheological testing of samples. The black circle demarcates the crossover between the storage modulus (G') and the loss modulus (G'') and hence the value that was used as the gelation temperature.

The T_g of the formulations ranged from 30.18 to 35.44°C and seemed to vary with the amount of CaCl_2 that had been added (Figure 4.8). This was confirmed by the surface response curve (Figure 4.9) which displayed the variation of gelation with both CaCl_2 and PMVE concentrations. Both these factors were found to have significant effects on the gelation of the implant formulations ($p=0.000$, Table 4.4), however the interaction between the two was not significant. As can be seen in the response surface plot the T_g decreased with the increased amount of salt in the formulation. As explained by the computational modeling (Chapter 4, Section 4.3.5), this effect was expected due to the CaCl_2 acting as a dessicant.

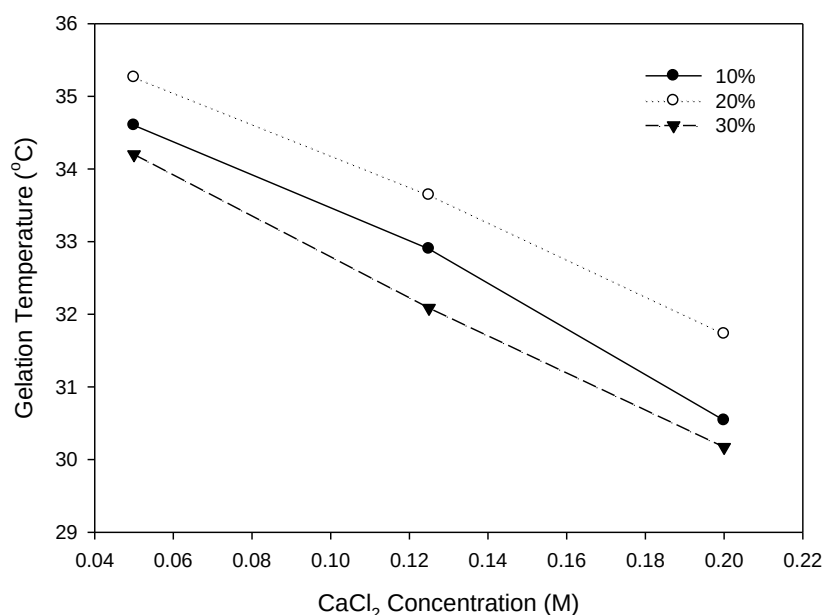


Figure 4.8: The effect of CaCl_2 on the gelation temperature of formulations ($n=3$, $SD < 0.35$).

The effect of salts on thermoresponsive polymers has been recorded by Kundu and Kundu (2001) in their work on methylcellulose where a decrease in gelation temperature has also been decreased with the addition of various salts.

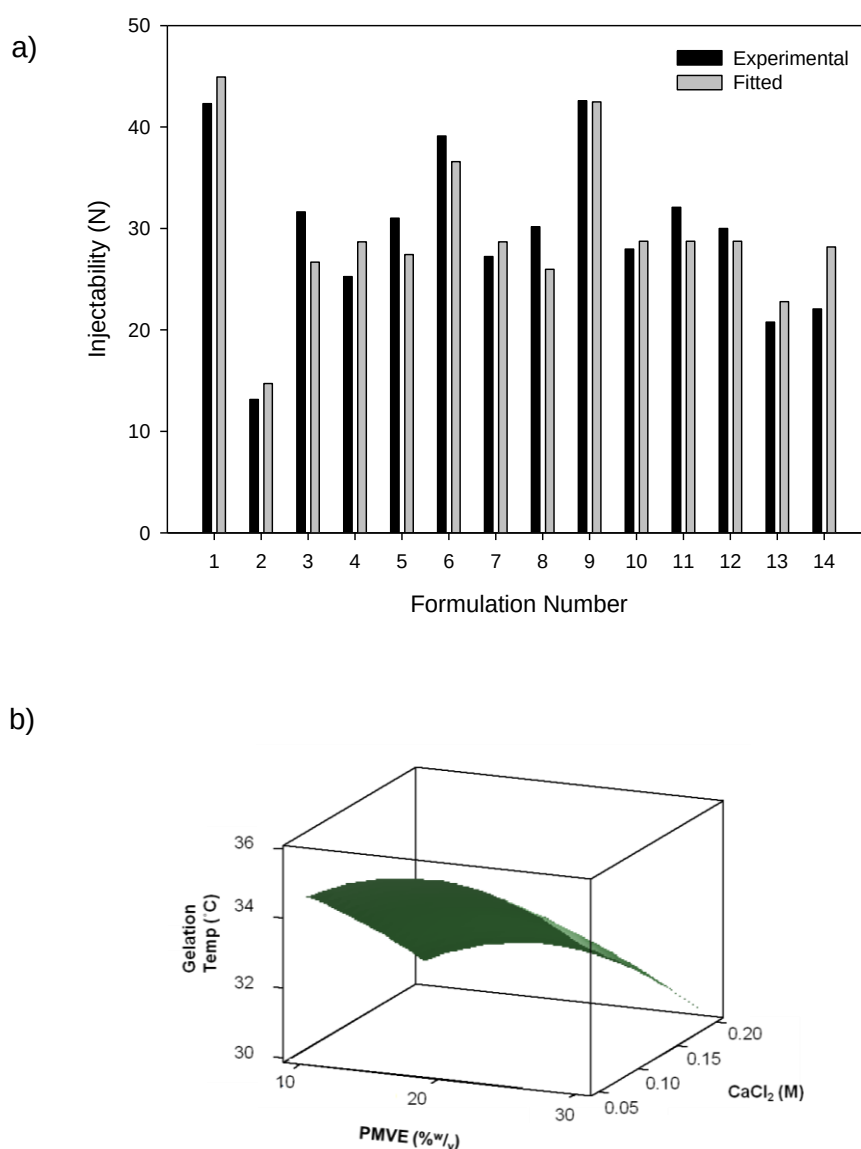


Figure 4.9: a) Bar chart for gelation temperature showing the experimental versus the predicted values ($R^2=0.9971$) and b) Response surface plot correlating the Gelation temperature with the concentration of PMVE and CaCl_2 present in the formulation.

4.3.3. Determination of the force of injectability: The force required to inject the ISFI formulations

In order to develop a formulation which will be clinically beneficial, the formulations must be easily injectable. PMVE, as supplied (50% w/v solution), is highly viscous and for this reason the force of injectability of the formulations was tested. The formulations all showed the typical profile displayed in Figure 4.10. Also shown is the comparison of

the test formulation to that of an air filled syringe. The maximum force recorded was used as the maximum injectability force.

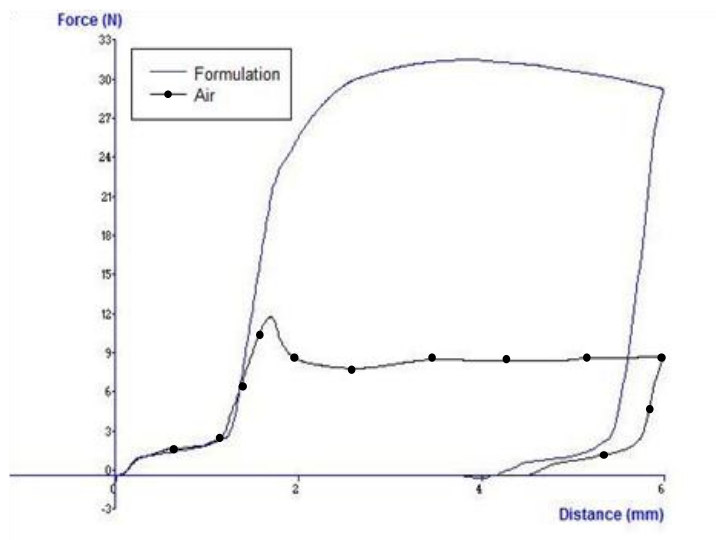


Figure 4.10: Typical profile obtained for a formulation filled syringe versus that of an air-filled syringe.

As expected, only the PMVE had a significant effect on the force of injectability ($p=0.001$, Table 4.4), while CaCl_2 had no significant effect ($p=0.542$, Table 4.4). As can be seen in the surface plot for force of injectability (Figure 4.11) the increase in the force of injectability has a very close relationship with the PMVE concentration. As expected the increase in PMVE concentration caused an increase in the force required. The CaCl_2 concentration had no apparent effect.

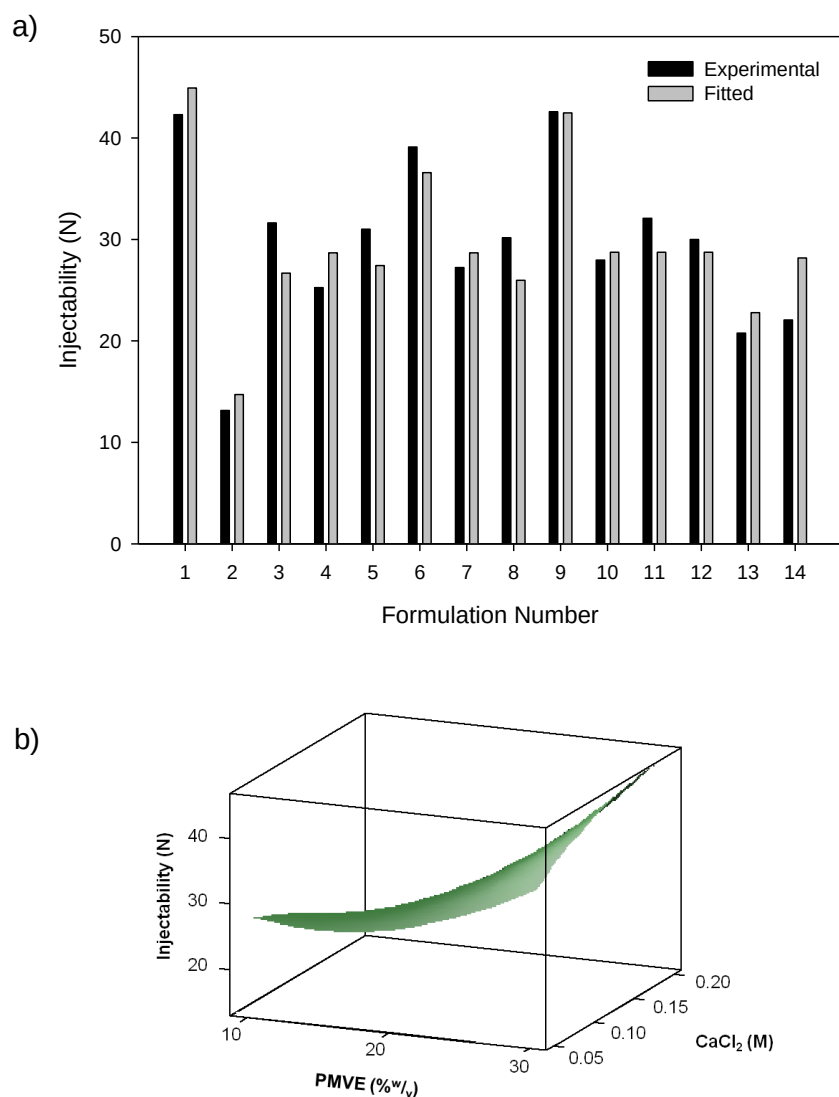


Figure 4.11: a) Graph showing the experimental values obtained for force of injectability compared to the predicted or “fitted” values ($R^2=0.9164$) and b) Response surface plot correlating the force of injectability with the concentration of PMVE and CaCl₂ present in the formulation.

4.3.4. Release of folic acid from the *ISFIs* over 32 days

As illustrated in Figure 4.12 the release of drug from all formulations exceeds a month. Further, the release appears dependant on the polymer concentration in the formulation, as shown in the figure the release is slowest from F1 (which is composed of 30%^w/_v PMVE) and quickest from the formulation containing 10%^w/_v PMVE (F5) (Figure 4.12(a)).

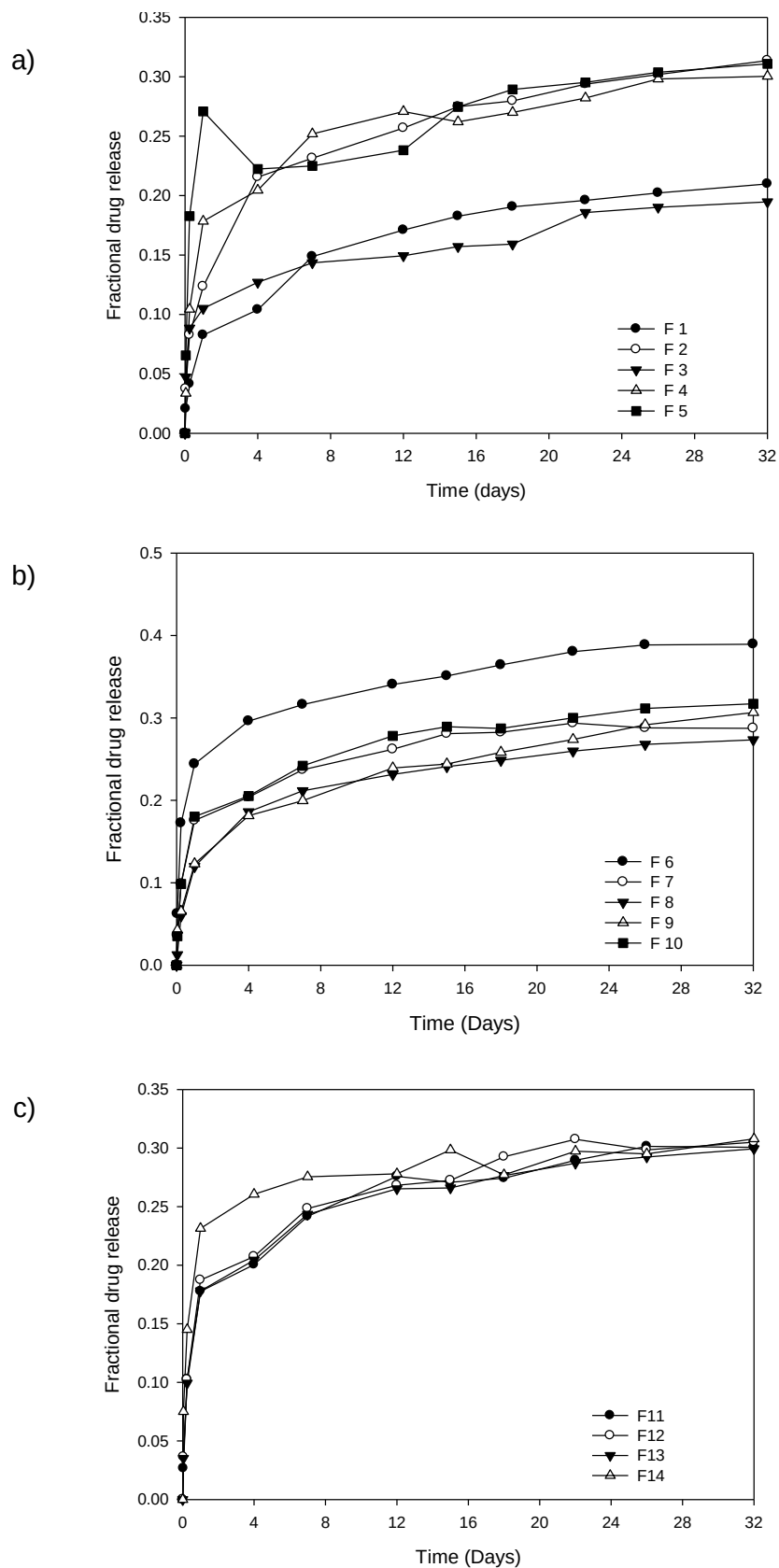


Figure 4.12: Drug release profiles from formulations over a period of 40 days where a) represents formulations 1-5 b) represents formulations 6-10 and c) represents formulations 11-14 (37°C, pH 6.75, n= 3, SD< 0.1).

As determined in preformulation studies, the addition of CaCl_2 results in a slower initial release of the drug, perhaps due to the lowering of the gelation temperature. However, this effect only appears important when the ISFI contains lower concentrations of PMVE. For formulations containing 10% $^w/v$ PMVE: the higher the concentration of CaCl_2 , the slower the release of folic acid from the formulation. As shown in Figure 4.12(a), F5 contains PMVE 10% $^w/v$ and 0.05M of CaCl_2 and shows a significant burst effect, whereas F2 which also contains 10% $^w/v$ of PMVE but 0.2M CaCl_2 shows almost no burst effect. This can also be seen in the response surface graph (Figure 4.13) obtained for MDT for the formulations. As can also be noticed in Figure 4.13, the same effect was not as pronounced with formulations having a higher concentration of PMVE, indicating that at low concentrations of the polymer, the amount of CaCl_2 in the formulation affected drug release whereas at higher concentrations of polymer, the concentration of polymer in the formulation was more important. As shown by the response surface plot (Figure 4.13) there exists a concentration at which the MDT was at its lowest point, indicating that at this point of a certain concentration of PMVE and CaCl_2 there is an optimal interaction between the polymer and the salt which delays the release of drug from the implant.

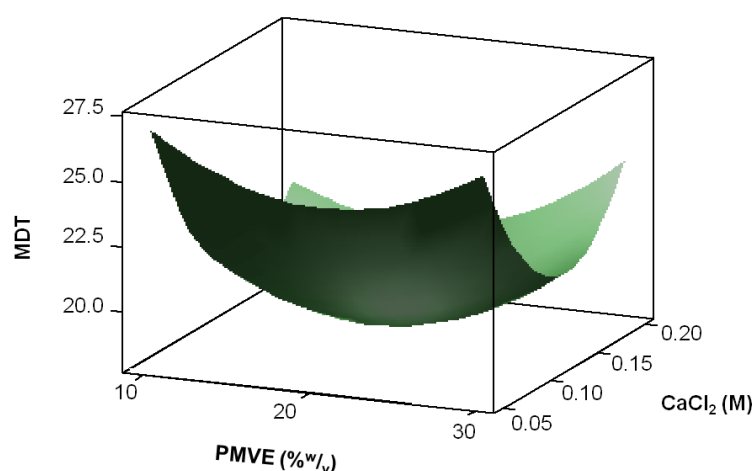


Figure 4.13: Response surface plot showing the relationship between MDT and the PMVE and CaCl_2 concentration in the ISFI formulations.

4.3.5. Computational modeling of the method of gelation and the effect of the addition of CaCl_2

PMVE consists of both hydrophobic (vinyl backbone and methyl side groups) and hydrophilic (ether oxygens) regions (Karayanni and Staikos, 2000). As a result the polymer is solvated at room temperature (surrounded by water molecules) due to the

presence of the ether oxygens on the polymer. However, at temperatures exceeding the LCST of the polymer, the hydration sheath surrounding the polymer chains is damaged and the interactions between the hydrophobic areas of the polymer such as the vinyl backbone and methyl groups on the polymer, become more important and as a result the water molecules are forced out, resulting in the “collapse” of the polymeric strands (precipitation of the sol or gel formation) (Richter, 2004; Karayanni and Staikos, 2000). As shown in Figure 4.14 the addition of the soluble salt is thought to have a “dehydrating” effect on the polymer as the salt interacts with the water molecules previously associated with the polymer. This would explain the decrease in gelation temperature observed in formulations with higher amounts of salt as the dehydration of the surrounding water sheath can occur at lower temperatures in the presence of CaCl_2 . It is thought that this is the reason for the prolonged release of the entrapped drug as the drug is lipophilic by nature and hence has the tendency to become entrapped in the core hydrophobic region of the gel. Figure 4.14 shows this hypothesized interaction using molecular models.

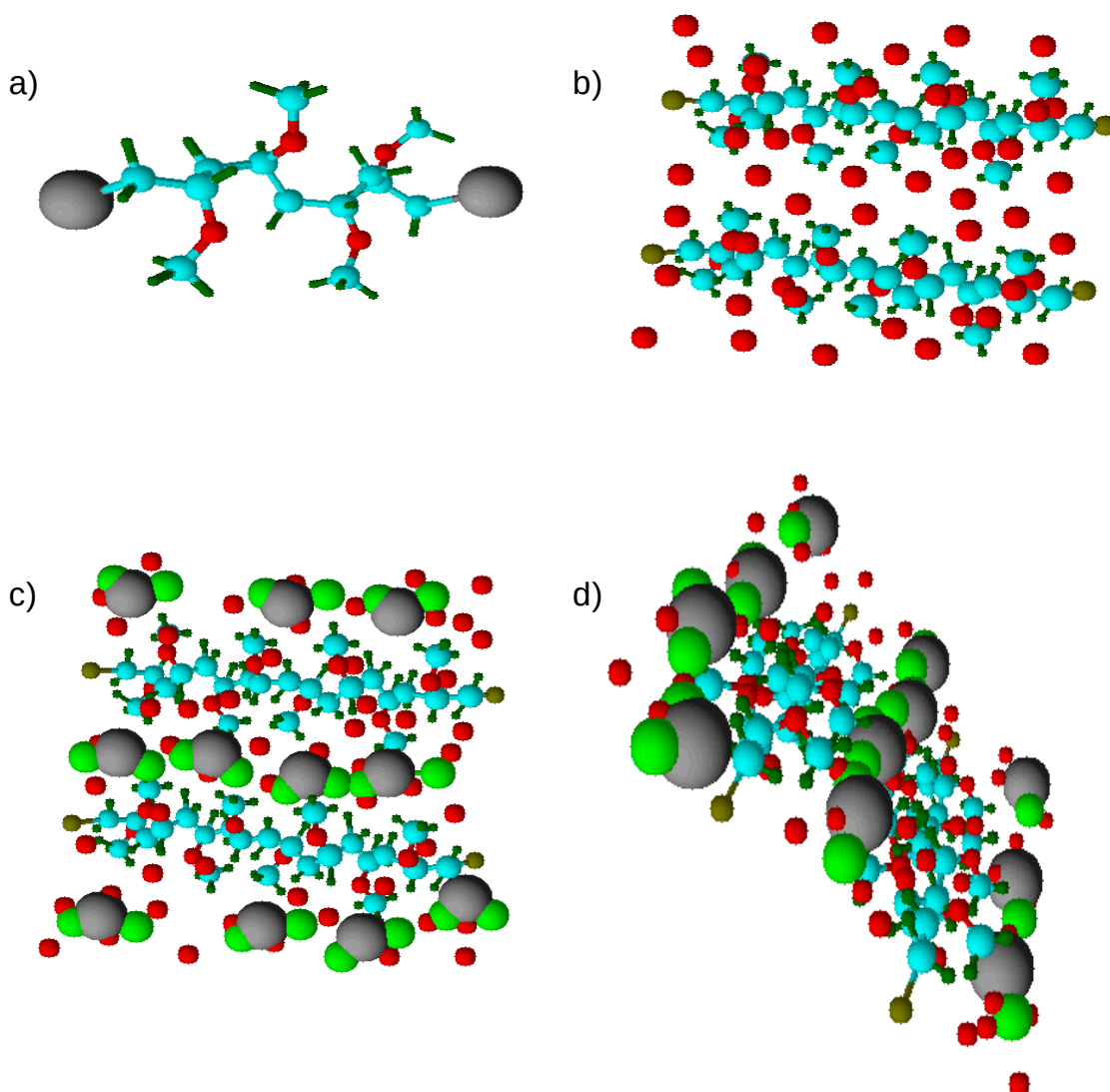


Figure 4.14: Computational modeling showing a) The PMVE Chain with R-representing chain elongation on both ends, the R is represented by large balls b) Water associated PMVE chains in the aqueous medium, only part shown, chain elongation R is shown in olive green. The -OCH₃ are in water association, red dots represent water molecules and oxygen atoms in the polymer chain as well. In c) CaCl₂ (the slate-green balls) in the medium, with water and PMVE chain as associated with water molecules, and around the polymer chain. Figure denotes size relationship of the salt with the polymer chain atoms (and not chain length and size). d) CaCl₂ in association with water molecules which are itself in association with the PMVE polymer methoxyl's oxygen and hereby giving a compacted matrix where in all the entities are in close proximity as well as in the limits of inter-atomic distances required for the physicochemical interaction. The salt, CaCl₂ is also in layered state between the two polymer strands and could not be inter-wined/embed into the polymer chain itself.

4.4. Optimization of ISFI Formulation Responses

The ISFI was optimized using Minitab V15 (Minitab® Inc, PA, USA) to determine the optimal level for each variable as summarized in Table 4.1. Table 4.6 summarises the

obtained values for the responses with the optimized formulation as well as the predicted values and desirability. The overall desirability for the all the responses in the design was 0.965. The optimal formulation was predicted to consist of 16.87%^{w/v} PMVE and 0.1482M CaCl₂.

Table 4.6: Summary of the obtained values for each of the responses and the predicted values and desirability.

Response	Obtained	Predicted	Desirability
Gelation Temperature	32.46°C	33.0°C	1.0000
Force of Injectability	24.7N	25.0N	1.0000
MDT*	21.23 (folic acid)	18.2357	0.89759

*MDT=Mean Dissolution Time

4.4.1. The release of MTX from the optimized formulation

Studies conducted thus far had been completed using the drug, folic acid due to its similarity with the chemotherapeutic drug methotrexate. With the determination of an optimized formulation, the release of MTX from the formulation had to be ascertained. To this end, a calibration curve of MTX was prepared in order to quantify the amount of drug that had been released using standard UV spectroscopy. A stock solution of methotrexate in phosphate-buffered saline was prepared at a concentration of 0.2%^{w/v}. Serial dilutions were then made to yield a concentration range of (0.00018-0.007 mg/mL). The solutions were then assayed using a UV Spectrophotometer (Specord 40, Analytik Jena, AG, Germany) using a wavelength of 306nm. The optimized formulation consisting of 16.87%^{w/v} PMVE and 0.1482M CaCl₂ was prepared and loaded with methotrexate (5mg/mL). Release studies as per Chapter 4, Section 4.2.6 of this chapter were conducted.

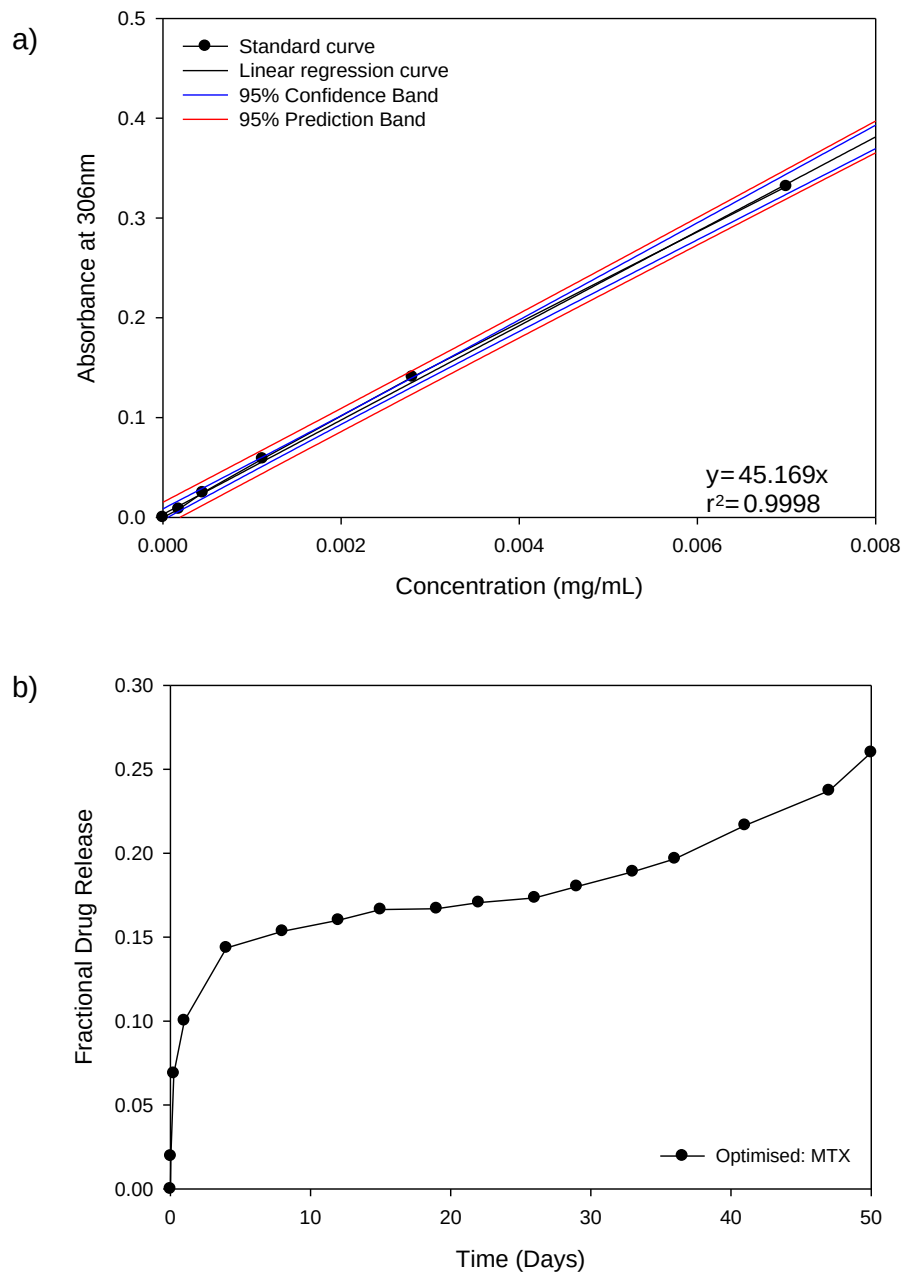


Figure 4.15: a) Calibration curve obtained for MTX and b) drug release profile from the optimized formulation containing MTX over a period of 50 days. (pH=6.75, n=3, SD< 0.024).

The calibration curve obtained showed a linear relationship between the concentration of MTX and the absorbance with the coefficient of determination being 0.9988. The release of MTX from the formulation followed a pattern similar to that obtained for folic acid, however the MDT was 20.65 whereas formulations containing folic acid had a slighter higher MDT of 21.23. The pattern of release was also considered satisfactory, in that the formulation appears to have a fast release initially and then a steady release

of drug followed by rapid release. Weinberg et al. (2007) have suggested that a formulation would prove the best for the delivery of drug to solid tumors if it is able to provide an initial burst release at the site and then show continuous slow release of drug. The ideal slow release of methotrexate in particular is also described by Kumar et al. (2008). Kumar and co-workers (2008) suggest that as MTX has a “time effect” i.e. the sensitivity of cells towards MTX increases with a time an implant releasing drug slowly will also be beneficial. The release of the drug *in vivo* will be discussed further in Chapter 6.

4.4.2. Further rheological characterisation of the optimized ISFI.

In addition to the determination of the gelation temperature of the optimized formulation using methods as described in Chapter 4, Section 4.2.4, a frequency sweep was also conducted. Briefly samples were exposed to fixed stress values of 1, 10 or 18Pa. as frequencies were ramped from 0.1 to 100Hz.

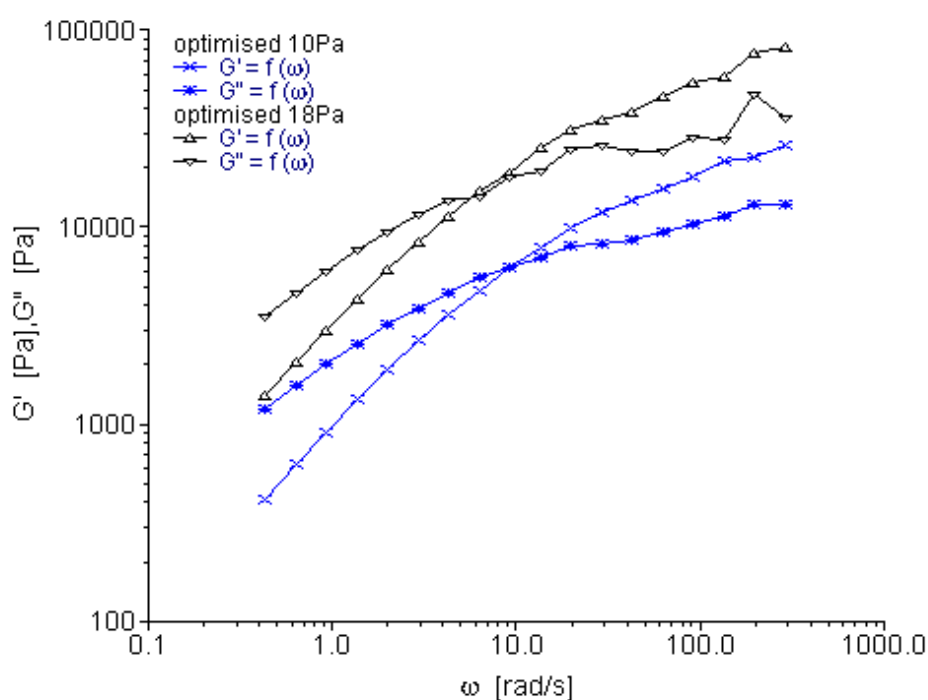


Figure 4.16: Rheological profile of the optimized ISFI at different stress values.

As shown in Figure 4.16, the formulation showed an increase in both G' and G'' in response to an increase in frequency, indicating a dependence on frequency.

4.4.3. Modelling of the release of drug from the implant

In order to postulate the mechanism of release of the drug from the implant various predictive models were considered. Table 4.7 shows the model as well as the coefficient of determination obtained when fitting the experimental data to the release model.

Table 4.7: Value of R^2 for different models of mechanisms of drug release

Model	R^2
Zero Order	0.1348
First Order	0.2295
Higuchi	0.7395
Hixson Crowell	0.1993
Korsmeyer-Peppas	0.9474

Higuchi's model showed the highest R^2 value (0.7395) and hence the data was fitted to the Korsmeyer-Peppas equation (Equation 4.5) which showed a R^2 value of 0.9474.

$$F = \left(\frac{M_t}{M} \right) = k_m t^n \text{ Equation 4.5}$$

Where F is the fraction of drug released at time t , M_t is the amount of drug released at time t , M is the total amount of drug in the dosage form, K_m is the kinetic constant, t is the time, and n is the diffusion or release exponent. n was estimated from linear regression of $\log \left(\frac{M_t}{M} \right)$ vs $\log t$ (Peppas and Sahlin, 1989, Jain et al., 2004 and Kabir et al., 2009). The diffusion or release coefficient (n) can be used to determine the type of release. When $n < 0.45$ this indicates release by fickian diffusion, while $0.45 > n < 0.89$ indicates anomalous diffusion and values of n exceeding 0.89 indicate release due to erosion of the polymer chain.

The value of n was found to be 0.231 for the release of MTX, indicating that the release of the drug occurred via diffusion of the drug out of the polymeric matrix and this also explains the relatively high value obtained for Higuchi's model, a model explaining release of drug via diffusion (Equation 4.6). Two factors explain the less than ideal fit of the drug release to the Higuchi model. Firstly the model describes release from a film and secondly the model describes release from a formulation containing undissolved drug (i.e. a dispersed system) (Siepmann and Siepmann, 2008).

$$Q = K_H t^{\frac{1}{2}} \dots \dots \dots \text{Equation 4.6}$$

4.4.4. The effect of autoclaving on the properties of the ISFI

4.4.4.1. Vibrational transition analysis

There was no difference observed between autoclaved and un-autoclaved formulations in terms of FTIR spectra as can be seen in Figure 4.17. This seems to indicate that the structure of the polymer did not change following autoclaving.

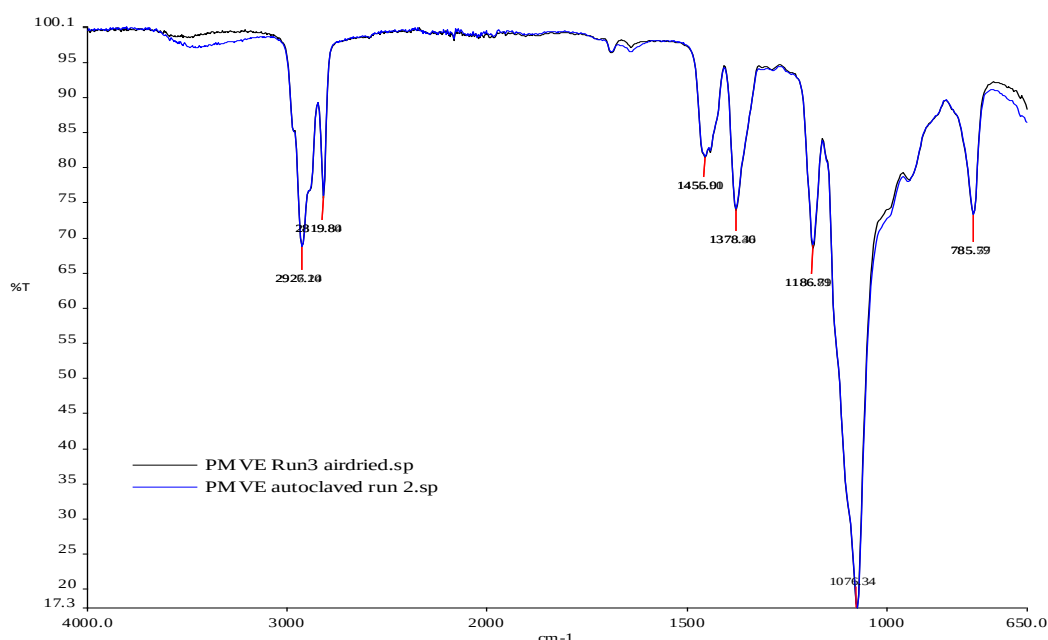


Figure 4.17: FTIR spectra comparing the autoclaved and unautoclaved formulations.

4.4.4.2. Rheological determination of the gelation temperature of the autoclaved formulation

Autoclaving did not appear to change the gelation temperature of the formulation either as depicted in Figure 4.18.

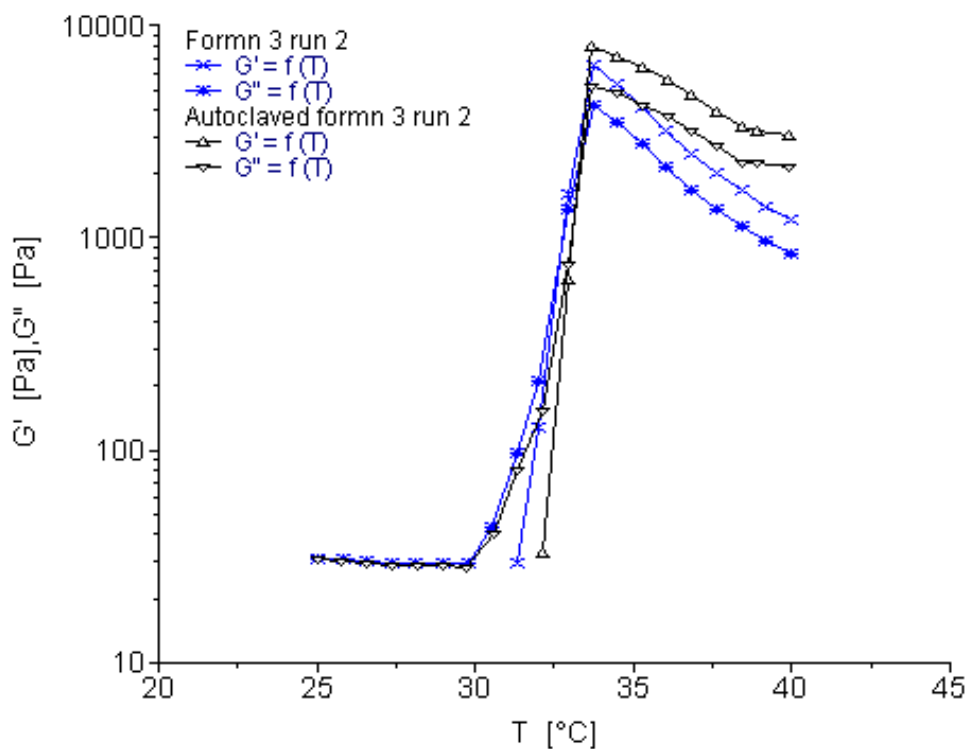


Figure 4.18: Rheological determination of gelation showed no apparent change between autoclaved and un-autoclaved formulations.

The gelation temperature of the formulations were very similar, 33.51°C for the un-autoclaved and 33.65°C for the autoclaved formulation. However this difference is well within the difference found between un-autoclaved formulations.

4.4.4.3. Release of folic acid from autoclaved formulations

Autoclaving of the formulations required the addition of water prior to the addition of the drug. As can be noticed in Figure 4.19, the release from the autoclaved formulation was faster than that from an un-autoclaved comparison formulation. Although the difference was only slightly greater than that observed between equivalent un-

autoclaved formulations, a possible reason for the difference is poor reconstitution of the formulation and indicative of the care that had to be taken during reconstitution.

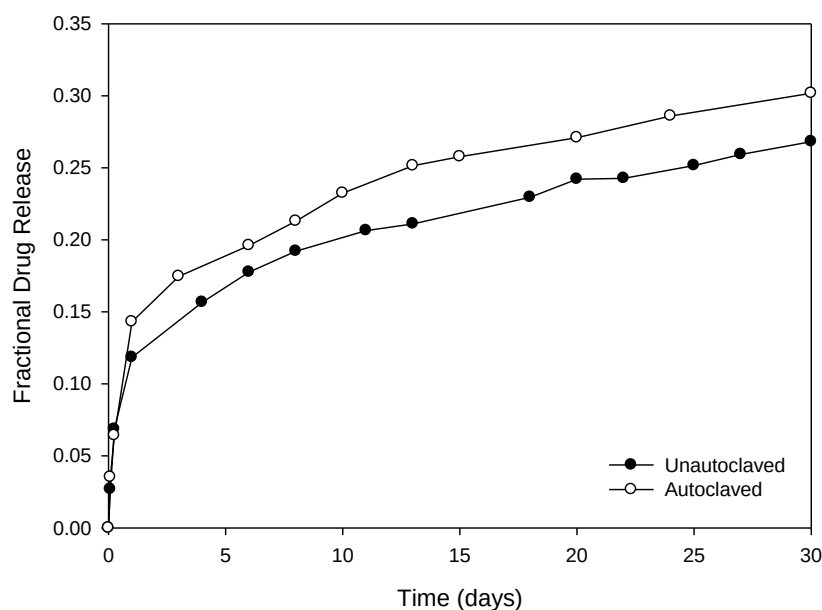


Figure 4.19: Release profiles showing the release of folic acid from autoclaved and un-autoclaved formulations (n=3, SD<0.067).

4.4.4.4. Sterility of the formulations by examination of agar plates

Agar plates were examined after 24 hours following incubation at 25°C and 37°C. Neither of the plates showed the presence of colonies indicating that the formulations were sterile. Plating of the formulations one month after autoclaving also showed the achievement of sterile formulations.

4.4.5. The effect of different temperatures on the release profile of folic acid from ISFI formulations

Formulations showed a slightly faster release at higher temperatures at least initially (Figure 4.20), while a markedly higher release at each point was obtained when the ice was applied. The faster release of drug following the application of ice is easily explained as the return of the formulation to a more liquid like phase and hence the drug is released. A further observation to be noted is that as the responsiveness of the implant to the ice appears to decrease with an increase in time. Release from the ISFI at higher temperatures is more of a challenge to explain and it is hypothesized that the increased release could be due to the lower viscosity shown by formulations at higher

temperatures. As shown in Figure 4.7 the viscosity of the implant drops slightly after the gelation temperature is achieved and this could possibly be the reason for such behaviour.

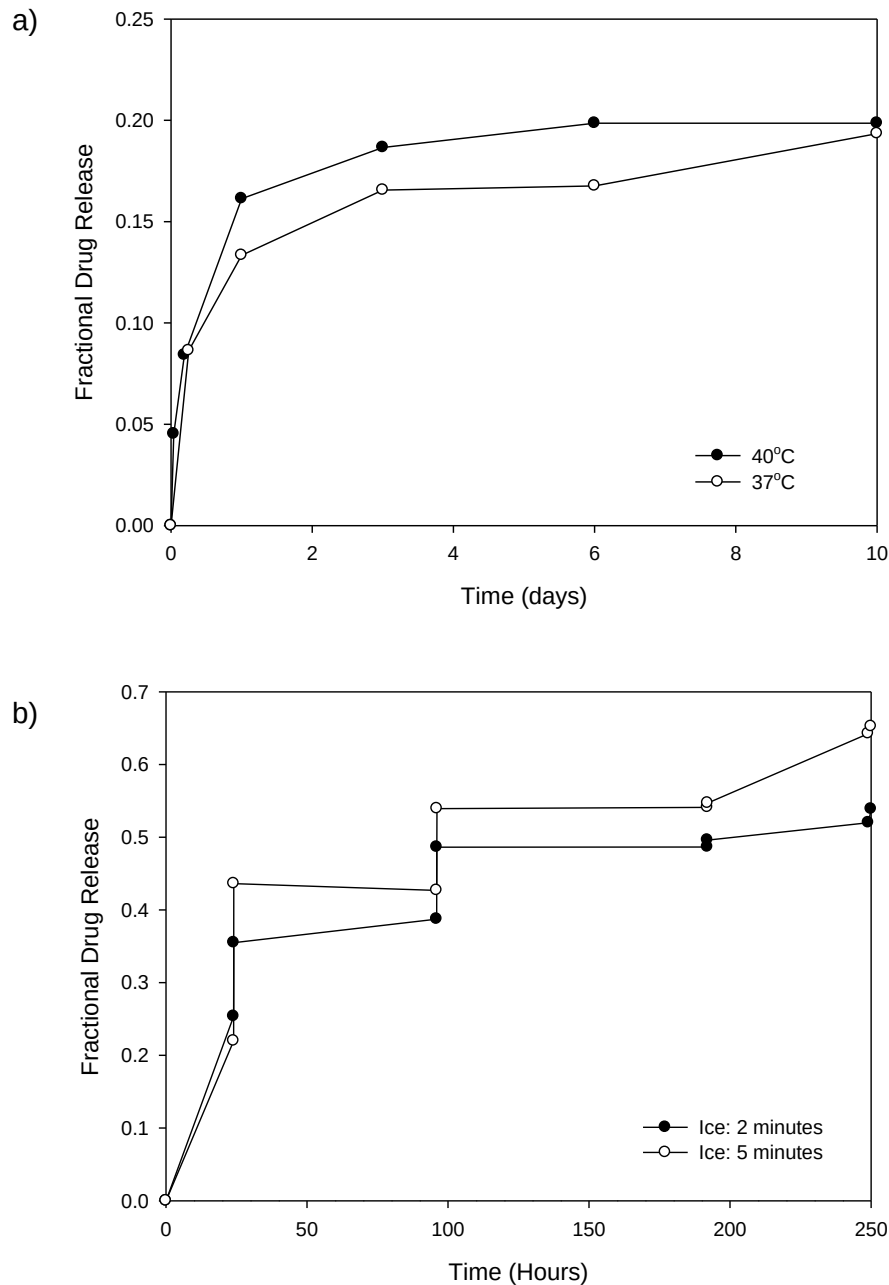


Figure 4.20: a) The effect of increased temperature on the release profile of folic acid from the ISFI over 10 days (pH 6.75, n=2, SD< 0.039) and b) the effect of ice application on the release profile from the ISFI over 10 days (pH 6.75, n=2, SD< 0.06).

4.5. Concluding Remarks

A thermoresponsive ISFI system was developed and the effect of CaCl_2 , on the physicommechanical properties of the polymer poly(methylvinyl ether) was examined. A Face-Centred Central Composite Design was used to optimize the formulation and proved to be quite effective in the selection of an optimized formulation which would be easily injectable, gel around body temperature and release drug from the formulation over a period exceeding 30 days. These drug release results demonstrated promise for the use of the ISFI for the prolonged delivery of drug in the treatment of solid tumors. In order to investigate improvement of the system, microparticles which were responsive to pH were developed as will be described in Chapter 5 of this dissertation.

CHAPTER 5

THE DESIGN AND OPTIMIZATION OF PH-RESPONSIVE MICROPARTICLES

5.1. Introduction

The use of responsive polymers in drug delivery systems has received much attention recently (Anal, 2007; Bajpai et al., 2008 and Bawa et al., 2009). Of the environmentally responsive polymers, pH responsive polymers in particular offer a multitude of advantages as they allow the controlled delivery of drugs at a certain pH. This is of considerable importance in the delivery of drugs to solid tumors as there are different pH conditions at the site of a solid tumor. The pH external to the solid tumor is approximately 6.75 whereas the pH within the tumor is about 5.6. The pH of normal tissue is about 7.4 (Martin and Jain, 1994). A combination of two pH responsive polymers allows the exploration of delivery of drug at different pHs.

Chitosan (CHT) is an abundant natural polysaccharide obtained from the deacetylation of chitin, a component of the external skeleton of many crustaceans and insects. It is a cationic polysaccharide that has one amino group and two free hydroxyl groups in every monomer unit (Sinha et al, 2004). The presence of the amino groups gives the molecule an overall positive charge. Poly(methacrylic acid-co-methyl methacrylate) (PMMA)- commercially available as Eudragit S100[®] and Eudragit L100[®]- has both carboxyl groups and ester groups. Eudragit S100[®], the polymer utilised in this study, has a ratio of carboxyl groups to ester groups is 1:2. The polymer hence carries an overall negative charge (Hilbrig and Freitag, 2007 and Huanbutta et al., 2008). In this study CHT and PMMA (Figure 5.1) were selected to formulate particles able to deliver drug at different pHs based on the interaction between the two polymers as a result of their opposing charges.

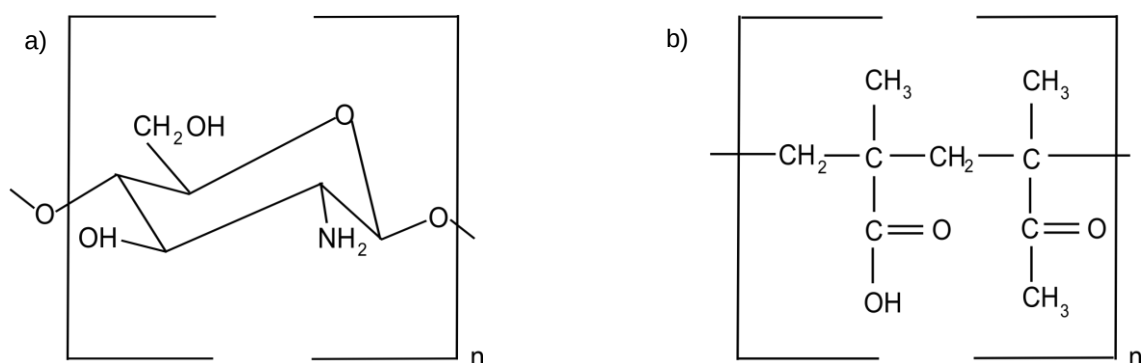


Figure 5.1: Chemical structure of a) chitosan and b) poly(methacrylic acid-co-methyl methacrylate).

The interaction that takes place between these two polymers was used to formulate microparticles. The aim of this study was to develop particles which were able to show different release profiles in response to different pHs. As outlined in Chapter 4 of this dissertation there are numerous benefits for utilising Design of Experiments to guide the formulator and to provide a method of statistically optimising a formulation. Once again a Face-Centred Central Composite Design was selected as two variables had been chosen for further investigation during preformulation studies.

5.2. Materials and Methods

5.2.1. Materials

Chitosan (medium molecular weight) (CHT), Glacial acetic acid, and methotrexate (>95% purity) were purchased from Sigma-Aldrich, Steinheim, Germany. Sodium hydroxide pellets (NaOH, $M_w=40\text{g/mol}$) were purchased from Rochelle Chemicals, South Africa. Poly(methacrylic acid-co-methyl methacrylate) (PMMA) in the form of Eudragit S100[®] was purchased from Evonik Röhm Pharma Polymers, Darmstadt, Germany. Other materials used were as described in Chapter 3, Section 3.2.1.

5.2.2. Preliminary investigations into the formulation of microparticles

5.2.2.1. Preparation of microparticles

Various methods of formulating particles were attempted and formulation A as described in Table 5.1 was the initial formulation found to provide desirable results.

Briefly, to produce formulation A the following method was used: a solution of medium molecular weight chitosan (0.2%^{w/v}) was prepared by adding 0.2g of chitosan to 100mL of a 1% acetic acid solution. An equivalent solution of PMMA (0.2%^{w/v}) was then prepared by dissolving 0.2g of PMMA in 100mL of a 0.01M NaOH solution. To 10mL of the PMMA solution, 30mg of folic acid was added. 10mL of the CHT solution was placed under a sonicator (Vibra-Cell Ultrasonicator, Sonics, USA) and 10mL of the second solution (PMMA and folic acid) was added to the chitosan solution during sonication. The following variables were then investigated as shown in Table 5.1.

Table 5.1: Investigation of variables for the formation of microparticles

Formulation	Chitosan(%^{w/v})	PMMA(%^{w/v})	Amplitude (%)	Time (mins)
A	0.2	0.2	60	30
A1	0.1	0.2	60	30
A2	0.2	0.1	60	30
A3	0.1	0.1	60	30
A4	0.5	0.5	60	30
A5	1	1	60	30
A6	0.2	0.2	30	30
A7	0.2	0.2	50	30
A8	0.2	0.2	80	30
A9	0.2	0.2	60	5
A10	0.2	0.2	60	15
A11	0.2	0.2	60	40

5.2.3. Statistical optimization of the microparticles

5.2.3.1. Design and optimization of the formulations using a central composite design

Preformulation studies identified polymeric concentration and sonication time to be two variables contributing to drug entrapment. A two factor Face-Centred Central Composite Design (FCCCD) was therefore employed for the determination of the effect of the two parameters (polymeric concentration (0.1- 0.5%^{w/v}) and sonication time(5-40 seconds) on the drug entrapment of the particles and on the mean dissolution time (MDT) at two pH values as described in Table 5.2.

Table 5.2: Formulation variables and responses applied in design

	Levels		Objective
	Upper	Lower	
Parameters			
Polymer concentration (% ^w / _v)	0.5	0.1	
Sonication time (minutes)	5	40	
Responses			
MDT ^{*1} (pH 6.75)			Maximize
MDT ^{*1} (pH 7.4)			Minimise
DEE ^{*2}			Minimize

^{*1} MDT: Mean Dissolution Time; ^{*2} DEE: Drug Entrapment Efficacy

The Face-Centred Central Composite Design provided the 14 formulations that were prepared as summarized in Table 5.3. Formulations were prepared as described in section 5.2.2.1 of this chapter. After the formulations were prepared, they were left for 12 hours to cure. The particles settled to the bottom and the supernatant was discarded. The particles were collected via centrifugation at 1000rpms for 3 minutes (Model TD5A-WS, Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., Shanghai, China) and resuspended in deionised water. The particles were centrifuged once more and collected. Particles were resuspended in deionised water and frozen for 24 hours. Hence the particles were only washed once as there was no need to remove organic solvents and the drug had been in solution and un-entrapped drug was likely to have been discarded in the initial supernatant. Formulations were then lyophilised (Labconco, Missouri, USA) for 48 hours.

Table 5.3: Composition of microparticle formulations in accordance with the FCCCD

Formulation	CHT / PMMA (% ^w / _v)	Time (minutes)
1	0.5	40
2	0.1	40
3	0.3	22.5
4	0.3	22.5
5	0.1	5
6	0.5	5
7	0.3	22.5
8	0.3	40
9	0.5	22.5
10	0.3	22.5
11	0.3	22.5
12	0.3	22.5
13	0.1	22.5
14	0.3	5

5.2.4. Determination of percentage of drug entrapped by the particles

To determine the quantity of drug entrapped within the microparticles, accurately weighed amounts of each sample (approximately 25mg) were placed in 100mL of 0.01M NaOH. Samples were left for 48 hours to ensure complete breakdown of the particles. Aluminium foil was used to cover the vessels to prevent degradation of the entrapped drug (Lund, 1994). Following centrifugation, the supernatant was assayed using UV spectrophotometry ($\lambda=280\text{nm}$) and drug concentration was determined. Equation 5.1 was used to determine Drug Entrapment Efficacy (DEE).

$$\text{DEE} = \frac{\text{Actual amount of drug in microparticles}}{\text{Theoretical amount of drug in microparticles}} \times 100 \dots\dots\dots \text{Equation 5.1}$$

A calibration curve of folic acid in sodium hydroxide (0.01M) was also constructed to determine amounts of drug entrapped in the particles. A stock solution was prepared by dissolving 1.65mg of folic acid in NaOH (50mL) in a volumetric flask. Serial dilutions were made and a UV spectrophotometer was used to measure the absorbance at a wavelength of 280nm.

5.2.5. Determination of the release of drug from the microparticles at various pHs

In order to determine the pH sensitivity of the particles, drug release studies were conducted using a dialysis tubing method as described by Moebus et al, 2009. Briefly, accurately weighed amounts of microparticles (~50mg) were placed in 6cm strips of dialysis tubing (molecular weight cut off: 12kDa) and the ends of the tubing were tied. The tubing was then placed into 100mL of phosphate buffered saline at an appropriate pH (6.75 or 7.4). At pre-determined time intervals, 10mL sample were drawn and replaced with an equal amount of pre-warmed buffer. In order to ascertain the fraction of drug being released at each time point a calibration curve of folic acid in PBS (7.4). The calibration curve for pH 6.75 can be found in Chapter 3, Section 3.3.1 of this dissertation. The Mean Dissolution Time (MDT) as described by Pillay and Fassihi (1998) was calculated for each of the formulations using Equation 4.1.

5.2.6. Examination of the morphology of the particles

In order to assess the size and shape of the prepared formulations, electron microscopy was undertaken using a scanning electron microscope, Phenom (FEI, USA). Samples were mounted onto stubs using carbon tape and sputter coated with gold under an argon atmosphere using a sputter-coater (SPI, USA) for 90 seconds.

5.2.7. Determination of the zeta potential of the optimised particles

In order to determine the stability of the microparticles, zeta potential was ascertained using a Zetasizer NanoZS (Malvern Instruments Ltd. Worcestershire, UK). The sample was diluted to less than a 1%^{w/v} solution with deionised water and placed in a capillary cell. Measurements were undertaken in triplicate.

5.3. Results and Discussion

5.3.1. Calibration curve of folic acid conducted to ascertain concentrations of folic acid entrapped in particles

A calibration curve was obtained as shown in Figure 5.2 and was utilised in the determination of the amount of drug entrapped in the microparticles.

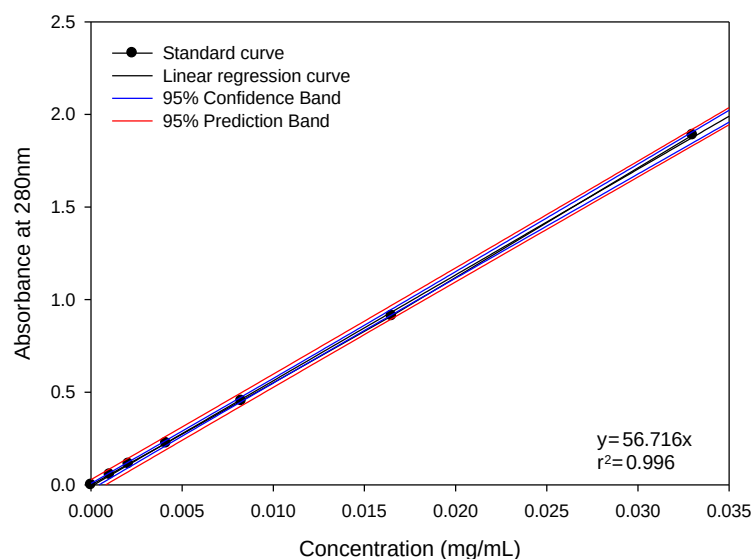


Figure 5.2: Calibration curve of folic acid in NaOH

5.3.2. Drug entrapment efficacy of preformulation microparticle formulations

The calibration curve was utilised together with the absorbances obtained in order to determine the drug entrapment efficacy of the various formulations of microparticles as shown in Table 5.4: Table listing formulations as well as the drug entrapment efficacy obtained for each formulation

Table 5.4: Table listing formulations as well as the drug entrapment efficacy obtained for each formulation (SD< 9.4)

Formulation	DEE
A1	45
A2	42
A3	61
A4	38
A5	48
A6	40
A7	62
A8	67
A9	62
A10	84
A11	81

The DEE of the formulations prepared during preformulation revealed some interesting trends and resulted in the variables being selected for experimental design. Altering the ratios of chitosan to PMMA (A1,A2) did not reveal desirable results. Increasing the amount of chitosan in the formulation decreased the DEE and although an increase in PMMA had a higher DEE, it was elected that the ratio of chitosan and PMMA would remain at 1:1 for the purposes of the design. Increasing the amounts of polymer in the formulation decreased the amount of drug being entrapped (A3-5), hence polymer concentrations were maintained between 0.1-0.5%^{w/v}. Although the higher amplitude (80%; A8) showed a high DEE, concerns for safety resulted in exclusion of this amplitude. For the purposes of the design the amplitude was maintained at 60%. Increased sonication time caused an increase in the DEE (A10-11). However, this was chosen as a variable as ideally the time was to be minimized. Hence, two variables were selected for further investigation and optimization of the particles formed. The concentration of the two polymers was to be varied between 0.1-0.5%^{w/v} and the sonication time was varied between 5-40 minutes. These values were then inputted into the two-factor Face-Centred Central Composite Design as described in Section 5.2.3 of this chapter.

5.3.3. Regression equations and examination of the FCCCD

The regression model for drug entrapment efficacy generated by the FCCCD showed very poor values for both the standard error of the model (S=5.938) and the coefficient of determination ($R^2= 17.9$). Slightly better values were obtained for the $MDT_{6.75}$ (S= 0.6003, $R^2 = 72.4$) and $MDT_{7.4}$ (S= 0.6388, $R^2=53.3$). These values are indicative of poor model fitting. Regression equations were generated (Equation 5.2, 5.3 and 5.4)

$$DEE = 65.7214 - 0.5543[CHT] - 2.1317[Time] + 2.55[CHT*CHT] + 0.5150[Time*Time] - 0.4775[CHT*Time] \dots \dots \dots \text{Equation 5.2}$$

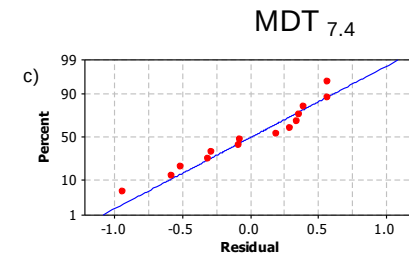
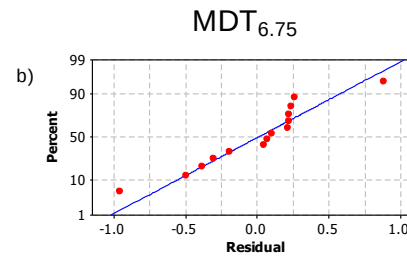
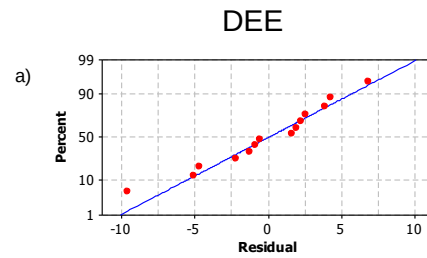
$$MDT_{6.75} = 66.7366 - 0.2121[CHT] + 0.3940[Time] + 0.5396[CHT*CHT] + 0.4241[Time*Time] - 0.0712[CHT*Time] \dots \dots \dots \text{Equation 5.3}$$

$$\text{MDT}_{7.4} = 37.4789 - 0.1558[\text{CHT}] + 0.3823[\text{Time}] + 0.5179\{\text{CHT}*\text{CHT}\} + 0.8353[\text{Time}*\text{Time}] - 0.0168[\text{CHT}*\text{Time}] \dots \text{Equation 5.4}$$

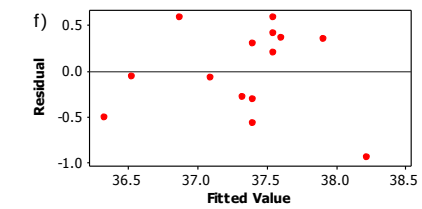
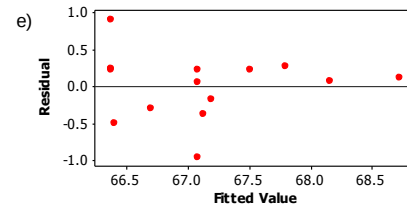
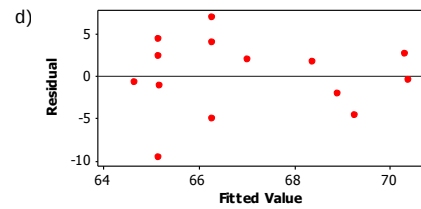
5.3.3.1. Residual error in the design

Figure 5.3 presents the various residual plots associated with the design and responses. The normal probability plots (a-c) for all the responses showed approximately straight lines indicating a normal distribution of residuals. Some outliers can be observed in graphs (a) and (b). The residuals versus fitted value scatter plots (d-f) showed random scatter of the residuals around 0 as expected. The plots of the residuals versus the order of the data (g-i) showed somewhat of a dependence for the early observation points. Histograms, plotting residuals against frequency (j-l), are bell shaped although somewhat skewed to the left (j) and (l).

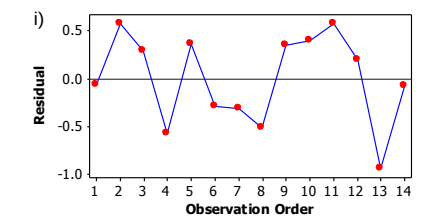
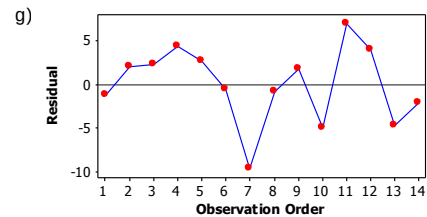
Normal probability plots of the residuals



Residuals versus the fitted value



Residuals versus the order of the data



Hisogram of the residuals

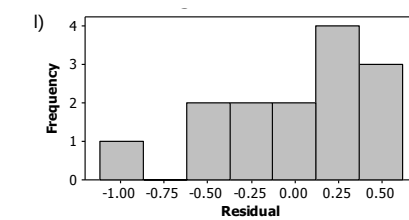
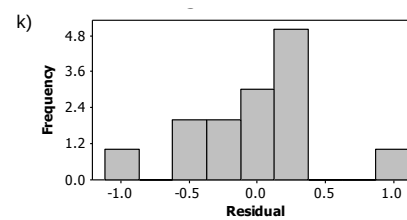
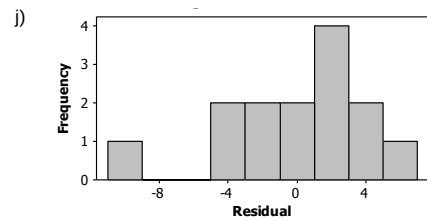


Figure 5.3: Residual plots obtained for the responses examined in the design.

5.3.4. Entrapment of drug within the design microparticles

Most of the formulations showed desirable entrapment ranging from 55-74%. As shown in the response surface plot (Figure 5.4) the DEE was higher at the extremes of polymer concentrations. Higher polymeric concentrations may allow more efficient entrapment of the drug within the formed particles. Higher entrapment efficacies observed with lower polymer concentrations could be due to surface adherence of the drug rather than entrapment within the polymeric complex.

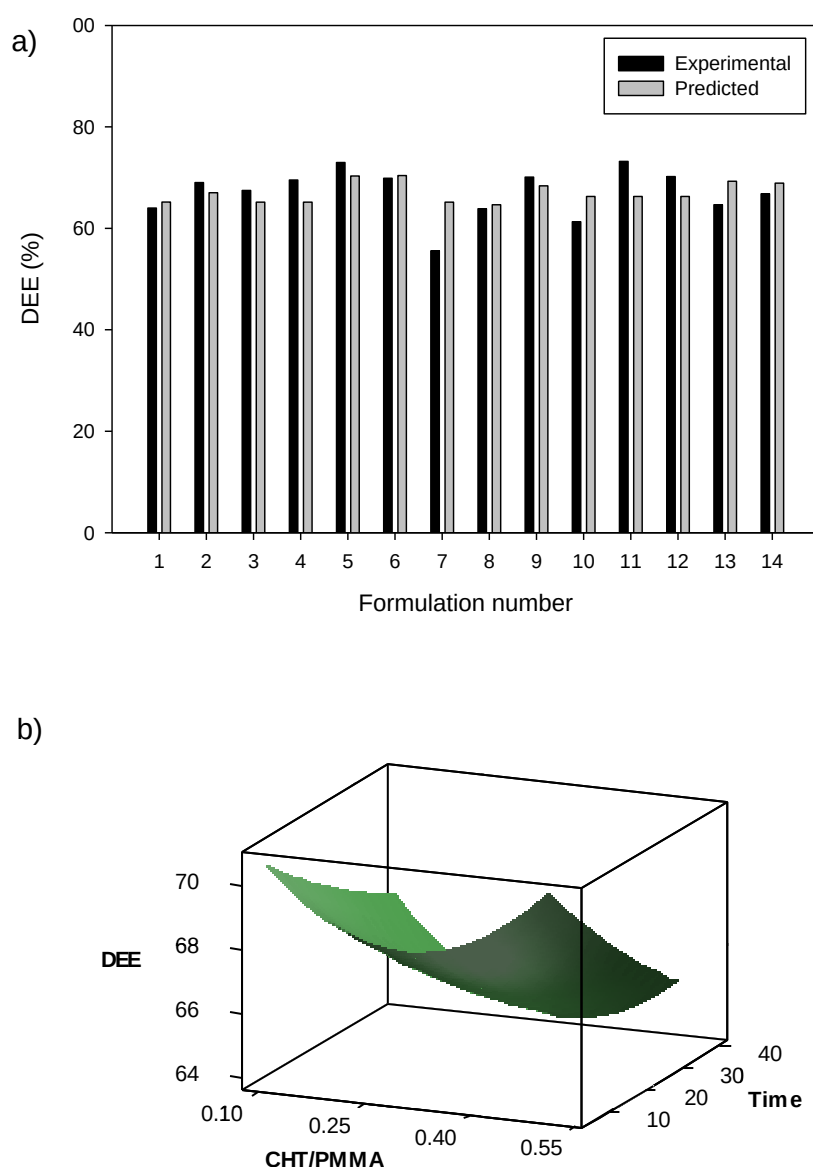


Figure 5.4: a) Bar graph showing the DEE: experimental and predicted values and b) response surface plot showing the relationship between the polymer concentration and DEE.

Interestingly a decrease of entrapment efficacy is noted with an increase in sonication time and the effect appears larger at high concentrations rather than lower ones. This could possibly be due to the heat generated by sonication. Following sonication, temperatures of the beaker were found to be 70°C. It is a possibility that this influenced the entrapment of the drug. It also possible that at these high temperatures some degradation of the drug occurred (Lund, 1994)

5.3.5. Release of folic acid from the optimised microparticle formulation

As shown in Figure 5.5, release of folic acid from the particles was conducted in buffered saline solutions, pH 6.75 and pH 7.4 and showed slightly different profiles. Release of the drug from the particles appeared to be pH responsive with the release of drug faster at pH 6.75 and slower at pH 7.4. The enhanced permeation and retention (EPR) effect was first described by Maeda et al.,2000. Observed in solid tumors, this effect describes the accumulation of macromolecules and particles at the site of the tumor for up to 100 hours (Matsumara and Maeda et al., 1986) and is a result of hyper-permeability of the tumoral vasculature and poor lymphatic drainage as described in Chapter 1 of this dissertation. Hence although release of the drug does occur at pH 7.4, it is not expected that much of the drug will reach the circulation as most of the particles are likely to be retained at the site of the tumor due to the EPR effect. Table 5.5 shows the various MDT values obtained for the formulations and demonstrates the faster release obtained at pH 6.75 compared to pH 7.4. Furthermore, it must also be noted that there are very small differences between the values.

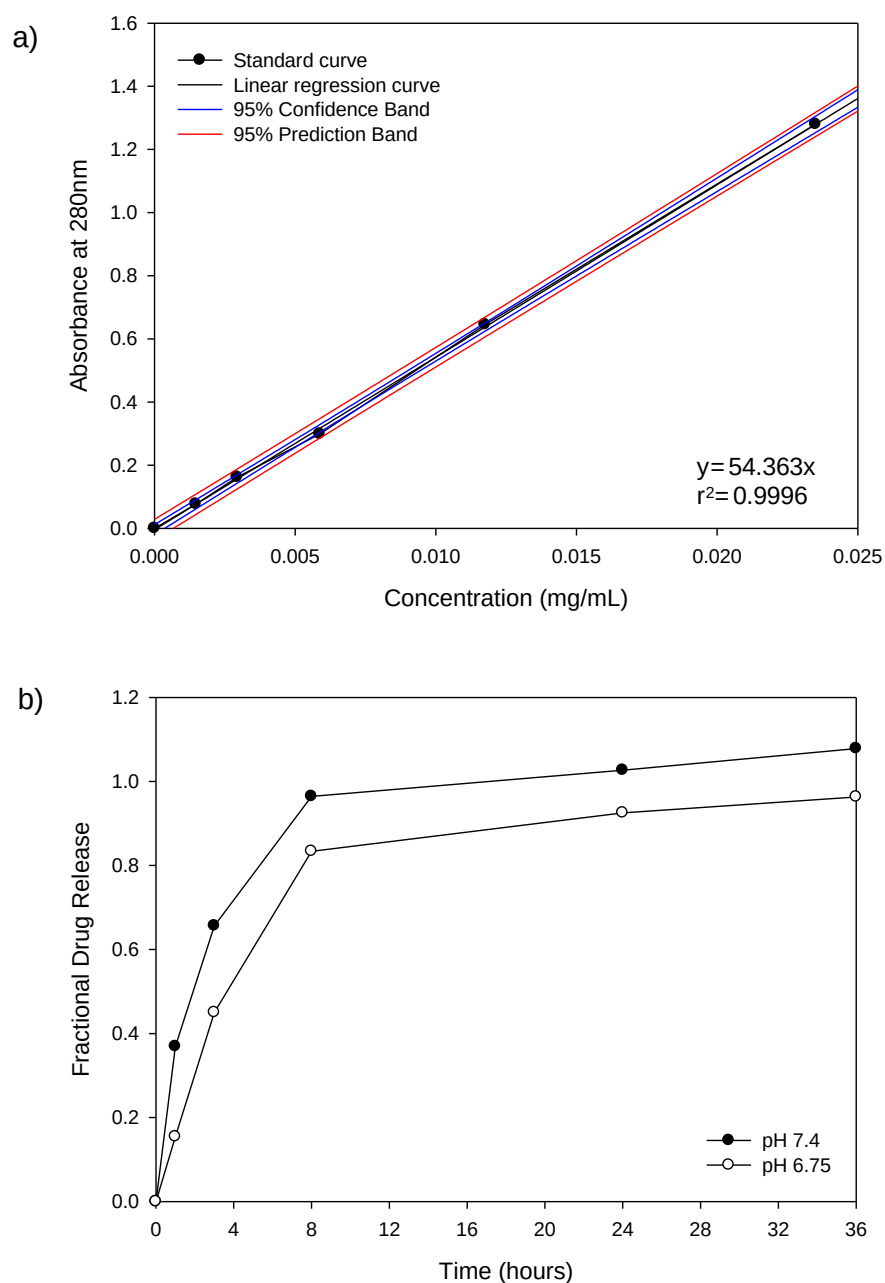


Figure 5.5: a) Calibration curve of folic acid in PBS, pH 7.4 and b) typical release profile obtained from a microparticle formulation.

Table 5.5: MDT values obtained for the formulation generated by the FCCCD

Formulation	MDT pH 6.75	MDT pH 7.4
1	68.84	36.47
2	68.24	37.46
3	67.14	37.70
4	67.32	36.83
5	67.75	37.97
6	68.07	37.05
7	66.13	35.83
8	67.02	37.10
9	66.76	38.26
10	66.60	37.95
11	67.27	38.13
12	66.63	37.75
13	66.41	37.29
14	65.92	37.02

Response surface plots as summarized in Figure 5.6 show the effect of the concentration of the polymers and the sonication time on the mean dissolution time at two pH values. At low sonication times the concentration of polymers does not appear to affect the $MDT_{6.75}$ as much as at high sonication times. Further $MDT_{6.75}$ appears to increase with sonication time with maximal release occurring at high sonication times and high polymer concentrations. The response plot for $MDT_{7.4}$ appears to be the inverse of $MDT_{6.75}$. A maximal release from formulation would be obtained at medium sonication times and with high polymer concentrations. As both the polymers were being used at the same concentrations, it is difficult to attribute the results to one polymer. However, the release profile suggest that the release is more dependent on the chitosan component. PMMA dissolves at pH 7 and as a result the release should have occurred rapidly from the formulations at this pH if PMMA was playing a larger part in the release. As these results appear to indicate, the release of folic acid is faster at lower pH values. This is concurrent with the reported pKa of chitosan (approximately 6.5), as at pH values below this value the polymer is soluble and above this pH the polymer is insoluble (Dehousse et al., 2010).

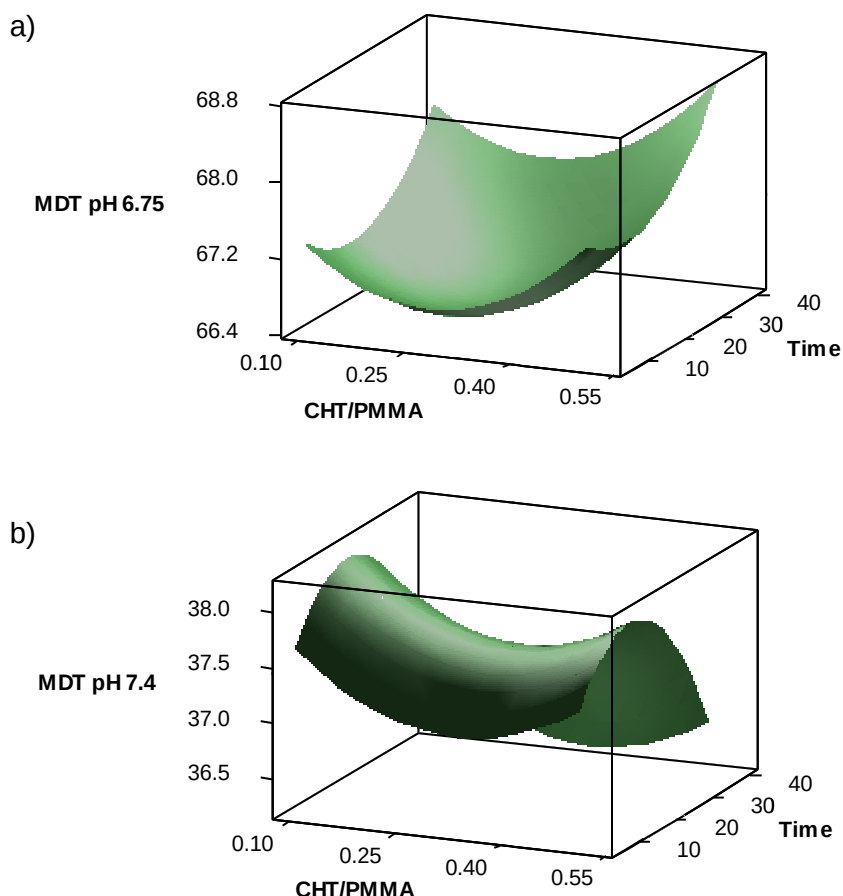


Figure 5.6: Response surface plots generated by the FCCCD analyzing the effect of CHT/PMMA concentrations and sonication time on the responses a) mean dissolution time at pH 6.75 and b) mean dissolution time at pH 7.4.

5.3.6. Determination of the morphology of the microparticles using SEM

As seen from the scanning electron micrographs (Figure 5.7) the microparticles had peculiar spindle like morphology. Note the single spindles in the lower micrograph as well as the clumps in the first micrograph. The size of the spindles ranged from 1.64 μm -9.88 μm in length and widths ranged from 0.29-0.6 μm . The reason for the formation of these spindle like particles has been hypothesized to be a result of the increasing crystalline structure of the particles.

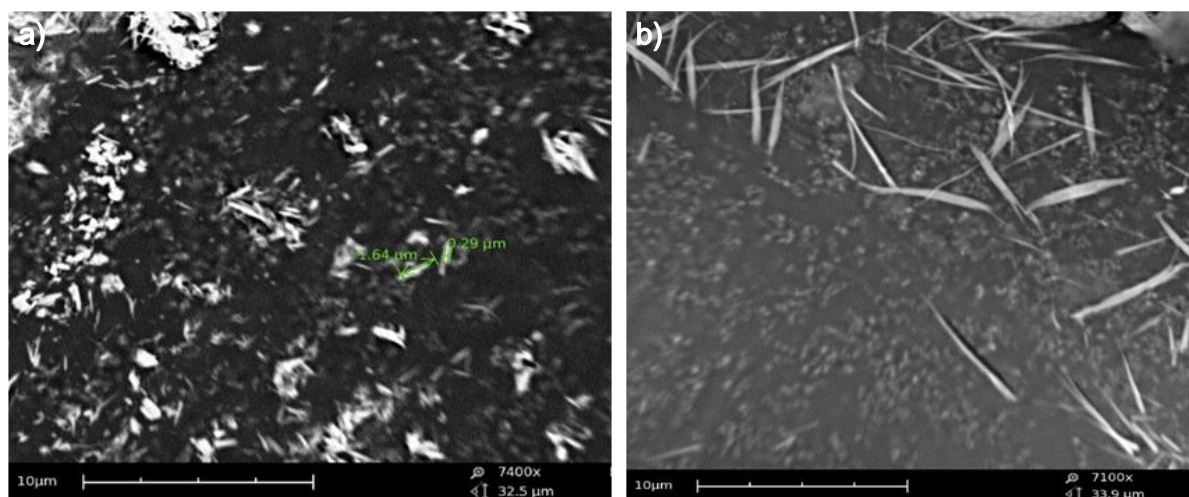


Figure 5.7: Electron micrographs showing the spindle like morphology of the microparticles.

5.4. Response optimization

The particle formulations were optimized using Minitab® V15 (Minitab Inc, PA, USA) to determine optimal levels of formulation components. The optimal formulation was found to consist of 0.4315%^{w/v} of PMAA and CHT with a sonication time of 40 minutes. Desirability values were satisfactory and obtained values were in close agreement with predicted values.

Table 5.6: Values obtained for each response as well as the model predicted values and the desirability.

Response	Obtained (folic acid)	Predicted	Desirability
DEE	64.89	64.60	1.00000
MDT _{6.75}	67.45	67.9745	0.99149
MDT _{7.4}	37.87	36.3717	0.82134

5.4.1. Loading of the optimised microparticle formulation with methotrexate

As mentioned in Chapter 1, Section 1.2.1 of this dissertation formulations were prepared with folic acid due to the high cost of the chemotherapeutic drug, methotrexate. Hence, only optimized formulations were loaded with methotrexate.

5.4.1.1. Determination of the drug entrapment efficacy of the microparticle formulation

To determine the amount of drug entrapped within the microparticles, 25mg of each sample (approximately) were placed in 100mL of 0.01M HCl. Samples were left for 48 hours to ensure complete solubilisation of the particles. Following centrifugation, the supernatant was assayed using UV spectrophotometry ($\lambda=306\text{nm}$) and drug concentration was determined. Equation 5.1 was used to determine the amount of drug entrapped in the formulation. A calibration curve of methotrexate in HCl (0.01M) (Figure 5.8) was also conducted to determine quantity of drug entrapped in the particles. Drug entrapment efficacy was very high for the optimized formulation with a DEE value of 95.28%.

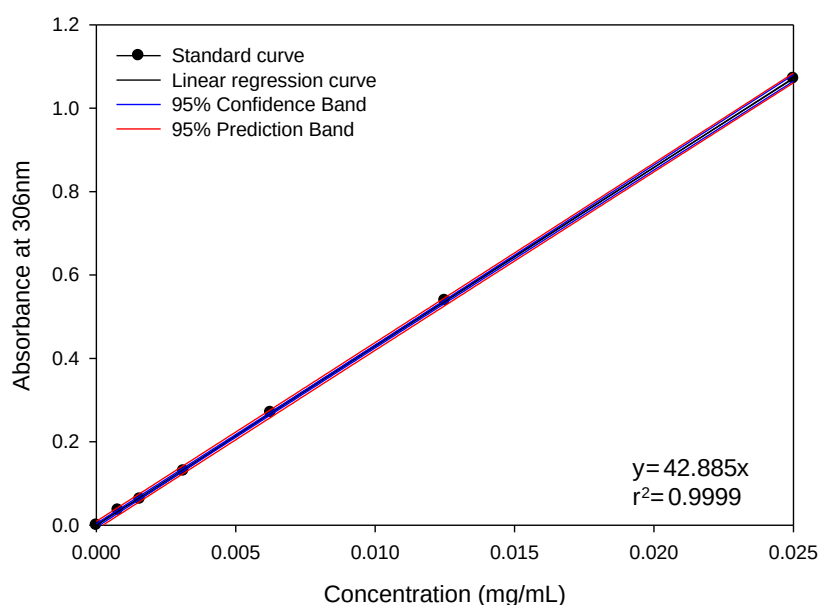


Figure 5.8: Calibration curve of MTX in hydrochloric acid.

5.4.1.2. Release of methotrexate from optimized microparticle formulations

Release of MTX from the optimized particles was conducted at three pH values. Although the size of the particles excluded use of the particles for intracellular targeting, the effect of the pH on the release was of interest. Figure 5.9 shows the release profile obtained from the optimized particles at three pH values.

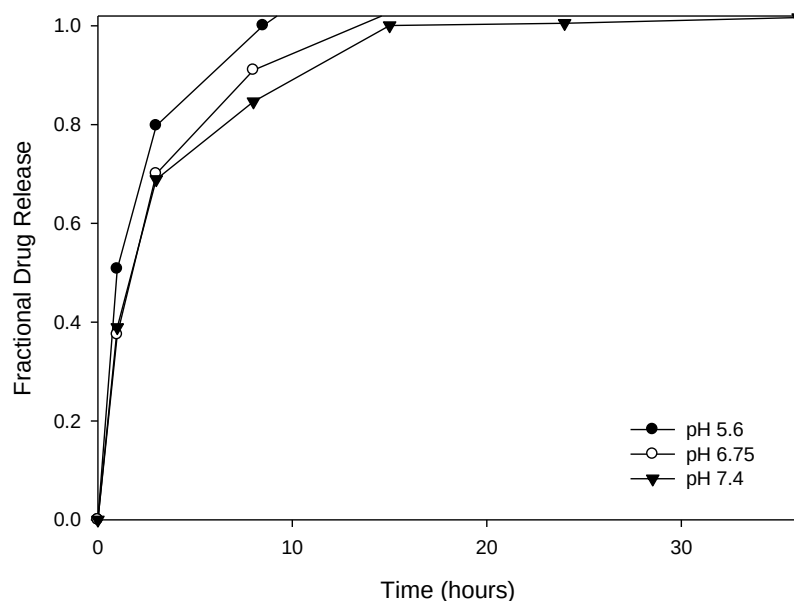


Figure 5.9: Release of MTX from the optimized formulation at three different pH values (37°C, n=3, SD<0.03).

As can be observed the pattern of release is similar to that observed for the design formulations with increasingly rapid release with a decrease in pH values. However the effect is not as great as that observed with folic acid. Had the size of the particles been smaller, the particles could have provided release of the drug intracellularly. However, at the present sizes as shown in the electron micrographs (Figure 5.7), it is unlikely that the particles would be taken up by cells. In addition, the MTX-loaded particles show a large burst release and it is possible that the washing step described in section 5.2.4., was not sufficient especially with the methotrexate loaded particles and hence a larger burst release was noted with these particles at all pHs possibly indicating the presence of surface drug.

5.4.1.3. Determination of the zeta potential of the particles

Zeta potential is used to refer to the cloud of charge surrounding particles. The charge around a particle is an important determinant of its stability. Weak surface charges (+30mV or -30mV) cause repulsion between the particles inhibiting aggregation. High forces between the particles or neutral charges cause attractive forces between particles leading to aggregation of the particles.

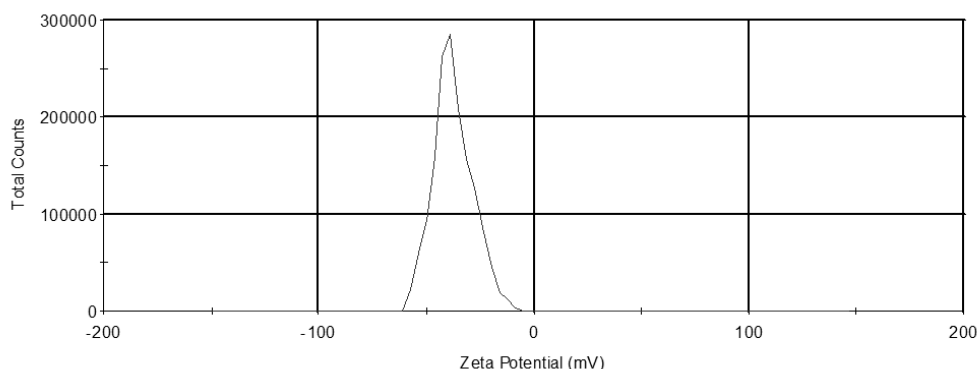


Figure 5.10: Zeta potential distribution obtained for the optimized formulation.

The results (Figure 5.10, -23mV $n=3$, $SD=5.03$) indicated that particles had a moderately high electrostatic charge associated with them indicating that the particles were unlikely to aggregate.

5.5. Investigation of the effect of microparticles in the ISFI

Optimized ISFI formulations were prepared as per Section 4.4 of Chapter 4 of this dissertation. Microparticles were loaded with MTX and prepared as described in Chapter 5, Section 5.2.2 with concentrations as per the optimized formulation described in Chapter 5, Section 5.4.

5.5.1. Determination of the release of MTX from the combination of MTX-loaded particles and the ISFI

The optimized ISFI (3mL) was mixed with 50mg of the optimized MTX-loaded particles. The formulation was then tested for drug release as described in Chapter 4, Section 4.2.6. It should be noted that contrary to the observed zeta potential values aggregation of the particles did occur and it was deemed unlikely that the formulation would pass through a 18G needle, making the injectability of the formulation a challenge.

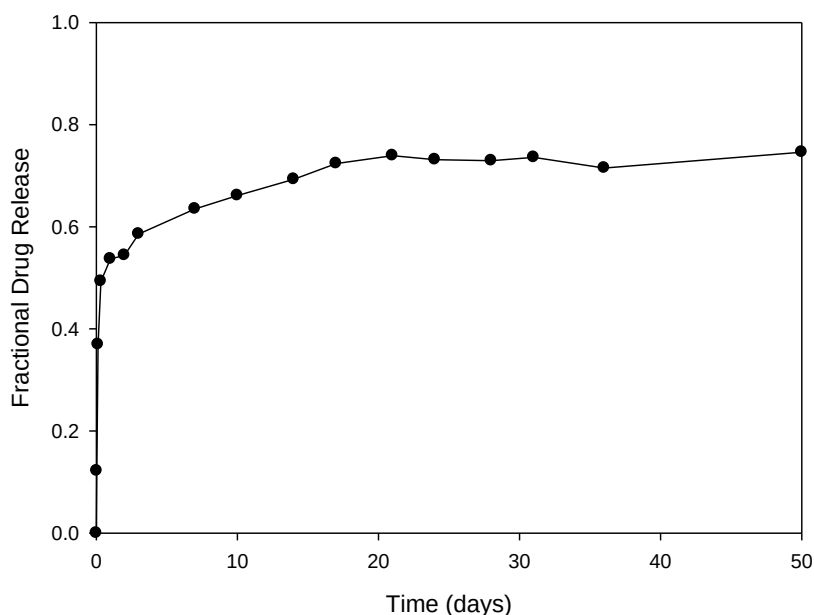


Figure 5.11: Release of MTX from drug loaded particles in the ISFI (37°C, pH 6.75, n=2, SD< 0.04)

Release of the drug occurred rapidly with the addition of the particles (Figure 5.11). However, as mentioned there was significant clumping of the particles observed upon addition of the particles to the ISFI solution i.e. clumping occurred at room temperature and before thermal gelation temperatures were reached. It is possible that when gelation did occur, the “clumps” of particles were pushed to the edges of the ISFI and hence release rate was increased. Another hurdle for utilization of the implant formulation containing drug-loaded microparticles, is the low amount of drug entrapped. The amount of drug that can be loaded is limited by the concentration of microparticles that can be added without creating a slurry. As a result 50mg of microparticles were added. This equates with the addition of 1.86mg of MTX. As a result this formulation cannot be used solely for the delivery of the drug to the solid tumor. Should the particles not create aggregation in the formulation, the possibility of a double loaded implant exists, i.e. an implant containing drug loaded in the implant and within the particles dispersed in the implant. This would offer a number of interesting possibilities such as the loading of the same drug with two rates of release or the loading of two different drugs. However, the significant clumping, the lowered gelation temperature and the difficulty of injecting the microparticle containing implant preclude use of this formulation.

5.5.2. Rheological investigation of the formulation

Rheological tests were carried out as first described in Chapter 3, Section 3.2.3 of this dissertation. As shown in Figure 5.12, the gelation temperature of the formulation is much lower than expected. This gelation temperature would be that samples would have to be refrigerated as normal handling could lead to gelation.

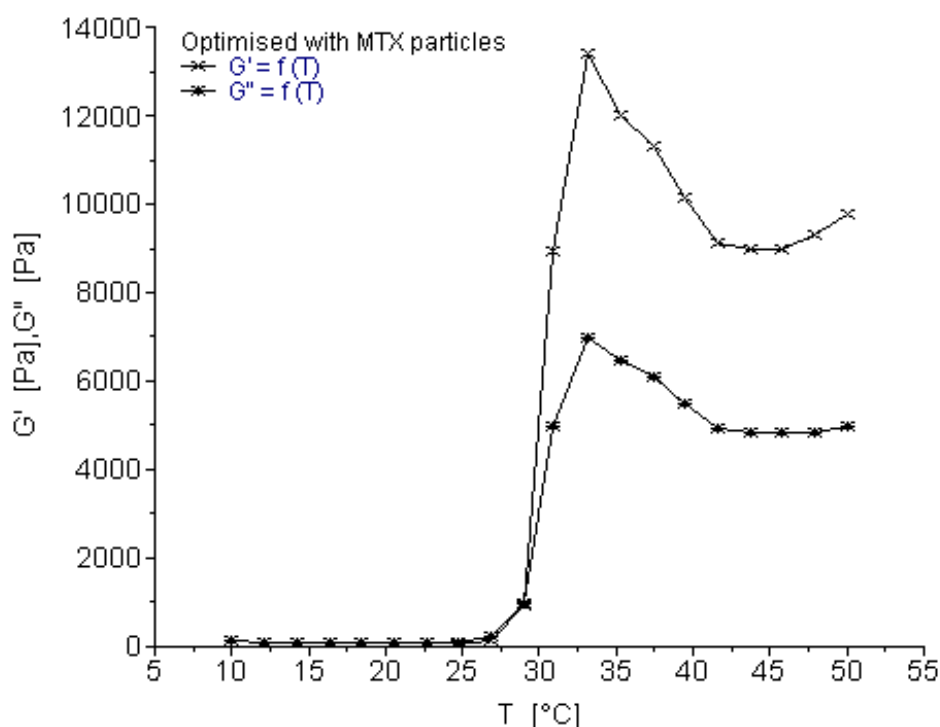


Figure 5.12: Rheological profile of the optimized implant containing MTX loaded particles.

5.6. Investigation of the effect of bicarbonate loaded microparticles

5.6.1. Preparation of the formulation

Particles were prepared as described in Chapter 5, Section 5.2.2. However, instead of adding the drug, 50mg of sodium bicarbonate as an erosion controlling substance as described in Chapter 3 of this dissertation, was added to the formulation. ISFI was prepared as described in Chapter 4, Section 4.4 as per the optimized formulation. Folic acid (15mg) was added to the ISFI and then the bicarbonate loaded particles (50mg) were added to create the formulation.

5.6.2. Determination of the effect of bicarbonate particles on the release of drug entrapped within the implant

Release from the implants loaded with bicarbonate particles and drug showed promising results (Figure 5.13). When the release from this formulation is compared to the addition of sodium bicarbonate directly to the implant as shown in Chapter 3, Figure 3.15 of this dissertation, a more pronged release was seen from the microparticle loaded formulation. Release from this implant must also be compared to that of the optimized ISFI (Figure 4.15) which showed release of approximately 25% of the drug after 50 days.

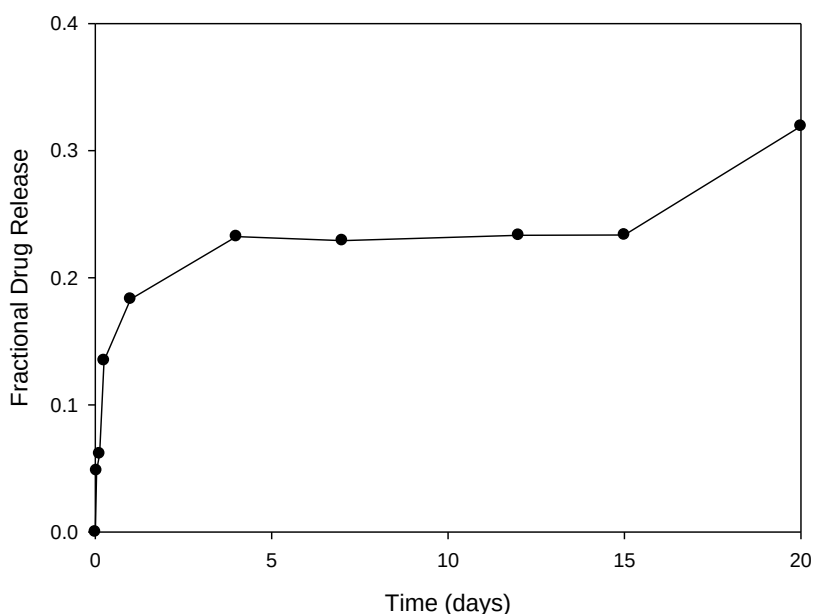


Figure 5.13: Release of folic acid from the ISFI loaded with sodium bicarbonate particles (37°C, pH 6.75, n=2, SD< 0.09).

It is possible that unlike observed with the addition of the salt in the powder form, the particles are not dissolved in the polymer matrix but rather remain suspended changing the pH surrounding the area and this could be the reason why the release of drug is more sustained. However the amount of bicarbonate that was entrapped within the particles could not be ascertained. This is a clear disadvantage and if the particles are to be utilised further, additional testing on these particles is required.

5.7. Concluding Remarks

This chapter described the development of microparticles for the enhancement of drug release from the ISFI. A FCCCD was utilized to achieve the optimization of the microparticle formulation. However, the FCCCD showed poor fitting indicating that the models generated were not ideal. Microparticles showed relatively high entrapment of the drug and pH responsive release of the drug. Particles were then optimized and loaded into the optimized ISFI. Two challenges were encountered, firstly the release of drug occurred rapidly and secondly the delivery of the combination of implant and microparticles proved difficult considering microparticle aggregation. For these reasons, particles were not tested *in vivo* although *in vitro* testing on cells was performed as described in Chapter 6 of this dissertation.

CHAPTER 6

***IN VITRO* TESTING OF THE ISFI AND MICROPARTICLES AGAINST VARIOUS CELL LINES**

6.1. Introduction

Chapters 4 and 5 described the *in vitro* release of the cytotoxic drug methotrexate from the implant and the microparticles respectively. This chapter aimed to determine the effect of MTX incorporated within the ISFI on a cancer cell line in order to determine whether the drug was being released in sufficient quantities over time to result in the death of the cancer cells. The optimised ISFI containing MTX as described in Chapter 4 of this dissertation and the optimised particles as described in Chapter 5 of this dissertation were selected for *in vitro* testing on the cell lines. In addition, the effect of the polymer, PMVE on the viability of the cancer cells was also determined. In order to achieve these aims, colon carcinoma cell lines (HT29 and DLD1) and a breast cancer cell line (MCF7), which were to serve as models for solid tumors for the purpose of this study, were grown. Both a MTT assay and flow cytometry were used to determine the percentage of living cells after treatment with elutes from the implant or from the particles.

MTT assays are colourimetric assay providing a measurement of cell viability by providing a measure of the proliferation of the cells. The tetrazolium salt, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added to the cells and is subsequently reduced to a purple formazan in the mitochondria of metabolically active (living) cells (Figure 6.1) Singh et al., 2007. A solubilisation solution such as dimethyl sulfoxide is then added to the cells to dissolve the formazan product to yield a coloured solution. Comparison of the treated cells with the untreated control wells provides an indication of the cell viability. Treated cells showing higher absorbances than the control cells is indicative of enhanced proliferation of the cells and lower absorbance values indicate of a reduction in cell growth.

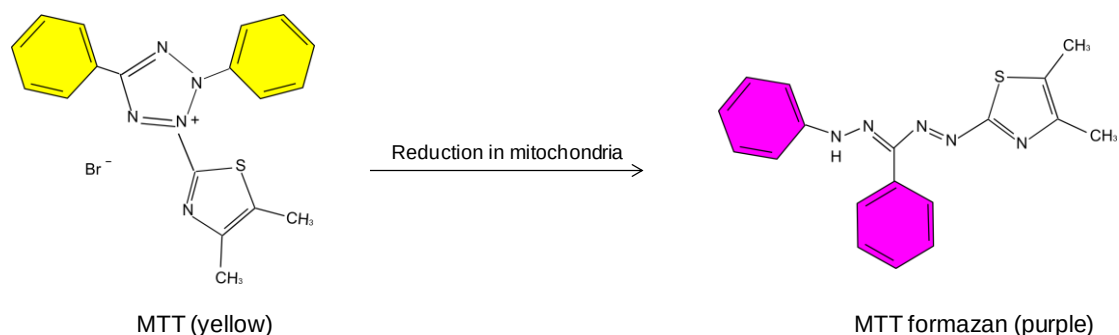


Figure 6.1: Yellow MTT is reduced to purple formazan crystals in the mitochondria of living cells.

Flow cytometry is a highly accurate way of determining the viable and dead cells in a population of cells. The fluidics system in a flow cytometer allows for individual characteristics of each cell to be determined as it causes the cells in a particular sample to flow in a single stream past the machine's detection system. The fluidics system (Figure 6.2) consists of two parts: A central core through which the sample will be aspirated via the application of pressurised air and a sheath surrounding the core through which sheath fluid flows. As the sheath fluid flows faster, this creates a drag effect on the narrowed central core and forcing the fluid and hence the cells to flow in a parabolic manner with faster flow in the centre and slower flow along the edges, hence forcing the cells into a single stream. This process is called hydrodynamic focusing. A higher sample pressure than sheath fluid pressure ensures that no mixing of the sample with the sheath fluid occurs (<http://www.bdbiosciences.com/support/training/index.jsp>, Accessed 2011-05-31).

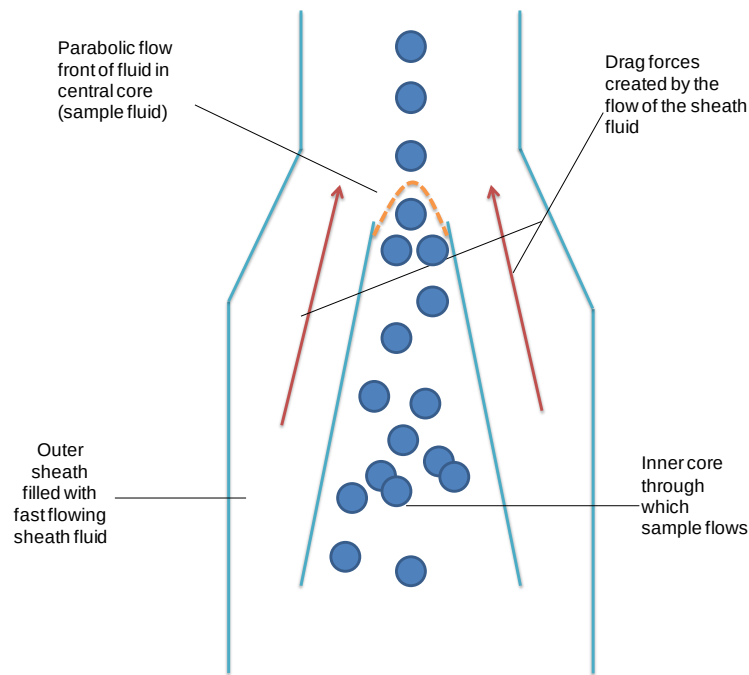


Figure 6.2: Schematic showing the principle of hydrodynamic focusing utilised by the flow cytometer within the flow cell to allow the flow of single cells past the detector.

Following hydrodynamic focusing, cells then flow past laser beams which are the light sources for scatter and fluorescence as shown in Figure 6.3. The light is then gathered by the optic system (consisting of various filters) and received by the detectors which convert the information from light intensity into voltage. Light that is scattered in the forward direction is collected by the forward scatter channel diode (FSC). As the amount of forward scatter produced by the cells is determined by the size of the cells, the forward scatter is a measure of the size of the cells. The light collected by the side scatter channel serves as a measure of the complexity of the cells as the granularity and structural complexity within the cell causes it to be scattered at larger angles. In order to gain information about the viability of the cells, fluorescent dyes are added to the cell sample. When laser light of the correct wavelength strikes the fluorescent dye, excitation occurs and the resulting fluorescence emission light is detected by the appropriate wavelength detector (<http://www.bdbiosciences.com/support/training/index.jsp>, Accessed 2011-05-31).

7-Amino-actinomycin (7-AAD) is a nucleic acid dye commonly used in flow cytometry and was selected for its ability to penetrate the cell membranes of dying or dead cells

and intercalate with DNA. It is effectively excluded by viable cells. Its fluorescence is detected in the far red range of the spectrum (650nm long-pass filter).

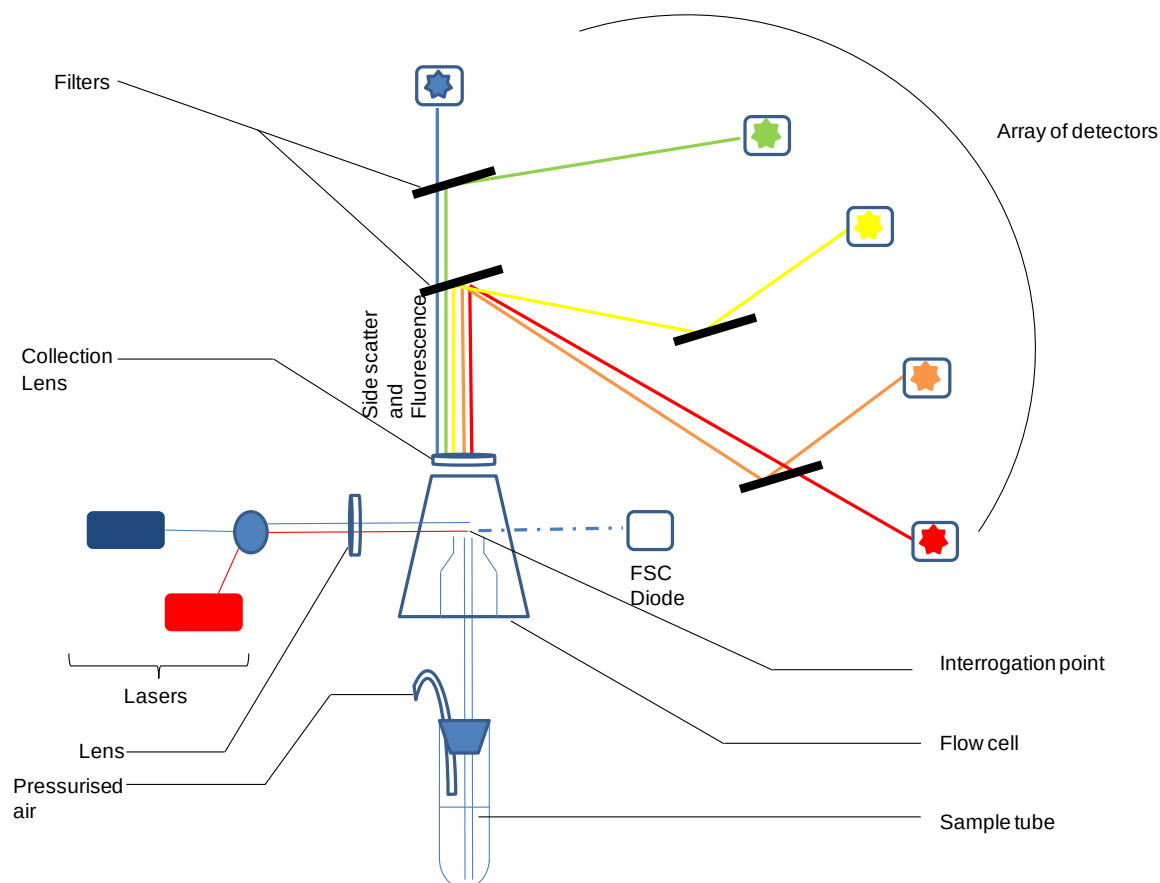


Figure 6.3: Figure showing a simplification of the fluidics and optic systems of the FACSCalibur (BD Biosciences, New Jersey, USA), the flow cytometer used in this study (adapted from: <http://www.bdbiosciences.com/support/training/index.jsp>).

6.2. Materials and Methods

6.2.1. Materials

HT29, MCF-7 and DLD1 cells were obtained from the ATCC (American Type Culture Collection). Dulbeccos Minimum Essential Medium (DMEM), fetal bovine serum (FBS), Penicillin-Streptomycin Mixture (Pen-Strep), Trypan Blue (0.4%_{v/v} solution), Trypsin-Versene[®] (0.5g/L trypsin:250 and 0.2g/L Versene[®] (EDTA)) and cryoprotective medium (15%_{v/v} DMSO) were all purchased from Whitehead Scientific, Brackenfell, South Africa. Phosphate buffered saline tablets were purchased from Sigma Aldrich (Steinheim, Germany) and once prepared were

autoclaved before use on the cells. Trypan Blue Solution (0.4%), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dimethyl sulphoxide (DMSO) were also purchased from Sigma Aldrich (Steinheim, Germany). Tubes used during flow cytometry analysis (BD™ Falcon tubes (5cm) with polypropylene lids) were purchased from The Scientific Group, Randburg, South Africa. Tissue cell flasks and other cell culture equipment was purchased from Lasec (South Africa).

6.2.2. Methods

6.2.2.1. Aseptic technique

Aseptic technique was utilised at all times in the culturing of cells. Briefly all surfaces of the horizontal laminar flow unit (Labotec, Midrand, South Africa) were wiped with 70% ethanol before commencing work. Laminar flow units are validated bi-annually. Gloves were worn at all times and were periodically sanitised with ethanol and when leaving the laminar flow unit. All tissue culture bottles and flasks were sanitised with 70% alcohol prior to placing them in the unit and were flamed on opening and closing. All used disposable flasks, pipettes and culture equipment was sent for incineration, and all non-disposable items were treated with bleach for 2 hours before being discarded to the drain with copious amounts of water. Cell medium was periodically tested for contamination by incubating media at 37°C for 24 hours.

6.2.2.2. Culturing and counting of cells

Standard methods were employed for the culturing of all cell lines. Cell lines were grown using DMEM (with 4.5g/L glucose and L-glutamine) supplemented with 10%^{w/v} fetal bovine serum and 0.2%^{w/v} Pen-Strep® in 25cm³ flasks. When cells had reached confluency, media in the culture flask was discarded and 3mL Trypsin-EDTA was added to each flask and the flasks were placed in the incubator for 3-4mins. When cells had detached, 3mL of fresh media was added and the entire volume was centrifuged at 1000rpm for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in 10mL of fresh media and plated into two flasks. When necessary, cells were frozen in a 1:1 mixture of cryoprotective medium (15%^{v/v} DMSO) and media at -70°C.

6.2.2.3. Cell counting using the trypan blue exclusion assay and a haemocytometer

Following trypsinisation as described in Chapter 6, Section 6.2.2.2., and the discarding of the supernatant, the cell pellet was resuspended in 3mL of fresh media. The number of cells per mL of media was then calculated using the trypan blue exclusion assay. Briefly, the cell suspension (10 μ L) was added to 20 μ L of trypan blue. The chamber of a disposable haemocytometer was then filled and the chamber was examined using light microscopy (Olympus CKS microscope, Olympus, Japan). The haemocytometer allowed the counting of the cells. Trypan blue is a stain which is taken up by dead cells and excluded by living ones and hence by counting stained cells as well as unstained cells allowed the estimation of dead cells in the sample. Only samples that showed viability of greater than 95% were utilised in subsequent testing.

6.2.2.4. The inclusion of controls into test runs

The use of controls is essential during the testing of samples especially when living cells are being utilised. For this reason positive and negative controls were used during the testing of samples. Table 6.1 summarises the types of controls used and the reason for the addition.

Table 6.1: Summary of the controls used in the study.

Type of control	Method	Reason for addition
Positive	Addition of a standard solution of MTX to cells	Determination of sensitivity of the cells to MTX
Negative	1. Cells allowed to proliferate without the addition of other substances	To ensure that cells are viable and that the tested substance is responsible for the death of the cells
	2. Incubation of blank medium with no cells	To verify that the media is uncontaminated

6.2.2.5. MTT assay

6.2.2.5.1. Preparation of the MTT solution

A 0.5%^{w/v} of MTT was prepared by adding 5g of MTT to 1000mL of phosphate buffered saline, pH 7.4. The solution was filtered through 0.22 μ m filters and stored in the fridge (4°C) in a foil covered flask until further use.

6.2.2.5.2. Calibration curve of HT29 cells using the MTT assay

A calibration curve for the MTT assay was conducted by starting with a cell suspension of 1×10^6 cells/mL. Serial dilutions in culture medium were then made yielding concentrations ranging from 1×10^6 cells/mL to 1×10^3 cells/mL. Cell suspension (100 μ L) was plated out in triplicate in the wells of a 96 well plate (NuncTM Surface 96-well plates, sterile, NuncTM, Roskilde, Denmark). Media (200 μ L) was added to each well of the plate. The plate was incubated for 48 hours in an incubator (37°C, 5% CO₂). MTT (40 μ L of a 0.5%^{w/v} solution) was added to all the wells and the plate was incubated for a further 4 hours. The plate was then centrifuged at 3000rpm for 5 mins. The supernatant (200 μ L) was removed and DMSO (150 μ L) was added to each well to dissolve the formazan crystals. Plates were maintained at 37°C and shaken for 5 minutes using an orbital plate shaker (MRC Thermo-Shaker, Hagavish, Israel). Plates were then read using a Labsystems iEMS Reader MF (Helsinki, Finland) with Ascent PC software at 540nm with a reference wavelength of 690nm. Figure 6.4 shows the obtained calibration curve for HT29 cells which was subsequently used to determine the number of cells to add to each well.

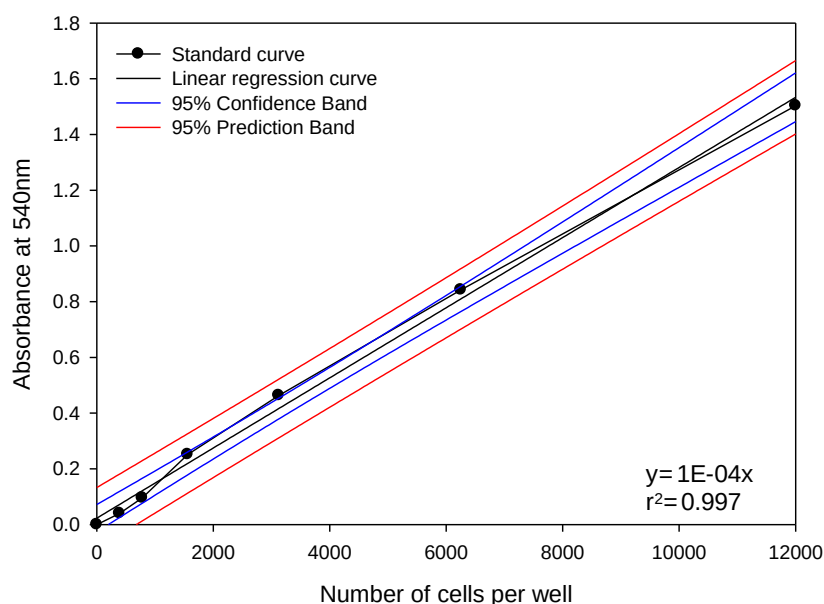


Figure 6.4: MTT calibration curve obtained for HT29 cells

6.2.2.5.3. Determination of the effects of the drug release elutes and microparticles on HT29 cells utilising the MTT assay

6.2.2.5.3.1. Preparation of test samples

As the amount of solution that can be tested when using an MTT assay is typically 100 μ L or less, direct elutes from drug release studies were not used. Instead the amount of drug which was determined to have been released at each period of time was prepared aseptically and added to the wells of the 96-well plate. Solutions of the polymer which had been autoclaved as described in Chapter 4, Section 4.4.4 of this dissertation were added directly to the wells of the plate in a concentration range of 8.435g/mL to 0.263g/mL. Microparticles were suspended in media at 2mg/100 μ L media. Solutions of the amount of drug believed to have been released after 6 hours and 24 hours were also prepared. A control formulation was prepared in accordance with the literature at a concentration of 55nM for HT29 cells (Leteurtre et al., 2004). However, at this concentration cells (examined using the trypan blue assay) did not show susceptibility. For this reason the concentration of MTX used as a control in this study against HT29 cells was 5.5 μ M.

6.2.2.5.3.2. Testing of samples

Culture media (100 μ L) was added to each well of the 96 well plate. As per the calibration curve obtained (Figure 6.4), cells were plated at a density of 6250 cells/well (62500 cells/mL) as this would result in a maximum possible absorbance of approximately one. Cells were allowed to attach and grow for 24 hours in an incubator (37°C, 5% CO₂). Test samples (100 μ L) prepared as described in Chapter 6, Section 6.2.2.6.1 were then added to the wells and the plate was incubated for 24 hours. Microparticles were added in two sets. The first set (24 hours) was added when the implant test samples were added. The second set was added 6 hours before the addition of MTT.

Positive and negative controls were used as described in Table 6.1. However, in this assay the negative control also served to compensate for background absorbance. In addition, to ensure that the media was uncontaminated, three wells were filled with media containing no cells. As outlined in Section 6.2.2.5.1 of this chapter, MTT was added to all the wells and the procedure of centrifuge, addition of DMSO, plate shaking and plate reading as described there was carried out.

6.2.2.6. Determination of the effects of the drug release elutes and microparticles on HT29 cells, MCF7 cells and DLD1 cells utilising flow cytometry

6.2.2.6.1. Preparation of test samples and controls

Drug release was conducted in sterile media as described by Decker et al., (1999). Briefly for each of the cell lines, four formulations of the optimized ISFI containing MTX and four formulations with no drug were placed in 4mL of sterile media each. The media was removed from the respective vessels at day 1, 7, 15 and 30 and added to the flasks containing cells. Microparticles (6mg) were added directly to the flasks containing cells. A control formulation was prepared at the following concentrations: 5.5µM for HT29 cells, 0.28µM for DLD1 cells (Calabresi, 1981) and 4.4µM for MCF-7 cells (Kohler et al., 2005).

6.2.2.6.2. Testing of samples

Cells were counted as outlined in Section 6.2.2.3 of this chapter and plated at a density of 220, 000 cells and placed in prepared media in a 25cm³ tissue cell culture flask. The flask was placed in a humidified incubator (37°C, 5% CO₂ atmosphere) and cells were allowed to attach and grow for 24 hours. The media in the flasks was discarded and replaced with elute containing media (4mL) and 1mL of sterile media. Elutes from the placebo implant were similarly treated, and microparticles (6mg) were added directly to the media and into the flasks. Control flasks received either the media containing methotrexate at the concentrations outlined in Section 6.2.2.6.1 of this chapter (positive control) or received sterile media (negative control). Cells were subcultured 24 hours later using standard trypsination methods. 7AAD (BD Via-Probe™, BD Biosciences, CA, USA) (10µL) was utilised as the stain for flow cytometry (FACSCalibur, BD Biosciences, New Jersey, USA). Counting beads obtained from BD Biosciences, CA, USA (BD™ Liquid Counting Beads) were also utilised so that exact numbers of dead cells could be determined. The beads were vortexed and a fixed volume of bead solution (50µL) was added to a fixed volume of cells (500µL). Equation 6.1 was used to calculate the number of viable and non-viable cells in the test sample, where NCI is the number of cells of interest.

$$NCI = \frac{\text{Number of events in region}}{\text{Number of beads in region}} \times \frac{\text{Number of beads(theoretical)}}{\text{Sample size}} \dots\dots\dots \text{Equation 6.1}$$

The number of events in the region refers to the number of events in the region of interest, i.e. the region where cells have not taken up the 7AAD dye and are hence viable could be the region of interest or alternatively the region where cells have taken up the dye and are therefore considered non-viable. These regions are shown with more clarity in Figure 6.8 in Chapter 6, Section 6.3.2 of this dissertation. The percentage of non-viable cells was then calculated as a percentage of all the cells tested.

6.3. Results and Discussion

6.3.1. The effects of methotrexate, polymer and microparticles on HT29 cells using the MTT assay

The MTT assay showed a decreasing viability of cells as expected (Figure 6.5). However two observations are of importance. Firstly it is important to note that the reported IC₅₀ did not in fact reduce the viability of the cells by 50%. This is suggestive of a cell line displaying some resistance and hence it should be noted that the effect of MTX might be higher in a different cell line. Secondly, the cumulative release was utilised in the determination of concentrations and the release of MTX from the implant is high initially and then slows as the implant reaches the plateau phase.

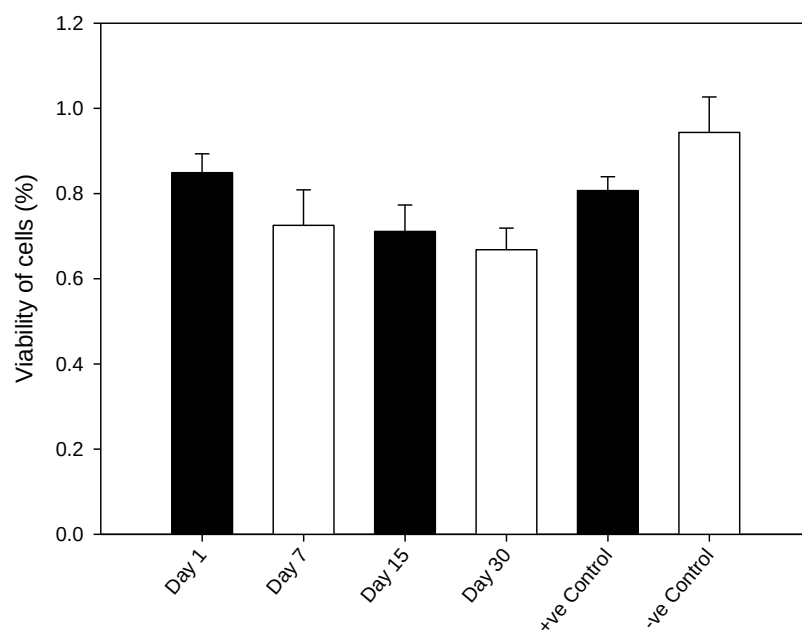


Figure 6.5: Bar graph illustrating the reduction in viability of the HT29 cells when treated with the drug release elutes of the optimized ISFI

The effect of the polymer on the HT29 cells was more difficult to determine than the effect of MTX. As demarcated in Figure 6.6, once the polymer solutions were incubated at 37°C, they formed solid deposits at the base of the wells. These could not be re-dispersed following the addition of DMSO despite vigorous shaking using an orbital plate shaker. Low concentrations of the polymer (0.451 and 0.263mg/mL) formed deposits that could be dispersed and showed absorbance that was equal or slightly higher than the negative control, indicating that the cells were viable.

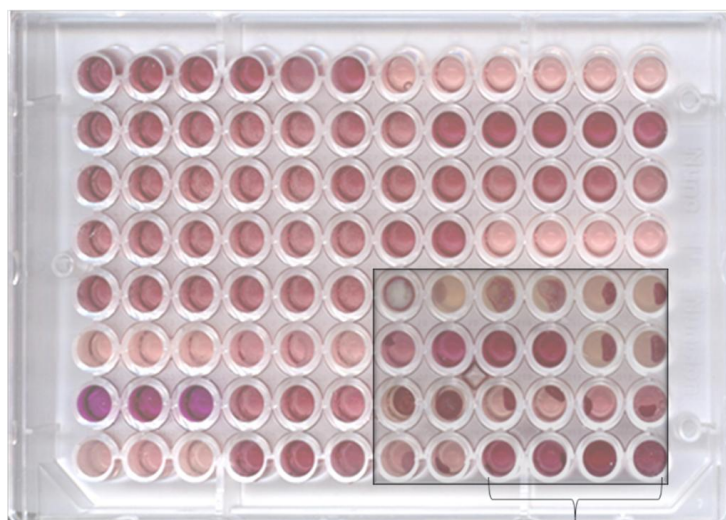


Figure 6.6: Digital image showing an MTT plate following addition of DMSO as the solubilising agent for formazan crystals. Note the highlighted area indicating the presence of undispersable polymer deposits and the final four wells showing no deposit.

An interesting observation was noted on the addition of microparticles directly to the cells. These appeared to show a greater cytotoxic effect when compared with the release elutes and with viability of the cells being decreased by a considerable amount as shown in Figure 6.7. While the size of the particles is too large for entry into the cells as discussed in Chapter 5 of this dissertation, perhaps the presence of the particles themselves caused some disruption in the formation of a confluent cell layer.

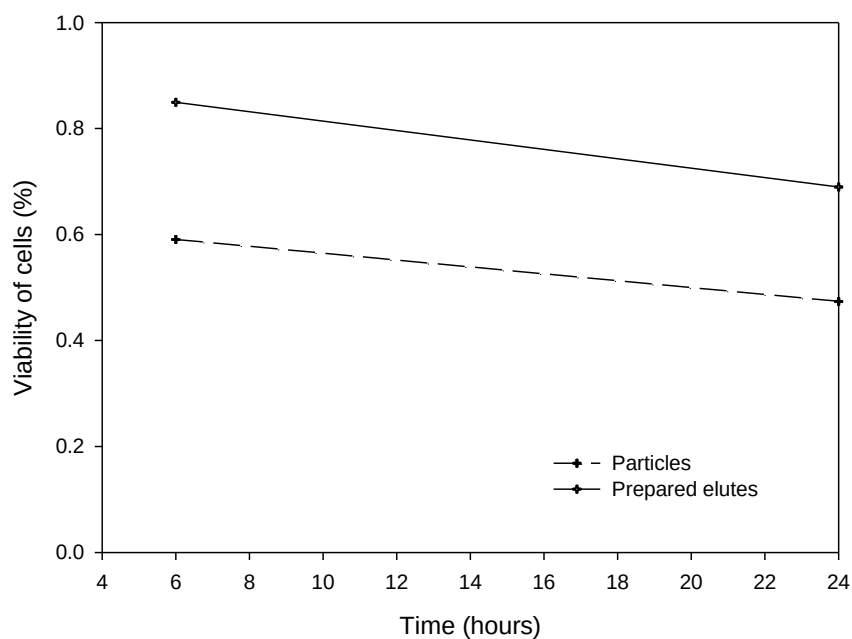
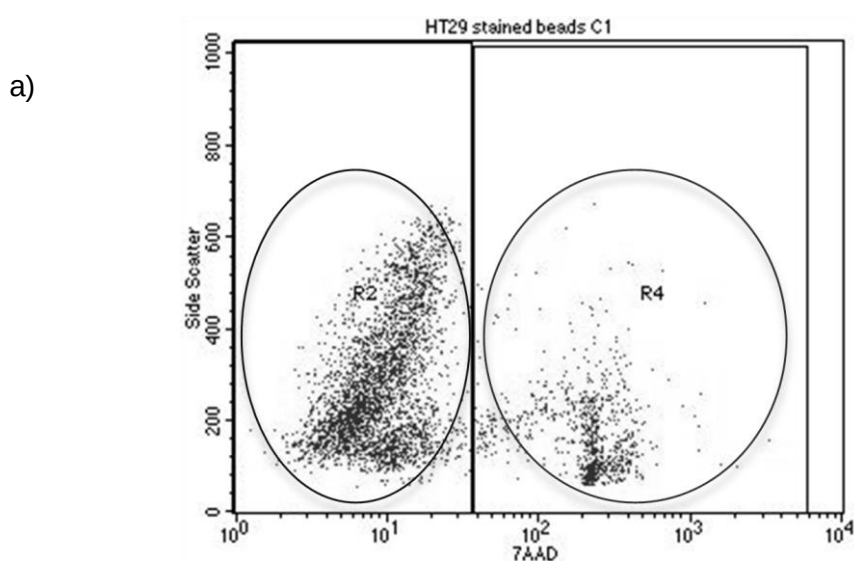


Figure 6.7: Plot showing the decrease in the viability of the cells following treatment with the particles versus treatment with the elutes prepared ($n=3$, $SD < 0.1$).

6.3.2. The effect of drug elutes, placebo elutes and microparticles on HT29, MCF7 and DLD1 cells determined using flow cytometry

Flow cytometry results correlated well with the MTT assay and showed that the amount of drug that was released over the 30 days was sufficient to cause toxicity to the cancer cells at the site. Figure 6.8 shows the dot plots obtained and the toxicity of the elutes against HT29 cells.



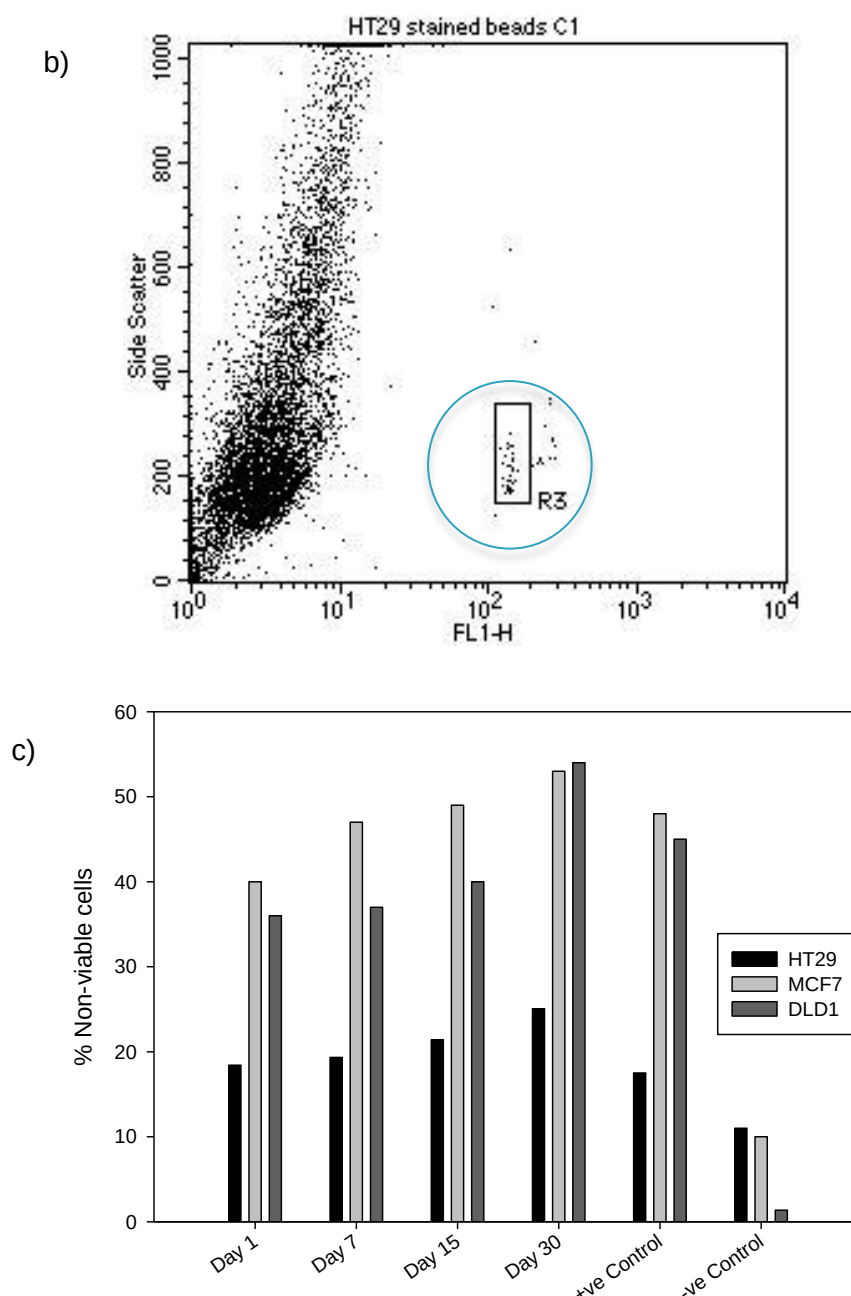


Figure 6.8: a) Typical dot plot obtained showing the viable cells (region R2), and cells which have taken up the 7AAD stain (region R4) b) counting beads are shown in region R3 and c) toxicity of elutes on HT29, MCF7 and DLD1 cells.

As observed with the MTT assay, the reported IC₅₀ was not achieved with the HT29 cells. Hence the percentage of non-viable cells both with the positive control and with the actual elutes was low. However, a notable difference was observed with the DLD1 and MCF7 cells. This is ascribed to the greater sensitivity of the DLD1 and MCF7 cells to the MTX.

The placebo elutes showed no cytotoxicity towards the cancer cells with values of non-viable cells being similar to non-treated controls. Due to the settling of the microparticles in one region at the base of the flask, the true toxicity of the particles could not be determined using flow cytometry. The MTT assay provides a more comprehensive account of the toxicity of the microparticles.

6.4. Conclusion

In conclusion, this study attempted to determine the effect of release of drug from the implant and microparticles on cancer cells using two different techniques of measuring cell viability. Both flow cytometry and the MTT assay showed that the release of MTX from the optimised ISFI was sufficient to cause cell death. In addition, the implant did not appear to cause any major cytotoxicity towards the cell lines. The testing of the pH-responsive microparticles showed that the particles were more toxic than the release elutes. Although this is promising, the particles were not utilised further mainly due to the clumping that occurred when the particles were added to the ISFI formulation. For this reason only the implant was tested *in vivo* as described in Chapter 7.

CHAPTER 7

***IN VIVO* TESTING OF THE ISFI IN THE SPRAGUE- DAWLEY RAT MODEL**

7.1. Introduction

Animal ethics approval for *in vivo* studies described in this chapter was obtained from WITS Animal Ethics Screening Committee, AESC Number: 2009/37/05 (Appendix C).

Although animal experimentation remains controversial, the testing of pharmaceuticals in animal models provides valuable information to researchers by allowing the study of formulations *in vivo* and hence providing important information which cannot be ascertained *in vitro*. A number of animals are commonly used for laboratory research such as rats, mice and larger animals such as primates, dogs and pigs. The majority of the animals used in research are mice and rats (80-95% of total animals used) (Lachman, 1992) Rats and mice are commonly used as they are readily available, associated with low cost due to their short gestation period and large litters. In addition they are small animals and hence easy to handle and easy to take care of both in terms of feed and housing. In particular Sprague-Dawley rats are calm and easy to handle (Sprague Dawley Rats, Harlan Laboratories, <http://www.harlan.com/models/spraguedawley.asp>, Accessed 2011-06-18). Due to the ease in obtaining large numbers of rats compared to larger animals and the larger volumes of blood obtained from rats versus mice, Sprague-Dawley rats were chosen for this study.

7.1.1. Biocompatibility of implanted materials

Essentially, a biomaterial is considered one if it is able to exist in the body without causing an unacceptable amount of harm (Williams, 2008). Determination of biocompatibility of an implant *in vivo* is of utmost importance in the avoidance of damage to the body. When a material is introduced into the body a number of responses may occur (Table 7.1).

Table 7.1: Responses to a foreign body that may occur (Williams, 2008)

Protein adsorption and desorption characteristics
Generalised cytotoxic effects
Neutrophil activation
Macrophage activation, foreign body giant cell production, granulation tissue formation
Fibroblast behaviour and fibrosis
Microvascular changes
Tissue/organ specific cell responses (e.g. osteoclasts and osteoblasts for bone, endothelial proliferation)
Activation of clotting cascade
Platelet adhesion, activation, aggregation
Complement activation
Antibody production, immune cell responses
Acute hypersensitivity/anaphylaxis
Delayed hypersensitivity
Mutagenic responses, genotoxicity
Reproductive toxicity
Tumor formation

Besides the damage caused at the site, implants being used for the delivery of a drug may be adversely affected by the response of the local tissue surrounding the area of implantation. The presence of inflammation and the associated oedema may cause an increase or a decrease in the release of the drug from the implant. Presence of granulation tissue and formation of a fibrous capsule will lead to a decrease in the amount of drug reaching the blood stream. The study had three main aims: firstly determination of the biocompatibility of the implant *in vivo*, secondly the determination of the release characteristics of the implant *in vivo* in the rat model and thirdly the effect of ice on the release profile of drug from the implant.

7.2. Materials and Methods

7.2.1. Materials

Ortho-phosphoric acid ($\text{H}_3\text{O}_4\text{P}$) (85-90%) purchased from Fluka (Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland), Methanol 215 (ROMIL-SpSTM Super Purity Solvent (MeOH), Assay>99.9%) and Acetonitrile 200 (ROMIL-SpSTM Super Purity Solvent (CH_3CN), Assay>99.9%) was purchased from Romil Pure Chemistry

(Cambridge, Great Britain) and double de-ionized water was obtained from a Milli-Q water purification system (Milli-Q, Millipore, Billerica, MA, USA). Liquid Nitrogen was purchased from Afrox, South Africa. Phosphate buffered saline tablets, formaldehyde (4% v/v), methotrexate ($M_w=431\text{g/mol}$) and pyrazinamide ($M_w=123.1\text{g/mol}$) were purchased from Sigma Aldrich, Steinheim, Germany. Trypton Soya Agar and Blood Agar was purchased from Oxoid (UK) and was sterilised and then poured into sterile petri dishes until use.

7.2.2. Receipt, marking and treatment of animals

Healthy Sprague Dawley rats (200-250g $\pm$ 5g) were obtained from the WITS Central Animal Service. On receipt, each animal was marked at the base of the tail with the number assigned to it, weighed and placed in a cage with 4 other animals. Animals were maintained on a 12 hour light/dark cycle and fed commercially available rat chow and allowed access to food and water *ad libitum*.

7.2.3. Biocompatibility studies in rats

Rats received the drug-loaded implant on Day 1 and were sacrificed after 1 hour, 4 hours and then on days 2, 4, 6, 8 and 10. The area around the implant including skin and underlying muscle was resected. The flank opposite to the side of implant implantation was utilised as the control. The samples were persevered in formaldehyde (4% v/v) were sent to IDEXX laboratories where they were analysed using routine histopathological methods. Briefly, sections of the samples were processed in an automated tissue histological processor according to a routine standard operating procedure. Following overnight tissue processing, wax blocks were prepared and 5 μm slides were cut on a microtome before the sections were mounted on a microscope slide. These sections were stained with the Haematoxylin and Eosin staining method and mounted before microscopy was performed.

7.2.4. *In vivo* studies on the rat model

In vivo studies comprised two parts. Firstly, the release of the drug over 10 consecutive days from the ISFI needed to be determined and the secondly the release of drug in response to the application of ice. An application to the University

of the Witwatersrand Animal Ethics Screening Committee (AESC) was made. A copy of the approval of the study (AESC clearance number: 2009/37/05) and the subsequent approval of modification to the study can be found in Appendix C.

7.2.4.1. Sterile preparation of formulations

As described in chapter 4, PMVE solutions were autoclaved. Due to the thermal sensitivity of methotrexate, the drug was added to the formulation under sterile conditions. Briefly, formulations were prepared under a horizontal laminar flow unit (Labotec®, Midrand, South Africa) fitted with a HEPA filter in a sterile room. Sterile settle plates of Trypton Soya Agar were placed at the corners of the laminar flow unit in order to determine the presence of possible contaminating micro-organisms. These plates were incubated at 37°C and 25°C for 24 hours to determine the presence of bacteria and fungi respectively. A small amount of the final formulation was also streaked onto a nutrient rich blood agar plate and incubated at 37°C for 24 hours to determine if the formulation was sterile.

7.2.4.2. Determination of thermally responsive drug release from the implant into the bloodstream and monitoring of body temperature of animals: pilot study

In order to determine the release of drug from the implant in response to the application of ice and hence the cooling of the implant a pilot study was conducted. Anaesthesia was induced using a 1:4 xylazine and ketamine (Anaket®) mixture (0.1mL/100g). Four animals were injected with the MTX-loaded implants (12mg/kg) delivered in 0.4mL formulation. As this was the pilot study, a higher dose of drug was used as the animals were to be sacrificed on the day of implantation of the ISFI and hence there were no concerns of toxicity. Due to concerns of hypothermia in the animals, body temperature was monitored in two ways. Firstly body temperature was monitored using a rectal thermometer (World Precision Instruments Inc, Florida, USA) and secondly a temperature probe (Physitemp Instruments Inc, New Jersey, USA) was inserted at the site of the injection to monitor local decreases in temperature. A lag time of 15 minutes was implemented following implantation before the application of ice. In order to determine the time taken for the MTX to cross over into the blood following the application of ice, animals were sacrificed at the following intervals: 15 mins, 1 hour, 2 hours and 3 hours. Blood samples were withdrawn via

cardiac puncture and were analysed using ultra performance liquid chromatography as subsequently described in Chapter 7, Section 7.2.7.3.

7.2.4.3. Determination of drug release from the ISFI over 10 days and the effect of the application of ice on the release of MTX from the ISFI

The main study involved the application of an ice pack to the area of implantation of the ISFI to determine the release of the entrapped drug from the implant in response to temperature. Animals were divided into 2 main study groups: baseline animals and ice treatment animals (group 1), and three control groups (groups 3, 4 and 5) as summarised in Figure 7.1 and Figure 7.2.

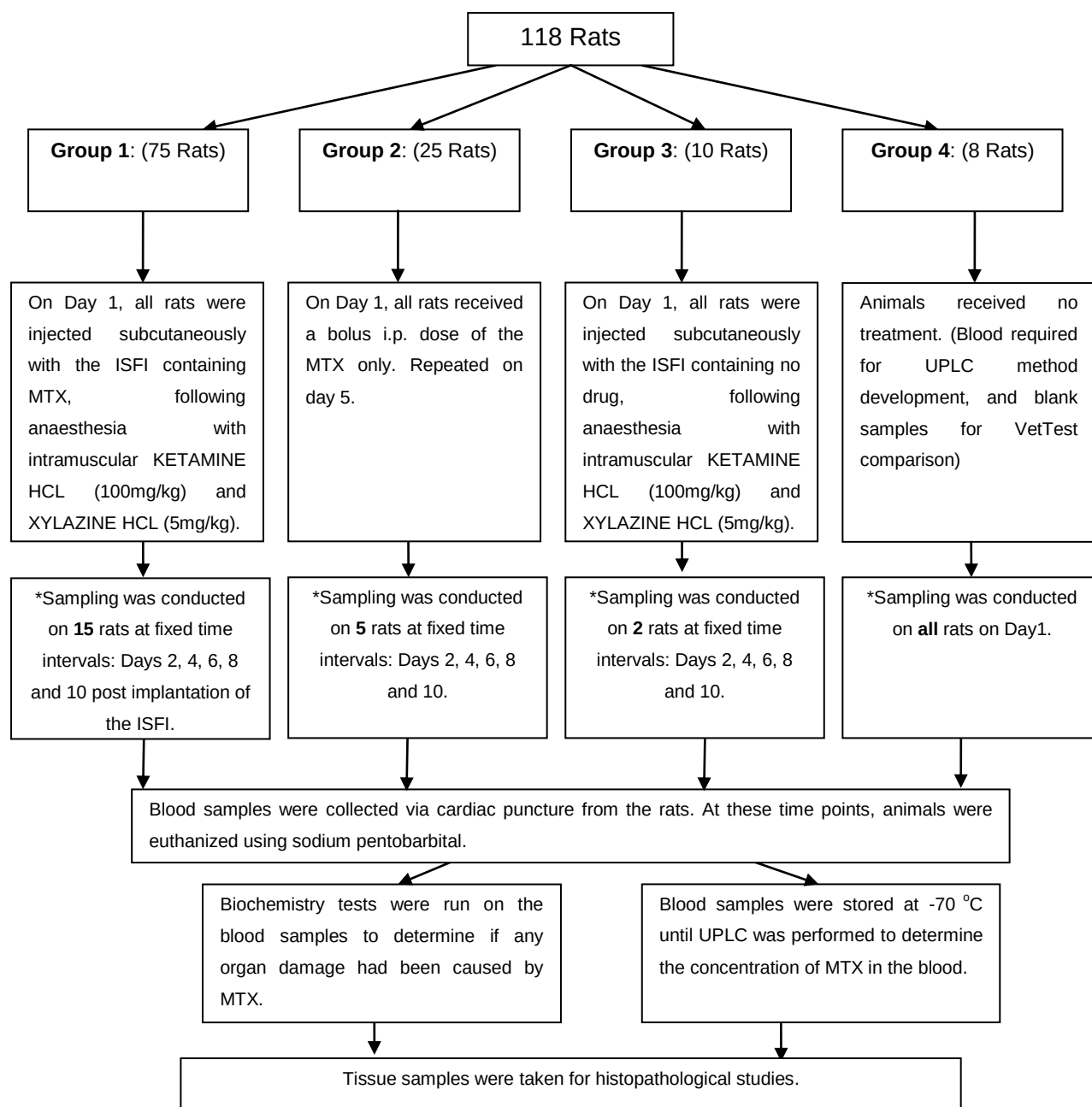


Figure 7.1: Flow diagram showing overall design of *in vivo* studies (*Sampling procedure is elaborated on in Figure 7.2).

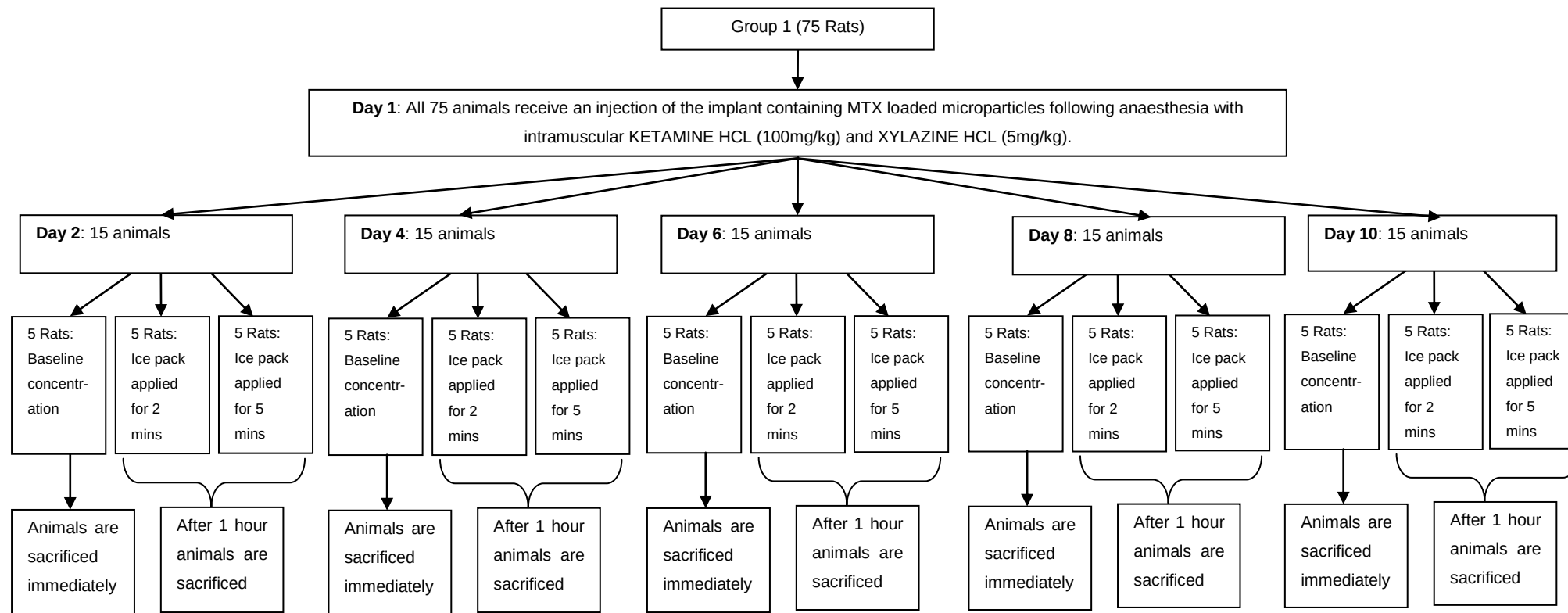


Figure 7.2: Flow diagram depicting the treatment each of the experimental groups (i.e. Both Group1 and Group 2) were to undergo.

7.2.4.3.1. Control animals

Positive control animals (group 2, Figure 7.2) received intra peritoneal injections of MTX as per the flow diagram (Figure 7.1) MTX solutions were prepared from a stock solution of Arbitrexate® (Pharmachemie, Netherlands) (50mg/2mL) which was diluted to an appropriate concentration of 2mg/mL under sterile conditions as described in Chapter 6, Section 6.2.2.1 of this chapter. Each animal received 0.5mL injections i.p. using a 1mL syringe and a 25G needle on Day 1 and then on Day 5, hence delivering a dose of 8mg/kg/10 days. Negative control animals (group 3, Figure 7.2) received an implant (0.4mL) containing no drug on day 1.

7.2.4.3.2. Baseline animals

Animals (25 SD rats) were injected with a dose of 8mg/kg/10 days in order to match the dose given to the control animals. The ISFI was aseptically prepared and injected into the flank area of the rats as depicted in Figure 7.3. The dose of 8mg/kg was delivered in a 0.4mL formulation. Five rats were sacrificed on each of the following days: day 2, 4, 6, 8 and 10.

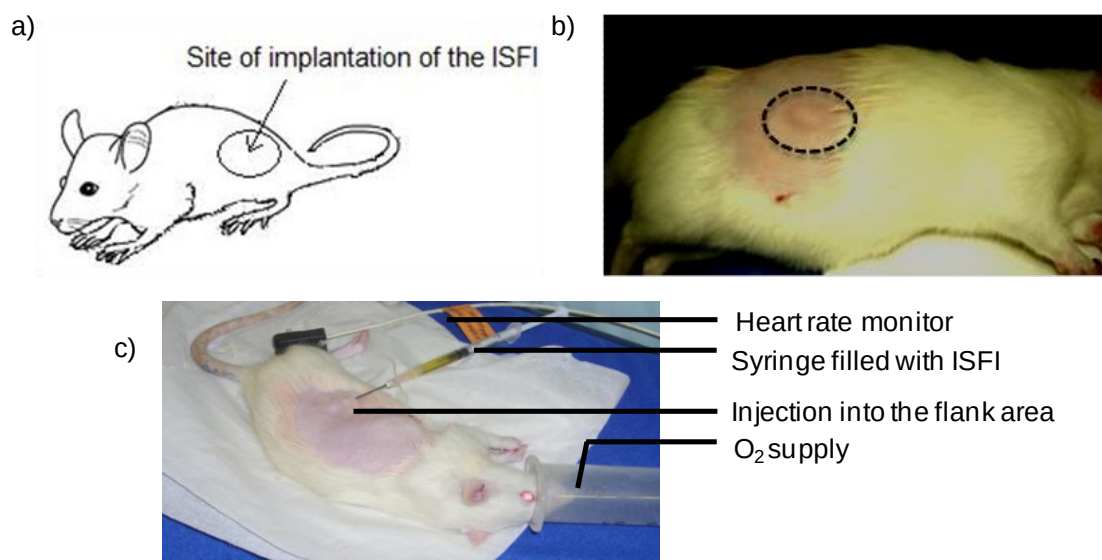


Figure 7.3: a) Sketch showing the proposed area of implantation of the ISFI in the flank of the animal b) Digital image showing the actual site of implantation and c) digital image showing the injection of the ISFI into the flank of the rat.

7.2.4.3.3. Animals receiving application of ice to region of implantation of the ISFI

As shown in the flow diagram (Figure 7.2) 50 animals were to receive ice treatment with 10 animals being sacrificed every two days. However, as discussed in the results section of this chapter (Section 7.3.2.4.1) some animals in the baseline group began experiencing toxic side-effects after day 6. Hence the ice study was conducted on twenty animals only. These twenty animals were injected the ISFI delivering a dose of 8mg/kg on day 1. On day 2, 5 animals received an application of ice to the area for 2 minutes and 5 animals received an application of ice to the area for 5 minutes. Information obtained from the pilot study was utilised to determine the time point after application of ice that the animal would be sacrificed and hence animals were sacrificed 1 hour after application of the ice and bloods were collected via cardiac puncture. The same procedure was repeated on day 4.

7.2.5. Evaluation of the toxicity of the drug *in vivo*

7.2.5.1. *Weighing of animals and cage activity*

Animals were weighed daily and animals that had lost more than 15% of their weight were to be removed from the study. Cage activity was observed twice a day on the day of implantation of the ISFI and then once a day thereafter.

7.2.5.2. *Analysis of blood obtained from treated animals*

In order to determine the toxicity of methotrexate on the animals that received doses over the period of 10 days, blood was extracted from two animals on day 2, 4, 6, 8 and 10 from baseline animals and positive control group animals via cardiac puncture. The blood sample, approximately 1mL was allowed to clot in a tube and then centrifuged using an IDEXX StatSpin VT centrifuge (Westwood, USA). The serum was collected and analysed using a VetLab Station (IDEXX Laboratories, Netherlands) and VetTest Kits (General Profile, IDEXX Laboratories, Netherlands). In order to assess liver function, ALKP (Alkaline phosphatase), ALT (aspartate transaminase); creatinine levels were examined. As the formulation contained a high concentration of CaCl_2 , blood concentrations of calcium needed to be determined. In addition the following tests were also undertaken: glucose, total bilirubin, total protein, urea, phosphate, albumin, cholesterol and amylase mostly to test possible toxicity of MTX on the liver and kidneys. Further, bloods from these animals were sent to IDEXX laboratories

(Johannesburg, South Africa) for a full blood count in order to determine haematological toxicity, if any.

7.2.6. Visualisation of the ISFI utilising ultrasound imaging

Ultrasound or sonography imaging utilises sound waves to produce images of underlying tissues. It is a topographic modality and as such it offers of non-invasive method of examination of a cross-sectional area of soft tissue (Anderson and Trahey, 2006). Ultrasound imaging involves the generation of a sound wave typically of between 2-20MHz from an electric pulse by a transducer and transmission of the sound wave through the tissue. Sound is reflected partially through the underlying layers and totally reflected by denser objects such as bone. Some of these reflections or echos return to the transducer and are converted from sound waves into electric pulses and relayed to the ultrasonic scanner for interpretation and conversion into digital images. The length of time required for return of the echo allows the deduction of the depth of the underlying tissue whereas the strength of the echo allows deduction of the density of the underlying tissue (Anderson and Trahey, 2006).

The animal was anaesthetised using xylazine and ketamine (1:4 mixture, 0.1mL/100g). It was placed on the stage fitted with a heating pad. A rectal thermometer was inserted to monitor the temperature of the animal. A tube delivering oxygen was placed over the snout of the animal. Following the disappearance of plantar reflexes, ultrasound gel was applied the area of implantation and the area was examined using an ultrasound imager (Vevo[®] 2100, VisualSonics Inc., Toronto, Ontario, Canada) and a 15Hz transducer to visualise the presence of the implant. To estimate the size of the ISFI, the transducer was fixed in position and a scan of the area of implantation was conducted.

7.2.7. Utilization of Ultra Performance Liquid Chromatography in the determination of the methotrexate concentration in blood and tissue samples obtained from rats

Chromatography is often utilised in the quantitative determination of separated substances and involves separation based on polarity. Liquid chromatography utilises a stationary phase and a mobile phase with the analyte of interest and other possible

contaminating substances being able to pass through the stationary phase and be eluted with the mobile phase at retention times in accordance with their affinity for either the mobile or the stationary phase. The retention time of the analyte of interest can be determined from a standard solution previously run and hence differentiated from other substances. As both height and peak areas vary linearly with an increase in concentration, the peak height or the peak area of the analyte can be used in comparison with a set of standards and hence can be used to quantify analyte concentrations in any sample (Skoog et al, 1992).

Both high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) utilise short columns which are densely packed with stationary phase particles and use pumps to pump the mobile phase through the column rather than gravity. UPLC (a specialised HPLC, first produced, marketed and trademarked by Waters[®]) differs from HPLC in that the packing particles are often less than 2µm and hence the pressures are much higher in UPLC (1000atm) whereas HPLC columns often have particles sizes of 5µm or greater, with pressures of 400atm (Nováková et al., 2006). While both these chromatography methods offer the advantages of high resolution and short elution times, UPLC is superior (Wren and Tchitcheff, 2006; Dongre et al, 2008).

Normal phase chromatography utilises a polar stationary phase with a non polar mobile phase whereas reverse phase chromatography, as used in this dissertation, consists of a non-polar stationary phase and a moderately polar mobile phase. The analyte of interest determines the type of chromatography to be used as the analyte is usually of similar polarity to the stationary phase with the mobile phase being of opposite polarity (Skoog et al. 1992). This is to ensure that the analyte is retained on the column for a sufficient period of time rather than being eluted as soon as the mobile phase is run through the column. As methotrexate is a non polar drug, reverse chromatography was chosen to be used in the quantitative analysis of MTX.

Stationary phases in reverse chromatography are often silica coated with a thin chemically or physically bonded organic phase to create the non polar phase and are named according to the number of carbons that are attached. In the analysis of MTX as

described in this dissertation, an Acquity UPLC[®] BEH Shield RP18 column (2.1x100mm, 1.7µm particle size) was successfully employed. This column consists of hybrid particles composed of bridged ethylsiloxane/silica as pure silica materials do not provide the mechanical strength or efficiency required in UPLC (Grumbach et al., 2005).

Besides the strength of interactions with the stationary phase, analyte retention times also depend on factors such as the ratio and composition of solvents used and the flow rate of the mobile phase used. Isocratic elution is the use of a single mobile phase whereas gradient elution utilises at least two mobile phases, often differing significantly in polarity. For the purposes of this study, gradient elution was used as it was found to improve separation efficacy.

Lastly due to the errors that can occur in sample preparation and equipment, the use of an internal standard is recommended. An internal standard is a substance added to all samples and standards in a fixed quantity and concentration and allows high precision as errors especially with injection volumes are accounted for (Skoog et al., 1992). Pyrazinamide (PYZ) was used as an internal standard in this study.

7.2.7.1. UPLC analysis

Analysis was conducted on an Acquity[®] Ultra Performance Liquid Chromatography system (Waters[®], Milford, MA, USA) fitted with an Acquity UPLC[®] BEH Shield RP18 1.7µm VanGuard precolumn (2.1x5mm) and an Acquity UPLC[®] BEH Shield RP18 column (2.1 x100mm, 1.7µm particle size). The purpose of the guard column as the name implies was to prevent proteinaceous material from reaching the column. Samples were run for 2 minutes and an injection volume of 15µL for plasma samples, or 10µL for tissue samples and implant samples was injected onto the column. Detection was via a photoiodide UV/Vis detector (PDA Detector). This allowed the recording of the full UV/Vis absorption spectra and determination of the highest absorption of the methotrexate as shown in Figure 7.4. Using this information, a wavelength of 310nm was used for the detection of methotrexate and pyrazinamide.

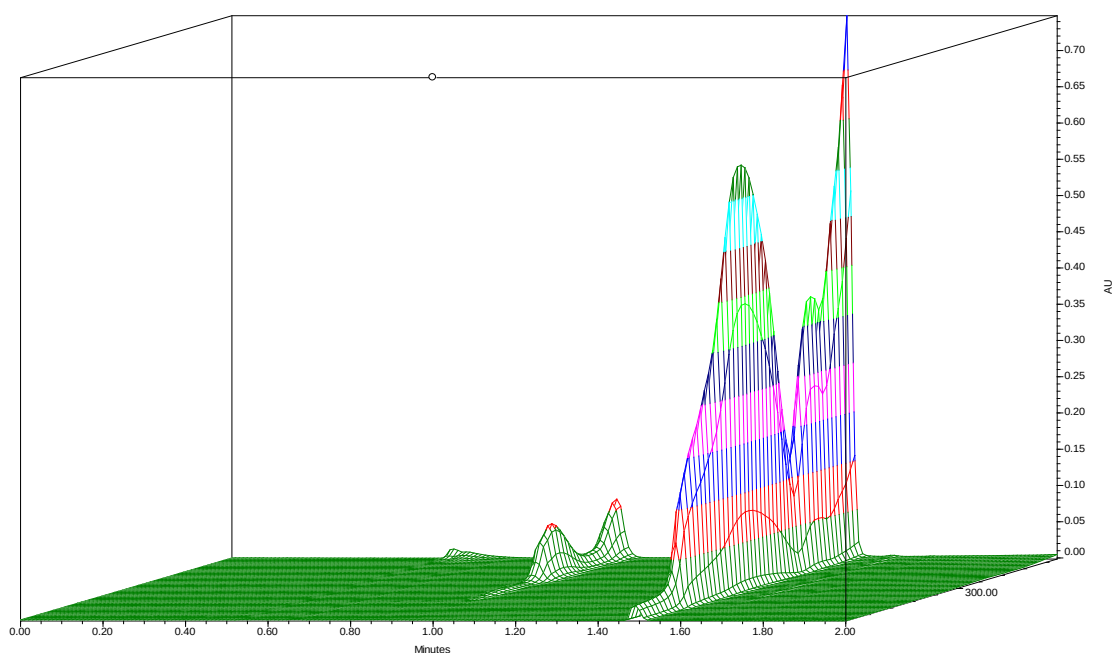


Figure 7.4: PDA plot showing the absorbance of MTX (retention time= 1.5mins) and PYZ (retention time= 1.1.mins).

7.2.7.2. Preparation of weak and strong washes and mobile phases

Considering the packing nature of the column, it can be easily understood that any particulate material in the system can cause blockages which would increase pressures and hence lead to failure of equipment. For this reason all solutions were prepared using double deionised water (DDW) (Milli-Q Gradient, Millipore, MA, USA, electrical conductivity 18.2MΩ.cm at 25°C) and all solutions were freshly prepared daily and filtered under vacuum using Durapore® membrane filters (Millipore, Ireland). Gloves were worn at all times to minimise contamination. Priming solutions consisted of a weak wash (10%^{v/v} acetonitrile) and a strong wash (90%^{v/v} acetonitrile). Gradient elution was achieved using acetonitrile and phosphoric acid (0.1%^{v/v}) as mobile phases in a method described in Table 7.2.

Table 7.2: UPLC method for the elution of methotrexate and internal standard pyrazinamide.

Time (min)	Flow Rate(mL/min)	% Phosphoric Acid	% Acetonitrile
Initial	0.400	95	5
0.5	0.400	85	15
1.0	0.400	75	25
1.5	0.400	65	35
1.6	0.400	95	5

Precision and accuracy were determined by replicate injections of three concentrations of MTX. Intraday accuracy and precision was determined by multiple injections (n=3) of the three concentrations of MTX during the period of analysis on one day and inter-day accuracy and precision were determined by injecting the same three concentrations of MTX over 3 non-consecutive days (n=3). Peak area and retention time were noted for each of the analyte runs. In order to determine the precision, the % RSD (Equation 7.1) for retention time and peak area was calculated. Accuracy was determined by comparing the obtained amount with the theoretical quantity.

$$\%RSD = \frac{\text{std dev} \times 100\%}{\text{mean}} \dots\dots\dots \text{Equation 7.1}$$

7.2.7.3. *Drug content in the blood*

7.2.7.3.1. Obtaining blood samples

On the day of termination, animals were sedated using a 1:4 xylazine and ketamine mixture (0.1mg/100g). Deep plantar reflexes were tested and when no response was obtained, a 22G needle attached to a 5mL syringe (flushed with Heparin-Sodium Fresenius, Bodene) was slipped into the heart via the intercostal space and 5mL of blood was drawn.

7.2.7.3.2. Treatment of blood samples following collection

Blood samples obtained from the cardiac puncture were collected in heparinised tubes (Improve® Improvacuter® Lithium Heparin collection tubes, GmbH, Hamburg, Germany). Bloods were immediately centrifuged at 3000rpm for 15minutes using a desktop centrifuge (Model TD5A-WS, Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., Shanghai, China). Plasma was collected and stored at -70°C until analysis.

7.2.7.3.3. Determination of the optimal method of extraction of drug from plasma samples

Before samples of blood can be injected onto the UPLC column, proteins must be removed. Two methods exist for achieving this: liquid-liquid extraction and solid phase extraction. Liquid-liquid extraction was chosen as solid phase extraction showed low

levels of MTX in the final elute. Methanol was selected as the deproteinising agent in this study as other agents such as acetonitrile and perchloric acid proved unsuccessful.

7.2.7.3.3.1. Determination of the optimal ratio of deproteinating agent

Extraction yield was determined by spiking 400µL blank plasma samples with fixed quantities of drug sample (100µL of a 0.1mg/mL MTX solution). To these samples differing quantities of methanol was added and UPLC analysis conducted on these samples was compared with samples of the drug solution (100µL of a 0.1mg/mL MTX solution) in the same quantity of methanol.

Comparison of these allowed the determination of the best recovery method, i.e. the amount of methanol that should be added to the plasma sample to recover the greatest amount of drug. The ratio of plasma to methanol in order to obtain a recovery of 99% was found to be 1:4.2, and subsequently plasma samples were treated in this way. Although recovery obtained was good, concern over the low amounts of drug in the actual plasma samples necessitated the concentration of samples. For this reason plasma samples were dried completely under a gentle stream of nitrogen gas and reconstituted in 500µL methanol or samples were dried to 500µL in a vacuum oven (Trade Raypa® Digital drying oven, Barcelona, Spain maintained at 50°C, -0.6bar). Comparison of these two samples determined that the samples dried to 500µL showed better recovery.

7.2.7.3.3.2. Treatment of actual plasma samples

Using the obtained ratio of 1:4.2, 400µL samples of blood were pipetted into suitable centrifuge tubes and 1800µL of methanol was then added. The samples were vortexed (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) for 15 seconds to ensure adequate mixing of the methanol with the plasma. The samples were then centrifuged at 1500rpm for 5 minutes (Model TG16-WS, Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., Shanghai, China) in order to precipitate the proteins. Supernatants were then decanted into Eppendorf microtubes (Eppendorf AG, Hamburg, Germany), placed in a vacuum oven and evaporated to 500µL. Samples were filtered using a 0.2µm filter (GHP Acrodisc filter, Pall Life Sciences, NY, USA). Pyrazinamide was used

as an internal standard and 2 μ L of a 0.5mg/mL solution was added to each sample before analysis.

7.2.7.3.3. Construction of a calibration curve in order to quantify amounts of drug in actual plasma samples

A calibration curve was generated in order to quantify the amount of drug in the plasma samples. Briefly, blank plasma samples were spiked with known quantities of MTX and the procedure outlined above was followed. The ratio of the area under the (AUC) of the chromatogram of drug to the internal standard was plotted against the corresponding drug concentrations (μ g/500 μ L). The least squares method was used to determine the linearity equation and correlation coefficient (r^2). The limit of quantification (LOQ) was defined as the concentration which produces chromatographic peaks with heights at least 3 times that of the baseline noise.

7.2.7.4. Determination of the residual drug in ISFIs

From day 4 and 10 animals, the area surrounding the implant was resected and the implant was dissected out (Figure 7.5). Care was taken to ensure that all visible implant was removed. The implant was then dissolved in phosphate buffered saline (PBS, pH 7.4). Samples were twice filtered through a 0.22 μ m Cameo Acetate membrane filter (Millipore Co., Bedford, Massachusetts) and a 500 μ L sample was placed in the sample vial for analysis. Pyrazinamide (0.5mg/mL) was used as the internal standard and 2 μ L was added to each sample.

7.2.7.4.1. Construction of a calibration curve in PBS

A calibration curve of MTX in PBS was also conducted. The ratio of the area under the (AUC) of the chromatogram of the drug to internal standard was plotted against the corresponding drug concentrations expressed in μ g/mL. The least squares method was used to determine the linearity equation and correlation coefficient (r^2).

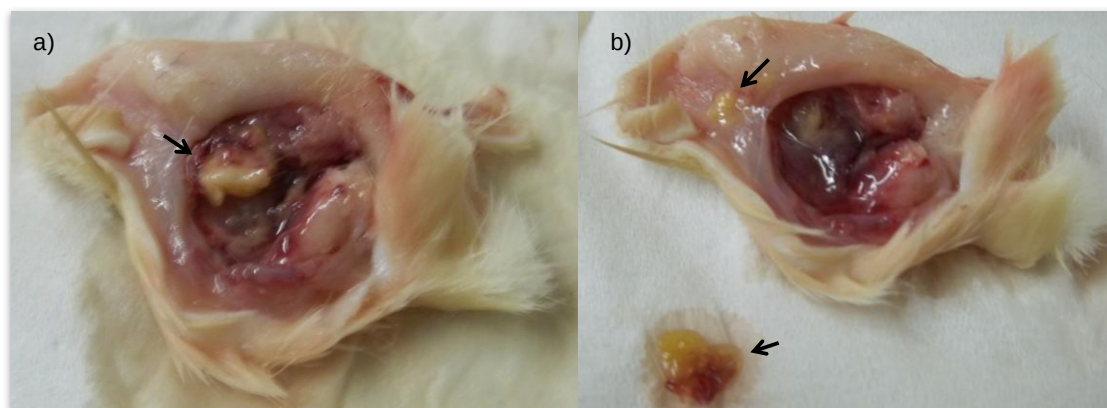


Figure 7.5: Digital images showing the resected tissue surrounding the implant with a) showing the implant *in situ* as indicated by the black arrow and b) shows the resected implant with some of the implant still remaining as indicated by the black arrow. This area was then scraped using a scalpel to collect the remaining implant.

7.2.7.5. Determination of the drug content in surrounding tissue

Tissue immediately surrounding the implant was collected from day 4 and day 10 rats following removal of the ISFI as described in Section 7.2.7.4 of this chapter. As shown in Figure 7.6 the entire area surrounding the implant was resected (approximately 4mm), yielding a sphere like sample. The sphere was cut open and the entire implant removed. Care was taken to ensure that all the implant was removed and the tissue was rinsed with PBS to ensure there was no drug that had diffused out of the implant and onto the tissue post removal. The implant was treated further as described in Section 7.2.7.4 of this chapter. The samples of tissue surrounding the implant were rinsed with PBS and then accurately weighed and cut into thin pieces using surgical scissors and/or a scalpel and placed in a mortar. Liquid nitrogen was then added to the mortar and a pestle was used to crush the tissue to a powder. PBS (5mL) was then added to the powder and the solution was homogenised for 15 seconds using a homogeniser (Polytron®, Kinematica Inc, Bohemia, NY, USA). The obtained solution was centrifuged at 3000rpm for 10 mins and the supernatant was collected and filtered using filter paper (Whatman filter paper, Kent, England) to remove any remaining pieces of flesh or fatty tissue and then refiltered using 0.22µm filters. To remove protein from the samples, 400µL of the obtained filtrate was treated with 800µL of methanol. At this ratio of methanol to tissue sample, protein precipitation was observed. Samples were then vortexed for 15 seconds and centrifuged for 5 minutes. Following final filtration of the supernatant through a 0.22µm filter into vials (Waters® LCMS certified vials with a pre-slit screw top, Waters, Milford, MA, USA) samples were injected onto the column.

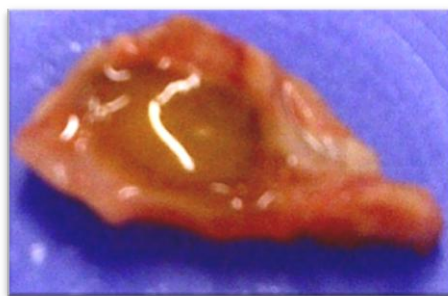


Figure 7.6: Digital image showing the resected implant with surrounding tissue.

7.2.7.5.1. Construction of a calibration curve in methanol

A calibration curve of MTX in methanol was also conducted. The ratio of the area under the (AUC) of the chromatogram of the drug to internal standard was plotted against the corresponding drug concentrations expressed in $\mu\text{g/mL}$. The least squares method was used to determine the linearity equation and correlation coefficient (r^2).

7.3. Results and Discussion

7.3.1. Biocompatibility of the ISFI in the rat model

7.3.1.1. *Implantation site and tissue necrosis*

After one hour of implantation of the ISFI, no inflammation was observed. Minimal to acute inflammation was detected 4 hours after implantation of the ISFI. The inflammatory response then varied from acute in the 2, 4- and 6- day specimens to subacute in the 8- and 10-day implantation sites. This is the expected response of the tissue to a foreign body. Chronic inflammation generally follows and is generally part of the healing process. It is persistent inflammation that presents a problem (Anderson and Langone, 1999). The inflammation was regarded as minimal to mild and no severe exudative inflammatory response could be detected as shown in Figure 7.7.

However, tissue necrosis, especially in the subcutaneous fatty tissue, was observed in almost all of the implantation sites. Poly(methyl vinyl ether) is reported by Nurkeeva et al. (2005) to be biocompatible and poly(methyl vinyl ether-co-maleic anhydride)

(PMVE/MA) has been used in a number of formulations (Oriente et al., 1998; Finne et al., 1989 and Cerchiara et al., 2005). In addition, some salts of PMVE/MA have been approved for use in the cosmetic industry (Burnett, 2011). PMVE/MA crosslinked by 1,9 decadiene, was tested in rabbits eyes and was found to be only a slight irritant (ISP (Australasia) ,1993). The same report by ISP also showed low toxicity of the polymer on the skin and had an oral lethal dose of more than 5g/kg. A locally available product, Correga[®]: a denture fixative, also contains PMVE/MA. However, to date reports of PMVE/MA or PMVE used as an implant system have not been found. This study however clearly shows necrosis associated with the ISFI. While the reports of safety associated with the copolymer seem promising and indicate that the polymer itself is not the likely cause of toxicity, the polymer needs to be examined further. It is a possibility that the polymer is safe and the high calcium content of the ISFI is then most likely the causation of the necrotic regions. Further investigation in this regard is warranted.

7.3.1.2. Tissue mineralization

Dystrophic calcification was found in most of the specimens and was likely the result of tissue damage and necrosis. It could also be attributed to the presence of CaCl₂ in the formulation. However, it was not severe and resorption was thought possible.

7.3.1.3. Granulomatous inflammation and fibroplasia

The granulomatous response appeared minimal to mild with only early fibroplasia and encapsulation observed in one of the sections (a day 8 animal). No well-developed capsule or any collagen formation could be detected in any of the specimens.

7.3.1.4. Focal skin ulceration

Some of the implantation sites showed foci of superficial skin necrosis and ulceration. It was the opinion of the pathologist (Dr WS Botha, IDEXX Laboratories) that this was most likely secondary induced by licking or biting at the implantation site based on the morphological findings. It was therefore regarded as an incidental observation and the biting may well have been associated with sensitivity and pain at the implantation site. However, several animals in the placebo group also experienced the skin ulceration and another possible explanation was the decrease in blood flow to the region due to

subcutaneous damage caused by the presence of the implanted material. Again the high concentrations of calcium in the implant must be questioned. Calcium is an important ion in the clotting of the blood (Hilgard, 1973). Hilgard (1973) investigated hypercalcaemia on clotting time and found a reduction in clotting time *in vitro*. *In vivo* and further *in vitro* experimentation revealed that hypercalcaemia may play a part in the formation of thrombi *in vivo* in the presence of other trigger mechanisms (Hilgard, 1973). Hence it is possible that the elevated concentrations of calcium may have caused coagulation of blood in the region inhibiting blood flow to the dermal layers and hence resulting in secondary ulceration.

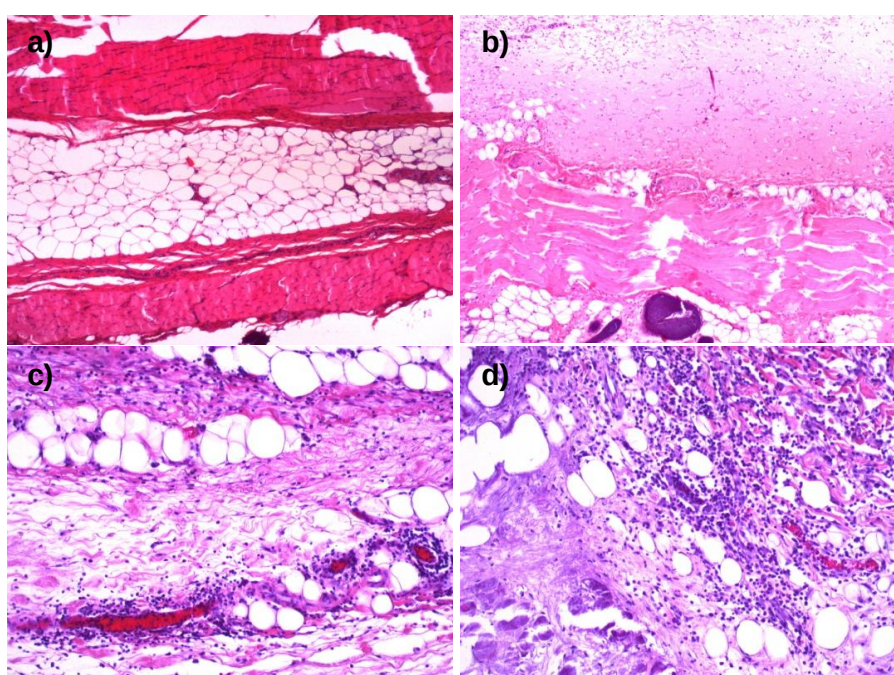


Figure 7.7: Histopathological analysis of local tissue surrounding the area of implantation of the implant in a) non-treated animal b) after 2 days of implantation c) after 6 days of implantation and d) after 10 days of implantation.

7.3.2. *In vivo* testing of the ISFI

7.3.2.1. Sterile preparation of ISFI

After incubation of the plates (settle plates and streaked formulation plate) for 24 hours, plates were examined visually to determine the presence of growth. None of the plates showed any growth indicating the sterility of the formulation prepared.

7.3.2.2. Construction of a calibration curve in order to quantify the amount of methotrexate in blood and tissue samples and precision and accuracy of the UPLC method

Figure 7.8 shows the typical chromatograms obtained for the drug, methotrexate and the internal standard, pyrazinamide. As shown in Figure 7.9, a calibration curve with a high coefficient of determination was obtained. Table 7.3 shows the calculated precision and accuracy of the method when three replicate injections of three samples with known concentrations were made on the same day and on three non-consecutive day. As should be noted the accuracy remained high even between days and %RSD remained relatively low indicating that the method had good precision and accuracy. The limit of quantification was determined to be 0.50ng/mL.

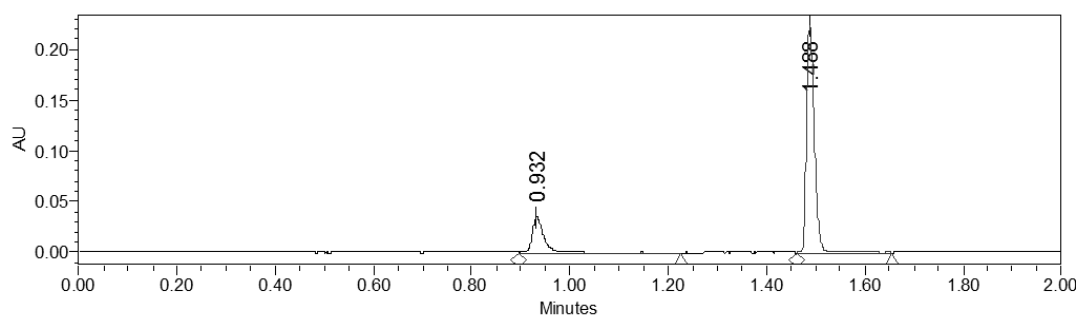


Figure 7.8: Typical chromatogram obtained for methotrexate (retention time=1.408min) and internal standard, pyrazinamide (retention time=0.932min)

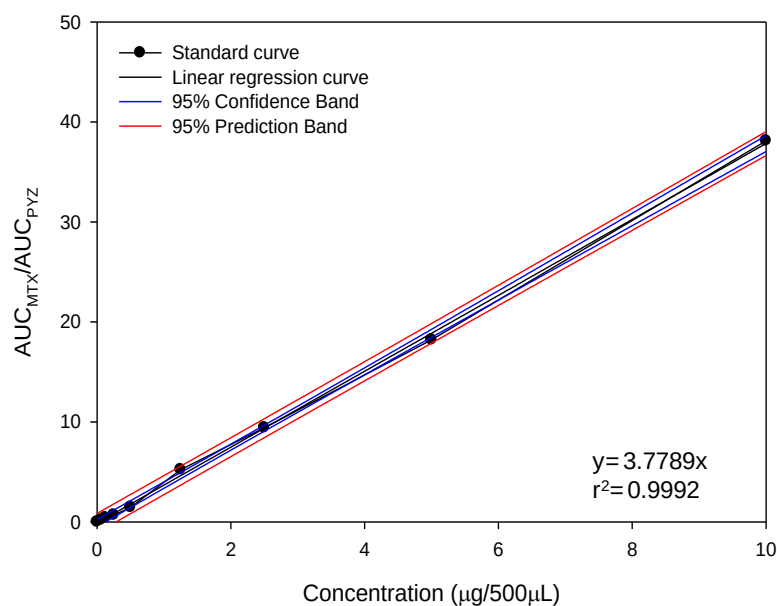


Figure 7.9: Calibration curve obtained of Methotrexate in blank plasma

Table 7.3: Inter- and intra- day precision and accuracy of the method were determined (only one set of interday data is shown).

	%RSD (T_R)	%RSD (AUC)	Accuracy
Intraday			
1	0.154	0.583	99%
2	0.143	0.433	99%
3	0.166	0.494	99%
Interday			
1	0.61	0.290	97%
2	0.54	0.540	98%
3	0.49	0.667	96%

%RSD= Percentage relative standard deviation; T_R = Retention Time; AUC= Area under the curve

7.3.2.3. Observations and findings of the pilot study

The pilot study determined two things: firstly the effect of the application of ice to the region on the body temperature was determined. Table 7.4 summarises the effect of ice on the temperature (body and at the site) of the rats.

Table 7.4: Table showing the effect of the application of ice on the rats on the local and systemic temperatures.

	Rat 1	Rat 2	Rat 3	Rat 4
Weight	234g	237g	227g	219g
Temp at site				
Before (°C)	33.0	33.9	32.8	31.6
After (°C)	26.7	28.0	24.1	23.7
Body Temp				
Before (°C)	32.7	34.3	34.5	33.2
After (°C)	30.7	33.7	32.4	32.7

Temp at site: Temperature at the site of implantation, Body temp: Body Temperature

As can be noticed, the body temperature (measured with the rectal thermometer) did not change by many degrees (the largest difference in temperature is 2.1°C). However, the temperature at the site decreased sufficiently. This was the desired outcome as it showed that the application of ice to the region for up to 5 minutes would not cause hypothermia in the animals.

Secondly, the pilot study was used to determine the time of sacrifice of the animals. Plotted in Figure 7.10 are the concentrations of MTX in the blood sacrificed 15 minutes, 1 hour, 2 hours or 3 hours after the application of ice for 5 minutes.

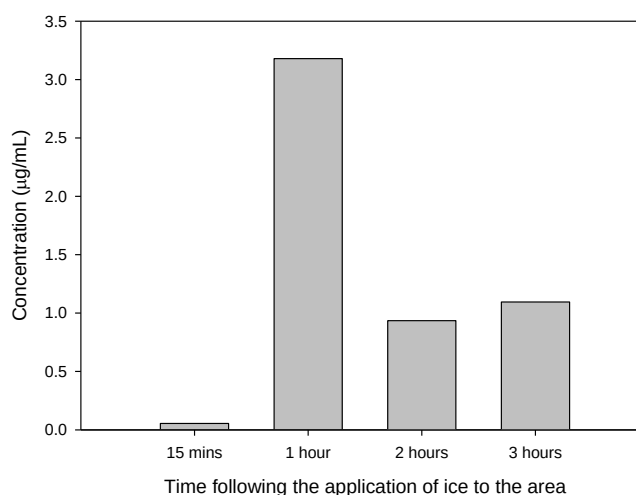


Figure 7.10: MTX levels in the blood of the pilot study animals clearly showing the peak of drug at 1 hour following the application of ice to the area

Hence, for studies that followed, animals were sacrificed 1 hour following the application of ice as this was when the highest concentration of drug was obtained. This was possibly the time of diffusion of the drug from the site of implantation to the blood stream. Following this, the amount of drug in the blood stream decreased due to diffusion to other tissues or excretion.

7.3.2.4. Main study

7.3.2.4.1. Baseline Animals

Animals were injected with a dose of 8mg/kg/10 days in order to equalize the dose given to the control animals. Animals were labelled as described in the flow chart (Figure 7.11)

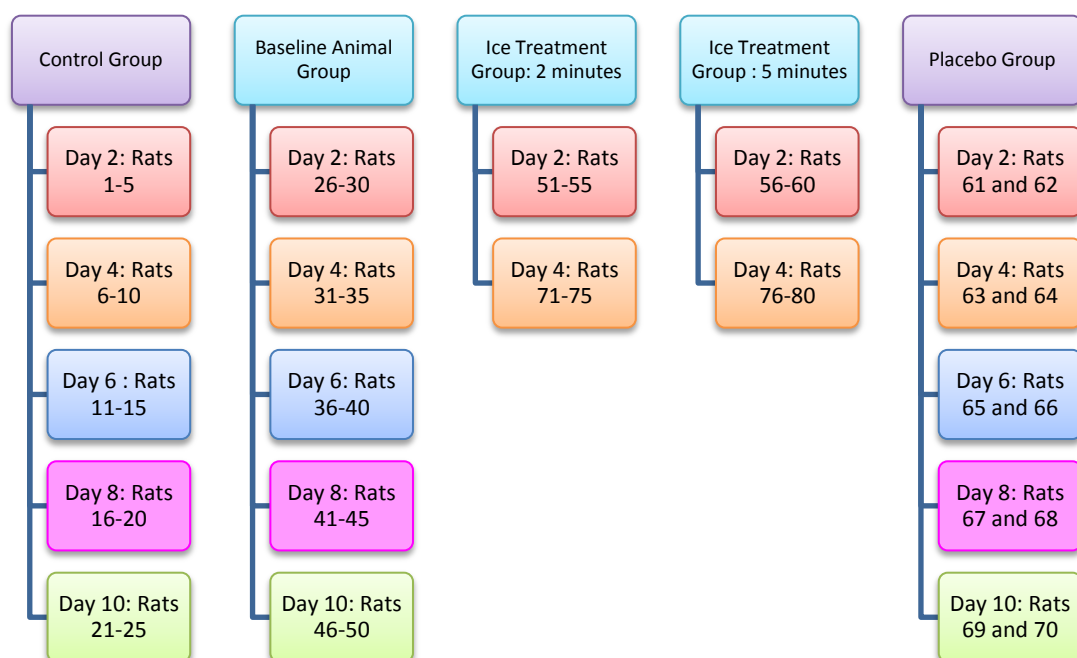


Figure 7.11: Flow chart demonstrating the numbering of the experimental animals.

Day 2 animals showed no toxicity and were sacrificed. However in the Day 4, 6, 8 and 10 groups, Rats 32, 33, 37, 40, 41, 47 and 49 developed diarrhoea and of these four animals, one animal (Rat 41) developed enterohaemorrhagic diarrhoea and subsequently died on the 8th day following implantation. Post mortem examination of the animal revealed severe haemorrhaging in the gastrointestinal tract and severe dehydration. In addition, all animals in the main and ice treatment branches of the study lost weight throughout the study. The weight loss and the diarrhoea were not

observed in animals that received the non-drug loaded placebo (Rats 61-70) and animals in the control group (Rats 1-25).

The significance of these observations is that the animals receiving the implant developed side effects and loss of weight indicating the toxic levels of drug were reached. Animals in the control group receiving intraperitoneal injections of the drug did not experience these side effects indicating that high levels of drug were not obtained in these animals. Animals receiving the placebo implant also did not develop these side effects, indicating that these were likely to have been drug induced and could not have been due to the polymeric material composing the implant.

7.3.2.4.2. Analysis of the toxicity of MTX *in vivo*

7.3.2.4.2.1. Analysis of blood biochemistry

Analysis of the blood revealed some toxicity in the animals. Several animals in the baseline study group showed a decrease in blood phosphate levels. When levels of calcium increase in the blood, one of the action of parathyroid hormone is to decrease the resorption of phosphate ions from the kidney tubules. This leads to a decrease in phosphate levels (Vander et al., 1990). Hence the drop in phosphate levels could be due to the increase in calcium levels due to the high calcium content in the implant. The parathyroid hormone is responsible for maintaining calcium homeostasis in the body. Calcium blood levels remained within normal limits over the period of study. Normal levels of phosphate and calcium were noted in the control animals. One animal in the Day 10 group of the baseline study showed an elevated urea concentration, which could be a result of kidney toxicity. Such an observation was made by Heringou et al. (1989) where rats treated with a cement delivering MTX over a prolonged period developed severe nephropathy with necrosis of the epithelium and the convoluted tubules. All other tests were marked as within normal ranges.

Full blood counts revealed low levels of haemoglobin in all animals, both the control group and the baseline animals. Some animals in each group also showed a decrease in platelet counts and white cell counts. Baseline animals also showed unsatisfactory readings for Red Blood Cell (RBC) counts. Some animals showed a decrease in the RBC count while others an elevated count. A high RBC count can easily explained by

the dehydration observed in several of the animals in the baseline study and is attributed to the diarrhoea experienced by the animals. Low RBC counts when coupled with low white cell counts could be indicative of bone marrow suppression. None of the animals in the control group showed a decrease or increase in RBC counts. Once again this could be indicative of the higher levels of MTX obtained in the animals receiving the implant and hence indicative of the release of drug from the implant.

7.3.2.4.3. Analysis of blood samples

7.3.2.4.3.1. Baseline animals and control animals

Control animals (n=25) received a dose of 8mg/kg/10 days of MTX in two divided doses. Blood levels of the drug fell below the LOQ and could not be determined except in one animal that showed a concentration of 56.6ng/mL on Day 6, the day following the i.p. injection of MTX. However, it was the only animal in the group to show a reading.

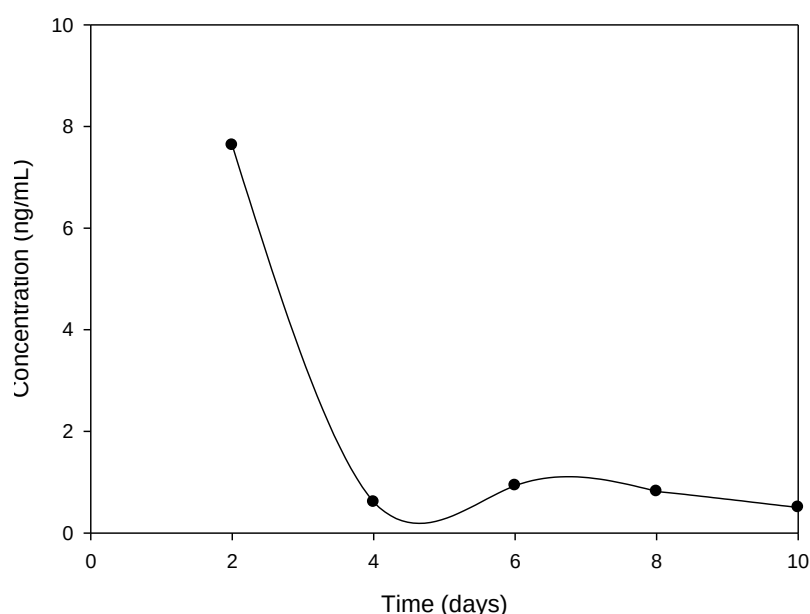


Figure 7.12: Blood methotrexate concentrations in baseline animals (SD< 0.034)

Figure 7.12 shows the drug content of the blood in the baseline animals. While n=5 for day 2 and day 4, it should also be noted that the final three values are obtained from 3 animals only as the other two animals in each group had levels of drug that were below

the LOQ. Further the final concentration was approximately the same as the LOQ and it is difficult to say with confidence that these values are accurate. Nevertheless it can be said that the amount of MTX in the blood is very low almost below quantifiable amounts when utilising standard UPLC methods. This could be indicative that the majority of the drug being released at later stages was retained at the site of implantation rather than distributing into the blood.

There was large variation in the blood concentration observed between animals. Possible reasons for this could be that the implant was not injected in one single depot area or the site of injection (subcutaneous versus subdermal) could have differed between animals. Certainly both of these are possible as the injections were not all performed by the same individual and the rat is a small animal, making distinguishing between subdermal and subcutaneous areas difficult. Further unlike during *in vitro* testing where the implant clumped together within the dialysis tubing regardless of method of injection due to the relatively large volume of the fluid within the tubing. *In vivo* this is not possible as there is spreading of the implant due to the pressure from the tissue surrounding the implant. Another reason for the variation between animals could be the temperature. During the pilot study the temperature of animals at the site of implantation was noted (Table 7.4). One animal showed a temperature reading of 31.6°C which is below the gelation temperature of the ISFI. It is possible that the implant released the drug faster in some animals due to the lower temperature.

The side-effect (diarrhoea) experienced by the animals was possibly the result of the sustained release of drug from the implant compared to the quick clearance of the MTX when the drug was given intraperitoneally to the control group. This is possibly due to an initial damage of the gastrointestinal tract (GIT) tissue due to the burst effect of the implant which is consistent with the high drug quantity in the blood observed on Day 2. The toxicity of MTX on the GIT as has been reported by Bajin-Katić et al., 2004. Although the exact mechanisms of the damage are unknown, damage to the precursor crypt cells occur and hence once mature cells are sloughed off, there are no cells to replace them leaving the intestinal tissue exposed to biliary salts and pancreatic secretions and hence autodigestion occurs. A similar toxicity profile has been reported in rats receiving MTX delivered by a surgically implanted acrylic cement implant

(Heringou et al.,1989). The rats in this study also developed gastrointestinal toxicity characterised by gastrointestinal haemorrhage after 4-6 days following implantation.

After Day 2 however, the drug concentration in the blood is extremely low indicating perhaps very little blood reaching the blood stream as desired. In a tumor bearing animal such a side-effect may not have been seen as the burst release of the drug would have been utilised by the rapidly growing tumor. However, this is a merely a hypothesis and must be confirmed in further work on tumor bearing animals.

7.3.2.4.3.2. The effect of the application of ice

As shown in Figure 7.13, application of ice to the region did cause an enhanced release of drug. However once again there was much variation between the implants, once again possibly due to different injection patterns in the animals. On day 4, the implant did not appear as responsive to ice as on day 2 following the implantation. This was as observed *in vitro*.

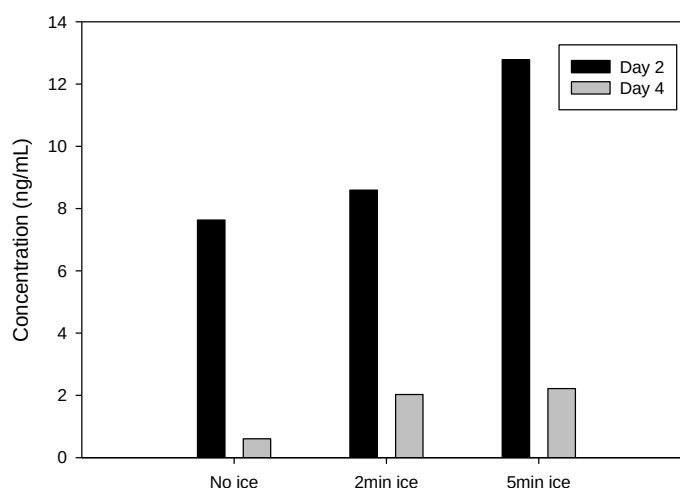


Figure 7.13: The effect of application of ice to the region of implantation (SD<0.06)

7.3.2.4.4. Analysis of methotrexate concentration remaining in implant and in surrounding tissue

Figure 7.14(a) and (b) show the calibration curves obtained for PBS and methanol respectively. Figure 7.14(c) shows the amount of drug remaining in recovered implants.

The two day 10 rats (Rats 49 and 50) show very different results. Rat 49 has very little drug remaining while Rat 50 has a quarter of the drug initially implanted, still present. Although to some degree this can be compared with the weight loss shown by the animals, the amount of variation between animals makes the observation of trends difficult. All animals (placebo and main study) lost weight on the day following injection of the implant. However, thereafter, placebo animals gained weight or eventually exceeded the original weight. It should be noted that Rat 70 (Figure 7.14(e)) suffered muscle injury during the administration of the anaesthetic before ultrasound imaging on Day 6 and as result appeared to have lost weight in the days following the procedure. However, it did regain the weight it had lost. Rat 67, a placebo animal sacrificed after 8 days shows the typical profile of weight gain showed by the animals (Figure 7.14(e)). It is worthy noting that Rat 50 gained weight after day 7 whereas Rat 49 continued to lose weight Figure 7.14(f). This is perhaps due to the increased release of drug from the implant in Rat 49 whereas the ISFI in rat 50 did not release as much MTX. This explains both the high amount of residual drug in the implant as well as the lack of weight loss observed in Rat 50. In addition, Rat 49 also presented with diarrhoea but Rat 50 did not. Of animals from day 4 to day 10 from the baseline study (n=20), 13 rats showed a loss of weight and 7 showed a gain of weight. The day 4 rats also showed low amounts of drug remaining in the implants and the animals examined had also lost weight over the 4 days and both suffered from diarrhoea. Hence animals that lost weight and developed diarrhoea had low levels of drug remaining in the implant while animals that did not develop diarrhoea showed higher amounts of drug remaining in the implant. This indicates that in some animals drug release from the implant occurred more rapidly than others. Once again this may be due to the lower temperature at the site in these animals or a variation in injection technique.

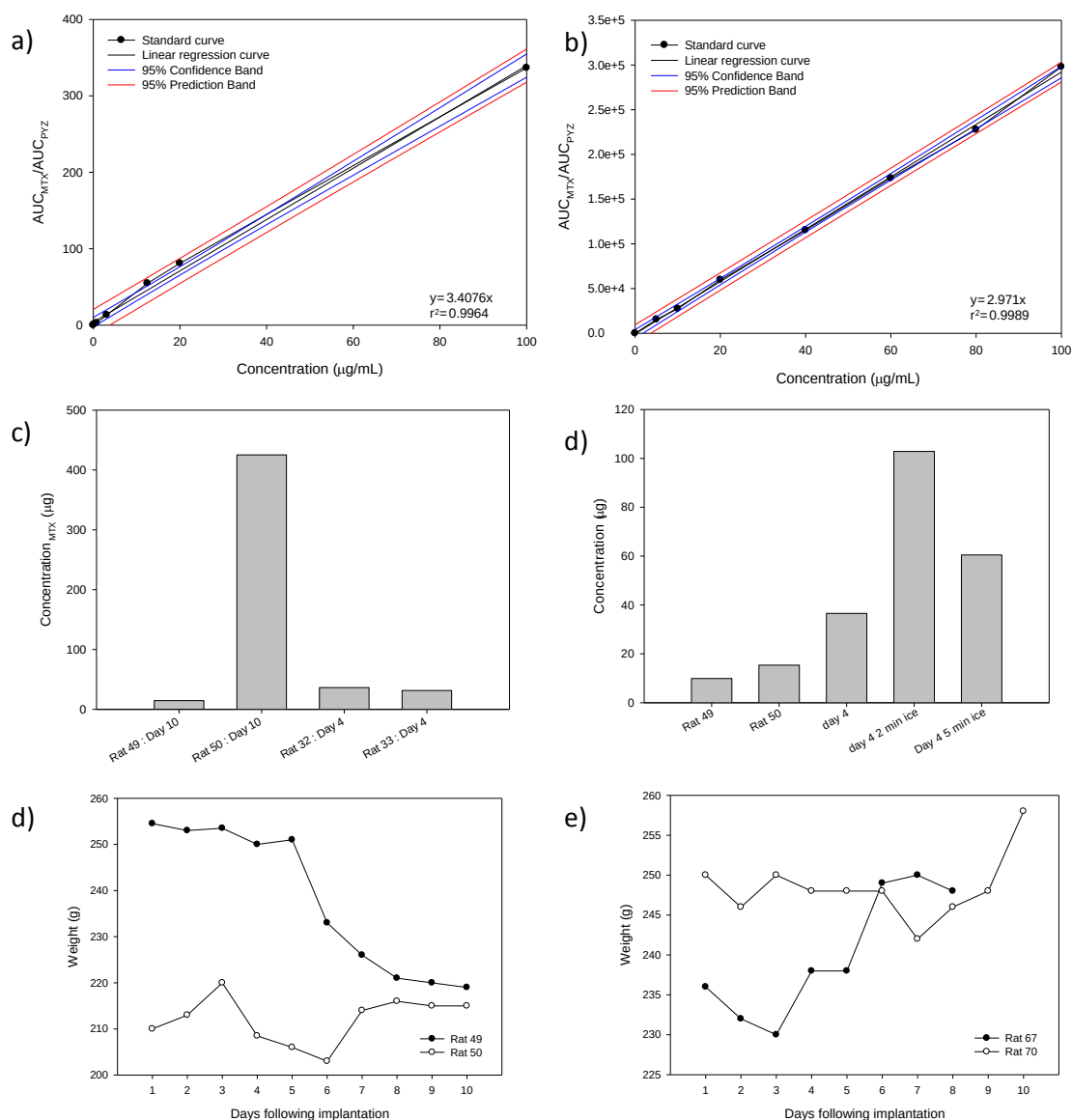


Figure 7.14: a) Calibration curve of MTX in PBS b) calibration of MTX in methanol c) remaining drug in the implant over 4 or 10 days and after the application of ice to the region d) drug in the surrounding tissue after 4 or 10 days and after the application of ice e) weight loss in rats carrying the MTX loaded implants for 10 days and f) weight loss in placebo receiving rats.

The area around the implant (Figure 7.14(d)) showed relatively high amounts of drug after the application of ice and as shown drug was still present at the site after 10 days even in animals with low amounts of drug remaining in the implant. Interestingly, although the application of ice showed higher amounts of drug in the blood with the application being for 5 minutes, the application of ice for 2 minutes appears to show higher amounts of MTX in the surrounding tissues. Further investigation is required in order to determine possible reasons for this occurrence.

7.3.2.4.5. Ultrasound imaging of the ISFI *in vivo*

Ultrasound imaging conducted revealed a solid mass in the subcutaneous tissue as shown in Figure 7.15. Note the absence of the mass in the placebo animal (a).

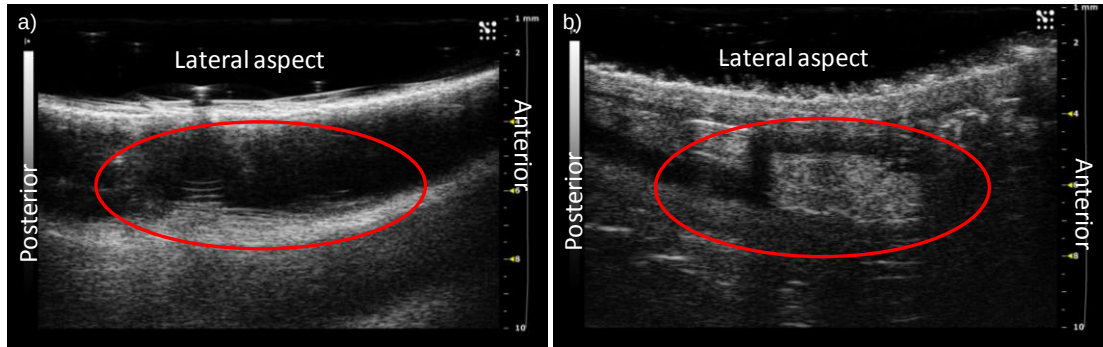


Figure 7.15: Ultrasound imaging showing a) the absence of the implant in a placebo rat and b) the presence of the ISFI in an animal from the main study.

The scan of the area of implantation in the rat was used to calculate the approximate volume of the implant. Figure 7.16 shows the images obtained during three dimensional plotting of the area of the implant. The area of the implant was determined to be 25mm^3 in the animal examined.

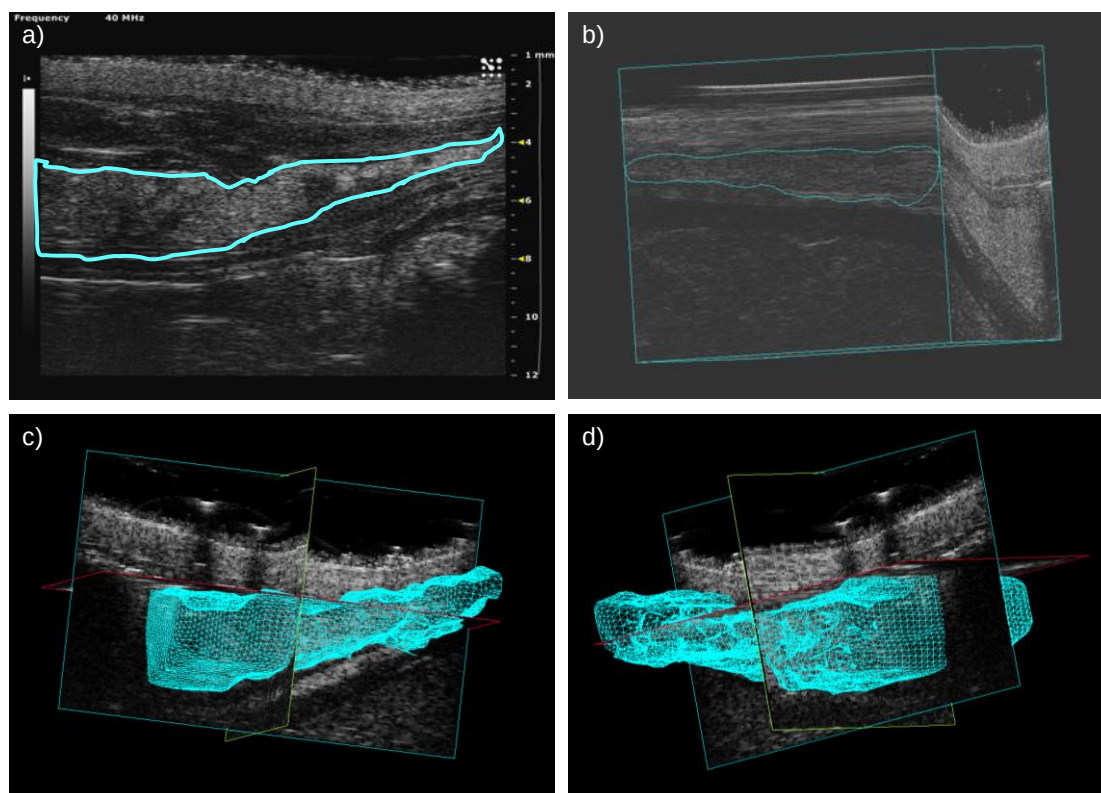


Figure 7.16: a) Image obtained using 2 dimensional ultrasound b) three-dimensional image obtained from scan of implant area showing the medial and sagittal planes and c) and d) three-dimensional plotting of the area of the implant. Note the tapering of the implant indicating the point of withdrawal of the needle from the flank leaving a “trail” of the implant.

7.4. Concluding Remarks

In conclusion, the ISFI while promising *in vitro*, has shown less promising results *in vivo*. *In vivo* results revealed that the ISFI formulation was able to deliver drugs to the site over a period of ten days. Animals receiving the implant showed side effects related to the chemotherapeutic drug indicating that doses of the drug were being delivered and maintained *in vivo*. This was confirmed by the blood levels of the drug determined using UPLC. Animals receiving the drug intraperitoneally did not show the same side-effects and blood levels of the drug were so low they could not be determined even at Day 2 indicating that the delivered drug was quickly cleared *in vivo*. Animals receiving the placebo implant also did not show this side-effect indicating that it was a side-effect of the drug and not of the implant material itself. While this indicates the superiority of the ISFI over the intraperitoneal delivery of drug, it was not considered likely that the ISFI would be able to deliver drug over a period of three months as envisaged.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. Conclusions

Considering the burden of cancer and the numerous problems associated with the systemic treatment of solid tumors in particular, the development of an *in situ* forming implant capable of delivering chemotherapeutics directly to tumors is certainly required. In order to formulate an ideal *in situ* forming implant, preformulation studies were conducted and the thermoresponsive polymer, PMVE in combination with CaCl_2 was selected for use in the development of an *in situ* forming implant. Statistical optimization utilizing a Face-Centered Central Composite design was employed for determining the optimal ISFI capable of being easily injectable into the body, forming an implant at the site of injection and of releasing the entrapped chemotherapeutic over a long period of time. The optimized formulation was found to comprise of 16.87%^{w/v} of PMVE and 0.1482M of CaCl_2 . Optimization of formulated pH-responsive microparticles was also conducted. Due to difficulty in injecting the formulations as a result of particle agglomeration, particles were only tested *in vitro* against cell lines. Microparticles were not tested *in vivo*.

Optimized ISFI formulations were prepared and release fractions tested for activity against cancer cell lines. *In vivo* testing of the implant was conducted in Sprague-Dawley rats. Results showed that several animals that received the ISFI loaded with methotrexate developed diarrhea. Animals in the control group receiving methotrexate intraperitoneally did not show this side-effect. Hence the side-effects were indicative of prolonged release of drug from the implant compared to the intraperitoneal delivery of methotrexate which was cleared rapidly. This was confirmed by the blood levels found in the animals receiving the implant. In addition, animals receiving the placebo implant did not show this side-effect either indicating that the diarrhea was a side-effect of the drug and not of the implant material itself.

In conclusion, *in vitro* work showed promising results with the release of drug from the implant exceeding a month while *in vivo* results revealed that further work is required to

improve the biocompatibility of the ISFI and to prolong the release of drug from the implant *in vivo*.

8.2. Recommendations

To date there is very little data on the pharmaceutical use of PMVE. It is recommended that the polymer be investigated further. The ISFI should be formulated without the addition of CaCl_2 , in order to provide basis to the possibility that CaCl_2 may be responsible for the local necrosis caused. In addition, it is recommended that should such a study be conducted, organs of each animal be collected for tracing of methotrexate concentrations.

A tumor bearing animal will also provide a more realistic model for testing rather than healthy animals. Tumor bearing models will offer two advantages: firstly the drug when released from the implant will be delivered to the cancerous tissue. This will allow the researcher to monitor the size of the tumor as a measure of drug release. Secondly the microenvironment of a solid tumor differs from that of a healthy animal. *In vitro* studies conducted attempted to mimic the solid tumor environment. It is possible that different results would be seen in a tumor bearing model.

Assuming that the ISFI is successfully formulated without the addition of the CaCl_2 , a different animal model should be selected. The peripheral temperature at the site of implantation in the rat model may have impacted the release behaviour of the drug from the implant due to a smaller body surface area.

Further, studies of PMVE in different pharmaceutical embodiments is also recommended. Utilization of PMVE for the formation of micro- or nano- particles among other uses may prove interesting.

Considering the calcification of tissues caused and the slow *in vivo* degradation of the implant, the implant (as currently formulated) is perhaps also suited for the potential delivery of cells (such as osteoblasts) to areas where bone formation is required.

REFERENCES

- Adams CP and Brantner VV. 2010. Spending on new drug development. *Health Economist*, 19:130–41.
- Al-Abd AM, Hong K-Y, Song S-C and Kuh H-J. 2010. Pharmacokinetics of doxorubicin after intratumoral injection using a thermosensitive hydrogel in tumor-bearing mice. *Journal of Controlled Release*, 142:101-7.
- American Cancer Society. 2010. Cancer Facts and Figures 2010. Atlanta: American Cancer Society [Available at: <http://www.cancer.org/Research/CancerFactsFigures/CancerFactsFigures/cancer-facts-figures-2011>, Accessed 2011-04-05.]
- Amiji MM, Lai PK, Shenoy DB and Rao M. 2002. Intratumoral administration of paclitaxel in an *in situ* gelling poloxamer 407 formulation. *Pharmaceutical Development and Technology*, 7:195-202.
- Anal A. 2007. Stimuli-induced pulsatile or triggered release delivery systems for bioactive compounds. *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery*, 1:83-90.
- Anderson JM and Langone JJ. 1999. Issues and perspectives on the biocompatibility and immunotoxicity evaluation of implanted controlled release systems. *Journal of Controlled Release*, 57:103-13.
- Anderson M and Trahey G. 2006. A seminar on k-space applied. [Available at: <http://dukemil.egr.duke.edu/Ultrasound/k-space/node2.html>, Accessed 2012-02-08.]
- Andriola Silva Brun-Graepi AK, Richard C, Bessodes M, Scherman D, Narita T, Ducouret G, Merten O-W. 2009. The effect of sterilization methods on the thermogelation properties of xyloglucan hydrogels, *Polymer Degradation and Stability*, 95:254-9.
- Argyriou AA, Antonacopoulou A, Iconomou G and Kalofonos HP. 2009. Treatment options for malignant gliomas, emphasizing towards new molecularly targeted therapies. *Critical Reviews in Oncology/ Hematology*, 69:199-210.
- Astaneh R, Nafissi-Varcheh N and Erfan M. 2007. Zinc–leuprolide complex: preparation, physicochemical characterization and release behavior from *in situ* forming implant. *Journal of Peptide Science*, 13:649–54.

Au JL-S, Jang SH, Zheng J, Chen CT, Song S, Hu L, and Wientjes MG. 2001. Determinants of drug delivery and transport to solid tumors. *Journal of Controlled Release*, 74:31-46.

Avendaño C and Menéndez JC. 2008. Antimetabolites in Medicinal Chemistry of Anticancer Drugs. Elsevier [e-book, Available at: <http://0-www.sciencedirect.com.innopac.wits.ac.za/science/book/9780444528247> , Accessed 2011-12-30].

Babich A. 2005. Tumor necrosis by controlled ebullism. *Medical Hypotheses*, 64:318–19.

Bajin-Katić K, Stankov K and Kovačević Z. 2004. Changes of biochemical parameters in rat intestinal mucosa induced by methotrexate and effects of enteral administration of glutamine. *Archive of Oncology*, 12:35-8.

Bajpai AK, Shukla SK, Bhanu S, Kankane S. 2008. Responsive polymers in controlled drug delivery. *Progress in Polymer Science*, 33: 1088–118.

Bawa P, Pillay V, Choonara YE and du Toit LC. 2009. Stimuli-responsive polymers and their applications in drug delivery. *Biomedical Materials*, 4: 1-15.

BD Biosciences Training [Available at: <http://www.bdbiosciences.com/support/training/index.jsp>, Accessed 2011-05-31]

Berrada M, Serreqi A, Dabbarh F, Owusu A, Gupta A and Lehnert S. 2005. A novel non-toxic camptothecin formulation for cancer chemotherapy. *Biomaterials*, 26: 2115–20.

Bokias G, Staikos G and Iliopoulos I. 2000. Solution properties and phase behaviour of copolymers of acrylic acid with N-isopropylacrylamide: the importance of the intrachain hydrogen bonding. *Polymer*, 41: 7399-405.

Bosch P, Del Monte F, Mateo JL and Levy D. 2000. Photopolymerization of hydroxyethylmethacrylate in the formation of organic-inorganic hybrid sol-gel matrices. *Journal of Polymer Science Part A: Polymer Chemistry*, 34:3289-96.

Bouhadir K, Alsberg E and Mooney DJ. 2001. Hydrogels for combination delivery of antineoplastic agents. *Biomaterials*, 22:2625-33.

Brem H, Piantadosi S, Burger PC, Walker M, Selker R, Vick NA, Black K, Sisti, M, Brem S, Mohr G, Muller P, Morawetz R and Schold SC. 1995. Placebo controlled trial of

safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas, *The Lancet*, 345:1008-12.

Brem H. 1996. Developmental therapeutics program. National Cancer Institute (USA). [Available at: http://images.google.co.za/imgres?imgurl=http://dtp.nci.nih.gov/timeline/images/subs/success_stories/SS_1/SS_1_Gliadel_72dpi_small.jpg&imgrefurl=http://dtp.nci.nih.gov/timeline/noflash/success_stories/S1_Carmustine.htm Accessed 2009-05-06]

British Pharmacopoeia. 1993. HMSO:London

Burnett CL, Bergfeld WF, Belsito DV, Hill RA, Klassen CD, Liebler DC, Marks JG, Shank RC, Slaga TJ, Snyder PW and Andersen A. 2011. Final report of the amended safety assessment of PVM/MA copolymer and its related salts and esters used in cosmetics. *International Journal of Toxicology*, 30: 128S

Calabresi P. 1981. Cancer: crab or chimera? The clinical Implications of cancer cell heterogeneity. *Transactions of the American Clinical and Climatological Association*, 92: 49–65.

Cegnar M, Premzl A, Zavasnik-Bergant V, Kristl J and Kos J. 2004. Poly (lactide-co-glycolide) nanoparticles as a carrier system for delivering cysteine protease inhibitor cystatin into tumor cells. *Experimental Cell Research*, 301:223–31.

Cerchiara T, Luppi B, Chidichimo G, Bigucci F and Zecchi V. 2005. Chitosan and poly(methyl vinyl ether-co-maleic anhydride) microparticles as nasal sustained delivery systems. *European Journal of Pharmaceutics and Biopharmaceutics*, 61: 195–200.

Chen F, Kuriakose MA, Zhou M, DeLacure MD and Dunn RL. 2003. Biodegradable polymer-mediated intratumoral delivery of cisplatin for treatment of human head and neck squamous cell carcinoma in a chimeric mouse model. *Head and Neck*, 25:7554 – 60.

Chen S and Singh J. 2005. Controlled delivery of testosterone from smart polymer solution based systems: in vitro evaluation. *International Journal of Pharmaceutics*, 295:183–90.

Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, Leroux JC, Atkinson BL, Binette F and Selmani A. 2000. Novel injectable neutral solutions of chitosan form biodegradable gels *in situ*. *Biomaterials*, 21:2155–61.

Chertin B, Spitz IM, Lindenberg T, Algur N, Zer T, Kuzma P, Young AJM, Catane R and Farkas A. 2000. An implant releasing the gonadotropin hormone-releasing hormone agonist histrelin maintains medical castration for up to 30 months in metastatic prostate cancer. *Journal of Urology*, 163:838-44.

Cho YI, Park S, Jeong SY and Yoo HS. 2009. *In vivo* and *in vitro* anti-cancer activity of thermo-sensitive and photo-crosslinkable doxorubicin hydrogels composed of chitosan-doxorubicin conjugates. *European Journal of Pharmaceutics and Biopharmaceutics*, 73:59-65.

Chopko BW. 2009. Intracavitary carmustine polymer implantation as an adjunct to palliative metastatic vertebral column tumors. *Surgical Neurology*, 72:553.

Chu E and Sartorelli AC, 2004. Cancer Chemotherapy. In Katzung BG (Ed.) Basic and clinical pharmacology. Ninth Edition. McGraw-Hill: USA

Cole MA, Voelcker NH, Thissen H and Griesser HJ. 2009. Stimuli-responsive interfaces and systems for the control of protein–surface and cell–surface interactions. *Biomaterials*, 30:1827-50.

Cowie JMG, Garay MT, Lath D and McEwen LJ. 1989. Formation of polymer-polymer complexes and blends in the system poly(acrylic acid)-poly(vinyl methyl ether). *British Polymer Journal*, 21:81-5.

De Anta JM, Real FX and Mayol X. 2005. Low tumor cell density environment yields survival advantage of tumor cells exposed to MTX *in vitro*. *Biochimica et Biophysica Acta- General Subjects*, 1732:98-106.

Decker S, Winkelmann W, Nies B and van Valen F. 1999. Cytotoxic effect of methotrexate and its solvent on osteosarcoma cells *in vitro*. *Journal of Bone and Joint Surgery (Britain)*, 81: 545-51.

Dehousse V, Garbacki N, Jaspart S, Castagne D, Piel G, Colige A and Evarard B. 2010. Comparison of chitosan/siRNA and trimethylchitosan/siRNA complexes *in vitro*. *International Journal of Biological Macromolecules*, 46:342-9

Dijkman GA, del Moral PF, Plasman JWMH, Kums JJM, Delaere KPJ, Debruyne FMJ, Hutchkinson FJ and Furr BJA. 1990. A new extra long acting depot preparation of the LHRH analogue Zoladex®: First endocrinological and pharmacokinetic data in patients with advanced prostate cancer. *The Journal of Steroid Biochemistry and Molecular Biology*, 37:933-6.

Dongre VG, Karmuse PP, Rao PP and Kumar A. 2008. Development and validation of a UPLC method for determination of primaquine phosphate and its impurities. *Journal of Pharmaceutical and Biomedical Analysis*, 46:236-42.

Dordunoo SK, Oktaba AMC, Hunter W, Min W, Cruz T and Burt HM.1997. Release of taxol from poly(ϵ -caprolactone) pastes: effect of water-soluble additives. *Journal of Controlled Release*, 1997;44:87-94.

Eliasz R and Kost J. 2001. Composition and method for forming biodegradable implants in situ and uses of these implants. US Patent: 6206920.

Elkharraz K, Faisant N, Guse C, Siepmann F, Arica-Yegin B, Oger JM, Gust R, Goepferich A, Benoit JP and Siepmann J. 2006. Paclitaxel-loaded microparticles and implants for the treatment of brain cancer: Preparation and physicochemical characterization. *International Journal of Pharmaceutics*, 314:127–36.

Eudragit® L100 and Eudragit® S100. Technical Information [Available at: <http://eudragit.evonik.com/product/eudragit/Documents/evonik-specification-eudragit-l-100-and-s-100.pdf>, Accessed 2011-11-05]

Exner AA, Krupka TM, Scherrer K and Teets JM. 2005. Enhancement of carboplatin toxicity by Pluronic block copolymers. *Journal of Controlled Release*, 106:188-97.

Felice P, Corinaldesi G and Lizio G. 2009. Implant prosthetic rehabilitation of posterior mandible after tumor ablation with inferior alveolar nerve mobilization and inlay bone grafting: a case report. *Journal of Oral and Maxillofacial Surgery*, 67:1104-12.

Finne U, Kyrrönen K and Urtti A. 1989. Drug release from monoisopropyl ester of poly(vinyl methyl ether-maleic anhydride) can be modified by basic salts in the polymer matrix. *Journal of Controlled Release*, 10:189-94.

Folkman J. 1990. What is the evidence that tumors are angiogenesis dependant. *Journal of the National Cancer Institute*, 82: 4-6.

Furnaletto S, Cirri M, Maestrelli F, Corti G and Mura P. 2006. Study of formulation variables influencing the drug release rate from matrix tablets by experimental design. *European Journal of Pharmaceutics and Biopharmaceutics*, 62:77-84.

Ganguly S and Dash AK. 2004. A novel *in situ* gel for sustained drug delivery and targeting. *International Journal of Pharmaceutics*, 276:83–92.

Gaze MN, Wheldon TE, O'Donoghue JA, Hilditch TE, McNee SG, Simpson E and Barrett A. 1995. Multi-modality megatherapy with [131I]metaiodobenzylguanidine, high dose melphalan and total body irradiation with bone marrow rescue: Feasibility study of a new strategy for advanced neuroblastoma. *European Journal of Cancer*, 31:252-6.

Geever LM, Devine DM, Nugent MJD, Kennedy JE, Lyons JG, Hanley A and Higginbotham CL. 2006. Lower critical solution temperature control and swelling behavior of physically crosslinked thermosensitive copolymers based on N-isopropylacrylamide. *European Polymer Journal*, 42:2540-8.

Globocan, 2008. International Agency for Research on Cancer (IARC), 2010. [Available at: <http://www.who.int/mediacentre/factsheets/fs297/en/index.html>, Accessed 2011-03-18]

Gopferich A. 1997. Bioerodable implants with programmable release. *Journal of Controlled Release*, 44:271-81.

Govender S, Pillay V, Chetty DJ, Essack SY, Dangor CM, Govender T. 2005. Optimization and characterisation of bioadhesive controlled release tetracycline microspheres. *International Journal of Pharmaceutics*, 306: 24-40.

Graves R, Freeman T and Mandal TK. 2007 *In vitro* dissolution method for evaluation of buprenorphine *in situ* gel formulation: A technical note. *AAPS Pharmaceutical Science and Technology*, 8:E1-E4, Article 62.

Grumbach ES, Wheat TE, Kele M and Mazzeo JR. 2005. Developing columns for UPLC design considerations and recent developments, Separation Science Redefined. May: 41-4. [Available at: <http://www.modernmedicine.com/modernmedicine/data/articlestandard/lcgc/242005/164652/article.pdf> . Accessed 2011-12-28]

Gupta P, Vermani K and Garg S. 2002. Hydrogels: from controlled release to pH-responsive drug delivery. *Drug Discovery Today*, 7:569-80.

Hacker MC, Klouda L, Ma BB, Kretlow JD and Mikos AG. 2008. Synthesis and characterization of injectable, thermally and chemically gelable, amphiphilic poly(N-isopropylacrylamide)-based macromers. *Biomacromolecules*, 9:1558–70.

Han HD, Song CK, Park YS, Noh KH, Kim JH, Hwang T, Kim T and Shin BC. 2008. A chitosan hydrogel-based cancer drug delivery system exhibits synergistic antitumor

effects by combining with a vaccinia viral vaccine. *International Journal of Pharmaceutics*, 350: 27-34.

Han J, Wang K, Yang D and Nie J. 2009. Photopolymerization of methacrylated chitosan/PNIPAAm hybrid dual-sensitive hydrogels as carrier for drug delivery. *International Journal of Biological Macromolecules*, 44:229–35.

He C, Kim SW and Lee DS. 2008. *In situ* gelling stimuli-sensitive block copolymer hydrogels for drug delivery. *Journal of Controlled Release*, 127:189-207.

He YC, Chen JW, Cao J, Pan DY and Qiao JG. 2003. Toxicities and therapeutic effect of fluorouracil controlled release implant on tumor bearing rats. *World Journal of Gastroenterology*, 9:1795-9.

Heller J. 1985. Controlled drug release from poly(ortho-esters) - a surface eroding polymer. *Journal of Controlled Release*, 2:167-77.

Hernigou PH, Thiéry P, Benoit J, Voisin MC, Leroux P, Hagege G, Delepine G and Gouttallier D. 1989. Methotrexate diffusion from acrylic cement: local chemotherapy for bone tumors. *The Journal of Bone and Joint Surgery*. 71B: 804-11.

Hiremath JG, Devi VK, Devi K and Domb AJ. 2008. Biodegradable poly(sebacic acid-co-ricinoleic-ester anhydride) tamoxifen citrate implants: Preparation and *in vitro* characterization. *Journal of Applied Polymer Science*, 107:2745–54.

Hilbrig F and Freitag R. Affinity Precipitation. In Subramanian G (Ed.). 2007. Bioseparation and Bioprocessing. 2nd edition. Wiley-VCH: Darmstadt [e-book, Available at:

<http://books.google.co.za/books?id=ins8uA6esDsC&printsec=frontcover&dq=bioseparation+and+bioprocessing&hl=en&sa=X&ei=vwRnT8veA4ulhQfZkbS8CA&ved=0CDgQ6AEwAA#v=onepage&q=bioseparation%20and%20bioprocessing&f=false>, Accessed 2009-06-07]

Hilgard P. 1973. Experimental hypercalcaemia and whole blood clotting. *Journal of Clinical Pathology*. 26:616-9.

Ho EA, Vassileva V, Allen C and Piquette-Miller M. 2005. *In vitro* and *in vivo* characterization of a novel biocompatible polymer–lipid implant system for the sustained delivery of paclitaxel. *Journal of Controlled Release*, 104:181–91.

- Hong L, Tabata Y, Miyamoto S, Yamamoto M, Yamada K, Hashimoto N and Ikada Y. 2000. Bone regeneration at rabbit skull defects treated with transforming growth factor-b1 incorporated into hydrogels with different levels of biodegradability. *Journal of Neurosurgery*, 92:315
- Hong LT, Joon P and Whan LJ. 2007. *In situ* controlled release drug delivery system. EP Patent: KR 20070100701.
- Hori Y, Winans AM and Irvine DJ. 2009. Modular injectable matrices based on alginate solution/microsphere mixtures that gel in situ and co-deliver immunomodulatory factors. *Acta Biomaterialia*, 5:969-82.
- Huanbutta K, Luangtana-Anan M, Sriamornsak P, Limmatvapirat S, Puttipipatkachorn S and Nunthand J. 2008. Factors affecting preparations of chitosan microcapsules for colonic drug delivery. *Journal of Metals, Materials and Minerals*, 18:79-83.
- Hughes GA. 2005. Nanostructure-mediated drug delivery. *Nanomedicine: Nanotechnology, Biology, and Medicine*, 1: 22–30.
- Hunter WL, Burt HM and Machan L. 1997. Local delivery of chemotherapy: a supplement to existing cancer treatments: A case for surgical pastes and coated stents. *Advanced Drug Delivery Reviews*, 26:199–207.
- Jackson JK, Gleave ME, Yago V, Beraldi E, Hunter WL and Burt HM. 2000. The suppression of human prostate tumor growth in mice by the intratumoral injection of a slow-release polymeric paste formulation of paclitaxel. *Cancer Research*. 60:4146-51.
- Jackson JK, Zhang X, Llewellyn S, Hunter WL and Burt HM. 2004. The characterization of novel polymeric paste formulations for intratumoral delivery. *International Journal of Pharmaceutics*, 270:185–98.
- Jain JP, Modi S, Domb AJ and Kumar N. 2005. Role of polyanhydrides as localized drug carriers. *Journal of Controlled Release*, 103:541-563.
- Jain R, Pandey A, Pandeya SS, 2004. Mechanism of dissolution of delayed release formulation of diclofenac sodium. *Chemistry*, 18:131-8.
- Jeong B and Gutowska A, 2002. Lessons from nature: stimuli-responsive polymers and their biomedical applications. *Trends in Biotechnology*, 20:305-11.

- Jeong B, Bae YH and Kima SW. 2004. Drug release from biodegradable injectable thermosensitive hydrogel of PEG–PLGA–PEG triblock copolymers. *Journal of Controlled Release*, 63:155–63.
- Jeyanthi R and Rao KP. 1990. Controlled release of anticancer drugs from collagen-poly(HEMA) hydrogel matrices. *Journal of Controlled Release*, 13:91-8.
- Jönsson B and Wilking N, 2007. A global comparison regarding patient access to cancer drugs. *Annals of Oncology*, 18:iii1-iii78.
- Jones DS, Lorimer CJ, Andrews GP, McCoy CP and Gorman SP. 2007. An examination of the thermorheological and drug release properties of zinc tetraphenylporphyrin containing thermoresponsive hydrogels, designed as light activated antimicrobial implants. *Chemical Engineering Science*, 62:990–9
- Kabir AKL, Jesmeen T, Biswas BK and Rouf ASS. 2009. Formulation and in vitro evaluation of esomeprozole magnesium hydrophilic matrix. *Turkish Journal of Pharmaceutical Sciences*, 6:1-10
- Kakinoki S, Taguchi T, Saito H, Tanaka J and Tateishi T. 2007. Injectable *in situ* forming drug delivery system for cancer chemotherapy using a novel tissue adhesive: Characterization and *in vitro* evaluation. *European Journal of Pharmaceutics and Biopharmaceutics*. 66:383–90.
- Kakinoki S and Taguchi T. 2007. Antitumor effect of an injectable in-situ forming drug delivery system composed of a novel tissue adhesive containing doxorubicin hydrochloride. *European Journal of Pharmaceutics and Biopharmaceutics*, 67:676–81.
- Kang HW, Tabata Y and Ikada Y. 1999. Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials*, 20:1339-44.
- Karayanni K and Staikos G. 2000. Study of the lower critical solution temperature behaviour of poly(vinyl methyl ether) aqueous solutions in the presence of poly(acrylic acid). The role of interpolymer hydrogen bonding interaction. *European Polymer Journal*, 36:2645-50.
- Kim S and Healy KE. 2003. Synthesis and Characterization of Injectable Poly(N-isopropylacrylamide-co-acrylic acid) Hydrogels with Proteolytically Degradable Cross-Links. *Biomacromolecules*, 4:1214-23.

- Klouda L and Mikos AG. 2008. Review article: Thermoresponsive hydrogels in biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*. 68:34–45.
- Kohler N, Sun C, Wang J and Zhang M. 2005. Methotrexate-modified superparamagnetic nanoparticles and their intracellular uptake into human cancer cells. *Langmuir*, 21:8858-64.
- Konishi M, Tabata Y, Kariya M, Suzuki A, Mandai M, Nanbu K, Takakura K and Fujii S. 2003. *In vivo* anti-tumor effect through the controlled release of cisplatin from biodegradable gelatin hydrogel. *Journal of Controlled Release*, 92:301–13.
- Konishi M, Tabata Y, Kariya M, Hosseinkhani H, Suzuki A, Fukuhara K, Mandai M, Takakura K, Fujii S. 2005. In vivo anti-tumor effect of dual release of cisplatin and adriamycin from biodegradable gelatine hydrogel. *Journal of Controlled Release*, 103:7-19.
- Koussathana M, Lianos P and Staikos G. 1997. Investigation of hydrophobic interactions in dilute aqueous solutions of hydrogen-bonding interpolymer complexes by steady-state and time-resolved fluorescence measurements. *Macromolecules*, 30:7798-802.
- Kumar C, Satish CS and Shivakumar HG. 2008. Formulation and evaluation of chitosan-gellan based methotrexate implants. *Journal of Macromolecular Science, Part A: Pure and Applied Chemistry*, 45:643-49.
- Kundu PP and Kundu M. 2001. Effect of salts and surfactant and their doses on the gelation of extremely dilute solutions of methyl cellulose. *Polymer*, 42:2015-20.
- Lachmann P. 1992. The use of animals in research. *British Medical Journal*, 305:1.
- Lalloo A, Chao P, Hu P, Stein S and Sinko PJ. 2006. Pharmacokinetic and pharmacodynamic evaluation of a novel *in situ* forming poly(ethylene glycol)-based hydrogel for the controlled delivery of the camptothecins. *Journal of Controlled Release*, 112:333–42.
- Lee LY, Ranganath SH, Fu Y, Zheng JL, Lee HS, Wang C-H and Smith KA. 2009. Paclitaxel release from micro-porous PLGA discs. *Chemical Engineering Science*, 64:4341-9.

Leteurtre E, Gouyer V, Rousseau K, Moreau O, Barbat A, Swallow D, Huet G, Lesuffleur T. 2004. Differential mucin expression in colon carcinoma HT-29 clones with variable resistance to 5-fluorouracil and methotrexate. *Biology of the Cell*, 96:145–151.

Lewis GA, Mathieu D and Phan-Tan-Luu R.1999. Pharmaceutical Experimental Design, Marcel Dekker Inc.: New York: USA. [e-book, Available at: <http://books.google.co.za/books?id=jgFZOyBtS98C&printsec=frontcover#v=onepage&q&f=false>) Accessed 2012-01-31]

Lin R, Ng LS and Wang C. 2005. *In vitro* study of anticancer drug doxorubicin in PLGA-based microparticles. *Biomaterials*, 26:4476–4485.

Liu L, Sakaguchi T, Kanda T, Hitomi J, Tabata Y and Hatakeyama, K. 2003. Delivery of interleukin-12 in gelatin hydrogels effectively suppresses development of transplanted colonal carcinoma in mice. *Cancer Chemotherapy and Pharmacology*, 51:53–7.

Lue SJ, Hsu J and Wei T. 2008. Drug permeation modeling through the thermo-sensitive membranes of poly(N-isopropylacrylamide) brushes grafted onto microporous films. *Journal of Membrane Science*, 321:146-54.

Lund, W (Ed.). 1994. The Pharmaceutical Codex, 12th Edition. The Pharmaceutical Press: London

Madan M, Bajaj A, Lewis S, Udupa N and Baig JA. 2009. *In situ* forming polymeric drug delivery systems. *Indian Journal of Pharmaceutical Sciences*, 71:242- 51.

Madbouly SA and Ougizawa T. 2005. Thermal Croslinking of Poly(Vinyl Methyl Ether). I. Effect of Crosslinking Process on the viscoelastic properties. *Journal of Macromolecular Science*, 43:471-87.

Maeda H, Wu J, Sawa T, Matsumura Y and Hori K. 2000. Tumor vasculature and the EPR effect in macromolecular therapeutics: a review. *Journal of Controlled Release*, 65:271-84.

Makrilia N, Lappa T, Xyla V, Nikolaidas I and Syrigos K. 2009. The role of angiogenesis in solid tumors: An overview. *European Journal of Internal Medicine*, 20:663-71.

Marceij R, Youqing S, Huadong T. 2007. Nanoparticles for cytoplasmic delivery of drugs to cancer cells. EP Patent: 2007001356.

Marriott C, 2004. Rheology. In Aulton ME (Ed.) 2004. Pharmaceutics: The science of dosage form design. Churchill Livingstone: USA

Martin G and Jain RK. 1994. Non-invasive measurement of interstitial pH profiles in normal and neoplastic tissue using fluorescence ratio imaging microscopy. *Cancer Research*, 54:5670–4.

Martínez-Ruvalcaba A, Beccerra-Bracamontes F, Sánchez-Díaz JC and González-Álvarez A. 2009. Polyacrylamide-gelatin polymeric networks: effect of pH and gelatin concentration on the swelling kinetics and mechanical properties. *Polymer Bulletin*, 62:539–48.

Matsumura Y and Maeda H. 1986. A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs, *Cancer Research*, 46:6387-92.

Meyer DE, Shin BC, Kong GA, Dewhirst MW and Chilkoti A. 2001. Drug targeting using thermally responsive polymers and local hyperthermia. *Journal of Controlled Release*, 74:213–24.

Moebus K, Siepmann J and Bodmeier R. 2009. Alginate–poloxamer microparticles for controlled drug delivery to mucosal tissue. *European Journal of Pharmaceutics and Biopharmaceutics*, 72:42–53.

Mok TS, Kanekal S, Lin XR, Leung TWT, Chan ATC, Yeo W, Yu S, Chak K, Leavitt R and Johnson P. 2001. Pharmacokinetic study of intralesional cisplatin for the treatment of hepatocellular carcinoma. *Cancer Chemotherapy and Pharmacology*, 91:2369–77.

Monga SP, Wadleigh R, Sharma A, Adib H, Strader D, Singh G, Harmon JW, Berlin M, Monga DK and Mishra L. 2000. Intratumoral therapy of cisplatin/epinephrine injectable gel for palliation in patients with obstructive esophageal cancer. *American Journal of Clinical Oncology and Cancer*, 23: 386–92.

Munn LL. 2003. Aberrant vascular architecture in tumors and its importance in drug-based therapies. *Drug Discovery Today*, 8: 396-403.

Murray JC and Carmichael J. 1995. Targeting solid tumors: challenges, disappointments, and opportunities. *Advanced Drug Delivery Reviews*, 17: 117-127.

NIST/Sematech, e-Handbook of statistical methods. [Available at: <http://www.itl.nist.gov/div898/handbook/pri/section2/pri24.htm>, Accessed 2012-02-06]

Nováková L, Matysová L and Solich P. 2006. Advantages of application of UPLC in pharmaceutical analysis. *Talanta*, 63:908-918.

Nukeeva Z, Mun GA, Khutoryanskiy VV and Dzhusupbekova AB. 2004. Hydrophilic films based on poly(acrylic acid)–poly(vinyl methyl ether) blends cross-linked by gamma-radiation. *Radiation Physics and Chemistry*, 69:205–9.

Nurkeeva ZS, Tyukova IS, Suvorova AI, Mun GA, Dzhusupbekova AB, Khutoryanskiy VV. 2005. Miscibility studies in poly(methyl vinyl ether)/hydroxypropylcellulose binary system in aqueous solutions and solid state. *Carbohydrate Polymers*, 62:80–6.

Obara K, Ishihara M, Ozeki Y, Ishizuka T, Hayashi T, Nakamura S, Saito Y, Yura H, Matsui T, Hattori H, Takase B, Ishihara M, Kikuchi M and Maehara T. 2005. Controlled release of paclitaxel from photocrosslinked chitosan hydrogels and its subsequent effect on subcutaneous tumor growth in mice. *Journal of Controlled Release*, 110:79–89.

Okino H, Nakayama Y, Tanaka M and Matsuda T. 2002. *In situ* hydrogelation of photocurable gelatin and drug release. *Journal of Biomedical Materials Research*, 59:233–45.

Ohya S and Matsuda T. 2005. Poly(N-isopropylacrylamide) (PNIPAM)-grafted gelatin as thermoresponsive three-dimensional artificial extracellular matrix: molecular and formulation parameters vs. cell proliferation potential. *Journal of Biomaterials Science, Polymer Edition*, 16: 809-27.

Ono K, Saito Y, Yura H, Ishikawa K, Kurita A, Akaike T and Ishihara M. 2000. Photocrosslinkable chitosan as a biological adhesive. *Journal of Biomedical Materials Research*, 49:289–95.

Orienti I, Gentilomi G, Bigucci F, Luppi B, and Zecchi V. 1998. Substituted Poly(Methyl Vinyl Ether-alt-Maleic Anhydride) for the Release Control and Targeting of Methotrexate. *Archiv der Pharmazie*, 331:347-51.

Packhaeuser CB, Schnieders J, Oster CG and Kissel T. 2004. *In situ* forming parenteral drug delivery systems: an overview. *European Journal of Pharmaceutics and Biopharmaceutics*, 58:445–455.

Park KH and Song SC. 2006. Morphology of spheroidal hepatocytes within injectable, biodegradable and thermo-sensitive poly(organophosphazene) hydrogel as cell delivery vehicle. *Journal of Bioscience and Bioengineering*, 101:238–42.

- Patan S, Munn LL and Jain RK. 1996. Intussusceptive microvascular growth in a human colon adenocarcinoma xenograft: a novel mechanism of tumor angiogenesis. *Microvascular Research*, 51:260–72.
- Peppas NA and Sahlin JJ. 1989. A simple equation for the description of solute release III. Coupling of diffusion and relaxation. *International Journal of Pharmaceutics*, 57:169-72
- Perry J, Chambers A, Spithoff K, and Laperriere N. 2007. Gliadel wafers in the treatment of malignant glioma: a systematic review. *Current Oncology*, 14:189–94.
- Pezron E, Leibler L, Ricard A and Audebert R. 1988. Reversible gel formation induced by ion complexation. 2. Phase diagrams. *Macromolecules*, 21:1126–31.
- Phillips GO, Takigami S and Takigami M. 1996. Hydration characteristics of the gum exudates from *Acacia senegal*. *Food Hydrocolloids*, 10: 11-9.
- Pillay V and Fassihi R. 1998. Evaluation and comparison of dissolution data derived from different modified release dosage forms: an alternative method. *Journal of Controlled Release*, 55:45-55.
- Puccetti G and Leblanc RM. 1996. Direct evidence of the liquid–solid transition of a sol–gel material using photoacoustic spectroscopy. *The Journal of Physical Chemistry*, 100:1731–7.
- Pulsipher MA, Bader P, Klingebiel T and Cooper LNJ. 2009. Allogeneic transplantation for pediatric acute lymphoblastic leukemia: The emerging Role of peritransplantation minimal residual disease/chimerism monitoring and novel chemotherapeutic, molecular, and immune approaches aimed at preventing relapse. *Biology of Blood and Marrow Transplant*, 15:62-71.
- Qiu B, Stefanos S, Ma J, Lalloo A, Perry BA, Leibowitz MJ, Sinko PJ and Stein S. 2003. A hydrogel prepared by in situ cross-linking of a thiol-containing poly(ethylene glycol)-based copolymer: a new biomaterial for protein drug delivery. *Biomaterials*, 24:11–8.
- Ranganath SH, Fu Y, Arifin DY, Kee I, Zheng L, Lee H-S, Chow PKH and Wang CH. 2010. The use of submicron/nanoscale PLGA implants to deliver paclitaxel with enhanced pharmacokinetics and therapeutic efficacy in intracranial glioblastoma mice. *Biomaterials*, 31: 5199-207.

Ravivarapu HB, Moyer KL and Dunn RL. 2000. Sustained activity and release of leuprolide acetate from an in situ forming polymeric implant. *AAPS PharmSciTech*, 1: article 1.

Richter S, 2004. Mechanical properties of poly(vinyl methyl ether) hydrogels below and above their volume phase transition. *Colloid and Polymer Science*, 282:1213–21.

Ruel-Gariépy E, Shive M, Bichara A, Berrada M, Le Garrec D, Chenite A, Leroux JC. 2004. A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel. *European Journal of Pharmaceutics and Biopharmaceutics*, 57: 53–63.

Ruel-Gariépy E and Leroux JC. 2004. *In situ* forming hydrogels-review of temperature-sensitive systems. *European Journal of Pharmaceutics and Biopharmaceutics*, 58:409-26.

Sambrook J, Fritsch EF, and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, volume 3, Appendix B.12, p693 [e-book, Available at: <http://books.google.co.za/books?id=G5RgAAAAMAAJ&q=Molecular+Cloning:+A+Laboratory+Manual+Sambrook,+Fritsch,+and+Maniatis&dq=Molecular+Cloning:+A+Laboratory+Manual+Sambrook,+Fritsch,+and+Maniatis&hl=en&sa=X&ei=UBZjT63BE4fOhAfsu6SmCA&ved=0CDEQ6AEwAA>, Accessed 2008-06-15]

Schiller G. 2008. High-Dose Consolidation Chemotherapy V: Bone Marrow Transplantation as Postremission Therapy in Adult Acute Myeloid Leukemia Medicine. [Available at: http://www.meds.com/leukemia/trends/mon_pt2.html, Accessed 2009-05-06]

Schmaljohann D. 2006. Thermo- and pH-responsive polymers in drug delivery. *Advanced Drug Delivery Reviews*, 58:1655-70.

Schuetz YB, Gurny R and Jordan O. 2008. A novel thermoresponsive hydrogel based on chitosan. *European Journal of Pharmaceutics and Biopharmaceutics*, 68:19–25.

Seong H, An TK, Khang G, Choi S, Lee CO and Lee HB. 2003. BCNU-loaded poly(D, L-lactide-co-glycolide) wafer and antitumor activity against XF-498 human CNS tumor cells *in vitro*. *International Journal of Pharmaceutics*, 251: 1-12.

Sharifi S, Mirzadeh H, Imani M, Rong Z, Jamshidi A, Shokrgorza M, Atai M and Roohpour N. 2009. Injectable in situ forming drug delivery system based on poly(ϵ -

caprolactone fumarate) for tamoxifen citrate delivery: Gelation characteristics, *in vitro* drug release and anti-cancer evaluation. *Acta Biomaterialia*, 5:1966-78.

Siepmann J and Siepmann F. 2008. Mathematical modeling of drug delivery. *International Journal of Pharmaceutics*, 364:328-43.

Singh B, Kumar R and Ahuja N. 2005. Optimizing drug delivery systems using systematic design of experiments. Part I: Fundamental Aspects. *Critical ReviewsTM in Therapeutic Drug Carrier Systems*, 22: 27-105.

Singh S, Webster DC and Singh J. 2007. Thermosensitive polymer: synthesis, characterisation and delivery of proteins. *International Journal of Pharmaceutics*, 341:68-77.

Sinha VR, Singla AK, Wadhawan S, Kaushik R, Kumria R, Bansal K and Dhawan S. 2004. Chitosan microspheres as potential carriers for drugs. *International Journal of Pharmaceutics*, 274:1-33.

Skoog DA, West DM and Holler FJ. 1992. Fundamentals of Analytical Chemistry, Sixth edition. Saunders College Publishing: USA

Smith J, Stock E, Orenberg EK, Yu NY, Kanekal S and Brown DM. 1995. Intratumoral chemotherapy with sustained-release drug delivery system inhibits growth of human pancreatic cancer xenografts. *Anti-cancer Drugs*, 6:717–26.

Sofou S and Yu YB. 2008. Delivery systems for the targeted radiotherapy of cancer. *Advanced Drug Delivery Reviews*, 60:1317-18.

Soo PL, Cho J, Grant J, Ho E, Piquette-Miller M, Allen C. 2008. Drug release mechanism of paclitaxel from a chitosan–lipid implant system: Effect of swelling, degradation and morphology. *European Journal of Pharmaceutics and Biopharmaceutics*, 69:149–57.

Sprague Dawley Rats, Harlan Laboratories [Available at: <http://www.harlan.com/models/spraguedawley.asp>, Accessed 2011-06-18]

Tabata Y and Ikada Y. 1999. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. *Biomaterials*, 20:2169-75.

Tauro JR and Gemeinhart RA. 2005. Matrix Metalloprotease Triggered Delivery of Cancer Chemotherapeutics from Hydrogel Matrixes. *Bioconjugate Chemistry*, 16:1133-39.

ten Brinke GT and Karasz FE. 1984. Lower critical solution temperature behavior in polymer blends: compressibility and directional-specific interactions. *Macromolecules*, 17:815-20.

Tomšič M, Prossnigg F and Glatter O. 2008. A thermoreversible double gel: Characterization of a methylcellulose and κ -carrageenan mixed system in water by SAXS, DSC and rheology. *Journal of Colloid and Interface Science*, 322: 41–50.

United States Pharmacopoeia and National Formulary XXII. 1999. The United States Pharmacopoeial Convention, Inc.: Rockville, MD, USA

Urtti A. 1985. Pilocarpine release from matrices of alkyl half esters of poly(vinyl methyl ether/maleic anhydride). *International Journal of Pharmaceutics*, 26:45-55.

Usov AI. 1998. Structural analysis of red seaweed galactans of agar and carrageenan groups. *Food Hydrocolloids*, 12:301–8.

Van Den Bulcke AI, Bogdanov B and De Rooze N. 2000. Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules*, 1:31–8.

Van Tomme SR, Storm G and Hennink WE. 2008. *In situ* gelling hydrogels for pharmaceutical and biomedical applications. *International Journal of Pharmaceutics*, 355:1–18.

Vander AJ, Sheman JH and Luciano DS. 1990. The kidneys and regulation of water and inorganic ions. In Human Physiology. 5th Ed. McGraw-Hill: New York

Vassileva V, Grant J, de Souza R, Allen M and Piquette-Miller M. 2007. Novel biocompatible intraperitoneal drug delivery system increases tolerability and therapeutic efficacy of paclitaxel in a human ovarian cancer xenograft model. *Cancer Chemotherapy and Pharmacology*, 60:907-14.

Verseput R. 2000. Digging into DOE. *Quality Digest*. [Online article. Available at: <http://www.qualitydigest.com/june01/html/doe.html>, Accessed 2012-12-31]

Vogelhuber W, Rotunno P, Magni E, Gazzaniga A, Spruß T, Bernhardt G, Buschauer A and Göpferich A. 2001. Programmable biodegradable implants. *Journal of Controlled Release*, 73:75–88.

- Wei H, He J, Sun L, Zhu K and Feng Z. 2005. Gel formation and photopolymerization during supramolecular self-assemblies of α -CDs with LA-PEG-LA copolymer end-capped with methacryloyl groups. *European Polymer Journal*, 41:948-57.
- Weinberg BD, Blanco E and Gao J. 2007. Polymer implants for intratumoral drug delivery and cancer therapy. *Journal of Pharmaceutical Sciences*, 97:1681-702.
- Wenig BL, Werner JA, Castro DJ, Sridhar KS, Garewal HS, Kehrl W, Pluzanska A, Arndt O, Costantino PD, Mills GM, Dunphy II FR, Orenberg EK and Leavitt RD. 2002. The role of intratumoral therapy with cisplatin/epinephrine injectable gel in the management of advanced squamous cell carcinoma of the head and neck. *Archives of Otolaryngology: Head and Neck Surgery*, 128:880-5.
- Wike-Hooley JL, Haveman J and Reinhold HS. 1984. The relevance of tumor pH to the treatment of malignant disease. *Radiotherapy and Oncology*, 2:343-66.
- Williams DF. 2008. On the mechanisms of biocompatibility. *Biomaterials*, 29: 2941-3.
- Winnik FM. 1990. Phase transitions of poly-(N-isopropylacrylamide) solutions: A study by non-radiative energy transfer. *Polymer*, 31:2125-34.
- Winternitz CI, Jackson JK, Oktaba AM and Burt HM. 1996. Development of a polymeric surgical paste formulation for taxol. *Pharmaceutical Research*, 13:368-75.
- Wong HL, Bendayan R, Rauth AM, Li Y and Wu XY. 2007. Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles. *Advanced Drug Delivery Reviews*, 59:491-504.
- Woolfson AD, McCafferty DF and Moss GP. 1998. Development and characterisation of a moisture-activated bioadhesive drug delivery system for the percutaneous local anaesthesia. *International Journal of Pharmaceutics*, 169: 83-94.
- Wren SAC and Tchlitcheff P. 2006. UPLC/MS analysis for the identification of β -blockers. *Journal of Pharmaceutical and Biomedical Analysis*, 40:571-80.
- Wyss HM, Larsen RJ and Weitz DA. 2007. Oscillatory Rheology, Measuring the Viscoelastic behaviour of soft materials. *G.I.T. Laboratory Journal* 3-4: 68-70. GIT VERLAG GmbH & Co. KG, Darnstadt [Available at: http://www.mate.tue.nl/~wyss/files/Wyss_GIT_Lab_J_2007.pdf, Accessed: 2012-01-06]

- Yahara T, Koga T, Yoshida S, Nakagawa S, Deguchi H and Shirouzu K. 2003. Relationship between microvessel density and thermographic hot areas in breast cancer. *Surgery Today*, 33: 243-248.
- Yu L, Chang GT, Zhang H and Ding JD. 2008. Injectable block copolymer hydrogels for sustained release of a PEGylated drug. *International Journal of Pharmaceutics*, 348:95–106.
- Yu Y, Li Z, Tian H, Zhang S and Ouyang P. 2007. Synthesis and characterization of thermoresponsive hydrogels cross-linked with acryloyloxyethylaminopolysuccinimide. *Colloid and Polymer Science*, 285:1553–60.
- Zentner GM, Rathi R, Shih C, McRea JC, Seo MH, Oh H, Rhee BG, Mestecky J, Moldoveanu Z, Morgan M and Weitman S. 2001. Biodegradable block copolymers for drug delivery of proteins and water-insoluble drugs, *Journal of Controlled Release*, 72:203–215.
- Zhang J and Misra RDK. 2007. Magnetic drug-targeting carrier encapsulated with thermo-sensitive smart polymer: Core-shell nanoparticle carrier and drug release response. *Acta Biomaterialia*, 3:838–50.
- Zhou Y, Yan D, Dong W and Tian Y. 2007. Temperature-Responsive Phase Transition of Polymer Vesicles: Real-Time Morphology Observation and Molecular Mechanism. *The Journal of Physical Chemistry B*, 111:1262–70.

APPENDICES

APPENDIX A

PUBLICATIONS

Expert Opinion

1. Introduction
2. *In situ* forming implants
3. Implants based on natural polymeric sources
4. Synthetic, biodegradable mono-polymer and co-polymers as implant sources
5. Synthetic, biodegradable triblock polymers used for implants
6. Synthetic non-biodegradable polymers
7. Surgical pastes
8. Other implants
9. Conclusions
10. Expert opinion

informa
healthcare

Recent advances in the design of drug-loaded polymeric implants for the treatment of solid tumors

Ameena Wadee, Vinees Pillay[†], Yahya E Choonara, Lisa C du Toit, Clement Penny, Valence MK Ndesendo, Pradeep Kumar & Caragh S Murphy

[†]University of the Witwatersrand, Department of Pharmacy and Pharmacology, Johannesburg, South Africa

Introduction: The effective treatment of solid tumors continues to be a great challenge to clinicians, despite the development of novel drugs. In order to improve the clinical efficacy of existing chemotherapeutic agents, researchers have considered the possibility of site-specific solid tumor treatment. The greatest advantage of localized delivery is the significantly fewer side effects experienced by patients. Recently, *in situ* forming implants have attracted considerable interest. These polymeric systems are injected as solutions into tumor sites and the injected solution forms an implant as a result of local environmental stimuli and hence removes the need for surgical implantation.

Areas covered: This review summarizes the attempts that have been made to date in the development of polymeric implants for the treatment of solid tumors. Both *in situ* forming implants and preformed implants, fabricated using natural and synthetic polymers, are described. In addition, the peri- or intra-tumoral delivery of chemotherapeutic agents based on implants inserted surgically into the affected region is also discussed along with a short coverage of implants having an undesirable initial burst release effect.

Expert opinion: Although these implants have been shown to improve the treatment of various solid tumors, the ideal implant that is able to deliver high doses of chemotherapeutics to the tumor site, over prolonged periods with relatively few side effects on normal tissue, is yet to be formulated.

Keywords: chemotherapy, implant, polymeric systems, solid tumors, stimuli-responsive

Expert Opin. Drug Deliv. (2011) 8(10):1323-1340

1. Introduction

Despite the advances in cancer therapy, solid tumors have remained to be difficult to treat successfully as there are a significant number of barriers to overcome during treatment [1]. One such barrier is the three-dimensional structure of the solid tumors, in which drug penetration into solid tumors is 5 – 10-fold less when compared with penetration into a single layer of cells [2]. In addition, despite the extensive angiogenesis that takes place around the tumor, blood perfusion of the region is often erratic. Tumors often consist of a well-perfused outer region and a poorly perfused necrotic center. Hence, systemically administered drugs may reach the exterior layers but rarely reach the central area [1]. This poor perfusion is mainly due to the low movement of drugs from the blood vessels into the stroma of the tumor and hence to the cancerous cells. Although the endothelium of the blood vessels in the area of the tumor does appear to 'leak' more than normal cells, drugs are not taken up well into the stroma. This is thought to be due to the high interstitial hydrostatic pressure in the stroma or a diminished vascular pressure [3]. In addition, the host (i.e., the patient) tolerability to the high doses of chemotherapy that are often used must also be considered [4,5]. Furthermore,

APPENDIX A (II)



Cum Laude

Academy of Pharmaceutical Sciences

Determination of the effect of the release of methotrexate from an in situ forming implant on colon carcinoma cells and in vivo studies in the rat model

Ameena Wadee^a, Viness Pillay^a, Yahya Choonara^a, Lisa Du Toit^a, Pradev Kumar^a, Clement Penny^b, Leith Meyer^c

^aDepartment of Pharmacy and Pharmacology, Wits Advanced Drug Delivery Platform, University of the Witwatersrand

^bDepartment of Medical Oncology, University of the Witwatersrand

^cCentral Animal Service, University of the Witwatersrand

Correspondence to: Viness Pillay, e-mail: viness.pillay@wits.ac.za

Keywords: methotrexate, colon carcinoma cells, rat model

APPENDIX B

CONFERENCE PROCEEDINGS

APPENDIX B (I)

Formulation approaches for interactive pH-responsive nanoparticles

Farhana Ravat, Ameena Wadee, Viness Pillay*, Yahya E. Choonara and Lisa C. du Toit

University of the Witwatersrand, Department of Pharmacy and Pharmacology, 7 York Road, Parktown, 2193,
Johannesburg, South Africa

*Correspondence: viness.pillay@wits.ac.za

Introduction

To compare the particle sizes, zeta potential and drug entrapment efficiency of nanoparticles comprising various polymeric permutations and utilizing different nanoparticle preparation approaches. Furthermore, the effect of pH on the zeta potential of the nanoparticles was also explored.

Methods

Two approaches of forming chitosan (CHT) nanoparticles were investigated namely, a coacervation/precipitation (CP) and an emulsion-droplet (ED) method. In each case the stabilizer used was polyvinyl alcohol (PVA) at a concentrations ranging from 1-5%^{w/v}. The effect of Span 80 was also investigated. Methacrylic acid nanoparticles were produced using a nano-precipitation method with PVA (0.4%^{w/v} or 1%^{w/v}) used as stabilizers. Diphenhydramine (DPH) was used as the model drug to investigate the drug entrapment efficacy (DEE) within the nanoparticles. The particle size distribution and zeta potential were measured using a ZetaSizer NanoZS (Malvern Instruments, UK).

Results

Using the CP method for preparing CHT nanoparticles mean particle sizes ranged between 499.7d.nm (Pdi=0.404) and 530d.nm (Pdi=0.373) with the use of 1%^{w/v} and 5%^{w/v} PVA respectively. The ED approach using CHT produced nanoparticles with a mean particle size ranging between 408nm (Pdi=0.436), 531nm (Pdi=0.711) and 166nm (Pdi=0.364) with the use of either 1%^{w/v} PVA, 5%^{w/v} PVA or Span 80 as a stabilizer respectively. Methacrylic acid nanoparticles had a mean particle size of 338nm (Pdi=0.141 at 0.4%^{w/v} PVA) and 384nm (Pdi=0.112 at 1%^{w/v} PVA). The zeta potential values for all nanoparticle formulations varied between 0.236mV to -0.162mV regardless of the stabilizer or the pH of the suspending fluid used. DEE for CHT using the CP approach had values of 9.20% (1%^{w/v} PVA) and 27.00% (5%^{w/v} PVA). DEE values for CHT using the ED method revealed values of 8.12 % (1%^{w/v} PVA), 6.99% (5%^{w/v} PVA) and 14.66% (Span 80). DEE values for the methacrylic acid nanoparticles were 2.47% (0.4%^{w/v} PVA) and 1.88% (1%^{w/v} PVA) for the w/o/w approach. The nanoparticle sizes varied significantly between the various approaches adopted and the type of stabilizer used. An increase in the stabilizer concentration yielded larger particles with greater polydispersity indices that were suggestive of agglomeration and the large particle size variation.

Conclusions

The CP method of preparing CHT nanoparticles displayed the most stability and efficiency in terms of entrapping highly soluble drugs such as DPH.

APPENDIX B (II)

The effect of CaCl_2 and Gum Arabic on the physicochemical properties and drug release profile of thermoresponsive poly(methyl vinyl ether) gels

Ameena Wadee, Viness Pillay*, Yahya E. Choonara, Lisa C. Du Toit

University of the Witwatersrand, Department of Pharmacy and Pharmacology, 7 York Road, Parktown, 2193, Johannesburg, South Africa, University of the Witwatersrand

*Correspondence: viness.pillay@wits.ac.za

Purpose:

To determine the effect of Gum Arabic on gel strength and drug release profile of thermosensitive poly(methyl vinyl ether) (PMVE) hydrogels. Further, to determine the interactions occurring between the polymers by means of FTIR. As the implant formed is intended for the prolonged treatment of solid tumors, conditions chosen mimic those found at a tumor site.

Methods:

Preparation of solutions: PMVE solutions were diluted to a 0.25% w/v solution. CaCl_2 was added to 20mL of PMVE in concentrations of 0.05M, 0.1M, 0.2M and 0.3M with or without the addition of Gum Arabic (4%).

Textural Analysis: Phosphate buffered solution (pH 6.75) was heated to $37^\circ\text{C} \pm 0.5^\circ\text{C}$ and 3mL of the polymeric blend being tested was immersed for 10 seconds. Gel strength and compressibility were then determined using a textural analyser (TA.XTplus Texture Analyser, StableMicroSystems, England) and the following parameters: Probe diam: 10mm; pre test speed: 1mm/s, test speed: 2mm/s; post test speed: 3mm/s; depth of compression: 2mm; trigger force: 0.001mN. All tests were conducted in triplicate.

Fourier Transformed Infrared (FTIR): Polymeric blends were examined using a FTIR Spectrophotometer (Perkin Elmer, USA).

Drug release: Folic acid -as the model drug- was added to prepared polymeric blends in the concentration of 1mg/mL. Solutions were stirred until the folic acid was evenly suspended in the solution. 5mL of the formulation was injected into a dialysis bag (MWCO: 2kDa) which had been prefilled with 3mL of phosphate buffer solution (pH6.75). The dialysis bag was then immersed in a jar containing 60 mL phosphate buffer solution (pH 6.75) and placed in an orbital shaker bath ($37^\circ\text{C} \pm 0.5^\circ\text{C}$; 25 rpms). 4mL samples were drawn at 0.5 hours, 1.5 hours, 3 hours, 4.5 hours, 6 hours, 24 hours, 36 hours and 60 hours and replaced with prewarmed buffer to maintain sink conditions. Drug content was assayed by UV spectroscopy at 280nm.

Results:

Textural analysis: The CaCl_2 containing gels were harder and the area under the curve for these gels were greater than those of PMVE gels without any additives. The addition of Gum Arabic to the CaCl_2 containing gels resulted in gels that were weaker.

FTIR: The profiles for the combinations of polymers showed no interaction between either the polymers, or polymer-salt or polymers-drug combinations.

Drug Release: Sustained release of folic acid was achieved from all the formulations. The PMVE-CaCl₂ gels released drug more slowly than the PMVE gels and release was slowed even further with the addition of Gum Arabic. Less than 20% of drug was released from the PMVE-CaCl₂ and PMVE-CaCl₂-GumArabic implants after 20 hours and approximately 35% of drug had been released from the PMVE gels at the same time point.

Conclusions:

The addition of CaCl₂ to PMVE formed gels that were harder and released drug more slowly than the PMVE gels with no additives. The addition of Gum Arabic to CaCl₂-PMVE gels retarded drug release further but the gels formed were weaker indicating that the hardness of the gels does not correlate with drug release from the implant.

APPENDIX B (III)

An Injectable *In Situ* Forming Implant for the Treatment of Solid Tumors Utilizing a Central Composite Design

Ameena Wadee¹, Vineess Pillay^{*1}, Yahya E. Choonara¹, Lisa C. du Toit¹, Clement Penny², Valence M.K. Ndesendo

¹University of the Witwatersrand, Department of Pharmacy and Pharmacology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

²University of the Witwatersrand, Department of Medical Oncology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

**Corresponding author: vineess.pillay@wits.ac.za*

Purpose:

Poly(methylvinyl ether) (PMVE) is a thermoresponsive polymer which when exposed to an increase in temperature converts from a solution to a gel. This can be exploited in the development of an injectable, *in situ* forming implant (ISFI) for the treatment of solid tumors.

Methods:

A two-factor face-centered central composite design (FCCCD) was employed to obtain an optimal ISFI formulation which was easily injectable, able to gel around body temperature and capable of delivering a chemotherapeutic over prolonged periods of time. PMVE solutions (15-30%^{w/v}) were diluted and CaCl₂ (0.08-0.5M) was added in accordance with 14 ISFI formulations generated by a FCCCD. In order to determine the response of the ISFI to increases in temperature, a Rheometer System was used to expose samples to fixed strain and oscillation (18Pa, 10Hz) while the temperature of the sample was increased. Model drug, folic acid, was added to ISFI formulations at a concentration of 5mg/mL. Drug release studies were performed and samples were drawn every 3 days for 30 days. Drug content was assayed by UV spectroscopy. A Textural Analyzer was utilized to determine the injectability of the ISFI formulations by comparing the maximum force required to depress the plunger of a syringe filled with a formulation to that of a water filled syringe.

Results:

ISFI formulations showed gelation temperatures of below 36°C with the formulation with the highest concentration of CaCl₂(0.5M) showing the lowest gelation temperature 32.1°C (±0.6°C). Prolonged release of folic acid was achieved from all of the ISFI formulations (≤ 45% of the drug over 30 days) with formulations containing higher amounts of salt (0.2-0.5M) showing lower initial burst release profiles. Lower polymer concentrations (15%^{w/v}) in the formulation resulted in easily injectable formulations.

Conclusion:

Utilization of a statistical design of the experiments has assisted in obtaining an optimal implant formulation which possesses the required properties and which may provide sustained release of entrapped chemotherapeutics.

APPENDIX B (IV)

The development of an *in situ* forming implant for the treatment of osteosarcoma: Autoclaving as a sterilization method

A. Wadee¹, V. Pillay^{*1}, Y.E. Choonara¹, L.C. du Toit¹, C. Penny²

¹University of the Witwatersrand, Department of Pharmacy and Pharmacology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

²University of the Witwatersrand, Department of Medical Oncology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

*Correspondence: vineess.pillay@wits.ac.za

Poly(methyl vinyl ether) (PMVE) is a thermoresponsive polymer which at increased temperatures converts from a solution to a gel. This can be exploited in the development of an injectable, *in situ* forming implant for the treatment of osteosarcomas and other solid tumors. A sterilization method which does not alter the gelation properties is required. **MATERIALS AND METHODS:** 1) *Preparation of solutions:* PMVE solutions were diluted to 35%^{w/v} solutions and CaCl₂ (0.5M) was added. Solutions were autoclaved at 121°C for 15mins and compared with unsterilized solutions. 2) *Rheology:* A Haake Modular Advanced Rheometer System (ThermoFisher Scientific, Germany) was used to expose samples to fixed strain and oscillation (18Pa, 10Hz) while the temperature of the sample was increased. 3) *FTIR:* A Fourier Transformed Infrared Spectrophotometer (PerkinElmer, USA) was used to determine the effect of autoclaving on the chemical structure of the polymer. 4) *Drug release:* Model drug, folic acid, was added to polymer solutions in the concentration of 5mg/mL. Drug release studies were carried out and samples were drawn every 3days for 30days. Drug content was assayed by UV spectroscopy. **RESULTS:** The gelation temperature of the autoclaved formulations remained the same at 33.6°C. FTIR revealed no change in the spectra. However, release of folic acid from the autoclaved formulation was slightly faster as 32% of the drug was released after 32 days (unsterilized formulations: 27%). **CONCLUSIONS:** Autoclaving does not appear to have changed the thermogelation properties. Observed differences between the unsterilized polymer and the autoclaved one can be explained by the incorrect making up to volume of the solution following autoclaving.

APPENDIX B (V)

The development of an *in situ* forming implant for the treatment of solid tumors: Determination of the effect of methotrexate release on colon carcinoma cells and *in vivo* studies in the rat model

Ameena Wadee¹, Viness Pillay^{*1}, Yahya E. Choonara¹, Lisa C. du Toit¹ and Clement Penny²

¹University of the Witwatersrand, Department of Pharmacy and Pharmacology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

²University of the Witwatersrand, Department of Medical Oncology, 7 York Road, Parktown, 2193, Johannesburg, South Africa.

***Corresponding author:** viness.pillay@wits.ac.za

Purpose:

An implant which forms at the site of a tumor and releases drug *in situ* provides many benefits. High local concentrations of drug at the site can be achieved as well as a reduction in systemic side-effects. Poly(methylvinyl ether) is a biocompatible, thermoresponsive polymer which converts from a solution to a solid with a change in temperature. As a result the polymer in solution form can be injected at the site of the tumor and will form a solid implant *in situ* due to its response to the increase in temperature. This study sought to determine whether the release of the entrapped chemotherapeutic, methotrexate (MTX) occurred over a month and whether the amount of drug being delivered was sufficient to cause cancer cell death. A pilot study in the non-tumor bearing rat model was also conducted.

Methods:

Preparation of implant formulations: Optimized formulations of poly(methylvinyl ether) (20%^{w/v}, Calcium Chloride: 0.125M) were prepared and loaded with MTX at a concentration of 5mg/mL. Formulations prepared for animals were prepared from autoclaved polymer and drug was added to the formulation under aseptic technique.

Determination of the release of MTX from the implant: Drug release studies were performed and samples were drawn every 3 days for 30 days. Drug content was assayed using UV spectroscopy. Drug elutes collected were also utilized to determine whether MTX was being released in sufficient quantities to inhibit the growth of cells.

Utilization of flow cytometry and an MTT assay for the determination of cell viability: Colon carcinoma cells (HT29) which were used as the model for solid tumors, were grown in Dulbecco's Modified Essential Medium (supplemented with 10% FBS and 1%Pen-Strep) to 75% confluency. Elutes collected from the drug release studies on days 1, 7, 15 and 30 were then added to cells and incubated over 24 hours. Flow cytometry and MTT assays were then conducted on the treated cells to determine cell viability.

Determination of the release of MTX in the rat model: Sprague-Dawley rats (200-250g) were divided into two groups of 25 rats each. Animals were followed over a period of 10 days. Control animals received two intraperitoneal injections (on days 1 and 5) delivering a total dose of MTX 2mg/rat/10 days, whereas the

study group received a once-off implant delivering a dose of 2mg/rat/10 days. Rats were monitored for toxicity throughout the study.

Results and Conclusions:

Release of MTX exceeded 30 days with mean fractional drug release after 30 days of 25% of the entrapped drug. The MTT assay showed that a significant percentage of cells were non-viable after treatment with elutes from the implant. This was confirmed by flow cytometry. Rats in the control group did not develop any significant toxicity as a result of the intraperitoneal injection possibly due to the rapid clearance of the MTX. Rats that received the implant began to develop significant toxicity to MTX after 6 days. This indicated that MTX was being released in sufficient quantities and in a sustained manner and shows promise for utilization of this formulation as an *in situ* forming implantable system delivering chemotherapeutics to the site of solid tumors.

**Ethics approval for *in vivo* studies obtained from WITS Animal Ethics Screening Committee, AESC Number: 2009/37/05.

APPENDIX C

ANIMAL ETHICS CLEARANCE

CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2009/37/05

APPLICANT: Ms A Wadee

SCHOOL: Pharmacy and Pharmacology

DEPARTMENT:

LOCATION:

PROJECT TITLE: "A prolonged release in situ reconfiguring device for the delivery of a chemotherapeutic drug to tumour bearing mice"

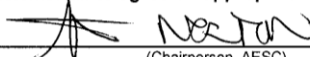
Number and Species

120 200-250g Nu/nu Mice

Approval was given for the use of animals for the project described above at an AESC meeting held on 27.10.09. This approval remains valid until 27.10.2011

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 to the following additional conditions:

- Approval is conditional to approval being provided by the appropriate committee for the "use of biohazardous materials" (form IBC1).
- Biochemistry is to be performed on blood samples from mice to ensure that methotrexate does not result in side effects including myelosuppression, hepatitis, a nephropathy or significant bleeding. This should be discussed with the CAS veterinarian to agree on appropriate tests.

Signed: 
(Chairperson, AESC)

Date: 16/11/2009

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: 16/11/2009

cc: Supervisor:
Director: CAS

Works 2000/In0015/AESCcert.wps

MODIFICATIONS TO STUDY: CLEARANCE

Which AESC 2009

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

- a. Name: Ameena Wadee.....
- b. Department: Pharmacy and Pharmacology.....
- c. Experiment to be modified / extended AESC NO:

Original AESC number	2009	37	05
Other M&E's :			

- d. Project Title: Original Title: A Prolonged release in situ reconfiguring device for the delivery of a chemotherapeutic drug to tumour bearing mice
New Title: An *in situ* reconfiguring device for the pulsatile delivery of a chemotherapeutic drug in rats

e.	Number and species of animals originally approved :	120	Nude Mice (<i>nu/nu</i>)
f.	Number of additional animals previously allocated on M&Es :		
g.	Total number of animals allocated to the experiment to date:		
h.	Number of animals used to date:		
i.	Specific modification / extension requested: - Nude mice (120 animals) model will be changed to MF1 mice (15 animals for add-on pilot study) and Sprague-Dawley rats (179 animals for full study) - Tumours will no longer be induced in the animals, instead healthy animals will be used (<i>continued on next page</i>) - Fifteen (15) mice are required initially for an add-on pilot study which implicates training on a new ultrasound microimaging system (see additional details at end of form under 'details of study'). Should this technique prove successful in mice, the ultrasound imaging will be applied to the rats in the full study.		
j.	Motivation for modification / extension: - Nude mice could not be obtained in sufficient numbers. - IBC clearance for the growth of tumours in mice was not obtained. - In order to minimise the number of animals being used, the study will be conducted over 10 days rather than 30. - For the add-on pilot study, the mice will be injected with the delivery system and visualised employing a novel ultrasound microimaging technology awarded through the 2009 NRF National Equipment Programme (Strategic Research Infrastructure Programme).		

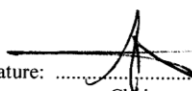
Date: 27/10/2010.....

Signature: Hardcopy signed.....

RECOMMENDATIONS:

Approved subject to conditions
listed below

Date: 04/02/2011.

Signature:  N. N. N.
Chairman, AESC

i. (continued)

Details of Study

Add-on Pilot Study MF1 Mice

- The pilot study will be conducted over 5 days. Three (3) mice will be subjected to microimaging daily.
- A high-frequency microultrasound imaging system (Vevo 2100[®], VisualSonics, Toronto, Canada) designed specifically for imaging small animals will be used in this study. This equipment was awarded through the 2009 NRF National Equipment Programme (Strategic Research Infrastructure Programme).
- Contrast mode images will then be acquired.
- Mice will be anaesthetised in a chamber with 2% isoflurane gas in oxygen. Body temperature will be maintained at 37°C using a heat pad. Mice are then placed supine on the imaging stage. Their fore- and hindlegs will be extended, placed on electrode gel and taped to the stage to monitor respiration (and minimize respiration motion), heart rate, ECG and temperature.
- The drug delivery system (~200µL), comprising a drug-free suspension, will be intravenously injected via the tail vein or subcutaneously injected e.g. in the flank of the mouse.
- The distribution of the nanoparticulate delivery system will be visualized in diverse organs in the mouse body (e.g. liver, stomach, lungs, brain).
- The mice will remain anaesthetized for a period of up to one hour.
- Anaesthesia is then reversed and the mice returned to the stock.
- Should this technique prove successful in mice, the ultrasound imaging will be applied to the rats in the full study, entitled: 'An *in situ* reconfiguring device for the pulsatile delivery of a chemotherapeutic drug in rats' for *in vivo* imaging of movement of nanoparticles for assessment of drug release, from the device.

Full Study in Sprague-Dawley Rats

- The study will be conducted over 10 days rather than 30 days
- In order to determine the release of the entrapped drug in response to an externally applied stimulus from the subcutaneously injected implant, an ice pack will be applied to the area of injection of the implant for 2 or 5 minutes (as described in attached document) on Day 2, 4, 6, 8 and 10 of the study and blood samples will be taken thereafter. This will provide the information required to assess the release of methotrexate from the subcutaneously injected implant. (Please see the attached document for further details on the design of the study)

- If further deaths occur during the imaging procedures that these procedures need to be reassessed before they continue
- The investigator should perform procedures in rats anaesthetized with ketamine and xylazine
- All procedures need to be monitored by CTS for untoward outcomes.
- The size of the ice-pack that will be used needs to be specified
- The body temperature is measured during the ice-pack application.

 N. N. N.