



<http://researchspace.auckland.ac.nz>

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.

<http://researchspace.auckland.ac.nz/feedback>

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.

Ocular Delivery of Antisense
Oligonucleotides using Colloidal Carriers:
Improving the wound repair after corneal surgery

Ilva Dana Rupenthal

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Auckland

New Zealand

February 2008

Abstract

Background and Aim: Clinical outcomes of refractive surgeries are variable due to individual wound healing responses, but might be improved by effective delivery of anti-inflammatory agents. Knockdown of connexin proteins, using an antisense oligonucleotide (AsON) approach, has been shown to significantly reduce the inflammatory response and increase the rate of wound closure after corneal laser surgery. The challenge remains to find delivery systems that are easy to apply, but can still effectively deliver the AsONs to the target site. This thesis aimed to evaluate the efficacy of several *in-situ* gelling formulations to deliver Cx43 AsONs to the wounded tissues of a rat corneal scrape wound model.

Methods: Formulations were characterised in terms of their rheological behaviour, microstructure and spreading ability. They were then evaluated for their irritation potential, precorneal retention and ability to control the release of the model hydrophilic drug pilocarpine hydrochloride both *in vitro* and *in vivo*. The stability of the AsONs in these formulations was assessed using Fluorescence Resonance Energy Transfer. Finally, formulations containing the stable AsONs were applied to a rat corneal scrape wound model and penetration depth, wound size after 12 hours and cellular dynamics underlying the wound healing response were analysed.

Results and Discussion: Systems based on gellan gum, xanthan gum, carrageenan and alginate underwent sol-to-gel phase transition upon addition of the cations present in tear fluid. All tested systems exhibited favourable contact angles and were found to be non-irritant. Systems based on gellan gum, xanthan gum and carrageenan showed the longest ocular retention and exhibited the slowest release characteristics both *in vitro* and *in vivo*. AsONs were found to be stable in all formulations apart from the chitosan system, where precipitation occurred. This formulation also exhibited the slowest wound healing rate due to induction of a pro-inflammatory response. Conversely, delivery of the AsONs by gellan gum and carrageenan formulations resulted in significant reduction in wound size, inflammatory response and Cx43 levels.

Conclusion: *In-situ* gelling systems based on gellan gum and carrageenan are able to successfully deliver Cx43 AsONs to the wounded tissues and therefore improve the healing response after corneal surgery.

Dedication

To my mum, my dad and my beloved sister

Acknowledgements

First of all, I would like to thank my supervisors, **Dr. Raid Alany** and **Professor Colin Green**, for their guidance, support and continuous encouragement, especially during the last couple of months. Without your valuable input, this work would have never been completed. Not only have you supported me in completing this PhD, you were my friends and never complained when I asked for extra time off after conferences to go and explore the world. You have made this last four years really enjoyable for me.

Special thanks to my flatmate and good friend **Laura**, who has spent countless hours proofreading this thesis. Without her help, this work would have never reached the current standards.

A big thank you also goes to **Jacqui Ross** and **Hilary Holloway** from the Biomedical Imaging Research Unit, who helped me with setting up the FRET experiments and taught me everything I have ever needed to know about microscopes.

Thanks to **Professor Thomas Rades** and his group for letting me use all the equipment during my stay in Dunedin. Special thanks also to **Liz Girvan**, who helped me with the Cryo FESEM imaging.

My sincere thanks also go to **Dr. Ibrahim Hassan** and **Dr. Michael Rutland** for sacrificing extra hours after work to help with the gamma scintigraphy study. Without their support, the precorneal retention study would have been impossible.

Special thanks also to my former colleagues and friends from the **R&E Department at Kettenbach**, who let me use their brand new Krüss Drop Shape Analyser to perform the contact angle measurements.

I would also like to thank **Linley Nisbet** and **Justine Stewart** for their support during the animal studies, **Thilini** for the countless hours she spent helping me with sectioning rat eyes, **Judy, James, Darren, Andreas, Stephanie, Friederike** and **Sebastian** for helping me with little parts of this study, and my former 'cage-mates' **Ines, Sri, Puneet** and **Hemant** for their moral support and encouragement.

Special thanks also to **Kaa-Sandra, Ally, Jie, Narme, Taras** and all the other people from the Department of Ophthalmology. Thanks for helping me out when I got lost in the lab. I had a great time up on 4th floor and really enjoyed my window seat.

I would also like to thank the University of Auckland for awarding me an International Doctoral Scholarship and financially supporting me to attend international conferences. Thanks also to Education New Zealand for the New Zealand Postgraduate Study Abroad Award, which enabled me to attend the FRET workshop in Virginia last year. Further thanks go to the New Zealand Federation of Graduate Women for awarding me the Harriette Jenkins Award to perform a short-term research project at the University of Otago. Thanks also to the Sir John Logan Campbell Trust, the Maurice & Phyllis Paykel Trust and the School of Pharmacy for their financial support, which allowed me to attend many, many conferences throughout my PhD.

Personal thanks go to my friends **Silke, Anja** and **Sushila**, who were always there for me when I needed someone to talk to. Thanks for believing in me, when I was ready to give up. Special thanks also to **Julia** for the countless conversations on Skype in the middle of the night. It really helped to have a friend in the same situation. Thanks also to my physiotherapist **Bryce** for helping release the constant tension in my shoulders.

My special thanks go to **Simon** for his constant encouragement and support. Without his food deliveries, cheer-up text messages and the little breaks every night, I would not be where I am today. Thanks for being your wonderful self.

My closing thanks go to my **mum**, my **dad** and my beloved little **sister**. Although 20,000 kilometres away they were always there for me through thick and thin. **Mum** you are the strongest woman I have ever known and I admire you for everything you have accomplished in your life. **Dad** you are the most creative cabinet maker in the world and I can only hope that I have inherited some of your creativity. **Anna**, my little sister, you are simply the best! Thank you very much for believing in me. I know it has been hard having your daughter/sister living at the other end of the world.

Finally, I would like to thank **my body**, for hanging in there during the last couple of weeks, while I thought I was collapsing. It has been a really stressful time with hardly any sleep and tons of chocolate. Thanks for not giving up on me. I promise I will make it up to you soon!

Publications arising from this thesis

Book Chapter

- **Rupenthal ID**, Alany RG. *Ocular Drug Delivery*. Invited book chapter to S. C. Gad (Ed.), *Pharmaceutical Manufacturing Handbook: Production and Processes* (pp. 729-767). New York: John Wiley & Sons, Inc., 2008

Refereed Journal Articles

- Franke S, Malcolmson C, Bunt CR, **Rupenthal ID**, Alany, RG. *Imaging techniques and their role in dosage form design and drug delivery research*. Current Pharmaceutical Analysis (Manuscript accepted)
- **Rupenthal ID**, Alany RG. *Fluorescence Resonance Energy Transfer – A New Tool to Quantify the Stability of Antisense Oligonucleotides*. Controlled Release Society Newsletter, 24(2), p.12-14, June 2007

Refereed Journal Articles (in preparation)

- **Rupenthal ID**, Green CR, Rades T, Alany RG. *Phase diagrams, rheological and microstructural characteristics of in-situ gelling systems for ocular use* (Manuscript in preparation)
- **Rupenthal ID**, Green CR, Alany RG. *Pre-corneal retention, in vitro and in vivo release characteristics of ion-activated in-situ gelling systems* (Manuscript in preparation)
- **Rupenthal ID**, Green CR, Alany RG. *Evaluation of various Fluorescence Resonance Energy Transfer approaches as a tool to quantify the stability of antisense oligonucleotides* (Manuscript in preparation)
- **Rupenthal ID**, Green CR, Alany RG. *Improved corneal wound healing after refractive surgery – delivery of anti-Cx43 oligonucleotides to the wounded rat cornea by in-situ gelling systems* (Manuscript in preparation)

Conference Abstracts

- **Rupenthal ID**, Green CR, Hassan IM, Rutland M, Alany RG. *Investigating the precorneal residence time of ophthalmic in-situ gelling systems using gamma scintigraphy* (35th Annual Meeting and Exposition of the Controlled Release Society, New York City, New York, USA, July 2008; podium)
- **Rupenthal ID**, Green CR, Alany RG. *Antisense oligonucleotide loaded in-situ gelling systems for improvement of the corneal wound healing after refractive surgery* (35th Annual Meeting and Exposition of the Controlled Release Society, New York City, New York, USA, July 2008; poster)

- **Rupenthal ID**, Hassan IM, Rutland M, Alany RG. *Gamma scintigraphy to monitor ocular drainage of in-situ gelling delivery systems* (Annual Scientific Meeting of the Australian and New Zealand Society of Nuclear Medicine, Auckland, New Zealand, October 2007; podium)
- **Rupenthal ID**, Green CR, Rades T, Alany RG. *Rheological and micro-structural characteristics of in-situ gelling systems for ocular drug delivery* (34th Annual Meeting and Exposition of the Controlled Release Society, Long Beach, California, USA, July 2007; podium)
- **Rupenthal ID**, Green CR, Alany RG. *Evaluation of FRET Sensitized Emission and Acceptor Bleaching to monitor the stability of antisense oligonucleotides in vitro* (Annual Conference of the Australasian Pharmaceutical Science Association, Adelaide, Australia, December 2006; podium)
- **Rupenthal ID**, Green CR, Rades T, Alany RG. *Phase behaviour and characterization of in-situ gelling systems for corneal delivery* (Annual Conference of the Australasian Pharmaceutical Science Association, Adelaide, Australia, December 2006; podium)
- **Rupenthal ID**, Green CR, Alany RG. *Different FRET applications as tool to monitor the stability of antisense oligonucleotides for ocular use* (Annual Meeting of the Australasian Society of Clinical & Experimental Pharmacologists & Toxicologists, Auckland, New Zealand, August 2006; poster)
- **Rupenthal ID**, Green CR, Alany RG. *In vitro and in vivo evaluation of various in-situ-gelling systems for ocular drug delivery* (33rd Annual Meeting and Exposition of the Controlled Release Society, Vienna, Austria, July 2006; poster)
- **Rupenthal ID**, Green CR, Alany RG. *FRET applications as a tool to monitor the stability of antisense oligonucleotides in vitro* (33rd Annual Meeting and Exposition of the Controlled Release Society, Vienna, Austria, July 2006; podium)
- **Rupenthal ID**, Green CR, Alany RG. *Using different FRET applications as a tool to determine the stability of antisense oligonucleotides in ocular delivery systems* (8th Conference on Formulation and Delivery of Bioactives, Dunedin, New Zealand, February 2006; podium; **Winner of prize for best oral student presentation**)
- Alany RG, **Rupenthal ID**, Green CR. *Corneal Delivery and Biological Effect of Antisense Oligonucleotides using Water-in-oil Microemulsions* (32nd Annual Meeting and Exposition of the Controlled Release Society, Miami Beach, Florida, USA, June 2005; poster)
- **Rupenthal ID**, Green CR, Alany RG. *Stability and Ocular Delivery of Antisense Oligonucleotides using Water-in-oil Microemulsions* (7th Conference on Formulation and Delivery of Bioactives, Dunedin, New Zealand, February 2005; podium)
- **Rupenthal ID**, Green CR, Alany RG. *Ocular Delivery of Antisense Oligonucleotides using Colloidal Carriers* (22nd Conference on Microscopy, Dunedin, New Zealand, February 2005; podium; **Recipient of the Young Microscopist Sponsorship**)
- Alany RG, **Rupenthal ID**, Green CR. *Oligonucleotide Stability in Colloidal Carriers: Assessment using Fluorescence Resonance Energy Transfer combined with Confocal Laser Scanning Microscopy* (Annual Conference of the Australasian Pharmaceutical Science Association, Melbourne, Australia, December 2004; podium)

Table of Contents

ABSTRACT.....	II
DEDICATION	III
ACKNOWLEDGEMENTS	IV
PUBLICATIONS ARISING FROM THIS THESIS.....	VI
TABLE OF CONTENTS	VIII
LIST OF FIGURES.....	XII
LIST OF TABLES.....	XVII
LIST OF ABBREVIATIONS	XIX
CHAPTER ONE INTRODUCTION	1
1.1 CHALLENGES IN OCULAR DRUG DELIVERY	1
1.1.1 Anatomical and physiological considerations	2
1.1.2 Pharmacokinetic considerations	7
1.1.3 Formulation considerations	10
1.2 FORMULATION APPROACHES TO IMPROVE OCULAR BIOAVAILABILITY	12
1.2.1 Conventional dosage forms	17
1.2.2 Polymeric delivery systems.....	18
1.2.3 Colloidal delivery systems	22
1.2.4 Other delivery approaches.....	26
1.3 CORNEAL WOUNDING AND WOUND HEALING	31
1.3.1 Photorefractive keratectomy and laser in situ keratomileusis	31
1.3.2 Corneal repair mechanisms following wounding.....	33
1.3.3 Molecular therapies in ocular wound healing	37
1.4 CONNEXINS, CONNEXONS AND GAP JUNCTION CHANNELS.....	38
1.4.1 Molecular structure of the gap junction channel	38
1.4.2 Functional diversity and regulation of connexin gene expression	41
1.4.3 Effects of connexin mutations and connexin gene knockouts.....	43
1.4.4 Role of gap junctional cellular communication during injury.....	44
1.4.5 Connexin expression in the cornea before and after wounding	46

1.5	THE ANTISENSE APPROACH	49
1.5.1	Mechanisms of action – oligonucleotides, ribozymes and siRNAs	51
1.5.2	Antisense oligonucleotides – design and chemical modifications	53
1.5.3	Antisense technology – a tool to improve corneal wound healing.....	56
1.5.4	Delivery systems for nucleic acids – cellular uptake and stability.....	57
1.6	OBJECTIVES OF THIS THESIS	60

CHAPTER TWO IN-SITU GELLING SYSTEMS: FORMULATION AND PHYSICO-CHEMICAL CHARACTERISATION..... 61

2.1	INTRODUCTION.....	61
2.2	MATERIALS.....	68
2.3	METHODS.....	69
2.3.1	Effect of ions on phase behaviour – partial ternary phase diagrams	69
2.3.2	Rheological characterisation – rotation and oscillation viscometry.....	70
2.3.3	Spreading ability – determination of the contact angle	71
2.3.4	Microstructure – Cryo field emission scanning electron microscopy	73
2.4	RESULTS	73
2.4.1	Effect of ions on phase behaviour – partial ternary phase diagrams	73
2.4.2	Rheological characterisation – rotation and oscillation viscometry.....	77
2.4.3	Spreading ability – determination of the contact angle	82
2.4.4	Microstructure – Cryo field emission scanning electron microscopy	83
2.5	DISCUSSION.....	86

CHAPTER THREE IN-SITU GELLING SYSTEMS: EVALUATION AS VEHICLES FOR OCULAR DRUG DELIVERY 95

3.1	INTRODUCTION.....	95
3.2	MATERIALS.....	101
3.3	METHODS.....	101
3.3.1	Evaluation of ocular irritation using a modified HET-CAM test.....	101
3.3.2	Precorneal clearance study using gamma scintigraphy	103
3.3.3	In vitro release of a model hydrophilic drug	105
3.3.4	In vivo pharmacodynamic study on New Zealand Albino rabbits.....	106

3.4	RESULTS	108
3.4.1	Evaluation of ocular irritation using a modified HET-CAM test.....	108
3.4.2	Precorneal clearance study using gamma scintigraphy	112
3.4.3	In vitro release of a model hydrophilic drug	115
3.4.4	In vivo pharmacodynamic study on New Zealand Albino rabbits.....	117
3.5	DISCUSSION.....	119

CHAPTER FOUR STABILITY OF ANTISENSE OLIGONUCLEOTIDES IN OCULAR DELIVERY SYSTEMS USING FRET 130

4.1	INTRODUCTION.....	130
4.2	MATERIALS.....	138
4.3	METHODS.....	139
4.3.1	pH-dependent fluorescence of FAM and TAMRA	139
4.3.2	Spectral scans to assess FRET.....	139
4.3.3	Acceptor Photobleaching FRET.....	140
4.3.4	Sensitized Emission FRET	141
4.4	RESULTS	143
4.4.1	pH-dependent fluorescence of FAM and TAMRA	143
4.4.2	Spectral scans to assess FRET.....	144
4.4.3	Acceptor Photobleaching FRET.....	147
4.4.4	Sensitized Emission FRET	148
4.5	DISCUSSION.....	151

CHAPTER FIVE IMPROVED CORNEAL WOUND HEALING IN THE RAT CORNEA: TREATMENT WITH CX43 ANTISENSE OLIGONUCLEOTIDES 159

5.1	INTRODUCTION.....	159
5.2	MATERIALS.....	162
5.3	METHODS.....	163
5.3.1	Corneal wounding procedure and treatment with Cx43 AsONs.....	163
5.3.2	Penetration study using Confocal Laser Scanning Microscopy.....	164
5.3.3	Macroscopic appearance of the wound area using fluorescein staining.....	165
5.3.4	Tissue collection and processing.....	166

5.3.5	Histology documentation of the corneal wound healing dynamics.....	166
5.3.6	Immunolabelling of Cx43 protein channels	167
5.4	RESULTS	168
5.4.1	Corneal wounding procedure and treatment with Cx43 AsONs	168
5.4.2	Penetration study using Confocal Laser Scanning Microscopy	169
5.4.3	Macroscopic appearance of the wound area using fluorescein staining.....	171
5.4.4	Histology documentation of the corneal wound healing dynamics.....	172
5.4.5	Immunolabelling of Cx43 protein channels	175
5.5	DISCUSSION.....	183
CHAPTER SIX GENERAL DISCUSSION, FUTURE DIRECTIONS AND		
FINAL CONCLUSION.....		189
6.1	GENERAL DISCUSSION	189
6.2	FUTURE DIRECTIONS	194
6.3	FINAL CONCLUSION.....	196
REFERENCES.....		197

List of Figures

CHAPTER ONE

Figure 1.1 Schematic cross section of the human eye and cornea.....	2
Figure 1.2 Structure of the precorneal tear film.....	3
Figure 1.3 Schematic illustration of the nasolacrimal drainage system.....	4
Figure 1.4 Histology of the adult rat cornea with emphasis on the central corneal epithelium	5
Figure 1.5 Schematic diagram of ocular absorption	8
Figure 1.6 Surgical techniques of PRK and LASIK	32
Figure 1.7 The corneal wound healing cascade	34
Figure 1.8 Molecular structure of a gap-junctional plaque.....	38
Figure 1.9 The 'x, y, z hypothesis'.....	48
Figure 1.10 ISIS [®] Product pipeline.....	50
Figure 1.11 Main mechanisms of antisense oligonucleotides	51
Figure 1.12 Sites for chemical modification of oligonucleotides	53
Figure 1.13 Chemical modifications of oligonucleotides	55
Figure 1.14 Proposed intracellular pathway of non-pegylated and pegylated lipoplexes	59

CHAPTER TWO

Figure 2.1 Chemical structures of gellan gum, xanthan gum, carrageenan and alginate	64
Figure 2.2 Chemical structures of HPMC, carbopol and chitosan	66
Figure 2.3 Gibb's triangle representing a three-component system.....	69
Figure 2.4 Contact angle formation on a solid surface by Young	71
Figure 2.5 Krüss Drop Shape Analyser setup with temperature and humidity controlled measuring chamber and examples of good and poor wetting of a liquid drop on a glass slide.....	72
Figure 2.6 Partial ternary phase diagrams showing the effect of K ⁺ and Ca ²⁺ on the sol- to-gel transition of gellan gum, xanthan gum, carrageenan and alginate.....	74

Figure 2.7 Representative polarised light micrographs showing birefringence and coloured textures of a 5% xanthan gum formulation, while exhibiting only faint lamellar textures for a 1% xanthan gum system.....	76
Figure 2.8 Rheogram showing near Newtonian flow for all selected formulations	78
Figure 2.9 Flow indices for a 1% (w/w) carrageenan formulation with different amounts of K^+ and Ca^{2+}	79
Figure 2.10 Dynamic stress sweep measurements for the different polymer systems showing the magnitude of G' and G''	80
Figure 2.11 Cryo FESEM micrographs for each polymer system at two different magnifications	83
Figure 2.12 Idealised structures for κ -, ι - and λ -carrageenan	87
Figure 2.13 Typical frequency sweeps for an entangled polymer network and a network of secondary bonds	92

CHAPTER THREE

Figure 3.1 Typical scintigraphy image with five regions of interest	100
Figure 3.2 Vascular responses used to score the test formulations.....	102
Figure 3.3 Set-up for precorneal clearance study	104
Figure 3.4 Schematic diagram of a standard Franz diffusion cell	105
Figure 3.5 Digital camera image showing the miotic response being measured with the ruler serving as a calibration scale.....	107
Figure 3.6 Viable and dead embryos during various developmental stages.....	108
Figure 3.7 Vascular responses after application of the various formulations at 0.5, 2 and 5 min	109
Figure 3.8 Protective effect of the chitosan formulation after application of 70% isopropyl alcohol across the whole CAM	112
Figure 3.9 Representative results for the ocular drainage of an aqueous solution with sequential pictures and their overlay as well as the curves for the radioactivity remaining over time.....	113
Figure 3.10 Precorneal drainage of ^{99m}Tc -DTPA incorporated into the various formulations.....	113
Figure 3.11 Cumulative amounts of PHCl released versus time	115
Figure 3.12 Cumulative amounts of PHCl released versus square root of time	117

Figure 3.13 Miotic response profiles for all formulations displayed as change in pupil diameter versus time curves	118
Figure 3.14 Radio-image of the whole rabbit showing the presence of radioactivity in the stomach 20 min after instillation of an aqueous solution containing 4 MBq of ^{99m} Tc-DTPA	121
Figure 3.15 Molecular structure of DTPA (Diethylene Triamine Pentaacetic Acid)..	124

CHAPTER FOUR

Figure 4.1 Simplified Jablonski diagram illustrating the non-radiative energy transfer between the donor emission and the acceptor absorbance	132
Figure 4.2 Exponential relationship between the energy transfer efficiency (E) and the distance (R) separating donor and acceptor.....	133
Figure 4.3 Relative orientation of donor and acceptor transition dipole moments.....	134
Figure 4.4 Molecular structures of FAM and TAMRA with the circles emphasizing the structural differences between the two fluorophores resulting in their different excitation and emission wavelengths	136
Figure 4.5 Principles of FRET and spectra of an intact and a degraded antisense oligonucleotide	137
Figure 4.6 Spectral profiles for the donor (FAM) and the acceptor (TAMRA) including quenched donor and sensitized acceptor emission profiles.....	137
Figure 4.7 Schematic example of the pre- and post-bleach images obtained for the donor and the acceptor fluorophore excited at 488 nm and 561 nm respectively .	140
Figure 4.8 Spectral profiles for FAM and TAMRA illustrating the variables of Equation 4.5.....	141
Figure 4.9 Fluorescence intensity versus pH profiles of FAM and TAMRA probes excited at 488 nm and 561 nm respectively	143
Figure 4.10 Wavelength scans of the FAM and TAMRA probes (2 μM) excited at 488 nm with the fluorescence intensity plotted against the wavelength in nm.....	144
Figure 4.11 Wavelength scans for the physical mixtures of the single tagged AsONs at a ratio of 1:1 and the double tagged AsON	145
Figure 4.12 Wavelength scans confirming the breakdown, and therefore the loss of FRET, upon addition of DNase I to the double tagged AsON incorporated into PBS	147

Figure 4.13 Wavelength scans confirming the FRET efficiencies measured by the FRET SE approach.....	148
Figure 4.14 Percentage FRET efficiency remaining for the FAM-TAMRA tagged AsON incorporated into the previously discussed <i>in-situ</i> gelling systems over a period of a week	150
Figure 4.15 Principal set-up of confocal microscopy	158

CHAPTER FIVE

Figure 5.1 Wounding procedure showing the tilted rat head with the tip of the dermal biopsy punch pointing towards the eye and the demarcated wound area after removal of the corneal epithelium.....	163
Figure 5.2 Fluorescein staining showing the wound area of an untreated and a Cx43 AsON treated eye 12 h after corneal surgery	165
Figure 5.3 3D-reconstruction of the optical slices obtained by CLSM showing the penetration depth of the fluorescently tagged AsONs into the corneal tissues	169
Figure 5.4 Frontal view of the z-stack demonstrating the measurement of the penetration depths of the Cy3-tagged AsONs at the wound site and along the epithelium adjacent to the wound edge	170
Figure 5.5 Wound areas of untreated and Cx43 AsON treated eyes for the various formulations 12 h after mechanical scrape wounding.....	171
Figure 5.6 Histological documentation of normal stromal thickness and keratocyte distribution in an unwounded cornea and a wounded cornea immediately after surgery.....	172
Figure 5.7 Histological documentation of the corneal healing dynamics 12 h after scrape wounding.....	173
Figure 5.8 Histological documentation of the corneal healing dynamics 24 h after scrape wounding.....	174
Figure 5.9 Cx43 protein expression in the unwounded cornea and the wounded cornea immediately after surgery.....	176
Figure 5.10 Cx43 protein expression in control and AsON treated corneas for gellan gum and carrageenan 8 h after surgery.....	178
Figure 5.11 Cx43 protein expression in control and AsON treated corneas for chitosan and PBS 8 h after surgery.....	179

Figure 5.12 Cx43 protein expression in control and AsON treated corneas for gellan gum and carrageenan 12 h after surgery.....	180
Figure 5.13 Cx43 protein expression in control and AsON treated corneas for chitosan and PBS 12 h after surgery	181
Figure 5.14 Cx43 protein expression in the untreated wound and the AsON treated corneas for all tested formulations 24 h after surgery	182

List of Tables

CHAPTER ONE

Table 1.1 Comparison of pharmacokinetic factors between the rabbit and the human eye.....	9
Table 1.2 Summary of conventional and novel drug delivery approaches	13
Table 1.3 Marketed ophthalmic delivery systems based on recent formulation approaches	16
Table 1.4 Human and homologous mouse connexins with representative tissues and cell types for mouse connexin family members.....	39
Table 1.5 Compatibilities of mammalian connexins.....	40

CHAPTER TWO

Table 2.1 Composition of simulated lacrimal fluid (SLF).....	63
Table 2.2 Composition of formulations selected for further investigations and viscosities measured for formulations containing 1% (w/w) of the model hydrophilic drug pilocarpine hydrochloride (PHCl)	77
Table 2.3 Values for the loss tangent ($\tan \delta$) at the beginning and the end of the measurement as well as the shear stress applied to achieve a cross-over of G' and G'' ($\tan \delta = 1$).....	81
Table 2.4 Results for the contact angle measurements	83
Table 2.5 Levels of cations in a Gelrite [®] sample	89

CHAPTER THREE

Table 3.1 Extra embryonic circulatory systems of the chicken egg utilised for ocular irritation testing	97
Table 3.2 Numerical time-dependent scores for each of the three irritant responses ..	103
Table 3.3 Classification of the cumulative scores in terms of their irritation potential	103
Table 3.4 Area under the curve ($AUC_{0-15\text{min}}$) and percentage activity remaining after 15 min ($a_{15\text{min}}$).....	114

Table 3.5 Tukey's pairwise comparison of the area under the curve ($AUC_{0-15min}$) and the activity remaining after 15 min (a_{15min})	114
Table 3.6 Area under the 'percentage-drug-released-versus-time' curve (AUC_{0-12h}) and percentages PHCl released after 2 h ($PHCl_{2h}$) and 12 h ($PHCl_{12h}$)	115
Table 3.7 Tukey's pairwise comparison of the area under the curve (AUC_{0-12h}) and the percentage PHCl released after 2 h ($PHCl_{2h}$) and 12 h ($PHCl_{12h}$).....	116
Table 3.8 Rate constants (k) for the release of PHCl from the different formulations according to the Higuchi model	117
Table 3.9 Area under the 'change-in-pupil-diameter-versus-time' curve ($AUC_{0-180min}$), time required to achieve peak miotic response (t_{max}) and miotic response at 120 min after administration ($action_{120}$)	118
Table 3.10 Tukey's pairwise comparison of the area under the curve ($AUC_{0-180min}$), the time required to achieve peak miotic response (t_{max}) and the miotic response at 120 min after administration ($action_{120}$).....	119

CHAPTER FOUR

Table 4.1 Fluorescence intensities and red-to-green ratios.....	146
Table 4.2 pH of the <i>in-situ</i> gelling formulations prepared in PBS before addition of the AsON probes	148
Table 4.3 FRET efficiencies for the FAM-TAMRA tagged AsON incorporated into the various <i>in-situ</i> gelling systems over a period of a week.....	149
Table 4.4 pH of the formulations and FRET efficiencies of the incorporated FAM-TAMRA tagged AsONs after a week of storage at 4 °C.....	150
Table 4.5 Control and FRET Images required for FRET data analysis using the algorithm proposed by Chen <i>et al.</i>	157

CHAPTER FIVE

Table 5.1 Number of eyes used for each investigation method at different time points after corneal surgery	168
Table 5.2 Penetration depths of the Cy3-tagged AsONs incorporated into the various systems at the wound site and along the epithelium at the wound edge	170

List of Abbreviations

A	Adenine
ANOVA	Analysis of variance
AP	Acceptor Photobleaching
ASBT	Acceptor spectral bleed-through
AsON(s)	Antisense oligonucleotide(s)
AUC	Area under the curve
C	Cytosine
°C	Degrees Celsius
Ca ²⁺	Calcium ions
CaCl ₂	Calcium chloride
CLSM	Confocal Laser Scanning Microscope
Cx	Connexin(s)
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DSBT	Donor spectral bleed-through
FAM	5-carboxyfluorescein
FESEM	Field emission scanning electron microscopy

FRET	Fluorescence Resonance Energy Transfer
G	Guanine
G'	Storage modulus
G''	Loss modulus
Gd-EDTA	Gadolinium Ethylene diamine tetraacetic acid
GJIC	Gap junctional intercellular communication
h	Hour(s)
H&E	Haematoxylin & Eosin
HET-CAM	Hen's egg chorioallantoic membrane test
HPMC	Hydroxypropyl methylcellulose
ι	Iota
κ	Kappa
K ⁺	Potassium ions
KCl	Potassium chloride
λ	Lambda
LASIK	Laser <i>in situ</i> keratomileusis
min	Minute(s)
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
μm	Micrometers
μM	Micromolar

N	Flow index
nm	Nanometers
NaOH	Sodium hydroxide
O.C.T.	Optimal cutting temperature
Pa	Pascal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHCl	Pilocarpine hydrochloride
PRK	Photorefractive keratectomy
R_0	Förster distance
RNA	Ribonucleic acid
RNase H	Ribonuclease H
ROI	Region of interest
SD	Standard deviation
SE	Sensitized Emission, Standard error
SLF	Simulated lacrimal fluid
T	Thymidine
Tc-DTPA	Technetium diethyl triamine pentaacetic acid
TAMRA	5-carboxytetramethyl-rhodamine
w/v	Weight per volume
w/w	Weight per weight