Ocular Delivery of Antisense Oligonucleotides using Colloidal Carriers:

Improving the wound repair after corneal surgery

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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

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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)

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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
ºC	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 *in situ* 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-rhodamie
w/v	Weight per volume
w/w	Weight per weight