Aston University

Some pages of this thesis may have been removed for copyright restrictions.

If you have discovered material in AURA which is unlawful e.g. breaches copyright, (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please read our Takedown Policy and contact the service immediately

The Tear Film and Contact Lens Wear

Helena Catherine Peach

Doctor of Philosophy

Aston University

April 2003

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and no quotation from the thesis and no information derived from it may be published without proper acknowledgement

Aston University

The Tear Film and Contact Lens Wear

Helena Catherine Peach

Submitted for the Degree of Doctor of Philosophy

April 2003

Summary

Contact lenses have become a popular method of vision correction for millions of people globally. As with all devices designed for use within the body, interactions occur between the implanted material and the surrounding biological fluid.

A common complaint of lens wearers is that they often experience symptoms of dry eye whilst wearing lenses. This sensation is often heightened towards the end of the day. Through the course of this study, various analytical techniques have been utilised including one dimensional electrophoresis and Western Blotting to study the protein profiles of tear samples. By studying the tears of non contact lens wearers, it was possible to analyse what could be considered normal, healthy, individuals. A clinical study was also undertaken which followed a population of individuals from the neophyte stage to one whereby they were accustomed lens wearers. Tears were monitored at regular intervals throughout the course of this study and worn contact lenses were also analysed for proteins that had been deposited both on and within the lens.

Contact lenses disrupt the tear film in a physical manner by their very presence. They are also thought to cause the normal protein profile to deviate from what would be considered normal. The tear film deposits proteins and lipids onto and within the lens. The lens may therefore be depriving the tear film of certain necessary components. The ultimate aim of this thesis was to discover how, and to what extent, lenses affected tear proteins and if there were any proteins in the tear fluid that had the potential to be used as biochemical markers. Should this be achievable it may be possible to identify those individuals who were more likely to become intolerant lens wearers.

This study followed the changes taking place to the tear film as an effect of wearing contact lenses. Twenty-eight patients wore two different types of silicone hydrogel lenses in both a daily wear and a continuous wear regime. The tear protein profiles of the lens-wearers were compared with a control group of non-lens wearing individuals. The considerable amount of data that was generated enabled the clearly observable changes to the four main tear proteins to be monitored.

Keywords: Tear proteins, Western Blotting, electrophoresis, contact lenses, discomfort

To my family

ACKNOWLEDGEMENTS

Firstly, a huge thank you must go to my supervisor, Brian Tighe. Without his constant help, support, and ability to find the positive side to everything, this thesis would not have been possible.

Thank you to all members of the Biomaterial Research Unit, both past and present. Steve, thanks for providing many an entertaining night out – especially when you spot the dancefloor! To Ais, Fiona, Ellie, Anisa, Raminder, Muriel and last but by no means least, Val. Huge thanks for reading and re-reading my chapters and everything else that you have sorted out for me since I joined the group! To all those who have now moved on to pastures new: Rich, Matt, Jifan, Rich, and Bev.

I would also like to say a big thank you to Chris. Your help on things both academic and otherwise is greatly appreciated.

To my friends who have been there for me throughout the duration of my PhD, especially Marie and Ragan.

To my flatmates, especially Michelle. You always manage to put a smile on my face. Thank you for the many late night chats in the infamous kitchen and all the highly entertaining nights out!

To Aaran, thank you so much for always being there for me, for all the moral support and encouragement you gave to me – and also for attempting to sort out my many computer related disasters! I cannot thank you enough for everything that you have done.

And finally, to my parents, Hannah, Maria and Richard. Thank you to you all for being there and for the support that you have shown to me.

List of Contents

	Page
Title page	**1**
Summary	**2**
Dedication	**3**
Acknowledgments	**4**
List of Contents	**5**
List of Figures	**11**
List of Tables	**14**
List of Symbols and Abbreviations	**17**
Chapter 1 Introduction	**20**
1.1 Introduction	21
1.2 Overview of the Structure of the Eye	22
1.2.1 The Conjunctiva	24
1.2.2 The Cornea	24
1.2.2.1 The Corneal Epithelium	25
1.2.2.2 Bowman's Epithelium	25
1.2.2.3 Stroma	26
1.2.2.4 Descemet's Membrane	26
1.2.2.5 Endothelium	26
1.2.2.6 Function of the Cornea	27
1.3 Tears: Structure and Function	28
1.3.1 Mucin layer	29
1.3.2 Aqueous layer	29
1.3.3 Lipid layer	31
1.4 The Roles of the Tear Proteins	32
1.4.1 Antibacterial agents	32
1.4.1.1 Lactoferrin	33
1.4.1.2 Lysozyme	33
1.4.1.3 β-lysin	34

1.4.2 Antibodies and Inflammatory Mediators 34
1.4.2.1 Antibodies and Immunological Mediators 34
1.4.2.2 IgM 35
1.4.2.3 IgA 36
1.4.2.4 IgG 37
1.4.2.5 IgE 37
1.4.2.6 IgD 37
1.4.2.7 The Complement System 37
1.4.2.8 Cytokines 41
1.4.2.9 The Kinin System 44
1.4.2.10 Vitronectin 45
1.4.2.11 Fibronectin 46
1.4.2.12 Mucosal Associated Lymphoid Tissues 47
1.4.3 Transfer/Carrier Proteins 50
1.4.3.1 Albumin 50
1.4.3.2 Lipocalin 50
1.5 Electrolytes 52
1.6 Summary of the Functions of the Tear Film 54
1.7 Tear States 55
1.8 Contact lenses and deposition 57
1.9 Complications of Contact Lens Wear 61
1.9.1 Dry Eye 61
1.9.2 Giant Papillary Conjunctivitis 62
1.9.3 Allergic Conjunctivitis 63
1.9.4 Contact Lens Acute Red Eye 63
1.9.5 Microbial Keratitis 64
1.10 Aims and Scope of Research 65

Chapter 2 Materials and Methods **67**

2.1 Introduction 68
2.2 Tear Collection 68
2.2.1 The Schirmer Test 68

2.2.2 Filter Paper 68
2.2.3 Sponge Collection 68
2.2.4 Microcapillary 69
2.3 Extraction of Proteins from Contact Lenses 69
2.3.1 Preparation of Contact Lens Extracts 69
2.3.2 Collection of the Tear Envelope 70
2.4 Sample Treatment 71
2.5 Ultra Violet Spectrophotometry (UV) 71
2.6 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) 72
2.6.1 Pouring the Polyacrylamide Gels 74
2.6.1.2 Hardware 75
2.6.1.3 Method 75
2.6.2 Loading and Running the Gels 76
2.6.2.1 Solutions 76
2.6.2.2 Hardware 76
2.6.2.3 Method 76
2.6.3 Visualisation of the Bands 77
2.6.3.1 Solutions 77
2.6.3.2 Hardware 77
2.6.3.3 Method 77
2.7 Western Blotting 78
2.7.1 Solutions 79
2.7.2 Hardware 79
2.7.3 Method 79
2.8 Immunostaining of Western Blots 80
2.8.1 Solutions 80
2.9 Analysis of One Dimensional Gels 81

Chapter 3 TotalLab™ Software 83

3.1 Introduction 84
3.2 Lane Creation 85
3.3 Background Subtraction 87

3.3.1 "Rubber Band" Option 87
3.3.2 "Minimum Profile" Option 87
3.3.3 "Rolling Ball" Option 87
3.3.4 Manual Background Subtraction 88
3.4 Band Detection 89
3.4.1 "Minimum Slope" 89
3.4.2 "Noise Reduction" 89
3.4.3 "Percentage Maximum Peak" 90
3.4.4 "Automatic Detection" and "Manual Editing" 90
3.5 Molecular Size Calibration 92
3.6 Quantity Calibration 94

Chapter 4 Western Blotting and Immunostaining 97

4.1 Background 98
4.2 Method 99
4.3 Protein Transfer from Gel to Membrane 99
4.4 Immunostaining for Albumin 103
4.5 Sensitivity of Western Blotting 105
4.6 Detecting IgE in Tear Fluid 106
4.7 Assessment of Western Blotting and Immunostaining 107

Chapter 5 Comparison and Evaluation of Tear Sampling Techniques 109

5.1 Introduction 110
5.2 Collecting the Tear Envelope 111
5.3 Effects of Contact Lenses on the Tear Film Using Two Distinct Techniques 112
5.3.1 Aims 112
5.3.2 Methods 113
5.3.2.1 Tear Analysis Following Collection with Conventional Microcapillaries 113
5.3.2.2 Collection of the Tear Envelope 113

5.4 Results 114

5.4.1 Conventional Tear Analysis Following Collection with Microcapillary 114

5.4.2 Tear Envelope 120

5.5 Discussion 122

5.5.1 Conventional Tear Analysis 122

5.5.2 Collection and Analysis of the Tear Envelope from Three Types of Daily Disposable Lenses

5.6 Diurnal Variation 125

5.6.1 Introduction 125

5.7 Method 126

5.7.1 Tear Collection Using Conventional Microcapillary 126

5.7.1.1 Experimental Parameters 126

5.7.2 Collecting the Tear Envelope to Observe the Effects of Diurnal Variation 127

5.8 Results 128

5.8.1 Tear Samples Collected Using Conventional Microcapillary 128

5.8.2 Preliminary Evaluation of the Lens Tear Envelope 135

5.9 Discussion 138

5.9.1 Diurnal Variations Observed Using Conventional Microcapillary Technique 138

5.9.2 Diurnal Variations Observed in the Tear Envelope 140

Chapter 6 Clinical Study on the Effects of Contact Lenses on the Tear Film **141**

6.1 Aim 142

6.2 Introduction 142

6.3 Parameters of the Clinical Study 143

6.4 Silicone Hydrogels 145

6.5 Patient Details 147

6.6 Results 153

6.6.1 Tear Samples Collected by Microcapillaries 153

6.6.1.1 Concentrations of the Four Major Tear Proteins in the Tear Film 154
6.6.2 Calculation of Minimum and Maximum Limits 166
6.6.3 Comparison of a Lens Wearing Population and a Control Population 172
6.6.4 Removal of the Tear Envelope from Lenses Worn in the Clinical Study 174
6.6.5 Lens Extractions 176
6.7 Discussion 177
6.7.1 Effects of Contact Lenses Upon the Tear Film 177
6.7.2 Analysing the Tear Envelope Collected from Lenses Worn in the Study 182
6.7.3 Contact Lens Extracts 183

Chapter 7 Discussion and Future Work **186**

7.1 Discussion 187
7.2 Future Work 197

References **201**

Appendix **217**

LIST OF FIGURES

Title		Page
Figure 1.1	The structure of the eye	23
Figure 1.2	The structure of the cornea	25
Figure 1.3	The structure of tears	28
Figure 1.4	The structure of an immunoglobulin	35
Figure 1.5	The complement pathway	40
Figure 1.6	Formation of plasma enzyme mediators following tissue damage	45
Figure 2.1	Collecting the tear envelope	71
Figure 2.2	Diagrammatic representation of electrophoresis	73
Figure 2.3	Diagrammatic representation of western blotting	79
Figure 3.1	Lane creation	86
Figure 3.2	Band detection	91
Figure 3.3	Calculation of molecular weights	93
Figure 3.4	Calculating protein concentrations	95
Figure 3.5	Typical data sheet obtained upon completion of analysis	96
Figure 4.1	Gel following electrophoresis	101
Figure 4.2	Gel following blotting with continuous buffer system	102
Figure 4.3	Gel following electrophoresis	103
Figure 4.4	PVDF membrane following immunostaining for albumin	103
Figure 4.5	PVDF membrane following immunostaining for IgM	105
Figure 4.6	PVDF membrane following immunostaining for IgE	106
Figure 5.1	Tear samples collected before and after a three-day wearing period (Patient 1: Soflens, Patient 2: Acuvue)	114
Figure 5.2	Tear samples collected before and after a three-day wearing period (Patient 1: Acuvue, Patient 2: Focus Dailies)	115
Figure 5.3	Tear samples collected before and after a three-day wearing period (Patient 1: Focus Dailies, Patient 2: Soflens)	116
Figure 5.4	Shows the changes in concentration of tear proteins of patient 1 following three days of lens wear (Acuvue)	117

Figure 5.5	Shows the changes in concentration of tear proteins of patient 1 following three days of lens wear (Focus Dailies)	117
Figure 5.6	Shows the changes in concentration of tear proteins of patient 1 following three days of lens wear (Soflens)	118
Figure 5.7	Shows the changes in concentration of tear proteins of patient 2 following three days of lens wear (Acuvue)	118
Figure 5.8	Shows the changes in concentration of tear proteins of patient 2 following three days of lens wear (Focus Dailies)	119
Figure 5.9	Tear envelope removed from Acuvue lenses	120
Figure 5.10	Tear envelope removed from Focus Dailies lenses	120
Figure 5.11	Comparison of the tear envelope as removed from three types of daily disposable lenses	121
Figure 5.12	Patient 1 tear samples collected over a two-day period	133
Figure 5.13	Patient 4 tear samples collected over a two-day period	133
Figure 5.14	Shows the changes in the ratio of lactoferrin: lipocalin of five subjects at different time points throughout the day	134
Figure 5.15	Shows the changes in the ratio of lactoferrin: lysozyme of five subjects at different time points throughout the day	134
Figure 5.16	Tear envelope removed from three different types of daily disposable lenses worn for more than eight hours	135
Figure 5.17	Tear envelope removed from 1-day Acuvue worn at different times of the day	136
Figure 6.1	Graphical representation showing the number of patients wearing each lens type and the corresponding wear modality	152
Figure 6.2	Tear samples collected pre lens wear	156
Figure 6.3	Tear samples following one week of lens wear (Focus® Night and Day™)	157
Figure 6.4	Tear samples following three weeks of lens wear (Focus® Night and Day™)	157
Figure 6.5	Tear samples following three months of lens wear (Focus® Night and Day™)	158
Figure 6.6	Tear samples following six months of lens wear (Focus® Night and Day™)	158

Figure 6.7	Tear samples following one week of lens wear (PureVision™)	161
Figure 6.8	Tear samples following two weeks of lens wear (PureVision™)	161
Figure 6.9	Tear samples following one month of lens wear (PureVision™)	162
Figure 6.10	Tear samples following three months of lens wear (PureVision™)	162
Figure 6.11	Tear samples of control patients (Visit 0)	164
Figure 6.12	Tear samples of control patients (Visit 4)	164
Figure 6.13	Tear samples of control patients (Visit 5)	165
Figure 6.14	Tear samples of control patients (Visit 6)	165
Figure 6.15	Bar chart showing the distribution of the lactoferrin: albumin ratio for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)	170
Figure 6.16	Bar chart showing the distribution of the lactoferrin: lipocalin for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)	170
Figure 6.17	Bar chart showing the distribution of the lactoferrin: lysozyme for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)	171
Figure 6.18	Gel scan showing tear envelope from worn lenses	174
Figure 6.19	Gel scan showing tear envelope from worn lenses	175
Figure 6.20	Gel image showing extracts from PureVision™ lenses worn in a continuous wear regime	176

LIST OF TABLES

Title		Page
Table 1.1	Summary of averaged reported concentrations of tear proteins	32
Table 1.2	Selected functions of some cytokines	43
Table 1.3	Tear film components with antibacterial properties	49
Table 1.4	Host defense mechanisms of the ocular surface	53
Table 1.5	FDA Classifications of Contact Lenses	58
Table 2.1	Composition of resolving gel solutions	75
Table 5.1	Characterisation of the daily disposable lenses worn	112
Table 5.2	Percentage of proteins present in tear samples before and after a three-day wearing period (Patient 1: Soflens, Patient 2: Acuvue)	114
Table 5.3	Percentage of proteins present in tear samples before and after a three-day wearing period of lens wear (Patient 1: Acuvue, Patient 2:Focus Dailies)	115
Table 5.4	Percentage of proteins present in tear samples before and after a three-day wearing period of lens wear (Patient 1: Focus Dailies, Patient 2:Soflens)	116
Table 5.5	Average centre thickness of 1-day Acuvue, Focus Dailies and Soflens 1-day (measured at –3.00D)	124
Table 5.6	Results of tear protein concentrations of five individuals over the course of the day	128
Table 5.7	Ratios of albumin, lipocalin and lysozyme to lactoferrin of five subjects averaged over a three-day period.	129
Table 5.8	Ratios of albumin, lipocalin and lysozyme to lactoferrin of five subjects over a three-day period as observed at each visit	130
Table 5.9	The ratios of albumin, lipocalin and lysozyme to lactoferrin of five individuals throughout the day (Averaged over a three-day period).	131

Table 5.10	The ratios of albumin, lipocalin and lysozyme to lactoferrin of control subjects involved in the clinical study (Averaged over a three-day period)	132
Table 5.11	Concentration of proteins within the tear envelope removed from 1-day Acuvue lenses worn at different times throughout the day as calculated using TotalLab™	137
Table 6.1	Characterisation of the lenses employed in the clinical study	146
Table 6.2	Patients wearing Focus® Night and Day™ on a daily wear basis	147
Table 6.3	Patients wearing Focus® Night and Day™ on a continuous wear basis	148
Table 6.4	Patients wearing PureVision™ on a daily wear basis	149
Table 6.5	Patients wearing PureVision™ on a continuous wear basis	150
Table 6.6	Control patients	151
Table 6.7	Summary of the number of people wearing each lens type and the corresponding wear modality	152
Table 6.8	Details of patient visits and sample collection and the corresponding time period they refer to	153
Table 6.9	Concentrations of the major tear proteins in the tear fluid of patients wearing Focus® Night and Day™ on a daily wear basis (Visit 0 – Visit 7)	154
Table 6.10	Concentrations of the major tear proteins in the tear fluid of patients wearing Focus® Night and Day™ on a continuous wear basis (Visit 0 – Visit 7)	156
Table 6.11	Concentrations of the major tear proteins in the tear fluid of patients wearing PureVision™ on a daily wear basis (Visit 0 - Visit 7)	159
Table 6.12	Concentrations of the major tear proteins in the tear fluid of patients wearing PureVision™ on a continuous wear basis (Visit 0 – Visit 7)	160
Table 6.13	Concentrations of the major tear proteins in the tear fluid of control patients	163

Table 6.14	Ratios of the major tear proteins for all control patients averaged out over all time periods (Visit 0 – Visit 7)	166
Table 6.15	Minimum and maximum limits for the protein ratios as being indicative of a healthy tear film	167
Table 6.16	Ratios of tear proteins of patients wearing Focus® Night and Day™ in a daily wear modality for all post lens visits	167
Table 6.17	Ratios of tear proteins of patients wearing Focus® Night and Day™ in a continuous wear modality for all post lens visits	168
Table 6.18	Ratios of tear proteins of patients wearing PureVision™ in a daily wear modality for all post lens visits	168
Table 6.19	Ratios of tear proteins of patients wearing PureVision™ in a continuous wear modality for all post lens visits	169
Table 6.20	Comparison of the tear protein ratios of a lens wearing population and a control population at Visit 0 and Visit 7	172
Table 6.21	Comparison of the protein ratios of the lens-wearing individuals according to lens type and wear modality	174

List of Symbols and Abbreviations

Ab	antibody
Ag	antigen
AL	axial length
AP	alkaline phosphatase
APC	antigen presenting cells
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N, N1-methylene-bis-acrylamide
C	closed eye
°C	degree Celsius
CALT	conjunctival associated lymphoid tissue
CBB	coomassie brilliant blue
CEE	closed eye environment
CIE	counter immunoelectrophoresis
CLARE	contact lens acute red eye
CLIPU	contact lens induced papillary conjunctivitis
CW	continuous wear
DTT	dithiothreitol
DW	daily wear
EGF	epidermal growth factor
ELISA	enzyme linked imunosorbent assay
EW	extended wear
Fab	fragment antigen-binding
Fc	fragment crystallisable
FDA	Food and Drug Administration
Fn	fibronectin
g	gram
GPC	giant papillary conjunctivitis
Hf	Hageman factor (factor XII)
HMWK	high molecular weight kininogen
IFN-α	interferon alpha

IFN-β	interferon beta
IFN-γ	interferon gamma
Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
J chain	joining chain
KCS	keratoconjunctivitis sicca
kDa	kiloDaltons
LMWK	low molecular weight kininogen
MA	methacrylic acid
MAC	membrane attack complex
MALT	mucosal associated lymphoid tissue
2-ME	2-mercaptoethanol
MK	microbial keratitis
MW	molecular weight
μg	micrograms
μl	microlitre
μm	micrometre
mg	milligrams
ml	millilitre
mm	millimetre
mM	millimole
NAG	N-Acetylglucosamine
NAM	N-acetylmuramic acid
NBT	nitro blue tetrazolium
ng	nanograms
NK cell	natural killer cell
nm	nanometre
O	open eye

OEE	open eye environment
PMFA	protein moving faster than albumin
PMMA	poly(methyl methacrylate)
PMN	polymorphonuclear
PVA	polyvinyl alcohol
PVDF	polyvinylidene difluoride
PVP	poly (vinyl pyrrolidine)
polyHEMA	poly (2-hydroxyethyl methacrylate)
SC	secretory component
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEALS	superior epithelial arcuate lesion
sIgA	secretory immunoglobulin A
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylenediamine
TGF-β	transforming growth factor-beta
TPA	tear specific prealbumin
TNF-α	tumour necrosis factor-alpha
TNF-β	tumour necrosis factor beta
Tris	Tris (hydroxylmethyl) aminomethane
UV	ultraviolet
Vn	vitronectin

Chapter 1

Introduction

1.1 Introduction

The development of materials suitable for use in the eye has proved to be a huge success over the past three decades. New materials and solutions are continuously being developed in the hope of increasing comfort, visual acuity and convenience for the wearer. A great deal of research has been carried out to date on the interactions that take place between the tear film and the contact lens upon insertion into the eye. One of the major problems of lens wear is the deposition that occurs almost instantaneously upon inserting a lens in to the eye. Such deposits can lead to discomfort, blurring and a reduction in visual acuity. This deposition can be severe enough in some cases to cause individuals to cease wearing lenses. These deposition processes and their consequence for the wearer have been the primary driver in the development of new materials.

At the same time as the lens is being coated with components of the tear film, the tear film itself may be being disrupted due to the lens adsorbing important elements of the tear film. The integrity of the tear film is vital for safe and comfortable contact lens wear and, as such, needs to be protected. Factors affecting deposition include the chemical composition of the lens (e.g. ionicity), the composition and pH of the tears, inadequate blinking and poor lens hygiene. Studies of the interactions taking place between the lens and the tear film are necessary to better understand the relationship between spoilation and clinical complications and to ultimately improve the physiological biocompatibility of biomaterials used in the manufacture of contact lenses.

Studies on tears and *in vivo* contact lens wear have many advantages over investigations of other materials designed for use within the body. The tear fluid is easily accessible and minimal trauma is caused to the subject when it is being collected. It can also be studied continuously without causing any harm. A complete knowledge of the structure of the tear film is of paramount importance. Proteins, and their assumed role in spoilation, are of particular interest as they are thought to be involved in immunological responses which may be heightened upon the introduction of a foreign body in to the eye, e.g. a contact lens.

1.2 Overview of the Structure of the Eye

The structure of the eye is very briefly described below. This thesis was more concerned with the structure and function of tears but it was considered important to understand the anatomy and function of the eye before the interactions between the tear fluid and contact lenses can be considered.

The eye is a spherical, fluid filled structure, approximately 2.5 cm in diameter,[1] enclosed by three layers. These are (from outermost to innermost):

- Sclera and cornea – the sclera is a fibrous outer protective coating of the eye – consisting almost entirely of collagen. It forms the visible white part of the eye. Anteriorly, the outer layer consists of the transparent cornea. Light rays pass through the cornea into the interior of the eye. The conjunctiva is the thin, transparent, mucous membrane that covers the posterior surface of the lids and the anterior surface of the sclera.
- Choroid, ciliary body and the iris – the choroid is the highly pigmented layer underneath the sclera. It contains many blood vessels that nourish the retina. The choroid layer becomes specialised anteriorly to form the ciliary body and the iris. The iris is a thin, pigmented smooth muscle. Light enters the interior portions of the eye through the pupil – the round opening in the centre of the iris. The radial muscles in the iris allow the pupil to increase in size when they contract to allow more light to enter the eye in dim conditions. Contraction of the circular muscles within the iris causes the pupil to get smaller. This has the effect of reducing the amount of light entering the eye.
- Retina – the retina consists of an outer, pigmented, layer and an inner layer of nervous tissue. The rods and cones are found in the layer of nervous tissue. These photoreceptors are responsible for converting light energy into nerve impulses.

Illustration removed for copyright restrictions

Figure 1.1 - The structure of the eye

The interior of the eye consists of two fluid-filled cavities separated by a lens. They are all transparent and allow light to pass through the eye from the cornea to the retina. The anterior cavity between the cornea and the lens contains the aqueous humour – a watery fluid. The aqueous humour carries nutrients to the cornea and the lens as they lack their own blood supply. The larger, posterior, cavity between the lens and the retina contains the vitreous humour – a semi-solid substance. The vitreous humour is important in maintaining the spherical shape of the eyeball.

Aqueous humour is produced at a rate of about 5ml per day by a capillary network within the ciliary body. The fluid drains into a canal at the edge of the cornea and eventually enters the blood. Glaucoma is a condition arising due to the intraocular pressure rising as a result of excess fluid in the anterior cavity.

1.2.1 The Conjunctiva

The conjunctiva, which is the thin transparent mucous membrane covering the posterior surface of the lids and anterior surface of the sclera, comprises an epithelium, which consists of up to five layers of stratified epithelial cells, and an underlying stroma.[2] The goblet cells are found within the conjunctival epithelium. They secrete the mucin component of the tear film.

The accessory lacrimal glands (the glands of Krause and Wolfring) are located in the conjunctival stroma. The stroma is made up of loosely arranged collagen bundles. It consists of fibroblasts, macrophages, lymphocytes and occasionally eosinophils and neutrophils.

1.2.2 The Cornea

The human cornea occupies about 7% of the eye's surface area. It has a diameter of approximately 12mm. Reports suggest that it is 0.52mm thick in the centre and 0.65mm at the periphery.[3] The cornea is a transparent tissue. It has five distinct layers:

- Epithelium
- Bowman's layer
- Stroma
- Descemet's membrane
- Endothelium

Aston University

Illustration removed for copyright restrictions

Figure 1.2 – The structure of the cornea

1.2.2.1 The Corneal Epithelium

The corneal epithelium is the outermost layer and provides an effective barrier against damage to the eye. It is approximately 50 - 60μm thick[4] and constitutes roughly 10% of the total thickness of the cornea.[5, 6] It consists of several layers of cells. They progress in shape from the basal columnar cells of the superficial layer to the squamous flattened cells. These basal cells form a single layer resting on a basement membrane. They gradually desquamate into the tear film and are continuously replaced by cell division in the basal cells. The multi-layered epithelium provides a permeability barrier to prevent the penetration of the tear fluid into the underlying stroma. The epithelium secretes Bowman's membrane to which it is attached.

1.2.2.2 Bowman's Layer

The Bowman's layer lies immediately beneath the basement membrane of the corneal epithelium. This is a thin layer (8 – 12 μm in thickness) consisting of a network of short collagen fibrils. It is a fairly durable layer and provides the first few microns of the connective tissue of the corneal stroma. The Bowman's layer merges with the stroma

1.2.2.3 Stroma

The stroma is an important part of the cornea and constitutes approximately 90% of the total thickness of the cornea. The stroma comprises collagen fibrils, stromal cells and ground substance (containing proteoglycans, glycoproteins, proteins and keratocytes). Water is particularly abundant in the ground substance and accounts for 78% of the total weight. The ground substance is jelly-like in consistency in its normal hydrated state. The collagen fibrils are very thin – approximately 32nm in diameter. It is the orderly arrangement of the collagen fibrils that is responsible for the transparency of the cornea.

Keratocytes are the most common type of cell found in the stroma. They build up the structure of the stroma by synthesising collagen fibrils. Keratocytes migrate to the site of injury when there is damage to the cornea.[7]

1.2.2.4 Descemet's Membrane

The Descemet's membrane lies on the posterior surface of the stroma and is the basement membrane of the endothelium. This layer is thin (approximately 10 μm in thickness) and consists mainly of collagen. It is secreted by the endothelium. It is produced throughout life and so increases with age. The membrane is 10 – 15μm thick[8] in the adult eye.

1.2.2.5 Endothelium

The endothelium is a monolayer of cells. The cell cytoplasm contains numerous mitochondria, a prominent endoplasmic reticulum and a Golgi apparatus. These are indicators that the endothelium plays an active role in the synthesis and transport of fluid. The corneal endothelium secretes its own basement membrane, the Descemet's membrane. The endothelial cells are packed together on this membrane. The endothelium is bathed in the aqueous humour. The endothelium pumps ions and fluids out of the stroma into the aqueous humour. This maintains corneal hydration and transparency.

1.2.2.6 Function of the Cornea

The main function of the cornea is to transmit light. A healthy cornea transmits about 90% of the light in the visible spectrum.[4] It also provides most of the eye's refractive power. Approximately 70% of the refraction that takes place does so at the air-cornea interface. The human cornea has a refractive index of 1.375. Together with the sclera it forms the physically robust outer layer of the eye thus protecting the fragile tissues within the eye. The cornea also provides a barrier to prevent the entry of pathogenic organisms.

1.3 Tears: Structure and Function

Human tears have a very complex chemical composition. At least 60 components have been identified including proteins, lipids, enzymes, salts, glucose and urea.[8] The tear volume has been estimated to be 7.0 ± 2.0μl.[9] The average rate of tear production has been estimated to be approximately 1μl/min. The technique employed in the sampling of the tears does affect this value however. The tear film has been described as a tri-laminar structure, 7 – 10μm in thickness.[10] These layers are not thought to be distinct layers as some degree of interaction occurs between them. This is a well-established model of the tear film, but modifications to this structure are regularly proposed. The three layers are: the inner mucin layer, the middle aqueous layer, and the outer lipid layer.

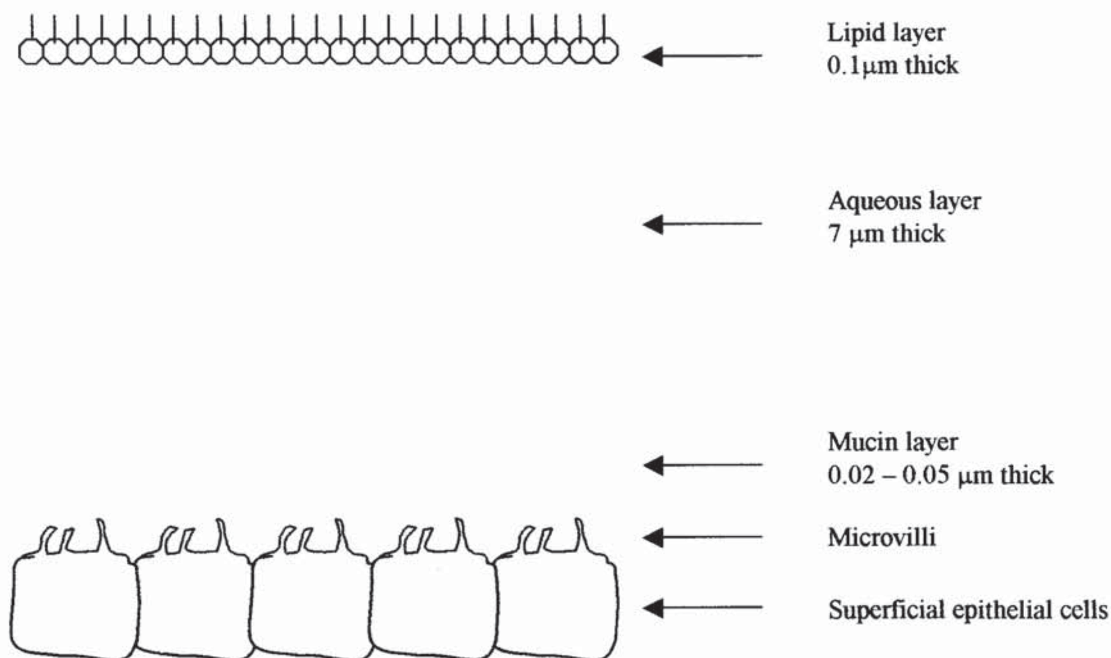


Figure 1.3 – The structure of tears

1.3.1 Mucin Layer

The mucins are made up of glycoproteins, free proteins and salts with an electrolyte content similar to that of serum. This mucous glycoprotein layer is probably essential in the maintenance of a stable tear film over the ocular surface. Goblet cells in the conjunctiva are believed to supply the mucin, along with the glands of Manz and the crypts of Henle. There are also mucous-secreting cells that have been found in the lacrimal gland.

The tear model is constantly under review and discussion. Previously, it was thought that the mucins were found only in this layer, which was about 0.02 – 0.05μm in thickness. Recent studies suggest that they may in fact be more widely dispersed through the tear film.[11, 12] This is referred to as the "mucous gel" model.

The mucins within the tear film appear to make the surface of the cornea more wettable due to their hydrophilic nature.[13] They are also thought to lower the surface tension of the tears. In doing so, they enable the tears to be spread over the cornea during the blink action.[14, 15]

This mucous layer is similar to that found in other parts of the body. The mucous in the respiratory tract traps microorganisms and other irritants. They can then be expelled from the body by sneezing. The mucous found in the eye acts to inhibit the adherence of pathogens to the ocular surface. They are then cleared through the normal tear turnover or are flushed by excessive lacrimation and blinking.[16] It is thought that this layer may also provide a means of allowing immunological agents such as IgA and lysozyme to anchor themselves. The mucous layer may therefore be thought of as making a contribution to local immunity.[17]

1.3.2 Aqueous Layer

The aqueous layer accounts for approximately 98% of the total thickness of the tear film. It is thought to be about 7.0μm in thickness and contains a variety of both organic and inorganic species. As many as sixty proteins have been detected in the aqueous layer as well as glycoproteins, urea, electrolytes and glucose.[8, 18] Some of the proteins are tear

specific originating from the main lacrimal gland and, to a lesser extent, the accessory glands of Krause and Wolfring. Others are plasma/serum derived as a result of vascular leakage or transport across the blood-tear barrier. The proportion of these serum derived components is increased during eye closure and inflammation.[19, 20] The concentration of tear components varies between individuals, even in the healthy eye. Age, gender, and the wearing of contact lenses are all factors that may affect the concentration. The collection technique employed also affects protein concentrations due to the degree of trauma the eye is subjected to. Invasive methods e.g. filter paper and sponges stimulate the conjunctiva. This results in a higher proportion of plasma proteins from the serum being detected. Differing results may also arise through the usage of different analytical techniques.

The tear film also contains many electrolytes; both cations and anions. The main cations are sodium and potassium. Magnesium and calcium are also present in lower concentrations. Chloride and bicarbonate are the main anions found within the aqueous layer. The electrolytes act to maintain the pH of the tears within normal limits and also add to the osmotic pressure.[21]

The proteins present in the tear film are of great importance because of their assumed role in immunological reactions. Contact lenses disrupt the structure of the tear film and may cause the concentrations of these proteins to deviate from the normal range. The main proteins of interest are: albumin, lactoferrin, lysozyme, lipocalin, the immunoglobulins (Ig) including IgA, IgG and IgE, and the cytokines. The role of these proteins is discussed in section 1.4.

The main functions of the aqueous layer can be summarised as follows:

- Supplies the corneal epithelium with essential nutrients
- Maintains the pH of the tears within a healthy range
- Improves the lubrication of the cornea to protect it from mechanical damage
- Allows the removal of foreign bodies from the ocular surface[22, 23, 24]

1.3.3 Lipid Layer

The outermost layer of the tear film is the lipid layer. It is only about 0.1μm thick. During the action of a blink this layer becomes compressed and so becomes even thinner. It is mainly composed of waxy esters and sterols. Together they comprise 90% of the total thickness of the lipid layer. The remainder is made up of triacylglycerols, cholesterol, polar lipids and free fatty acids.[25, 26] A great deal of variation is seen between individuals. The lipid layer is secreted by the meibomian glands in the lower and upper lids and also the glands of Moll and Zeiss.[27] The lipid layer is anchored at the orifices of the meibomian glands and so acts completely independently of the aqueous layer beneath it. The lipids are orientated in such a manner so as to expose their hydrophilic ends to the aqueous layer.[28, 29]

The lipid layer has many important functions, which can be summarised as follows:

- Reduces evaporation from the underlying aqueous layer. A four-fold increase is seen in evaporation if the lipid layer is absent
- Acts as a lubricant allowing the lids to move smoothly over the surfaces of the conjunctiva and cornea
- Reduces the surface tension of the tear film enabling it to be spread more effectively following a blink
- Prevents wetting of the skin of the lids adjacent to the eye

1.4 The Roles of the Tear Proteins

Tear proteins have been very important in this study due to their assumed role in immunological responses and hence their involvement in the protection of the eye. They also serve many other functions including:

- Metal transport
- Control of infectious agents
- Osmotic regulation

Table 1.1 (see below) provides a summary of the main tear proteins and their relative characteristics.

Protein	Average concentration (mg/ml)	Molecular weight (kDa)	Origin	Net charge
Lysozyme	1.3	14.6	Tear	+
Lactoferrin	1.4	82	Tear	+
s-IgA	0.3	160	Tear/plasma	+
IgG	0.13	150	Plasma	+
Lipocalin	1.23	21	Tear	+
Albumin	1.3	68	Plasma	-

Table 1.1 - Summary of averaged reported concentrations of tear proteins

1.4.1 Antibacterial Agents

The eye is constantly exposed to the external environment and as such is exposed to many organisms and foreign bodies that could be detrimental to ocular health. Many mechanisms exist to protect the eye from such ocular assault. These mechanisms range from the simple

blink to the more complex e.g. the presence of antibacterial agents, which can interact with pathogens and ultimately render them harmless.

1.4.1.1 Lactoferrin

Lactoferrin (molecular weight 82 kDa)[30] is an iron binding protein found in most body fluids including saliva, tears and nasal secretions.[31] It is thought that lactoferrin acts as a defense protein against bacterial invasion due to its bacteriostatic properties. It binds to essential metal ions making them unavailable for microbial metabolism. Lactoferrin may also assist lysozyme in the killing of Gram negative bacteria by altering the outer membrane thus exposing the peptidoglycan. This leads to the lysis of the bacteria.[14, 16] The main and accessory lacrimal glands are the major sources of tear lactoferrin. Values obtained for the concentration of lactoferrin in tears range from 1 – 2 mg/ml depending on the collection technique employed. The average concentration found in unstimulated tears is 1.2 mg/ml. A great deal of variation is found between individuals.[32] The concentration also declines with age. Diseases that affect the lacrimal gland e.g. keratoconjunctivitis sicca and Sjögrens syndrome result in drastically lowered concentrations of lactoferrin.[33, 34]

1.4.1.2 Lysozyme

Alexander Fleming first identified lysozyme in 1922 as an antibacterial enzyme. It was isolated in nasal secretions and was then later discovered in tears. Lysozyme accounts for approximately 20% of the total tear protein. It is present in most tissues and secretions but not in high enough concentrations to be anti-bacterial – with the exception of tears, nasal secretions and white blood cells. In the tear fluid, lysozyme (molecular weight 14.6 kDa) is derived from the lacrimal gland. The concentration of lysozyme in tears is 0.5 – 4.5 mg/ml depending on collection technique with an average concentration of 1.3 mg/ml.[10] Lysozyme acts by breaking the $\beta 1 \rightarrow 4$ glycosidic bond between repeating glycan units of the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) backbone structure of the peptidoglycan outer coat of gram positive bacteria thus disrupting the cell wall.[18] Gram negative bacteria are not affected by lysozyme as they have an extra outer membrane containing lipopolysaccharide that prevents their cell wall being attacked. They may become more susceptible if this outer layer becomes damaged in some way.[18] Lysozyme is

said to have both bacteriostatic (prevents bacteria from multiplying and growing but is not toxic) and bactericidal (kills bacteria) activity.[35] The levels of lysozyme decrease with age and also in dry eye states e.g. Sjögrens syndrome. This enables the lysozyme concentration to act as an indicator of tear dysfunction.

1.4.1.3 β-lysin

β-lysin is thought to be derived mainly from platelets. It is an enzyme that causes cell lysis by disrupting the cell membrane found beneath the cell wall.[18] It is successful in combating infections caused by bacterial cocci. Many of these are resistant to the actions of lysozyme. The two enzymes work together to destroy bacteria. Lysozyme initially breaks the cell wall thereby allowing β-lysin access to the cell membrane.[18,] It is found in many body fluids and is likely to be found in tears as a result of transport from the blood. The level of β-lysin in tears is reported to be approximately 150μg/ml.[36]

1.4.2 Antibodies and Inflammatory Mediators

Many other protein species are found within the ocular environment that act to protect the eye from infection in addition to the antibacterial enzymes described above. Other proteins are involved in inflammatory responses and wound healing. Such proteins include the antibodies (immunoglobulins), cytokines, kininogen, and members of the complement cascade.

1.4.2.1 Antibodies and Immunological Mediators

Approximately 20% of the total tear protein is made up of immunoglobulins. These proteins, synthesised by plasma cells, are vital for ocular defense. There are five classes of antibody: immunoglobulin (Ig) A, D, E, G and M. All are antigen specific. They are similar in structure but have different amino acid sequences in the constant region of their heavy chain allowing them to perform different functions. Each class plays a different role in host defense.[37] This humoral immunity is the first line of defense against entry of pathogens.

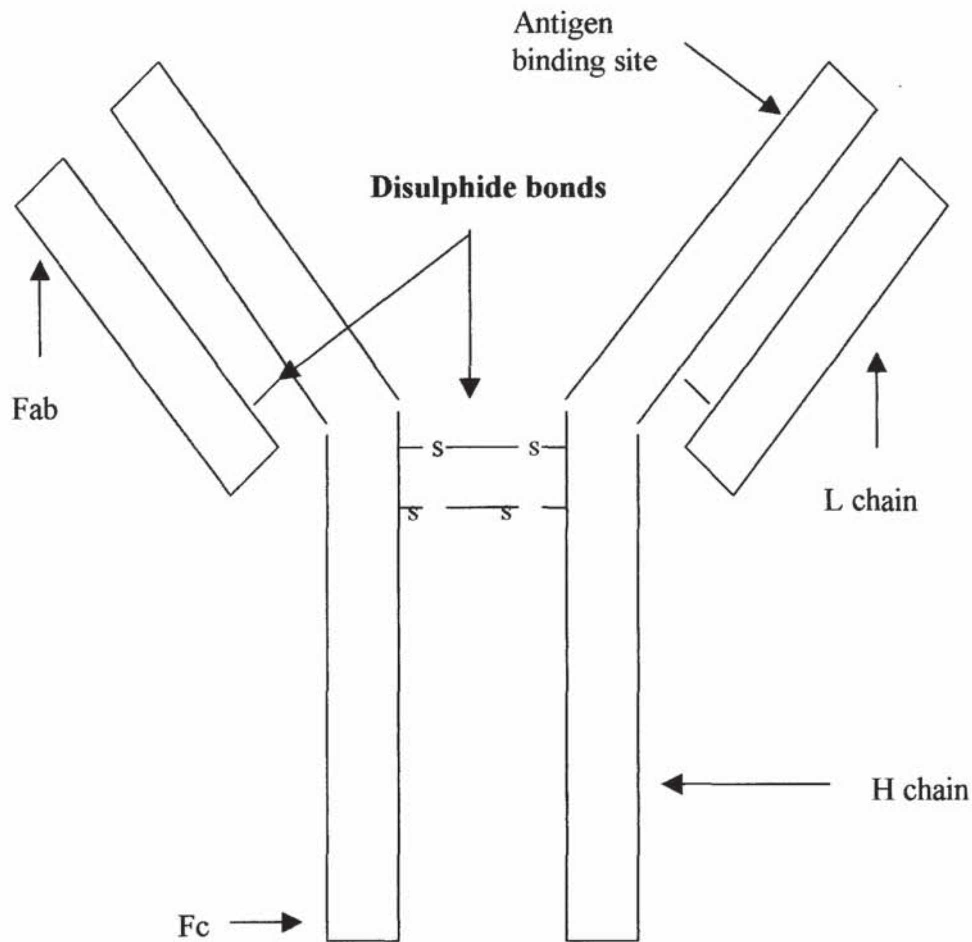


Figure 1.4 - Structure of an immunoglobulin

1.4.2.2 IgM

IgM provides the first line of defense. It is the first immunoglobulin to be produced in a primary response to an antigen and is also the first immunoglobulin synthesised by a neonate.[38] During the host's first encounter with an organism, IgM provides the main humoral immune response. This stimulates an increase in IgA and IgG. 5 – 10% of the total serum immunoglobulin consists of IgM.

The secreted form of IgM is a pentamer. It consists of five separate IgM molecules linked by a polypeptide called a J chain. Secreted IgM therefore has ten antigen-binding sites making it highly successful in aggregating antigens.[39]

IgM is unable to diffuse into the central stroma of the cornea due to its size. It is therefore restricted to the periphery of the ocular surface. The concentration of IgM in tears has recently been reported to be 5.6 μg/ml using enzyme linked immunosorbant assays.[40]

In a healthy individual, only a trace amount of IgM is detected in the tear fluid – if any at all. This level rises in some individuals who are deficient in serum IgA. A possible theory for this is that serum derived IgM is secreted in some way so as to compensate for this deficiency.[40, 41]

1.4.2.3 IgA

IgA is the main immunoglobulin present in tear fluid.[42] It is the main class of antibody in a number of secretions including milk, saliva and in respiratory and intestinal secretions.[39] The IgA found in external secretions is different both chemically and immunologically from serum IgA.[43] In tears, it is attached to an antigenic fragment called secretory component.[18] Serum IgA is a 150kDa monomer. Secretory IgA is a dimer with a molecular weight of 385kDa. This additional weight is due to the addition of a joining (J) chain and a secretory component. IgA is probably synthesised by the interstitial plasma cells and then coupled to secretory component following entry into the intercellular spaces. It is then secreted through the blood-tear barrier as secretory IgA (s-IgA). Secretory-IgA may inhibit the adherence of pathogens to mucosal sites and thus reduces the colonisation of pathogenic organisms.[44, 45] It can also cross-link large antigens with multiple epitopes. This binding of s-IgA to the surface of viral and bacterial antigens prevents the attachment of such pathogens to mucosal cells. IgA is the main protective antibody of the mucosal and secretory immune system. Values have been reported in the literature ranging from 0.04 – 0.85mg/ml. The average concentration is accepted to be approximately 0.3mg/ml.[46, 47] The method of tear collection and analytical technique does affect this value.

1.4.2.4 IgG

IgG (molecular weight 150kDa) accounts for approximately 80% of serum antibody.[38] It is the second immunoglobulin to be produced as a result of an antigenic challenge, IgM being the first. Following the successful combat of an infection, IgM specific for that antigen will eventually disappear. IgG molecules directed against the organism will however persist in the serum. IgG will be able to react faster than IgM upon a secondary exposure due to the retained memory from the prior encounter. IgG is present in normal tears - albeit at a lower concentration than in serum. The average concentration in the tear fluid is 0.13mg/ml.[10] It is found in both the precorneal tear film and the aqueous humour.

1.4.2.5 IgE

IgE occurs in very low concentrations in the precorneal tear film at approximately 100 – 700ng/ml.[10] IgE antibodies mediate hypersensitivity reactions responsible for the symptoms seen in hay fever, asthma and anaphylactic shock. IgE binds to Fc receptors on the membranes of basophils and mast cells.[48] Degranulation of these cells is then induced. This results in a variety of mediators present in the granules to be released, giving rise to allergic manifestations. The presence of IgE may represent a local immune system reaction.

1.4.2.6 IgD

IgD constitutes approximately 0.2% of total serum immunoglobulin. It is a membrane bound immunoglobulin expressed by B cells and it may therefore activate B cells by antigen. To date, no other role has been identified for IgD.[38]

1.4.2.7 The Complement System

The complement system is the major effector of the humoral branch of the immune system. It consists of nearly 30 serum and membrane proteins. These proteins and glycoproteins are synthesised largely by liver hepatocytes - although significant amounts of complement components are also produced by blood monocytes, tissue macrophages and epithelial cells of the gastrointestinal and genitourinary tracts. These components circulate in the serum in

inactive forms. Following initial activation, the various components interact in a highly regulated enzymatic cascade to generate reaction products that enable the generation of an inflammatory response. There are two pathways by which the complement system is activated. The two pathways share a common terminal reaction sequence, which results in the generation of a membrane attack complex (MAC). This complex is able to lyse a variety of cells, bacteria and viruses.[38]

The complement reaction products amplify the initial antibody-antigen reaction and converts the reaction into a more effective defense mechanism. A variety of reaction products are released during the activation of complement, inducing localised vasodilation and the attraction of phagocytic cells chemotactically. This results in an inflammatory reaction. As antigen becomes coated with complement reaction products it is then more readily phagocytosed by phagocytic cells that carry receptors for these complement products.

Complement activation by the classical pathway is usually initiated by the formation of soluble antigen-antibody complexes (immune complexes) or by the binding of antibody to an antigen on the surface of a target e.g. a bacterial cell. IgM and certain subclasses of IgG can activate the classical complement pathway. The classical pathway is a specific immune response. The initial stages of activation involves C1, C2, C3 and C4 in a sequence of activation reactions. These are all present in the serum in an inactive form. The result of these components being activated is the alteration to the conformation of C5. This is cleaved into C5a, which diffuses away, and C5b – which attaches to the antigenic surface. The bound C5b initiates the formation of the membrane attack complex.

Bound C5b can also be generated by the second major pathway of complement activation – the alternative pathway. The alternative pathway is initiated in most cases by various cell-surface constituents that are foreign to the host. Both Gram positive and Gram negative bacteria have constituents in their cell wall that can activate the alternative pathway. This pathway involves four serum proteins: C3, factor B, factor D and properdin.[38]

Serum C3 undergoes hydrolysis to yield C3a and C3b. The C3b component can bind to foreign surface antigens (e.g. those found on bacterial cells or viral particles) or to the host's own cells. Most mammalian cells have high levels of sialic acid which allows bound

C3b molecules on the host cells to be rapidly inactivated. Many foreign antigenic surfaces (bacterial cell walls, yeast cell walls and certain viral envelopes) have low levels of sialic acid, C3b bound to these surfaces remains active for longer periods of time. Bound C3b can also bind another serum protein called factor B. The binding of C3b to factor B exposes a site on factor B that serves as the substrate for factor D. Factor D cleaves the C3b-bound factor B to release a small fragment (Ba) which diffuses away and generates C3bBb. This can then go on to generate more C3b. C3bBb3b complex is generated by the C3 convertase activity of C3bBb – similar to the classical pathway. The non-enzymatic C3b component binds C5. The C3bBb component subsequently hydrolyses the bound C5 to generate C5a and C5b. It is C5b that later binds to the antigenic surface.

The membrane attack complex is formed following the terminal sequence of complement activation whereby C5b, C6, C7, C8 and C9 interact in sequence. This complex displaces the phospholipids found in the membrane of invading pathogens. A large transmembrane channel is then formed which disrupts the membrane and enables ions and small molecules to diffuse through it freely.

The membrane attack complex is capable of lysing a broad spectrum of microorganisms, viruses, and erythrocytes. The alternative pathway generally occurs without an initial antigen-antibody interaction and so is an important innate system of non-specific defense. The classical pathway provides a more specific defense mechanism, as it requires an initial antigen-antibody reaction. The complement system plays a crucial role in host defense against viruses. It is vital in containing the spread of viruses during acute infection and protects against re-infection.

Various peptides generated during the formation of the membrane attack complex also play a role in the development of an effective inflammatory response. C3a, C4a and C5a are called anaphlatoxins. They bind to receptors on mast cells and basophils and induce degranulation to release histamine and other mediators. These mediators induce smooth muscle contraction and increased vascular permeability.

C3a and C5a can act together to induce monocytes and neutrophils to adhere to vascular endothelial cells and migrate to the site of complement activation in the tissues. Activation

of the complement system therefore results in the influx of fluid that carries antibody and phagocytic cells to the site of antigen entry.

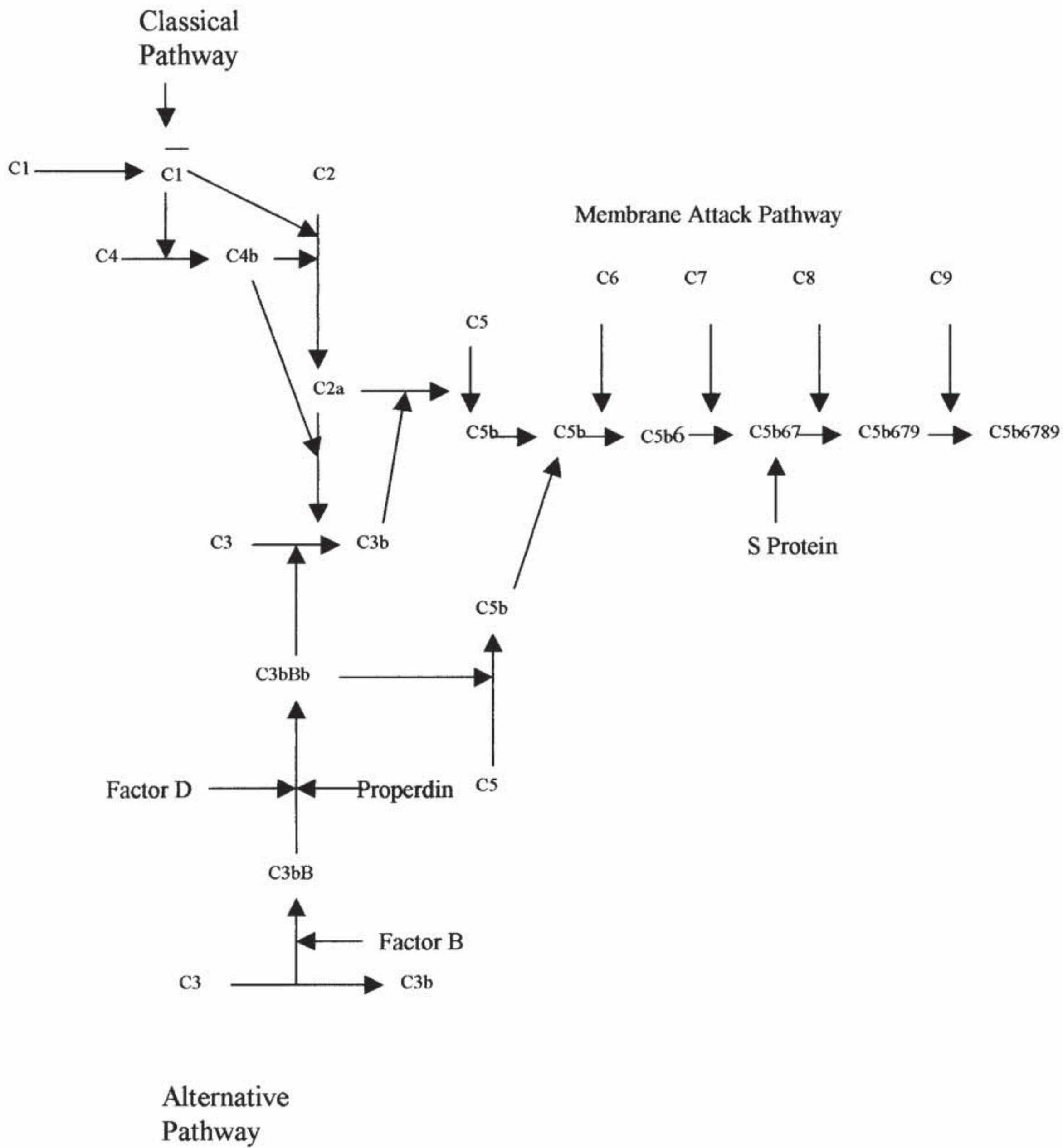


Figure 1.5 – The complement pathway

1.4.2.8 Cytokines

Cytokines are low molecular weight proteins (usually less than 30kDa) secreted by white blood cells and a variety of other cells in the body in response to a number of stimuli. The two main producers are the T_H cell and the macrophage. Cytokines released from these two cells activate an entire network of interacting cells. They are involved in communication between cells and they assist in regulating the development of immune effector cells.[49] Some cytokines possess direct effector functions of their own. Cytokines tend to be secreted following the activation of a particular cell. The secretion is short-lived and can last for anything from a few hours to a few days. They bind to specific receptors on the membrane of target cells thus triggering signal transduction pathways, which ultimately alter the gene expression in the target cell. Cytokines and their receptors exhibit a very high affinity for each other so pico-molar concentrations are sufficient to produce a biological effect. The term cytokines encompasses those cytokines secreted by lymphocytes (lymphokines) and those secreted by monocytes and macrophages (monokines). Many of the cytokines are referred to as interleukins (IL). This indicates that they are secreted by leukocytes and that they act upon other leukocytes. Interleukins 1 – 17 have been identified to date.

Among the numerous physiological responses that require cytokine involvement are the development of cellular and humoral immune responses, induction of the inflammatory response, regulation of haematopoiesis[50] (production of red blood cells), control of cellular proliferation and induction of wound healing.[51]

Cytokines involved in the immune response play an important role in determining which cells or class of antibody will be involved in the host defense process. Interferon gamma (IFN-γ) and interleukin-2 (IL-2) induce the formation of cellular immunity mediated by T helper cells. Interleukin-4 (IL-4) is involved the immediate hypersensitivity responses by stimulating Th2 lymphocytes that regulate the production of IgE by B cells.

The presence of cytokines in the ocular environment is clearly of great value. It has been demonstrated that cytokines are present in basal, uncompromised, tears (see section 1.7, tear states), as well as in incidences of infection.[50, 52, 53] Various cytokines have been shown to be present in subjects with intraocular inflammation. Ocular cells that have been shown

to produce cytokines include the retinal pigment epithelium, lens epithelial cells, corneal stromal and corneal epithelial cells.

Epidermal Growth Factor (EGF) is the most studied of the cytokines. EGF plays a crucial role in epithelial and endothelial wound healing. The function of EGF varies according to which other cytokines are present. It is found in very low concentrations in a basal tear (approximately 5.3 ng/ml)[52]. This is sufficient to trigger an amplified cascade of other responses however. Sjögrens syndrome results in a decreased concentration of EGF.

A summary of some selected functions of some cytokines is shown in Table 1.2.

Cytokine	Secreted by	Target cells/tissues	Activity
IL-1	Monocytes, macrophages, B cells	T_H cells, B cells	Co-stimulates activation Promotes clonal expansion Enhances activity
		Natural killer cells	Induces proliferation
IL-2	T_H1 cells	Antigen primed T_H and T_C cells	
IL-3	T_H cells, mast cells	Haematopoietic cells, mast cells	Stimulates growth & histamine secretion
IL-6	Monocytes, macrophages, T_H2 cells,	Proliferating B cells, plasma cells	Promotes terminal differentiation into plasma cells, stimulates antibody secretion
IL-8	Macrophages	Neutrophils	Induces adherence to vascular endothelium
IL-10	T_H2 cells	Macrophages	Suppresses cytokine production
IFN-α	Leukocytes	Uninfected cells	Inhibits viral replication
IFN-β	Fibroblasts	Uninfected cells	Inhibits viral replication
IFN-γ	T_H, T_C and natural killer cells	Uninfected cells, macrophages, inflammatory cells	Inhibits viral replication, enhances activity, mediates various effects important in hypersensitivity reactions
TNF-α	Macrophages, mast cells	Tumour cells, inflammatory cells	Cytotoxic effect, induces cytokine secretion
TNF-β	T_H and T_C cells	Tumour cells, macrophages and neutrophils	Cytotoxic effects, enhances phagocytic activity

Table 1.2 – Selected functions of some cytokines

1.4.2.9 The Kinin System

The kinin system is an enzymatic cascade that begins when Hageman factor (a clotting factor) is activated following tissue injury. The activated Hageman factor then activates prekallikrein to form kallikrein. This then cleaves kininogen to produce bradykinin. This inflammatory mediator is a potent vasoactive peptide that increases vascular permeability, induces pain and the contraction of smooth muscle.[54, 55]

The interaction of a variety of species (including Hageman factor, prekallikrein and high molecular weight kininogen) with negatively charged species results in Hageman factor being activated and ultimately cleaved. The complement system can also be activated by kallikrein. Bradykinin is released as a result of the digestion of high molecular weight kininogen by kallikrein. This results in the cleavage of C5 into C5a and C5b. The C5a complement component is an anaphylatoxin that induces mast cell degranulation. This results in the release of a number of inflammatory mediators from the mast cell.

Kininogens are single chain glycoproteins found in two forms. High molecular weight kininogen (HMWK) has a molecular weight of 120 kDa. Low molecular weight kininogen has a molecular weight of 68 kDa.[56] They have a heavy chain in common but they each possess a unique light chain. Low molecular weight kininogen (LMWK) has a much shorter light chain.[57] HMWK produces bradykinin upon cleavage whereas LMWK produces kallidin (lysyl-bradykinin). They are responsible for triggering the inflammatory responses described earlier.

Very little work has been carried out so far on the significance of kininogen within the ocular environment. HMWK has not been detected in open eye basal tears. This indicates that it is not present in the tears of normal, non-contact lens wearing, individuals. It has, however, been detected in deposits extracted from lenses worn in both a daily wear and an extended wear regime. The implications of these findings as yet remains to be determined.[58, 59]

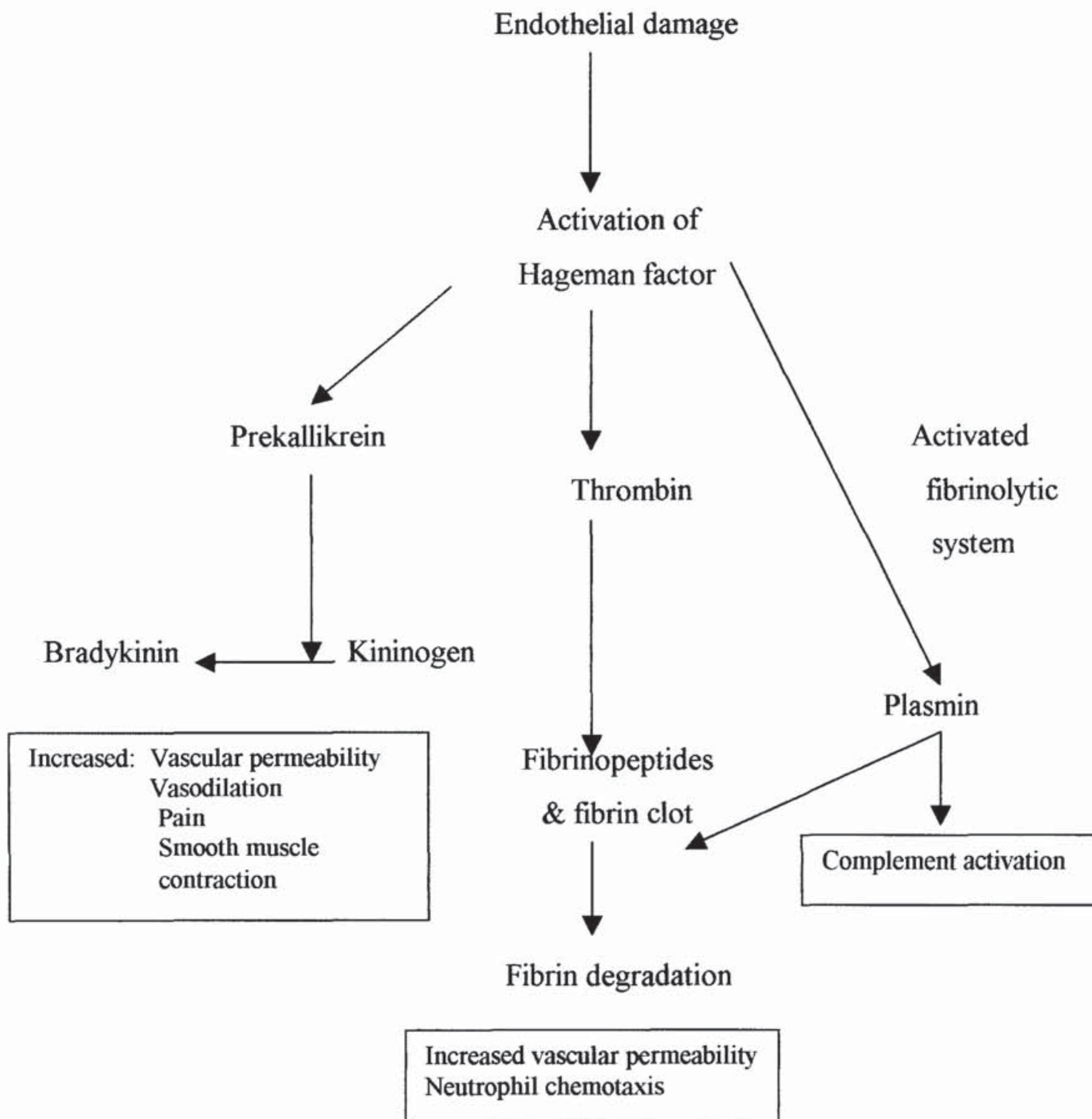


Figure 1.6 - Formation of plasma enzyme mediators following tissue damage

1.4.2.10 Vitronectin

This glycoprotein may play a role in inhibiting complement and plasmin-mediated damage in other tissues. It is likely that it also protects the ocular surface against autolytic damage.[60] It is known that vitronectin potentiates microbial phagocytosis by polymorphonuclear cells and enhances the efficiency of opsonisation and processing of

microorganisms by monocytes and macrophages. It is found in human tear fluid in increased concentrations during the closed eye state. Eye closure is associated with a change in the pre-ocular tear film. The tear fluid changes from one considered to be in a state of dynamic equilibrium to one that contains high concentrations of secretory IgA.[61] Complement activation occurs, serum albumin builds up and polymorphonuclear (PMN) cells enter the tear fluid. These changes indicate a state of sub-clinical inflammation.[62] This is thought to protect the closed eye from the entrapment and proliferation of pathogens as it is in a more vulnerable state during the closed eye state than it is in the open eye state. Vitronectin is considered to be mainly a liver derived serum glycoprotein. It is also distributed in loose connective tissue. Other suggested functions of vitronectin include its ability to control haemolytic processes (it can bind factors such as heparin), cell migration, tagging of damaged cells for phagocytic processing through vitronectin-specific receptors, and also cellular adhesion due to direct interaction with the cell binding domain of vitronectin.

1.4.2.11 Fibronectin

Fibronectin is a high molecular weight glycoprotein (reported to be 500 kDa)[63] found in various body fluids – including plasma and amniotic fluid.[64] It has a number of functions including cell adhesion, migration and differentiation[65], and enhancement of phagocytic activity by leukocytes and macrophages.[66] It also plays an important role in corneal wound healing.[67] Fibronectin in the tear fluid may originate from the conjunctival blood vessels through the tear film. It may be secreted from the lacrimal gland, or from the ocular surface epithelium – either corneal or conjunctival. It appears at the site of injury and disappears upon the healing of the wound.

Tear fibronectin concentration may increase during the closed eye state, as it is known that fibronectin accumulates during inflammation. This has been observed in inflammation of the lungs, joints and vitreous. Levels of fibronectin are shown to increase following cataract surgery. The concentration of fibronectin in the tear film has been estimated to be 3 – 9 μg/ml

1.4.2.12 Mucosal Associated Lymphoid Tissues

The mucous membranes lining the digestive, respiratory and urogenital systems have a combined surface area of about $400m^2$. They are also the major sites of entry for most pathogens. The defense of these membranous surfaces is provided by a group of organised lymphoid tissues collectively known as mucosal-associated lymphoid tissue (MALT). These tissues range from loose clusters of lymphoid cells with little organisation to organised structures such as the tonsils, appendix, and Peyer's patches. There is a high population of antibody-producing cells found within MALT.

The epithelial cells of mucous membranes play an important role in promoting the immune response by delivering small samples of foreign antigen from the lumina of the respiratory, digestive and urogenital tracts to the underlying mucosal-associated lymphoid tissue. Specialised cells called M cells carry out this antigen transport. They are flattened epithelial cells. They contain a deep invagination in the basolateral plasma membrane, which is filled with a cluster of B cells, T cells and macrophages. Luminal antigens are endocytosed into vesicles that are transported from the luminal membrane to the underlying pocket membrane. The vesicles then fuse with the membrane of the pocket and deliver the antigens to the clusters of lymphocytes found within it.

M cells are located in inductive sites – small regions of a mucous membrane that lie over organised lymphoid follicles. Antigens that are transported across the mucous membrane by M cells activate B cells within these follicles. The activated B cells differentiate into plasma cells, which then leave the follicles and secrete IgA. IgA is then transported across the epithelial cells and is released as secretory IgA into the lumen where it can interact with antigens.

Mucous membranes provide an effective barrier against the entrance of most pathogens. Mucosal epithelial cells are cemented to one another by tight junctions that make it difficult for pathogens to penetrate.[38]

The conjunctival system is called the conjunctival associated lymphoid tissue (CALT). The overlying epithelium is highly specialised. Antigens are taken up at this site. The lymphoid aggregates consist of small and medium sized lymphocytes. There are no plasma cells

present. To process foreign antigens they exert their response in conjunction with lymphocytes of the B and T cell populations.

Langerhans' cells are found in the epidermal and mucosal epithelial surface. They process antigen and present it to T cells via epithelial surface. The Langerhans' cells can present antigen to activate secretory IgA and also to produce a T-cell mediated response. These antigenic signals bring about a response in T cells and B cells in the lymph nodes. They then migrate to the mucosal site via the bloodstream. The B cells migrate to the sites beside the lacrimal and accessory gland epithelia where they differentiate into plasma cells capable of producing immunoglobulins. The main antibody to be produced is secretory IgA although IgG, IgM and IgE can also be produced. T cells migrate to the sub-mucosal sites of the conjunctiva. They produce cytokines.[59]

Table 1.3 (shown overleaf) gives a summary of components present in the tear fluid that possess antibacterial properties and are important for ocular protection.

Component	Function
Lysozyme	Antibacterial Inhibition of C3 convertase
Lactoferrin	Iron binding
Peroxidase	Bactericidal, virucidal, fungicidal
Ceruloplasmin	Chelates copper ions Acts as superoxide dismutase
Lipocalin	Enhances lysozyme activity
Fibronectin	Facilitates phagocytosis
Collagenase	Collagen degradation
Sialin	Binds microorganisms
Plasminogen activator	Chemoattractant for leukocytes
α_1-antitrypsin	Modulation of tissue degrading enzymes
α_1-antichymotrypsin	Modulation of tissue degrading enzymes
α_2-macroglobulin	Protease inhibitor
β-lysin	Antibacterial activity
Cytokines	Recruitment of immunocompetent cells
Complement	Opsonisation, perforins
Antibodies (IgA, sIgA, IgG, IgM, IgE)	Histamine release

Table 1.3 - Tear film components with antibacterial properties [14]

1.4.3 Transfer/Carrier Proteins

Proteins present in the ocular environment carry out essential functions such as the transportation of metabolites from the vascular areas that are well supplied to the avascular areas (e.g. the cornea). Lipocalin and albumin are two such examples of these proteins.

1.4.3.1 Albumin

Serum albumin (molecular weight 68 kDa) is a relatively minor component of tears. Albumin found in tears is derived from the serum. Its role in tears is not well understood although it is known that its presence is due to the permeability of the blood-tear barrier. In plasma, albumin acts as a transport protein for free fatty acids. It is postulated that it may perform the same function in the tear fluid. In a normal, open, eye albumin is found at concentrations of approximately 1.3 mg/ml. In contrast, the concentration of albumin in the serum is 50 mg/ml. Epithelial damage or vascular leakage can dramatically increase this value in tears. Elevated concentrations of albumin in tears can therefore be an indicator of inflammation.[68]

1.4.3.2 Lipocalin

Many terms have been given to this protein over the years including Protein Moving Faster than Albumin (PMFA), Anodal protein and Tear Specific Prealbumin. In more recent times, it was determined that this protein is actually a member of the lipocalin family.[69] There are a number of lipocalins present in tears.[70]

Tear lipocalins are the main lipid binding proteins in tears. They have the ability to bind a variety of lipids including fatty acids, cholesterol, phospholipids and glycolipids.[71] It is thought that tear lipocalins increase the solubility of lipids in the tear film and may enable a lipid layer to establish itself on the surface of the tear film. Tear lipocalins have a very high affinity for the relatively insoluble long chain fatty acids and phospholipids. They are the only protein component found in tears that can bind to lipid components in any significant

way. For this reason, they are often thought of as being "lipid scavengers."[72] An important function they may perform is the removal of lipids from the surface of the cornea.[13]

Tear lipocalins are found in all normal tear samples. They constitute 15 – 33% of total tear protein and are found at a concentration of approximately 1.7 mg/ml.[70] The concentration of tear lipocalin seems to correlate with the stability of the tear film. In conditions where there is destruction of the lacrimal glands the concentration of tear lipocalin is lowered.

1.5 Electrolytes

The electrolytes present in the tear film are responsible for the osmolality of the tears. They also play an essential role in maintaining epithelial integrity.

The predominant cation found in the aqueous phase is sodium.[73] It is found in similar concentrations to that of serum at 145 mmol/ml. Potassium is another cation found in tears. However, this is found in much higher concentrations than it is in serum. It has been shown that the concentration of potassium is 3 – 6 times higher than it is in serum. Magnesium and calcium are also found at low concentrations in the tear fluid. Calcium has also been implicated in lens deposits.

Chloride and bicarbonate ions are the main anions found in tears.[73] They are found at concentrations similar to that of serum.

A great deal of variation in the concentration of electrolytes is observed between individuals and also between the open and closed eye states.

As has already been discussed, there are many mechanisms of defense that exist to protect the ocular environment. Some of these mechanisms are summarised below in Table 1.4.

Non specific	Specific
Eyelids	Immunoglobulins - Tear - Cornea - Comjunctiva
Tears	Complement (classical pathway) - Tears - Conjunctiva - Cornea
Complement (alternative pathway) - Tears - Cornea - Conjunctiva - Aqueous humour	Lymphocytes
Epithelia of ocular surface - Cornea - Conjunctiva	Macrophages
Normal flora of the external eye	
Macrophages	
Natural killer cells	

Table 1.4 - Host defense mechanisms of the ocular surface [14]

1.6 Summary of the Functions of the Tear Film

- Maintenance of vision – the tears provide a smooth optical surface by filling in irregularities of the corneal surface[23]
- Protection of the ocular environment – the presence of antibacterial agents in the tear fluid help to reduce the risk of infection. Irritants and foreign bodies can also be washed away. In addition, waste products of corneal metabolism can be removed[18, 74]
- Lubrication – reduces the friction of the eyelids passing over the ocular surface[24]
- Oxygen delivery to be delivered to the avascular cornea. Oxygen from the ambient air dissolves in the tear fluid and is transferred to the corneal epithelium[23]
- Delivers nutrients which are transferred from the tear fluid to the cornea

1.7 Tear States

A great deal of variation can be observed in the protein composition of the tear film between individuals. However, variation of an individual's tear film can be observed in response to different stimuli. It is important to note the tear state that is actually being sampled when any analysis is being carried out on tears due to the difference in tear composition in various states.[75, 76, 77]

- Basal tears – this type of tear reflects most accurately the normal levels of tear proteins. This is the tear flow rate needed to maintain a lacrimal film on the corneal surface for optical, mechanical and lubricative purposes. Microcapillary pipettes are usually the preferred method of collection for this type of tear as it enables tears to be collected with a minimal degree of stimulation. Sampling should be at the normal rate of tear flow and no excess tearing should occur. Tears collected using this method have been shown to be rich in lactoferrin, lysozyme and lipocalin.[78, 79]
- Reflex tears – higher quantities of albumin, IgG and other leakage proteins are observed.[79, 80] Methods such as the Schirmer filter strip test result in this type of tear being collected. The abrasion of the conjunctival surface is thought to be responsible for the influx of these proteins as they show an increase in plasma leakage. A reflex tear is a flow produced in response to a physical stimulus. This type of tear washes out foreign bodies and irritating secretions from the ocular surface.
- Externally stimulated tears – excess lacrimation is observed following the inhalation of ammonia or other lachrymatory chemicals. The tears show lower protein levels as they are effectively diluted as a result of the excess watering.
- Emotional tears – also known as psychogenic tears. These are the tears of sadness, happiness and other emotional states. This type of tear does not appear to serve the eye in any way.[81] As such, their purpose is not truly understood. This type of tear is believed to be unique to humans. A higher protein level is observed in this tear type than in a stimulated tear sample.[82] Emotional tears also contain a higher concentration of manganese as this ion is thought to have an important function in affecting mood.[83]
- Open and closed eye tears – the term "state of sub-clinical inflammation"[19, 84] is commonly used when describing a closed eye tear sample. There is a reduced rate of flow, an increase in plasma leakage proteins (e.g. albumin), a decrease in lacrimal gland

secretion and the recruitment of massive numbers of polymorphonuclear cells into the tear fluid in the closed eye state[84, 85] This may reflect an alteration in the defense mechanism of the ocular environment during eye closure. Collection of a closed eye tear sample is almost impossible to achieve. A novel technique that is being developed may go some way towards achieving a closed eye tear sample with a greater degree of success than has been possible to date.

1.8 Contact Lenses and Deposition

The 1950s saw glass lenses being replaced by poly-methyl methacrylate (PMMA). This material was used for years as the standard 'hard' contact lens material. It offered a similar appearance and ease of fabrication to glass, had an acceptable surface wettability and was also extremely durable.[86] Despite these advantages, it was later discovered that PMMA lenses were essentially a barrier to oxygen transport. Thus its properties were far from ideal. A decade later came hydrophilic lenses made of poly- 2-hydroxyethyl methacrylate (polyHEMA). They differ considerably from PMMA lenses in that they are able to absorb a great deal of water (30 – 85% by weight) and become soft and flexible. Their ease of fitting and the increase in possible wearing time ensured a rapid growth in the market share. The rise in popularity and consumer demand for a lens that was easier to take care of led to the development of lenses used for overnight wear. This required a balance of properties to accommodate the three requirements of the ocular environment; namely the cornea, the eyelid and the tears.[87] The cornea is avascular and so the need to ensure oxygen transport to the corneal surface governs the permeability requirements of the hydrogel. The eyelid dictates the range of acceptable mechanical properties e.g. the upper and lower limits of elasticity.

There are many different hydrogel contact lens materials currently available, containing 30 – 80% by weight of water based on lightly cross linked (0.5 – 1.0%) combinations of various monomers including HEMA, vinyl pyrrolidone, methoxyethyl methacrylate, methyl methacrylate and methacrylic acid. Silicone hydrogel lenses have also recently been introduced. These contain novel silicone-based monomers and are designed to enhance oxygen permeability. The level of oxygen permeability is high enough to permit overnight wear.

Hydrogel lenses have been categorised by the Food and Drug Administration (FDA) in to four groups. High water content lenses are those with a water content greater than 50%. Those lenses with a water content less than 50% are classed as being low water content lenses. Ionic lenses have more reactive surfaces than non-ionic lenses.

FDA group	Lens properties
I	Low water content, non-ionic
II	High water content, non-ionic
III	Low water content, ionic
IV	High water content, ionic

Table 1.5 - FDA Classification of Contact Lenses

The introduction of high water content extended wear lenses in the 1970s introduced other problems that were not previously encountered. Hydrogel lenses have been associated with adverse reactions and immunological host responses previously insignificant with PMMA lenses.[88, 89] Deposition is a major factor implicated in these detrimental responses. These deposits arise from both individual tear chemistry and environmental exposure. Tear components include proteins and lipids. Environmental deposits may arise from the use of cosmetics, airborne pollution and lens handling.[90]

During contact lens wear, the tear film acts to maintain an optically uniform interface between the air and the anterior lens surface, to remove debris from the front and from beneath the lens and to ensure the eyelids move smoothly over the front surface of the lens. The introduction of a lens into the eye disrupts this otherwise "ordered" film. Biocompatibility has been, and remains, a major problem associated with the wearing of contact lenses. The interaction of the tear film with a contact lens is comparable to the interactions that occur in other body sites in response to an implanted biomaterial.[91, 92] Unfortunately, the interfacial interactions between tissues or fluids and the biomaterial are poorly controlled and not very well understood.[93] Proteins and lipids from the tears can form deposits both on the surface of contact lenses and within the matrix.[94] This process begins within minutes of being inserted into the eye and continues throughout the period of wear.[95] It has been estimated that as much as 50% of the surface of the lens is soiled within thirty minutes. This can drastically reduce the wettability of the lens surface leading to discomfort and reduction in visual acuity.

Deposits forming on the surface of contact lenses often hinder successful contact lens wear. They cause irritation for the wearer and may also reduce the wearing time.[96] There are various types of deposits e.g. proteins, lipids, mucins and microorganisms.[97] Muco-protein lipid deposits (white spots) are one of the most complex manifestations occurring as a result of interactions between the tear film and contact lenses. These 'white spots' are approximately 1mm in diameter and found on the anterior lens surface.

The type of material, the quality/composition and pH of tears, inadequate blinking, lens wear modality and lens hygiene are all factors that affect the formation of lens deposits.[98] These deposits could lead to a variety of symptoms ranging from mild discomfort, itching, blurred vision and giant papillary conjunctivitis, red eye, increased irritation and conjunctivitis.[99 - 104]

Surfactant and enzymatic cleaners are able to remove most of the protein accumulating onto the surface of the lenses. They are not, however, able to prevent this build-up in the lens. The patient also has to be relied upon to be compliant with the cleaning schedule in order to maintain hygienic lenses. A lack of compliance has been shown to be a major implicating factor in the development of many adverse symptoms experienced by lens wearers.

This problem of deposition and the associated clinical effects led to the introduction of disposable contact lenses in the mid 1990s. They were thought to offer many advantages to lens wearers when compared to conventional soft lenses including better visual performance, lower incidence of deposition, and the removal of the necessity for a cleaning schedule.[105] This in turn meant that there was less patient handling of the lens and, theoretically, less opportunity for microbial contamination.

Studies carried out on patients in the daily disposable wearing regime have shown that the introduction of lenses worn in such a modality has significantly reduced the symptoms described above e.g. infectious and allergic conjunctivitis.[106 - 109]

Contact lenses remain a popular method of vision correction and are worn by approximately 85 million people globally.[110] New materials are being continually developed in the hope of improving vision and comfort during lens wear.[90] However, the host-material interactions which follow the implantation of any device or prosthetic is a complex, and poorly

understood, series of events. A clearer understanding of the nature, complexity and significance of these events should lead to the development of improved materials for use within the body.[111, 112]

1.9 Complications of Contact Lens Wear

The composition of the tear film must be qualitatively and quantitatively maintained within fairly narrow limits in order to maintain a functional visual system. Abnormalities of the tear film can very quickly lead to serious dysfunction of both the eyelids and conjunctiva. This can eventually lead to a loss of corneal transparency.

1.9.1 Dry Eye

"Dry eye" is one such disorder of the tear film that has been of particular interest in the field of contact lens research. The term "dry eye" encompasses a wide range of diseases that cause desiccation of the ocular surface. It covers a multitude of disorders ranging from a marginal dry eye to Sjögrens syndrome. It has been suggested that the distribution and/or character of ocular surface mucins may alter in dry eye sufferers. A dry eye is said to exist when the quality and/or quantity of the precorneal tear film is not sufficient to maintain the ocular health of the ocular epithelial surface. Sufferers of dry eye often show poorer tear film stability and greater damage to the ocular surface compared to normal eyes.

There is a very high prevalence of dry eye symptoms in contact lens wearers. The creation of an adequate tear film at the surface of the contact lens and the surrounding areas is impaired upon insertion of a contact lens.

The formation of the mucous layer relies on it being spread during the blink action. Its coverage will be limited, irregular and less stable during contact lens wear. A limited supply of mucin will lead to areas of instability and hence a rapid break-up of the tear film.

If there is an adequate supply of tears, the aqueous phase will form a regular film a few microns thick above the mucous layer. If there is a reduced tear supply, the mucous layer will only be covered with a thin and unstable aqueous layer, which will dissipate quickly by evaporation. This, in turn, will lead to a reduced break up time and an increased rate of deposition.

An even and regular lipid layer should form at the surface of the aqueous phase to reduce evaporation and to provide the tear film with greater stability – provided there is an

adequate supply from the meibomian glands. If the lipid coverage is inadequate, this will lead to increased evaporation.[112]

The loss of water from the tear film increases tear osmolarity and causes dry eye. This could be due to conditions that decrease tear production or by those that increase tear evaporation. Tear secretion can be decreased by autoimmune diseases that cause damage to the lacrimal gland or by conditions that decrease corneal sensation such as the long term wear of hard contact lenses.

Patients with dry eye from either the reduced production of tears or from increased evaporation will often complain of a gritty sensation in their eyes. At night, eye closure forms a watertight seal over the tear film, which allows the ocular surface to recover. Evaporation will begin again upon opening of the eye. Tear film osmolarity will increase throughout the course of the day hence symptoms get worse as the day progresses.

1.9.2 Giant Papillary Conjunctivitis

Giant papillary conjunctivitis (GPC) is fairly common amongst contact lens wearers. Soft lenses are most often implicated in this condition although any contact lens material can cause it. Patients with asthma, hay fever or other allergies are often at increased risk. It is thought that GPC has an immunological origin and that lens deposits, especially proteins, may be a triggering factor in bringing this condition about.

This condition may arise months or even years after beginning lens wear. The patient will often complain of the eyes itching when the lenses have been removed, an increased production of mucous in the morning, increased photophobia and a reduced level of tolerance to lens wear.

Improving lens hygiene and reducing the length of time the patient wears their lenses are usually sufficient to treat this condition.[113, 114, 115]

1.9.3 Allergic Conjunctivitis

Thiomersal is a preservative formerly used in contact lens care solutions. It tends to be used far less often nowadays as 10% of all lens wearers experienced allergic responses to it. They were therefore unable to use solutions containing this preservative.

Patients would present with redness, burning and itching soon after inserting lenses. These symptoms may not develop until months after the initial exposure had taken place.

Avoiding solutions containing Thiomersal and using a 3% hydrogen peroxide system would normally be sufficient to treat this condition.[114]

1.9.4 Contact Lens Acute Red Eye

Contact lens acute red eye (CLARE) is a corneal infiltrative event that often has a rapid onset. It has been observed during extended wear of hydrogel contact lenses. Lenses are often found to have elevated levels of Gram negative bacteria during incidences of CLARE. It would seem that CLARE is an acute inflammatory response to toxins including lipopolysacharide, enzymes and other bacterial products.

Symptoms of CLARE include:

- No symptoms before eye closure
- Irritation to moderate pain
- Redness
- Burning sensation
- Photophobia

It is necessary to discontinue lens wear if this condition arises. Anti-inflammatory medication or antibiotics are usually not required.[113, 114, 116]

1.9.5 Microbial Keratitis

This is the most serious, but fortunately a very rare, complication associated with contact lens wear. It may result in permanent visual damage to the cornea.

Contact lenses are known to be an implicating factor in the development of this disease in otherwise previously normal eyes as they affect the corneal epithelium. Lenses worn on an extended wear basis are thought to increase the risk although it can also occur with lenses worn on a daily wear modality. The adhesion of *Pseudomonas aeruginosa* to soft contact lenses is an important initial stage in the pathogenesis of microbial keratitis.[117, 118]

Microbial keratitits (MK) is a sight threatening condition and requires urgent identification and eradication of the causative organism.

Symptoms of MK include:

- Moderate to severe pain that may have a rapid onset
- Severe redness
- Blurred vision
- Photophobia
- Puffiness of eye lids

The patient will require a course of antibiotics and will be taken out of lens wear until the condition abates. Improved attention to lens hygiene is important to reduce the build up of deposits on the surface of the lens. Extended wear lenses should not be worn for longer than is prescribed as this has been implicated as being a major risk factor for damaging ocular health.[113, 114, 119, 120]

1.10 Aims and Scope of Research

Dry eye is a problem experienced by many contact lens wearers. It can often lead to an inability to wear lenses due to the discomfort that is experienced. Research is constantly being undertaken to formulate new materials that will improve the biocompatibility of lenses to minimise adverse effects.

It has long been thought that proteins may play a key role in dry eye due to their central role in immunological responses. This thesis is concerned with obtaining a clearer understanding of the tear protein profile using various analytical techniques, including sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting. The sensitivity of Western Blotting has made it an invaluable tool in the detection of very low concentrations of specific proteins in the tear film. IgE is of particular importance due to its known role in hypersensitivity reactions. Following modification and optimisation of these techniques, they will be applied to studies which aim to determine whether contact lens wear causes the tear film to deviate from a normal base line.

The central feature of this work is the application of these techniques to the study of the transition from the non-lens wearing eye to the lens-wearing eye.

A clinical study will be undertaken which will follow a group of subjects from the neophyte stage to the time at which they are adapted lens wearers. Neophytes will be taken and studied for the first twelve months of lens wear. The commercial availability of silicone hydrogels that can be worn both on a continuous wear basis (e.g. overnight) and a daily wear basis means that the wear modalities can be studied in parallel. There are currently two materials available with significant differences in their surface and stiffness.

The study is designed around four equal groups of lens wearers. They will be wearing:

- Focus® Night and Day™ – daily wear basis
- Focus® Night and Day™ – continuous wear basis
- PureVision™ – daily wear basis
- PureVision™ – continuous wear basis

- Control group – they will be non-lens wearers and will be used to enable the effects of lens wear to be compared against a group of normal, healthy, eyes over the same time period.

During this time, clinical measurements will be made to assess the ocular health of the participants. Tear samples will also be obtained at specified intervals allowing for changes taking place to the tear film from a biochemical and immunological point of view to be monitored. The overall aim is to identify proteins that can be used as biochemical markers and, as such, used to predict detrimental changes taking place to the tear film. This will enable those people likely to become intolerant lens wearers to be identified.

Participants will also complete a questionnaire that has been specifically designed for this study. This will enable their subjective response regarding comfort to be compared with the clinical and laboratory data that will be generated. This study will allow for a variety of parameters to be investigated including: lens material, wear modality, and patient variability.

Chapter 2

Materials and Methods

2.1 Introduction

This chapter outlines the techniques utilised in the collection and analysis of tear samples and contact lens extracts. Further details are provided in the relevant results chapters.

2.2 Tear Collection

There are a number of methods available for the collection of tears. It is widely reported in the literature that the collection technique employed can greatly affect the values of the proteins within the tear sample depending on the degree of conjunctival stimulation.

Amongst the many techniques available for the collection are the Schirmer test, the filter paper method, sponge collection and microcapillaries.

2.2.1 The Schirmer Test

The Schirmer test utilises a strip of filter paper being inserted into the lower conjunctival sac. The strip is then allowed to simply absorb the tears. This is a simple and inexpensive test but usually results in reflex tears being collected as excess watering occurs. This dilutes the normal tear protein levels and causes plasma leakage. Plasma derived proteins, including albumin, would be present in elevated concentrations using this technique.

2.2.2 Filter Paper

The filter paper method is similar to the Schirmer test in that the filter paper is placed in direct contact with the eye. The Schirmer test has the added advantage however that it can also calculate the tear flow rate – which cannot be done with this method.

2.2.3 Sponge Collection

The sponge collection method uses a sponge 1.5cm in length. It collects tears from the inferior marginal strip to the medial canthus. The sponge opens out as the tears are adsorbed. Large volumes of tears are required. This technique is often used when lipid analysis is to be carried out on the tear sample.

2.2.4 Microcapillary

Throughout this study, tears were collected using narrow bore glass microcapillary pipettes (The Binding Site, Birmingham). Previous work undertaken within the group had found this to be most suitable method. Tears collected this way were more representative of the normal tear fluid. The tear fluid was found to be rich in proteins derived from the lacrimal gland with minimal plasma leakage. This is also a less traumatic technique to the subject compared to the techniques described earlier.

The tip of the capillary is placed in the lateral canthus and/or the inferior marginal strip. Tears are then drawn into the capillary. A plunger is inserted into the top of the capillary to transfer the tear fluid into an eppendorf.

2.3 Extraction of Proteins from Contact Lenses

When a contact lens is inserted into the eye, it accumulates proteins, lipids and other components of the tear film on the surface. Some of these species can also penetrate the matrix of the lens. This can occur within a minute of the lens being placed into the eye. Such deposits have been implicated in allergic and inflammatory reactions, mechanical irritation and microbial contamination. Protein deposition was the area of central importance in this study as proteins are known to be involved in immunological reactions as part of the host defense mechanism.

2.3.1 Preparation of Contact Lens Extracts

Solutions (for all recipes see Appendix 1)

Extraction solution (1)

The concentration of total protein deposited on the lens was initially determined by ultra-violet (UV) spectrophotometric analysis (Section 2.5). The lens was then placed into 200µl of an extraction solution (1) containing 40% urea, 1% SDS, 1 mM DTT and 100mM Tris solution (pH 8.0). The lens was then heated to 90°C on a heated stirrer for 3 hours in a 1 ml

eppendorf. The solution was then allowed to cool before being transferred to a fresh eppendorf and stored at 4°C until required.

2.3.2 Collection of the Tear Envelope

The extraction technique described above uses very harsh reagents at a high temperature for a long period of time. As a result, many protein species will be denatured. Following SDS-PAGE, it was observed that it was not possible to identify individual components that had been removed from the lens.

A novel technique was developed which allowed the tear envelope attached to the lens to be removed. It arose as a result of discussion within the Biomaterials Research Unit; preliminary attempts to use it have been described by Olsen.[121, 122]

Solutions (for all recipes see Appendix 1)
Sample treatment buffer (2)

The freshly removed lens was placed into a 0.5 ml eppendorf containing 40μl of sample treatment buffer (2). Tweezers were used to place the lens into the eppendorf, as a minimum of handling was required. It was imperative to ensure that the lens was completely immersed in the treatment buffer. A vortex mixer was then used to ensure that the lens surface was thoroughly washed by the solution. An incision was made in the bottom of the eppendorf, which was then placed inside a larger, 1.5ml, eppendorf. This was then centrifuged at 5000 rpm for 10 minutes.[121] The resulting eluate contained the tear envelope and was ready for analysis. A diagrammatic representation of this technique is shown below. (See Figure 2.1).

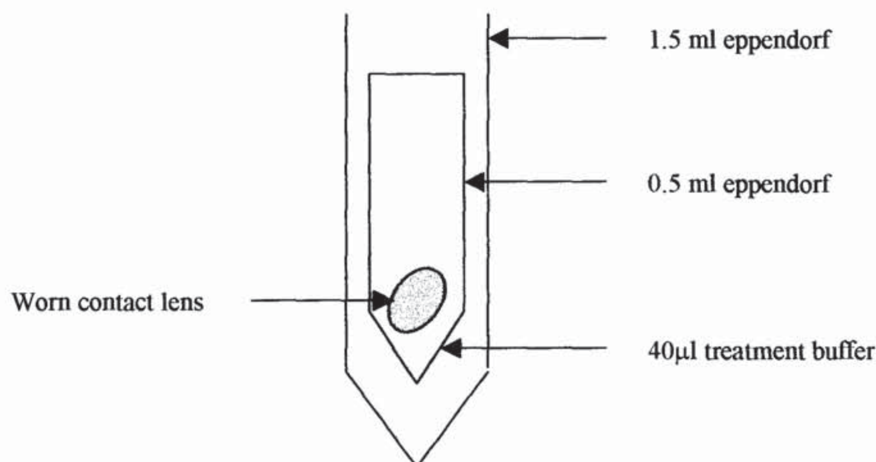


Figure 2.1 Collecting the tear envelope

2.4 Sample Treatment

The sample to be analysed (tear sample or contact lens extract) was transferred to a 0.5ml eppendorf. An equal volume of treatment buffer (2) was then added. The resulting solution was then boiled for ten minutes prior to being analysed by SDS-PAGE. Tear proteins are very sensitive to changes in sample buffer temperature and incubation time with buffer. It is reported that they are more susceptible to change than are serum proteins. To obtain comparable results, the above protocol needs to be carefully adhered to.[123, 124]

2.5 Ultra Violet Spectrophotometry (UV)

A Hitachi U2000 spectrophotometer was used to study the amount of protein deposited onto the surface of a worn contact lens. This technique takes advantage of the fact that most proteins display an absorbance peak at 280 nm due to intrinsic aromatic amino acids, which can be measured and translated into protein concentration.

The lens to be analysed was inserted carefully into the bottom of the quartz cuvette in solution. It was placed in an upright position facing the light beam against the wall of the

cell. The cuvette containing the lens was then put into the absorbance cell sitting on an 8mm high rubber mount. This was to ensure the light beam hit the lens centrally. The lens was measured whilst in distilled water. The instrument was auto-zeroed prior to each lens being analysed with only distilled water in the cuvette and the reference cell. The absorbance measurement and calculated concentration was obtained for each lens upon pressing the "Start" button.

The absorbance of each lens plus deposition could be read directly from the instrument. To calculate the relative concentration, a linear calibration was performed. A calibration graph was set up of absorbance versus concentration using a set of standards of known concentration of the analyte under investigation. The absorbance of the unknown analyte was then measured and the concentration could then be calculated from the calibration graph.

2.6 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins are built up from amino acids. The primary structure is the chain of amino acids. The secondary structure is the way that the chains are helically coiled. The way that the helically coiled chains are folded is the tertiary structure of the protein.

Electrophoresis is a powerful technique that is relatively easy and inexpensive. It can be described as the transport of particles through a solvent by an electrical field. It enables protein mixtures to be resolved into individual components. The electrophoretic mobility of a protein is determined by its size, shape and net charge. SDS-PAGE works on the basis of denaturing the proteins and coating them with a uniform charge in order to minimise the effects of shape and charge. This then allows the proteins to be separated by size through a molecular sieve. (See Figure 2.2)

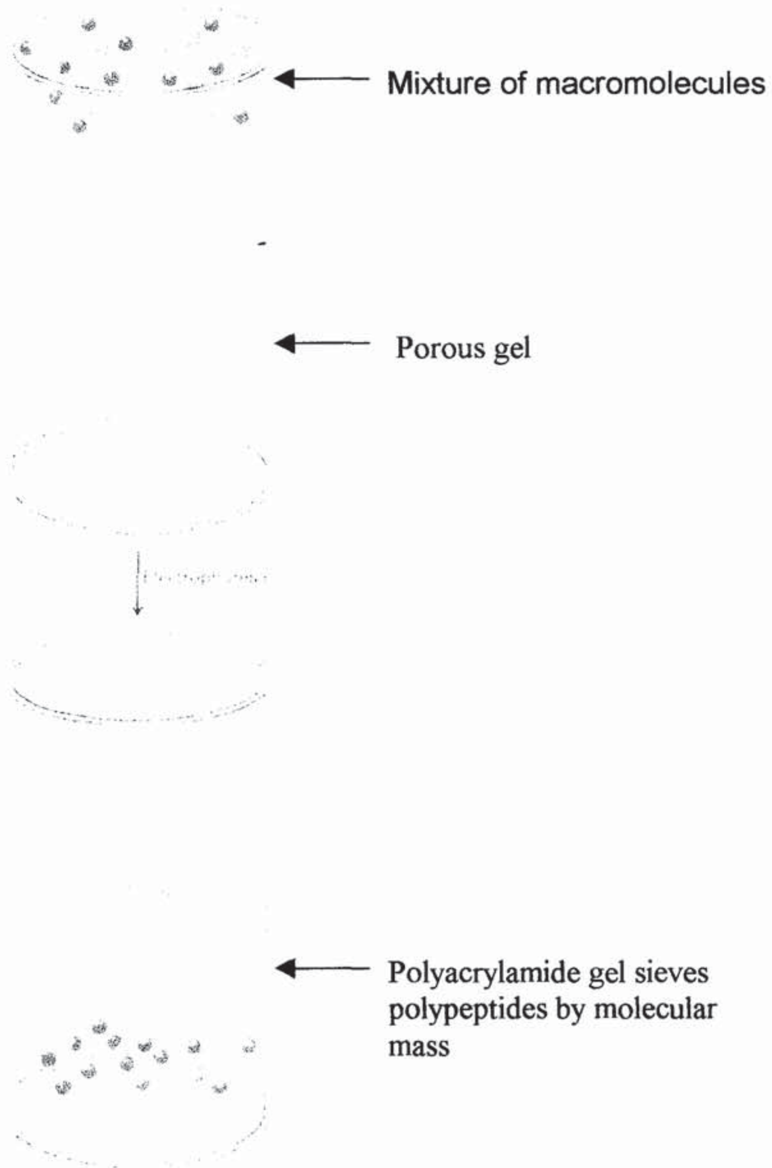


Figure 2.2 – Diagrammatic representation of electrophoresis[37]

The samples are boiled in a sample buffer as described earlier (2). The buffer contains β-mercaptoethanol (which reduces the disulphide linkages) and SDS. This anionic detergent denatures the polypeptides and covers them with a uniform negative charge. The treatment buffer also contains glycerol (to make the sample denser), tris (acts as a buffer) and bromophenol blue (a dye which allows the progress of the separation to be monitored). The result is a mixture of linear polypeptides with a negative charge that is proportional to their length (which is roughly proportional to their molecular weight). The mixture is then electrophoresed through a matrix of polyacrylamide, which acts as a molecular sieve. The smaller polypeptides move through the Polyacrylamide gel more rapidly than do the larger polypeptides.

Polyacrylamide gels are formed from the polymerisation of 2 compounds: acrylamide and N, N1-methylene-bis-acrylamide (Bis). Bis is a cross linking agent for the gels. The polymerisation is initiated by the addition of ammonium persulphate and N,N,N1,N1,-tetramethylethylenediamine (TEMED).

The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size is determined by 2 factors: the total amount of acrylamide present (%T) and the amount of cross linker (%C). As the total amount of acrylamide increases, the pore size decreases. 5%C gives the smallest pore size

2.6.1 Pouring the Acrylamide Gels

2.6.1.1 Solutions (for all recipes see Appendix 1)

Acrylamide stock (3)
1.5M Tris HCl (4)
10% SDS (5)
7.5%T gel solution (6)
20%T gel solution (7)
10% APS (8)
TEMED (9)
Water-saturated n-butanol (10)

2.6.1.2 Hardware

SE 245 casting cradle (Hoefer)

Notched alumina plates (Hoefer)

10 x 8 cm glass plates (Hoefer)

1.5 mm spacers (Hoefer)

Combs (Hoefer)

Cams (Hoefer)

2.6.1.3 Method

10ml of the resolving gel solution was prepared using a method supplied by Hoefer. It was possible to produce gels with varying properties but previous work carried out within the group had found a gradient gel of 7.5-20% gave optimal separation. (See Table 2.1)

Solution	7.5%T	10%T	12.5%T	15%T	20%T
Acrylamide stock	2.5ml	3.3ml	4.2ml	5.0ml	6.6ml
1.5M Tris HCl	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
Distilled water	4.9ml	4.1ml	3.2ml	2.4ml	0.8ml
10% APS	50μl	50μl	50μl	50μl	50μl
TEMED	5μl	5μl	5μl	5μl	5μl
Final volume	10.0ml	10.0ml	10.0ml	10.0ml	10.0ml

Table 2.1 Composition of resolving gel solutions

Two gel sandwiches were constructed. Each consisted of a notched alumina plate, two 1.5mm spacers, and one 10 x 8cm rectangular plate. The gel sandwiches were inserted into the casting clamps of a Hoefer Mighty Small SE 245 Dual Gel Caster with the alumina

plates facing the back. The screws of the clamps were then tightened to secure the sandwiches. Each clamp was then placed in to the casting cradle, with the screw facing outwards. Cams were inserted into each side of the casting cradle and rotated 180°.

The gel solution was then pipetted into the sandwich to just below the level of theglass plate. The gradient was produced by pouring 5ml of the highest percentage solution into the sandwich first (7). This solution also had 1.5g sucrose added to increase its density. 5ml of the lower percentage solution (6) was then poured on top of this. This solution had a higher concentration of APS, which allowed the gel to set from the top to the bottom.

A comb was then inserted into each gel sandwich. This enabled wells to be formed in the gel into which the samples would be loaded. The gels were then allowed to fully polymerise before the combs were removed. Following polymerisation, the combs were removed and the surfaces of the gels were rinsed with distilled water. Water-saturated n-butanol (10) was then overlaid to prevent oxidation of the gels. They were then covered with parafilm and stored at 4°C until required.

2.6.2 Loading and Running the Gels

2.6.2.1 Solutions (for all recipes see Appendix 1)

Running buffer (11)

2.6.2.2 Hardware

SE 250 electrophoresis unit (Hoefer)

Power pack

2.6.2.3 Method

A Hoefer SE250 electrophoresis unit was used throughout. The gel sandwiches were removed from the casting cradle and clamped into position in the unit. The upper and lower buffer chambers were then filled with running buffer (11). 5µl of the samples under investigation were loaded into the wells using a pipette. A molecular weight marker was always added alongside the samples so as to make it easier to determine the molecular

weights of the components separated. The gels were then allowed to run at 40mA (20mA per gel) constant current until the tracking dye had reached the bottom of the gel.

The running buffer was then poured off and the sandwiches unclamped. A Hoefer Wonderwedge was used to separate the plates.

2.6.3 Visualisation of Bands

2.6.3.1 Solutions (for all recipes see Appendix 1)

Preconditioning medium (12)
Working stain solution (13)
Destain solution (14)
Fixative solution (15)
Developing solution (16)

2.6.3.2 Hardware

Janke and kunkel 501 D flat bed shaker
Glass containers

2.6.3.3 Method

Following the separation of the sample under investigation into its individual components, the bands needed to be visualised. The most widely used method of visualising bands is Coomassie Brilliant Blue (CBB) due to its ease of use and its ability to detect low concentrations of proteins. CBB (13) is a dye, which forms non-covalent bonds with proteins. The gels were immersed in CBB for approximately 2 hours. At this point, they were transferred to a destain solution (14). The destain solution was changed about every 30 minutes until the bands were seen clearly with little background staining.

An alternative method of staining was to use a silver staining technique. This has the added benefit of being more sensitive than CBB. It does, however, require more steps in the process and can often leave a high background, which affects visualisation.

The gels were placed in a glass container that had been thoroughly cleaned to remove laboratory detergent. It was placed on a Janke and Kunkel 501 D flat bed shaker. 400ml of fixative solution was then added and left for 20 minutes with gentle agitation before being discarded and replaced with 400ml distilled water to rinse the gels. The gels were washed for 2 x 10 minute periods.

The gels were then transferred to 100ml developing solution. They were developed for about 20 minutes at which point the bands could be visualised without excess background staining. The development reaction was stopped by adding 400ml of a 5% acetic acid solution. The gels were then rinsed in distilled water.

2.7 Western Blotting

Antibodies are able to bind to their corresponding antigen with high specificity and affinity. Immunoglobulins are antigen-binding proteins found on the membrane of B cells. They are secreted by plasma cells and circulate in the blood. In order to create the specificity an antibody has for an antigen, B cells are able to rearrange and modify their chromosomal DNA and hence create a vast range of antibody molecules.

Many techniques employed in laboratories make use of antigen-antibody interactions. Western Blotting is one such example. This technique enables a specific protein in a complex mixture to be identified.

SDS-PAGE separates proteins electrophoretically but results in a complex pattern of protein bands. After this initial separation, it is possible to transfer the proteins to a membrane under the influence of an electrical current. Nitrocellulose is the membrane most commonly used during Western Blotting. It was discovered however that polyvinylidene difluoride (PVDF) was less prone to tearing so this was the membrane of choice utilised throughout this study.

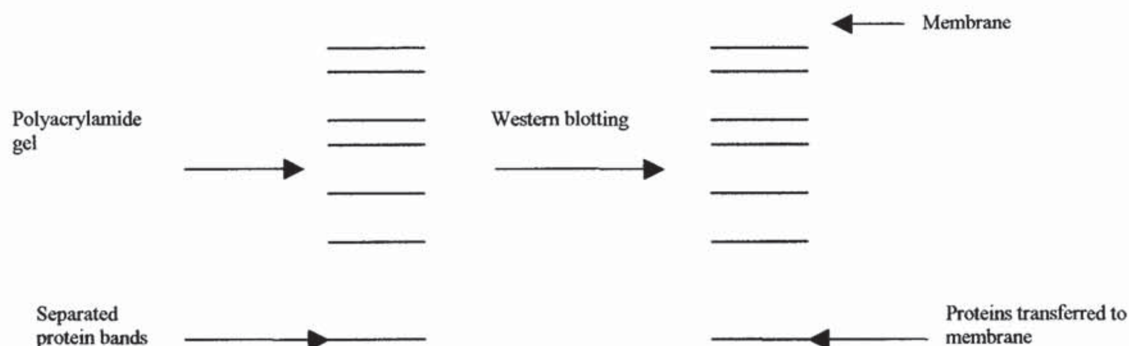


Figure 2.3 – Diagrammatic representation of Western Blotting

2.7.1 Solutions (for all recipes see Appendix 1)

Anode solution 1 (17)

Anode solution 2 (18)

Cathode solution (19)

2.7.2 Hardware

Kem-En-Tec horizontal semi-dry blotter

Whatman filter papers (125mm)

PVDF membrane

2.7.3 Method

A Kem-En-Tec horizontal semi-dry blotter (Anachem) was utilised. Semidry blotting is becoming increasingly popular as it is fast and uses less buffer than traditional Western Blotting methods. A discontinuous buffer system was used. This uses buffers of different composition and pH to create a discontinuous voltage and pH gradient.

In order to transfer the proteins from the gel to the membrane a series of filter papers (Whatman) were soaked in various buffer solutions. Twelve were soaked in anode solution

1 (17) and then placed on to the lower electrode. It was imperative to ensure that no air bubbles were introduced at any stage, as this would have disrupted the transfer to the membrane. A further three filter papers were then soaked in anode solution 2 (18) and placed on top.

A piece of PVDF membrane was cut to the same size as the slab gel. This was then wetted with methanol and placed on top of the filter papers. The gel was carefully placed on top of this. It was finally sandwiched by a further fifteen filter papers soaked in cathode solution (19). The upper part of the apparatus was then fixed in place. The semi-dry blotter was then allowed to run for 4 hours – the voltage was adjusted automatically according to the time of run.

Following the completion of the transfer, the membrane and gel were removed. The gel was stained in CBB to determine the extent of transfer whilst the membrane was immersed overnight in a blocking solution to block any non-specific adsorption of antibodies to the membrane.

2.8 Immunostaining of Western Blots

2.8.1 Solutions (for all recipes see Appendix 1)

Tris buffer saline (20)
Blocking solution (21)
BCIP/NBT tablets (22)

Anti-human IgA (α - chain specific)	(Sigma I-1261)
Anti-human IgD (δ chain specific)	(Sigma I-1386)
Anti-human IgE (ε chain specific)	(Sigma I-0632)
Anti-human IgG (Fab specific)	(Sigma I-9010)
Anti-human IgG (γ chain specific)	(Sigma I-1136)
Anti-human IgM (μ chain specific)	(Sigma I-1636)
Anti-human lactoferrin	(Sigma L-3262)
Goat IgG (whole molecule)	(Sigma A-4187)
Rabbit IgG (whole molecule)	(Sigma A-3687)

Antibodies specifically directed towards a given protein were used. The membrane was incubated with the primary antibody (diluted 1:1000 in blocking solution) for an hour. Excess antibodies were washed from the membrane during 3 x 5 minute washes in Tris Buffer Saline (TBS). The bound antibody, which remains on the membrane, was detected using a secondary antibody directed against the first. The secondary antibody is one that has been previously labelled with alkaline phosphatase. This was also diluted 1:1000 in blocking solution. Again, the membrane was incubated for an hour and then washed for a further 3 x 5 minutes in TBS.

The bands were then visualised using two 5-bromo-4-chloro-3-indolyl phospahete (BCIP/NBT) tablets dissolved in 20ml distilled water. Alkaline phosphatase reacts with BCIP to give BCI and free phosphate. BCI then reacts with NBT to give an insoluble blue colour thus allowing the protein to be visualised. The reaction can be stopped by immersing the membrane in distilled water. The membrane can then be stored indefinitely if not exposed to light.

2.9 Analysis of One Dimensional Gels

A software package (TotalLab™) was procured from Nonlinear to enable further analysis of the gels following SDS-PAGE. Although SDS-PAGE is a very useful technique and enables an estimation of the molecular weights of the protein components separated, it is also highly subjective. High levels of accuracy are not obtainable when the human eye alone is being used to identify the unknown species using a molecular weight marker and standard proteins of known molecular weight. Another drawback is that it is not a quantitative technique.

Upon completion of the staining stage, the gels were scanned into a PC using a commercial scanner. This image was then available for analysis using the TotalLab™ software. The software allowed for comparisons to be made within, and also between, gels. The background was subtracted to reduce the effects of differences in staining. It automatically detected the bands and, using a molecular weight calibration, it was also possible to determine the molecular weight of each separated protein. Another feature of this program, which made it so valuable, was its ability to normalise quantities against a known quantity.

This enabled changes in the concentrations of tear proteins occurring over time to be monitored.

Full experimental protocol for this software package is given in Chapter 3.

Chapter 3

TotalLab™ software

3.1 Introduction

TotalLab™ is a software package designed for the analysis of gels following electrophoresis. An extensive literature search suggests that it has not previously been applied to the study of tear proteins. This program has revolutionised the way in which data obtained from tears and lenses can be processed and evaluated. For this reason, a brief account of the software's capabilities and application to tear proteins is given here. SDS-PAGE is a valuable technique that allows complex protein molecules to be separated into their individual polypeptide components. The use of molecular weight markers and standard proteins allows the molecular weights of the separated components in unknown samples to be estimated. However, it is impossible to identify the exact molecular weight. This is obviously of paramount importance when monitoring changes that are taking place to the tear film over time. Another drawback to traditional methods of analysis was the inability to monitor changes in the concentrations of proteins in the tear film. It has been thought for many years that contact lenses may cause a departure from the normal in the concentrations of certain proteins. This may ultimately be responsible for the development of the dry eye symptoms so prevalent in many contact lens wearers. In order to monitor changes taking place in the concentrations of the protein components of the tear samples, it was previously only possible to make a qualitative assessment based upon differences in the degree of staining. The introduction of TotalLab™ has permitted accurate identification of the molecular weights of the tear samples obtained from the clinical study and also the relative concentrations of the proteins within the sample.

Following electrophoresis and staining as described in Chapter 2, the gel is scanned into a computer. This image is then used for subsequent analysis of the experimental data.

3.2 Lane Creation

This mode is used to identify the position of the lanes of the gel. (See Figure 3.1). The location of the lanes to be analysed need to be identified before analysis can commence. This is principally an automated software-driven process but needs manual adjustment to ensure that all lanes are located thus it is possible to edit the lanes that are marked out by resizing them or moving the boundaries. This ensures that the effect of any "smiles" or other distortions can be taken into consideration, which is important for any densitometry that is to be carried out.

The lanes are then automatically numbered. Lanes can also be added or deleted as necessary. This ensures that analysis is not carried out on lanes that have not had any samples loaded into them, or for lanes that the investigator does not wish to analyse.

The lanes can be created automatically by simply pressing the "Create" button. The program will draw two red lines across the top and bottom of the lanes as well as outlining the defined lane areas. (See Figure 3.1). Alternatively, lanes can be created manually by dragging out a rectangle in the image window over the gel. This shows the lanes and their size. The width of the lanes can be adjusted by moving the cursor over the perimeter of the rectangle to the desired location at the edge of the lane. This also makes it possible to adjust the position of the lane to counteract any warping that may have taken place.

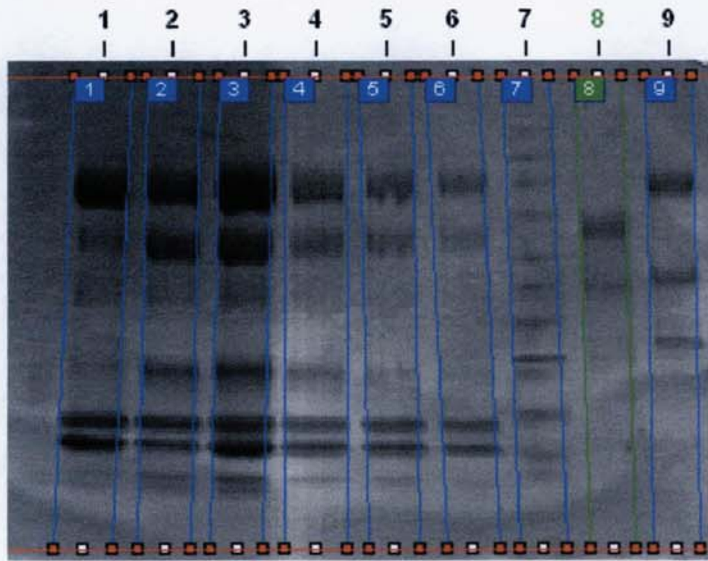


Figure 3.1 – Lane creation

3.3 Background Subtraction

It can be seen by inspection that the differences in intensities of the lanes and individual bands vary considerably. Thus a systematic background subtraction is vital for identifying and quantifying the data points. This stage was carried out to minimise the effects of over or under staining of the gel. Gel images always have a background intensity due to the colour of the gel material. This background needs to be removed so the calculated band volume then represents the volume of the material of interest within the band, rather than the volume of all material – including the gel material – at that position.

Once in the "Background" mode in the program, the "Remove Background" option was selected. The various methods for subtracting the background can then be selected. There are three automatic and one manual method for subtracting the background. Manual removal of the background was never used in this work. If using the automatic subtraction, the required option is simply selected. Within the automatic subtraction option are: "Rubber band," "Minimum profile," and "Rolling ball." They are all described below.

3.3.1 "Rubber Band" Option

This option does not work effectively if the intensity at the ends of the profile are lower than that of the rest of the profile. If the bands are not well separated, this method is not the most reliable one to use. For this reason, this feature was not used in this study.

3.3.2 "Minimum Profile" Option

This method takes the lowest value on the profile of each lane as the background for that lane. This method is not recommended when the beginning or end of the lane is the lowest point as this depends on the lane boundaries. This makes it hard for repetitions of the same gel. This made it an unsuitable option for use with this work.

3.3.3 "Rolling Ball" Option

After preliminary experimentation and discussion with the software suppliers the "Rolling ball" option was the method chosen for removing the background within this work. A value

needs to be entered for the "rolling ball". It calculates the background as if a disc, with the diameter as entered, were rolling under the lane profile. The larger the radius of the disc, the less the background rises with the profile. This method was found to consistently give the best results when the background subtraction trace was analysed. Following experimentation and advice provided by the software suppliers a value of 200 was used as the radius. (The maximum possible value that can be used is 10,000). Once the value is added, the subtract value needs to be pressed at the top of the navigation menu. The value of 200 was found to be the most suitable and was used throughout the work done.

3.3.4 Manual Background Subtraction

This method, which was not used, relies on the average intensity within a rectangular area on the image being taken as the background intensity. A rectangle is drawn on the image window. The background intensity is applied and calculated to all lanes from this rectangle.

3.4 Band Detection

The bands within each individual lane are automatically detected. (See Figure 3.2). This was an extremely valuable function and hugely extended the scope of gel analysis of tears. It was possible to detect bands that were extremely difficult to identify with the naked eye. It is also possible to manually edit individual bands. The two main points of this stage are to detect the peaks and also the edges of the bands.

The peak of a band is the point where the image intensity is at its maximum both laterally down a lane and widthways. This is used to define the band's position in the lane. The extent of the band must also be taken into account so the volume can be measured. These edges are found where the software locates the troughs in the lane profile at either side of the band's peak (both widthways and lengthways).

Automatic band detection finds the peaks in the profile to declare them as bands and the troughs to declare them as the edges.

Certain parameters need to be set even though this is an automatic method. (3.4.1 – 3.4.4)

3.4.1 "Minimum Slope"

This determines how pronounced the band needs to be from the surrounding area in the lane in order for it to be identified as being a separate band. The range is 0 –999.

The higher the value, the transition from background lane intensity to a band's peak intensity must be sharp. The gradient has to be less severe the lower the value. More bands are generally detected the lower the value. The value recommended for use is 100. This was shown to give satisfactory band detection for this purpose.

3.4.2 "Noise Reduction"

This represents the degree to which small local peaks should be ignored on the profile. It therefore eliminates any noise on the image. This does not have any effect on the profile itself – only the number of bands that are detected. The higher the value for noise

reduction, the fewer the peaks that are detected. The range is 0 – 20. A noise reduction value of 5 has been shown to be adequate.

3.4.3 "Percentage Maximum Peak"

The purpose of the percentage maximum peak is to discard peaks of under a certain size in relation to the highest peak on the gel. Fewer peaks will be detected as this value increases. The sizes of the peaks are calculated after any background has been subtracted. The range is 0 – 100. The value recommended for use was 3, and was found to be applicable here.

3.4.4 "Automatic Detection" and "Manual Editing"

The automatic detection option is the final step of the detection process and is activated when all the parameters described previously have been set. The software then detects any bands within the lanes in accordance with the parameters that have been set when the "Automatic detection" option is selected.

Bands can then be manually added and removed in the image window. This was accomplished by moving the cursor to the position in the image window where a band needed to be edited. The cursor changes to a drawing tool indicating that it is possible to add or delete a band at that position. The band was then edited by clicking the left mouse button. It was found that it was never necessary to add bands – although bands were sometimes deleted if they were believed to be an artefact of staining.

A diamond then appears centred on the chosen position (or nearest peak to the position). Horizontal red lines are seen above and below the diamond. This will indicate the edges within which volume measurements were made. (See Figure 3.2).

To delete a band, the cursor was moved over the diamond shape of the band that needs to be deleted. By right clicking on the band it then disappears. The measurements and analysis windows are then automatically updated.

It was also possible to zoom in on the image. This makes it much easier when carrying out band detection as it enables true bands to be distinguished from those that are present as a result of staining or other anomalies.

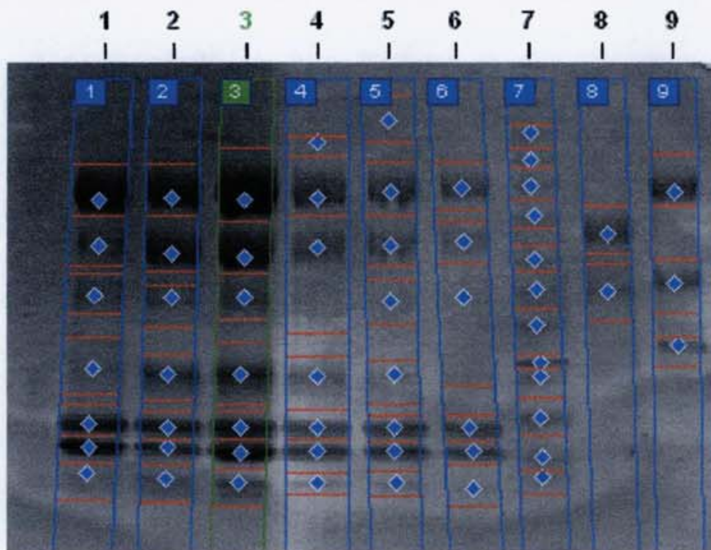


Figure 3.2 – Band detection

3.5 Molecular Size Calibration

After detection, the "processed" bands are quantified. The Molecular Size calibration mode was used in conjunction with a gel that has molecular weight markers loaded in it. It can then be used to establish the molecular weight of the bands in the lanes of the gel.

The first step is identification of the molecular weights of all the bands within the standard lane. When the known values for these bands in the standard lanes are entered, TotalLab™ can then interpolate and extrapolate contour lines of known molecular weights horizontally along the gel.

Once the horizontal contours have been determined, a curve-fitting algorithm was applied to determine the relationship of molecular size to position along the lane for all points in all the lanes. This allows the molecular size of any given band in the lanes to be determined.

There are several sets of standards already programmed into the package. Additional standards can be added if they do not match the spread of the markers used in the gel. For the purposes of this study, the standard was edited according to the molecular weights as supplied by Sigma.

The lane that contains the standards was then selected by clicking on it when asked. Standard values can be added or deleted from the gel image by selecting or de- selecting them from the list of values.

Before performing the molecular size calculation the parameters must first be set. The selected curve was used to work out the values between the bands on the standard lane and between points of the molecular size contours and the other lanes. If the Rf value is selected, the molecular weight contours will conform to relative lane position and not the absolute position. When the standard, curve type and propagation method is selected, the "compute" icon is pressed. The analysis window is updated and a figure is displayed. (See Figure 3.3).

Figure 3.3 – Calculation of molecular weights

3.6 Quantity Calibration

The software program allows for the concentrations of the separated bands to be calculated in one of two ways: quantity "calibration" and "normalisation". It is possible to either "normalise" the values or quantity calibrate – not both. Both of these options are described below. Throughout the course of this work, it was found preferable to "normalise" between the volumes of separated bands. This was deemed to be the more versatile option and allowed for more accurate comparisons to be made between gels.

The "quantity calibration" option allows the band volume in terms of image intensity to be related to what the software describes as real world volumes (i.e. external reference data). The real world volumes are initially entered for bands with known values. A calibration curve is then computed from these values.

The other available option is "normalisation". Normalisation was used to normalise the volumes in a gel image to a specific value derived from a standard protein present in known concentration. Albumin was selected for use in this work and was loaded into a reference lane in each gel. All other volumes are re-calculated relative to that value. This is useful when comparing the volumes across lanes or gels where the loading may be different.

The bands that have been used for the purpose of normalisation are shown in the image window as yellow diamonds. Those that are not included are shown as blue diamonds. (See Figure 3.4).

In the process of normalisation, a band or bands in the gel that are used as the basis of quantification (in this case an albumin reference) must be individually selected together with the reference value (i.e. quantity that has been loaded onto the gel). The allowed range for the normalised volume is 0.0001 – 1,000,000. There are ten units to be chosen from ranging from milligrams to attomoles.

When the normalising parameters had been set, the "normalise" button at the top of the menu was selected. The data was then displayed in the measurements table. (See Figure 3.4).

As previously stated, it was found that normalisation was preferable to quantity calibration for the purposes of this work. Along with the molecular weight markers, the gel was always loaded with albumin at 2 mg/ml and lactoferrin at 1.25 mg/ml. Other bands that were detected on the gel were then normalised against the concentration of albumin.

Figure 3.4 – Calculating protein concentrations

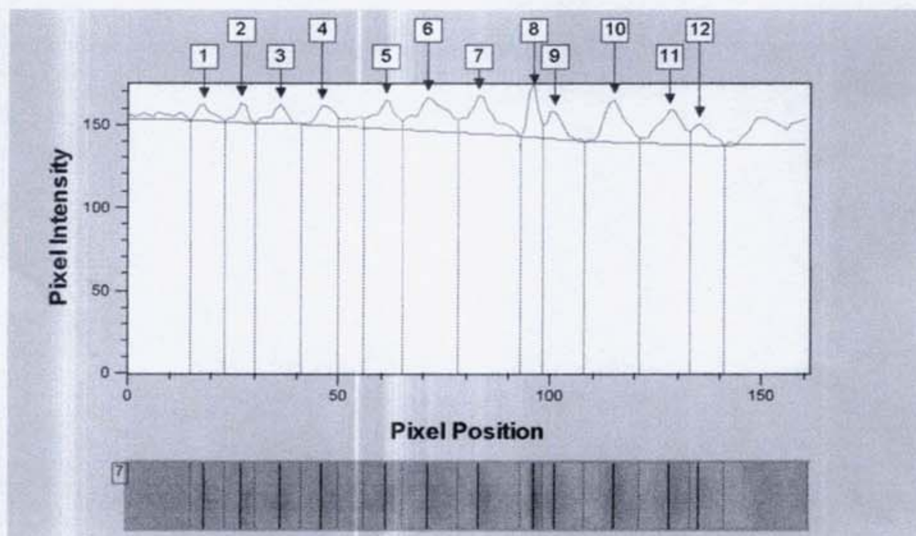
A typical print out that is available upon completion of the analytical stages is shown overleaf (See Figure 3.5).

Lane Report : csfocus1v0.tif, Lane 7

Lane: 7
Gel Path: C:\Documents and Settings\Helena\My Documents\csfocus1v0.tif
Date: 24/03/2003

Background Type : Rolling Ball 200
Minimum Band Slope : 100
Band Noise Factor : 5
% Maximum Peak : 3
Edge Detection Type : Automatic
Band Positions Edited : Yes

Additional Notes:



Band	Volume	Norm'd Vol(mg)	MW
1	647.60	0.001	116
2	599.40	0.001	97
3	807.00	0.001	84
4	851.62	0.001	66
5	1,499.17	0.003	55
6	2,647.50	0.005	45
7	2,629.62	0.005	36
8	1,300.67	0.002	29
9	1,237.33	0.002	24
10	2,316.27	0.004	20
11	2,181.55	0.004	14
12	1,063.18	0.002	7

TotalLab 1D Gel Analysis v2.0 Lane Report : csfocus1v0.tif, Lane 7 Page: 1

Figure 3.5 – Typical data sheet obtained upon completion of analysis

Chapter 4

Western Blotting and Immunostaining

4.1 Background

One-dimensional electrophoresis is a useful technique as it permits the separation of a complex mixture of proteins into its individual components. The disadvantage of such a technique is its inability to provide a definite identification of the separated protein species. An estimation can be made as to the identity of the proteins separated using molecular weight markers and standard protein samples loaded alongside the unknown sample. A graph can then be constructed of the mobility of these standards, thus allowing the molecular weights of the unknown proteins to be estimated by the relative distance they have travelled in the gel. Although useful to a certain degree, such calculations only allow the investigator to suggest what the protein is likely to be. Western Blotting is a technique that enables a definitive identification of a specific protein in a complex mixture whilst simultaneously determining its molecular weight.

The procedure can be broken down into a series of steps:

- Size separation of the proteins in a mixture by SDS-PAGE
- Transfer of the separated proteins to a membrane whilst retaining their relative position
- Detecting the protein under investigation by its specific reaction with an antibody (Immunostaining)

As previously stated, it is possible that a departure from the normal protein profile as seen in a normal, healthy, eye may be responsible for the dry eye symptoms so often reported amongst the contact lens wearing population. The main advantage of Western Blotting is that immunostaining can be performed which allows the investigator to determine whether a protein of interest is present or absent within any clinical samples.

Western Blotting involves the separated proteins being transferred from the gels in which they were run to a membrane under the influence of an electric current. There are two major methods used to transfer proteins to a membrane: semi-dry blotting and tank transfer blotting. Semi-dry blotting involves two plate electrodes providing a uniform electrical field over a short distance. Sandwiched between these electrodes is a gel/membrane/filter paper assembly all soaked in transfer buffer. Tank transfer blotting uses large volumes of

buffers and is therefore quite wasteful. Semi-dry blotting is now becoming the more popular method of choice due to the many advantages that it has over the traditional tank transfer. Firstly, it has the benefits of using smaller volumes of buffer than the traditional tank buffer system. The transfer buffer is the liquid contained in the gel and filter papers in the assembly. A semi-dry system would require approximately 200ml buffer compared to as much as five litres for some commercial tank transfer kits. Additionally, several gels can be blotted simultaneously and the electrodes are usually carbon blocks

The rationale behind transferring proteins to membranes from gels is to enable more efficient access with various probes. Polyacrylamide is not very amenable to the diffusion of large molecules. Antibodies are commonly used to probe for the immobilised proteins. Probes for the detection of antibody binding can be conjugated anti-immunoglobulins (e.g. goat-anti rabbit/human). Other possibilities for probing include the use of fluorescent or radioisotope labels. Proteins can be detected at nanogram concentrations compared to about 0.001mg for Coomassie Blue staining.

It was hoped that this technique could be utilised in the identification of unknown proteins separated by SDS-PAGE whilst at the same time giving credence to the presence of proteins within tears that had previously been reported using other techniques available within the Biomaterials Research Unit

4.2 Method

A full experimental protocol is described in Chapter 2, section 2.7.

4.3 Protein Transfer from Gel to Membrane

The initial stage involved optimisation of all running conditions to ensure maximum transfer of proteins from the gel to the membrane. These variables included: the length of time over which the transfer was allowed to take place, the buffer system to be utilised, and the membrane onto which the proteins were to be transferred.

The apparatus used in this technique was a horizontal semi-dry blotter supplied by Anachem. It was so called a "semi-dry" blotter as it uses filter papers soaked in various

anode and cathode buffer solutions. The use of filter papers in this way reduces the volume of buffer required for protein transfer to take place.

In order for these parameters to be investigated two gels were always prepared and loaded in an identical manner. Following the completion of electrophoresis, one gel was stained in the usual way with Coomassie Blue whilst the other was subjected to Western Blotting. The gel that had been blotted was then stained and de-stained in the usual manner once blotting was completed to assess the extent of transfer.

In initial experiments, the membrane was also stained with Ponceau S solution (Sigma). Once blotting was completed, the membrane was briefly immersed in this solution. This allowed the bands that had been transferred from the gel to the membrane to be visualised. The membrane could then be rinsed with distilled water to remove the Ponceau S. This solution did not interfere with any subsequent immunostaining reactions. This step was later omitted as it was found that it was impossible to remove all the background red stain from the membrane. The subsequent staining and destaining of the gel once blotting was complete made it possible to evaluate the efficiency of the transfer.

The time over which the gels were blotted to transfer the proteins from the gel to the membrane was also investigated and evaluated. Again, gels were prepared in an identical manner and the transfer allowed to run for varying time periods. It was found that the best results were obtained following a transfer time of four hours. A shorter time span than this resulted in an incomplete transfer from the gel. It is of paramount importance that the vast majority, if not all, of the bands were transferred if this technique was to be used in monitoring protein changes in the tears of contact lens wearers as part of a clinical study.

Traditionally, a nitrocellulose membrane is used. It was decided to compare the results obtained using a PVDF (Polyvinylidene difluoride) membrane against those obtained using a nitrocellulose membrane. It was found that the nitrocellulose membrane showed the bands with an equally high resolution as the PVDF membrane but it was a great deal more fragile and, as such, was prone to tear under stress. As a result, PVDF was the membrane of choice throughout this study.

It was also possible to use either a continuous or a discontinuous buffer system. The discontinuous buffer system employed three different buffer solutions: anode solution 1, anode solution 2 and a cathode solution. (See Appendix for chemical compositions). These buffers all have a different pH to assist the migration of the protein through the matrix of the gel to the surface of the membrane. The continuous buffer system used only one buffer solution. The continuous buffer system was found to be both quicker and easier to use but resulted in the loss of resolution of the bands following staining. This made it preferable to use the discontinuous buffer system.

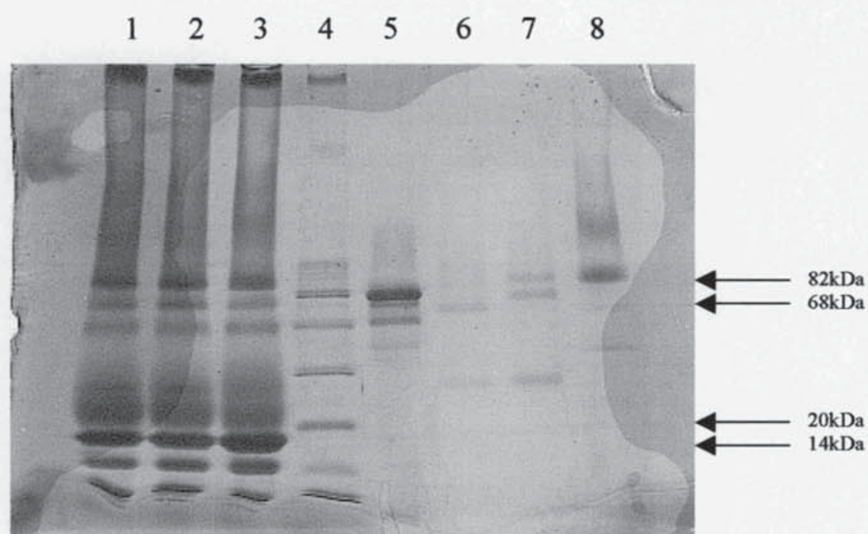


Figure 4.1 – Gel following electrophoresis

Lane 1 – subject 1 tear sample, Lane 2 – subject 2 tear sample, Lane 3 – subject 3 tear sample, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 6 – IgG (1 mg/ml), Lane 7 – IgA (1 mg/ml), Lane 8 – Lactoferrin (1.25 mg/ml)

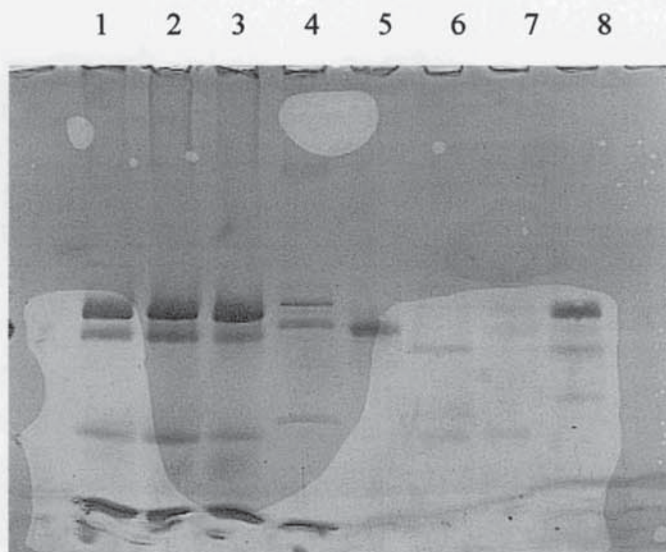


Figure 4.2 Gel following blotting with continuous buffer system

Lane 1 – subject 1 tear sample, Lane 2 – subject 2 tear sample, Lane 3 – subject 3 tear sample, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 6 – IgG (1 mg/ml), Lane 7 – IgA (1 mg/ml), Lane 8 – Lactoferrin (1.25 mg/ml)

Figure 4.2 clearly shows that there are still many bands in evidence on the gel following blotting for 4 hours using the continuous buffer system. This indicates that an incomplete transfer has taken place. The above gels are both identical in composition having being poured at the same time, loaded with the same samples and then subjected to electrophoresis simultaneously. One gel was then transferred to Coomassie Blue following electrophoresis whilst the other was blotted. Following blotting, the second gel was then stained and de-stained in the same way as the first. This enabled the progress of the transfer to be monitored.

4.4 Immunostaining for Albumin

The initial work of determining the parameters was carried out using albumin. This was the protein of choice as it is known to be present in tears at fairly high concentrations of approximately 1.3 mg/ml. An albumin standard was always loaded alongside unknown clinical samples as it enabled the molecular weights of the unknowns to be determined

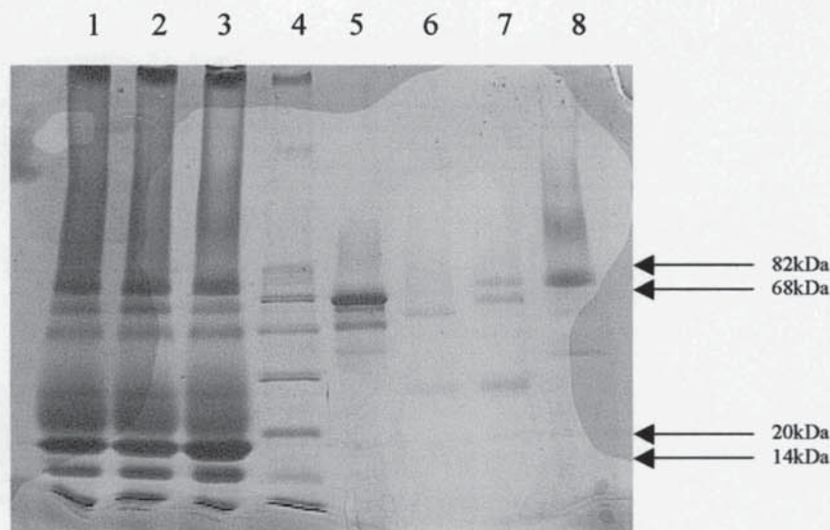


Figure 4.3 – Gel following electrophoresis

Lane 1 – subject 1 tear sample, Lane 2 – subject 2 tear sample, Lane 3 – subject 3 tear sample, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 6 – IgG (1 mg/ml), Lane 7 – IgA (1 mg/ml), Lane 8 – Lactoferrin (1.25 mg/ml)

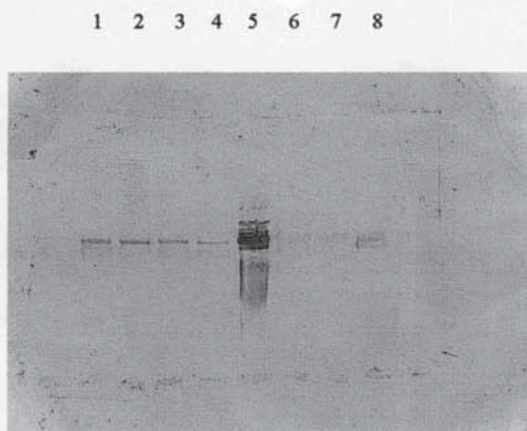


Figure 4.4 – PVDF membrane following immunostaining for albumin

Figure 4.4 shows a PVDF membrane that has undergone immunostaining to detect albumin. It can be clearly seen that albumin has been detected in the three tear samples (as shown in lanes 1 – 3). The albumin standard that was loaded in lane 5 has stained far more intensely indicating that it is at a higher concentration than the albumin detected in the tears. This made it possible to observe concentration differences in a qualitative manner although absolute values could obviously not be determined by observation. A slight degree of staining is observed in lane 4. This lane contains the molecular weight marker. This is to be expected, however, as albumin is one of the components of the molecular weight marker.

The transfer membrane was blocked overnight (5% non-fat milk powder) to prevent further non-specific binding of proteins. It was then possible to commence immunostaining. The immunostaining procedure was carried out adding antibody that had been raised against human albumin. (The antibody used throughout had been raised in goats – although it is possible to obtain antibodies that had been raised in other host animals). The antibody was added in a 1:1000 dilution e.g. 20μl antibody in 20ml blocking solution. Following incubation for an hour, the membrane underwent washing for 3 x 5 minutes. The secondary antibody was then added – at the same dilution as the first. This antibody had an alkaline-phosphatase (AP) side chain attached. The AP would then subsequently react with BCIP/NBT to give an insoluble blue precipitate whenever there was a positive reaction. Full experimental details are given in Chapter 2, section 2.8.

4.5 Sensitivity of Western Blotting

Western Blotting was widely used throughout this study to demonstrate the presence or absence of a range of proteins commonly found within tears. An immunoglobulin that is known to be present in tears, albeit at very low concentrations, is IgM. Reported values for this protein are approximately 5 μg/ml. It has also been said that only trace amounts of IgM, if at all, can be detected in the tear fluid of a healthy individual. IgM is the antibody known to be produced in the host's primary encounter with an antigen.

Figure 4.5 – PVDF membrane following immunostaining for IgM
Lane 1 – subject 1 tear sample (left eye), Lane 2 - subject 1 tear sample (right eye), Lane 3 – subject 2 tear sample, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 7 – IgG (1 mg/ml), Lane 8 – IgA (1 mg/ml)

These results illustrate the ability of this technique to detect proteins that are present in the tear fluid at very low concentrations. Such sensitivity is obviously important when the samples are of low volumes and contain low concentrations of proteins.

4.6 Detecting IgE in Tear Fluid

IgE is well documented for its role in hypersensitivity reactions. It was hoped that Western Blotting would enable the presence of this protein to be used as an indicator of adverse reactions of the host to the wearing of contact lenses. Furthermore, it may possibly be an implicating factor in the development of the dry eye symptoms often experienced by contact lens wearers.

Tear samples were initially taken from healthy, non-contact lens wearers. It was seen that, in many instances, IgE was also detected in these samples. IgE is also present in very low concentrations of 100 – 700 ng/ml[10] in the normal tear fluid.

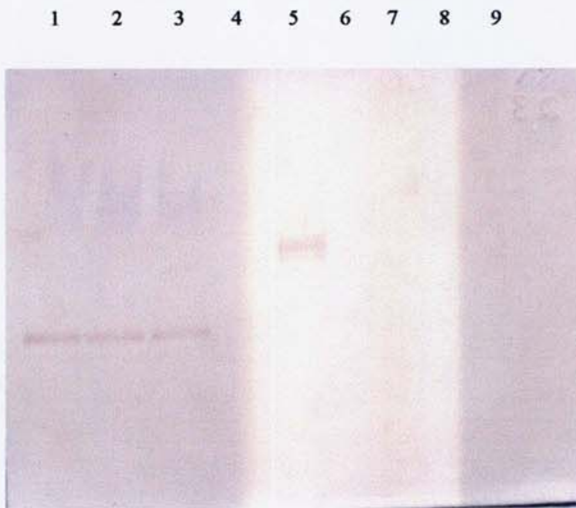


Figure 4.6 – PVDF membrane following immunostaining for IgE
Lane 1 – subject 1 tear sample, Lane 2 – subject 2 tear sample, Lane 3 – subject 3 tear sample, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 7 – IgG (1 mg/ml), Lane 8 – IgA (1 mg/ml), Lane 9 – lactoferrin (1.25 mg/ml)

It is clearly seen from the PVDF membrane that there are distinct positive reactions in the lanes containing the tear samples (Figure 4.6, lanes 1 – 3). There also appears to have been a reaction in the lane that contained albumin (lane 5). This "cross-reactivity" occurred in a few cases. Reasons for this and the implications will be discussed in a following section.

4.7 Assessment of Western Blotting and Immunostaining

The role and importance of proteins in the tear film cannot be overstated. As already mentioned, it is thought that disturbances in the normal concentrations of tear proteins may be integral to the development of dry eye symptoms and other forms of discomfort experienced by contact lens wearers.

Immunoglobulins in tears are of particular importance due to their role in immune responses. An elevation in the level of a plasma-derived immunoglobulin, such as IgG, would indicate an increase in the permeability of the blood-tear barrier. This is known to happen during inflammatory responses.

The sensitivity of this technique has been demonstrated clearly by its ability to detect immunoglobulins known to be present at only very low concentrations in the tear fluid. IgD and IgE are two such examples. IgE is known to be involved in allergic responses and other hypersensitivity reactions. The function of IgD in the tears, or indeed anywhere else, is still unknown.

One area that gave cause for concern was the "cross-reactions" that were occasionally seen to occur. Unfortunately, this is unavoidable to a certain extent. Immunoglobulins consist of four domains. Two of these domains are common to every class of immunoglobulin. It was seen that such cross reactions often occurred between IgA and IgG when they were both loaded onto a gel. When immunostaining for IgA was carried out it was seen that IgG stained with equal intensity. This problem was overcome by using an antibody specific for the heavy chain of the immunoglobulin in question. It is the heavy chain that gives the immunoglobulin its name e.g. α, δ, ε, γ and μ. Antibodies that have been raised against these specific subunits should show no cross reactivity between any other immunoglobulins. The reactions should be completely specific. Unfortunately, not all of the immunoglobulin subunits have corresponding antibodies that are available commercially at this time. Nonetheless, the utilisation of Western Blotting has proven to be an invaluable tool in the analysis of tear samples and complements data obtained from SDS-PAGE.

Kininogen has been shown to be a protein of considerable interest within the tear film due to its role in inflammatory responses. It was unfortunate to discover that this technique was unable to detect its presence in samples that gave positive results using other immunological techniques available within the group (e.g. Counter Immunoelectrophoresis). A possible reason for this is that it is present at such low concentrations it is below the threshold for detection by this technique.

Another drawback that was observed was the inability of proteins to migrate from a polyacyrlamide gel to a PVDF membrane after the gel had been stained with Coomassie Blue. This became particularly important when the samples being analysed were obtained as part of the clinical study.

The volumes of tears that were collected for analysis were often so low that there was only just the minimum volume required to load a well in the gel. This meant that it was not possible to run two gels in duplicate. It was obviously important to obtain data initially by SDS-PAGE in order that changes to the tear profile could be monitored visually over time. However, this then meant that once the gel had been stained it was then unsuitable for use in Western Blotting. The addition of the TotalLab™ software made it possible for the molecular weights and quantities of the separated proteins to be accurately determined. Another area of interest would have been to monitor changes in immunoglobulins, for instance IgE, as the subject went from being a neophyte to an accustomed lens wearer. (Fortunately, this information was obtainable by CIE).

This technique is still highly valuable, especially when used in conjunction with one-dimensional electrophoresis. It enables a specific protein to be simultaneously detected by means of its antigenicity and its molecular mass: proteins are first separated by mass during SDS-PAGE and then specifically detected by immunoassay.

Chapter 5

Comparison and Evaluation of Tear Sampling Techniques

5.1 Introduction

It is well documented that concentrations of proteins in the tear fluid are affected by the methods used to collect the sample and also by the subsequent analytical techniques employed. This is discussed more fully in Section 2.2. Tears collected by the Schirmer strip results in a higher concentration of plasma derived proteins, as the insertion of a strip of filter paper into the eye causes a degree of conjunctival stimulation. This results in a reflex tear being collected as excess watering occurs. Throughout this study, tear samples have been collected using microcapillaries. Previous work carried out within the Biomaterials Research Unit determined that this method of collection resulted in a "truer" tear, as the subject feels little to no stimulation. The flow can also be controlled to ensure that a stimulated tear is not being collected.

Studies on the tear film have the advantage that the fluid is readily accessible and no trauma is caused to the patient thereby allowing samples to be readily obtained. The main drawback that faces researchers in this field is the low volumes available for sampling. One of the main areas of interest is the interaction that occurs between the tear film and contact lenses upon insertion into the eye. Blood proteins and other proteins found within all body fluids interact with any surface that they encounter.[125] This interfacial phenomenon is as common an occurrence with contact lenses as it is with any prosthesis device implanted into the body. A great deal of research has been carried out over the past three decades since contact lenses became commercially available on the nature of these interactions. Proteins from the tear film are adsorbed onto the surface of the lens, and also within the matrix, within minutes of a lens being inserted into the eye. The adsorption of proteins onto a lens means that important components of the tear film may be present in much lowered concentrations, if at all, within the tear fluid. The exact mechanisms involved in such interactions are not fully understood. A major complaint of contact lens wearers is the discomfort that they often experience, particularly at the end of the day. Symptoms that are often cited include a gritty sensation and the eyes becoming very itchy. Materials are being continually developed to improve the level of comfort and visual acuity that can be attained whilst wearing contact lenses.

Proteins are thought to be of paramount importance in adverse reactions experienced when wearing lenses, as they are known to play a role in immunological reactions in other parts

of the body. A better understanding of the changes that take place in the tear film during contact lens wear is needed in order to remedy this problem. This would allow materials to be developed that are more compatible with the tear fluid.

5.2 Collecting the Tear Envelope

As mentioned previously, one of the major problems with studying the tear film is the low volumes that are available. This is often heightened in contact lens wearers. A sensation of dryness is a major factor that causes many people to cease wearing lenses. Other factors that are thought to be significant in contact lens drop-outs are an unacceptable level of visual acuity, discomfort and inconvenience. Reports have estimated that 10 – 50% of lens wearers cease lens wear within the first 2 – 3 years of commencing lens wear.

A technique was developed that would allow the lens to be used in a novel way to instantaneously sample the tear film by collecting the tear envelope that was attached to the lens at the time of removal. The advantage of this technique over conventional tear collection using microcapillaries was that even when the lens wearer was experiencing dry eye symptoms, such as would be expected at the end of the day, the lens would still carry the intact tear envelope from the eye for analysis.

This technique was seen to have the potential to be a highly valuable tool in contact lens and tear analysis as it enabled the tear film to be sampled immediately upon removing the lens, even in cases where no tear sample could be obtained from the subject via conventional methods. A full experimental protocol for this technique is given in Section 2.3.2

5.3 Effects of Contact Lenses on the Tear Film Using Two Distinct Techniques

5.3.1 Aims

The aims of this study were to compare the effects the lens material had upon the tear film, and also to evaluate the degree of deposition that took place onto the lens. A randomised, controlled, study was undertaken to enable preliminary analysis to be carried out. The lenses that were investigated held the majority share of the market for daily disposable lenses. The participants were employees of Aston University. The subjects were occasional wearers and were asked to wear each of the three different types of daily disposable lenses. Each particular lens type was worn for three days. At the end of this three day period they were asked to refrain from wearing lenses for the next two days to ensure that the eyes and tear film had recovered before the next lens type was tested.

Lens Name	Manufacturer	FDA Classification	% Water Content	Ionicity	Composition
Acuvue	Vistakon	Group IV	58	Ionic	HEMA, MA
Focus Dailies	Ciba Vision	Group II	69	Non ionic	HEMA, PVA
Soflens one day	Bausch and Lomb	Group II	70	Non ionic	HEMA, NVP

Table 5.1 – Characterisation of the daily disposable lenses worn
HEMA = 2-hydroxyethyl methacrylate, MA = methacrylic acid, NVP = N-vinyl pyrrolidone, PVA = polyvinyl alcohol

5.3.2 Methods

5.3.2.1 Tear Analysis Following Collection with Conventional Microcapillaries

Each subject was randomly assigned a particular lens type. Tear samples were then collected from each patient using a 5μl microcapillary before they commenced lens wear and then again upon completion of the three day wearing period. The same protocol was observed for each of the different lens types. The tear fluid was expressed into an eppendorf and stored at 4°C until required.

5.3.2.2 Collection of the Tear Envelope

Immediately upon removal of the lens it was placed into an eppendorf containing 40μl of sample treatment buffer. This was stored at 4°C until required. Following centrifugation (as detailed in Section 2.3.2), the tear envelope could then be analysed.

5.4 Results

5.4.1 Conventional Tear Analysis Following Collection with Microcapillary

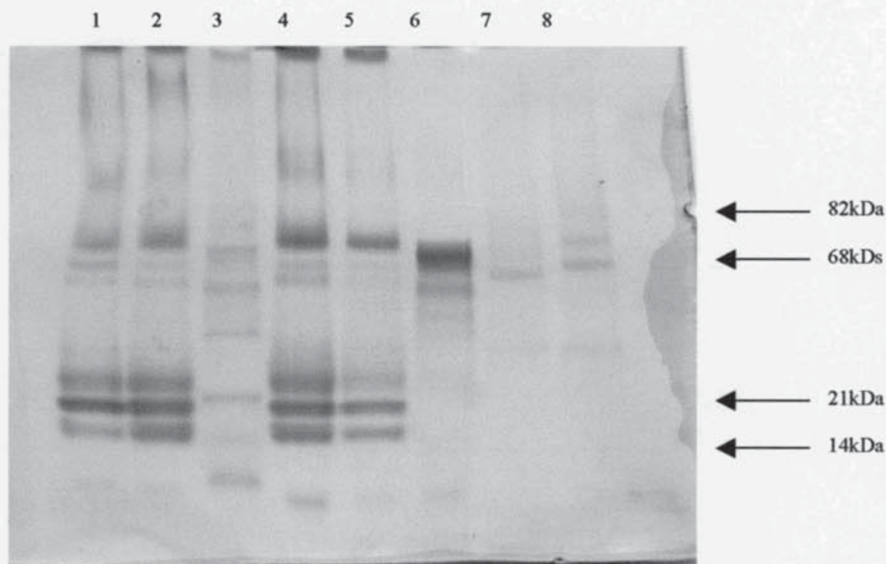


Figure 5.1 – Tear samples collected before and after a three-day wearing period (Patient 1: Soflens, Patient 2: Acuvue)

Lane 1 – patient 1 pre lens wear, Lane 2 – patient 1 post lens wear (Soflens), Lane 3 – molecular weight marker, Lane 4 – patient 2 pre lens wear, Lane 5 – patient 2 post lens wear (Acuvue), Lane 6 – albumin (2mg/ml), Lane 7 – IgG (1mg/ml), Lane 8 – IgA (1mg/ml)

	Patient 1		Patient 2	
Molecular weight (kDa)	**Pre lens wear (%)**	**Post lens wear (%)**	**Pre lens wear (%)**	**Post lens wear (%)**
82	4.8	3.5	6.1	5.3
68	14.3	15.4	18.2	21.1
63	4.8	3.8	3.0	5.3
58	4.8	3.8	3.0	5.3
28	23.8	23.1	27.3	21.1
21	23.8	23.1	18.2	21.1
14	14.3	19.2	18.2	15.8
6	9.5	7.7	6.1	5.2

Table 5.2 – Percentage of proteins present in tear samples before and after a three-day wearing period (Patient 1: Soflens, Patient 2: Acuvue)

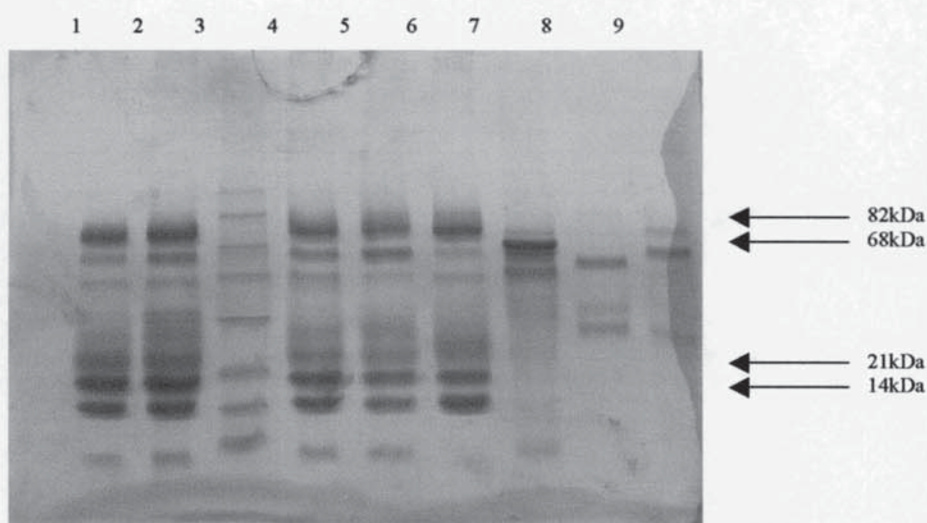


Figure 5.2– Tear samples collected before and after a three-day wearing period (Patient 1: Acuvue, Patient 2: Focus Dailies)

Lane 1 – patient 1 pre lens wear, Lane 2 – patient 1 post lens wear (Acuvue), Lane 3 – molecular weight marker, Lane 4 – patient 2 pre lens wear, Lane 5 – patient 2 post lens wear (Focus Dailies), Lane 6 – patient 3 post lens wear (Focus Dailies), Lane 7 – albumin (2 mg/ml), Lane 8 – IgG (1 mg/ml), Lane 9 – IgA (I mg/ml)

	Patient 1		**Patient 2**	
Molecular weight (kDa)	**Pre lens wear (%)**	**Post lens wear (%)**	**Pre lens wear (%)**	**Post lens wear (%)**
82	17.1	20.0	20.9	22.0
68	7.3	9.1	9.3	12.2
58	7.3	7.2	9.3	7.3
32	-	-	11.6	17.1
24	31.7	32.7	14.0	12.2
21	19.5	16.4	16.3	14.6
14	14.6	12.7	14.0	9.7
6	2.4	1.8	4.7	4.9

Table 5.3 – Percentage of proteins present in tear samples before and after a period of lens wear (Patient 1: Acuvue, Patient 2: Focus Dailies)

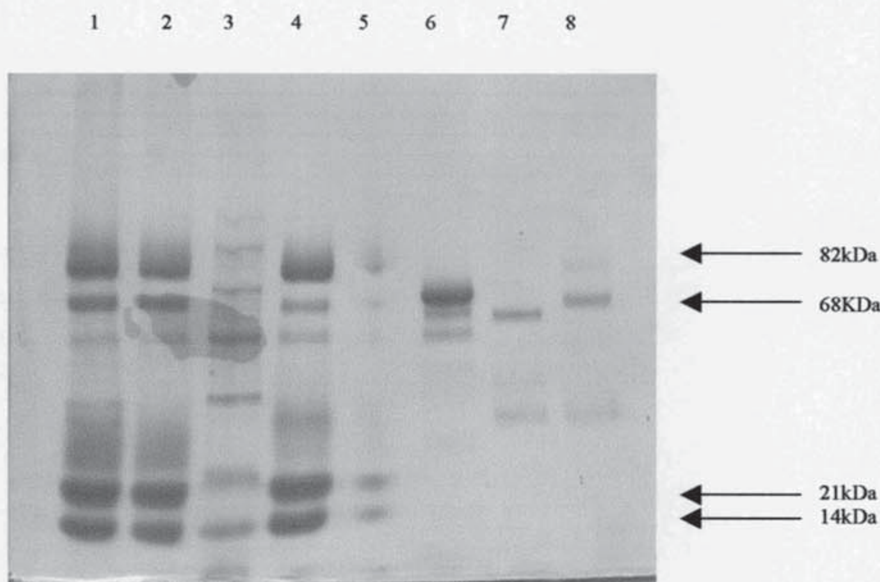


Figure 5.3 – Tear samples collected before and after a three-day wearing period (Patient 1: Focus Dailies, Patient 2: Soflens)

Lane 1 – patient 1 pre lens wear, Lane 2 – patient 1 post lens wear (Focus Dailies), Lane 3 – molecular weight marker, Lane 4 – patient 2 pre lens wear, Lane 5 – patient 2 post lens wear (Acuvue), Lane 6 – albumin (2 mg/ml), Lane 7 – IgG (1 mg/ml), Lane 8 – IgA (1 mg/ml)

	Patient 1		Patient 2	
Molecular weight (kDa)	**Pre lens wear (%)**	**Post lens wear (%)**	**Pre lens wear (%)**	**Post lens wear (%)**
82	20.3	17.2	20.8	6.3
68	9.4	10.9	12.5	10.3
58	3.1	9.4	4.2	6.3
32	1.6	-	6.3	-
24	23.4	21.9	20	14.8
21	23.4	23.4	25.0	21.0
14	18.8	17.2	25.0	22.9

Table 5.4 – Percentage of proteins present in tear samples before and after a period of lens wear (Patient 1: Focus Dailies, Patient 2: Soflens)

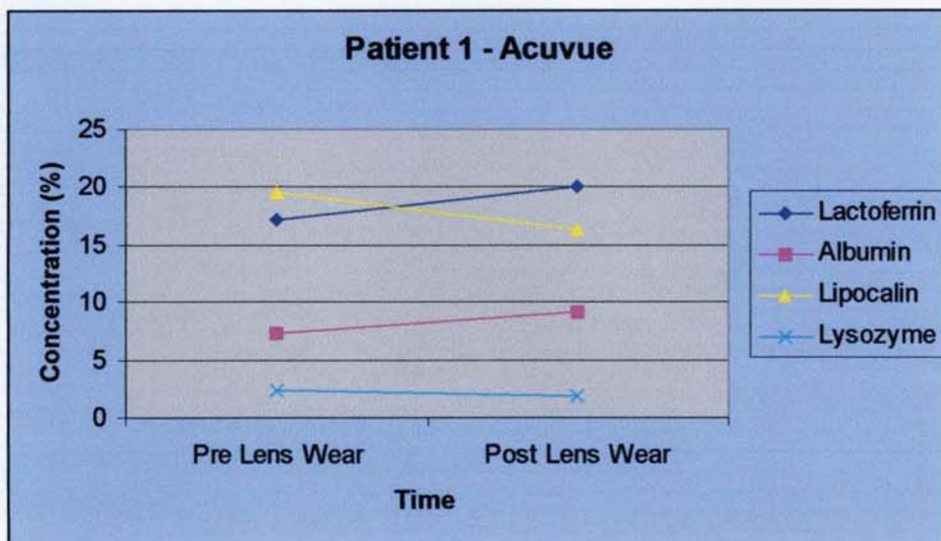


Figure 5.4 – Shows the changes in concentration of tear proteins of patient 1 following three days of lens wear (Acuvue)

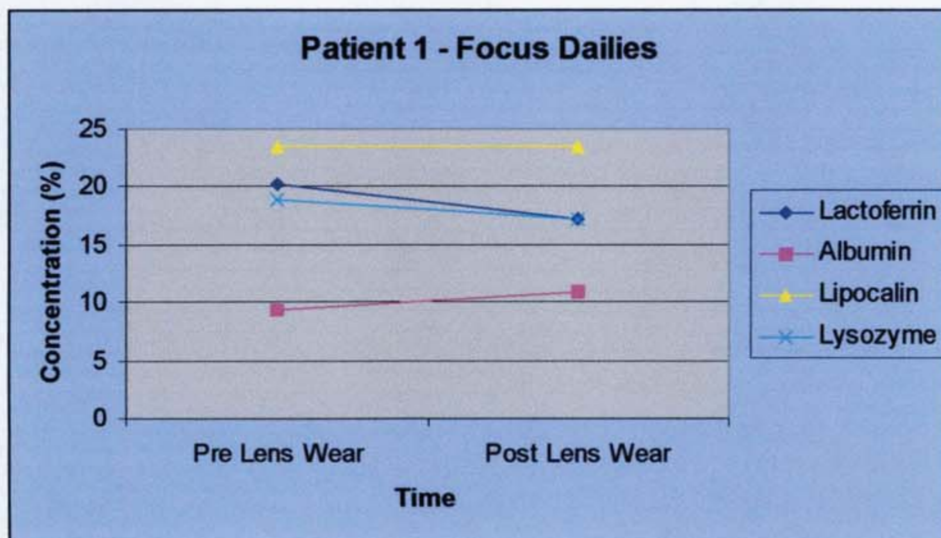


Figure 5.5 – Shows the changes in concentrations of tear proteins of patient 1 following three days of lens wear (Focus Dailies)

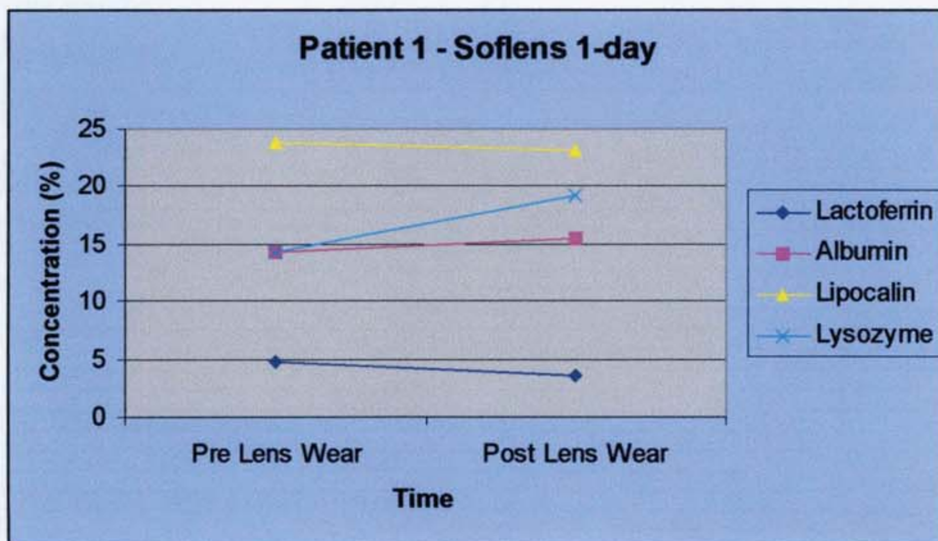


Figure 5.6 – Shows the changes in concentrations of tear proteins of patient 1 following three days of lens wear (Soflens)

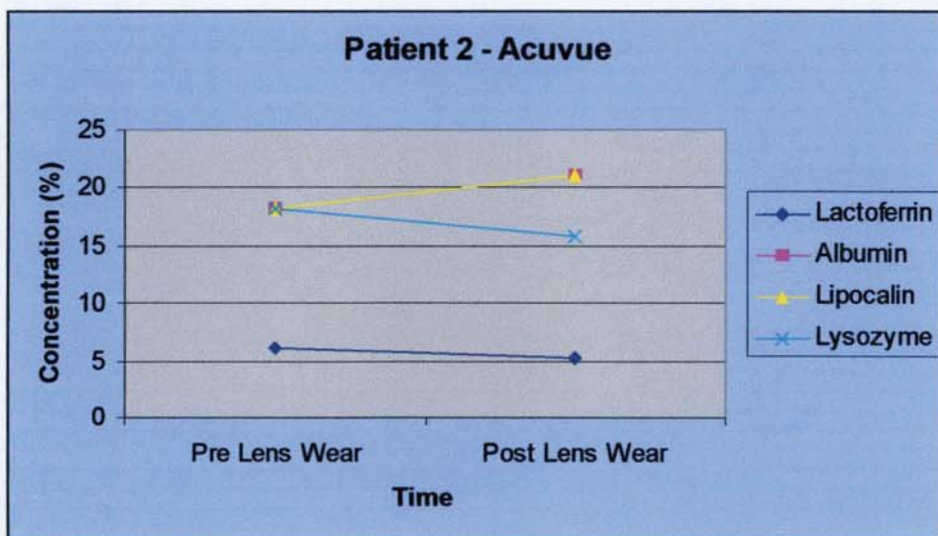


Figure 5.7 – Shows the changes in concentration of tear proteins of patient 2 following three days of lens wear (Acuvue)

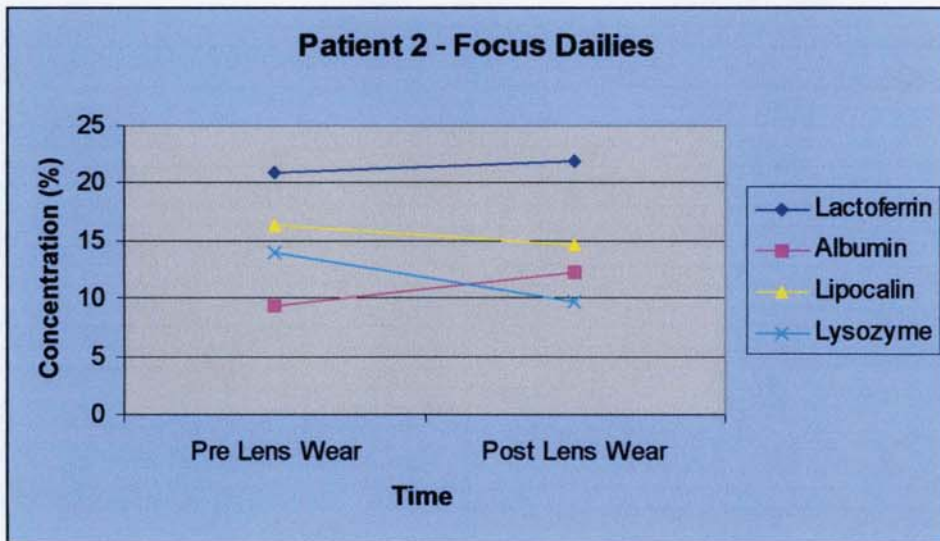


Figure 5.8 – Shows the changes in concentration of tear proteins of patient 2 following three days of lens wear (Focus Dailies)

5.4.2 Tear envelope

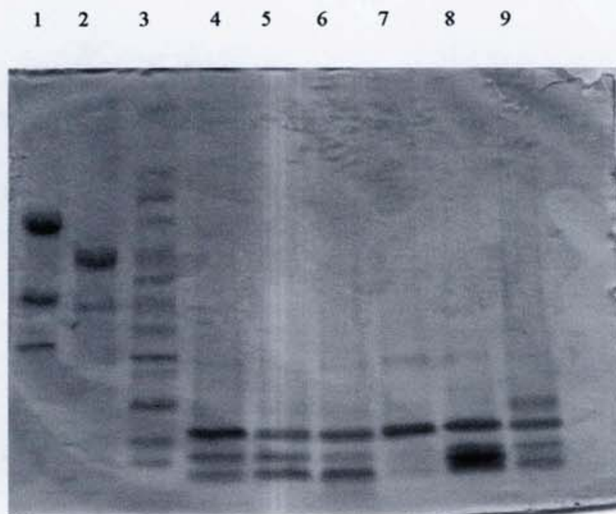


Figure 5.9 – Tear envelope removed from Acuvue lenses

Lane 1 – lactoferrin (1 mg/ml), Lane 2 – albumin (2 mg/ml), Lane 3 – molecular weight marker, Lane 4 – patient 2 tear envelope day 3, Lane 5 –patient 2 tear envelope day 2, Lane 6 – patient 2 tear envelope day 1, Lane 7 – tear envelope patient 1 day 3, Lane 8 – patient 1 tear envelope day 2, Lane 9 – patient 1 tear envelope day 1.

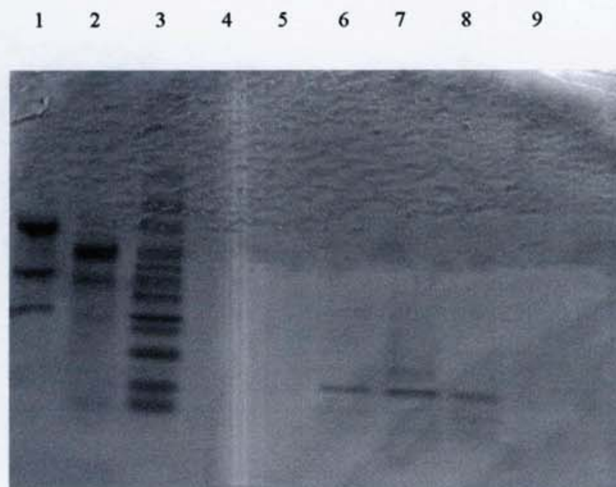


Figure 5.10 – Tear envelope removed from Focus Dailies lenses

Lane 1 – lactoferrin (1 mg/ml), Lane 2 – albumin (2 mg/ml), Lane 3 – molecular weight marker, Lane 4 – patient 2 tear envelope day 3, Lane 5 –patient 2 tear envelope day 2, Lane 6 – patient 2 tear envelope day 1, Lane 7 – tear envelope patient 1 day 3, Lane 8 – patient 1 tear envelope day 2, Lane 9 – patient 1 tear envelope day 1.

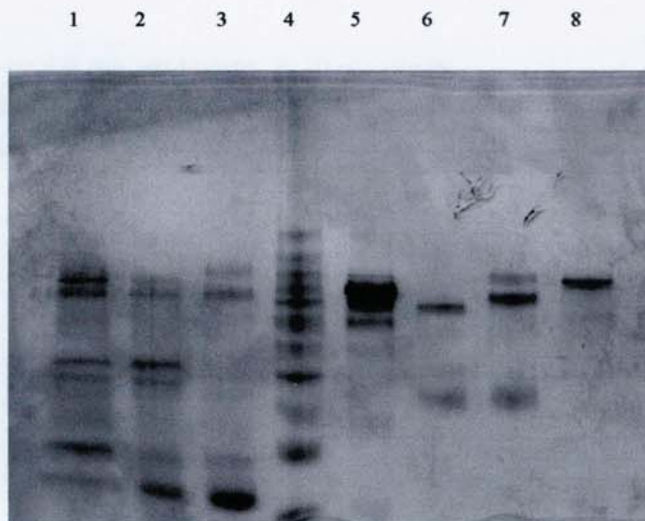


Figure 5.11 – Comparison of the tear envelope as removed from three types of daily disposable lenses

Lane 1 – Acuvue, Lane 2 – Focus Dailies, Lane 3 – Soflens, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 6 – IgG (1 mg/ml), Lane 7 – IgA (1 mg/ml), Lane 8 – lactoferrin (1.25 mg/ml)

5.5 Discussion

5.5.1 Conventional Tear Analysis

The initial work carried out revealed that the wearing of contact lenses caused a change to the tear film after a very short time. The initial aim of this work was to determine if, and how, contact lenses caused the tear film to deviate from normal. The secondary aim was to then see if the lens material affected this change to any large extent. The final aim of this work was to compare the three different lens materials for their ability to maintain an intact tear envelope.

The first point that is noticed is that the concentration of albumin is seen to increase following lens wear in all cases. Albumin is a plasma-derived protein. It is likely that the conjunctiva is being stimulated by the contact lenses thus causing a leakage of albumin, and possibly other plasma derived proteins, into the tear fluid.

It was not possible to make any judgements on the effects lens material had upon the tear film based on data that was collected over a relatively short period of time. In order for any definite conclusions to be reached, it would have been necessary to follow each subject over a longer time period than three days to allow the eye and tear film to become de-sensitised to the initial sensation of wearing lenses. The primary aim of this exercise was successful in that it clearly demonstrated that contact lenses do indeed affect the normal protein profiles of wearers. It is not clear whether the tear film would eventually revert back closer towards its normal, pre-lens wearing, state as the eye becomes adapted to lens wear or whether the tear film remains altered until lens wear is ceased.

5.5.2 Collection and Analysis of the Tear Envelope from Three Types of Daily Disposable Lenses

The preliminary work that was carried out on the tear envelope found that it was necessary to submerge the worn lens into 40μl of sample treatment buffer. Volumes lower than this resulted in the lens not being sufficiently covered. Once the lens was in the eppendorf, it

was then vortex mixed thoroughly to re-wash the lens before being centrifuged at 5000 r.p.m for ten minutes prior to analysis.

A disadvantage of studying the tears of contact lens wearers is they often appear to have a reduced tear volume and it is sometimes very difficult to obtain a sample that is of sufficient volume for it to be analysed by conventional methods. The electrophoresis unit used throughout this study required a minimum volume of 5μl of the analyte – to which a further 5μl of sample treatment buffer was added. It was often not possible to collect such volumes from individuals. This technique made it possible to analyse tear samples from these individuals to a certain extent. Previous methods that existed for analysing proteins that had deposited on lenses required very harsh chemicals, which were likely to denature proteins. Proteins would not necessarily exist in their native state. Lens extractions also contained proteins that had deposited into the matrix of the lens. Being able to extract the tear envelope would allow for a more accurate representation of the tears of contact lens wearers to be obtained.

It was found that the tear envelopes obtained from the Acuvue lenses were those most representative of a conventional tear sample obtained by microcapillary. Figure 5.11 (see above) showed that many proteins had been separated following electrophoresis. The tear envelope that was collected from the Focus Dailies lenses did not show the presence of as many protein bands as the Acuvue lenses. Attempts to collect the tear envelope from Soflens 1-day did not prove to be as successful as with the other two lens types.

Many attempts were made to remedy this problem but a clearer, more comprehensive, protein profile was never obtained. The non-ionic, high water content of this lens may have prevented them from sustaining a tear film as thick as that of an Acuvue lens and hence the tear film broke up more quickly. An estimation of the tear envelope volume that is attached to a lens at the time of removal would be in the order of 2μl. Along with this is the dilution of any tears that are removed by the required volume of treatment buffer. All of these are limiting factors for this technique.

The Soflens 1-day is a much thicker lens than either 1-day Acuvue or Focus Dailies. It appears that the thinner the lens, the better the tear envelope that can be removed from the

lens. The average centre thicknesses for the three different types of lenses used in this study are shown below in Table 5.5.

Lens type	Average centre thickness (mm)
1-day Acuvue	0.084
Focus Dailies	0.10
Soflens 1-day	0.17

Table 5.5 – Average centre thickness of 1-day Acuvue, Focus Dailies and Soflens 1-day (measured at –3.00D)

Figure 5.11 shows that the tear envelope following SDS-PAGE can be resolved into more protein components the thinner the lens. This would lead to the conclusion that thinner lenses are better able to sustain a tear envelope on the surface of the lens.

Although it proved difficult to remove the tear envelope from the Soflens 1-day, which technique still has the potential for studying areas of tear analysis that have so far not proved possible. Overnight wear of lenses is known to be associated with an increased risk of microbial contamination. It may in the future be possible to utilise this technique to analyse the tear envelope for lactoferrin and lysozyme concentrations, or indeed other proteins that are known to possess bacteriostatic or bactericidal properties. Another possibility would be to attempt to analyse a "closed eye" tear sample using this technique. This has been an area that has received a great deal of attention in the past but it has been found extremely difficult to sample such tears since the tear film begins to revert back to its normal open eye state immediately upon eye opening. Removing the tear envelope that is attached to the lens immediately upon opening the eyes may give a truer representation of a closed eye tear than has previously been possible.

5.6 Diurnal Variation

5.6.1 Introduction

Tear protein levels are known to alter during pathological conditions such as ocular inflammation and infections, with age and also during overnight closure. Observed deficiencies of certain lacrimal gland derived proteins (such as lysozyme and lactoferrin) are thought to be useful in the diagnosis of an aqueous deficient dry eye.[126] An understanding of the normal protein profile is therefore imperative if distinctions are to be made between a healthy ocular environment and a diseased eye.

It is well documented that differences are observed in tear protein levels depending on the collection technique employed. Research has also been carried out on the changes that take place to the tear film during a period of prolonged eye closure such as during sleep. However, surprisingly little attention has been given to the variations that occur in the tear film naturally throughout the course of the day. To date, diurnal variations in tear pH, osmotic pressure, lysozyme and IgA have been studied. It was found that the pH of the tears varies throughout the day, and also day to day. The studies carried out on lysozyme and lactoferrin were found to produce inconsistent results. Of the few studies that have been carried out on diurnal variations of the major tear proteins, no definitive conclusion was reached.[127]

A study was undertaken to investigate whether there were any discernible differences that could be observed in the tear protein profile throughout the day. It was decided to use both the conventional capillary collection of tears and also to try and extract the tear envelope from the lens to monitor changes in the tear film as a function of time.

5.7 Method

5.7.1 Tear Collection Using Conventional Microcapillary

Tear samples were collected from members of the Biomaterials Research Unit after obtaining informed consent. In order to minimise the effects environment may have had upon the subjects, all samples were collected during the working day. A small time period was given to allow the subjects to accustom to their environment. Samples were then collected in the morning, again at lunchtime, and finally before going home. To reduce any added variations in time, samples were collected from the participants at the same time. This was repeated over a period of three days.

5.7.1.1 Experimental Parameters

The tear samples were collected from the subjects using 5μl microcapillaries. The following criteria was fulfilled to reduce the introduction of any additional variables

- All female
- Aged in their twenties and thirties
- All worked in the same environment to minimise the impacts that environment may have upon the tear film
- Samples were collected from the subjects at the same time every day

The clinical study that was undertaken (refer to Chapter 6 for further details) involved a population of lens wearers being compared with and monitored against a control population of non-lens wearers. The data obtained from these control patients has also been used here as it enables tear protein profiles from this subset to be analysed over the course of a year to determine whether there are any significant changes that can be observed taking place to the tear film with time.

5.7.2 Collecting the Tear Envelope to Observe the Effects of Diurnal Variation

In order to assess the impact that diurnal variation had upon lens wearers, the participating subjects were asked to wear one lens in the left eye as a control lens. The lens that was worn in the right eye was removed every three hours and replaced with a fresh one. The removed lenses were then stored in sample treatment buffer at 4°C until required.

5.8 Results

5.8.1 Tear Samples Collected Using Conventional Microcapillary

		Day 1			Day 2			Day 3		
Patient No.	**Protein (%)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	Lactoferrin	31.8	28.6		25.0	33.3	23.1	23.1	23.1	30.0
	Albumin	38.6	15.2		12.5	11.1	7.7	23.1	15.4	10.0
	Lipocalin	9.1	8.7		25.0	22.2	23.1	15.4	15.4	20.0
	Lysozyme	9.1	8.7		25.0	22.2	23.1	23.1	23.1	30.0
2	Lactoferrin	30.0	34.9	23.5	32.6	31.1		15.4	18.2	20.0
	Albumin	24.0	27.9	17.6	23.9	43.7		7.7	18.2	10.0
	Lipocalin	12.0	11.6	14.7	13.0	9.2		15.4	27.3	20.0
	Lysozyme	10.0	9.3	14.7	15.2	7.6		38.5	18.2	20.0
3	Lactoferrin	7.5	25.0	25.2	26.2	24.8	33.3	28.6	28.6	33.3
	Albumin	1.0	6.3	9.1	12.6	15.7	21.3	28.6	28.6	40.0
	Lipocalin	53.2	33.9	26.3	17.5	16.5	16.7	14.3	14.3	6.7
	Lysozyme	38.3	34.8	34.3	32.0	27.3	19.4	14.3	14.3	6.7
4	Lactoferrin	47.8	45.0	57.1	25.0	39.2	40.6	49.4	64.3	62.6
	Albumin	26.1	20.0	14.3	20.0	27.2	20.3	31.1	7.8	14.5
	Lipocalin	17.4	5.0	7.1	11.0	7.2	8.6	3.7	7.0	5.6
	Lysozyme	8.7	15.0	14.3	24.0	6.4	18.0	9.1	13.2	11.2
5	Lactoferrin	20.0	40.0		45.1	44.0	43.4	28.6	42.9	45.0
	Albumin	6.7	10.0		22.6	24.0	17.4	14.3	21.4	30.0
	Lipocalin	36.7	16.7		9.7	4.0	8.7	28.6	14.3	10.0
	Lysozyme	33.3	30.0		16.1	16.0	21.7	28.6	14.3	10.0

Table 5.6 – Results of tear protein concentrations of five individuals over the course of the day

(Shaded areas indicate where no samples could be obtained).

The results shown in Table 5.6 were then used to calculate the ratio of lactoferrin to albumin, lipocalin and lysozyme. The ratio of albumin: lipocalin has been suggested to alter with contact lens wear[112] and so was also calculated to enable comparisons to be drawn between non-lens wearers and lens wearers. These results are presented below.

Patient	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
1	1: 0.61	1: 0.64	1: 0.75	1: 1.04
2	1: 0.58	1: 0.43	1: 0.46	1: 0.73
3	1: 0.70	1: 0.86	1: 0.95	1: 1.22
4	1: 0.42	1: 0.38	1: 0.28	1: 0.40
5	1: 0.47	1: 0.41	1: 0.55	1: 0.88

Table 5.7 – Ratios of albumin, lipocalin and lysozyme to lactoferrin of five subjects averaged over a three-day period.

The average ratios shown in Table 5.7 are interesting but it was deemed necessary to consider variability for two reasons:

1. To discern a pattern emerging for individuals or the group as a whole relating to diurnal change.
2. To assess the extent to which individual data points might be judged to be flawed, in that they represent unreasonably abnormal behaviour.

The data generated from each individual patient was then used to calculate the protein ratios as observed at each collection point. These results are displayed in Table 5.8.

		Day 1			Day 2			Day 3		
Patient	**Protein (Ratio to Lactoferrin)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	Albumin	1.21	0.53		0.5	0.33	0.33	1.00	0.67	0.33
	Lipocalin	0.29	0.30		1.00	0.67	1.00	0.67	0.67	0.67
	Lysozyme	0.29	0.30		1.00	0.67	1.00	1.00	1.00	1.00
2	Albumin	0.80	0.80	0.75	0.73	1.41		0.50	1.00	0.50
	Lipocalin	0.40	0.33	0.63	0.40	0.30		1.00	1.50	1.00
	Lysozyme	0.33	0.27	0.63	0.47	0.24		2.50	1.00	1.00
3	Albumin	0.13	0.25	0.36	0.48	0.63	0.64	1.00	1.00	1.20
	Lipocalin	7.09	1.36	1.04	0.67	0.67	0.50	0.50	0.50	0.20
	Lysozyme	5.11	1.39	1.36	1.22	1.10	0.58	0.50	0.50	0.20
4	Albumin	0.55	0.44	0.25	0.80	0.69	0.50	0.63	0.12	0.23
	Lipocalin	0.36	0.11	0.12	0.44	0.18	0.21	0.07	0.11	0.09
	Lysozyme	0.18	0.33	0.25	0.96	0.16	0.44	0.18	0.21	0.18
5	Albumin	0.34	0.25		0.50	0.55	0.40	0.50	0.50	0.67
	Lipocalin	1.84	0.42		0.22	0.09	0.20	1.00	0.33	0.22
	Lysozyme	1.67	0.75		0.36	0.36	0.50	1.00	0.33	0.22

Table 5.8 – Ratios of albumin, lipocalin and lysozyme to lactoferrin from five subjects over a three day period as observed at each visit

The ratios of albumin, lipocalin and lysozyme to lactoferrin were then calculated for each subject according to the time of day samples were taken. These results are displayed in Table 5.9.

Patient	Protein (Ratio to Lactoferrin)	AM	MID	PM
1	Albumin	1: 0.93	1: 0.49	1: 0.33
	Lipocalin	1: 0.62	1: 0.54	1: 0.81
	Lysozyme	1: 0.72	1: 0.64	1: 1.00
2	Albumin	1: 0.71	1: 1.07	1: 0.63
	Lipocalin	1: 0.52	1: 0.57	1: 0.80
	Lysozyme	1: 0.82	1: 0.42	1: 0.80
3	Albumin	1: 0.68	1: 0.65	1: 0.77
	Lipocalin	1: 1.36	1: 0.83	1: 0.54
	Lysozyme	1: 1.36	1: 0.97	1: 0.66
4	Albumin	1: 0.63	1: 0.37	1: 0.31
	Lipocalin	1: 0.26	1: 0.13	1: 0.13
	Lysozyme	1: 0.34	1: 0.23	1: 0.27
5	Albumin	1: 0.47	1: 0.44	1: 0.54
	Lipocalin	1: 0.80	1: 0.28	1: 0.21
	Lysozyme	1: 0.83	1: 0.48	1: 0.36

Table 5.9 – The ratios of albumin, lipocalin and lysozyme to lactoferrin of five individuals throughout the day.
(Averaged over a three-day period).

As part of the clinical study (described in Chapter 6), which was undertaken to assess the impacts that contact lenses had upon the tear film, a population of control individuals were recruited. They were all non-lens wearers and were free from ocular disorders. (Full patient details are described in Section 6.4). This enabled observations to be made regarding changes to the tear film taking place as a direct result of lens wear. The results of the tear ratios for these control patients are also displayed below (see Table 5.10). These data have been presented here because it enabled a clearer picture of how tear films that are considered to be stable may be susceptible to variation over a longer period of time. It must be remembered that these samples were taken as part of the clinical study and therefore

other variables may influence any variations observed including: environmental effects, the time of sampling, and the lapsing intervals between sampling.

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme
64	1: 0.52	1: 0.46	1: 0.79
65	1: 0.41	1: 0.46	1: 0.46
67	1: 0.36	1: 0.40	1: 0.52
68	1: 0.50	1: 0.50	1: 0.52
69	1: 0.53	1: 0.49	1: 0.55
72	1: 0.39	1: 0.44	1: 0.44

Table 5.10 – The ratios of albumin, lipocalin and lysozyme to lactoferrin of control subjects involved in the clinical study.
(Averaged over all visits)

The tear samples that were collected by microcapillary as part of the investigation into the effects of diurnal variation were then subjected to SDS-PAGE. Figures 5.12 and 5.13 shown below are typical images that were produced as a result of this study.

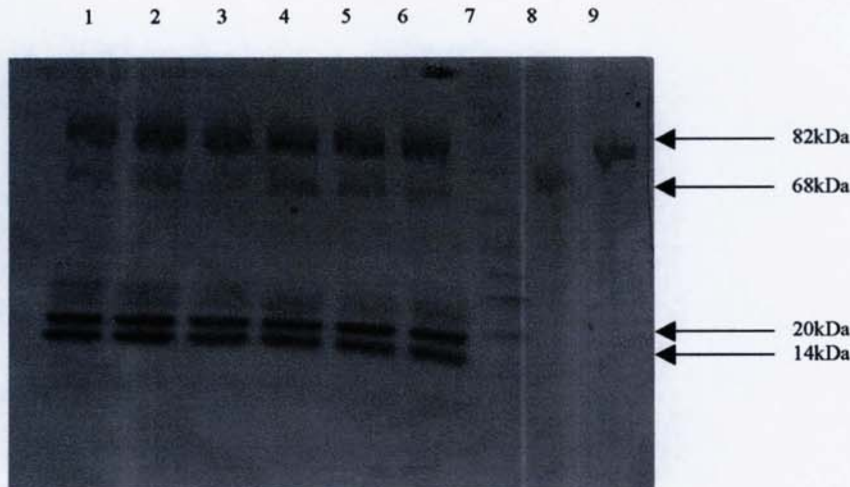


Figure 5.12 - Patient 1 tear samples collected over a two day period
Lane 1 – day 2 (am), Lane 2 – day 2 (mid), Lane 3 – day 2 (pm), Lane 4 – day 3 (am), Lane 5 – day 3 (mid), Lane 6 –day 3 (pm), Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

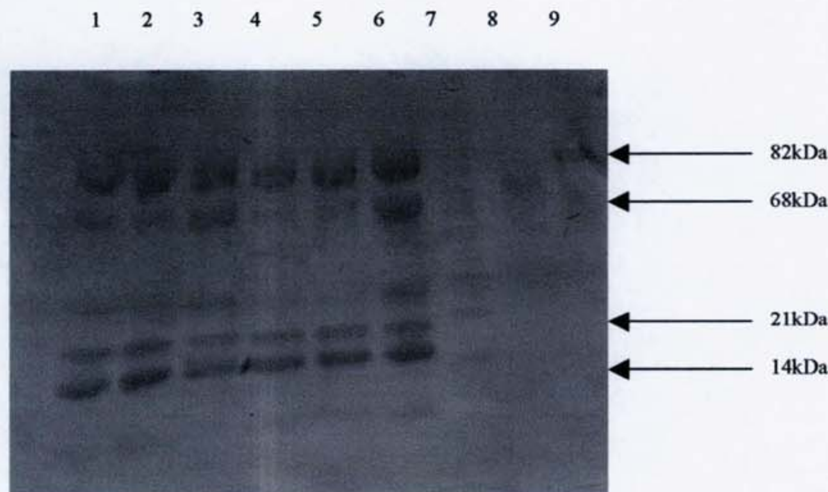


Figure 5.13 – Patient 4 tear samples collected over a two day period
Lane 1 – day 1 (mid), Lane 2 – day 1 (pm), Lane 3 – day 2 (am), Lane 4 – day 2 (mid), Lane 5 – day 2 (pm), Lane 6 – day 1 (am), Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

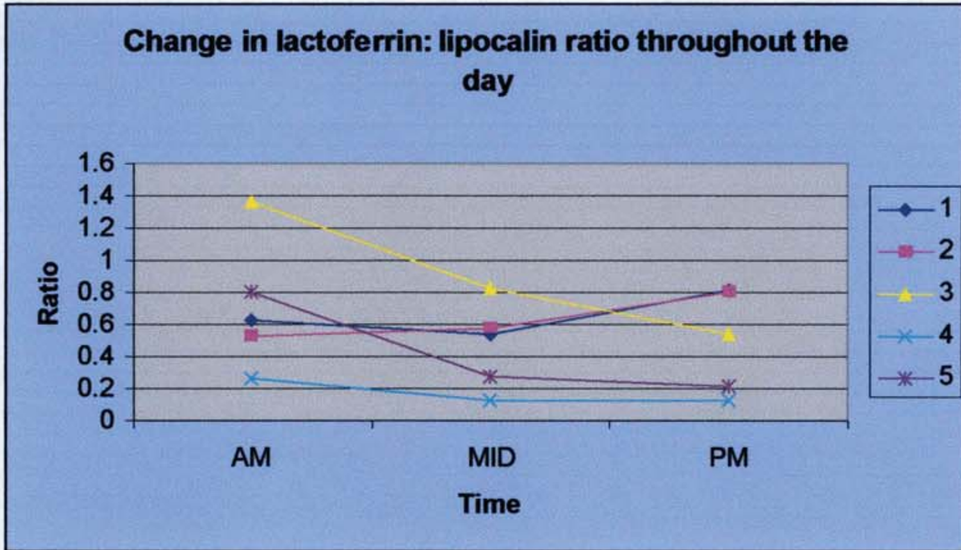


Figure 5.14 – Shows the changes in the ratio of lactoferrin: lipocalin of five subjects at different time points throughout the day
(Averaged over a three day period)

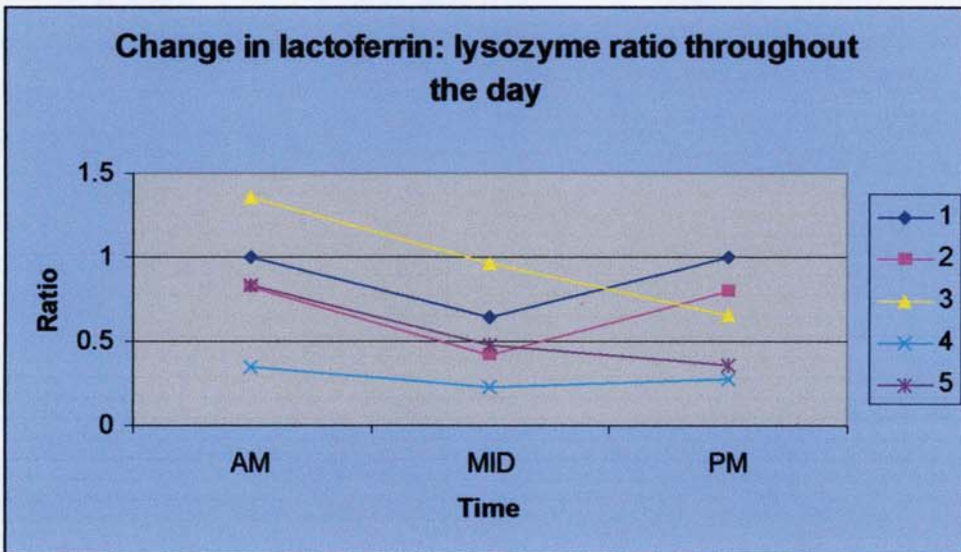


Figure 5.15 – Shows the changes in the ratio of lactoferrin: lysozyme of five subjects at different time points throughout the day
(Averaged over a three day period)

5.8.2 Preliminary Evaluation of the Lens Tear Envelope

In order to assess whether diurnal variation may have had any impact on the tear envelope that could be collected from the surface of a lens, the same three daily disposable lenses that were employed e.g. 1-day Acuvue, Focus Dailies and Soflens 1-day. The lenses were worn at different times of the day to ascertain whether any differences in the resulting profile could be observed. A full experimental protocol is detailed in Section 5.7.2.

Figure 5.16 (shown below) is an example of the tear envelope that could be collected from the three lens types following a wearing time of eight hours or more.

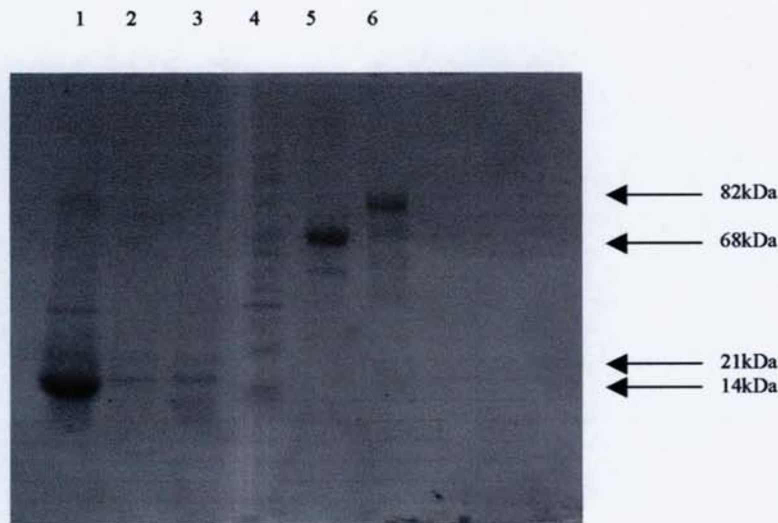


Figure 5.16 – Tear envelope removed from three different types of daily disposable lenses worn for more than eight hours.
Lane 1 – tear envelope removed from 1-day Acuvue, Lane 2 – tear envelope removed from Focus Dailies, Lane 3 – tear envelope removed from Soflens 1-day, Lane 4 – molecular weight marker, Lane 5 – albumin (2 mg/ml), Lane 6 – lactoferrin (1 mg/ml)

Figure 5.17 is the result of the tear envelope that was collected from 1-day Acuvue lenses that had been worn at different times of day. In order to ascertain whether it was possible to observe any differences in the tear envelope obtained from lenses that had been worn at different times of the day, the lens in the right eye was worn for the whole day (this served as the control lens) whilst the lens in the left eye was removed every three hours and

replaced with a fresh lens. The removed lens was immediately placed into an eppendorf containing treatment buffer.

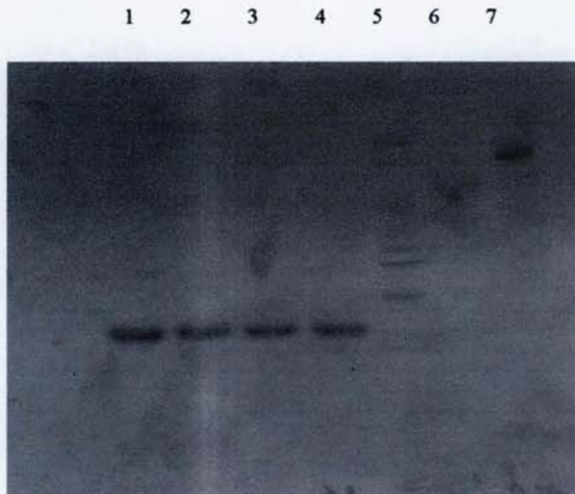


Figure 5.17 – Tear envelope removed from 1-day Acuvue worn at different times of the day
Lane 1 – control lens, Lane 2 – lens worn for 0 – 3 hours, Lane 3 – lens worn for 3 – 6 hours, Lane 4 – lens worn for 6 – 9 hours, Lane 5 – molecular weight marker, Lane 6 – albumin (2 mg/ml), Lane 7 – lactoferrin (1 mg/ml)

The resulting gel image was then analysed using the TotalLab™ software. There only appears to be one band present in lanes 1 – 4. The software package used the albumin standard that had been loaded into lane 6 as a reference point from which to calculate the concentrations of the unknowns in these lanes. This is presented below in Table 5.11.

Time of lens wear	Protein concentration (mg/ml)
Control (eight hours or more)	0.006
0 – 3 hours	0.009
3 – 6 hours	0.006
6 – 9 hours	0.007

Table 5.11 – Concentration of proteins within the tear envelope removed from 1-day Acuvue lenses worn at different times throughout the day as calculated using TotalLab ™

5.9 Discussion

It is not only contact lens wearers that are affected by experiences of end of day dryness. Many non-lens wearers also feel that their eyes become very tired and dry towards the end of the day. Many factors are thought to be responsible for this. A great deal of work is now carried out using computers and it is thought that sitting close to a screen for the vast majority of the day would affect the tear film. This may in part be due to the subject not performing proper blinks at regular intervals and thus not allowing the tear film to reform properly ensuring that the cornea is constantly lubricated. Air conditioning present in many work places is also thought to be a triggering factor in the end of day dryness. Other factors known to be responsible for causing a drying out of the tear film include smoking and the consumption of alcohol due to the subsequent effects of dehydration that ensue.

5.9.1 Diurnal Variations Observed Using Conventional Microcapillary Technique

This exercise has shown how relatively unreliable some of the quoted values of tear composition are. Variables associated with sampling, health, time of day and environment cause massive fluctuations in results obtained from individual patients. The subjects involved in this study were all free from ocular disease. Effects of environmental influences were minimised as far as was possible but the results generated still show a great deal of variation both within an individual and also between all subjects.

Patient 1 was unable to provide an evening tear sample on one occasion due to a drying out of the tear film. Using the results presented in Figure 5.7, whereby the data for each ratio was averaged out over the three day testing period, this patient was seen to have the highest ratio of lactoferrin: albumin of all the subjects. The ratio of albumin to lactoferrin has been used in the past as a diagnostic tool for ocular diseases including keratoconjunctivitis sicca (KCS). Patients suffering from primary Sjögrens syndrome are still able to produce tears. It has been reported that having an albumin to lactoferrin ration above 2: 1 is significantly more common in those patients suffering from primary Sjögrens syndrome than it is in healthy individuals.[128] The ratio observed in patient 1 was not elevated to this magnitude but could still possibly suggest that there was a slight degree of dry eye in this subject at the time of testing.

Tear samples were collected from Patient 3 at each of the time intervals. It was seen, however, that it was possible to collect a maximum of only 3µl. Any further attempts at collecting a sample would have resulted in a stimulated tear being produced. Such a tear is known to have an increased concentration of plasma derived proteins e.g. albumin. Previous work carried out on optimisation of this technique had found that a minimum sample volume of 5µl was required for reliable analysis. A low sample volume also made further analysis using the TotalLab™ software very difficult. Although it may have been possible to detect and quantify the proteins that have been studied in this exercise, as they are each known to be present in concentrations of approximately 1.3mg/ml, it may not have been possible to detect any other proteins present in significant concentrations. This would subsequently affect any calculation of percentages that were made.

Patients 2, 4 and 5 produced results that were most similar. The ratio of lactoferrin to lipocalin was especially similar and a range of 1: 0.38 to 1: 0.43 was observed between the three subjects.

It must be borne in mind that this investigation into diurnal variation was carried out on a relatively small population of individuals over a short period of time. Should this study be carried out over a longer period of time with a larger population it might be possible to determine variations that occur in the tear film as a result of diurnal variation.

Data generated from the clinical study (See Section 6.6) was concerned with sampling tears at regular intervals over the course of a year. The results showed that these individuals yielded more consistent results as a population over this time period. However, this study did not consider the effects that time of sampling might have had upon the tear samples. It therefore introduced other variables that could not be controlled, such as differences in the time of day and a varying environment. It does however illustrate the point that there are consistencies in certain protein ratios over time. This is discussed more fully in Chapter 6.

5.9.2 Diurnal Variations Observed in the Tear Envelope

The results concerning diurnal variation investigated using the novel tear envelope technique proved to be quite disappointing. The results that were presented in Figure 5.17 were obtained using 1-day Acuvue. It was not possible to collect the tear envelope from either Focus Dailies or Soflens 1-day that were of a high enough quality to be analysed by TotalLab™. Using the 1-day Acuvue suggested differences could be observed in the protein concentration at different times of day. Unfortunately, there did not appear to be any stable pattern emerging in the changes that took place. Until this has been repeated several times, with the participation of more subjects, it is impossible to categorically predict what changes are likely to take place.

Although unfortunate, it must be remembered that the lens is being submerged in 40μl of sample treatment buffer. It is estimated that only 2μl of the tear envelope would be attached to the lens at the time of removal. This represents a high dilution factor, but it was found in previous work that this was the minimum volume of buffer that could be used to ensure that the lens was completely immersed once retained in the eppendorf. It is therefore quite encouraging to demonstrate that such minute volumes of sample can be analysed using the techniques employed in this study.

SDS-PAGE has been shown to be one of the optimal electrophoretic techniques for the study of tear proteins. Nonetheless, it appears that systematic and unsystematic variations are so large that they make the observation of one variable (e.g. diurnal variation) unreasonably difficult using this technique.

In conclusion, this investigation into diurnal variation was conducted over a very short time period with a limited number of samples. Although it has not been possible to identify specific changes to the tear protein profile as a result of diurnal variation using either conventional methods of collection or the novel tear envelope sampling technique, it could be demonstrated that the larger population of control patients involved in the clinical study showed stable and unchanging ratios over the twelve month period confirming the presence of stable tear films.

Chapter 6

Clinical Study on the Effects of Contact Lenses on the Tear Film

6.1 Aim

Following on from a baseline study previously carried out on the effects of the tear film as a result of contact lens wear a unique, collaborative, clinical study was undertaken between the Biomaterials Research Unit and the Vision Sciences Department at Aston University. Following approval by the Human Sciences Ethical Committee, a population of previous non-contact lens wearers was recruited and randomly assigned to wear one of two lens types in a given wear modality. The key aspects of the study was that both the lens materials used were silicone hydrogels therefore allowing a direct comparison of the effects of daily wear and extended wear with not one but two materials. Clinical and biochemical changes were monitored over a two-year period. This was to enable the investigators to study the effects contact lenses, lens material, patient variation and wear regime had upon tear physiology and ocular health.

6.2 Introduction

The fact that contact lenses immediately come into contact with a biological fluid, the tear film in this case, upon insertion into the eye has been discussed. Components from the tear film are adsorbed onto the surface of the lens or into its matrix. This deposition onto the lenses does not only affect the visual performance of the lens but it can also disrupt the normal balance of the tear fluid.[125, 129, 130, 131]

This study was concerned with monitoring changes taking place in the tear protein profile over time. Tear samples were collected using a microcapillary prior to beginning lens wear to enable a base profile to be established for each person. The tears were then sampled at regular intervals (see section 6.3 for full experimental protocol) in order to investigate if, and how, contact lenses caused deviations in the tear protein profile. Tear proteins are of such interest due to their assumed role in immunological reactions. It was hoped that this study would enable predictions to be made regarding the suitability of a patient for lens wear according to the presence/absence of certain proteins in the tear film.

6.3 Parameters of the Clinical Study

In order to be eligible to participate within the study, the subjects had to fulfil certain criteria:

- Previous non-contact lens wearers
- Normal binocular vision
- A low to moderate level of refractive error and astigmatism
- No previous ocular health complications including either dry eye or highly atopic individuals
- 19 – 25 years old

The measurement of ocular parameters that were monitored included:

- Refractive error
- Axial length and anterior chamber depth
- Corneal topography
- Corneal thickness profile
- Slit lamp grading of anterior eye health
- Tear film with clinical measures such as slit lamp, tear prism height and the Tearscope
- Tear film changes following laboratory analysis

This data allowed the analysis of tear proteins in the hope of identifying particular proteins that could potentially be useful as biochemical predictive markers for the development of contact lens complications. Ocular biometry and refractive changes following a period of silicone hydrogel contact lens wear were monitored so clinical changes could also be followed and assessed. The examinations that were performed enabled objective measurements of ocular health to be made. A questionnaire designed specifically for this study allowed a comparison to be made between the participant's subjective opinions regarding comfort and the objective results obtained from clinical and biochemical data. The questionnaire can be found in the Appendix.

The subjects were initially examined prior to commencing lens wear. Tear samples collected by microcapillary were also obtained at this time. This allowed a base profile for

each individual to be established. The subjects were then examined at the following intervals:

- Initial visit
- After one week
- After two weeks
- After three weeks
- After one month
- After three months
- After six months
- After twelve months
- After twenty four months

Tear samples and worn lenses were collected at each examination for laboratory analysis. The tear samples were collected by microcapillaries and expressed into eppendorfs. They were then stored at 4°C until required. Worn lenses were placed in vials containing distilled water and stored at 4°C. The lenses were then analysed for lipid deposition and extracted to allow a determination of total protein and lipid to be made. This lens analysis does not form part of the work that has been presented within this thesis.

6.4 Silicone Hydrogels

A great deal of interest has centred on silicone hydrogels in the past few years regarding their suitability for use as contact lens materials. One of the most important requirements for a material designed for use in the eye is that it offers a high permeability to oxygen to ensure that oxygen is delivered to the avascular cornea. It was discovered that the oxygen transmissibility of hydrogels could be greatly enhanced when structural elements of silicone rubber were incorporated into their structures. This is because oxygen is more soluble in silicone rubber than it is in water.

The FDA has only recently given approval for silicone hydrogels to be worn for up to thirty days of continuous wear. They have been prescribed for use in Europe in this wear modality since 1999.[132] These materials are quite different from conventional contact lens materials. Silicone hydrogels have a high oxygen transmissibility (Dk/t) and it was thought that they would eliminate problems that were common with conventional hydrogels including contact lens-induced hypoxia.[133] The relatively recent introduction of these materials however means that there is not much data available on the effects they have upon the ocular environment.

The subjects were randomly assigned to one of the following groups:

1. Bausch and Lomb PureVision™ worn as a daily wear (DW) lens. The lens was to be worn for eight hours or more per day.
2. Bausch and Lomb PureVision™ worn as a continuous wear (CW) lens for 30 days.
3. Ciba Vision Focus® Night and Day™ worn as a DW lens for eight hours or more per day.
4. Ciba Vision Focus® Night and Day™ worn as a CW lens for 30 days.
5. Control group – no contact lenses.

A summary of the main characteristics of the lenses employed in this study is shown below (see Table 6.1).

Lens name	**Manufacturer**	**FDA classification**	**% water content**	**Ionicity**
Focus® Night & Day™	Ciba Vision	I	24	Non-ionic
PureVision™	Bausch & Lomb	III	35	Ionic

Table 6.1 – Characterisation of the lenses employed in the clinical study

6.5 Patient Details

The patients that were recruited into the study were then randomly allocated a particular lens type and wear modality. Tables 6.2 – 6.6 provide details on the wear modality and the lens type that individual patients wore throughout the course of this study. Any clinical observations that were made at clinical visits are also indicated within these tables.

Patient number	Incidences	Gender	Ethnicity
5	Strong vascular response after 18 hours. CLIPU (superior quadrant) at 3 month visit	M	Caucasian
6	Drop out after first month due to dry eyes and discomfort	F	Caucasian
7	Arthritis. Medication includes Nimesulid	F	Caucasian
9	Suffers from Crohn's Disease	F	Caucasian
13	Hay fever, asthma	F	Asian
22	Sensitive to Alcon, hay fever – prescribed Claritin	F	Asian
25	Recurrent meibomian gland dysfunction. Symptoms of dry eye	F	Asian
27		F	Asian-African
33	Dry eyes – uses rewetting drops, hay fever – prescribed Claritin.	F	Asian
37		F	Asian
62	Suffers from hay fever – takes systemic anti-histamines	F	Asian
71		M	Caucasian
73	Meibomian gland dysfunction	M	Caucasian

Table 6.2 - Patients wearing Focus® Night and Day™ on a daily wear basis

Patient number	Incidences	Gender	Ethnicity
1		F	Asian
2	SEAL on superior quadrant of the cornea at six month visit	F	Caucasian
3	Mucin balls at all routine visits; recurrent episodes of CLIPU and CLARE; meibomian gland dysfunction	M	Caucasian
11	Meibomian gland dysfunction, dryness and tired eyes when wearing lenses	M	Hong Kong Chinese
12	Meibomian gland dysfunction. Left eye resolved CLIPU after first month, experiences dry eyes so uses rewetting drops	M	Hong Kong Chinese
16	Experiences dry eyes so uses rewetting drops	M	Hong Kong Chinese
19	Recurrent meibomian gland dysfunction	M	Asian
26	Very thick lipid layer (acne problems) at initial visit. Lipid layer thinned at month visit	M	Chinese
28	CLIPU in inferior quadrant of cornea at six month visit	F	Malaysian-Chinese
38	Takes medication once monthly due to menstrual pains; Medication Mifenamic acid (pain killer), co-codamol, mycrogynon (steroids)	F	Caucasian
48	Hay fever, meibomian gland dysfunction	F	Caucasian
71		M	Caucasian

Table 6.3 – Patients wearing Focus® Night and Day™ on a continuous wear basis

Patient number	Incidences	Gender	Age
17	Suffers from hay fever, squint when child (corrected by surgery)	F	Caucasian
21	Experiences dryness and itching – uses rewetting drops	F	Hong Kong Chinese
24	Heavy smoker, meibomian gland dysfunction	F	Caucasian
32	Takes contraceptive pill	F	Caucasian
44	Drop out at 3 month visit due to blurred vision and discomfort	M	Caucasian
50	Suffers from hay fever, allergies to dust and pollen. Experiences end of day dryness	M	Chinese
51		M	Asian
53		M	Asian
54		M	Asian
57	Occasionally lenses become very dry and drop out	F	Asian
61	Variable AL	F	Asian

Table 6.4 – Patients wearing PureVision™ on a daily wear basis

Patient number	Incidences	Gender	Ethnicity
29	Dry eyes – uses rewetting drops	F	Asian-African
30	Change from a thick tear film at initial visit to a more aqueous tear film at month visit	M	Asian
31	Uses rewetting drops	F	Chinese
35	Asthmatic	F	Caucasian
36	Lens deposits (thick lipid layer), mucin balls	M	Asian
41	Meibomian gland dysfunction	M	Caucasian
43		F	Caucasian
46	Variable AL	F	African
47	Mucin balls at several routine visits. SEAL on left eye at six month visit	M	Caucasian
49	Bacterial conjunctivitis on left eye in 4th month of wear, tear film contaminated with make up, mucin balls, lenses dry and fall out, sensitivity to Alcon, asthmatic	F	Caucasian
52	Allergy to dust. Prescribed antibiotics for acne	F	Asian
56	Suffers from hay fever	F	African
58	Allergies to dust and pollen. Asthmatic – uses inhaler	M	African
59	Heavy smoker	F	Caucasian

Table 6.5 – Patients wearing PureVision™ on a continuous wear basis

Patient number	Incidences	Gender	Ethnicity
18	Variable AL	M	Asian
34	Variable AL	F	Asian
40	Meibomian gland dysfunction. Mucous and/or lipid strands on tear film	M	Hong Kong Chinese
45	Meibomian gland dysfunction	M	Asian
55	Slight dry eye, meibomian gland dysfunction	F	Asian
63		F	Asian
64	Variable AL	M	Asian
65	Variable AL	F	Caucasian
66	Variable AL	F	Caucasian
67	Slight dry eye (uses rewetting drops), meibomian gland dysfunction.	F	Asian
68	Meibomian gland dysfunction	F	Caucasian
69		M	Asian
70		M	Asian

Table 6.6 – Control patients

The patients participating in this study along with their corresponding lens type and wear modality are summarised below.

Lens type and wear modality	Number of patients
Focus® Night and Day™ – daily wear	13
Focus® Night and Day™ – continuous wear	12
PureVision™ – daily wear	11
PureVision™ – continuous wear	14
Control patients	13

Table 6.7 – Summary of the number of people wearing each lens type and the corresponding wear modality

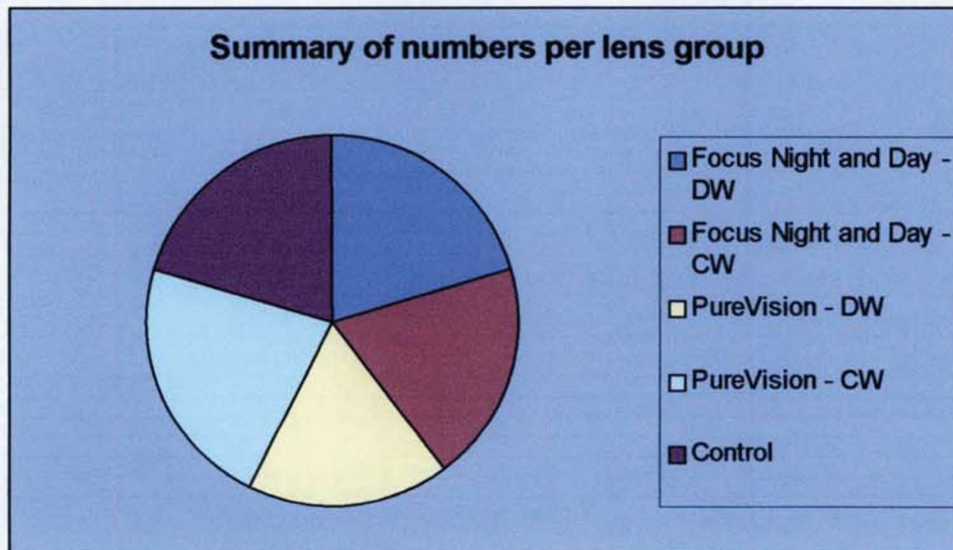


Figure 6.1 – Graphical representation showing the number of patients wearing each lens type and the corresponding wear modality

6.6 Results

6.6.1 Tear Samples Collected by Microcapillaries

Tear samples from the participants in the clinical study (see Tables 6.2 – 6.6) were collected at specified time intervals as described above (see section 6.3 and Table 6.8) and then analysed using SDS-PAGE. The resulting protein profiles were then subjected to further analysis using the TotalLab™ software package. (For full experimental protocol refer to section 2.6 and Chapter 3). Unfortunately, it was not possible to carry out western blotting and immunostaining on these samples due to the low volumes of samples that were obtained.

The visit numbers referred to throughout this chapter and their corresponding time period are summarised below in Table 6.8.

Visit Number	Time period
0	Pre lens wear
1	Week 1
2	Week 2
3	Week 3
4	Week 4
5	Month 3
6	Month 6
7	Month 12

Table 6.8 – Details of patient visits and sample collection and the corresponding time period they refer to

The results for each of the four major tear proteins are presented below (see Tables 6.9 – 6.13) as the percentage of the total protein present within the tear sample as calculated by TotalLab™. The results are presented according to lens material and wear modality.

6.6.1.1 Concentrations of the four major tear proteins in the tear film

		Visit Number							
Patient Number	**Protein (%)**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
5	Lactoferrin	33.3	29.3	26.9	29.5			22.8	34.3
	Albumin	12.8	9.8	13.5	17.9			14.1	14.3
	Lipocalin	14.9	17.1	23.1	12.6			23.9	14.3
	Lysozyme	9.2	29.3	1.9	18.9			-	12.9
7	Lactoferrin	38.5			21.9			22.6	26.7
	Albumin	12.5			8.2			11.3	17.8
	Lipocalin	17.7			13.7			25.8	17.8
	Lysozyme	3.1			30.1			-	20.0
9	Lactoferrin	37.1	21.4	32.4	17.7	16.7	36.9	25.0	23.4
	Albumin	16.1	14.3	13.5	15.0	4.3	3.6	10	12.8
	Lipocalin	17.7	21.4	25.7	9.7	19.7	25.0	20	14.9
	Lysozyme	3.2	28.6	2.7	21.2	26.5	29.8	-	19.1
13	Lactoferrin	-	7.1	9.5	43.5		18.2	13.0	13.6
	Albumin	-	7.1	4.8	-		9.1	4.3	3.9
	Lipocalin	7.7	35.7	47.6	21.7		27.3	23.2	27.2
	Lysozyme	7.7	50.0	28.6	13.0		27.3	21.7	46.6
25	Lactoferrin	31.8	33.3	10.0	23.3		28.6	19.2	26.7
	Albumin	-	5.6	10.0	13.3		28.6	12.1	11.1
	Lipocalin	31.8	33.3	40.0	23.3		28.6	17.2	24.4
	Lysozyme	36.4	27.8	40.0	-		14.3	25.3	33.3
27	Lactoferrin	20.0		18.2	23.1	64.7	65.2	30.0	31.1
	Albumin	7.3		9.1	26.9	8.8	13.0	9.2	18.9
	Lipocalin	38.2		36.7	19.2	8.8	18.7	10.6	17.6
	Lysozyme	27.3		36.7	21.2	8.8	13.0	4.9	25.7

Table 6.9 – Concentrations of the major tear proteins in the tear fluid of patients wearing Focus® Night and Day™ on a daily wear basis (Visit 0 – Visit 7)

		Visit Number							
Patient Number	**Protein (%)**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	Lactoferrin	41.1	27.8	24.1	30.3		28.8	30.4	28.6
	Albumin	4.4	11.1	13.8	11.8		10.8	6.5	7.1
	Lipocalin	20.0	22.2	24.1	18.4		19.8	32.6	21.4
	Lysozyme	5.6	25.0	-	18.4		32.4	-	28.6
3	Lactoferrin	35.4	28.6	24.1	26.8	12.2	27.2	23.6	19.4
	Albumin	28.5	28.6	13.8	21.1	2.4	10.9	14.5	30.6
	Lipocalin	6.3	28.6	24.1	14.6	28.5	25.2	21.8	13.9
	Lysozyme	6.3	14.3	-	12.2	20.3	29.3	-	22.2
11	Lactoferrin	40.8	26.0	29.3	20.5	24.7	22.3	27.8	18.2
	Albumin	14.3	14.0	14.7	14.5	8.4	14.0	12.5	10.7
	Lipocalin	18.4	18.0	24.0	14.5	18.2	21.5	27.8	21.2
	Lysozyme	2.0	24.0	-	14.5	21.4	8.3	-	30.3
12	Lactoferrin	20.0	32.7	30.0	28.3	37.5	29.7	37.3	35.7
	Albumin	4.0	26.5	18.0	20.0	25.0	28.4	28.4	42.9
	Lipocalin	8.0	14.3	14.0	11.7	12.5	10.8	7.4	7.1
	Lysozyme	20.0	24.5	24.0	21.7	12.5	8.9	14.9	7.1
16	Lactoferrin	33.3	50.0	42.9	50.0	43.75	48.6	48.9	
	Albumin	8.3	11.1	28.6	25.0	31.3	28.6	27.7	
	Lipocalin	16.7	11.1	7.1	7.1	6.3	5.7	6.4	
	Lysozyme	25.0	16.7	10.7	10.7	12.5	8.6	8.5	
19	Lactoferrin			18.2				22.1	33.3
	Albumin			9.1				9.1	11.1
	Lipocalin			27.3				20.8	11.1
	Lysozyme			27.3				23.4	38.9
26	Lactoferrin			50.0			22.4	22.1	38.2
	Albumin			35.7			16.3	9.1	50.0
	Lipocalin			7.1			12.2	20.8	2.9
	Lysozyme			7.1			16.3	23.4	2.9

48	Lactoferrin			41.9				4.7	72.4
	Albumin			25.8				5.5	13.8
	Lipocalin			6.5				22.0	3.4
	Lysozyme			16.1				17.3	3.4

Table 6.10 - Concentrations of the major tear proteins in the tear fluid of patients wearing Focus® Night and Day™ on a continuous wear basis (Visit 0 – Visit 7)

Below are shown some typical gel images representative of those that were obtained throughout the clinical study.

Figure 6.2 (shown below) illustrates the protein profiles of the tear samples that were collected from the patients, who were subsequently recruited into Focus Night and Day lenses, prior to commencing lens wear.

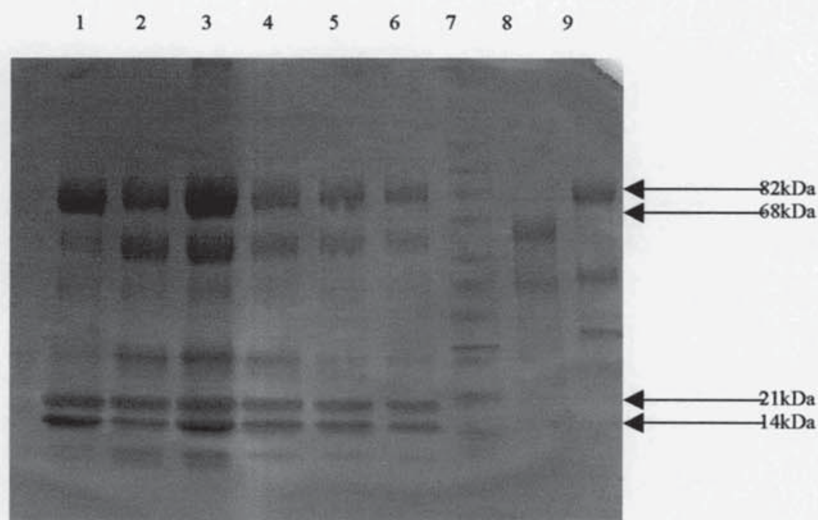


Figure 6.2 – Tear samples collected pre lens wear
Lane 1 – patient 1, Lane 2 – patient 3, Lane 3 – patient 5, Lane 4 – patient 7, Lane 5 – patient 9, Lane 6 – patient 11, Lane 7 –molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

Figures 6.3 – 6.6 shown overleaf are the tear protein profiles that were collected at subsequent visits upon commencement of lens wear (Focus Night and Day). Patients illustrated in these images belong to both the daily wear regime and the continuous wear regime. For full details of patients and their corresponding wear modality, refer back to Tables 6.2 and 6.3.

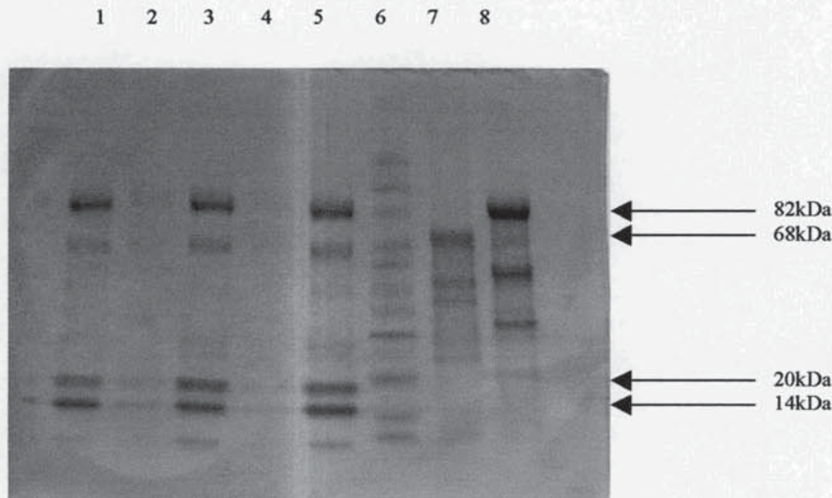


Figure 6.3 – Tear samples following one week of lens wear (Focus® Night and Day™)
Lane 1 – patient 5, Lane 2 – patient 9, Lane 3 – patient 1, Lane 4 – patient 3, Lane 5 – patient 11, Lane 6 – molecular weight marker, Lane 7 – albumin (2 mg/ml), Lane 8 – lactoferrin (1 mg/ml)

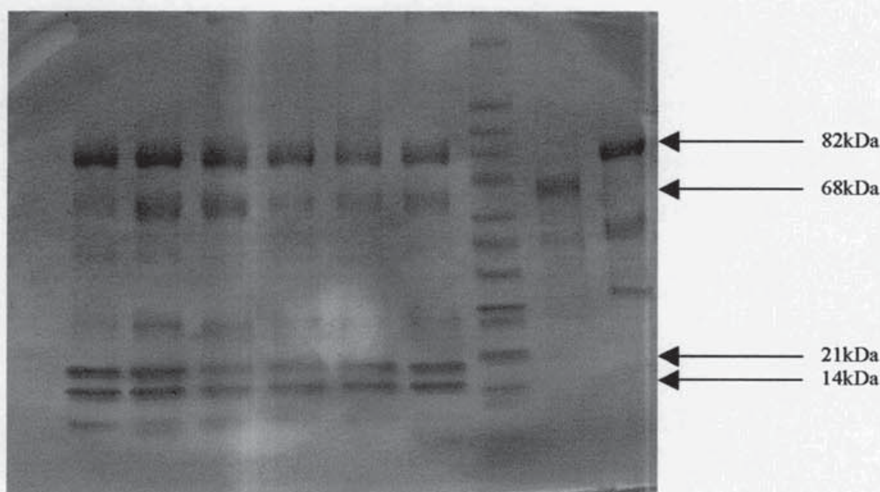


Figure 6.4 – Tear samples following three weeks of lens wear (Focus® Night and Day™)
Lane 1 – patient 1, Lane 2 – patient 3, Lane 3 – patient 5, Lane 4 – patient 7, Lane 5 – patient 9, Lane 6 – patient 11, Lane 7 –molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

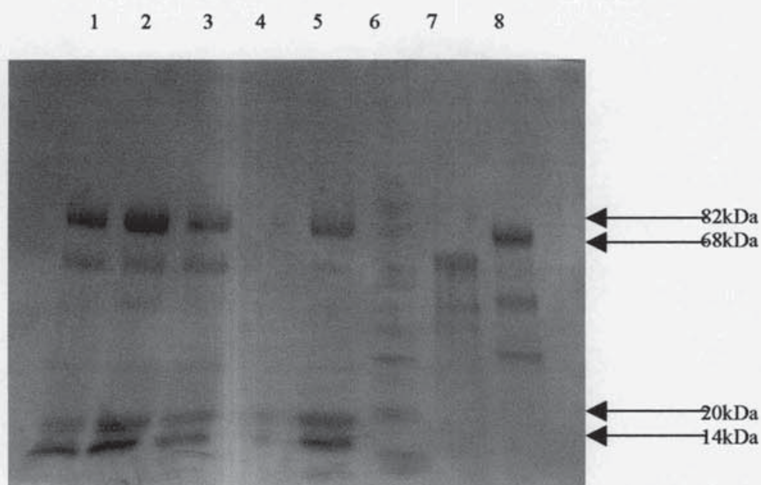


Figure 6.5 – Tear samples following three months of lens wear (Focus® Night and Day™)
Lane 1 – patient 1, Lane 2 – patient 3, Lane 3 – patient 11, Lane 5 – patient 9, Lane 6 – molecular weight marker, Lane 7 – albumin (2 mg/ml), Lane 8 – lactoferrin (1 mg/ml)

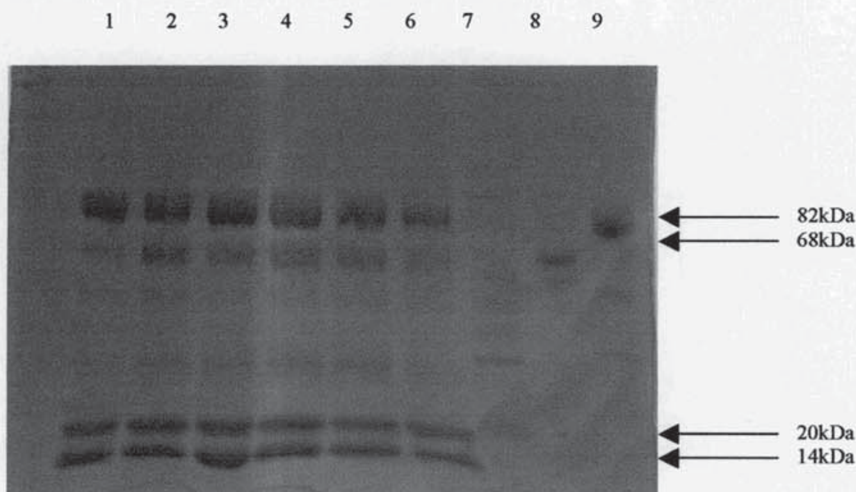


Figure 6.6 – Tear samples following six months of lens wear (Focus® Night and Day™)
Lane 1 – patient 1, Lane 2 – patient 3, Lane 3 – patient 11, Lane 4 – patient 5, Lane 5 – patient 7, Lane 6 – patient 9, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

Patient Number	Protein (%)	Visit Number 0	1	2	3	4	5	6	7
17	Lactoferrin	52.2	21.3			25.0	31.1	33.3	35.1
	Albumin	39.1	10.6			4.2	5.6	12.6	18.9
	Lipocalin	-	19.1			26.4	23.3	23.0	16.2
	Lysozyme	-	25.5			8.3	6.7	18.4	21.6
21	Lactoferrin	65.0	29.3			37.3	38.3	38.8	41.7
	Albumin	20.0	9.8			6.8	6.2	14.4	16.7
	Lipocalin	-	18.3			15.3	16.0	22.4	16.7
	Lysozyme	-	26.8			3.4	3.7	20.0	16.7
24	Lactoferrin	25.6				42.0	44.6	41.2	30.3
	Albumin	7.7				16.0	16.9	11.8	9.1
	Lipocalin	25.6				10.0	7.7	17.6	18.2
	Lysozyme	-				18.0	18.5	23.5	18.2
32	Lactoferrin	34.3	46.2			20.8	24.4	34.6	30.4
	Albumin	11.4	12.8			9.1	9.3	11.5	30.4
	Lipocalin	25.7	17.9			15.6	17.4	23.1	17.4
	Lysozyme	-	23.0			5.2	12.8	17.9	13.0
50	Lactoferrin	30.0	19.2	37.5	24.0	41.7	33.3	34.2	38.5
	Albumin	10.0	3.8	6.25	32.0	16.7	14.3	8.2	23.1
	Lipocalin	33.3	30.8	25.0	22.0	16.7	19.0	13.7	15.4
	Lysozyme	26.7	34.7	31.25	16.0	16.7	23.8	10.3	23.1
51	Lactoferrin	20.8	38.5	23.8	20.9		36.4	18.0	
	Albumin	16.7	11.5	4.8	14.0		13.6	10.0	
	Lipocalin	29.2	15.4	33.3	23.3		18.2	22.0	
	Lysozyme	33.3	30.8	33.3	25.6		27.3	22.0	
53	Lactoferrin	22.2	41.2	5.9	18.4		22.2	15.6	
	Albumin	14.8	11.8	11.8	8.2		11.1	7.8	
	Lipocalin	29.6	-	29.4	18.4		16.7	18.8	
	Lysozyme	33.3	47.1	47.1	28.6		33.3	25.0	

Table 6.11 - Concentrations of the major tear proteins in the tear fluid of patients wearing PureVision™ on a daily wear basis (Visit 0 – Visit 7)

		Visit Number							
Patient Number	Protein (%)	0	1	2	3	4	5	6	7
29	Lactoferrin	19.4	33.3			36.6	37.2	37.8	28.1
	Albumin	13.9	11.1			4.2	4.9	18.9	18.8
	Lipocalin	22.2	16.7			21.1	20.6	15.1	12.5
	Lysozyme	-	27.8			9.9	20.6	20.8	18.8
30	Lactoferrin			43.3				20.0	
	Albumin			20.0				6.0	
	Lipocalin			13.3				26.0	
	Lysozyme			13.3				30.0	
31	Lactoferrin	43.8	28.9			35.1	34.7	36.2	43.5
	Albumin	13.4	13.3			10.6	10.8	22.4	21.7
	Lipocalin	18.9	19.3			19.1	19.8	15.5	8.7
	Lysozyme	25.0	25.3			21.3	21.6	10.3	8.7
35	Lactoferrin			46.8				8.5	35.5
	Albumin			27.7				2.9	16.1
	Lipocalin			8.5				27.0	19.4
	Lysozyme			10.6				22.2	25.8
47	Lactoferrin			44.8				17.2	46.6
	Albumin			27.6				6.9	25.9
	Lipocalin			10.3				24.1	10.3
	Lysozyme			13.8				25.9	13.8
58	Lactoferrin	25		50.0	33.3		50.0	18.8	53.8
	Albumin	25		20.0	46.7		16.7	31.3	30.8
	Lipocalin	25		10.0	6.7		8.3	6.3	7.7
	Lysozyme	25		10.0	6.7		8.3	6.3	7.7
59	Lactoferrin	5.9	79.3	37.0			52.8	40.0	
	Albumin	5.9	10.8	22.2			30.6	13.3	
	Lipocalin	-	1.0	11.1			5.6	20.0	
	Lysozyme	29.4	2.7	14.8			2.8	26.7	

Table 6.12 - Concentrations of the major tear proteins in the tear fluid of patients wearing PureVision™ on a continuous wear basis (Visit 0 – Visit 7)

Shown below (see Figures 6.7 – 6.10) are gel images illustrating the tear protein profiles of the tear samples that were collected from the subjects wearing PureVision™ lenses. Both wear modalities are illustrated here. Information regarding an individual's wear modality is detailed in Tables 6.4 and 6.5.

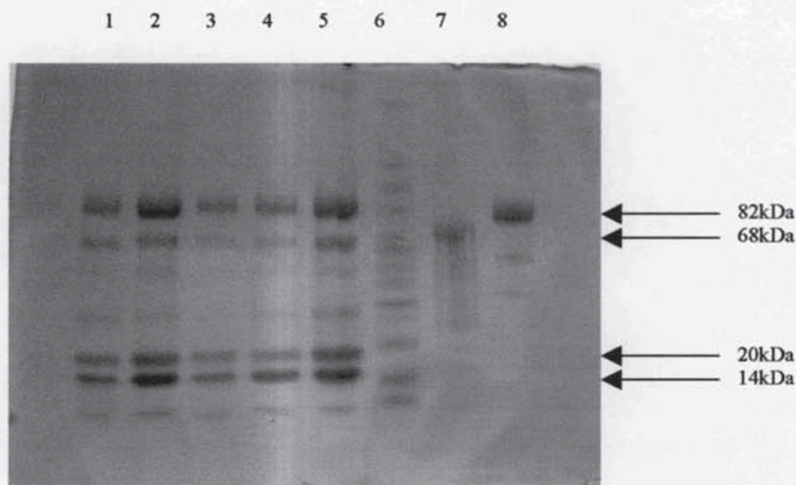


Figure 6.7 – Tear samples following one week of lens wear (PureVision™)
Lane 1 – patient 17, Lane 2 – patient 21, Lane 3 – patient 32, Lane 4 – patient 29, Lane 5 – patient 31, Lane 6 – molecular weight marker, Lane 7 – albumin (2 mg/ml), Lane 8 – lactoferrin (1 mg/ml)

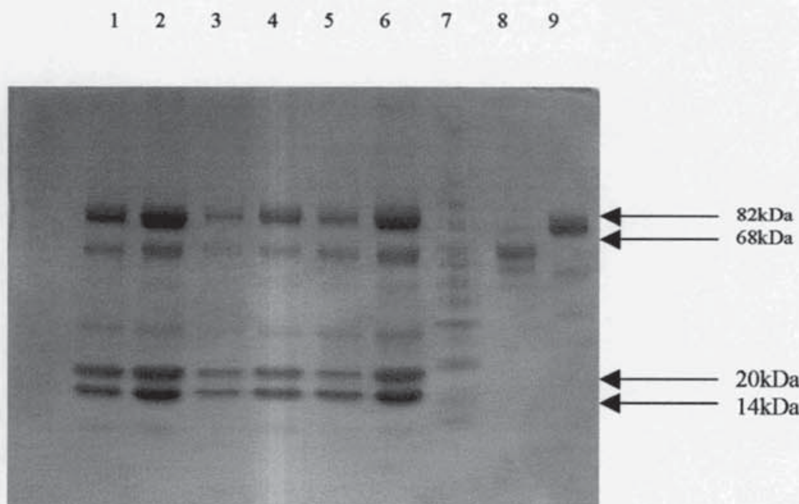


Figure 6.8 – Tears samples following two weeks of lens wear (PureVision™)
Lane 1 – patient 17, Lane 2 – patient 21, Lane 3 – patient 32, Lane 4 – patient 24, Lane 5 – patient 29, Lane 6 – patient 31, Lane 7 - molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

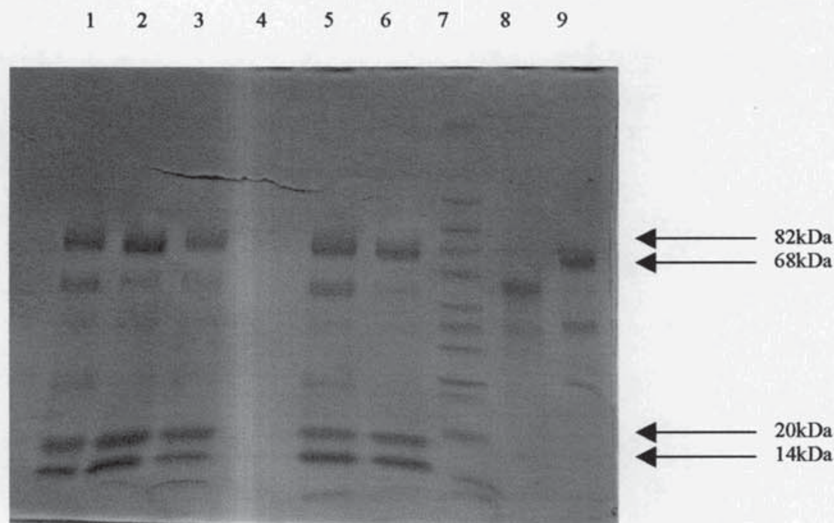


Figure 6.9 – Tear samples following one month of lens wear (PureVision™)
Lane 1 – patient 17, Lane 2 – patient 21, Lane 3 – patient 32, Lane 4 – patient 24, Lane 5 – patient 29, Lane 6 – patient 31, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

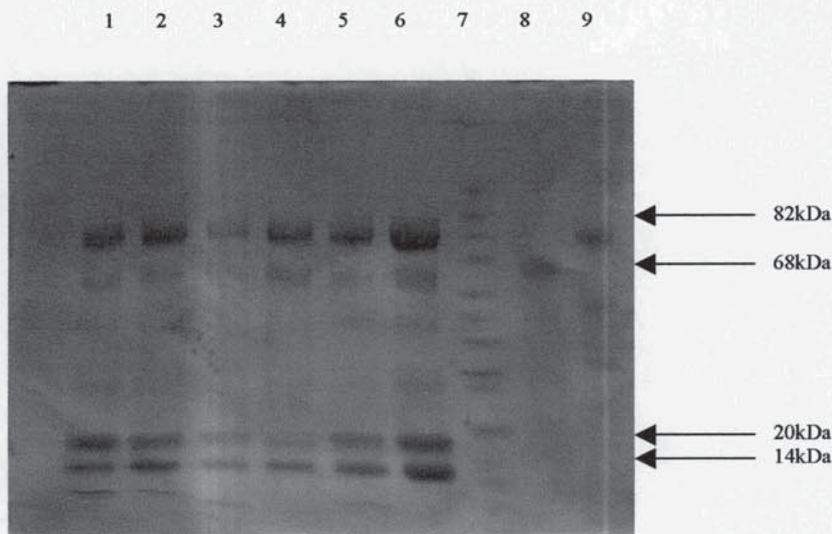


Figure 6.10 – Tear samples following three months of lens wear (PureVision™)
Lane 1 – patient 17, Lane 2 – patient 21, Lane 3 – patient 32, Lane 4 – patient 24, Lane 5 – patient 29, Lane 6 – patient 31, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

Patient Number	Protein (%)	Visit Number 0	1	2	3	4	5	6	7
64	Lactoferrin	23.7				30.5	44.6	29.3	22.7
	Albumin	15.5				10.2	24.6	14.6	13.6
	Lipocalin	2.1				25.4	10.8	12.2	18.2
	Lysozyme	-				-	12.3	31.7	27.3
65	Lactoferrin	39.7				37.5	47.8	28.0	39.1
	Albumin	6.3				6.3	32.6	12.0	21.7
	Lipocalin	20.6				31.3	3.3	20.0	13.0
	Lysozyme	28.6				-	5.4	24.0	13.0
67	Lactoferrin	34.2				30.8	48.1	16.7	
	Albumin	13.2				15.4	29.9	5.6	
	Lipocalin	18.4				25.6	5.2	22.2	
	Lysozyme	23.7				-	7.8	33.3	
68	Lactoferrin	28.7				33.3	47.4	19.2	44.1
	Albumin	16.1				4.8	28.9	15.4	20.6
	Lipocalin	20.7				33.3	5.2	15.4	11.8
	Lysozyme	20.7				-	7.2	34.6	8.8
69	Lactoferrin	30.4				31.7	44.4	26.7	44.4
	Albumin	7.1				14.6	41.1	10.0	22.2
	Lipocalin	21.4				26.8	3.3	26.7	8.3
	Lysozyme	28.6				-	8.8	26.7	13.9
72	Lactoferrin	29.8				34.6	35.0	48.1	42.9
	Albumin	10.6				7.7	22.0	14.8	20.0
	Lipocalin	19.2				34.6	7.3	11.1	11.4
	Lysozyme	26.0				-	7.3	22.2	11.4

Table 6.13 - Concentrations of the major tear proteins in the tear fluid of control patients

Figures 6.11 – 6.14 are illustrative of the tear samples that were collected from the control subset of individuals in the clinical study at differing time periods.

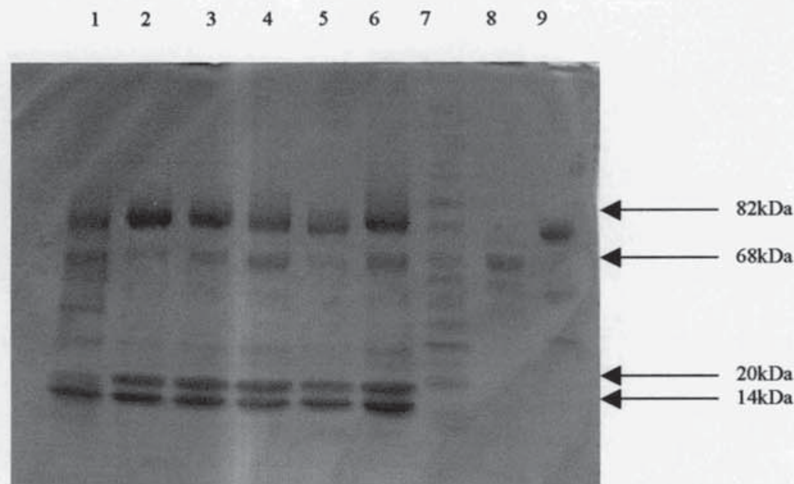


Figure 6.11 – Tear samples from control patients (Visit 0)
Lane 1 – patient 64, Lane 2 – patient 65, Lane 3 – patient 67, Lane 4 – patient 68, Lane 5 – patient 69, Lane 6 – patient 72, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

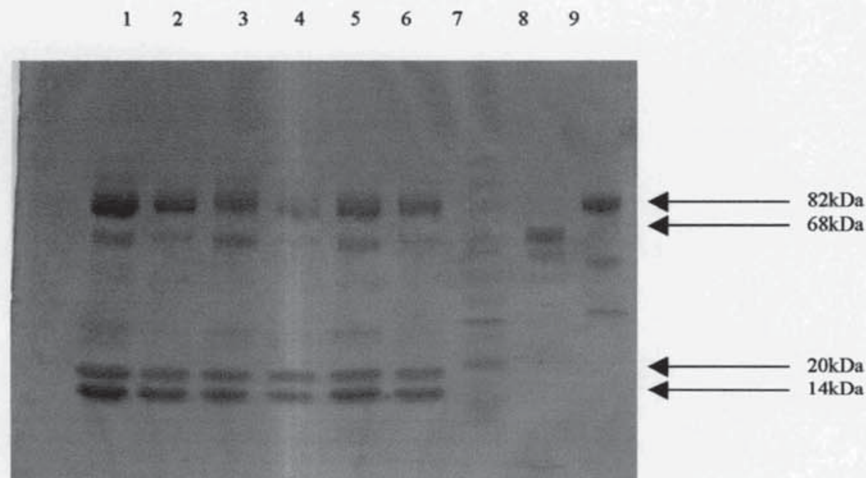


Figure 6.12 – Tear samples from control patients (Visit 4)
Lane 1 – patient 64, Lane 2 – patient 65, Lane 3 – patient 67, Lane 4 – patient 68, Lane 5 – patient 69, Lane 6 – patient 72, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml), Lane 9 – lactoferrin (1 mg/ml)

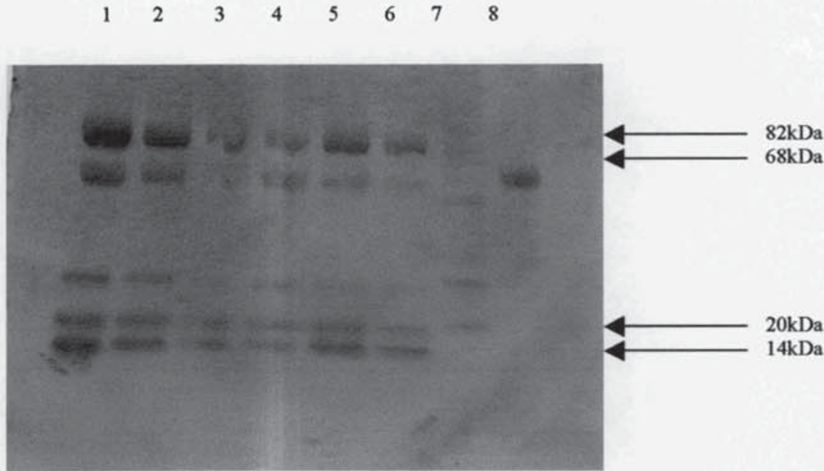


Figure 6.13 – Tear samples from control patients (Visit 5)
Lane 1 – patient 64, Lane 2 – patient 65, Lane 3 – patient 67, Lane 4 – patient 68, Lane 5 – patient 69, Lane 6 – patient 72, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml)

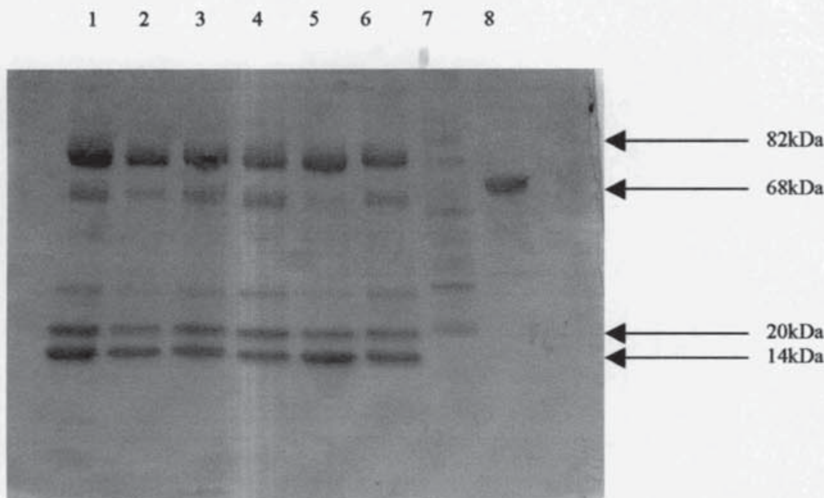


Figure 6.14 – Tear samples from control patients (Visit 6)
Lane 1 – patient 64, Lane 2 – patient 65, Lane 3 – patient 67, Lane 4 – patient 68, Lane 5 – patient 69, Lane 6 – patient 72, Lane 7 – molecular weight marker, Lane 8 – albumin (2 mg/ml),

6.6.2 Calculation of Minimum and Maximum Limits

In order for distinctions to be made between tolerant and intolerant lens wearers, any changes taking place in the tear film protein concentrations of the lens wearers in this study (shown in Tables 6.9 – 6.12) must first be compared with those obtained from the control population. This was achieved by calculating the ratio of lactoferrin: albumin, lactoferrin: lipocalin and also albumin: lipocalin of all control patients averaged over Visit 0 – Visit 7. The tear proteins of these patients, being all non-lens wearers, would not be expected to fluctuate greatly. This data is shown below in Table 6.14.

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
64	1: 0.52	1: 0.46	1: 0.79	1: 0.88
65	1: 0.41	1: 0.46	1: 0.46	1: 1.12
67	1: 0.36	1: 0.40	1: 0.52	1: 1.10
68	1: 0.50	1: 0.50	1: 0.52	1: 1.01
69	1: 0.53	1: 0.49	1: 0.55	1: 0.91
72	1: 0.39	1: 0.44	1: 0.44	1: 1.11

Table 6.14 – Ratios of the major tear proteins for all control patients averaged out over all time periods (Visit 0 – Visit 7)

The information presented above shows the distribution of the ratios obtained from the control patients. This population is representative of a normal tear fluid. All values that are shown for each patient, and each protein ratio, appear to be contained within reasonably tight limits.

In order to identify any disturbances taking place to the tear films of the lens wearers it is vital to establish limits that are considered to be normal. Table 6.15 shows the values that are considered to be representative of this control population as they encompass most data points that were calculated.

Protein ratio	Minimum limit	Maximum limit
Lactoferrin: Albumin	1: 0.40	1: 0.50
Lactoferrin: Lipocalin	1: 0.40	1: 0.50
Lactoferrin: Lysozyme	1: 0.45	1: 0.55
Albumin: Lipocalin	1: 0.90	1: 1.10

Table 6.15 – Minimum and maximum limits for the protein ratios as being indicative of a healthy tear film

The ratios were then calculated for all lens wearers in the study. This was calculated by taking the average of the protein concentrations from Visit 1 – Visit 7, e.g. all visits following commencement of lens wear. This data for all patients is shown below (see Tables 6.16 – 6.19) according to lens type and wear modality.

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
5	1: 0.49	1: 0.64	1: 0.55	1: 1.31
7	1: 0.52	1: 0.80	1: 1.06	1: 1.54
9	1: 0.42	1: 0.79	1: 0.86	1: 1.86
13	1: 0.33	1: 1.74	1: 1.78	1: 5.21
25	1: 0.57	1: 1.18	1: 1.20	1: 2.07
27	1: 0.46	1: 0.61	1: 0.61	1: 1.33

Table 6.16 – Ratios of tear proteins of patients wearing Focus® Night and Day™ in a daily wear modality for all post lens visits
(Average data for all visits)

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
1	1: 0.36	1: 0.81	1: 0.92	1: 2.27
3	1: 0.75	1: 0.97	1: 0.85	1: 1.29
11	1: 0.53	1: 0.86	1: 0.82	1: 1.64
12	1: 0.82	1: 0.37	1: 0.47	1: 0.41
16	1: 0.54	1: 0.15	1: 0.24	1: 0.29
19	1:0.40	1: 0.80	1: 1.22	1: 2.02
26	1: 0.84	1: 0.32	1: 0.37	1: 0.39
48	1: 0.38	1: 0.27	1: 0.31	1: 0.71

Table 6.17 – Ratios of tear proteins of patients wearing Focus® Night and Day™ in a continuous wear modality for all post lens visits
(Average data for all visits)

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
17	1: 0.36	1: 0.74	1: 0.49	1: 2.08
21	1: 0.29	1: 0.49	1: 0.38	1: 1.65
24	1: 0.33	1: 0.43	1: 0.53	1: 1.29
32	1: 0.47	1: 0.58	1: 0.46	1: 1.25
50	1: 0.46	1: 0.62	1: 0.68	1: 1.37
51	1: 0.39	1: 0.82	1: 1.01	1: 2.08
53	1: 0.49	1: 1.01	1: 1.75	1: 2.05

Table 6.18 - Ratios of tear proteins of patients wearing PureVision™ in a daily wear modality for all post lens visits
(Average data for all visits)

Patient Number	Lactoferrin: Albumin	Lactoferrin: Lipocalin	Lactoferrin: Lysozyme	Albumin: Lipocalin
29	1: 0.33	1: 0.50	1: 0.57	1: 1.49
30	1: 0.41	1: 0.46	1: 0.50	1: 1.10
31	1: 0.44	1: 0.46	1: 0.46	1: 1.05
35	1: 0.51	1: 0.60	1: 0.65	1: 1.18
47	1: 0.56	1: 0.41	1: 0.49	1: 0.74
58	1: 0.74	1: 0.28	1: 0.28	1: 0.38
59	1: 0.37	1: 0.18	1: 0.22	1: 0.40

Table 6.19 - Ratios of tear proteins of patients wearing PureVision™ in a continuous wear modality for all post lens visits
(Average data for all visits)

The ratios that are displayed above in Tables 6.16 – 6.19 are presented graphically below to illustrate the proportion of lens wearers that are considered to be within the minimum and maximum levels deemed to be representative of a tolerant lens wearer. Those patients that appear to be significantly out of the limits, as set with reference to the control patients, may be more likely to suffer from an impaired tear film.

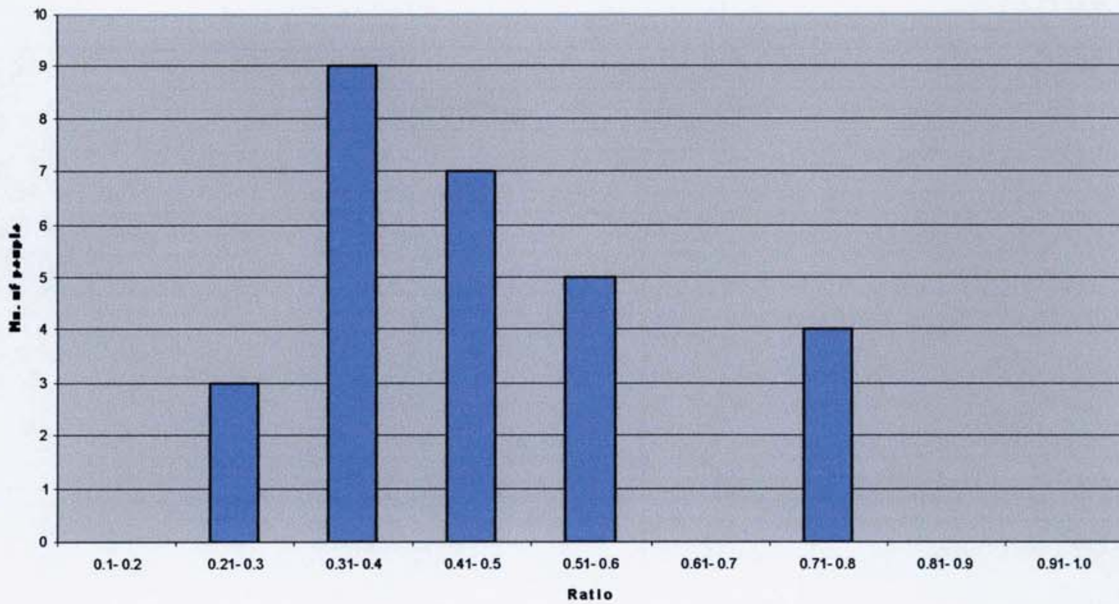


Figure 6.15 – Bar chart showing the distribution of the lactoferrin: albumin ratio for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)

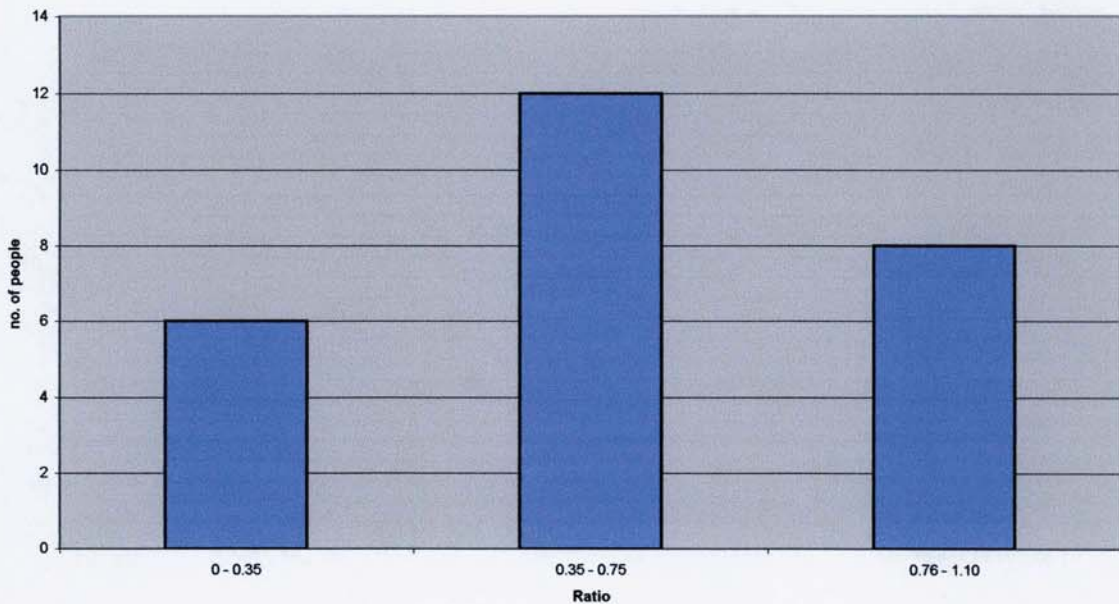


Figure 6.16 - Bar chart showing the distribution of the lactoferrin: lipocalin for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)

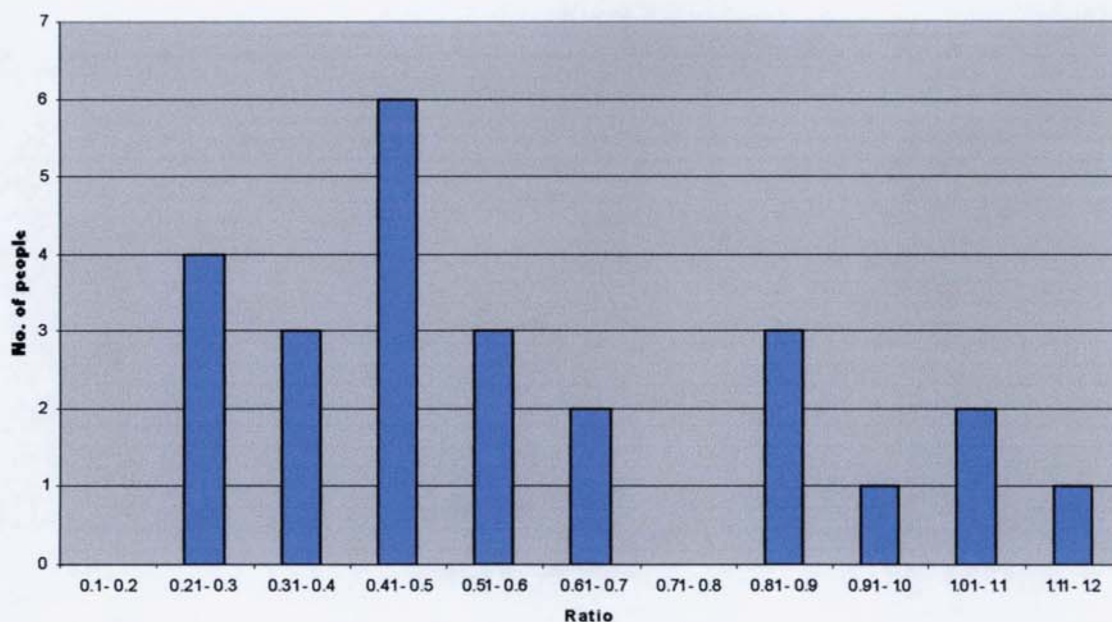


Figure 6.17 - Bar chart showing the distribution of the lactoferrin: lysozyme for all lens wearers in the clinical study, post lens wear (Visit 1 – Visit 7)

The graphical representation in Figure 6.15 above indicates that the majority of the lens wearing population has a lactoferrin: albumin ratio not too dissimilar from the non-lens wearing, control, population. Figures 6.16 and 6.17 show that the distribution of the ratios for these proteins is much more widely spread in the lens wearing population.

6.6.3 Comparison of a Lens Wearing Population and a Control Population

The data were then used to calculate the ratios as described above for the lens wearing population at Visit 0, and then again at Visit 7. This was then compared with the ratios calculated for the control patients over the same period. At this point, no distinction was made between either the lens material or the wear modality.

Group	Ratio	Visit 0	Visit 7
Lens wearing population (n = 28)	Lactoferrin: Albumin	1: 0.49	1: 0.58
	Lactoferrin: Lipocalin	1: 0.65	1: 0.43
	Lactoferrin: Lysozyme	1: 0.52	1: 0.59
	Albumin: Lipocalin	1: 1.64	1: 0.74
Control population (n = 6)	Lactoferrin: Albumin	1: 0.37	1: 0.51
	Lactoferrin: Lipocalin	1: 0.55	1: 0.32
	Lactoferrin: Lysozyme	1: 0.82	1: 0.39
	Albumin: Lipocalin	1: 1.49	1: 0.64

Table 6.20 - Comparison of the tear protein ratios of a lens wearing population and a control population at Visit 0 and Visit 7

The ratios were then calculated for Visit O and Visit 7 for each individual wearing each lens and each wear modality. These results are shown in Table 6.21.

Group	Ratio	Visit 0	Visit 7
Focus DW	Lactoferrin: Albumin	1: 0.30	1: 0.50
	Lactoferrin: Lipocalin	1: 0.74	1: 0.74
	Lactoferrin: Lysozyme	1: 0.49	1: 1.01
	Albumin: Lipocalin	1: 2.47	1: 1.47
Focus CW	Lactoferrin: Albumin	1: 0.34	1: 0.68
	Lactoferrin: Lipocalin	1: 0.41	1: 0.33
	Lactoferrin: Lysozyme	1: 0.35	1: 0.54
	Albumin: Lipocalin	1: 1.16	1: 0.33
PureVision DW	Lactoferrin: Albumin	1: 0.48	1: 0.56
	Lactoferrin: Lipocalin	1: 0.80	1: 0.48
	Lactoferrin: Lysozyme	1: 0.87	1: 0.53
	Albumin: Lipocalin	1: 1.68	1: 0.85
PureVision CW	Lactoferrin: Albumin	1: 0.43	1: 0.55
	Lactoferrin: Lipocalin	1: 0.65	1: 0.28
	Lactoferrin: Lysozyme	1: 0.79	1: 0.36
	Albumin: Lipocalin	1: 1.50	1: 0.52

Table 6.21 – Comparison of the protein ratios of the lens-wearing individuals according to lens type and wear modality

6.6.4 Removal of the Tear Envelope from Lenses Worn in the Clinical Study

A small number of worn lenses became available for the analysis of the tear envelope. This technique is described in more detail in section 2.3.2

Attempts at removing the tear envelope from silicone hydrogels had not been carried out prior to this. The results of the attempts to remove the tear envelope from the lenses are illustrated below.

(The lenses were removed from patients at the time of examination and were immediately placed into an eppendorf containing treatment buffer. The eluate that was obtained following centrifugation was subjected to SDS-PAGE).

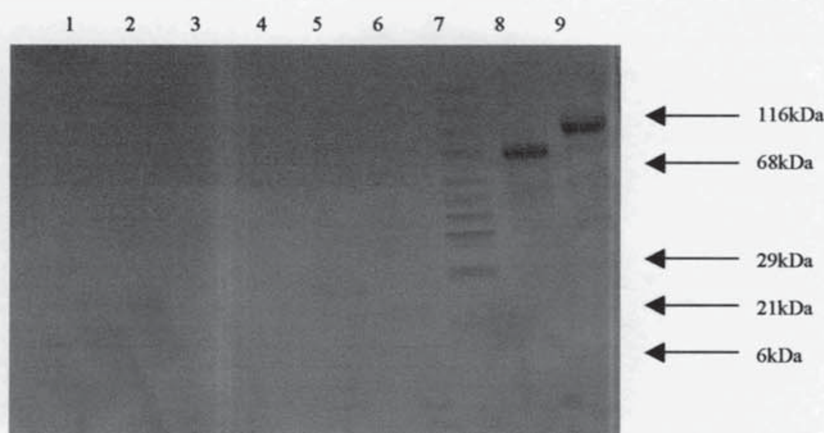


Figure 6.18 – Gel scan showing tear envelope from worn lenses
Lane 1 – patient 48, Lane 2 – patient 47, Lane 3 – patient 38, Lane 4 – patient 27, Lane 5 – patient 57, Lane 6 – patient 53, Lane 7 – molecular weight marker, Lane 8 – albumin (2mg/ml), Lane 9 – lactoferrin (1.25mg/ml)

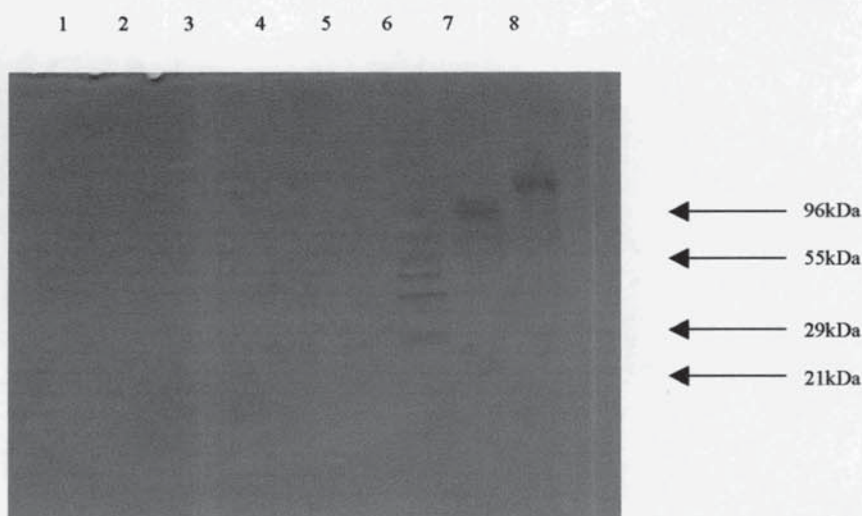


Figure 6.19 – Gel scan showing tear envelope from worn lenses
Lane 1 – patient 43, Lane 2 – patient 37, Lane 3 – patient 50, Lane 4 – patient 31, Lane 5 – patient 35, Lane 6 – molecular weight marker, Lane 7 – albumin (2mg/ml), Lane 8 – lactoferrin (1.25mg/ml)

Unfortunately, because the lenses were to be analysed for both lipid and protein deposition, it was not possible to attempt to remove the tear envelope from any other lenses that had been worn by participants in the clinical study. It was hoped that the utilisation of this technique would give a clearer representation of the tear film of those patients unable to provide tear samples by microcapillary that were of a high enough volume to ensure consistent and reliable results following analysis by SDS-PAGE and TotalLab™.

6.6.5 Lens Extractions

Analysis was carried out on Focus® Night and Day™ and PureVision™ lenses that had been worn in both a continuous wear and daily wear modality. They were extracted in order to ascertain whether it was possible to detect any of the protein that had deposited onto the lens. The results shown below in Figure 6.20 are those obtained from PureVision™ lenses worn on a continuous wear basis. This image is representative of all lens extracts from the silicone hydrogels worn in this study and analysed by SDS-PAGE.

Figure 6.20 – Gel image showing extracts from PureVision™ lenses worn in a continuous wear regime

It was not possible to detect the presence of any proteins on the gel following electrophoresis (Figure 6.20). This could be because the proteins extracted from the lenses were present in very low quantities and could not be detected using this technique. This, and other possible reasons for the results obtained are discussed more fully in Section 6.7.3.

6.7 Discussion

6.7.1 Effects of Contact Lenses Upon the Tear Film

Proper functioning of the tear film is essential for the eye as it nourishes the cornea and protects the eye from external infections. Contact lens wear has been shown to interfere with most of the functions of the tear film. They are often associated with dry eye symptoms, tear film instability, inflammation, infection and corneal erosions.[134, 135] The incidence of vision threatening, contact lens related, conditions are known to be higher in subjects with dry eye than those with a normal tear film. Contact lens wear can induce, or exaggerate, dry eye conditions. The mechanisms involved are unclear. A decreased tear volume is associated with contact lens induced dry eye. Symptomatic contact lens wearers have a lower tear flow rate and reduced tear film stability than asymptomatic lens wearers. Physiological changes to the tear film depend on the contact lens material (e.g. soft hydrogel and silicone hydrogel) and the wear regime. Individuals also differ in their susceptibility, so changes may not be uniform in their occurrence.

The lenses that were used throughout the clinical study were silicone hydrogels. To date, very little work has been carried out on such lenses. They have very different characteristics from a conventional hydrogel lens and, as such, are not thought to accumulate as much protein and lipid onto the surface. This may also result in a reduced tear volume being attached to the lens after removal. The silicone hydrogels used in this study have been approved for a thirty-day continuous wear lens in Europe. Deposition appears to be more common towards the end of the thirty-day wearing period – although no significant differences have been observed in the level of deposition onto the lens when worn either as a daily wear or a continuous wear lens.[136]

The patients that were involved in this study wore lenses either in a daily wear regime or a continuous wear regime. Conventional hydrogels, when worn on a continuous wear basis, have been associated with a range of changes to the cornea including inflammatory conditions and infections such as microbial keratitis. The number of people wearing lenses in this modality declined as a result. There has been a renewed interest in continuous wear lenses since silicone hydrogels were introduced. These materials have an increased permeability to oxygen and, as such, do not cause the cornea as much physiological stress

during periods of prolonged eye closure. Such lenses are now becoming a popular choice of vision correction for many people.[137, 138]

The data that was generated as a result of the clinical study was very interesting. A study such as this whereby subjects were followed closely over a twelve month period to monitor changes taking place to the tear film as they progress from being non lens wearers to being accustomed lens wearers is not thought to have been carried out before.

One problem that was encountered was the very low volumes of tear fluid that were available for analysis from some of the participants. Unfortunately, this is an inherent problem associated with tear analysis. Contact lenses are known to disrupt the tear film and cause a drying in some patients. This makes it very difficult to attempt to identify any potential biochemical markers in the tear film that could be used as indicators of potential problems the wearer might experience. There were often cases whereby no sample was available for analysis. This made it very difficult to monitor changes to the tear fluid when there were so many data points missing.

The TotalLab™ software proved to be invaluable when analysing the gels following SDS-PAGE. By being able to calculate the concentrations of proteins within the tear fluid it was possible to calculate ratios of proteins that were deemed to be of particular importance in this study. One flaw was the fact that low volumes could produce very spurious results. The data produced could be misleading if the experimental conditions were not considered when analysing results. This became more evident when the normalised volumes as calculated by the software were converted into percentages.

A huge amount of data was generated from this study. It was decided that the best approach would be to concentrate on the four main proteins in the tear film. The normalised volumes that were produced by the software were initially converted into percentages as this made it easier for comparisons to be drawn upon first observation. There appeared to be a great deal of variation between individuals in the relative percentages that were calculated for the four proteins.

The next logical step was to reference all the percentages against one protein in order to calculate the ratios of the proteins. Lactoferrin was chosen as the protein against which all

other proteins would be referenced. There seemed to be more consistencies amongst the calculated percentages for lactoferrin than there were amongst the other proteins. Lactoferrin is also present in reasonably high concentrations in the tear fluid at approximately 1.4 mg/ml. Lactoferrin is involved in ocular defence as it possesses both bacteriostatic and bactericidal properties and is therefore a very important tear protein. It has also been implicated in the fouling of contact lenses.[139]

The results shown in Figure 6.20 show that the ratio of lactoferrin: lysozyme had increased in those individuals wearing Focus Night and Day in both a daily wear and continuous wear modality. When the lens wearing population was analysed as a whole (See Table 6.19), it was found that there was indeed a slight increase in lysozyme concentration at visit 7 (after 12 months) compared with visit 0 (pre lens wear). This is in agreement with studies that have previously been carried out which found that the regular wearing of contact lenses does alter the concentration of tear lysozyme. Studies that have been undertaken have found that levels of lysozyme increase as a result of lens wear. It is thought that this may be due to the continuous stimulation of the conjunctiva by the contact lens.[140] Other proposed explanations include an increase in tear osmolarity, simulating a dry eye condition, which is associated with contact lens wear. Tear osmolarity may be increased due to an increase in evaporation. This may ultimately mean that the concentrations of various tear components are increased.

Tear lipocalin and any subsequent changes taking place to the concentration in the tear fluid as a result of lens wear was very important throughout the course of this study. Tear lipocalins are members of the lipid-binding protein super-family. They are able to bind long chain fatty acids, glycolipids, phospholipids and cholesterol. They may also scavenge harmful lipids (e.g. lipid products of inflammation). Tear lipocalin is the main lipid binding protein in tears and may exist to protect the eye. It may scavenge lipid from the cornea to prevent dry spots from forming. The concentration of tear lipocalin is decreased in dry eye disease. The literature suggests that the lipocalin concentration increases in intolerant lens wearers as it is associated with an increase in lipid degradation.[141, 142] Glasson carried out a study on symptomatic and intolerant lens wearers and compared their tear film lipid features with those of tolerant wearers. Intolerant lens wearers had a greater amount of degraded lipid in their tear film along with an elevated lipocalin concentration.[143] The results obtained in this study are contrary to this. In many cases, the lipocalin concentration

was found to decrease following lens wear. The data obtained from comparing lipocalin against both albumin and lactoferrin highlighted some very interesting patients. When the ratios obtained for albumin: lipocalin are examined, it can be seen that there are a few patients that have ratios markedly different from other participants. Patients 12, 16, 26 and 48 were seen to have a very low concentration of lipocalin compared with albumin in their tear fluid. These patients all suffered from meibomian gland dysfunction. Three of these patients regularly used re-wetting drops as they often experienced dryness.

A ratio of 1: 40 to 1: 50 was considered to be an acceptable value for lactoferrin: albumin as it encompassed most of the subjects involved in the study. Tables 6.14 – 6.18 show that five of the patients were quite considerably outside the normal boundaries that were considered normal. These were: patients 3, 12, 25, 48 and 58. Four of these patients were observed to have recurrent meibomian gland dysfunction. The resulting reduction in the lipid layer would have led to an increased evaporation of the tear film. Wearing lenses would have exacerbated the situation.

The ratio of lactoferrin: albumin has been used in the past as an indicator of dry eye. A ratio of 1:2 has been said to be indicative of primary Sjögrens syndrome.[128] None of the subjects involved in this study had a ratio as high as this, but clearly an elevated lactoferrin: albumin ratio is indicating those patients who have dry eye symptoms. An elevation in albumin concentration can occur as the result of vascular leakage or epithelial damage. It can therefore be used as an indicator of inflammation.

When looking at differences that existed between the different types of lenses worn by patients in this study, it can be seen that there is a rise in the ratio of lactoferrin: albumin in both lens types and both wear modalities. A similar decrease was observed in the ratio of lactoferrin: lipocalin. When comparing the ratios obtained for lactoferrin: lysozyme it was seen that there was an increase in lysozyme in those people wearing Focus® Night and Day™, and a decrease in the lysozyme concentration in those wearing PureVision™. PureVision™ is a low water content, ionic lens and is therefore classified as a group III lens. The difference in lysozyme concentration described above may be due to tear lysozyme adsorbing onto the lens. There may therefore be less lysozyme in the tear fluid as a result of this.

Patient 59 was chosen for analysis as the clinical patient details stated that this individual was a heavy smoker. There has not been a great deal of work carried out on the effects smoking has on the tear fluid and tear proteins. However, cigarette smoke has been implicated in several eye diseases including glaucoma and cataracts. The toxic and oxidative effect of cigarettes may be an implicating factor in damaging ocular tissues. Smoking is also known to cause symptoms of dry eye. It has been demonstrated that exposure to cigarette smoke leads to a reduction in the tear break up time – indicating that the tear film is less stable. Studies that have been carried out comparing the tears of smokers and non-smokers have found that there are differences. There appeared to be a degree of fragmentation in the proteins of tears derived from smokers.[144]

The initial problem that was encountered whilst analysing this individual was the very low volumes that were available for analysis from every visit. Table 6.9 shows the percentage of the four proteins that were present in the tear sample at each visit. The general trend for lipocalin can be seen to increase over time. This may be a result of the subject suffering dry eyes. There may be a break up of the lipid layer which results in a higher concentration of lipocalin being required by the eye in an attempt to "scavenge" this lipid and prevent it from causing dry spots on the cornea to cause further desiccation of the corneal surface. Comparison of the protein ratios shown in Table 6.17 indicates that this patient has very low volumes of all proteins within the tear film. This would lead to this patient being selected as one that would be likely to develop complications associated with lens wear.

It was thought that contact lens wear caused the concentrations of tear proteins to deviate from normal. Many studies have been carried out on the effects lens wear has upon total tear protein, but very little has been done regarding changes to the concentrations of particular proteins as opposed to total protein. There is a great deal of variation between reported values in the literature due to differences in tear collection methods, sample treatment and analytical techniques. This study has shown that there is indeed a change in tear protein concentration before and after a period of lens wear. It is unfortunate that it was not possible to identify a specific protein that could be used as a biochemical marker to identify those patients more likely to develop complications or become intolerant wearers. However, by analysing the four proteins as described here it was possible to identify those people who had an abnormally high or low concentration of protein present in their tears.

Physiological variations that occur between individuals made it impossible to predict how these proteins were likely to deviate. There were certain individuals (patients 12, 26, 58 and 59) who all showed an elevation in their lactoferrin: albumin ratio. At the same time, the ratio of lactoferrin: lysozyme was seen to decrease. It could be that as the concentration of one or more protein increases, there is a corresponding decrease in the concentration of other proteins in an attempt to counteract any detrimental effects an elevated total protein concentration might have upon the ocular environment.

6.7.2 Analysing the Tear Envelope Collected from Lenses Worn in the Study

The results shown in Figure 6.21 and 6.22 are those obtained from extracting the tear envelope from the worn lens. The lenses that were analysed were all from the twelve-month visit. Unfortunately, very little can be visualised on these gels. There could be a variety of reasons for this. It was found in initial experimental work on optimising this technique that 40 µl of treatment buffer was required to ensure that the lens was completely submerged. A consequence of this requirement is that any of the tear envelope that is attached to the contact lens at the time of removal is considerably diluted. It is thought that a maximum of 2 µl of tear fluid would be present on the lens so there is a very high dilution factor involved.

In order for the tear envelope to be analysed it is important to ensure that the lens is immediately placed in the eppendorf after removal. A minimum amount of handling is needed, as this would remove any tears that were attached to the lens. Tweezers were used to insert the lens into the eppendorf and ensure that the buffer completely covered the surface. The eppendorf was mixed thoroughly prior to analysis to rewash the lens surface. However, if the lens was not completely immersed at the time of removal any of the tear envelope that was attached to the lens would have evaporated.

Attempts to remove the tear envelope from the surface of silicone hydrogel lenses had not been made prior to this clinical study. Although the factors described above may have reduced the chances of being able to collect tears attached to the lens at the time of removal, it may be that the composition and properties of the lens materials of these two lens types simply did not support a sustainable tear envelope on their surface. They are known to be

less prone to deposition than conventional hydrogels. The tear envelope may be attached to a layer of deposited protein on the surface of contact lenses. If this is the case, this more "deposit resistant" feature of silicone hydrogels may inhibit the adherence of the tear film.

6.7.3 Contact Lens Extracts

Very little information could be obtained regarding the total amount of protein that was deposited on lenses using the extraction technique detailed in Section 2.3.1. Although both Focus® Night and Day™ and PureVision™ lenses that had been worn in both modalities were extracted and then subjected to electrophoresis, no significant data was obtained. The resulting gels were stained using both Coomassie Blue and silver stain. Silver stain is capable of detecting protein present in nanogram concentrations. Nevertheless, it was not possible to obtain any positive identification. Western blotting was also used to check for the presence of certain proteins that may have been present on the acrylamide gel but in concentrations too low to be detected even by silver staining. (The sensitivity of western blotting has been demonstrated by its ability to detect IgM in tear samples – known to be present in trace amounts, if at all, in the normal tear fluid). It was not possible to detect either albumin or lactoferrin using this technique. These two proteins were chosen, as they are present in fairly high concentrations in tear fluid. Albumin is found at about 1.3 mg/ml and lactoferrin at about 1.4 mg/ml.

The ability to extract proteins from contact lenses is important when it is used in conjunction with other immunological techniques, such as counter immunoelectrophoresis. This enables either the presence or absence of certain proteins to be identified. The reagents used in the extraction technique are very harsh and, as such, cause the proteins to denature. SDS-PAGE may not be able to separate the proteins into their constituent polypeptides as a result of this denaturation. Another possible explanation may be that SDS-PAGE is not sensitive enough to detect the presence of any proteins that have been extracted. The lens is also extracted in 200μl of extraction solution. This is a very high dilution factor and, as such, reduces the likelihood of electrophoretic separation and identification of proteins species that may have been present in the eluate.

This thesis was concerned with the development of techniques that could be applied to tear protein analysis. A novel technique has been devised to remove the tear envelope from the surface of the lens. The application of TotalLab™ to the study of tears has not been reported in the literature and is therefore also a novel approach in tear analysis.

It is important to remember that due to the timing of the study and the mass of samples that needed to be analysed, this had to be the main focus of the investigative work that was carried out.

This clinical study has highlighted a couple of areas where the techniques can be seen to produce spurious results. Analysis of people and all living organisms usually means that there will be a great deal of inherent variation that already exists between the subjects before analysis has even commenced. The reliability of these techniques has been demonstrated by their ability to provide reproducible results e.g. when standard proteins are run on gels, or when the sample is pooled over time and so can be loaded into several gels.

There are a few criteria that could be suggested in order to ensure that results are as accurate and as reproducible as possible. There is always a slight degree of experimental induced variation which could result from a variety of factors, including: the initial sampling technique resulting in a different type of tear being collected, a flaw in the gel and unsuitable running conditions which are sometimes seen to give distortions of the gel.

Throughout the course of this work, there have been samples provided for analysis, which, under normal conditions, would be rejected. The timing of the sample was important in this work as it aimed to monitor protein changes as the wearer became accustomed to lens wear. These samples that were often of very low volume but were still analysed.

In order to be certain that the results generated are accurate, it is not advisable to use a low sample volume. The electrophoresis unit used in this work required a minimum of a 5μl sample. The software package is able to make adjustments for distortions in the gel, but these results are not likely to be as accurate as results collated from a gel that had completed the run under optimum conditions. The fact that there were always protein standards loaded into a gel resulted in the knowledge of when an unknown sample was producing misleading

results. Throughout the course of this work, it was seen that lactoferrin concentrations in tear samples were most commonly observed in the range of 30 – 50% of the total tear protein. In instances where this protein deviated far from these boundaries, it is likely that there is a high degree of error within the sample. This would be more likely than it was the result of a disrupted tear film when dealing with a large sample base. Awareness of these parameters is essential in order to utilise these techniques to their maximum capability.

The final conclusion to be drawn from this study is that the techniques utilised in this work have determined that the tear film and tear proteins are affected by lens wear. The extent to which proteins are affected seems to be more dependent on individual physiological variations than it is on the lens material or wear regime.

Chapter 7

Discussion and Future Work

7.1 Discussion

Contact lenses are, and will continue to be, a major method of vision correction for millions of people globally. Major improvements have been made to the materials, choice of wear modality and cleaning regimes since the introduction of contact lenses some thirty years ago. However, along with the introduction of a new material comes the introduction of a host of complications that may have not been encountered previously.

One problem that became a major issue with the advent of hydrogel contact lenses was the deposition of proteins, lipids, microorganisms and other extraneous substances onto the surface of the lens, and also within its matrix. This had not been an apparent problem with PMMA lenses.

Deposition remains a problem to this day. The interactions taking place between the contact lens and the tear fluid are extremely complex and are still not fully understood. Proteins are of particular significance, as they are known to be involved in immunological reactions both within the eye and other parts of the body.

There are many techniques available for the purpose of tear protein analysis. It is well documented that the techniques employed can affect the values obtained. Different methods of tear collection result in different types of tear being collected due to the degree of stimulation experienced. Subsequent treatment and analysis of the collected samples also affects the results obtained.

This work was mainly concerned with initially optimising techniques that would enable the tear protein profiles of "normals" to be analysed. The techniques of choice that were utilised were one dimensional electrophoresis and Western Blotting. There are many other techniques that are available for tear analysis including: counter immunoelectrophoresis (CIE), High Performance Liquid Chromatography (HPLC), Enzyme Linked Immunosorbant Assays (ELISA), and two dimensional electrophoresis. All of the aforementioned techniques have their relative advantages and disadvantages.

SDS-PAGE and Western Blotting were the techniques of choice as they complement each other in their mode of action and the results they are capable of generating. One-

dimensional electrophoresis is used initially to separate a complex mixture of proteins into its constituent polypeptides. A molecular weight marker is always loaded alongside the samples being investigated, which allows the molecular weights of the unknowns to be calculated. This allows an estimation to be made regarding the protein species that are present within the sample. Western Blotting involves the transfer of proteins from within a gel to the surface of a membrane under the influence of an electric current. Western Blotting was therefore utilised to confirm the presence of the protein of interest within the sample.

The main advantage of SDS-PAGE is that unlike other techniques, whereby it is only possible to determine the presence or absence of a certain protein, is the ability to obtain semi-quantitative data. This was of particular importance to the study of tear samples. It is possible to determine differences between protein concentrations according to the intensity of staining. This technique is not without its flaws however. It is not possible to make exact judgements as to a protein's exact molecular weight, nor is it possible to obtain truly quantitative data regarding the concentration of a protein species.

In order to analyse samples using the mini electrophoresis unit utilised throughout this study, it was found that a minimum volume of 5μl was required – to which a further 5μl of sample treatment buffer was added. This is a much lower volume than is required by many other operating systems. However, the study of tear fluid often presents the problem that it is only possible to collect very low volumes. This is often seen to increase in contact lens wearers and people experiencing a degree of dry eye symptoms. There are methods that have been described in the literature that can be used to overcome this problem. These include using an eye flush method that has been seen to increase the tear volume that can be collected. It was decided that this would not be carried out in this study as the aim was to collect a tear sample that was considered to be representative of the actual tear film of the subject without the influence of stimulation, be it mechanical or chemical.

Throughout the course of the study it was found that low volumes did affect the results of the ensuing tear analysis. Data that was obtained was always correlated with the standard of the sample that was obtained. Should the study of dry eye patients become more important, it would be necessary to devise a method whereby a higher sample volume could be obtained. This was not necessary in the course of this work as all subjects had to be

treated in the same manner in order to allow comparisons to be drawn between lens wearers and non-lens wearers. Introducing other variables would have made such a comparison more difficult to achieve.

Western Blotting was used in conjunction with electrophoresis as it enabled a protein to be positively identified due to the specificity of the antibodies involved. The transfer of the proteins from a polyacrylamide gel to a membrane makes them more accessible. Polyacrylamide is not very amenable to the diffusion of large molecules. There are numerous advantages associated with the application of antibody analysis in the study of tear proteins. Antibody-antigen interactions are highly specific and sensitive. Results are also easy to interpret.

Once the proteins had been transferred to the membrane, it was possible to immunostain for a wide range of proteins and immunoglobulins. The primary antibody binds to the protein of interest. Following incubation with the primary antibody for the required period of time and subsequent washing stages, the membrane was then incubated with the secondary antibody. The secondary antibody is one that has an alkaline phosphatase side chain attached to it. When the final staining stages were carried out, the alkaline phosphatase conjugated antibody would react with NBT/BCIP to produce an insoluble blue precipitate. This was conclusive of a positive reaction as the antibodies only bind to one particular protein, or subunit of this protein.

Optimisation of this technique determined that this technique required a minimum of four hours running time. There have been suggestions in the literature that the running time could be reduced to ninety minutes. It was found through the course of this thesis that a running time of less than four hours resulted in an incomplete transfer of proteins from the polyacrylamide gel to the membrane. It is obviously extremely important to ensure that as complete a transfer as possible is achieved. By not allowing adequate time for the proteins to be transferred to the membrane, there was the potential for the production of misleading results regarding the presence or absence of a protein within a sample.

An unforeseen problem that was encountered during the immunostaining of tear proteins was the cross reactivity that occurred. This became particularly apparent when the immunoglobulins were being studied. The reason for this is that there are certain subunits

that are common to all immunoglobulins. This was later overcome by using antibodies directed against the unique subunits of each immunoglobulin e.g. the gamma chain of IgG.

One major drawback that was encountered through the use of antibody analysis for the study of tear proteins was the lack of commercial availability of some of the proteins and their corresponding antibodies that were deemed to be of particular interest in this work. Lipocalin was one such example of a protein that was considered to be particularly important in the clinical study. Lipocalin functions as a lipid scavenger in the ocular environment. One of its assumed roles is that it exists to remove any lipid spots that may form on the cornea. Lipid deposition onto the surface of the cornea would cause the cornea to dry out in these areas. An increased lipocalin concentration could, and is thought to be, an indicator of a dry eye condition.

There were a few patients within the clinical study that were identified as having a disturbed tear film. These patients were only able to produce low volumes of tear samples. They regularly used re-wetting drops and stated in the questionnaire that they often experienced symptoms of dryness and discomfort. It would have been interesting to be able to analyse their tear samples and subsequently carry out immunoblotting in an attempt to identify the presence of lipocalin within their tears and any subsequent increases or decreases in the concentration of lipocalin as the period of lens wear increased. Unfortunately, neither tear lipocalins nor its antibodies were available commercially at the time of this study. This therefore places a restriction upon the amount of analysis that can be performed utilising antibody-antigen techniques.

The antibodies that were available for the study of tear proteins showed that there was a high level of sensitivity and specificity associated with this technique. It was demonstrated that it was able to detect IgE and IgM in the tears of healthy individuals. These immunoglobulins are known to be present only in trace amounts in the tears of healthy individuals, if at all.

These two techniques, when used in conjunction, allow for a greater amount of information regarding the tear protein profile to be examined. One-dimensional electrophoresis allows the tear proteins within tears to be separated. This enables comparisons to be drawn between those samples that are considered normal and those that appear to deviate from a

typical protein profile. Western Blotting can then be used to determine whether the "deviant" sample has a protein that may or may not be present in the tears of a healthy individual.

The introduction of TotalLab™ revolutionised the way in which data generated from these techniques could be analysed. This became extremely important during the clinical study. This program was able to calculate the molecular weights of unknown bands separated by SDS-PAGE and was then able to calculate the concentration following normalisation to a standard protein of known concentration. It was found that it was not possible to carry out Western Blotting on the tear samples collected during the clinical study. It was quite usual to have the bare minimum volume of tears required for SDS-PAGE. It was not possible to repeat samples. It was therefore necessary to stain the gels using Coomassie Blue. It was found that it was not possible to blot the gels after they had been stained. The dye was obviously bound to the proteins and prevented them from being transferred from the polyacrylamide gel to the membrane.

TotalLab™ became an invaluable addition to the techniques that were available. The most important applications of this technique were firstly in analysing the tear proteins of a population of non-lens wearing individuals to assess the impact that diurnal variation had upon the relative concentrations of the main tear proteins. The second application was in the course of the clinical study whereby the tear samples of a population of initial non-lens wearers was analysed and then monitored as they progressed from neophyte lens wearers to adapted lens wearers. By simultaneously monitoring a control subset group of individuals it became feasible to identify changes that were taking place in the tear film as a result of lens wear, the lens material and also the wear regime.

The initial aim of the clinical study was to be able to identify a certain protein that could, by its presence or absence from the tear fluid, be used as a potential biochemical marker that would enable those patients more likely to become intolerant lens wearers to be identified. A huge amount of data was produced as a result of this clinical study. The variations that were seen amongst individuals regardless of the lens type or wear regime made it unfeasible to study each patient individually. The approach that was deemed to be most suitable was to convert the values of the concentrations calculated by the software into percentages.

This made it easier for initial observations to be made with respect to changing protein concentrations.

The percentages that were calculated as described above were then converted into ratios, using lactoferrin as a reference point. This reduced the anomalous results that were occasionally observed in the patients where the tear sample was of very low volume and so had produced spurious results.

The ratios that were calculated showed that there was indeed a change in tear protein – although they did not change uniformly for all the participants in the clinical study. Definite trends could be observed however, which allowed those patients that had a disrupted tear film to be identified from laboratory results. When the results obtained through laboratory analysis were compared with clinical data, it was seen that these patients were suffering from meibomian gland dysfunction. These patients were all seen to have an elevated lactoferrin: albumin ratio and a decreased lactoferrin: lipocalin ratio when compared with other patients. An increase in the lactoferrin: albumin ratio is used clinically as an indicator for dry eye. A ratio above 2: 1 is considered to be indicative of dry eye. Although none of the patients had a ratio that would be considered to be in this category, they did indeed show signs that they were suffering from symptoms of dry eye.

The tear volume that is available for analysis is an important consideration when analysing samples. It was seen that 5μl produced optimum results. When the volume was below this, an incomplete profile was observed. This would then affect the data produced using the software.

To be sure that the observable changes to the tear sample was representative of the tear fluid and not a result of an insufficient volume, the concentration of lactoferrin was used to judge the quality of the sample. A lactoferrin concentration of 30 – 50% was taken as being acceptable within a tear sample. Samples that deviated from this range were likely to be the result of low sample volume.

This study was primarily concerned with the optimisation and application of various techniques to the study of tear proteins. Certain criteria were adhered to through the course of this study. They can be summarised as follows:

- A minimum volume of 3µl was required. Although it is always preferable to obtain as high a volume as possible, without causing a stimulated tear to be collected, it was found that samples less than 3µl did not produce results that were deemed to be reliable.
- Gels should always be monitored while they are running. If the samples showed signs of distortion then it was imperative to reduce the current immediately.
- Lactoferrin concentrations were used as an indicator of the reliability of the sample. A concentration of 30 – 50% of the total protein was taken as being representative of a reliable sample.

The preliminary work that was carried out prior to lens wear showed that there was a change in protein concentration in individuals over a very short time period. These subjects only wore lenses for three days at a time and this was found to be sufficient to cause the tear film to depart from normal. More than this, it was found that the lens material that was being worn caused the tear film to change in differing ways. The effects observed in the clinical study were not as dramatic as this although general trends were observed between the two lens types. There was an overall increase in the lysozyme concentration of those patients wearing Focus Night and Day lenses and an overall decrease in the lysozyme concentrations in those wearing PureVision. The wear modality did not appear to have a major influence on the protein profile however.
The effects of diurnal variation upon the tear film were also investigated. This involved tear samples being taken at three time points during the day e.g. morning, mid-day and in the evening. The subjects were all researchers in the Biomaterials Unit. These subjects were chosen, as it would minimise the effects of environment on the results.

The results that were obtained showed that no discernible changes occurred to the tear film common to all individuals. Those individuals who were experiencing feelings of dry eye were distinguished as being markedly different to the other participants. However, it must be remembered that this study involved looking at a small population of individuals over a short period of time. Only four proteins were investigated, which is not to say that there are no other proteins that may be more susceptible to diurnal variation.

Another technique was devised that allowed for the tear film attached to the contact lens at the time of removal to be analysed. This attached tear film was termed the tear envelope. There were many benefits that were provided by this technique.

Up until this point, it had been possible to extract proteins adsorbed either on to the surface of the lens, or within the matrix of the lens. These contact lens extracts are routinely analysed within the Biomaterials Research Unit using other biochemical and immunological techniques that were not described in this work.

The extraction method itself uses very harsh procedures whereby the proteins are placed into an eppendorf containing 200µl of extraction solution and then heated at 90°C for three hours. This is very likely to denature the proteins that are present in the extracted solution either through the use of such harsh chemicals, or the temperature to which the proteins are being subjected.

When the lenses were extracted it was found that it was rarely possible to obtain any information using SDS-PAGE. This may be due to the denaturation that has taken place or because the proteins are present in very dilute concentrations and are below the threshold of sensitivity of this technique.

The tear envelope was designed to remove the proteins that were attached to the lens at the time of removal without the harsh conditions described above. This would then be a truer representation of the tears of a lens wearer. The contact lens extracts may also show a higher concentration of particular proteins than would be expected to be present in the tear film because of their affinity for the lens material. Group IV lenses are known to adsorb a great deal of lysozyme onto their surface because of the ionic nature of the lens material.

This novel, yet simple, technique for the removal of the tear envelope was carried out by immediately inserting the lens into an eppendorf containing a certain volume of treatment buffer. An incision was made in the bottom of this eppendorf, which was then placed into a larger eppendorf. Upon centrifugation, the liquid was drawn from the eppendorf containing the lens (and therefore the tear envelope) into the larger eppendorf. The resulting eluate was then analysed by SDS-PAGE.

Optimisation of this technique revealed that the optimum volume of sample treatment buffer was 40μl. A higher volume than this resulted in the dilution factor being too high for SDS-PAGE to detect the proteins present. Lower volumes resulted in the lens not being completely immersed in the buffer. This resulted in the tear envelope not being washed from the surface of the lens.

Various centrifugation speeds and times were also investigated. It was found that the best results were achieved following centrifugation of the eppendorf containing the lens and treatment buffer at 5000 r.p.m. for ten minutes. This was the protocol that was followed when analysing the tear envelope.

This technique yielded promising results in that it allowed a tear protein profile to be obtained that could be considered comparable with conventional tear sampling techniques (e.g. microcapillary collection) from certain lens types. The 1-day Acuvue consistently produced clear protein profiles. It was found, after many attempts, that a comparable profile could not be obtained from the Soflens 1-day. This was thought to be because this is a much thicker lens than the Acuvue lens and, as such, inhibited the formation of a thicker tear film.

Although this technique was very useful in that it enabled the tears of a contact lens wearer to be analysed, it was disappointing to find that it was only applicable when particular lens types were being worn. This was attempted during the course of the clinical study. There was a minimum number of lenses available for this analysis and it was found that very little could be obtained from the lenses that had been worn by the patients.

There are a couple of possible explanations for this:

1. The lens was handled too much prior to being immersed in buffer. It was important to ensure there was a minimum level of handling of the lens. Tweezers were used to place the lens in the eppendorf, but as this was quite hard to accomplish for a non-experienced person, this may not have been carried out sufficiently.
2. The lens was not immersed completely in the treatment buffer immediately after removal. If the lens was not completely submerged, the tear envelope that may have been attached to the lens at the time of removal could have evaporated.

3. There is only an estimated 2μl of tear envelope attached to the lens at the time of removal. When the lens is placed in 40μl of treatment buffer the resulting dilution factor that ensues is very high. However, there has to be a balance between these two requirements and the conditions that were chosen were found to be the most suitable overall.
4. The proteins that are present in the tear envelope may be below the sensitivity of the techniques used to analyse the sample.

The work presented in this thesis has highlighted many areas of interest which, with future work, may help to provide further insight into protein changes as a result of lens wear and how these changes impact on the development of intolerance to lens wear

7.2 Future Work

1. Investigation of other techniques available for the study of protein analysis.

The combination of SDS-PAGE and Western Blotting has proven to be very useful techniques in the study of tear protein profiles. They enabled tear samples from healthy eyes to be analysed and compared with those from a lens wearing population, who are often thought of as having a disturbed tear film. However, there are many other techniques that are available for the study of tear proteins. They each have their advantages and disadvantages. Exploring their capabilities and limitations would be an invaluable exercise. Techniques and methodologies that are already available are also continually being improved as the need to build on existing knowledge is increased. One of the limiting factors that became apparent as a result of the clinical study was the volume of tear fluid that was required for analysis. When presented with lower volumes from patients experiencing symptoms of dry eye, it became impossible to carry out reliable analysis of these samples. The influence contact lenses had upon the tear film was an integral part of this study. As contact lenses are known to disrupt the tear film and lead to a reduced tear volume, this was shown to be an important obstacle that needs to be overcome. This could possibly be achieved by exploring other methods of collecting tears e.g. using an eye flush or externally stimulated tears as would be collected when ammonia or other noxious chemicals are inhaled. These methods would undoubtedly result in a different type of tear being collected but, as long as the samples were all treated in an identical manner, this would still enable comparisons to be drawn between different groups of people.

2. Studying the effects contact lens wear has upon the immunoglobulins present in tears.

The long-term effects of lens wear on albumin, lipocalin and lactoferrin have been studied as part of this thesis. It would have been interesting to also monitor changes that may take place to the immunoglobulins present in tears. IgE was of particular importance at the beginning of this study as it is known to be involved in allergic reactions and, as such, may be an indication of a detrimental host reaction in response to lens wear. It was unfortunate that this could not be carried out in this work, but the low tear volumes that were available for analysis meant that it was only possible for the samples to be analysed using electrophoresis and then blotted without visualising the bands as it was found that it was not

possible to transfer the proteins from the gel to a membrane after they had been stained with Coomassie Blue. It was felt that this approach would result in the loss of too much valuable information and so it was deemed preferential that the samples were analysed electrophoretically and stained. This meant that immunoglobulins were not a main focus of the work carried out here.

3. Identification of all separated protein bands identified following electrophoresis.

There are at least eleven bands that are evident in a tear sample following electrophoresis. They have not all been conclusively identified in the work that has been carried out in our group to date. As the commercial availability of antibodies increases, it will become possible for these unknown, or previously unconfirmed, bands to be positively identified. Once all proteins present in tear samples have been identified, it should hopefully lead to a better understanding of the nature of interactions that take place between the tear film and a contact lens after insertion into the eye.

4. Diurnal Variation

A study was carried out to see whether any changes in diurnal variation could be observed in individuals, and whether these changes were common in a population. This study showed that there was no discernible pattern that could be found. However, this study was carried out over a very short time period. Should this work be repeated using a larger number of samples over a longer time period it may be possible to discover whether there is any correlation between the tear protein composition and the time of day. It is likely that there is a change of some description taking place to the tear fluid as many people experience discomfort towards the end of the day. It would be very interesting to monitor immunoglobulin concentrations simultaneously to see if they are able to provide some insight into why this discomfort occurs.

5. Utilising the tear envelope to monitor closed eye tear samples

Very little work has been carried out on the effects overnight lens wear has upon the ocular environment. This is largely due to the complications associated with collecting a true closed eye tear sample. The tear film begins to revert back to its normal open eye state as

soon as the eyes are opened. Studies that have been carried out to date have relied on samples that have been collected as soon as the eye is opened. It may be possible to apply the technique of collecting the tear envelope from the eye immediately after the eyes have opened. This is likely to be a truer representation of a closed eye tear sample than is collecting a tear sample by conventional methods, which is also more time consuming than removing a contact lens is and so would not allow the tear film as great an opportunity to revert back to its open eye state. The tear envelope that is collected following a period of eye closure could then be compared with one that has been worn throughout the day to determine whether any differences between the two can be seen.

6. Defining parameters required for analysis of tear proteins

This thesis was concerned with the development of methods that enabled quantitative data of tear protein concentrations to be generated. A certain degree of variability is always likely when studying a biological fluid such as tears because of inherent differences amongst individuals.

However, a further study is necessary to determine the variability associated with the methodologies involved beginning with the actual collection of the sample. Using two or three experienced personnel to collect the tears from a group of subjects it would be possible to determine the degree of variability involved in collecting the tear sample. It is important that a sample representative of a normal, basal, tear is collected. Stimulation is known to affect the type of tear that is obtained. Certain individuals may be more proficient in collecting non-stimulated tears than others.

By pooling the tears collected from an individual and running them on different gels, it would be possible to determine the extent of variation that may exist in laboratory analysis. This may occur in the gel itself or during electrophoresis. Gels sometimes distort as a result of the current being too high. Although TotalLab™ has features that are designed to allow for this, it is likely that a distorted gel may produce results slightly different from a gel that has been run under optimum conditions.

By observing the defined parameters it would ensure that any observable changes taking place to the tear film is a true representation of the tear film and not a result of experimental errors.

References

1. Forrester J.V., Dick A.D., McMenamin P.G., and Lee W.L. *The Eye. Basic Sciences in Prcatice*. 2nd ed., W.B. Saunders

2. Batterbury M. and Bowling B. *Opthalmology*. Churchill Livingstone

3. Vaughan D., Asbury T. and Kiordan-Eva P. *General Ophthalmology*. 15th ed, Appleton and Lange

4. Arffa R.C. *Grayson's Diseases of the Cornea*. 4th ed. Mosby

5. Friend J. *Physiology of the Cornea*

6. Kenjon K.R. *Anatomy and Pathology*

7. Fatt I. And Weissman B.A. *Physiology of the Eye: An Introduction to the Vegetative Functions*. Butterworth-Heinemann. (1992)

8. Gachon A.M., Verelle P., Betail G and Dastague B. Immunological and electrophoretic studies of human tear proteins. *Exp. Eye Res*; **29**: 539 – 553 (1979)

9. Port M.J.A. and Asaria T.S. The assessment of human tear volume. *J. Br. Contact Lens Assoc* **13**: 76 – 82 (1990)

10. Bright A.M. and Tighe B.J. The composition and interfacial properties of tears, tear substitutes and tear models. *Journal BCLA*; **16**: 2: 57 – 66 (1993)

11. Prydal J.I., Artal P., Woon H. and Campbell F.W. Study of the precorneal tear film thickness and structure using laser interferometry. *Invest. Ophthalmol. Vis. Sci.* **33**: 2006 – 2011 (1992)

12. Tiffany J.M., Winter N. and Bliss G. Tear film stability and tear surface tension. *Curr Eye Res*. **8**: 507 – 515 (1989)

13. Holly F.J. and Lemp M.A. Wettability and wetting of corneal epithelium. *Exp. Eye Res.* **11**: 239 – 250 (1971)

14. Smolin G. The defense of the outer eye. *Trans. Ophthalmol. Soc. UK* **104**: 363 – 366 (1985)

15. Holly F.J. Physical chemistry of the normal and disordered tear film. *Trans. Ophthalmol. Soc. UK* **104**: 374 – 380 (1985)

16. Hayashi K. Local immune responses in ocular virus infection and their implications for future immunotherapy. *Ophthalmologica*; **211**: (suppl 1) 45 – 52 (1997)

17. McClellan K.A. Mucosal defense of the outer eye. *Surv. Ophthalmol.* **42**: 233 – 246 (1997)

18. Pleyer U. and Baatz H. Antibacterial protection of the ocular surface. *Ophthalmologica*; **211** (suppl 1): 2 – 8 (1997)

19. Tan K.O., Sack R.A., Holden B.A. and Swarbrick H.A. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* **12**: 1001 – 1007 (1993)

20. Stapleton F., Willcox M.D.P., Morris C.A. and Sweeney D.F. Tear changes in contact lens wearers following overnight eye closure. *Curr. Eye Res.* **17**: 183 – 188 (1988)

21. Carney L.G. and Hull R.M. The contact lens environment: tear proteins in perspecive. *Am. Opt. Assoc.* **56**: 202 – 204 (1985)

22. Berman E.R. *Biochemistry of the Eye*. Plenum Press, New York (1991)

23. Blades K. and Craig J. Structure and function of the tear film. *Optician.* **5588**: 15 – 21 (1997)

24. Holly F.J. and Hong B. Biochemical and surface characteristics of human tear proteins. *Am. J. Optom and Physiol. Optics.* **59**: 43 – 50 (1982)

25. Tiffany J.M. Individual variations in human meibomian lipid composition. *Exp. Eye Res.* **27**: 289 – 300 (1978)

26. Stevens-Andrews J. Human tear film lipids I: Composition of the principal non-polar components. *Exp. Eye Res* **27**: 289 – 300 (1978)

27. Sariri R. Tear protein interactions with hydrogel contact lenses. *PhD Thesis.* Aston Universoty (1995)

28. Bron A.J. Prospects for the dry eye. *Trans Ophthalmol. Soc. UK* **104**: 801 – 826 (1985)

29. Tiffany J.M. The role of meibomian secretion in the tears. *Trans. Ophthalmol. Soc. UK.* **104**: 396 – 401 (1985)

30. Broekhuyse R.M. Tear lactoferrin: a bacteriostatic and complexing protein. *Invest. Ophthalmol.* **13**: 550 – 554 (1974)

31. Janssen P.T. and van Bjsterveld O.P. The relations between tear fluid concentrations of lysozyme, tear-specific prealbumin and lactoferrin. *Exp. Eye Res.* **36**: 773 – 779 (1983)

32. Kijlstra A., Jeurissen S.H.M. and Koning K.M. Lactoferrin levels in normal human tears. *Br. J. Ophthalmol.* **67**: 199 (1983)

33. Janssen P.T. and van Bijsterveld O.P. Comparison of electrophoretic techniques for the analysis of human tear fluid proteins. *Clin. Chim. Acta* **610**: 67 – 86 (1981)

34. Janssen P.T. and van Bijsterveld O.P. Lactoferrin versus lysozyme in the sicca syndrome in "The Preocular Tear Film: In health, Disease and Contact Lens Wear" F. J. Holly ed., Dry Eye Institute, Lubbock, TX, USA, 167 (1986)

35. Selsted M.E. and martinez R. Isolation and purification of bactericides from human tears. *Exp. Eye Res.* **34**: 305 – 318 (1982)

36. Ford L.C., Delange R.J. and Petty R.W. Identification of a non-lysozymal bactericidal factor (beta-lysin) in human tears and aqueous humour. *Am. J. Ophthalmol.* **81**: 30 – 33 (1976)

37. Stryer L. *Biochemistry.* 4th ed. Freeman and Company, New York (1995)

38. Kuby J. *Immunology.* 3rd ed. W. H. Freeman and Company (1997)

39. Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J.D. *Molecular Biology of the Cell.* 3rd ed. Garland Publishing Ltd. (1994)

40. Coyle P.K. and Sibony P.A. Tear immunoglobulins measured by ELISA. *Invest. Ophthalmol. Vis. Sci.* **27**: 622 – 625 (1996)

41. Kuizenga A., Stolwijk T.R., van Aglmaal E.J., van Haeringen N.J. and Kijlstra A. Detection of secretory IgM in tears of IgA deficient individuaks. *Curr. Eye Res.* **9:** 997 – 1005 (1990)

42. McGill J.I., Liakos G.M., Goulding N. and Seal D.V. Normal tear protein profiles and age related changes. *Br. J. Ophthalmol.* **68**: 316 – 320 (1984)

43. McClellan B.H., Whitney C.R., Newman L.P. and Allansmith M.R. Immunoglobulins in tears. *Am. J. Ophthalmol.* **76**: 89 – 101 (1973)

44. Willcox M.D. and Lan J. S-IgA in tears: functions and changes during contact lens wear. *Clin and Exp. Optom.* **82**; 1: 1 – 3 (1999)

45. Gibbons R.J. Bacterial adherence to the mucosal surface and its inhibition by secretory antibodies. *Adv. Exp. Med. Biol.* **45**: 315 – 325 (1974)

46. Gachon A.M., Richard J. and Dastugue B. Human tears: normal protein pattern and individual protein determinations in adults. *Curr. Eye Res.* **2**: 301 – 308 (1982)

47. Little J.M., Centifanto Y.M. and Kaufman H.E. Immunoglobulins in human tears. *Am. J. Ophthalmol.* **68**: 898 – 905 (1969)

48. Bucci F.A., Kurt J., Moody O.D., Jay B., Tanner O.D. and Myers P.J. The temporal correlation of elevated tear IgE levels with the onset of symptoms in patients wearing daily wear and extended wear contact lenses. *ICLC*; **24**: September/October (1997)

49. Balkwill F.R. and Burke F. The cytokine network. *Immunol. Today*. **10**: 299 – 304 (1989)

50. Hoekzema R., Murray P.I. and Kijlstra A. Cytokines and intraocular inflammation. *Curr. Eye Res.* **9**: 207 – 211 (1990)

51. Pflugfelder S.C., Jones D., Ji Z., Afonso A. and Monroy D. Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. *Curr. Eye Res.* **19**: 201 – 211 (1999)

52. van Setten G.B., Viinikha L., Tervo T., Pesonen K., Tarkkanen A. and Perheentupa J. Epidermal growth factor is a constant component of normal human tear fluid. *Graefe's Arch. Clin. Exp. Ophthalmol.* **227**: 184 – 187 (1989)

53. Ohashi Y., Motokura M., Kinoshita Y., Mano T., Watanabe H., Kinoshita S., Manabe R., Oshiden K. and Yanaihara C. Presence of epidermal growth factor in human tears. *Invest. Ophthalmol. Vis. Sci.* **30**: 1879 – 1882 (1989)

54. Stites D.P. and Terr A.L. *Basic and Clinical Immunology*. 7th ed; Appleton and Lange (1991)

55. Mann A.M., Jones L.W. and Tighe B.J. The application of counter immunoelectrophoresis (CIE) in ocular protein studies. Part I: Time dependent deposition patterns of immunoregulatory proteins on anionic hydrogel contact lenses. *Contact Lens and Anterior Eye*. **25**: 73 – 80 (2002)

56. DeLaCadena R.A. and Coleman R.W. Structure and function of human kininogens. *TIPS*. **12**: 272 – 275 (1991)

57. Margolius H.S. Kallikreins and kinins: some unanswered questions about system characteristics and roles in human disease. *Hypertension*. **26**: 221 – 229 (1995)

58. Mann A.M. and Tighe B.J. The application of counter immunoelectrophoresis (CIE) in ocular protein studies. Part II: kinin activity in the lens wearing eye. *Contact Lens and Anterior Eye*. **25**: 81 – 88 (2002)

59. Mann A.M. Immunological and Biochemical Techniques in the Analysis of Tear Proteins. *PhD Thesis*. Aston University. (1998)

60. Sack R.A., Underwood P.A., Tan K.O. and Morris C. Vitronectin in human tears. In *Lacrimal gland, tear film and dry eye syndromes*. (1994)

61. Sack R.A., Underwood P.A., Tan K.O., Sutherland H. and Morris C.A. Vitronectin – possible contribution to the closed eye external host defense mechanism. *Ocular Immunology and Inflammation*. **1**: 4: 327 – 356 (1993)

62. Sack R.A., Underwood A., Tan K.O. and Morris C. Vitronectin in human tears – protection against closed eye induced inflammatory damage. *Lacrimal gland, tear film and dry eye syndromes*. (1994)

63. Fukuda M. Dry eye and closed eye tears. *Cornea*. **19** (suppl 1): 544 – 548 (2000)

64. Jensen O.L., Glund B.S. and Eriksen H.O. Fibronectin in tears following surgical trauma to the eye. *Acta Ophthalmol*. **63**: 346 – 350 (1985)

65. Grinnell F., Ho C. and Wysocki A. Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting and cell adhesion assays. *Journal of Invest. Dermatol*. **98**; 4: 410 – 416 (1992)

66. Fukuda M., Fullard R.J., Willcox M.D.P. Baleriola-Luca C., Bestawros F., Sweeney D. and Holden B.A. Fibronectin in the tear film. *Invest. Ophthalmol. and Vis. Sci*. **37**; 2: 459 – 467 (1996)

67. Nishida T. Role of fibronectin in corneal epithelial wound healing. *The cornea: transactions of the world congress on the cornea*. New York. Raven Press. (1988)

68. Janssen P.T. and vam Bijsterveld O.P. Origin and biosynthesis of human tear fluid protein. *Invest. Ophthalmol. Vis. Sci*. **24**: 623 – 630 (1983)

69. Delaire A., Lassagne H. and Gachon A.M. New members of the lipocalin family in human tear fluid. *Exp. Eye Res*. **55**: 645 – 647 (1992)

70. Fullard R.J. and Kissner D.M. Purification of the isoforms of tear specific prealbumin. *Curr. Eye Res*. **10**: 613 – 628 (1991)

71. Abduragimov A.R., Gasymov O.K., Yusifov T.N. and Glasgow B.J. Functional cavity dimensions of tear lipocalin. *Curr. Eye Res*. **21**; 4: 824 – 832 (2000)

72. Glasgow B.J., Marshall G., Gasymov O.K., Abduragimov A.R., Yusifov T.N. and Knobler C.M. Tear lipocalins: potential lipid scavengers for the corneal surface. *Invest. Ophthalmol. Vis. Sci*. **40**: 3100 – 3107 (1999)

73. van Haeringen N.J. Clinical biochemistry of tears. *Surv. Ophthalmol*. **26**; 2: 84 – 96 (1981)

74. Effron N. Contact lens associated tear film dysfunction. *Optician*. **5676**: 16 – 25 (1998)

75. Stuchell R.N., Feldmann J.J., Farris R.L. and Mandel I.D. The effect of collection technique on tear composition. *Invest. Ophthalmol. Vis. Sci*. **25**: 374 – 377 (1984)

76. Jones D.T., Monroy D and Pflugfelder S.C. A novel method of tear collection: comparison of glass capillary micropipettes with porous polyester rods. *Cornea*. **16**: 450 – 458 (1997)

77. van Agtmal E.J., van Haeringen N.J., Bloem M.W. and Schreurs W.H.P. Recovery of protein from tear fluid stored in cellulose sponges. *Curr. Eye Res*. **6**: 585 – 588 (1987)

78. Stuchell R.N., Farris R.L. and Mandel I.D. Basal and reflex human tear analysis II: chemical analysis: lactoferrin and lysozyme. *Am. Acad. Ophthalmol*. **88**: 858 – 861 (1981)

79. Fullard R.J. and Snyder C. Protein levels in non stimulated and stimulated tears of normal human subjects. *Invest. Ophthalmol. Vis Sci*. **31**: 1119 – 1126 (1990)

80. Fullard R.J. and Tucker D.L. Changes in human tear protein levels with progressively increasing stimulus. *Invest. Ophthalmol. Vis Sci*. **32:** 2290 – 2301 (1991)

81. Murube J., Murube L. and Murube E. Orogon and types of emotional tearing. *European Journal of Ophthalmol*. **9**; 2: 77 – 84 (1999)

82. Frey W.H., Desota-Johnson D. and Hoffman C. Effect of stimulus on the chemical composition of human tears. *Am. J. Ophthalmol*. **92**: 559 – 567 (1981)

83. Sack R.A., Tan K.O. and Tan A. Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid. *Invest. Ophthalmol Vis. Sci*. **33**: 626 – 640 (1992)

84. Stapleton F., Willcox M.D.P., Morris C.A. and Sweeney D.F. Tear changes in contact lens wearers following overnight eye closure. *Curr. Eye Res*. **17**: 183 – 188 (1998)

85. Tighe B.J., Franklin V.F. and Tonge S. Contact lens care. Part III – Contact lens materials and their interactions with tears. *Optician*. **5803**; 221: 22 – 29 (2001)

86. Franklin V.J., Bright A.M. and Tighe B.J. Hydrogel polymers and ocular poilation processes. *TRIP*. **1**; 1: 9 – 15 (1993)

87. Garret Q. and Milthorpe B.K. Human serum albumin adsorption on hydrogel contact lenses *in vitro*. *Invest. Ophthalmol. Vis. Sci.* **37**; 13: 2594 – 2602 (1996)

88. Bowers R.W.J. and Tighe B.J. Studies of the ocular compatibility of hydrogels: A review of the clinical manifestations of spoliation. *Biomaterials.* **8**: 83 – 88 (1987)

89. Sottys-Robitaille C.E., Ammon D.M., Valint P.L. and Grobe G.L. The relationship between contact lens surface charge and *in vitro* protein deposition levels. *Biomaterials.* **22**: 3257 – 3260 (2001)

90. Bruce A.S., Mainstone J.C. and Golding T.R. Analysis of tear film break up on Etafilcon A hydrogel lenses. *Biomaterials.* **22**: 3249 – 3256 (2001)

91. Allansmith M.R. Immunological effects of extended wear contact lenses. *Ann. Ophthalmol.* **21**: 465 – 474 (1989)

92. Gachon A.M., Bilbaut T. and Dastugue B. Adsorption of tear proteins on soft contact lenses. *Exp. Eye Res.* **40**: 105 – 116 (1985)

93. Kidane A., Szabocsik J.M. and Park K. Accelerated study on lysozyme deposition on poly(HEMA) contact lenses. *Biomaterials.* **19**: 2051 – 2055 (1998)

94. Maissa C., Franklin V., Guillon M. and Tighe B. Influence of contact lens material surface characteristics and replacement frequency on protein and lipid deposition. *Optom. And Vis. Sci.* **75**; 9: 697 – 705 (1998)

95. Garret Q., Chatalier R.C., Griesser H.J. and Milthorpe B.K. Effect of charged groups on the adsorption and penetration of proteins onto and into carboxymethylated poly(HEMA) hydrogels. *Biomaterials.* **19**: 2175 – 2186 (1998)

96. Lin S.T., Mandell R.B., Leahy C.D. and Newell J.O. Protein accumulation on disposable extended wear lenses. *CLAO Journal.* **17**; 1: 44 – 49 (1991)

97. Kok J. and Kijlstra A. The role of tears in preventing protein deposition on contact lenses. *Curr. Eye Res.* **8**; 2: 185 – 189 (1988)

98. McArthur S.L., McClean K.M., St. John H.A.W. and Greisser H.J. XPS and surface-MALDI-MS characterisation of worn HEMA-based contact lenses. *Biomaterials.* **22**: 3295 – 3304 (2001)

99. Tomlinson A. and Caroline P. Comparative evaluation of surface deposits on high water content hydrogel content lens polymers. *CLAO Journal* **16**; 2: 121 – 127 (1990)

100. Poggio E.C and Abelson M.B. Complications and symptoms with disposable contact lenses and conventional soft daily wear contact lenses. *CLAO Journal.* **19**; 2: 95 – 102 (1993)

101. Jones L., Mann A., Evans K., Franklin V. and Tighe B. An *in vivo* comparison of the kinetics of protein and lipid deposition on Group II and Group IV frequent-replacement lenses. *Optom. And Vis. Sci.* **77**; 10: 503 – 510 (2000)

102. Allansmith M.R., Korb D.R., Greiner J.V., Henriquez A.S., Simon M.A. and Finnemore F.M. Giant papillary conjunctivitis in contact lens wearers. *Am. J. Ophthalmol.* **83**: 697 – 708 (1997)

103. Mandell R.B. *Contact lens practice*. 4th ed. C.C. Thomas, Springfield (1989)

104. Boswall G.J., Ehlers W.H., Luistro A., Worrall M. and Donshik P.C. A comparison of conventional and disposable extended wear contact lenses. *CLAO Journal.* **19**; 3: 158 – 165 (1993)

105. Liesegang T.J. Contact lens related microbial keratitis: Part I: Epidemiology. *Cornea.* **16**: 125 – 131 (1997)

106. Schaumberg D.A., Snow K.K. and Dana M.R. The epidemic of *Acanthamoeba keratitis*: where do we stand? *Cornea.* **17**: 3 – 10 (1998)

107. Sern G.A. Contact lens associated bacterial keratitis: past, present and future. *CLAO Journal* **24**: 52 – 56 (1998)

108. Porazinski A.D. and Donshik P.C. Giant papillary conjunctivitis in frequent replacement contact lens wearers: a retrospective study. *CLAO Journal* **25**: 142 – 147 (1999)

109. Willcox M.D.P., Harmis N., Cowell B.A., Williams T. and Holden B.A. Bacterial interactions with contact lenses; effects of lens material, lens wear and microbial physiology. *Biomaterials.* **22**: 3235 – 3247 (2001)

110. Hunt J.A., McLaughlin P.J. and Flanagan B.F. techniques to investigate cellular and molecular interactions in the host response to implanted biomaterials. *Biomaterials.* **18**: 1449 – 1459 (1997)

111. Furness E.L., Ross A., Davis T.P. and King G.C. A hydrophobic interaction site for lysozyme binding to polyethylene glycol and model contact lens polymers. *Biomaterials.* **19**: 1361 – 1369 (1998)

112. McGill J., Liakos G., Seal D., Goulding N. and Jacobs D. Tear film changes in health and dry eye conditions. *Trans. Ophthal. Soc U.K.* **103**: 313 – 317 (1983)

113. Holden B.A., Sankaridurg P.R. and Jalbert I. *Silicone hydrogels: The rebirth of continuous wear contact lenses.* Oxford, Butterworth-Heinemann: 151 – 206 (2000)

114 Kanski J.J. *Clinical Ophthalmology. 4th ed.* Butterworth-Heinemann. (1999)

115. Michaud L. and Giasson C.J. Overwear of contact lenses: increased severity of clinical signs as a function of protein adsorption. *Optom and Vis Sci* **79**; 3: 184 – 192 (2002)

116. Holden B.A., Hood D., Grant T., Newton-Howes J., Baleriola-Lucas C., Willcox M.D.P. and Sweeney D.F. Gram negative bacteria can induce contact lens related acute red eye (CLARE) responses. *CLAO J* **22**; 1: 47 – 52 (1996)

117. Bruinsma G.M., Rustema-Abbing M., de Vries J., Stegenga B., van der Mei H.C., van der Linden M.L., Hooymans J.M.M. and Busscher H.J. Influence of wear and overwear on surface properties of Etafilcon A contact lenses and adhesion of *Pseudomonas aeruginosa*. *Invest. Ophthalmol. and Vis. Sci* **43**; 12: 3646 – 3652 (2002)

118. Szliter E.A., Morris C.A., Carney F., Gabriel M.M. and Hazlett L.D. Development of a new extended wear contact lens model in the rat. *CLAO J* **28**; 3: 119 – 123 (2002)

119. Radford C.F., Minassian D.C. and Dart J.K.G Disposable contact lens use as a risk factor for microbial keratitis. *Br. J. Ophthalmol;* **82**: 1272 – 1275 (1998)

120. Bruinsma G.M., van der Mei H.C. and Busscher. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. *Biomaterials.* **22**: 3217 – 3224 (2001)

121. Olsen M.R. Comparison and evaluation of various techniques for analysing tear proteins and contact lens extracts. *PhD Thesis.* Aston University. (2001)

122. Peach H.C., Mann A.M. and Tighe B.J. Immunoblotting and tear sampling techniques for the study of contact lens induced variations in tear protein profiles. *In Lacrimal gland, tear film and dry eye syndromes 3.* kluwer Academic?Plenum Publishers (2002)

123. Schmut O., Horwath-Winter J., Zenker A. and Trummer G. The effect of sample treatment on separation profiles of tear fluid proteins: qualitative and semi-quantitative protein determination by an automated analysis system. *Graefe's Arch. Clin. Exp Ophthalmol* **240**: 900 – 905 (2002)

124. Bjerrum K.B. and Prause J.U. Collection and concentration of tear proteins studied by SDS gel electrophoresis – presentation of a new method with special reference to dry eye patients. *Graefe's Arch. Clin. Exp. Ophthalmol* **232**: 402 – 405 (1994)

125. Sariri R. and Sabbaghzadeh R. Competitive adsorption of proteins on hydrogel contact lenses. *CLAO J.* **27**; 3: 159 – 162 (2001)

126.Ng V., Cho P.,Wong F. and Chan Y. Variability of tear protein levels in normal young adults: (diurnal (daytime) variation. *Graefe's Arch. Clin. Exp. Ophthalmol.* **239**: 257 – 263 (2001)

127.Huth S.W., Miller M.J. and Leopold I.H. Calcium and proteins in tears – diurnal variation. *Arch. Ophthalmol* **99**: 1628 – 1633 (1981)

128.Tear fluid analysis, development of a new method for tear analysis in dry eye. *Acta Ophthalmologica Scandinavica:* 26 – 29 (2000)

129.Brennan N.A. and Coles M.L.C. Deposits and symptomology with soft contact lens wear. *ICLC.* **27**: 75 – 99 (2000)

130.Pritchard N., Fonn D. and Weed K. Ocular and subjective responses to frequent replacement of daily wear soft contact lenses. *CLAO J.* **22**; 1: 53 – 59 (1996)

131.Pritchard N., Fonn D. and Brazeau D. Discontinuation of contact lens wear: a survey. *ICLC.* **26**: 157 – 161 (1999)

132.Orsborn G. and Edwards K. Patient and practioner perspectives on PureVision continuous wear lenses. *ICLC.* **27**; 175 – 180 (2000)

133.Brennan N.A., Coles M.L.C., Jaworski A. and Rosario D. Proposed practice guidelines for continuous contact lens wear. *Clin. and Exp. Optom.* **84**; 2: 71 – 77 (2001)

134.Thakur A. and Willcox M.D.P. Contact lens wear alters the production of certain inflammatory mediators in tears. *Exp. Eye Res.* **70**: 255 – 259 (2000)

135.Liesegang T.J. Physiological changes of the cornea with contact lens wear. *CLAO J.* **28**; 1: 12 – 27 (2002)

136. du Toit R., Stern J. and Sweeney D. Surfaces of silicone hydrogel contact lenses. *ICLC.* **27**: 191- 192 (2000)

137. Iruzubieta J.M., Ripoll J.R.N., Fernandez O.E., Alvarez J.J.R., Delgado F., Villa F. and Traverso L.M. Practical experience with a high Dk Lotrafilcon A fluorosilicone hydrogel extended wear contact lens in Spain. *CLAO J.* *27*; 1 (2001)

138. Nilsson S.E.G. Seven day extended wear and 30 day continuous wear of high oxygen transmissibility of silicone hydrogel contact lenses – a randomised 1-year study of 504 patients. *CLAO J.* **27**; 3: 125 – 134 (2001)

139. Meagher L. and Griesser H.J. Interactions between adsorbed lactoferrin layers directly measured with the atomic force microscope. *Colloids and Surfaces B: Biointerfaces.* **23**: 125 – 140 (2002)

140. Temel A., Kazokoglu H and Taga Y. Tear lysozyme levels in contact lens wearers. *Ann. Ophthalmol.* **23**: 191 – 194 (1991)

141. Glasgow B.J., Abduragimov A.R., Gasymov O.K. and Yusifov T.N. Tear lipocalin: structure, function and molecular mechanisms of action. *In Lacrimal gland, tear film and dry eye syndromes 3.* Kluwer Academic Plenum Publishers (2002)

142. Abduragimov A.R., Gasymov O.K., Yusifov T.N. and Glasgow B.J. Functional cavity dimensions of tear lipocalin. *Curr. Eye Res.* **21**; 4: 824 – 832 (2000)

143. Glasson M.J., Stapleton F. and Willcox M.D.P. Lipid, lipase and lipocalin differences between tolerant and intolerant lens wearers. *Curr. Eye Res.* **25**; 4: 227 – 235 (2002)

144. Grus F.H., Sabuncuo P., Augustin A. and Pfeiffer N. Effect of smoking on tear proteins. *Graefe's Arch. Clin. Exp. Ophthlmol.* **240**: 889 – 892 (2002)

Appendix

APPENDIX 1

Solution 1 – Extraction solution

Chemical	Amount
Urea	40%
SDS	1%
DTT	1mM
Tris	100mM

Solution 2 – Sample treatment buffer

Chemical	Amount
Tris Cl, pH 6.8	2.5 ml
10 % SDS	4.0 ml
Glycerol	2.0 ml
2-mercaptoethanol	0.2 ml
Bromophenol blue	0.2 mg
Distilled water	to 10 ml

Solution 3 – Acrylamide stock

Chemical	Amount
Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Distilled water	100 ml

Filter mixed solution (0.45 μm filter)

Solution 4 – Tris HCl

Chemical	Amount
Tris base	36.1 g
Distilled water	150 ml

Filter mixed solution (0.45 μm filter)

Solution 5 – 10% SDS

Chemical	Amount
SDS powder	10.0 g
Distilled water	to 100 ml

Solution 6 – 7.5%T solution

Chemical	Amount
Acrylamide stock	2.5 ml
1.5M Tris HCl	2.5 ml
10% SDS	0.1 ml
Distilled water	4.9 ml
10% APS	50 μl
TEMED	5 μl
Final volume	10.0 ml

Solution 7- 20%T solution

Chemical	Amount
Acrylamide stock	6.6 ml
1.5M Tris HCl	2.5 ml
10% SDS	0.1 ml
Distilled water	0.8 ml
10% APS	50 μl
TEMED	5 μl
Final volume	10.0 ml

Solution 8 – TEMED

Chemical	Amount
TEMED	200 μl
Distilled water	2.5 ml

Solution 9 – 10% APS

Chemical	Amount
Ammonium persulphate	0.15 g
Distilled water	to 2.5 ml

Solution 10 - Water-saturated n-butanol

Solution 11 – Running buffer

Chemical	Amount
Tris base (pH 8.3)	1.16 g
Glycine	6.0 g
SDS	0.4 g
Distilled water	400 ml

Solution 12 – Preconditioning medium

Chemical	Amount
Methanol	45.0 ml
Acetic acid	10.0 ml
Distilled water	45.0 ml

Solution 13 – Working stain solution

Chemical	Amount
Preconditoning medium	8.0 ml
Reagent A	8.0 ml
Reagent B	8.0 ml
Distilled water	16.0 ml

Solution 14 – Destain solution

Chemical	Amount
Methanol	45.0 ml
Acetic acid	10.0 ml
Distilled water	45.0 ml

Solution 15 – Fixative solution

Chemical	Volume
Methanol	200 ml
Acetic acid	40 ml
Fixative enhancer	40 ml
Distilled water	120 ml

Solution 16 – Developing solution

Chemical	Volume
Distilled water	35 ml
Silver complex solution	5 ml
Reduction moderator solution	5 ml
Image development reagent	5 ml
Development accelerator solution	50 ml

Solution 17 – Anode solution 1

Chemical	Amount
0.3 M Tris	36.3 g
20% (v/v) methanol	200 ml
Distilled water	800 ml

Solution 18– Anode solution 2

Chemical	Amount
0.025 M Tris	3.03 g
20% (v/v) methanol	200 ml
Distilled water	800 ml

Solution 19 – Cathode solution

Chemical	Amount
0.04 M 6-amino-n-hexanoic acid	5.2 g
20% (v/v) methanol	200 ml
Distilled water	800 ml

Solution 20 – Tris buffer saline

Chemical	Amount
Tris(aminoethane)	12.1 g
NaCl	29.22 g
Glycine	0.75 g

Above solution dissolved in 800 ml and adjusted to pH 8.0 before being made up to 1000 ml with distilled water

Solution 21 – Blocking solution

Chemical	Amount
Marvel (dry milk powder)	40 g
TBS	1000 ml

Solution 22 – BCIP/NBT

Chemical	Amount
BCIP/NBT tablets	2 tablets
Distilled water	20.0 ml

Patient Number: ________
Date ________
Time ________

NON-CONTACT LENS WEARER QUESTIONNAIRE (DEQ)

1. Have you worn contact lenses at least 3 days a week, eight hours a day, in the past month?

   1 Yes (please stop and fill out the contact lens wearer questionnaire)
   2 No

2. If you have worn contact lenses in the past, have you ever worn any of the following types of lenses?

	Yes	No
a. Rigid gas permeable	1	2
b. Soft daily wear (lenses replaced after 1 year or longer)	1	2
c. Frequent replacement (lenses replaced after 1 month or longer)	1	2
d. Disposable (lenses replaced daily or every 2 weeks)	1	2
e. Extended wear (lenses worn overnight)	1	2

3. How important was each of the following issues in your decision to stop wearing contact lenses?

	Not at All Important				Very Important	Not Applicable
a. I never got used to the lenses	1	2	3	4	5	0
b. The lenses were uncomfortable all day	1	2	3	4	5	0
c. The lenses were most uncomfortable when first put in	1	2	3	4	5	0
d. The lenses became more uncomfortable later in the day	1	2	3	4	5	0
e. My eyes felt dry	1	2	3	4	5	0
f. The lenses felt scratchy and irritating	1	2	3	4	5	0
g. My vision was not clear enough	1	2	3	4	5	0
h. My vision fluctuated throughout the day	1	2	3	4	5	0
i. Wearing contact lenses was too much trouble	1	2	3	4	5	0
j. Contact lenses were too expensive	1	2	3	4	5	0
k. I couldn't put the lenses in easily	1	2	3	4	5	0
l. I couldn't sleep in the lenses	1	2	3	4	5	0
m. I had red or infected eyes	1	2	3	4	5	0
n. Other reason (please specify below)	1	2	3	4	5	0

____

3d. What is your age?

____

3e. What is your gender?

1 Male **2** Female

Copyright© Trustees of Indiana University, 2000, all rights reserved

4. Questions about **EYE COMFORT**:

a. During a typical day in the past week, how often did your eyes feel uncomfortable?

1 Never (please skip to question 5)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt discomfort, how intense was this feeling of discomfort...

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

5. Questions about **EYE DRYNESS**:

a. During a typical day in the past week, how often did your eyes feel dry?

1 Never (please skip to question 6)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt dry, how intense was this feeling of dryness...

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

6. Questions about **BLURRY VISION**:

a. During a typical day in the past week, how often did your vision change between clear and blurry? (This is often described as "foggy or steamy vision that clears up when you blink.")

1 Never (please skip to question 7)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your vision was blurry, how noticeable was this blurry vision...

b. Within the first two hours of getting up in the morning?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Noticeable				Very Noticeable	Not Sure
1	2	3	4	5	0

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Noticeable				Very Noticeable	Not Sure
1	2	3	4	5	0

7. Questions about **EYE SORENESS** and **IRRITATION**:

a. During a typical day in the past week, how often did your eyes feel sore and irritated?

1 Never (please skip to question 8)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt sore and irritated, how intense was this feeling of soreness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

8. Questions about **EYE GRITTINESS** and **SCRATCHINESS**:

a. During a typical day in the past week, how often did your eyes feel gritty and scratchy (as if a piece of sand was in your eye)?

1 Never (please skip to question 9)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt gritty and scratchy, how intense was this feeling of grittiness and scratchiness…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	2	3	4	5	0

9. Questions about **FEELING LIKE SOMETHING IS IN YOUR EYE**:

a. During a typical day in the past week, how often did you have the feeling that "something" was in your eye (as if a piece of sand was in your eye)?

1 Never (please skip to question 10)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When you felt as if something was in your eye, how intense was this feeling…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

10. Questions about **EYE BURNING** and **STINGING**:

a. During a typical day in the past week, how often were your eyes burning and stinging?

1 Never (please skip to question 10)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes were burning and stinging, how intense was this feeling of burning and stinging…

b. Within the first two hours of getting up in the morning?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of the day (within the last 2 hours before you went to bed)?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

Patient Number: ______
Date ______
Time ______

CONTACT LENS WEARER QUESTIONNAIRE (CLDEQ)

1. Have you worn contact lenses at least 3 days a week, eight hours a day, in the past month?

   1 Yes **2** No (please stop and fill out the non-contact lens wearer questionnaire)

2. Do you currently wear any of the following types of lenses?

	Yes	No
a. Rigid gas permeable	**1**	**2**
b. Soft daily wear (lenses replaced after 1 year or longer)	**1**	**2**
c. Frequent replacement (lenses replaced after 1 month or longer)	**1**	**2**
d. Disposable (lenses replaced daily or every 2 weeks)	**1**	**2**
e. Extended wear (lenses worn overnight)	**1**	**2**

3a. How many days per week did you wear your lenses during a typical week during the past month?

______ days

3b. How many hours per day did you wear your lenses during a typical day during the past week?

______ hours

3c. How many hours have you worn your contact lenses today?

______ hours

3d. What is your age?

______

3e. What is your gender?

1 Male
2 Female

Copyright© Trustees of Indiana University, 2000, all rights reserved

4. Questions about **COMFORT**:

a. During a typical day in the past week, how comfortable did your contact lenses feel?

Very Uncomfortable				Very Comfortable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

b. During a typical day in the past week, how often did your eyes feel **uncomfortable**?

1 Never (please skip to question 5)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt uncomfortable, how intense was this feeling of discomfort…

c. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

e. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

5. Questions about **EYE DRYNESS**:

a. During a typical day in the past week, how often did your eyes feel dry **while wearing your contact lenses?**

1 Never (please skip to question 6)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt dry, how intense was the feeling of dryness…

b. In general?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

e. During a typical day in the past week, how often did your eyes feel dry **while not wearing your contact lenses?**

1 Never (please skip to question 6)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

f. In general, how intense was the feeling of dryness **while not wearing your lenses**?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

6. Questions about **BLURRY VISION**:

a. During a typical day in the past week, how often did your vision change between clear and blurry **while wearing your contact lenses**? (This is often described as "foggy or steamy vision that clears up when you blink.")

1 Never (please skip to question 7)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your vision was blurry, how noticeable was this blurry vision...

b. Within the first two hours of putting in your lenses?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Noticeable				Very Noticeable	Not Sure
1	**2**	**3**	**4**	**5**	**0**

7. Questions about **EYE SORENESS** and **IRRITATION**:

a. During a typical day in the past week, how often did your eyes feel sore and irritated **while wearing your contact lenses**?

1 Never (please skip to question 8)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt sore and irritated, how intense was this feeling of soreness and irritation...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

8. Questions about **EYE GRITTINESS** and **SCRATCHINESS**:

a. During a typical day in the past week, how often did your eyes feel gritty and scratchy **while wearing your contact lenses**?

- **1** Never (please skip to question 9)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your eyes felt gritty and scratchy, how intense was this feeling of grittiness and scratchiness...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

9. Questions about **FEELING LIKE SOMETHING IS IN YOUR EYE**:

a. During a typical day in the past week, how often did you have the feeling as if "something" was in your eye **while wearing your contact lenses** (as if a piece of sand was in your eye)?

- **1** Never (please skip to question 10)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When you felt as if something was in your eye, how intense was this feeling...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

10. Questions about **EYE BURNING** and **STINGING**:

a. During a typical day in the past week, how often were your eyes burning and stinging **while wearing your contact lenses**?

- **1** Never (please skip to question 11)
- **2** Infrequently
- **3** Frequently
- **4** Constantly
- **0** Not Sure

When your eyes were burning and stinging, how intense was this <u>feeling of burning and stinging</u>...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

11. Questions about **LIGHT SENSITIVITY**:

a. During a typical day in the past week, how often did your eyes feel unusually sensitive to bright lights **while wearing your contact lenses**?

1 Never (please skip to question 12)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt unusually sensitive to bright lights, how intense was this <u>unusual sensitivity</u>...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

12. Questions about **EYE ITCHING**:

a. During a typical day in the past week, how often did your eyes itch **while wearing your contact lenses**?

1 Never (please skip to question 13a)
2 Infrequently
3 Frequently
4 Constantly
0 Not Sure

When your eyes felt itchy, how intense was this <u>feeling of itchiness</u>...

b. Within the first two hours of putting in your lenses?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

c. In the middle of the day?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

d. At the end of your wearing time?

Not at All Intense				Very Intense	Not Sure
1	**2**	**3**	**4**	**5**	**0**

13a. How often during the past month, did your eyes *bother you so much* that you felt as if you needed to stop whatever you were doing and take out your contact lenses? Please choose the answer that is closest to your situation.

1 Never (please skip to question 14)
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily
6 Several times a day

13b. When your eyes bothered you so much that you felt as if you needed to stop what you were doing and take out your *contact lenses*, how much did each of the following symptoms contribute to this feeling?

	Not At All				Very Much	Not Sure
a. Eye discomfort	1	2	3	4	5	0
b. Eye dryness	1	2	3	4	5	0
c. Eye soreness and irritation	1	2	3	4	5	0
d. Eye grittiness and scratchiness	1	2	3	4	5	0
e. Eye burning and stinging	1	2	3	4	5	0
f. Light sensitivity	1	2	3	4	5	0
g. Eye itching	1	2	3	4	5	0

13c. How often during the past month did your eyes bother you so much that you **did** remove your contact lenses? Please choose the answer that is closest to your situation.

1 Never
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily
6 Several times a day

14a. How many hours during a typical workday do you use a computer?

__________ hours

14b. How many hours during a typical day when you're not working do you use a computer?

__________ hours

15. Are you currently taking any of the following medications?

	Yes	No
a. Thyroid medications	1	2
b. Blood pressure medications	1	2
c. Diabetes medications	1	2
d. Diuretics	1	2
e. Accutane	1	2
f. Heart condition medications	1	2
g. Depression medications	1	2
h. Ulcer medications	1	2
i. Oral contraceptives / hormone replacement therapy	1	2

16a. Do you have any of the following allergies?

	Yes	No	Don't Know
a. Seasonal allergies (Hayfever, "sinus")	1	2	3
b. Skin allergies (Dermatitis)	1	2	3
c. Asthma	1	2	3
d. Allergies to animals	1	2	3
e. Allergies to pollen & mold	1	2	3
f. Allergies to food	1	2	3
g. Allergies affecting your eyes	1	2	3
h. Allergies to contact lens solutions	1	2	3
i. Allergies to eye drops	1	2	3

16b. If you have taken any of the following medications for your allergies in the past year, how often did you take them?

	Less than once a week	Once a week	2-3 times a week	Daily	More than once daily	Do Not Take
a. Antihistamine pills or liquid	1	2	3	4	5	0
b. Antihistamine eye drops	1	2	3	4	5	0
c. Decongestant pills or liquid	1	2	3	4	5	0
d. Decongestant eye drops	1	2	3	4	5	0
e. nasal inhalants	1	2	3	4	5	0

17. In the past month, how often have you experienced dryness of the mouth, nose or vagina? Please choose the answer that is closest to your situation

1 Never
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily

18a. Have you been told you have dry eye(s)?

1 Yes **2** No **3** Don't Know

18b. If you use any of the following treatments, how much help do they provide?

	No Help At All				Complete "Cure"	Do Not Use
a. Artificial tears	**1**	**2**	**3**	**4**	**5**	**0**
b. Contact lens rewetting drops	**1**	**2**	**3**	**4**	**5**	**0**
c. Warm compresses and/or eyelid cleaning	**1**	**2**	**3**	**4**	**5**	**0**
d. Punctal plugs or cauterization..............	**1**	**2**	**3**	**4**	**5**	**0**
e. Removal of your contact lenses	**1**	**2**	**3**	**4**	**5**	**0**
f. Other (lease specify below)	**1**	**2**	**3**	**4**	**5**	**0**

____

19. Do you think you have dry eye(s)?

1 Yes **2** No **3** Don't Know

20. Have you used artificial tears in the last month?

1 Yes (please answer questions 21, 22, and 23 below)
2 No (***Thank You,*** that completes this survey)
3 Don't Know (***Thank You,*** that completes this survey)

21. In the last month, about how many times per day did you use artificial tears or contact lens rewetting drops?

__________ times

22. In the last month, about how many bottles of artificial tears or contact lens rewetting drops did you use?

__________ bottles

23. In the last month, about how much money did you spend on the purchase of artificial tears or contact lens rewetting drops?

__________ dollars

QUESTIONNAIRE

Myopia questionnaire

Please state the average time spent (hrs per day) performing the following tasks:

- Driving:
- Reading and writing:
- Meetings:
- Computers/ VDT:
- Sports:
- Video games:
- TV:
- Crafts:

Eye Health questionnaire

- Are you using re-wetting drops? If so, how often?
- What CL solution are you currently using? Opti-Free or ReNu?
- Do you CLs get dry and spilt and/or fall out of the eye? If so, how often?
- Are you taking long-term medication? If so, can you state what are you taking?
- How would you classify your diet? 0= Worst; 10= Best.

PATIENTS' SUBJECTIVE JUDGMENT

Ref: **No. Visit:**

Do you suffer from any of the symptoms below? Please mark the position on the scale that most adequately describes if you have experienced any of the following symptoms. Parts of the scale are labelled but you are not restricted to only using those positions:

Symptom	No symptom — Symptom unbearable	Symptom	No symptom — Symptom unbearable
• Blurred vision	|—————|	Dryness	|—————|
• Variable vision	|—————|	Burning	|—————|
• Glare	|—————|	Itching	|—————|
• Photophobia	|—————|	Excess secretion	|—————|
• Lens handling problems	|—————|	Excessive tearing	|—————|

Please mark the position on the scale that most adequately describes your subjective judgment of the following: Parts of the scale are labelled but you are not restricted to only using those positions:

Item	Worst — Best
• Overall visual quality	|—————|
• Comfort	|—————|
• Convenience	|—————|
• Ocular health	|—————|
• Patient appearance	|—————|
• Quality of life	|—————|
• Overall satisfaction	|—————|

Did you experience any other problems?

If you are in the DW regime, how many hours a week do you wear your contact lenses?

If you are in the CW regime, how many times do you remove your lenses a week or a month?