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TOWARDS AN IMPROVED OCULAR DRUG DELIVERY
SYSTEM

ANNE MARGARET BRIGHT

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

May, 1992

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Submitted for the Degree of Doctor of Philosophy

May 1992

SUMMARY

The ultimate aim of this project was to design new biomaterials which will improve the efficiency of ocular drug delivery systems. Initially, it was necessary to review the information available on the nature of the tear fluid and its relationship with the eye. An extensive survey of the relevant literature was made. There is a common belief in the literature that the ocular glycoprotein, mucin, plays an important role in tear film stability and, furthermore, that it exists as an adherent layer covering the corneal surface. If this belief is true, the muco-corneal interaction provides the ideal basis for the development of sustained release drug delivery.

Preliminary investigations were made to assess the ability of mucin to adhere to polymer surfaces. The intention was to develop a synthetic model which would mimic the supposed corneal/mucin interaction. Analytical procedures included the use of microscopy (phase contrast and fluorescence), fluorophotometry, and mucin-staining dyes. Additionally, the physical properties of tears and tear models were assessed under conditions mimicking those of the preocular environment, using rheological and tensiometric techniques. The wetting abilities of these tear models and ophthalmic formulations were also investigated. Tissue culture techniques were employed to enable the surface properties of the corneal surface to be studied by means of cultured corneal cells. The results of these investigations enabled the calculation of interfacial and surface characteristics of tears, tear models, and the corneal surface.

Over all, this work cast doubt on the accepted relationship of mucin with the cornea. A corneal surface model was designed, on the basis of the information obtained during this project, which would possess similar surface chemical properties (i.e. would be biomimetic) to the more complex original. This model, together with the information gained on the properties of tears and solutions intended for ocular instillation, could be valuable in the design of drug formulations with enhanced ocular retention times. Furthermore, the model itself may form the basis for the design of an effective drug-carrier.

Key words: ocular drug delivery, tear film, tear models, mucin, corneal surface.

For my family

ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

Professor Brian Tighe, my supervisor, for his advice, encouragement and unceasing enthusiasm over the course of this work.

Miss Helen Fitton for maintaining the corneal cell culture, and for preparing the cell layers used during this project.

Dr. Val Franklin for her assistance during the early part of this work, and Dr. Helen Oxley for help and advice concerning the hydrogels used.

All the staff and students in the Speciality Materials Research Group for making my time spent at Aston so happy.

Smithkline Beecham Pharmaceuticals for generous financial help throughout the course of this research programme, and for access to analytical equipment in the Formulation and Drug Development laboratories in Worthing.

SERC for financial support.

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CHAPTER 1

Literature Survey:

**The Composition and Properties of the Tear Film, and Current
Ocular Drug Delivery Systems.**

1.1 Introduction

In recent years there has been an explosion in research aimed at creating new drug delivery systems. New and more complex drugs, such as proteins, are becoming available and often require more complex delivery systems than conventional drugs. At the same time, there is a growing awareness that drug release patterns (continuous versus pulsatile) associated with current drug delivery systems significantly affect therapeutic responses. It has generally become accepted that the development of improved delivery systems, which increase the efficiency of conventional drugs, may, in many instances, be a better investment than the high cost involved in creating a new drug. Advances in materials science has seen the creation of novel polymers with increased biocompatibility, and these have great potential as drug carriers.

The eye provides a unique site for the study of biomaterials. This is because the tear fluid is easily accessible to the researcher and possesses a complexity which is in its own way comparable with the internal environment provided by other organs. It is, for example, a simple procedure to introduce a contact lens into the tear film, which re-establishes its structure with each blink, and to remove it after a certain length of time. The nature and extent of the interaction between tear fluid and lens material is reflected by the degree of spoilation (the amount of adsorbed tear lipids and proteins on the lens). This may be assessed by various techniques, including HPLC, electrophoresis and fluorescence spectroscopy.

Despite the advantages presented by this environment to the researcher, they have not been fully exploited. The level of understanding of the tear fluid is far from

adequate if we are to be able to interpret the effect of introducing biomaterials into the system. It is essential to fully appreciate the nature of the tear fluid, its origins and its fate, and the interactions occurring both within the tear film and between it and the surface of the eye. Only with this information as a working base will it be possible to develop new biomaterials which are less disruptive to tear stability and better tolerated by the environment. At present attempts are being made to produce biomaterials for very specific purposes by studying their interactions with a complex and poorly understood biological fluid. This chapter presents a critical review of the information available on the precocular environment.

1.2 Structure of the Tear Film

The tear film covering the cornea was first suggested to have a three-layered structure by Wolff in 1954¹. It is generally believed that this structure, as illustrated in Figure 1.1, consists essentially of the superficial lipid layer, the aqueous below this, and an adsorbed mucin layer which adheres to the epithelium². The superficial lipid layer is believed to comprise about 1% of the total thickness (about 100nm thick); the middle aqueous layer represents over 98% of the tear film and is 7 μ m thick; and the inner mucoid layer is most commonly quoted as comprising under 0.5% of the film, being 0.02 to 0.05 μ m thick^{3,4}. A study by Creeth in 1978⁵, however, suggests a much thicker mucoid layer of 0.6 to 1.0 μ m over the cornea and of 2.0 to 7.0 μ m over the conjunctiva. Using optical techniques (reflectance and confocal microscopy), rather than invasive methods for estimating tear film thickness, Prydal (1989)⁶ gives an estimate of between 40 and 44 μ m for the total film thickness. This is considerably greater than most estimates, which give values of less than 10 μ m for the tear film thickness, and is believed to result from the

inclusion of the mucus layer in the estimate, since bathing the eye in acetyl cysteine (removes mucin) reduced the thickness to $12\mu\text{m}$ ⁶. If the aqueous portion of the tear film is taken as $10\mu\text{m}$, this infers a mucus layer at least $30\mu\text{m}$ thick. Rather than being a distinct layer, however, observations have shown that mucins become diluted towards the aqueous layer and interact with and mask the hydrophobicity of the lipids⁷, making the delineation of different tear film layers somewhat arbitrary.

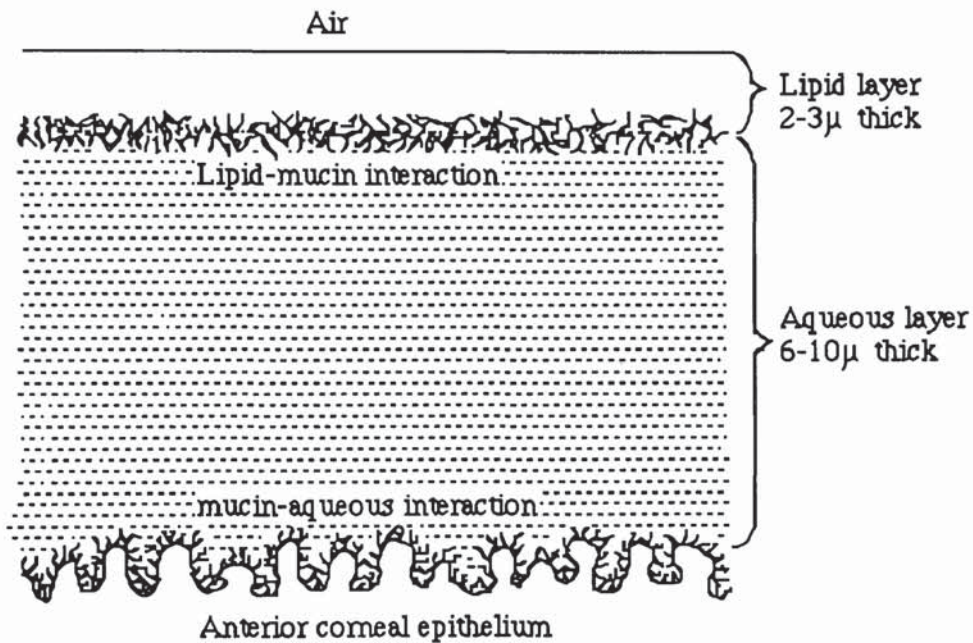


Figure 1.1 The tear film in cross-section.

Normal tear volume is between about 7.0 and 10.0 μl ^{8,9,10}, and there is a continual secretion (nonreflex) rate of about 1 μLmin^{-1} (the range of flow rates being 0.5 - 2.2 $\mu\text{lmin.}^{-1}$)¹¹ from the accessory lacrimal glands¹² (Wolfring's glands and glands of Krause, illustrated in Figure 1.2). The normal ocular tear volume can be divided into the exposed tear volume (ETV) which is interfaced with air, and the unexposed tear volume (UTV) which is not⁹. The distribution of the tear film can be visualised by slit-lamp fluorophotometry after the instillation of a drop of fluorescein or sulphorhodamine B into the eye¹³. The ETV consists of tears on the cornea, the exposed bulbar conjunctiva and the marginal tear strips (also referred to as tear menisci, lacrimal prisms, or rivi) along the lid margins. The UTV is contained by the upper and lower fornices. One large study¹² gave mean values for the respective volumes contained in these areas as 1.1 μl over the exposed bulbar conjunctiva and cornea, 4.5 μl in the fornices, and 2.9 μl in the marginal tear strips. Thus, slightly under half (47%) of a total tear volume of 8.5 μl is exposed to the external environment. Of this ETV, the most recent estimates suggest between 81.5% (Kwok, 1984¹⁴; Bron, 1985¹⁵) and 90% (Guillon and Guillon, 1988¹⁶) is contained in the rivi. Figure 1.1 gives a diagrammatic view of the precorneal tear film in cross section.

1.3 The Tear Components and their Origins

Human tears are formed by a group of glands collectively known as the lacrimal system (Figure 1.2.).

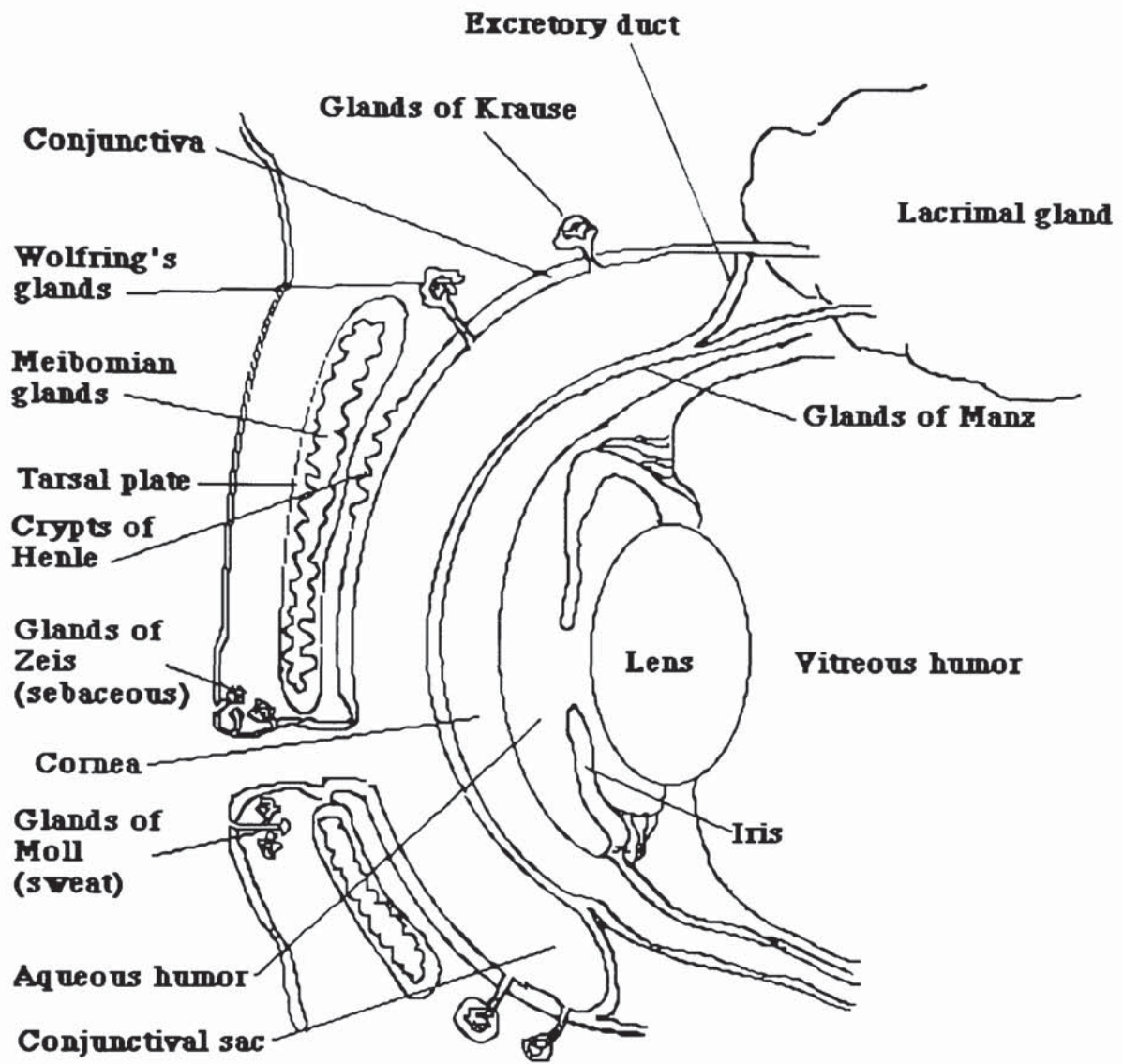


Figure 1.2 Cross-section through the anterior of the eye to show the location of the main tear-forming glands.

The secretory apparatus consists of the main and accessory lacrimal glands (source of the aqueous tear components), the meibomian glands (secrete lipids), and the conjunctival goblet cells (secrete mucous glycoproteins). The accessory lacrimal glands supply the minute-to-minute supply of tears, while the main lacrimal gland functions as a back-up reflex system by flooding the eye in response to injury, irritation or emotions (reflex tearing).

The various secretions contribute to a complex mixture which include proteins, enzymes, lipids, metabolites, electrolytes, hydrogen ions, and also drugs excreted in tears.

1.3.1 Protein Components

These are found chiefly in the middle aqueous layer which originates from the main lacrimal gland located in the superior temporal aspect of the orbit, and from accessory lacrimal glands (glands of Krause and of Wolfring, the infraorbital gland, and the glands of the plica and the caruncle) lining the surface of the conjunctiva. The total concentration of proteinaceous substances in the tear fluid is variously estimated to be 0.3 to 7.0% (800 mg/100ml¹⁸, 2200 mg/100ml¹¹), and as 0.6 to 1.0 %¹⁹. Using electrophoretic techniques, more than 60 protein components can be detected in normal tear fluid. Of these, more than 20 have been shown to be secreted by the lacrimal gland²⁰. Evidence for the secretory activity of the lacrimal gland was obtained by immunofluorescence²¹ and by measurement of specific proteins, such as lysozyme, in tear fluid of patients suffering from degeneration of the tear gland²².

The incorporation of radioactive label in the proteins secreted from the lacrimal gland shows them to be the product of local synthesis. For lysozyme this is certainly the case, confirming that it originates in the lacrimal gland itself. An incorporation of radioactive label was also detected in the serum proteins IgG and, particularly, IgA^{21,22}. The possibility of local synthesis by plasma cells is strongly supported by the results of immunofluorescence localisation of IgG and IgA in the lacrimal gland^{21,24}. The strong incorporation of radioactive label in IgA, detected with antisera against IgA- α -chain and secretory component, indicates that it is synthesized in both the conjunctiva and the lacrimal gland²¹. The concentrations of tear proteins varies depending on whether the tears are unstimulated (22g/l), emotional (6g/l), or irritant-induced (4g/l)¹¹. The sources of variability in tear composition are discussed in part 1.4 of this chapter.

1.3.2 Lipid Components

The superficial lipid oily layer is secreted by the meibomian glands and the glands of Moll and Zeis which are situated at the edge of the eyelids. The composition of meibomian lipids in humans has been found to vary considerably among individuals²⁵. The principal nonpolar component of the tear film lipid has been identified as a mixture of waxes and sterol esters, with the sterol esters accounting for nearly one third of the mixture¹¹. Chromatographic analysis demonstrated the presence of all lipid classes in the meibomian secretion: hydrocarbons, wax esters, cholesterol esters, triglycerides, and, in lesser amounts, diglycerides, monoglycerides, fatty acids, cholesterol, and phospholipids^{26,27}.

1.3.3 Mucous Component

The inner mucoid layer is secreted by specialised conjunctival cells in the Crypts of Henle and the glands of Manz¹⁷. The composition of the mucus layer, which is the innermost layer of the tear film, has been analysed to be⁵:

Salts	1.0%
Free protein	0.5 - 1.0%
Glycoprotein	0.5 - 1.0%
Water	>95%

The electrolyte content resembles that of serum or bile. Other constituents include immunoglobulins, salts, urea, glucose, leucocytes, tissue debris, and enzymes such as betalysin, peroxidase and lysozyme²⁸. The glycoproteins, or mucins, are 50 to 80% carbohydrate, with the oligosaccharides attached to a protein backbone of molecular weight $2.0 - 15.0 \times 10^6$ daltons, which is composed of about 800 amino acid residues. Serine and threonine comprise 40% of the total amino acid and form the O-glycosidic link with the side chains which cover approximately 63% of the backbone²⁹. The sugar side chains are composed of up to 12 sugar residues, which in human mucus secretions are made up of the same five monosaccharides: galactose, N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylneuraminic acid (sialic acid). The link with the protein backbone is formed by galactose, and the terminal sugar group is either fucose or sialic acid²⁹. Other sugar residues may bear ester sulphate groups, and these, together with the sialic acid groups, provide mucin with its overall negative charge. The structure, which is illustrated in Figure 1.3, means that areas of both sugar and protein are exposed to the solution, although the former predominate. The presence of cysteine amino acids enables the formation of disulphide bridges between or within molecules, and proline

residues allow the molecules to bend and assume random structures in solution. Cross-linking through disulphide bridges produces polymers of high molecular weight that bind to similar polymers by weak interactions of the carbohydrate chains to form a gel²⁸. The importance of mucin to corneal wettability and tear stability is discussed in Chapter 4.

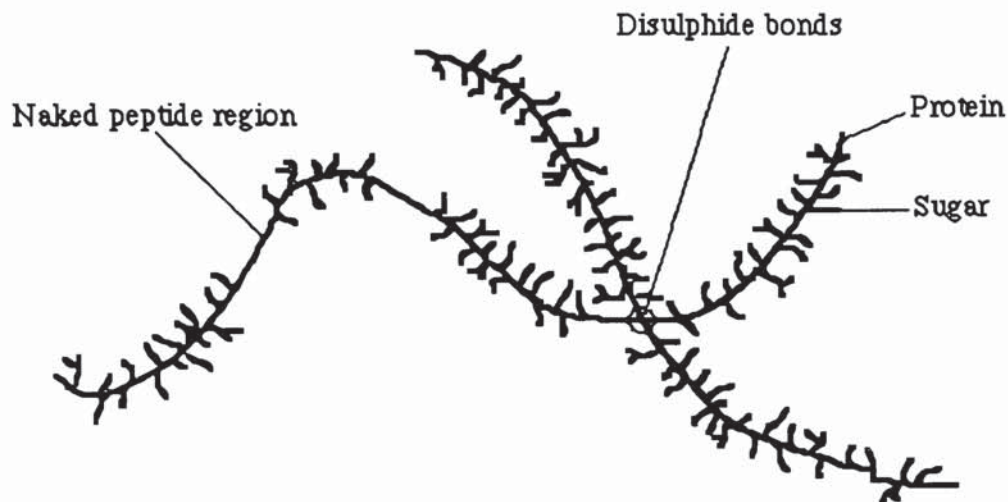


Figure 1.3 Structure of the main glycoprotein in mucus gel²⁹.

Table 1.1 summarises the various components found in the tear fluid and their sources.

Table 1.1 Components of the tears and their origins.

TEAR FLUID COMPONENT	ORIGIN
1. PROTEINS ^{4,17,21,18,5,30}	
Tear-Specific Prealbumin	Lacrimal Gland
Lysozyme, Lactoferrin.	Lacrimal Gland (In the secretory duct acinar cells.)
Transferrin	
Ceruloplasmin	Secreted from the serum by the conjunctiva and lacrimal glands.
Immunoglobulin A,	Modified and secreted from the serum
Immunoglobulin G	by the lacrimal glands. Tests using
Immunoglobulin E	immuno-fluorescence suggest local synthesis of IgA and IgG in plasma cells of the lacrimal gland.
2. GLYCOPROTEINS ^{18,5,30}	
Mucous	
(GP1 and GP2/GP3M)	Conjunctival goblet cells in acini of lacrimal glands.
3. ANTIPROTEINASES ^{18,5,30}	
α 1-Antitrypsin, α 2-M (Macroglobulin)	
α -1 - Antichymotrypsin	Lacrimal glands (or derived from plasma by a general increase in vascular permeability).
Prostaglandin F	
4. ENZYMES ^{18,5,30}	
Glycolytic, TCA Cycle Enzymes, including Lactate and Malate Dehydrogenases.	Corneal epithelium and Conjunctival secretion following irritation.
Lysosomal Enzymes, Plasminogen Activator, Angiotensin Converting Enzyme	Conjunctival secretions and the Lacrimal gland.
Peroxidase, Amylase	Lacrimal gland.
5. LIPIDS ^{4,17,25,26,30,31}	
Cholesterol Esters, Wax Esters, Triglycerides, Fatty Acids & Sterols.	Meibomian glands and glands of Moll and Zeis.

(Table 1.1 continued)

TEAR FLUID COMPONENT	ORIGIN
6. METABOLITES ^{18,5,30,32,33,34}	
Glucose, Lactate, Urea	Unrestricted passage across blood-tear barrier of conjunctival and corneal epithelium.
Histamine, Prostaglandins	Present in normal tears, suggesting passive entry through the blood-tear barrier and at elevated levels in inflammatory eye diseases (increase in vascular permeability).
7. ELECTROLYTES ^{18,5,30,32}	
Sodium	Passive secretion from blood.
Potassium	Active secretion.
Calcium	Present at lower concentrations.
Chloride, Carbonate	Unrestricted secretion from blood.

1.4 Tear Composition - Reliability and Self-Consistency of the Literature

The techniques used to identify and quantify the different protein components present in normal human tears include electrophoresis, immunochemistry and enzymatic assay. By far the most widely used analytical techniques are based on electrophoresis, but no single technique has been used on all proteins. Also, analyses are complicated by the fact that the collection methods used to obtain tears are often traumatic to the eye, resulting in reflex tearing, and it is now well established that different types of tear (e.g. basal, stimulated, emotional) have different compositions. For example, greater quantities of serum proteins such as albumin and immunoglobulins are present in tears collected by Schirmer filter strip, which causes reflex tearing, than are found in unstimulated tears

collected by microcapillary pipette. The Schirmer-strip method is believed to abrade the conjunctival tissue and cause leakage of serum proteins into the tear fluid. As would be expected if this is the case, a similar increase in serum proteins are found in tears of patients with inflammatory diseases²¹. Tear fluid collected following nasal stimulation with irritants such as ammonia gas or onion vapour also differs from nonstimulated (basal) tears^{35,36,37}. The major serum proteins, albumin, transferrin and IgG were shown to be present at significantly higher concentrations in nonstimulated tears³⁶.

There is some disagreement regarding additional factors affecting normal tear secretion⁹. Most workers (e.g. Zappia and Milder, 1972³⁸; Norn, 1965³⁹; Brandt et al., 1967⁴⁰; Furukawa and Polse, 1978¹⁰) find a decrease in secretion rate with age. Mishima et al. (1966)¹², on the other hand, and later Lamberts et al. (1979)⁴¹ and Arrata et al., (1982)⁴² who found no correlation of tear secretion with age, suggest that these findings could be related to older eyes becoming less sensitive to stimuli. Variations in tear secretion have also been attributed to eye colour, people with pale irides showing a higher secretion rate than those with darker irides⁴³, and to diurnal patterns: a higher tear secretion rate has been measured in the morning hours⁴⁴. Furthermore, significant differences in composition between the tear film in the closed and open eyes have been measured (Sack et al., 1992⁴⁵). The study by Sack and his co-workers⁴⁵ revealed a progression in composition from reflex-to-open-to-closed eye tear samples, with reflex tears and closed eye tears representing two opposite extremes both in terms of composition and origin. Total protein was found to increase from 6mg/ml (reflex) to 18mg/ml (closed eye), with individual protein variations indicating a different origin for these tear types. For example, while the concentrations of the major reflex tear proteins (lysozyme, lactoferrin, tear-

specific prealbumin) remain constant, the concentration of secretory IgA increases dramatically (from <0.23 for reflex tears, to 0.85 in the open eye, to 8.4mg/ml in the closed eye), and serum albumin also increases (from 0.02 to 0.06 to 1.1mg/ml). The authors attribute this variation to the cessation of reflex secretion upon eye closure, with a slower constitutive lacrimal flow composed almost exclusively of IgA. They postulate that the increase in albumin, which is secreted as a result of the subclinical inflammation of conjunctival tissue during eye closure, and IgA play a critical role in protecting the closed eye from pathogens⁴⁵. Presumably the majority of tear analyses have previously been carried out on tears obtained during the day, so the marked difference between daytime and nocturnal tears will not have impinged significantly on the reported values.

Other variations in particular tear component concentrations have been reported and attributed to age, sex, health and contact lens wear. The concentration of lysozyme and lactoferrin, for instance, show a gradual decline with age, while IgG tends to increase^{36,46}. Tear osmolarity, and the concentrations of some tear proteins, has been found to be higher in contact lens wearers than in non-wearers¹¹. Another factor not normally taken into account is the sex of the person contributing the tears. Tapaszto (1973)⁴⁷ reported significant differences between male and female subjects for a number of tear components, including some of the major tear proteins. Serum albumin in female tears was found at concentrations almost twice those occurring in tears from male subjects, while the concentration of lysozyme was somewhat lower in tears from females. Tapaszto also reported higher concentrations of lipids in the tears of women than of men⁴⁷. Age also exerts an influence, as mentioned above, as, of course, do eye infections and the general health of the person. Thus, the figures quoted in the literature for any particular protein

concentration are highly variable and not absolute.

The proteins lysozyme, lactoferrin and tear-specific prealbumin are secreted by both the main and accessory (Wolfring's glands and the glands of Krause) lacrimal glands, so their concentrations should remain reasonably constant in both stimulated and nonstimulated tears. Thus, the wide range of values quoted for the concentration of these proteins in normal tears must be largely due to the different sensitivities of alternative analytical tests. For example, enzymatic assays of lysozyme, which are the most commonly used technique to quantify this protein, may over estimate the concentration of this bacteriolytic enzyme since the tear fluid has been shown to contain factors which may enhance lysozyme activity. Gel electrophoretic methods, and other techniques which make use of stains such as Coomassie brilliant blue, make the assumption that the intensity of protein stain is independent of protein type, but this has been shown not to be the case. However, new, more sensitive analytical techniques are being developed. For example, capillary electrophoresis has several advantages over polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC), including its ability to rapidly detect tear proteins in nanolitre sample volumes, thereby eliminating the requirement for large tear volumes and reducing the tear variability arising from lacrimal stimulation⁴⁹. For the majority of tear proteins, however, their concentrations are reported to be greater in nonstimulated tears than in stimulated tears.

Table 1.2 lists the major tear components and the concentrations at which they occur in the tear fluid, taking average values resulting from a survey of over one hundred published reports.

Table 1.2 Concentrations of Some of the Major Components in Tears,
mg/100ml (Averages Derived from References 69-96)

Component	Average	Max. Value	Min. Value	No. of Ref.
	conc.	quoted.	quoted.	Sources.
Total Protein	786	960 ⁵²	652 ⁴⁷	9
Lysozyme	185.5	555 ³³	65 ³⁵	16
Albumin	130	390 ¹⁷	1.03 ⁵³	9
Tear-specific Prealbumin	123	184 ⁵¹	52 ⁴⁷	5
Lactoferrin	210.5	340 ³⁷	81 ⁵⁴	9
Ig A	29.0	85 ⁵⁵	4.1 ⁵⁶	17
Ig G	13.1	79 ⁵⁷	trace ^{55,57}	15
Ig M	1.39	5.0 ⁵⁸	0 ^{59,55,60}	9
Ig E	0.017	0.025 ⁶¹	0.003	3
Total Lipid	205.5	240 ⁴⁷	180 ⁶²	3
Cholesterol	64.3	190 ⁶³	8.0 ⁶⁴	4
Glucose	10.7	65 ⁶⁵	0 ^{66,61}	9
Ascorbic Acid	6.8	23 ⁶⁷	0.14 ⁶¹	2
Chloride	469.6	512.9 ⁶⁸	85 ³	15
Sodium	337.8	354 ⁴⁷	326.6 ⁶¹	7
Calcium	3.94	8.0 ³⁰	1.2 ³⁰	7
Potassium	101.4	137 ⁴⁷	58.7 ⁶¹	10

It should, again, be emphasized that the reliability of the values given in Table

1.2 is variable; while some tear components, such as lysozyme, have been well studied with at least ten reliable sources upon which to base the average, for others, such as immunoglobulin-E, it is necessary to rely upon only three measurements. As can be seen from Table 1.2, there is frequently a large range between the highest and lowest values quoted for the tear components. For example, values for lysozyme range from 65mg/100ml²¹ to 555mg/100ml³³. The source of these large errors lies in the collection methods and analytical techniques used. In Table 1.3 the different techniques used to estimate lysozyme concentration in tear fluid have been separated to illustrate the different values each obtains. Nevertheless, as Table 1.4a and 1.4b show, the ratios of some the proteins (serum albumin, lysozyme and lactoferrin) obtained in different studies remain fairly constant for tears collected atraumatically. For tears collected by the Schirmer strip method the concentration of serum albumin is dramatically increased⁵¹. It would, therefore, make sense to consider tear protein ratios in addition to actual concentration values when interpreting the importance of tear analyses. This would eliminate any differences in sensitivity between the various techniques used to quantify the tear components.

Also questionable is the interpretation of tear analyses. For instance, although lysozyme is widely assumed to be by far the most abundant tear protein, the range of values quoted by those who have analysed tears hardly supports this. Indeed, the fact that analytical studies of deposited lenses frequently show lysozyme as the major protein may, rather, reflect the strong adsorption of proteins such as lactoferrin to the lens surface, making them difficult to remove.

Table 1.3 Average lysozyme concentrations estimated for normal tears by different analytical techniques.

<u>Collection method</u>	<u>Analytical Technique</u>	<u>Lysozyme mg/100ml.</u>	<u>Ref.</u>
Microcapillary pipette:	Enzyme activity assay	555	33
basal tears	Enzyme activity assay	240	104
basal tears	Enzyme activity assay	188	106
basal tears	Enzyme activity assay	176	106
basal tears	Enzyme activity assay	330	36
basal tears	Enzyme-linked immuno-	330	36
Filter discs	sorbent assay (ELISA)	100	46
Microcapillary pipette:	SDS-PAGE +		
stimulated tears	Coomassie Brilliant Blue	201	51
	Immuno-turbidimetric		
basal tears	analysis	168	107
stimulated tears	Crossed immuno-	213	37
stimulated tears (filter-strip)	electrophoresis	150	11
basal tears		71	11
	Single radial immuno-		
basal tears	diffusion (SRID)	130	108

Table 1.4a. Concentration ratios of albumin, lysozyme and lactoferrin in tears analysed by different techniques.

<u>Serum albumin</u>	<u>Lysozyme</u>	<u>Lactoferrin</u>	<u>Method</u>	<u>Ref</u>
0.56	1.0	1.41	Crossed immunoelectrophoresis (Schirmer)	37
0.008	1.0	1.60	Crossed immunoelectrophoresis (stimulated tears)	37
0.12	1.0	1.11	SDS-PAGE (stimulated tears)	51

Table 1.4b. Concentration ratios of lysozyme and lactoferrin in tears.

<u>Lysozyme</u>	<u>Lactoferrin</u>	<u>Technique</u>	<u>Ref</u>
1.0	1.59	Crossed immunoelectrophoresis (naso-stimulated tears)	37
1.0	2.1	Crossed immunoelectrophoresis (basal tears)	35
1.0	1.4	ELISA (filter-disc collected tears)	46
1.0	1.1	SDS-PAGE (stimulated tears)	51
1.0	0.53	ELISA (basal tears)	36

In producing the average values for this report (shown in Table 1.2), the variable sensitivity of different techniques employed were ignored, although only values for "normal" and "healthy" and, where specified, unstimulated tears were used. Figure 1.4 uses these average concentrations, and values reported for serum, to compare the composition of human tears with serum. Overall, the concentration of protein is greater in serum than in tear fluid by a factor of 10, although the concentrations of individual proteins show varying degrees of difference. This may arise through the greater permeability of the conjunctiva to certain proteins and to the production and secretion of proteins by the lacrimal gland. The relative proportions of some of the major tear components are illustrated by the pie chart in Figure 1.5.

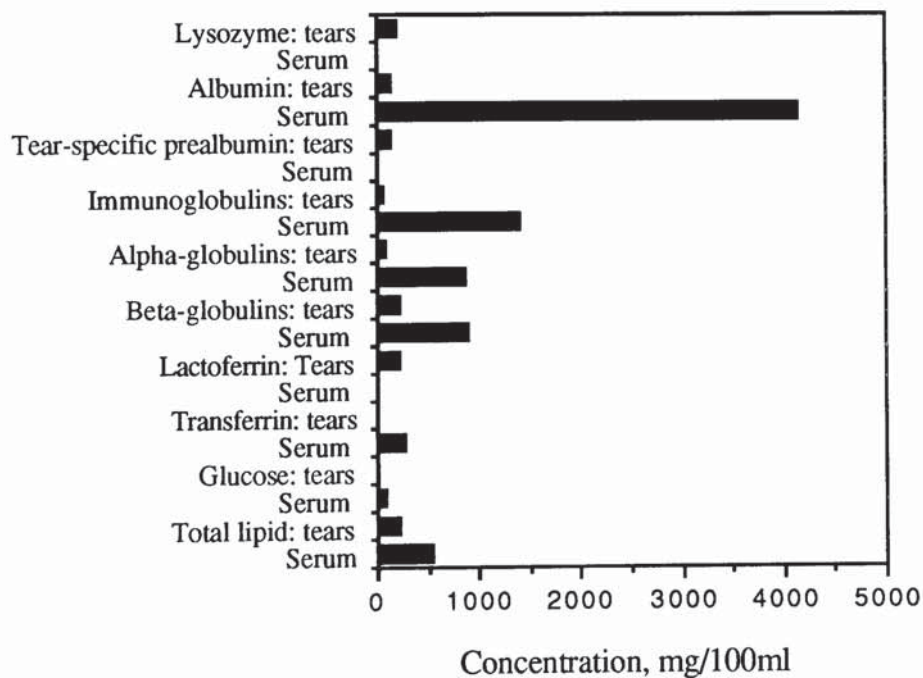


Figure 1.4 Concentrations of some of the major components present in human tears⁶⁹⁻⁹⁶ and serum^{59-61,69,70,84,86,93,97-103}.

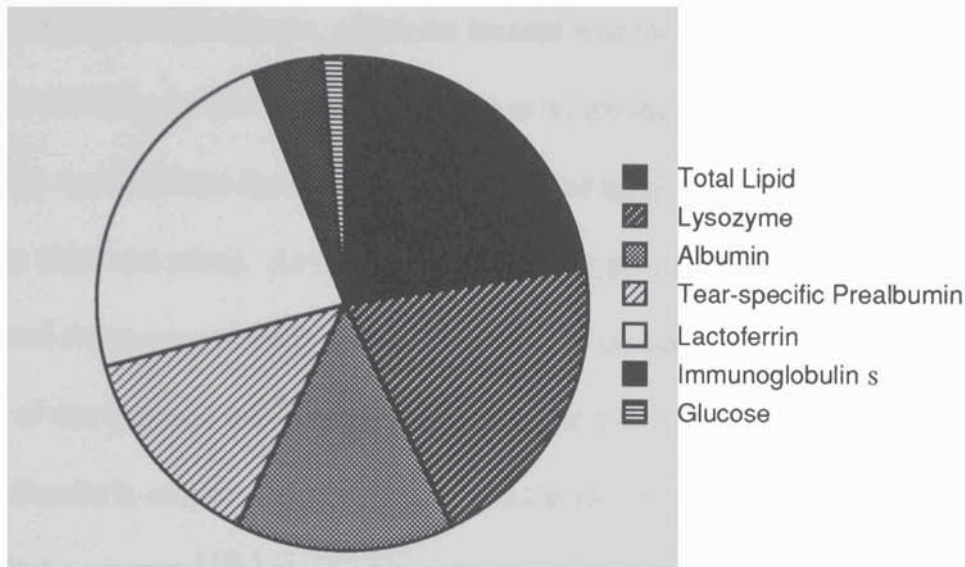


Figure 1.5 Relative concentrations of some of the major tear components.

1.5 Fate of the Tears

The excretory apparatus, responsible for tear drainage, consists of the puncta, the lacrimal duct, the lacrimal sac, and the nasolacrimal duct. When considering instilling a solution into the eye, particularly if- it is a drug together with drug-carrier, it is important to establish where it is likely to end up so that any systemic effects can be foreseen.

Normal tear volume is between about 7.0 and 10.0 μ l^{8,9,10}, and there is a continual secretion (nonreflex) rate of about 1 μ lmin⁻¹ (0.5 - 2.2 μ lmin⁻¹)¹¹ from the accessory lacrimal glands¹². To prevent a progressive increase in tear fluid volume, tears must exit the eye at a similar rate.

Most of the movement of tears occurs in the lacrimal river, along the eyelid

margins, with the general movement being from the upper outer quadrant of the eye across the surface down to the medial aspect of the eye³. Excess tear fluid drains out through the superior and inferior puncta, which are located near the medial canthus, into the canaliculi. These normally join in a common duct from which the fluid enters the lacrimal sac, drains into the nasolacrimal duct leading to the inferior nasal meatus and, finally, to the valve of Hasna (lacrimal plica). As blinks occur, tears are propelled along the superior and inferior lacrimal rivers toward the puncta by the action of the obicularis muscle^{3,109}. An estimated 75% of the exposed tear film occurs in this tear meniscus⁴. Some evidence suggests that the obicularis causes a contraction of the canaliculi and possibly the nasolacrimal sac, causing a vacuum^{110,111,112,113}. Key experiments were performed between 1917 and 1929 by both Fraberg and Rosengren in which the lacrimal sac and the canaliculi were cannulated from both the punctal and nasal ends, and direct measurements made of the pressure changes occurring during a blink. A substantial pressure rise occurred in the canaliculi and, to a lesser extent, in the lacrimal sac during the closing phase of a blink. A negative pressure was created as the lids opened, with the suction lasting up to several seconds after the lids started to open. This early hypothesis, that tears are drawn through the punctal openings into the canaliculi during the opening phase of the blink is supported by high-speed photography¹¹⁴. If carbon granules are used as a marker in the tear film, a pulse-like flow of tear fluid is seen to enter the puncta for 1-2 seconds as the lids open. The amount of fluid drained is proportional to the strength and completeness of the blink. The process of tear drainage is summarised below:

1. Upper lid makes its downward movement; lower lid moves in a horizontal nasal direction, often dropping by 1-2 mm.
2. Inferior nasal tear strip also moves nasally, with the lower lid, resulting in some fluid being deposited in the region of the medial canthus and near to the punctal openings.

3. The nasal corners of the lid margins meet before the upper lid is halfway to being fully closed so that any further closure acts to squeeze the walls of the canaliculi and the lacrimal sac.
4. There may be a concurrent constriction of the proximal portion of the canaliculi near the punctal openings which helps prevent any significant reflux of tear fluid from inside the system.
5. Tear fluid is forced down the canaliculi, then into and through the lacrimal sac^{109,115}. Much of the fluid is absorbed by the epithelial lining of the lacrimal sac. Any fluid remaining drains down the nasolacrimal canal, which extends inferiorly from the bottom of the sac and opens into the nasal cavity.
6. Complete blinks result in 1-2 μ l being drained per blink. Since the nonreflex secretion of tear fluid is approximately 1 μ l per minute, and the average blink rate is 14 per minute, the drainage system is capable of handling 14-28 μ l per minute at normal blink rates. As the secretion rate is only 1 μ l per minute, and evaporation between blinks accounts for about 7% of the tear film, the drainage channels operate nearly empty most of the time^{109,113,116}.
7. After the lids open sufficiently to expose the puncta, the suction developed by the expanding canaliculi draws in the fluid from the lacrimal river in preparation for the positive pressure of the next lid closure to force the fluid inwards¹⁰⁹.
8. It has been suggested that there must be a valve-like structure at or near the junction of the common canaliculus and lacrimal sac in order to prevent air and fluid entering the canaliculi from the nasal cavity. Strong evidence to support this has been obtained from direct measurement of pressure differentials between the lacrimal sac and canaliculi, and from X-ray scintillography where the isotope technetium was mixed in the tear fluid and

observed as it passed down the drainage pathway¹¹⁷. A small positive pressure is required to drive the fluid inward through the valve, whereas even the generation of a high pressure within the lacrimal sac and nose (e.g. by sneezing) is not able to force air or fluid back into the canaliculi.

Although both superior and inferior puncta are important in drainage, should one become occluded, the remaining punctum is sufficient under normal conditions. In fact, drainage through the puncta is negligible under normal circumstances since the absorption rate of fluid by the conjunctiva can reach 2 μ l per minute and can, therefore, lead to a steady state. In an experiment performed by Vidic and colleagues, the commonly held belief that the upper lacrimal duct plays a subordinate role was disproved. In the test, 40 eyes were studied following artificial occlusion of the upper, and then the lower, lacrimal ducts. Schirmer's No.1 test revealed that higher quantities of fluid were discharged from the upper ducts of 17 eyes, and from the lower ducts of 15. In 8 eyes there was an equal discharge¹¹⁸.

1.6 Tear Distribution

There is a general movement of tears from the upper outer quadrant of the eye across the surface down to the medial aspect of the eye. As described in the previous section, most of this movement occurs in the lacrimal river (along the lid margins), with the general medial movement being propelled by the blink mechanism³. The eyelids and tear meniscus (prism) adjacent to the edges of the lid ensure an even distribution of tear fluid over the cornea, with tear flow occurring only in the meniscus. The eyelids will also act to deposit materials, particularly mucin, on the surface of any biomaterial present in the

preocular tear film. This is of importance in the development of sustained drug delivery systems as will be discussed below.

1.7 Tear Film Stability and Interaction with the Cornea

The mucin component which forms the bottom layer of the tear film, adjacent to the corneal epithelium, is thought to be the principal factor in spreading the precorneal tear film¹⁰⁹. The mucin acts as a wetting agent, and serves as a bridge between the hydrophobic surface of the outermost epithelial cell layer and the tear film's middle aqueous layer. The strong effects of surface tension allow the tear film to maintain itself in a vertical plain. There is no significant flow of fluid along the cornea, and the film has been suggested to have a stable gel-like nature¹¹⁴.

1.7.1 Contribution of Mucin to Tear Film Stability

It appears that the temporary stability of aqueous films, such as that of the tear film, over an epithelial surface is achieved by coating the superficial epithelium with a thin layer of lipids having low polarity. On opening the eye, lipid secreted by the meibomian glands at the lid margins spreads directly on the freshly-swept aqueous surface and covers the exposed precorneal film before the surface can be contaminated by surface active macromolecules (principally mucus glycoproteins). An interaction then takes place between mucus dissolved in the aqueous tears and the polar groups of the innermost lipid layer. A glycoprotein-lipid interaction at the lipid-aqueous layer boundary will increase the lipid film pressure and thus enhance the stability of the tear film¹¹⁹. It has been demonstrated that mucin and other proteins dissolved in the aqueous layer of the tear film are capable of increasing the film pressure of the superficial lipid layer threefold¹¹⁹, so that

the surface tension of the tear film will be under 40mNm^{-1} . The consequent reduction in surface free energy, achieved firstly by the spreading lipid and, secondly, by the formation of interfacial complexes, is believed to make a significant contribution to the overall stability of the tear film²⁵.

It has been suggested that, in addition to increasing lipid film pressure, the primary role of the conjunctival glycoproteins in the eye is to transform the low-energy corneal surface into a higher energy surface via adsorption. Mucin is more surface active than simple proteins such as albumin or serum globulins, and mucin molecules are capable of lowering the water-interfacial tension by providing abundant sites for hydrogen-bonding¹¹⁹. These combined effects are believed to be sufficient, and essential, to achieve the complete wetting of the corneal surface². The properties of mucin are summarised in Table 1.5.

Table 1.5 Properties of mucin.¹¹⁹

PROPERTY	MUCIN
Molecular weight	High, 10^5 - 6×10^6 gmol ⁻¹
Hydrophilicity	Hydrophilic
Micelle formation	No
<u>Effect on:</u>	
water tension:	Decreases to 38mNm ⁻¹
water-oil interfacial tension:	Decreases to 15mNm ⁻¹
superficial lipid layer	Increases film pressure from 10 to 30 mNm ⁻¹
Integrity of cellular surface	No effect

Many workers have used Lemp and Holly's¹²⁰ measurements of the critical surface tension of the corneal surface as evidence that an adherent layer of mucin is required for tear film stability. Low values (28mNm⁻¹) for the critical surface tension were obtained for excised rabbit corneas which had been wiped clean. Measured values for the critical surface tension of the corneal epithelium are shown in Table 1.6:^{119,121}

Table 1.6 Effects of mucin on the critical surface tension of corneal epithelium

<u>Epithelial surface</u>	<u>Critical surface tension</u>
Mucin-coated	38mNm ⁻¹
Clean cornea (no excess mucin)	28-31mNm ⁻¹
Completely mucin free	28mNm ⁻¹
<u>(endothelial surface)</u>	

Changes in the nature of the epithelial surface or its mucous covering unbalance the surface-chemical equilibrium established by the surface lipid spreading and the interaction of macromolecules with polar lipids. The effects of such changes may be exemplified by clinical problems such as dry eye, where a relatively short tear break up time occurs. At present, tear break up time (BUT) is the only clinical measurement of tear film stability¹²². Break-up is in part resisted by the viscosity conferred on the film by its soluble mucous content. The composition of the tear film may in turn be affected by changes in the output of proteins and electrolytes by the lacrimal gland, by leakage of serum components from conjunctival vessels, or by the products of commensal or invasive micro-organisms¹²².

The role of mucin in stabilising the tear film by its interaction with the corneal epithelium is of central importance to the production of improved biomaterials. If a biomaterial can be developed which is muco-adhesive in the presence of the tear fluid, it will be retained for extended periods of time at the corneal surface. Thus, if harnessed to a

drug while maintaining this characteristic, it would allow sustained exposure of the eye to the drug. This would increase the efficiency of many areas of ocular therapy.

1.7.2 Relationship of Mucin to the Corneal and Conjunctival Epithelia.

Work which has thrown light on the properties of the corneal and conjunctival surfaces, and on the nature of the mucus layer, are discussed below.

The corneal epithelium is the most regularly arranged of all squamous epithelia in the body. This is a stratified epithelium mounted on a fine basement membrane. Columnar basal cells lie next to the basement membrane. Mounted on these are 2 to 3 rows of smaller interlocked wing or umbrellar cells. With increased displacement from the basal cells the wing cells become thinner and are capped by 2 or 3 layers of surface squamous cells. In all, the epithelial layer is around 70 μ m thick and has a turnover rate of about 7 days in man¹²⁸.

Microvilli and microplicae, 0.5-1.0 μ m long and 0.15 μ m wide occur in random reticular or corrugated pattern. However, the older desquamating cells have lost this membrane specialisation and, with it, the ability to retain the tear film¹²⁹.

Mucous exists in solution in the tear film as clusters of granules, as granular sheets, and as strands, the latter often arranged as a network. Mucous strands are common to epithelial mucous from various sites, but the mode of strand formation is obscure. In the conjunctiva, the presence of a surface mucous layer, the tendency for strands to form at the margins of mucous sheets, and the shearing effect of blinking can be combined in the

suggestion that strands are formed by the rolling up of a surface layer as a result of shearing forces. If the mucous layer is more adherent to the goblet cells than the epithelial cells, the strands would tend to remain interconnected at such areas of adherence, thus forming a network. The absence of significant strand formation on the corneal surface is explained by the immediate removal of any strands formed during a blink by their adhesion to the mucous network of the undersurface of the upper lid during the same blink. The absence of both clusters of granules and mucous network from the corneal surface may be explained by the absence of corneal goblet cells. A relatively loosely adherent surface mucous layer simultaneously removed (by strand formation) and replaced (by fresh mucous from the under surface of the lids) during each blink would represent an effective system for the removal from the ocular surface of desquamated cells and cell debris, foreign and infective particles, and lipid-contaminated mucous, and would also allow for the maintenance of a continuous precocular tear film¹²⁹.

Nichols et al.²⁸ used tannic acid staining to show a mucous layer of 0.8 μ m thick on the cornea and 1.4 μ m thick on the conjunctiva. These values are greater than those most frequently quoted (0.02-0.05 μ m), although Creeth et al. used a similar method to obtain figures of 0.6-1.0 μ m and 2.0-7.0 μ m for the thickness of the mucin layer over the cornea and conjunctiva, respectively. Prydal (1989)⁶ gives an even greater for the thickness of mucin over the ocular surface. Using a non-invasive technique to estimate total tear thickness, a mucus layer at least 30 μ m thick was indicated.

In the study by Nichols et al., the glycocalyx, to which the mucous is theoretically bound, was shown to extend 0.3 μ m from the microvilli tips into the region

occupied by the mucous in the tear film. The mucous was shown to extend down to the tips of the microvilli and therefore occupy the same area as the glycocalyx, thus indicating an interaction between the carbohydrate groups on the glycoprotein and the oligosaccharides of the glycocalyx. Dilly also used silver granule staining to show chains and clumps of mucous. The sites of adhesion were found to be most concentrated on the microvilli. Transverse sections of the microvilli showed that the filaments radiate outwards to form a complete corona that can extend outwards a micrometer or more. The long mucoprotein chains that are anchored to the cell membrane appear to extend in amongst the mucous that is secreted from the goblet cells. Thus, these mucoprotein anchor sites would seem to be the physical attachment of the mucous to the underlying epithelial cell membranes. Using Thiery's method and Ruthenium red to stain the mucoprotein, Dilly showed that the anchor site distribution in the cell membrane is of a similar dimension to that of the stained patches within subsurface vesicles observed in conjunctival epithelial cells. Since subsurface vesicles can often be seen fusing with the cell membrane above them, the role of the vesicles may be to present membrane-anchored mucoprotein to the cell surface. The mucus secreted by the goblet cells may bond to the conjunctival epithelium by interacting with the secretions of the subsurface vesicles.

Any foreign material which is introduced into the tear film will disrupt the balance of the tear components by interacting with particular components. An example is the build up of protein and lipid deposits on soft contact lenses. That these destabilise the tear film is apparent from the discomfort and reduced toleration often experienced by wearers of spoiled lenses. A greater understanding of the processes involved would facilitate the production of improved biomaterials, showing greater biocompatibility, which do not destabilize the tear film. The properties required differ according to the intended

uses of the biomaterial. For ocular drug delivery systems, for instance, the drug carrier would ideally interact specifically with the mucin at the corneal surface in order to extend drug residence time and thereby improve the efficiency of eye medication. For contact lens materials, on the other hand, the materials which show least interaction with tear proteins and lipids have been selected, since deposits reduce visual clarity of the lens in addition to destabilising the tear film and causing discomfort. It is possible, however, that a material which maintains an adherent layer of mucin on its surface might create the ideal contact lens. This conjecture is based on the observation that the mucin-coated corneal surface resists contamination by other tear components, making it reasonable to assume that a mucoadhesive contact lens may mimic this corneal property.

Ocular drug delivery systems, their efficacy, and their implications for tear film stability are discussed the following section.

1.8 Topical Ocular Drug Delivery.

Topically applied ophthalmic drugs are in concurrent contact with three absorptive membranes - the cornea, the conjunctiva, and the nasal mucosa - resulting in both systemic and ocular absorption. To date, few advances have been made in minimising the ratio of systemic to ocular absorption of topical drugs; typically, 10% or less of a topical dose is absorbed into the eye, with the remaining 90% available for entry into the bloodstream where it may exert systemic side-effects. However, the design of ocular drug delivery systems is becoming more sophisticated due both to the increasing understanding of the constraints imposed by the eye, and to the development of polymers which encompass a wide range of properties, particularly biodegradability and bioadhesiveness.

The constraints in topical ocular drug delivery include the highly selective permeability of the cornea and diversion of foreign chemicals to the systemic circulation via the conjunctiva, solution drainage, tear turnover and lacrimation. The tendency of the eye to reduce instilled fluid volumes in excess of 7-10 μ l by drainage, in particular, considerably limits the performance of liquid dosage forms such as solutions, suspensions, and liposomes. This has led to the recent explosion of interest in methods aimed at increasing the residence time of ocular drugs. These have concentrated largely on reducing solution drainage by increasing formulation viscosity, and on developing mucoadhesive drug-carrying polymers which will interact with the mucus layer covering the ocular surface. The efficacy of the different systems of ocular drug delivery are discussed below. Other approaches to enhancing ocular drug therapy are to increase corneal permeability by either modifying the structure of the drug^{130,132}, or by transiently altering the integrity of the cornea¹³³. However, these are outside the scope of this project and will not be discussed further.

1.8.1 Conventional Ocular Drug Delivery Systems

Administration of drugs to the anterior surface of the eye is most commonly accomplished by periodic application of liquid drops of solution directly from a squeeze-bottle. Both the concentration and duration of exposure of a medication in the eye are determined by the character and dynamics of the precocular tear film. Topical applications of an ophthalmic solution to the eye in a conventional manner result in extensive loss of therapeutic agents by drainage through the puncta¹³⁴. Reasons for this rapid loss of therapeutic agent are primarily the reactions induced by the instillation of eye drops, namely reflex tearing, blinking and lacrimal drainage. Conventional droplet administration of

timolol, for instance, results in 80% of the drug being absorbed into the systemic circulation via the vascularised nasal mucosa¹³⁵. Tear turnover has been estimated to be about 16% under normal physiological conditions, but this increases several fold upon stimulation¹³⁰, such as may arise by introducing ophthalmic solutions, particularly if they are non-isotonic and at a physiological pH. This method of drug delivery is, therefore, only effective and practical when small quantities of material are sufficient to treat the problem since instilled volumes in excess of 7 to 10 μl usually disappear from the tear film within 1 to 2 minutes in humans¹³⁰. The drug levels reach a maximum quickly, and then fall rapidly to a minimum, when further administration becomes necessary. If these concentration maxima and minima fall above or below the toxic level or minimum effective level, respectively, alternating periods of toxicity or inefficiency can result.

Studies have shown that under normal circumstances the half-time of residence ranges from 10 seconds to 4 minutes, depending on the degree of reflex tearing elicited¹³⁶. On average, the concentration has been shown to drop to around half of the applied topical dose in one minute, with the level decreasing to one-thousandth of the initial value within 8 minutes⁶⁷. Further investigations have suggested that the elimination rate is also dependent on the droplet size instilled. The rate of elimination is insensitive to the droplet size over a range of 1 to 10 μl , and sometimes to 20 μl ¹³⁴. However, conventional ophthalmic drug applicators dispense droplets averaging about 50 μl ⁸. A drop this size can displace most of the tear film, since the maximum volume of fluid held by the cul-de-sac is 20 to 30 μl , washing away the surface oils and hence reducing BUT.

Ingredients mainly associated with the bulk of the solution, including water,

will stay in the palpebral fissure for only a short time. On the other hand, surface-active ingredients that tend to be adsorbed on the epithelial surface or at the lipid/aqueous interface, or in both places, stay in the eye for a much longer time. This time depends on the degree of distribution of the material between the interface and the bulk, and on the concentration dependence of this distribution coefficient. A surface-active substance can exist at a much higher concentration at the interface than in the bulk and yet be undetectable even by sophisticated microanalysis. Often the presence of such substances can only be surmised through their effect on interfacial tension or film stability¹³⁶.

1.8.2 Viscous Ophthalmic Vehicles

The viscosity of eye drops is often increased by the addition of water-soluble polymers in order to extend their residence time in the eye. Polymers used for this purpose include dextran, polyvinyl alcohol (PVA), hydroxypropylmethyl cellulose (HPMC), hydroxypropyl cellulose (HPC), hydroxyethyl cellulose (HEC), and hyaluronic acid (HA). The use of polymers such as hydroxypropylmethyl cellulose and polyvinyl alcohol as vehicles for instilled substances increases their duration of residence within the tear film¹³⁷. Three mechanisms by which polymer solutions may achieve this effect are:

- i) By increasing the volume of fluid available in the marginal tear strip.
- ii) By adsorption of polymers at either the corneal/aqueous or the mucin/aqueous interface.
- iii) By a film of polymer solution at the air/tear interface supporting a layer of water beneath it or by this film dragging a layer of water along with it as it spreads out over the ocular surface with each blink.

An example of viscous ophthalmic solution is HealonTM, an artificial tear

replacement, which contains 1% hyaluronic acid. This solution is widely used by people suffering from dry eye since it is fairly effective in increasing BUT. Hyaluronic acid has also been used successfully to increase the area under the miosis-time curve and the duration of miosis (contraction of the pupil) when added to solutions of the glaucoma treatment drug, pilocarpine. This effect has been related to its capacity to retain water, its pseudoplastic behaviour and, possibly, adhesion to the epithelial cell surface ¹³⁸. The effect of sodium hyaluronate concentration on half-time of residence of solutions in the tear film is shown in Table 1.7¹³⁹.

Table 1.7 Effect of Na-hyaluronate on solution residence time in tear film.

<u>Solution</u>	<u>Half-time of residence (min.)</u>	<u>Tear film type</u>
Buffered saline	0.83 +/- 0.9	KCS
	0.98 +/- 1.1	Normal
0.2% Na-hyaluronate	11.1 +/- 9.7	KCS
	5.2 +/- 5.4	Normal
0.3% Na-hyaluronate	23.5 +/- 15.6	KCS
	12.5 +/- 17.5	Normal

In Table 1.7, KCS refers to keratoconjunctivitis sicca, a disease characterised by diminished tear volume and elevated tear osmolarity. The difference in retention time between normal and KCS eyes underlines the importance of taking the targetted tear film characteristics into account when assessing the efficacy of ophthalmic solutions. In this case, the improved solution performance in the diseased eyes may arise because of the

smaller tear volume initially present (4.8 μ l versus 6.5 μ l in normal eyes¹⁴⁰). The excess volume, normally rapidly expelled by the drainage system described above, is less in the eyes with KCS.

Low viscosity solutions (2.5 mPa.s) cause a significant decrease in tear elimination coefficient over that of phosphate buffer solution¹⁴¹. Residence time is further increased as viscosity is increased to 7 mPa.s, but increasing viscosity to 25 mPa.s results in only a small decrease in the tear elimination coefficient, k. There is no linear relationship between viscosity and elimination rate, and optimal viscosities of artificial tear solutions are reported to be 1.2-1.5 mPa.s¹³⁹. Solutions with viscosities greater than 20 cP (2 Pa.s) are uncomfortable, probably because they increase shear forces during blinking. In addition, highly viscous solutions have been reported to cause a blurring of vision when an excessively thick, uneven precorneal film is formed, and can also produce deposits along the lid margin¹⁴². Properties other than viscosity also affect the efficacy of this type of formulation. Dropsize has been mentioned, but solution temperature and pH are other factors to consider. For example, Mishima and colleagues¹² stated that the tear turnover rate in the eye could increase to 100% per minute or more with any minor irritation, and recommended that eye drops be warmed to body temperature before instillation. The slight acidity is another source of minor irritation, and this can cause some commercial preparations to stimulate tear flow and hasten wash-out. Liquifilm, for instance, containing 1.4% PVA, is supposedly pH 6 but may actually have a pH as low as 4-5¹⁴².

Dextran and HEC which reduce surface tension of the vehicle only slightly are

best tolerated by the eye since they mix easily with the lacrimal fluid and do not cause irritation. The reduced surface tension of solutions of PVA, HPMC and HPC, on the other hand, does not seem to facilitate mixing with the resident tear fluid, and destabilisation and irritation is caused for the first 15 to 60 seconds following instillation ¹⁴¹.

Viscous drug vehicles are intended to increase drug availability through maintaining a greater concentration of drug per unit area at the conjunctival and epithelial surfaces. A major disadvantage with this system is that the conjunctiva makes up about 90% of the ocular surface and, since it is vascularised, most of the drug is lost to the systemic circulation ¹⁴¹. Ocular absorption of lipophilic drugs with an n-octanol/buffer partition coefficient of greater than 10 was found not to be increased significantly by increasing vehicle viscosity from 1 to 90 cps¹⁴³. This is explained by the ease with which the more lipophilic drugs already partition into the corneal epithelium so that a transient increase in the residence time of a solution would have little effect.

1.8.3 Aqueous Suspensions

Aqueous suspensions of relatively insoluble drugs, such as anti-inflammatory steroids, have been shown to be more effective than solutions of the soluble counterparts. For example, this is the case for a suspension of dexamethasone acetate versus a solution of dexamethasone sodium phosphate. This effect may be explained by the aqueous vehicle becoming a saturated solution of the therapeutic agent. Instillation results in an initial rapid delivery of dissolved drug followed by a prolonged slower delivery as the entrapped drug particles are dissolved. A major draw back with this system is that the suspensions tend to precipitate and require resuspension to provide an accurate dose ¹⁴².

1.8.4 Ointments

Simple ointments consist of an oil-soluble drug dissolved in an ointment base. The most common are petrolate bases and mineral oil, with or without hydrophilic lipids such as lanolin or polyethylene glycols. Petrolates are inert and do not appear to be absorbed across epithelial surfaces. Ideally, an ointment should become a liquid with low viscosity at the temperature of the eye in order to spread readily as a thin film on the surface of the tear film. Drug particles are then brought to the ointment-tear interface by the shearing action of the lids. The peak concentration of drug in the eye resulting from a drug-in-ointment suspension is similar to that from a drug-in-aqueous suspension, but the duration is longer. Ointments and drugs suspended in oil, for instance, while remaining in the precorneal tear film for relatively long periods by forming drug depots in the conjunctival cul-de-sac and associating with the epithelial surfaces, rapidly diminish BUT¹⁴⁴. The PTF breaks up after a few seconds leading to damage of the deep mucous layer and underlying epithelial cells.

Generally, only water-in-oil emulsions are used since oil-in-water types tend to be irritants. Each drug-filled aqueous droplet is a concentrated solution that is brought to the ointment-tear interface by the shearing action of the lids. The free drug is then able to mix with the aqueous portion of the tears. Disadvantages with this system include the initial delay in drug delivery following instillation, reduction in tear film stability and difficulty in achieving accurate dosages. Ointments are often unstable at high temperatures and may precipitate or phase separate ¹⁴².

1.8.5 Controlled-Release Drug Delivery Systems

In a controlled release system, the active agent is incorporated into a carrier, which is generally a polymeric material. The rate of release of the drug is determined by properties of the polymer and is only slightly dependent on environmental conditions, in complete contrast to the more conventional drug delivery systems described above. While conventional drug formulations lead to concentration fluxes between maxima and minima in the tear fluid, controlled-release systems aim to maintain the drug concentration at an effective level with a single dose. Ideally, the drug is released continuously in a fixed, predetermined pattern for fixed time period, so that, in total, smaller dosages are required.

Controlled-release formulations were first considered for use in the medical field during the 1960s and, by the 1970s, controlled release formulations were designed for large molecular weight drugs such as polypeptides. There are four main categories of controlled-release systems:

- i) diffusion-controlled: reservoirs (in this system, polymeric membranes separate a core of therapeutic agent from the biological environment. E.g. microcapsules, hollow fibres, liposomes), and matrices (here, the bioactive agent is dispersed or dissolved into the polymer phase);
- ii) chemically-controlled: erodible inserts, pendant chain systems;
- iii) solvent-activated: drug release is controlled by osmotic pressure;
- iv) magnetically-controlled systems.

Most ophthalmic applications are diffusion-controlled, and some of these systems are outlined below.

1.8.6 Matrices

The most successful and widely-used drug matrix is the ocular therapeutic system, Ocusert™, designed to release pilocarpine to control intraocular pressure. This system was patented as Ocusert Pilo-20 and Ocusert Pilo-40, and marketed in 1974 in U.S.A., 1975 in W.Germany, and in 1976 in the Netherlands and Switzerland. The action of this delivery method depends on a drug-containing reservoir located between two synthetic rate-controlling polymer membranes consisting of an ethylene-vinyl acetate copolymer. The excess drug, as a saturated solution, within the reservoir maintains a constant release rate due to diffusion across a concentration gradient. Pilo-20, for example releases pilocarpine at a rate of 20µg/hr for 7 days, with 3.4mg drug in total being released from a reservoir of 5mg. This is equivalent to instilling a 2% solution 4 times daily. Pilocarpine base, rather than pilocarpine-HCl, is used since it has both hydro- and lipophilic properties¹⁴⁵. A new ophthalmic delivery system (NODS) has been designed by Smith and Nephew. In this system, a water-soluble PVA film dissolves in the tear film to release a "flag" (22 mm², 20µ thick) within which the drug is dispersed. This flag hydrates and the drug diffuses into the tear film. It has been reported to achieve an 8-fold greater bioavailability of pilocarpine compared to conventional eyedrop delivery¹⁴⁶.

Although largely replaced by conjunctival inserts, contact lenses may be used as therapeutic systems to treat epithelial defects, particularly since they act as a shield to protect the surface of the eye from the abrasive lid action. Uptake and elution rate of a drug varies depending on membrane pore size, drug concentration and molecular size, and the ocular environmental conditions. Nevertheless, Kaufman et al.¹⁴⁷ reported that a soft lens combined with 1% Pilocarpine drops was more effective in lowering intraocular pressure

(I.O.P.) than 8% eyedrops alone¹⁴¹. A soft lens soaked in a hypertonic solution can be used to treat corneal oedema since it will reclaim liquids from the corneal tissue¹⁴⁸. More recently, Leshner and Gunderson¹⁴⁹ report that ciprofloxacin-HCl may be maintained at therapeutic levels in the tear film for longer periods if released from a high water content disposable contact lens than can be attained with conventional drops¹⁴⁹.

A different form of ophthalmic disc is used in U.S.S.R. to deliver antibiotics (e.g. Neomycin), antivirals (e.g. Florenal), and alkaloids (e.g. Pi-HCl). The polymer discs are made by evaporating a 10% polyacrylamide + 1.5% plasticizer (oligoether) solution together with the drug. The final water content of the membrane carrier is 5-7%. A constant drug delivery for 24 hours is maintained when the disc is inserted beneath the eyelid¹⁴⁵.

Other ophthalmic insert systems of drug delivery being developed include a soluble drug insert (SODI), made from acrylamide, vinyl pyrrolidone, and ethacrylate, which dissolves in the tear film while releasing 2.7mg of pilocarpine-HCl daily, and degradable films made from collagen¹⁵⁰ or chitosan¹⁵¹.

1.8.7 Nanoparticulate and Liposomal Systems

Nanoparticles are colloidal particles, 10 to 1000 nm in diameter, in which the drug may be entrapped, encapsulated, and/or adsorbed. Alkyl cyanoacrylates such as polymethyl methacrylate (PMMA), lipophilic polybutylcyanoacrylate (PBCA) or polyhexylcyanoacrylate (PHCA), are used to produce nanoparticles. An average sized particle of 300nm may accommodate up to 40% w/w solid content¹⁵². In an experiment

polyalkyl-2-cyanoacrylates were labelled with carbon-14¹⁹⁴. The half-life of clearance of the particles was measured as 15-20 minutes, comparing favourably with a half-life of clearance of less than 2 minutes for aqueous solutions. Approximately 1% of the instilled nanoparticles was estimated to adhere to the conjunctiva and cornea, with 0.1% of the initial volume still associated with the corneal surface after 6 hours. The corresponding figure for the conjunctival surface was 0.6%¹⁹⁴. Aqueous nanoparticulate formulations of pilocarpine salt have been made, but the buffer system, electrolyte concentration, pH and temperature all effect the physical characteristics of the final product. Of the polymers investigated, only cellulose acetate hydrogen phthalate (CAP) shows sufficiently low buffer capacity to gel in the eye. CAP starts to dissolve at pH5 and rapidly coagulates once placed in the cul-de-sac of the eye (pH7.2). In this way it forms a microreservoir of drug which cannot be washed away¹⁵².

Liposomes are microscopic membrane vesicles made from phospholipids. Although large liposomes (and probably all liposomes) are prevented from crossing into the anterior chamber as long as the corneal epithelium is intact, topical application of an agent that does not require corneal penetration may prove to be beneficial if retention of liposomes on the corneal surface can be sufficiently prolonged. In most cases, however, the mechanical movement of the tear layer by blinking leads to poor retention of liposomes on the corneal surface¹⁵³.

Positively charged liposomes have shown increased retention times on the cornea¹⁸¹. These are prepared by covalently linking amino acid molecules to an amphipathic lipid such as phosphatidyl - ethanolamine (PE) or cholesterol. Amphipathic lipids are characterised by a long hydrophobic tail and a polar head group which may be

charged, and possess the ability to form both hydrophilic and hydrophobic interactions within the molecule. A hydrophobic drug may be added to the resulting amphipathic lipid, and if this is suspended in aqueous solution, with a high water:lipid ratio, membrane-bound vesicles are produced. The positively-charged amino acid groups are exposed at the surface to the polar solvent, while the drug is enclosed within the membrane.

Experimental work, which involved monitoring the retention of a ^{125}I -PE tracer incorporated into liposomes, indicates that retention times are dependent on the charge density and rigidity of the lipid bilayers¹⁵⁴. If liposomes formed with these charged analogues in the lipid layer could be formulated to have predictable and desirable rates of drug release *in vivo* (i.e. while bound to the corneal surface), then a biodegradable slow-release formulation might be possible. As yet, however, no successful preparation of this type has been shown.

A major draw back of these reservoir systems is that water-soluble drugs may be discharged from the vehicle more rapidly than the nanoparticles or liposomes are cleared from the cornea¹³⁰.

1.9 Mucoadhesive Polymers For Drug Delivery

The ocular surface is coated with a protective layer of mucus. By developing drug vehicles which will interact with this glycoprotein layer, it is anticipated that the residence time of drugs in the tear film will be extended. If a mucoadhesive drug vehicle can be specifically directed to the corneal surface, loss of drug to the systemic system, via the blood vessels in the conjunctiva, may be minimised.

Four mechanisms have been proposed for the process of mucoadhesion: the wetting, diffusion, electronic, and adsorption theories. The wetting theory relates the ability of the adhesive to spread spontaneously on mucin (i.e. produce a small interfacial tension) to the establishment of intimate contact and formation of bonds between mucoadhesive and mucin. The diffusion theory stresses the importance of molecular weight, chain flexibility and segment mobility of both adhesive and mucin on determining the degree of interpenetration and subsequent semipermanent bond formation. The third, electronic, theory suggests that, when contact between mucoadhesive and mucin is made, transfer of electrons will create an electrical double layer at the interface with subsequent adhesion. Finally, the adsorptive theory places importance on secondary bond formation, such as van der Waals attractions and hydrogen bonds, which may occur between mucoadhesive and biological tissue. However, all these proposed mechanisms are conjectural and derive from observations of different systems.

Carbopol-934 (poly acrylic acid) is believed to have mucoadhesive properties, probably arising from its negative charge and simple, flexible, chain structure. Interestingly, the adhesive nature of this polymer has been found to decrease with increasing crosslinking agent concentration. This may be due to the increase in crosslinker causing a reduction in chain-segment mobility, diffusion coefficient, and extent of mucin-polymer interpenetration. In an experiment (Davies et al. 1988 ¹⁵⁶) 23.5% of the instilled amount of Carbopol-934 remained after 3270 seconds, compared to 13.6% of equiviscous (60 cps) PVA and 9% of PBS solution.

Bioadhesion, as opposed to mucoadhesion, may occur at an epithelial surface

when the continuity of the mucus layer is physically interrupted or chemically altered. The qualities required for bioadhesion are similar to those for mucoadhesion, and bioadhesive polymers are usually macromolecules with numerous hydrophilic functional groups that can form hydrogen-bonds with the cell surface glycoproteins, such as carboxyl, hydroxyl, amide, and sulphate groups. For both mucoadhesion and bioadhesion to occur, sufficient water at the interface is required to hydrate and expand the adhesive, mobilise the flexible polymer chains, and expose the sites for secondary bond formation.

Bioadhesives have several potential uses. They may maintain the continuity of the mucus layer and provide a protective covering for the underlying cells against physical and chemical damage. The effective constituents of artificial tears, namely polymers such as polyvinyl alcohol and hyaluronic acid, are thought to act in this way. More important in the context of this work is the potential bioadhesives have to act as a platform for drug delivery to local tissues.

1.10 Scope and Objectives of Work

The objective of this work was to develop a corneal model which could be used to improve drug delivery systems, either as a drug carrier itself or as part of an *in vitro* model for the study of cornea/tear/drug formulation interactions. As the literature review presented in this chapter has highlighted, the understanding of the nature of the tear film and its precise interaction with the ocular surface is incomplete. Therefore, much of the investigatory work presented in the following chapters is concerned with gaining a clearer insight into the properties of the tear film and its components. In particular, the ocular glycoprotein, mucin, is believed to play a significant role in achieving tear film stability, yet

its relationship with the corneal surface has not been established. Indeed, much of the work published in this area assumes a role for mucin which is based on the results of an unsatisfactory technique used to estimate the surface properties of corneal epithelia. Therefore, further investigations into the properties of mucin were carried out for this work in order to gain a better understanding about its likely relationship with the cornea.

Because the cornea resembles a highly hydrated hydrogel in terms of its structural properties, it is this class of polymers which holds most potential in the search for a corneal model. The assumption was made that the most important property that such a model would possess would be an ability to retain an adherent film of mucin. Work was planned, therefore, using fluorescence spectroscopy and microscopy, to assess a range of hydrogels for mucoadhesivity. Whereas most other tests used to measure mucoadhesion of insoluble polymers have measured the force required to separate an isolated tissue layer and the polymer following physical contact of the two surfaces, this work was looking for the ability of mucin in solution to specifically adhere to the sample polymer. This system was chosen since it was thought that it would provide a better indication of which polymer, when introduced into the tear film, would form an adhesive interaction with the inner layer of mucus.

In considering the nature of the tear film, particularly in terms of drug delivery, it is important to consider the range of materials to which it is commonly exposed and the effects that these have. Contact lens materials and new hydrogels are important, and this work also included studies of the rheological, surface and interfacial properties of solutions, such as contact lens wetting and soaking solutions and artificial tears, designed for instillation into the ocular environment. The information gained from these

investigations is related to the behaviour of these solutions in the tear film, and may help explain the poor performance of many of these ophthalmic solutions in the eye. A qualitative assessment of the behaviour of current ophthalmic solutions that are designed to remain in the eye, providing comfort or other physiological effects, is seen as a useful study in this respect.

To help relate the results of these studies to the ocular environment, the possibility of studying the surface properties of cultured corneal cells is considered to provide a principal experimental objective, and may lead to a design for a corneal model.

CHAPTER 2

Materials and Methods

2.1 **Introduction**

This chapter describes the materials used for this work and gives an outline of the experimental procedures undertaken in the course of this project. Where necessary, further details will be given in subsequent chapters.

2.2 **Materials**

The materials used and their origins and, where appropriate, composition and chemistry are described below.

2.2.1 **Mucins**

The mucins used were porcine stomach mucin (PSM) and bovine submaxillary mucins (BSM):

MUCIN	SUPPLIER
Bovine submaxillary mucin:	Sigma Chemical Company Ltd.
Type 1-S	
Type 1	
Porcine stomach mucin:	Sigma Chemical Company Ltd.
Type II - crude	
Type III - partially purified	

2.2.2 **Hydrogels**

A range of hydrogel materials were used. These are polymers or copolymers, first developed in 1960 by Wichterle and Lim⁵⁰, which swell in water but are not water-

soluble. Their structure is that of a loose polymer network which may be crosslinked by the addition of agents such as ethylene glycol dimethacrylate (EGDM). Hydrogels used for this work included many which were made at Aston by members of the Speciality Materials Research Group. The method of preparation of one typical hydrogel polymer is given later in this chapter.

The equilibrium water content (EWC) is of primary importance in determining the physical properties of a hydrogel. It is defined as the weight fraction of water in the gel expressed as a percentage. The hydrogel component monomers are listed in Table 2.1 with their commonly used abbreviations. Among the various hydrogels used were some contact lenses. These are listed in Table 2.2.

Table 2.1 Hydrogel component monomers and abbreviations used in the text.

Hydrogel component	Abbreviation
2- ethoxyethyl methacrylate	EEMA
ethylene glycol dimethacrylate	EGDM
ethylene oxide	EO
2- hydroxyethyl methacrylate	HEMA
hydroxy propyl acrylate	HPA
hexapropoxylated hydroxypropylmethacrylate	HPHPMA
methacrylic acid	MAA
methoxy polyethylene glycol methacrylate	MPEGMA
methyl methacrylate	MMA
styrene	ST
vinyl pyrrolidone	VP

Table 2.2 Sources, components and equilibrium water content (EWC) of the contact lenses used.

Lens Type	Supplier	Principle Components	Water Content
Permathin Aquaflex	UCO Optics	HEMA/VP/MMA	42.5
Hydron HEMA	Hydron	HEMA	38.0
Permafex	Coopervision Optics	MMA/VP	74.0
Permalens	Coopervision Optics	HEMA/VP/MMA	42.5
Kelvin Eurothin	Kelvin Lenses	HEMA	38.0
Acuvue	Johnson & Johnson	MMA/VP	58.0

Table 2.3 The effect of increasing HPHPMA content on EWC

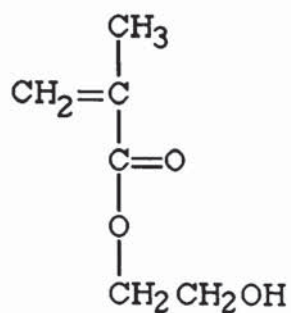
Membrane Composition	EWC (%)
HEMA:EGDM (99:1)	36.9
HEMA:EGDM (99:1) + 5% HPHPMA	37.1
HEMA:EGDM (99:1) + 10% HPHPMA	35.0
HEMA:EGDM (99:1) + 15% HPHPMA	34.2
HEMA:EGDM (99:1) + 20% HPHPMA	33.6

Table 2.4 The effect of polyether content on the EWC of HEMA:EGDM (99:1)

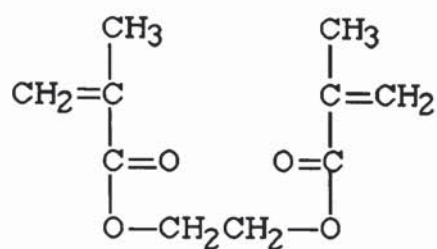
Membrane Composition	EWC (%)
HEMA:EGDM (99:1) + 5% MPEG 200 MA	37.6
HEMA:EGDM (99:1) + 10% MPEG 200 MA	38.8
HEMA:EGDM (99:1) + 15% MPEG 200 MA	41.1
HEMA:EGDM (99:1) + 20% MPEG 200 MA	43.0
HEMA:EGDM (99:1) + 5% MPEG 400 MA	39.0
HEMA:EGDM (99:1) + 10% MPEG 400 MA	40.6
HEMA:EGDM (99:1) + 15% MPEG 400 MA	42.6
HEMA:EGDM (99:1) + 20% MPEG 400 MA	46.4
HEMA:EGDM (99:1) +5% MPEG 1000 MA	41.0
HEMA:EGDM (99:1) +10% MPEG 1000 MA	44.4
HEMA:EGDM (99:1) +15% MPEG 1000 MA	48.5
HEMA:EGDM (99:1) +20% MPEG 1000 MA	49.4
HEMA:EGDM (99:1) +5% HEMA 4,5 EO	37.7
HEMA:EGDM (99:1) +10% HEMA 4,5 EO	39.9
HEMA:EGDM (99:1) +15% HEMA 4,5 EO	40.6
HEMA:EGDM (99:1) +20% HEMA 4,5 EO	41.4
HEMA:EGDM (99:1) + 5% PEGMA 10 EO	38.4
HEMA:EGDM (99:1) + 10% PEGMA 10 EO	39.6
HEMA:EGDM (99:1) + 15% PEGMA 10 EO	41.0
HEMA:EGDM (99:1) + 20% PEGMA 10 EO	42.1

Table 2.5 Other hydrogels used and their water contents.

<u>Hydrogel Composition</u>	<u>Water Content</u>
HEMA/EGDM (90:10)	24.3
MMA/VP (70:30)	67.8
MMA/VP (80:20)	76.7
MMA/VP (Vistagel)+ 1% MA	60.0
MMA/VP (Vistagel)+ 2% MA	60.0
HPA/EEMA/ST (60:30:10)	13.9
HEMA/MAA (75:25)	30.0



**2 - hydroxyethyl methacrylate
(HEMA)**



**ethylene glycol dimethacrylate
(EGDM)**

Figure 2.1 Structure of the 2 - hydroxyethyl methacrylate monomer and the crosslinking agent, ethylene glycol dimethacrylate.

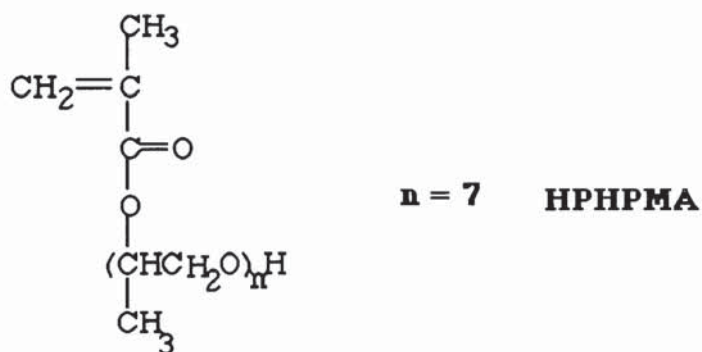
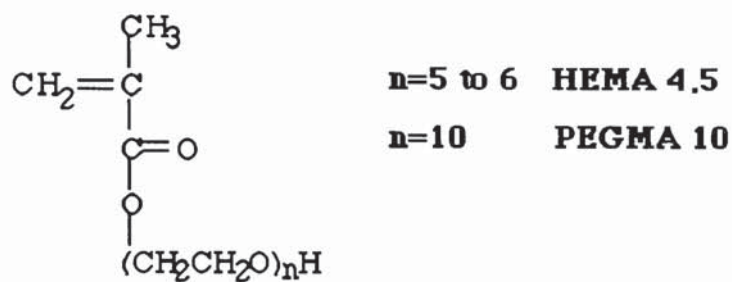
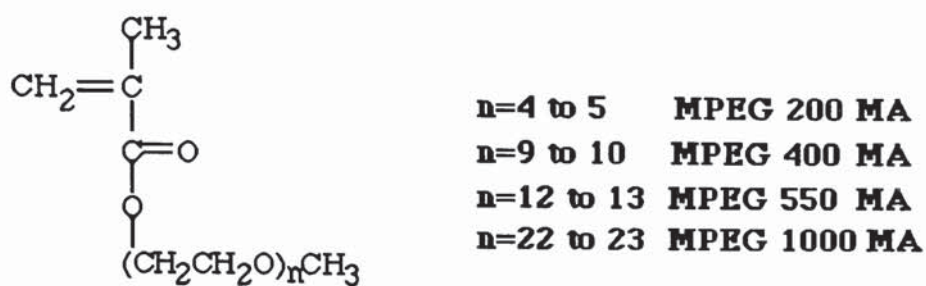


Figure 2.2 Structures of the poly(ethylene oxide) monomers present in some of the hydrogels used in this work.

2.2.3 Other Materials Supplied:

Foetal Calf Serum (FCS)	GIBCO
Mucic Acid	Sigma Chemical Co. Ltd.
Schiff's Reagent	Sigma Chemical Co. Ltd.
Basic Yellow, C.I. 48055	Sigma Chemical Co. Ltd.
Alcian Blue 8 GX, C.I. 74240	Sigma Chemical Co. Ltd.
Minimum Essential Medium (MEM)	
+ Hank's Salts	Flow Labs
Dulbecco's Phosphate Buffered	
Saline (PBS)	GIBCO

2.3 Instruments used for Monitoring Mucin and SIRC Cells

Two main techniques were used to detect mucin during the course of this research, fluorescence spectroscopy and microscopy. Microscopy was also used to examine corneal epithelial cells. The instruments used, and the principles on which the methods are based, are outlined below.

2.3.1 Fluorophotometry

An Aminco - Bowman fluorospectrophotometer (SPF - 125STM) was used during this study. This machine, which consists of an optical unit and a xenon lamp power supply, automatically scans the excitation or emission wavelengths from 200 to 800 nanometers (nm). A diagram of the fluorospectrophotometer is shown in Figure 2.3.

An excitation spectrum shows the dependence of the emission intensity, at a particular wavelength, upon the excitation wavelength. It gives an indication of the wavelength the subject should be excited at in order to provide the maximum emission peak at this wavelength. An emission spectrum shows the wavelength distribution of the emission at a single excitation wavelength. The xenon lamp provides the excitation energy to the first (excitation) monochromator. This monochromator selects and transmits the excitation wavelength to the sample under test. The resulting fluorescence or phosphorescence (emission) wavelengths are applied to a second (emission) monochromator. This monochromator selects and transmits the emission wavelength to a photomultiplier (PM) tube detector. The PM tube converts the incident emission radiation into a corresponding electrical signal which is amplified and displayed on the relative intensity meter. An output voltage proportional to the wavelength is transmitted to a chart recorder which records the characteristic spectrum of the sample.

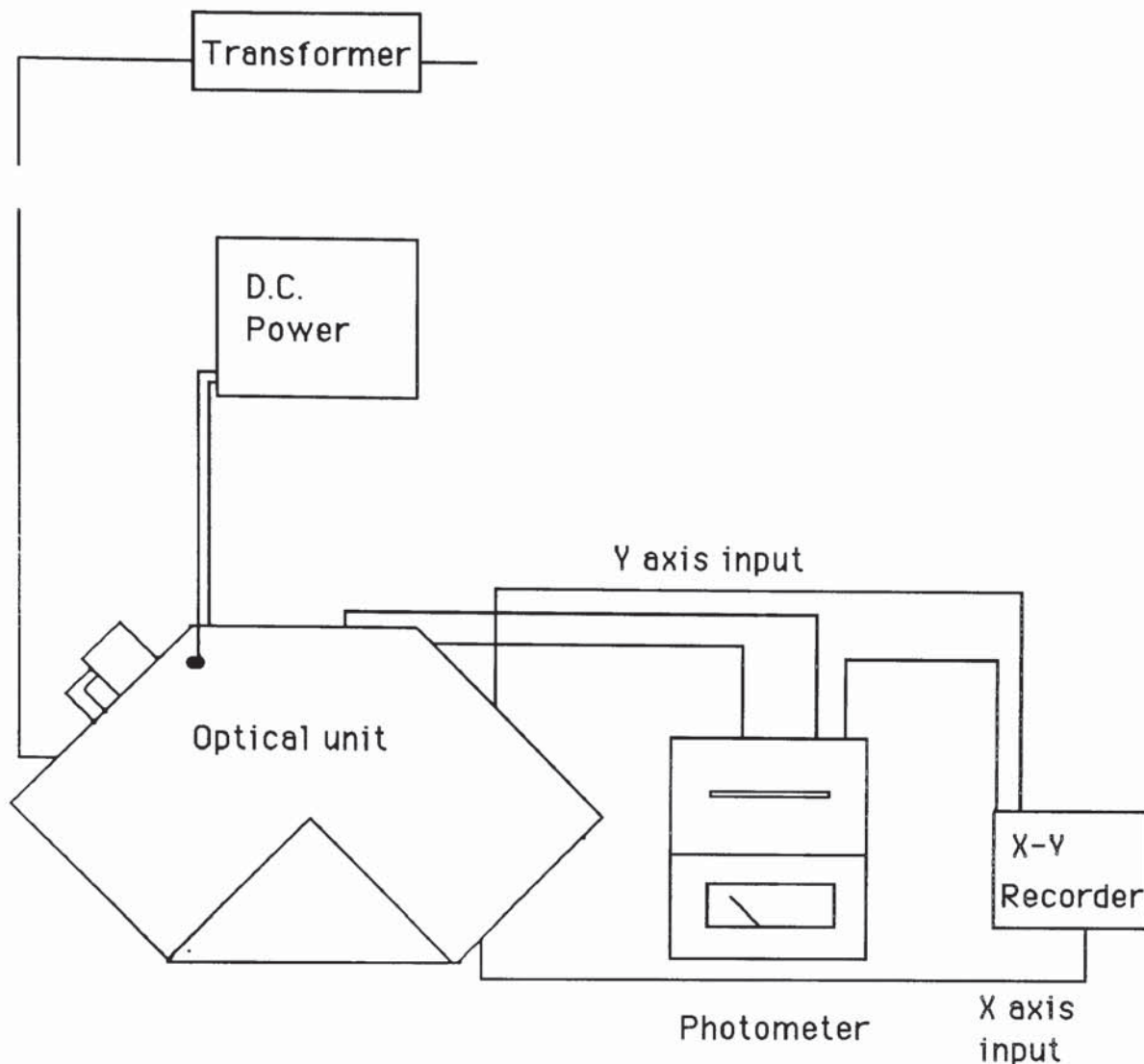


Figure 2.3 The Aminco Bowman Fluorospectrophotometer

The sample for analysis is placed in a round-sided quartz cuvette and this is put into the cell holder in the cell compartment. The cuvette is aligned so that light from the light source (a xenon bulb) will be directed through the flat surface (if the sample is adsorbed onto, or absorbed within) of, for instance, hydrogel matrix. For samples with unknown fluorescent properties it is necessary to obtain the excitation spectrum. To do this, the emission wavelengths are scanned while incrementing the excitation wavelengths. The emission monochromator is set to the excitation wavelength (200 nm, increasing by

increments of 20 nm for subsequent scans), and then the scan controls are set to EM and Drive. The excitation and emission wavelengths at which peak indications occur are noted.

The procedure described above was carried out for mucin solutions (stained and unstained) and for solutions of the protein and lipid species present in the Aston Tear Model (used in spoilation studies). When assessing the presence of these substances on contact lenses or other hydrogel materials the samples were excited at a wavelength that produced a peak relative intensity reading. For proteins and mucin, the excitation wavelength used was 360nm, while a wavelength of 280nm produced distinct peaks for proteins and lipids. Generally, an excitation slit width of 2mm was used since this gives optimum stability, while the emission slit width was set at 0.2mm or 0.5mm (increasing the slit width gives greater sensitivity). The "percent full scale" multiplier switch was set to 0.3, or to 0.1 to increase the relative intensity reading. The contact lens materials and other hydrogels used during fluorescence investigations did not fluoresce at the excitation wavelengths employed.

2.3.2 Phase Contrast Microscopy

A Leitz Dialux 20 microscope was used, together with an attached Wild MPS 15/11 camera. For the observation of unstained and stained cells, and mucin stained with Alcian blue, phase contrast microscopy was used. It is particularly useful in permitting the observation of living, unstained cells. For this technique, contrast is generated by converting differences in refractivity (i.e. differences in refractive index x thickness) into variations of light intensity perceptible by the eye. This is achieved by creating a difference of 180° between the light rays which are deflected by the specimen and those which pass

straight through it. When these deflected rays are recombined with the direct undeflected ones by the lenses of the microscope to form the final image of the specimen, they undergo destructive interference with the undeflected ones, because of their 180° phase difference, thus decreasing the light intensity and generating "phase contrast". Objects with a greater refractive index appear darker than areas of the specimen with lower refractive index.

In order to generate phase contrast, an annular aperture, the condenser phase annulus, is inserted into the condenser of the light microscope. This limits the amount of light which is permitted to reach the specimen from the lamp into a hollow cone. This cone of light comes to focus at the specimen, and the direct undeflected light rays travelling straight through the specimen thus emerge as an inverted cone of light which enters the objective lens. Light deflected by the specimen enter the objective lens at different angles, achieving a physical separation of the deflected and undeflected light rays.

A second modification to the light microscope required to generate phase contrast is the creation of the 180° phase difference between the deflected and undeflected light rays. This is achieved by the objective phase plate at the upper focal plane of the objective lens. The objective phase plate is a transparent glass plate onto which is deposited a semi-transparent phase ring. This phase ring is concentric with the ring of light transmitted by the phase annulus and is just large enough to allow all the undeflected light rays in this ring of light to pass through. The phase of the undeflected rays passing through it is advanced by 90° ($1/4$ wavelength), relative to the deflected light rays which pass through the supplementary areas of the phase plate, inside and outside the phase ring. The nature of refractive deflection of light by a transparent object mean that the deflected

rays emerging from the specimen are retarded by 90° relative to the undeflected beam, creating the 180° phase difference required. A second function of the phase ring is to reduce the intensity of the undeflected rays, originally much brighter in total intensity than the deflected light rays, so that the destructive interference which will occur when these two sets of rays are combined will cause a significant reduction (greater than 50%) in the recombined intensity of the light from those regions of the specimen which were highly refractile. For less strongly refractile areas of the specimen, the proportion of deflected light will be smaller and hence the final amount of destructive interference less, so that these areas of the specimen will appear less dark. A transparent object, such as an epithelial cell, thus appears in various shades of grey, depending on the product of its thickness and the difference between the refractive indices of the object and the medium. Figure 2.4 shows the path of light rays through the condenser, the specimen and the objective lens in phase contrast microscopy.

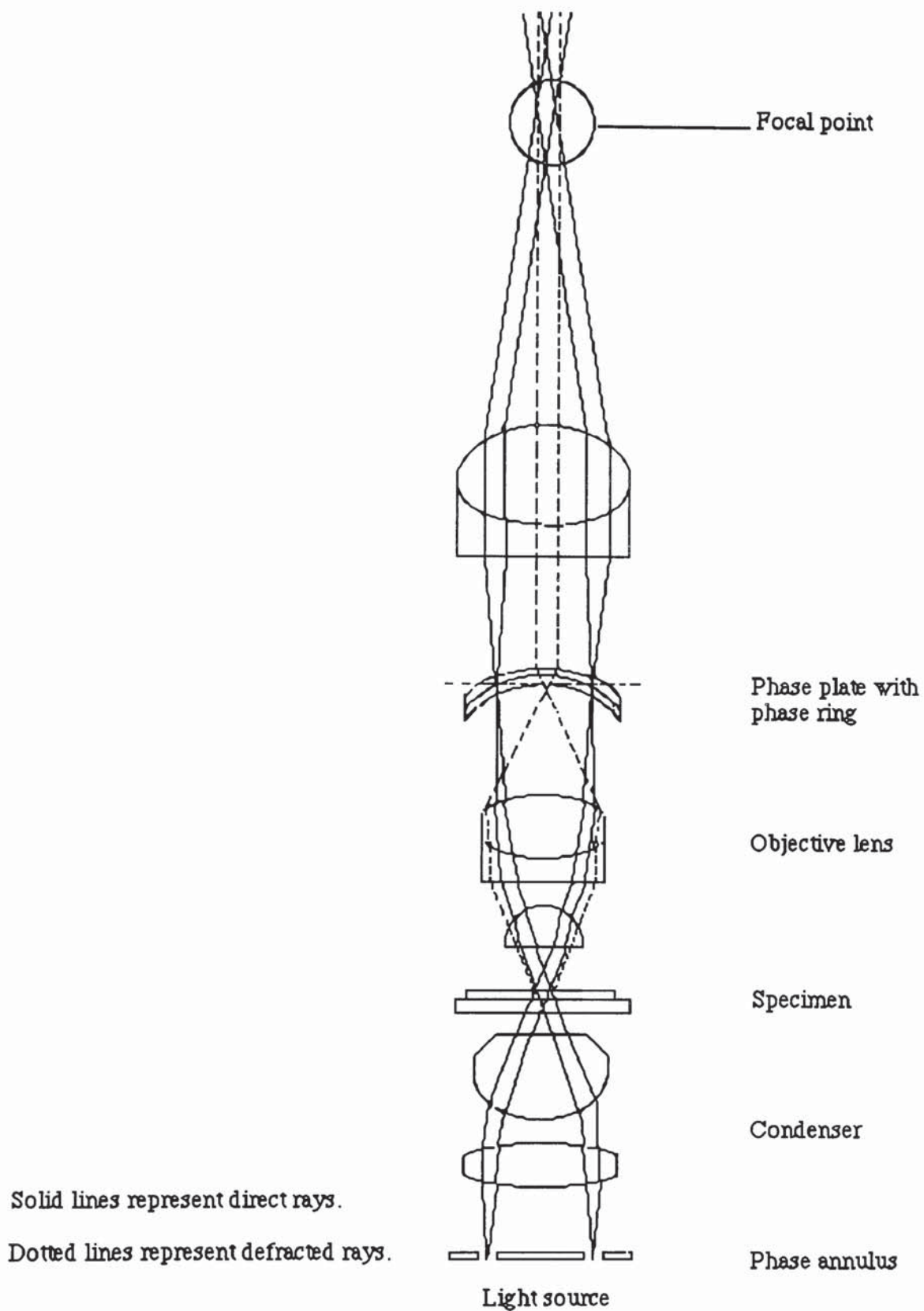


Figure 2.4 The path of light in phase contrast optics.

2.3.3 Fluorescence Microscopy

In fluorescence microscopy specimens are examined under ultraviolet (u.v.) light, near the visible spectrum, and the components are recognised by the fluorescence they emit in the visible region. Two types of fluorescence may be studied: natural fluorescence (autofluorescence), which is produced by parts of the specimen itself, and secondary fluorescence, which is induced by staining with fluorescent dyes.

For the fluorescence microscopy carried out during the course of this work, the u.v. light source was used with the Leitz Dialux 20 microscope, in conjunction with either an I-2 or an H-2 filter in order to restrict the wavelengths of ultraviolet light illuminating the specimen. The I-2 filter block has a narrow excitation wavelength range, 450 - 490nm, which increases the contrast produced by areas of different absorbance in the specimen. The H-2 filter block has a wider excitation range, 390 - 490nm, and is useful for detecting low levels of fluorescence.

2.4 Methods used to Study Mucins: Staining Techniques

Bovine submaxillary mucin (BSM) and porcine stomach mucin (PSM) solutions were generally made up by dissolving 0.5% w/v in either distilled water or in PBS.

When sterile conditions were necessary, Dulbecco's buffered saline was used. For other uses phosphate buffered saline (PBS) solution was prepared by combining the following:

NaCl	87.5g
NaHPO ₄ .12H ₂ O	23.25g
KH ₂ PO ₄	2.15g
H ₂ O	1000ml

2.4.1 Native Fluorescence Detected by Fluorophotometry.

Bovine submaxillary mucin - no fluorescence;

Porcine stomach mucin - Fluoresces in 400-500nm region.

The technique of fluorophotometry is described in Section 2.3.1 of this Chapter.

2.4.2 Alcian Blue Stain

Alcian blue has a high affinity for acid mucopolysaccharides and has been used clinically, in a 1% solution, to demonstrate corneal filaments, adsorbed mucus on the globe and the mucus thread in the lower fornix¹⁵⁷. The dye most commonly used, Alcian blue 8GX, is a water-soluble copper phthalocyanin (CuPC) precursor of the insoluble dye Monastral Fast Blue, into which it can be converted by treatment with alkalis. Solubilising groups such as sulphonic acid, carboxylic acid and chloromethyl groups are introduced during the preparation of dyes¹⁵⁸. Sulphonyl groups enter in the 3-positions, but less is known about the positions taken by carboxylic acid and chloromethyl groups. Figure 2.5 shows the basic structure of copper phthalocyanin.

Alcian blue is one of the chloromethyl-substituted CuPC dyes in which the chloromethyl groups have been reacted with thiourea or alkylthioureas to give isothiuronium salts which are very soluble in water. Alcian blue 8GX has four

tetramethylisothiuroneum groups¹⁵⁹; one of the side chains is shown below, in Figure 2.6, the position substituted in each aromatic ring being uncertain.

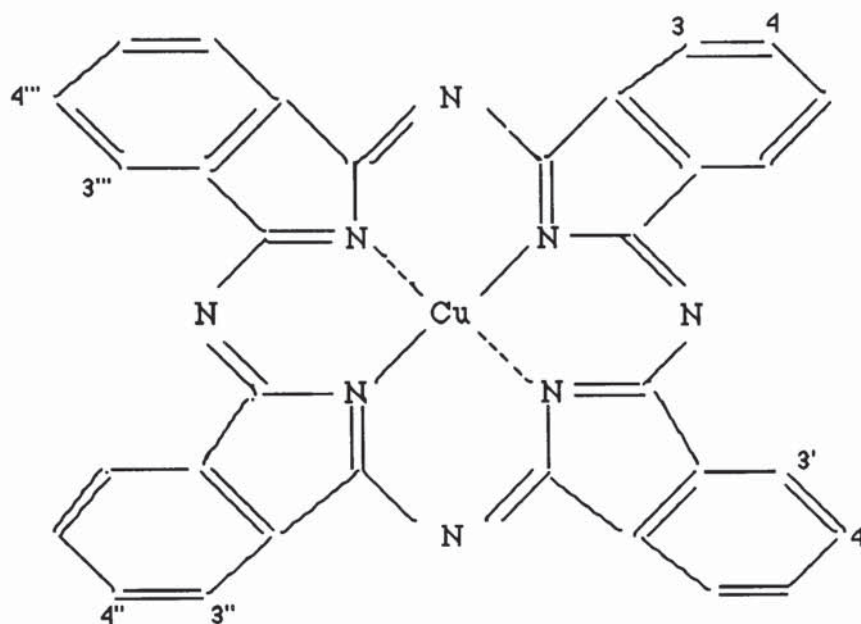


Figure 2.5 Copper phthalocyanin

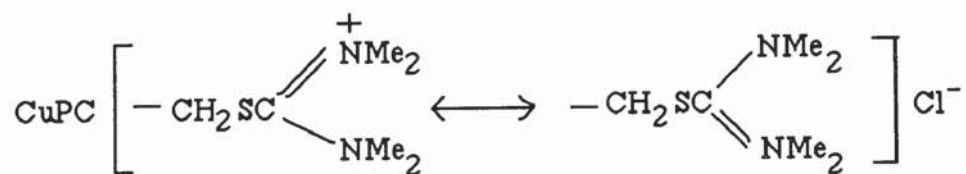


Figure 2.6 Structure of one of the four tetramethylisothiuroneum groups substituted into CuPC to produce Alcian blue.

2.4.2.1 Staining mechanism

The method depends on the fact that water-soluble polyanions or cations are quantitatively separated from solution as complexes by reagents of the opposite charge. The counter ion of the soluble polymer is exchanged for the reagent. Increasing the concentration of counter-ion reverses the process, at a rate depending on the relative affinities of the reagent and counter-ion for the charged sites of the soluble polymer. Differential staining of mixtures of polymers with different charged groups can be achieved on the basis of such varied affinities¹⁵⁸.

Alcian blue is considered to carry at least two, and possibly four, positive charges. Scott et al.¹⁵⁹ postulated that it would combine with polyanionic complexes by forming salt linkages. Sulphated mucosubstances and glucosaminoglycans bind Alcian blue in situ in the presence of low concentrations (less than 0.3M) of electrolytes; only sulphated mucosubstances do so at higher concentrations.

Various mechanisms of Alcian blue staining have been proposed. Goldstein¹⁶⁰ (1961,1962) suggested that there is an inverse relationship between the ionic weight of a basic dye and the density of its substrate. The least dense tissue component, in terms of weight rather than charge (acid mucosubstances), would take up the dye cations whose ionic weight exceeds 1000 (Alcian blue 1342). The denser nucleic acids, on the other hand, would be better stained by dyes with a cationic weight less than 280. Scott (1973)¹⁵⁹, however, explained the staining mechanism on the basis of intercalation of the dye followed by electrostatic and hydrophobic bond formation. This would explain why Alcian blue does not stain the poly deoxyribonucleic acid (DNA) of cell nuclei, for instance; although its chromophore has a high avidity for the base pairs of DNA, its bulky side

chains sterically hinder intercalation into these components.

2.4.2.2 Alcian blue - Periodic Acid/Schiff's (PAS) method for staining fixed mucin

Fresh 0.5% solutions of BSM and PSM were prepared, and smears were made on glass slides as described above. Lillie's fixative was chosen as suitable for fixing mucin smears, and this was made up as follows:

Lead nitrate	8g
40% formaldehyde	10ml
Water	10ml
Ethanol	80ml

The prepared smears were left to fix in this solution for 24 hours at room temperature. The smears were then rinsed briefly in 3% aqueous acetic acid and stained for 2 hours in 1% Alcian blue 8GX solution (the Alcian blue 8GX used has a dye content of approximately 57%, so for a 1% solution 1.75g Alcian blue was dissolved in 100 ml 3% acetic acid.). After removal from the dye they were rinsed briefly in water and then in 3% acetic acid, running water and distilled water. The stained slides were then oxidised in 1% periodic acid (aqueous) at room temperature for 10 minutes and washed in running water for 5 minutes. They were next immersed in Schiff's reagent for 10 minutes, washed once more in running water for 2 minutes, and finally rinsed in 3 changes of 0.5% sodium bisulphite, 1 minute each. The stained smears were dehydrated in 70%, 90% and absolute alcohol, and cleared in xylene.

2.4.2.3 Alcian blue - general method for staining mucin.

For staining mucin, a 1% w/v solution was generally used, prepared as above. For staining cells a 0.1% solution of Alcian blue was made up in 0.05M sodium acetate buffer with added 0.2M MgCl₂.

2.3.3 Basic Yellow Fluorescent Stain for Mucins.

This stain causes sulphomucins to fluoresce in the red or green region, between 500 and 550nm. The structure of Basic yellow is shown in Figure 2.7.

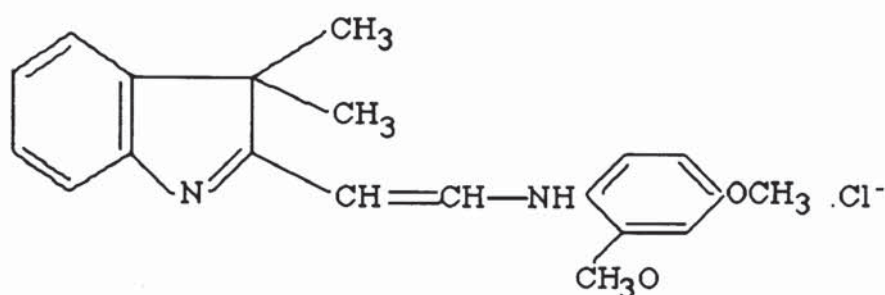


Figure 2.7 Structure of Basic yellow dye.

The Basic yellow dye supplied by Sigma Chemical Company contains 40% dye (w/w), so 0.25g were dissolved in 100ml distilled water in order to make a 0.1% solution. This was further diluted to make a 0.01% solution, as required. The concentration used varied according to the conditions and materials being studied.

Initially samples were analysed with the standard staining procedure for any induced fluorescence. This method is the iron aluminium sulphate (ferric alum) - Basic yellow (coriphosphine) fluorescent method for sulphomucins. The procedure is described

below.

2.4.3.1 Ferric alum-Basic yellow method for staining mucin.

The specimen, was immersed in 4% aqueous iron aluminium sulphate for 10 minutes; washed twice in distilled water; stained in 0.01% Basic yellow (0.025g of 40% stain in 100 ml water); rinsed in distilled water, dehydrated in 3 changes of isopropyl alcohol and cleared in xylene. The treated samples were examined for any fluorescence under the microscope.

2.4.3.2 Labelling of mucin solutions with Basic yellow

Mucin solutions, particularly those of porcine stomach mucin, were fluorescently labelled by adding a measured amount of Basic yellow dye. A solution of 0.5% PSM/ 0.1% BY (3:1) was found to exhibit maximum fluorescence in the region 500-550nm. The instrumentation used to measure and record the fluorescence has been outlined in Section 2.3. This method is described more fully in Chapter 4. For direct staining for mucin on a surface, however, it was sufficient in most cases to apply a few drops of 0.01% Basic yellow solution.

2.5 Synthesis of a Hydrogel Membrane

A 2- hydroxyethyl methacrylate (HEMA)/methyl methacrylate (MMA) (80:20) was prepared as outlined below.

Materials for HEMA/MMA (80:20):

80% HEMA (wt/wt).....3.2g

20% MMA (wt/wt).....0.8g

0.5% AZBN (wt/wt).....0.02g

1% EGDM (wt/wt).....0.04g

The materials were mixed together, with the free radical initiator, azobisisobutyronitrile (AZBN), added first. The mixture was degassed for 10 minutes by bubbling through nitrogen gas.

The membrane moulds were prepared as follows. Two glass sheets were sprayed with adhesive on one side. A sheet of clean melinex was then stuck to each glass sheet. The two sheets were clipped together, melinex faces together, with two gaskets (made with clean melinex) between. The monomer mixture was injected into the mould, and this was incubated at 60 C for 3 days and then at 90 C for 2-3 hours. The membrane was removed carefully from the mould and soaked in distilled water for 1 week so that any unreacted monomers were leached out.

2.6 Mucin Adhesion Studies

Mucin was tested for adhesion to a range of polymers, chiefly hydrogels. Samples of the polymers were left to soak in mucin solutions, some of which were fluorescently tagged with Basic yellow. After a given time the polymer was stained with Basic yellow (if it had been in an unlabelled solution) and examined for fluorescence using the fluorospectrophotometer.

2.6.1 Mucin Adhesion to Spoilt Lenses

The lens material examined was MMA/NVP with 1%,2% or 5% MA added. They were spoilt by boiling in an artificial tear solution. Spoilt lenses were placed in 0.5% porcine mucin solutions. The mucin solutions were changed every 24 hours. After a given length of time the lenses were tested for fluorescence, particularly at 530 nm which would indicate the presence of mucin on the lens surface, in the fluorospectrophotometer. Values given are percentages of maximum peak height, measured from the spectra obtained.

2.6.2 Investigation of Mucin Adhesion to Rabbit Cornea

Fresh rabbit cornea was obtained and treated as follows:

- i) half of cornea (1) was placed in a 0.1% Basic yellow solution;
- ii) cornea (2) plus the other half of cornea (1) was placed in a fluorescently-tagged PSM solution (3:1, PSM/BY). The corneal samples were examined by fluorescence microscopy and by fluorophotometry using an Aminco-Bowman fluorospectrophotometer.

2.7 Cell Culture Work: SIRC Cells

2.7.1 Preparation of a Confluent Cell Layer

SIRC cells were grown in minimum essential medium (MEM) with Hanks' salts, 10% foetal calf serum, 2mM L-glutamine, non-essential amino acids and antibacterial and antimycotic agents. The corneal cell culture was freshly passaged in order to provide cells in the active growth phase.

2ml trypsin was added to the cell culture flask and rinsed out. 4ml trypsin then added to the cells. The flask was agitated for several minutes until the cells were observed

to have come away from the base of the flask. The resulting solution was transferred to a centrifuge tube and spun at 1.5×10^3 rps.

The trypsin was decanted off and 10ml culture medium was added to the plug of cells which were then dispersed. A cell count was performed using a haemocytometer in order to obtain an estimate of cell population density. Approximately 5×10^5 were introduced into each TCP dish compartment which contained the polymer sample on which the cell layer was to be grown. 1 ml tissue culture medium was added to each of the compartments and to two standards (cells + medium). The cells were incubated for 24 hours at 37°C.

2.7.2 Staining of SIRC Cells

2.7.2.1 Basic yellow stain for mucins on cell surfaces

Confluent cell layers were prepared on 1cm discs of HEMA/MMA (80:20) copolymers. Each disc was examined under a few drops of buffered saline using phase contrast microscopy. In an attempt to stain for glycoproteins the PBS was withdrawn by pipette and replaced with a few drops of 0.01% Basic yellow. The cells were then studied under the u.v. illumination of the fluorescence microscope. This procedure was repeated, but this time the PBS was replaced with a solution of 0.5% PSM/0.1% Basic yellow (3:1). The sample discs were subsequently rinsed with PBS and reexamined using the phase contrast microscope.

2.7.2.2 Alcian blue stain for mucin on cell surfaces

A few drops of 1% Alcian blue solution were applied to the cell layer for about

30 seconds. The cells were rinsed with Dulbecco's buffered saline and examined under the phase contrast microscope.

2.8 Contact Angle Measurements

The contact angle that a drop of liquid makes at a surface provides an indication of how well that solution wets the particular surface. The simplest method for measuring this, known as the sessile drop method, involves observing the formation of a drop of liquid, normally delivered by hypodermic syringe, on a solid surface. The contact angle is the angle enclosed by the tangent to the droplet-surface interface and the solid surface. If the drop of liquid spreads on the surface, making a contact angle angle of 0° , complete wettability by the liquid is inferred. A hydrophilic surface is one on which water exhibits a contact angle of zero. The larger the angle, the less wettable that surface is by the liquid in question.

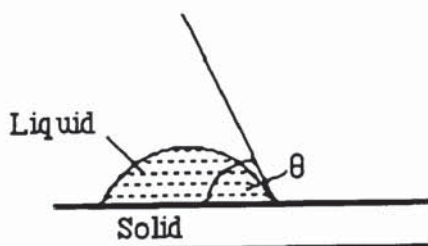
2.8.1 Sessile Drop and Captive Bubble Techniques

The most commonly used method of measuring a contact angle is by constructing a tangent to the profile of a liquid drop (sessile drop) on a surface at the point of contact. This technique was employed in order to find the critical surface tension of a number of polymer surfaces. Two liquids with very different polar and dispersive contributions to surface tension were chosen, water and methylene iodide, and the contact angles they made at various polymer surfaces were measured using a Rame-Hart goniophotometer. All such angles measured were taken to be equilibrium advancing contact angles.

There are a number of experimental difficulties with this technique. In

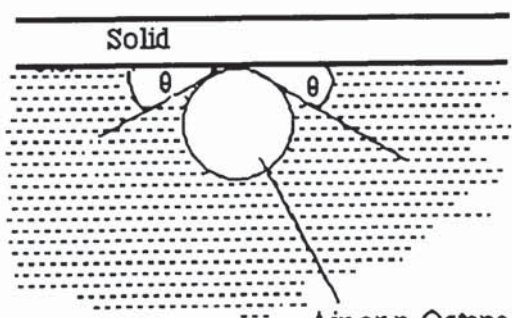
particular, contact angle measurements are very sensitive to surface contamination, so all samples have to be carefully cleaned and dried before measurements are taken. Any surface irregularity also makes accurate measurement difficult. In order to overcome the further difficulties these constraints imposed, particularly where the sample needed to be kept in a hydrated state, the captive bubble technique was employed. This follows the same principles as the sessile drop technique except that the sample is suspended in water. A curved syringe needle facilitates the application of a bubble to the surface of the sample. Contact angle measurements were taken for both air and n-octane. The measurements taken were used to calculate the critical surface tension, and its polar and dispersive components, using the Young's and the Owens and Wendt equations¹⁶¹ (outlined below).

a) Sessile Drop Method



θ is the contact angle made by the liquid at the solid surface

b) Captive Bubble Method



Air or n-Octane
For n-octane, contact angle $(180-\theta)$

Figure 2.8 Sessile drop and captive bubble methods of contact angle measurement.

Young's equation (2.1) describes the forces acting on a drop of liquid, which is at equilibrium, on a solid surface.

$$\gamma_{sv} - \gamma_{sl} = \gamma_{lv} \cos\theta \quad 2.1$$

where

γ_{sv} is the surface tension at the solid-vapour interface,

γ_{sl} is the surface tension at the solid-liquid interface,

γ_{lv} is the surface tension at the liquid-vapour interface, and

θ is the contact angle.

The critical surface tension of a solid can be obtained by measuring the contact angles made at its surface by a range of liquids of known surface tension. If the results are plotted, $\cos\theta$ against surface tension, a line can be extrapolated to a point where $\cos\theta = 1$ (zero contact angle). This corresponds to the surface tension of a liquid which will just wet the substrate, and is called the critical surface tension of the substrate¹⁶². It is a useful concept in the design of, for example, contact lens materials which must be wettable by tears which have a surface tension of about 46mNm^{-1} .

As described in equations 2.2 and 2.3, the surface tension of a solid or liquid may be divided into its polar and dispersive components. Measurement of the interfacial tensions between two liquids of known surface tension enables these components to be determined for the liquids.

$$\gamma_1 = \gamma_{1p} + \gamma_{1d} \quad 2.2$$

$$\gamma_{1,2} = \gamma_1 + \gamma_2 - 2(\gamma_{1p} \gamma_{2p})^{1/2} - 2(\gamma_{1d} \gamma_{2d})^{1/2} \quad 2.3$$

Where γ_1	= Total surface tension, liquid 1
γ_{1p}	= Polar component of γ_1
γ_{1d}	= Dispersive component of γ_1
γ_2	= Total surface tension, liquid 2
γ_{2p}	= Polar component of γ_2
γ_{2d}	= Dispersive component of γ_2
$\gamma_{1,2}$	= Interfacial tension between liquids 1 and 2

Equation 2.4, developed by Owens and Wendt¹⁶¹, shows the relationship between the contact angle and the components γ^p and γ^d for the liquid and solid.

$$1 + \cos\theta = 2/\gamma_l [(\gamma^{sd}\gamma^{ld})^{1/2} + (\gamma^{sp}\gamma^{lp})^{1/2}] \quad 2.4$$

where γ_l is the free energy of the liquid against its saturated vapour (surface tension),
 γ_{ld} is the dispersive component of the surface tension of the liquid,
 γ_{ds} is the dispersive component of the critical surface tension of the solid,
 γ_{pl} is the polar component of the surface tension of the liquid,
 γ_{ps} is the polar component of the critical surface tension of the solid.

By using liquids for which the surface tension, and its polar and dispersive components, is known, these values and the measured contact angle can be substituted into

the above equation in order to calculate the critical surface tension of the solid.

Two different liquids with known polar and dispersive components must be chosen, and the contact angles they make with the solid measured. In this way two simultaneous equations can be generated and γ^{sd} and γ^{sp} determined. This procedure was employed in an attempt to find values for the critical surface tension of corneal epithelial cells, as described in Chapter 5.

2.9 Rheological Measurements

Rotational methods of measuring viscosity were used. Both concentric cylinder and cone and plate instruments are useful for studying the flow behaviour of non-Newtonian liquids¹⁶².

The concentric cylinder technique achieves an approximation to uniform shear rate throughout the sample by shearing a thin film of the liquid between two cylinders. The outer cylinder can be rotated at a constant rate and the shear stress, measured in terms of the deflection of the inner cylinder, which is suspended by a torsion wire. Alternatively, the inner cylinder can be rotated with the outer cylinder remaining stationary, and the resistance offered to the motor measured.

A Carri-Med CS Rheometer with coaxial cylinder attachments was used for the work presented here, connected to an IBM computer and printer. The solution sample is pipetted into the larger cylinder before the smaller diameter cylinder is placed within it. The coaxial cylinder system depends on having laminar flow conditions throughout the gap (a

1mm gap was used during this study). Breakdown in laminar flow to give vortices can occur at high shear rates, and the point at which this occurs can be calculated using the Taylor Number which is dependent on the relative radii of the inner and outer cylinders, rotation speed, sample density and viscosity. The relationship between viscosity, shear stress and shear rate is expressed in equation 2.5.

$$\sigma = \eta\gamma \quad 2.5$$

where η is the viscosity, σ is shear stress, and γ is the shear rate.

The torque of a system is a measure of the internal frictional forces which exert an angular velocity, Ω , on individual particles in solution. These forces arise because different parts of a particle are situated in different layers of the fluid which carry different velocities. The angular velocity of a particle is of such magnitude that

$$\Omega = 1/2.\gamma \quad 2.6$$

and the total torque is zero. The Carri-Med CS Rheometer allows the maximum torque to be applied to a solution to be set. By setting a high torque, the shear rate is increased in order to satisfy equation 2.6.

However, end effects may arise due to viscous drag on the ends of the cylinder, and these become more pronounced as the speed of rotation increases. These effects can give rise to errors of 20-30% in the torque calculation. A further problem is that viscous heating may occur at high shear rate resulting in an apparent decrease in viscosity as time

and shear rate increase, although in this work the run times were short so that this effect is unlikely to be significant. Nevertheless, all these effects may cause apparent non-Newtonian behaviour to be exhibited by Newtonian dilute solutions. Advantages that this system has over other instrumental techniques include the relatively large torque it can exert at a given shear rate, and the ease of temperature control (the instrument used has an internal temperature gauge). Because the cylinders are enclosed, liquids are not expelled even at high shear rates. Cylinder 5222 and cylinder 5200 were each used, with a 1mm gap. although it was found that the 5200 cylinder being slightly smaller than the 5222 cylinder did not allow such high shear rates to be achieved. A diagram of a concentric cylinder rheometer is shown in Figure 2.9. Maximum shear rate achieved was 2500 sec^{-1} for some samples, but mostly shear rates in the order of 1000 sec^{-1} were reached.

In addition to the concentric cylinder method of measuring viscosity, a cone and plate instrument, a Contraves Rheomat, was used, which achieved shear rates up to 3970 sec^{-1} . This technique, illustrated in Figure 2.10, allows the velocity gradient to be kept constant throughout the sample, reducing the effects caused by viscous drag. It is particularly useful for highly viscous materials since it is less sensitive at low shear stresses. The instrument used for this work was a Contraves Rheomat. Shear rate was set manually by means of a dial. The dial was rotated through the fifteen speed settings, corresponding to shear rates from 63.1 to 3970 sec^{-1} . Scale readings were taken at each shear rate, and these were converted to viscosity by multiplying by the absolute viscosity factor for that shear rate, as described by Equation 2.7.

$$\eta = s.\eta\% (+/- 8\%)$$

2.7

where

η = the viscosity (centipoise),

s = the scale reading, and

$\eta\%$ = the viscosity factor.

The absolute viscosity factor and shear rate corresponding to each speed setting are shown in Table 2.6.

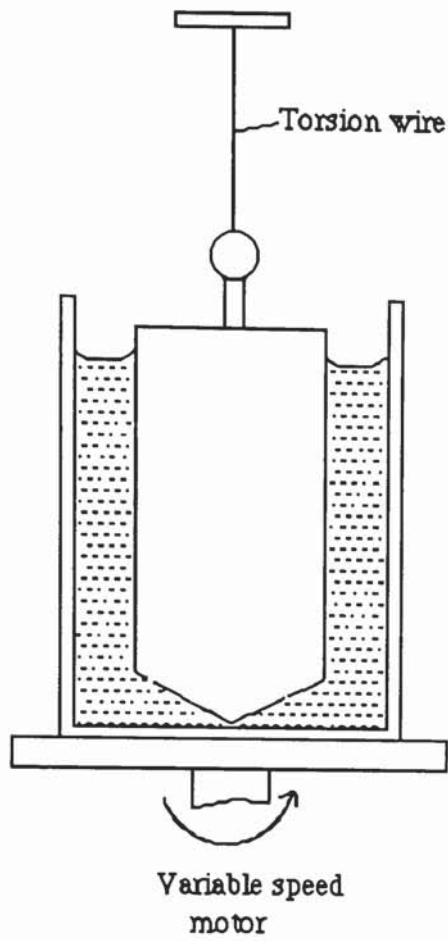


Figure 2.9 A concentric cylinder viscometer.

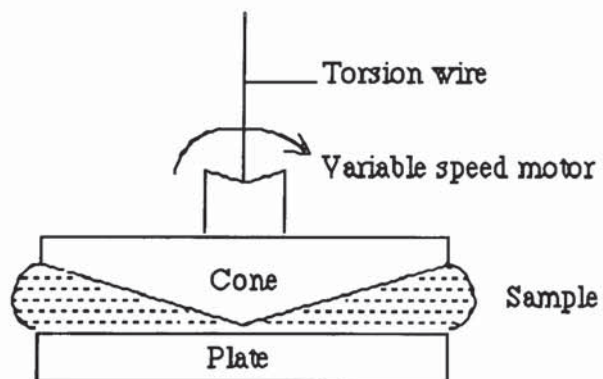


Figure 2.10 A cone and plate viscometer.

Table 2.6 Calibration of the Epprecht-Rheomat 15 cone and plate viscometer.

<u>Speed setting</u>	<u>Shear rate</u>	<u>Viscosity factor</u>
1	63.1	15.8
2	84.8	11.8
3	112	8.96
4	149	6.72
5	196	5.10
6	283	3.53
7	380	2.63
8	500	2.00
9	667	1.50
10	880	1.14
11	1280	0.783
12	1720	0.583
13	2260	0.443
15	3970	0.252

2.10 Interfacial Tension Measurements

Spinning drop tensiometry is a convenient method for the study of interfacial tension between a small droplet of the target fluid and a continuous phase of known surface tension characteristics and density. One of the advantages of this technique is that the interface is not in contact with a third phase such as glass or metal which is the case for most other methods for measuring interfacial tensions. A Kruss Site 04 Spinning Drop Tensiometer, shown in Figure 2.11, was used for this work. The interfacial tension between a light phase (i.e. less dense) droplet, which is located in the centre of a capillary

tube spinning at high revolutions, and the more dense heavy phase is described by equation 2.8.

$$I = \frac{1}{4} r^3 \cdot \Delta\rho \cdot w^2 \quad 2.8$$

where

I = interfacial tension between the phases,

r = droplet radius,

$\Delta\rho$ = density difference between light and heavy phase,

w = angular velocity.

For the Site 04 SDT, equation 1 can be written more fully as equation 2.9.

$$I = e (vd)^3 \cdot n^2 \cdot \Delta\rho \quad 2.9$$

where

e = a constant (3.427×10^{-7}),

v = magnification factor,

d = droplet diameter, in scale divisions (SDV),

n = capillary rotational speed (rpm),

$\Delta\rho$ = phase density difference (gcm^{-3}).

Thus, the interfacial tension can be simply calculated by measuring and observing d and n .

Before taking measurements, the temperature of the thermostat was set to 32.7°C , to resemble the temperature of the tear film²⁵. Because the test solutions (tears,

artificial tears, etc) were only available in small quantities, for these studies the lighter phase (methylene iodide) was used as the main phase. It was poured into the supply vessel and allowed to flow through the capillary tube to the waste outlet. Making sure that there were no air bubbles in the capillary tube, the outlet valve was closed and the rotary motor turned on. The capillary speed knob was adjusted to give a rotational speed of approximately 2000 r.p.m. The outlet valve was reopened and about 2 μl of the heavier phase was injected into the capillary tube by means of the syringe. As soon as a single droplet was in view of the microscope eyepiece, the outlet valve was closed and the capillary assembly was adjusted, using the inclining adjustment, to stabilise the drop. The rotational speed (n) was next increased until the length of the droplet was four times its diameter (Equation 2.9 requires the length to be at least 4 times its diameter). The diameter of the droplet (d) was measured by using the micrometer adjustment to obtain scale readings for the top and bottom of the drop and calculating the difference between the two readings. Several readings were recorded for each droplet at different rotational speeds.

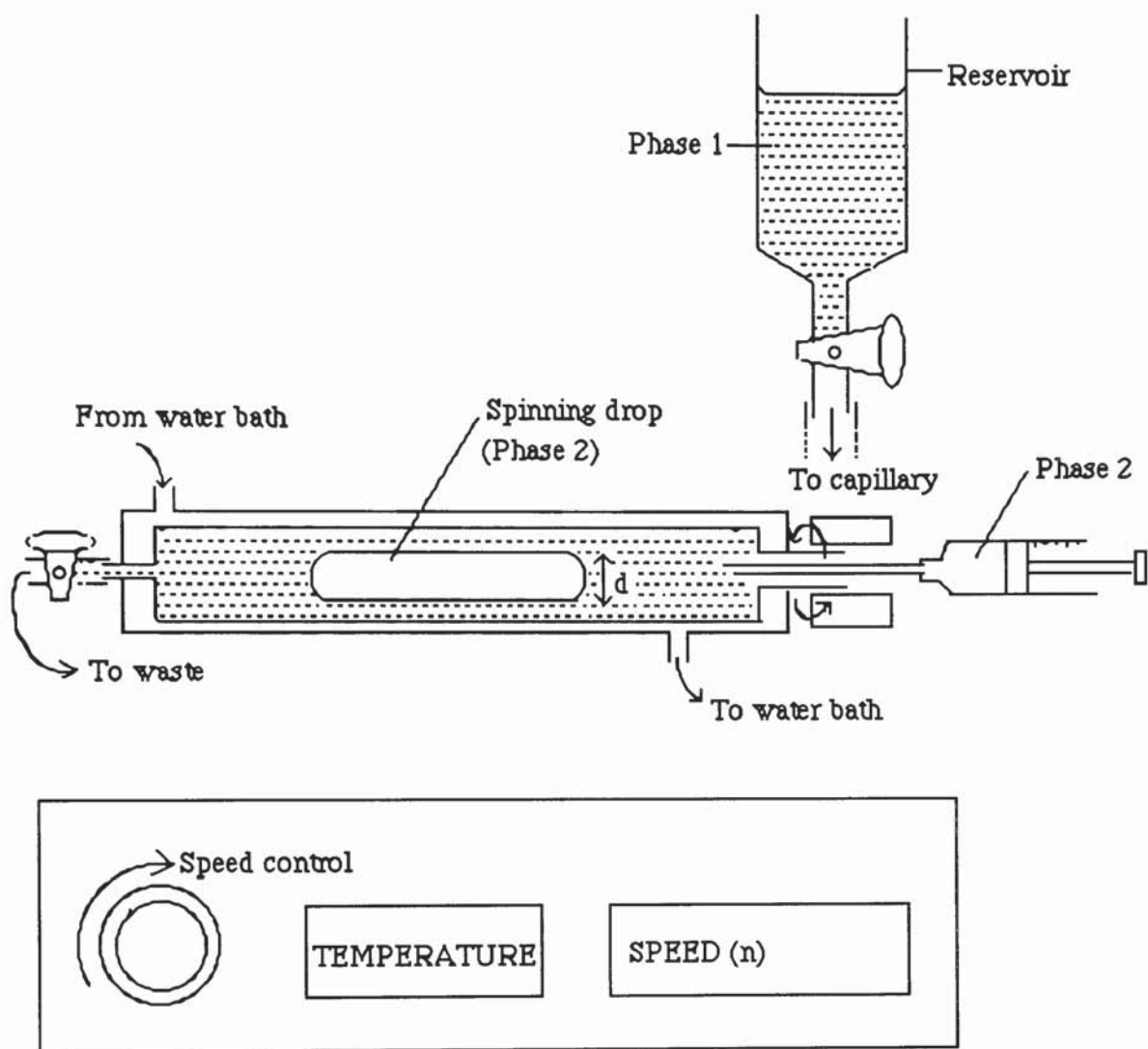


Figure 2.11 The Kruss Site Spinning Drop Tensiometer.

2.10.1 Calibration of the Spinning Drop Tensiometer

The density of the light (methylene iodide) and heavy (test solutions) were measured using a pycnometer vessel and a water bath at room temperature. The density difference ($\Delta\rho$) between the two phases was calculated.

A standard steel wire (1mm) was used to calibrate the eyepiece. It was inserted

into the capillary tube and allowed to equilibrate in the methylene iodide phase before its diameter was measured at several points along its length. The parameter, "v", was calculated as the reciprocal of the average diameter of the steel wire ($= 0.272$).

CHAPTER 3

**Properties of Tears, Artificial Tears, Tear Models,
and their Role in Biomaterials Development.**

3.1 Introduction

This chapter presents the results of an investigation into the surface, rheological and wetting properties of tears, artificial tears and tear models. The properties of these solutions are then related to their behaviour in the eye. A variety of solutions are introduced into the precocular tear film, and to fulfill a variety of functions. These include the so-called "artificial tears" and therapeutic eye drops, and also contact lens care solutions. The interactive behaviour of tears and the cornea with these instilled solutions needs to be investigated in order to be able to assess the effect they may have on corneal integrity and tear film stability. At the same time, the effect of the ocular environment on the properties of the solution should be understood. Ophthalmic solutions are designed to fulfill specific functions in the eye. These include delivering a drug, maintaining contact lens comfort and wettability, and acting as a surrogate tear film in cases of dry eye syndrome. Differences in the chemical structure and physicochemical properties of the constituent polymers of these solutions are reflected in differences in their rheological properties. The study of the interfacial properties of tears is a complex but important part of understanding the behaviour of solutions instilled into the eye. Such studies may also facilitate the development of more efficient drug delivery systems.

The best known use of biomaterials in the ocular environment is for contact lenses. However, despite their widespread use, and although the mechanical properties have improved, there has been little improvement in the biocompatibility of the materials used since the 1960s which saw the introduction of soft lens materials such as poly HEMA. The frequency of occurrence of problems such as lens deposition, oedema and dry eye illustrates the need for better biocompatibility. It is necessary to understand the

mechanisms behind the effects of introducing a foreign material into a given biological environment in order to be able to develop new biomaterials. This is equally true in contact lens development as it is in the development of new blood contact devices.

While the early stages of the interactions of contact lens, tear fluid and cornea can be studied relatively easily, it is difficult to interpret the effects fully without the required detailed knowledge of the nature and the interfacial properties of tears. It is a widely held principle in scientific research that information obtained with "model" systems (e.g. simplified synthetic equivalents) can throw light on the behaviour of the more complex original. Investigations into the interfacial and surface properties of artificial tear solutions and tear models were carried out.

3.2 Artificial Tears and Contact Lens Solutions

In the present context artificial tears and those contact lens solutions designed to be instilled into the eye fall into the category of model solutions. In addition they are often taken as paradigms for the development of ocular drug delivery systems.

Artificial tears and contact lens wetting solutions are dilute polymer solutions, and are routinely prescribed to increase the comfort of "dry-eye" sufferers and contact lens wearers. It is generally accepted that contact lens wetting solutions have one main function to perform. This is to assist in the wetting of the lens by the tears, but they may also act as a mechanical buffer between the lens and the cornea, and. Artificial tears are designed to fulfill similar functions except that here the solution is intended to be a buffer between cornea and eyelid, and to help maintain a coherent tear film over the cornea.

Dry eye symptoms, which include a short tear break-up time (TBUT) and a sensation of grittiness, may be caused by different disorders of the lacrimal system. They can arise, for example, where there is a deficiency in aqueous fluid due to malfunctioning of the lacrimal glands or the secretory ducts, and in cases of mucin deficiency. Despite the different underlying causes of dry eye, little consideration of this is taken in treatment, and the prescribed tear substitutes used do not always provide relief.

The ideal tear substitute would promote the formation of a functional tear film, preserve visual acuity and restore and maintain the health of the cornea. The solutions commercially available have numerous constituents which may be conveniently generalised as:

- 1) preservatives,
- 2) surface active agents,
- 3) polymer to give the required viscosity (most commonly contain methyl cellulose (MC), hydroxypropylmethyl cellulose (HPMC) or polyvinyl alcohol (PVA)),
- 4) sodium chloride,
- 5) buffering agents, and
- 6) water.

Only (1), (2), and (3) will have any appreciable affect on the wetting and flow properties of the solution. The polymer is intended to mimic the ocular mucins by having a strong affinity for the ocular surface, prolonged retention time, and increase the thickness and stability of the tear film.

The artificial tears currently prescribed have been designed to work by⁴¹:

- i) increasing the volume of fluid available at the tear margins;
- ii) adsorption of the polymer at the corneal surface or by its interaction with the mucin component of the tears ;
- iii) creating a film of polymer solution at the air/tear interface which supports a layer of water beneath it.

Whether they actually behave in any of these ways is questionable. In fact, some tear substitutes contain preservatives such as benzalkonium chloride which have powerful surfactant properties. These may not only disperse the lipid layer but also the mucin component, and destabilise the tear film further. Tears also contain components which act as surfactants (glycoprotein and protein components) and stabilise the tear film¹⁶³, and it has been argued that to alleviate the problem of tear deficiency there will be little difference between the effect of instilling a small drop of an aqueous solution or a similar amount of an artificial tear with low surfactant activity. Neither will have an appreciable effect on the surface tension of the tear film. Indeed, Golding et al.¹⁶⁴ reported that there is little difference in effect between instilling saline or artificial tears into the eye in terms of tear break-up time. This can be explained by the non-linear relationship between surfactant concentration and surface tension of a solution. The instilled saline solution simply makes up for the insufficient tear volume in these dry eye sufferers, while the active tear constituents provide the natural wetting and spreading properties. Any beneficial effects experienced by artificial tear users could simply be due to the lubricating effect of a temporary increase in tear volume and viscosity, thereby facilitating the normal functioning of the eye's native surfactants.

Another growing area of interest is in the use of synthetic polymers for

sustained ocular drug delivery. Current ocular drug delivery systems are inefficient and require frequent application. Topical applications of an ophthalmic solution to the eye in a conventional manner result in extensive loss of therapeutic agents¹³⁴. It is now well established that a large proportion of an eye drop is rapidly excreted through the puncta due to the reactions induced by instillation. These may be described as two processes which occur following instillation :

- i) the added volume in excess of the normal tear volume drains;
- ii) the solution is diluted by basal tearing and any reflex tearing elicited.

Studies have shown that under normal circumstances the half-time of residence of a solution in the eye ranges from 10 seconds to 4 minutes, depending on the degree of reflex tearing elicited¹³⁴. The elimination rate is also dependent on the droplet size instilled. The rate of elimination is insensitive to the droplet size over a range of 1 to 10 μ l, and sometimes to 20 μ l,¹³⁴ but conventional ophthalmic drug applicators dispense droplets averaging about 50 μ l⁸. A drop this size can displace most of the tear film, since the maximum volume of fluid held by the cul-de-sac is 20 to 30 μ l, washing away the surface oils and hence reducing tear film stability. On average, the concentration has been shown to drop to around half of the applied topical dose in one minute, with the level decreasing to one-thousandth of the initial value within 8 minutes⁶⁷. Both the concentration and duration of exposure of a medication in the eye are thus determined by the character and dynamics of the precorneal tear film.

It has been known for a number of years that the surface tension of tears, in common with other biological fluids, is lower than that of water due to the presence of

natural surfactants. Indeed, one of the underlying principles found in nature is the establishment of low interfacial tensions between coexisting solid and liquid surfaces, e.g. blood and blood vessel wall. As mentioned above, many of the tear components act as surface active agents (surfactants). In simplistic terms, lipids may be thought of as playing a major role at the tear-air interface, whereas the glycoprotein or mucin components act preferentially at the tear-cornea interface.

An ophthalmic solution with particular rheological properties in *in vitro* conditions may behave quite differently in the eye. For instance, it is unrealistic to expect viscosity measurements made at low shear rates at room temperature to reflect the behaviour of a solution in the eye where the action of the eyelid creates shear rates of several thousand per second. Studies of solutions, designed for ocular purposes under *in vitro* conditions, which mimic those of the eye, might provide answers as to why many of these ophthalmic solutions do not perform as expected *in vivo*. The behaviour of a series of artificial tears and wetting solutions were, for this reason, studied at various shear rates and at temperatures resembling those of the tear film.

Other factors, as well as surface chemistry and rheology, must also be considered, however, since these may play a major role in the effect of the solution in the eye. Probably most important is the effect on tear film stability of these solutions. Many contact lens solutions and ocular drug solutions are designed primarily to fulfill stringent antimicrobial requirements, with relatively little importance placed upon their physical properties. As a result, most solutions include preservatives, such as benzalkonium chloride and thiomerosal, which tend to have strong surfactant ability. Introducing

surfactants into the eye can disrupt the stability of the tear film by solubilising tear lipids and damaging the epithelial cell surfaces of the cornea and conjunctiva. The effects are initially manifested as a short tear break up time and dry eye syndrome. The incorporation of surfactant preservatives into artificial tear solutions which are instilled regularly and frequently into the eye can, therefore, exacerbate the very problem the solution is designed to treat.

The surface and interfacial properties of tears are, therefore, important factors to consider in the design of solutions for ocular purposes. In particular, to produce materials which mimic biology (i.e. are biomimetic) in this sense, the polar and dispersive components contributing to the surface tensions of the appropriate biological fluids and the biomaterial must be known. These properties were investigated for tears, a tear model, and for some artificial tear solutions.

3.3 Surface and Interfacial Tensions

The surface tension of a liquid is often defined as the force acting at right angles to any line of unit length on the liquid surface. The molecules located in the bulk of a liquid are, on average, subjected to equal forces of attraction in all directions. Those located at the surface, the liquid-air interface, however, experience an imbalance of attractive forces resulting to a net inward pull. Since there is no tangential force as such acting at the surface of a liquid, a more accurate definition is to describe surface tension and surface free energy as the work required to increase the area of a surface isothermally and reversibly by a unit amount. At the interface between two liquids there is a similar imbalance of intermolecular forces, the magnitude of which usually lies between the individual surface tensions of the

particular liquids.

3.3.1 Measurement of Surface Tension

The surface tension of most liquids decreases with increasing temperature in a more or less linear fashion, becoming very small as the critical temperature is approached. For this reason, during this study, surface tensions of the different liquids and solutions were measured at the same temperature wherever possible. The method used was the platinum ring method in which the platinum ring was suspended from the arm of a torsion balance which measured the force required to detach the ring from the surface of the liquid. Prior to each reading the platinum ring was carefully cleaned in chromic acid, and the balance calibrated to zero.

Table 3.1 gives surface tension values for water, a typical animal serum (foetal calf serum), and a 0.5% mucin solution in comparison to that of tears and the Aston Tear Model, which is representative of the total tear composition and is based on a stable lipid, glycoprotein, protein composition which is modified with appropriate electrolytes. The literature values for water and for tears are 72.8 mNm^{-1} and 43.6 mNm^{-1} , respectively.

Table 3.1 Surface tension measurements using the platinum ring method.

Solution	Surface Tension	Temperature
	mNm^{-1}	$^{\circ}\text{C}$
Water	72.9	21.8
Aston Tear Model	54.1	21.1
0.5% Mucin	45.2	22.8
Serum	54.3	22.6

3.3.2 Interfacial Tension Measurements

Interfacial tensions between test solutions and methylene iodide (to represent the hydrophobic cornea or the lipid layer of the tear film) were measured using a Kruss Site 04 Spinning Drop Tensiometer, as described in the Materials and Methods Chapter. Table 3.2 shows the intermediate parameters and calculated interfacial tensions for a selection of fluids, including tears and three commercial artificial tear solutions. The values given for the calculated interfacial tensions in Table 3.2 are averages, and therefore do not give any absolute indication of the rotational stability of the solutions studied. Of the solutions analysed, however, water, tears, the Aston Tear Model and the mucin solution were observed to be stable under high rotational speed whereas the artificial tear solutions were rotationally unstable, and gave apparently greater interfacial tensions at higher speeds. The lowest interfacial tensions were exhibited by mucin and the commercial artificial tear solutions. Human tears and the Aston Tear Model showed similar interfacial tension values with the methylene iodide.

Table 3.2. Interfacial tension measurements using a spinning drop tensiometer.

Solution	n	d	$\Delta \rho$	I.T.	Temp.°C
Water	6619	3.48	2.26	24.6	32.7
Aston Tear Model	5672	3.14	2.24	13.1	32.7
0.5% mucin	3684	3.68	2.25	9.0	32.7
0.5% mucin :1 + Tear Model :5	5531	3.23	2.24	13.6	32.7
Tears	4948	3.39	2.25	12.6	32.7
Tears Naturale	3724	4.32	2.25	14.8	32.6
Liquifilm Tears	4464	3.28	2.25	9.3	32.7
Sno Tears	4329	3.45	2.25	10.2	32.7
Ilube	4377	3.48	2.25	10.7	32.7

Methylene iodide was used as the "oil" phase.

$$\text{Interfacial Tension} = 3.427 \times 10^{-7} (v.d)^3.n^2 \times \Delta\rho$$

Calibration: v = magnification factor, 0.258,

d = measured diameter of drop of aqueous solution,

n = rotational speed, sec⁻¹,

$\Delta\rho$ = density difference between light and heavy phase.

Calibration of the spinning drop tensiometer is outlined in Chapter 2. The information obtained from our experimental work presented here, enables the calculation of the polar and dispersive components of the surface tension of tears and the "model" solutions to be measured. The polar and dispersive components of methylene iodide are 1.3 mNm^{-1} and 49.0 mNm^{-1} , respectively. By substituting these, as δ_{1p} and δ_{1d} , into equations 3.1 and 3.2, together with the calculated interfacial tension for tears shown in Table 3.2, and taking the surface tension of tears to be 43.6 mNm^{-1} , the polar and dispersive components of tear surface tension may be calculated. These first reported values for these surface tension components of tears are compared in Table 3.3 with values calculated for our Tear Model and the known values for water and a hydrocarbon.

$$\gamma_1 = \gamma_{1p} + \gamma_{1d} \quad 3.1$$

$$\gamma_{1,2} = \gamma_1 + \gamma_2 - 2(\gamma_{1p} \gamma_{2p})^{1/2} - 2(\gamma_{1d} \gamma_{2d})^{1/2} \quad 3.2$$

Where γ_1	= Total surface tension, liquid 1
γ_{1p}	= Polar component of γ_1
γ_{1d}	= Dispersive component of γ_1
γ_2	= Total surface tension, liquid 2
γ_{2p}	= Polar component of γ_2
γ_{2d}	= Dispersive component of γ_2
$\gamma_{1,2}$	= Interfacial tension between liquids 1 and 2

Table 3.3 Polar and dispersive components of tears (mNm^{-1}), compared with those of a tear model, water, and a hydrocarbon.

Solution	γ_p	γ_d
Tears	26.1	17.5
Tear Model	31.9	22.1
Water	51.0	21.8
n-Hexane	0	18.4

From these results, the surface tension of tears is seen to have more uniform polar and dispersive components than does water. Water and tears have dispersive forces in the region of 20 mNm^{-1} , as does the hydrocarbon n-hexane. However, unlike hexane, the surface tensions of water and tears also have considerable polar components. Taking the reliability of these calculations to lie within plus or minus 1 mNm^{-1} , the dispersive component of tears appears to be closer in magnitude to that of the hydrocarbon than it is to that of water and reflects the presence of surface active in the tear fluid. However, it should be pointed out that these calculations are based upon reported values of tear fluid surface tension in the literature. Most of the techniques normally used to measure the surface tension of tears are invasive, e.g. the Wilhelmy plate method, and require the tear fluid to be at "equilibrium" (i.e. static). Tear fluid contains a number of surface active components which will normally be dispersed throughout the tear fluid, but which will become preferentially concentrated at the film surface, and depress the surface tension,

when the solution is left undisturbed for some time. Hence, surface tension measurements by surface-invasive techniques are likely to underestimate the actual surface tension of aqueous tears.

The main contributions to the surface tension of tears result from the presence of mucins, proteins, and the sodium salts of fatty acids. It is questionable, in fact, whether measurements of the surface tension of tear fluid do actually measure the surface tension of "aqueous" tear fluid. Most techniques which have been used to measure the surface tension, such as the Wilhelmy or ring methods, are invasive in the sense that they involve disturbing the surface of a static solution. Because tear fluid contains surface active components which will be more concentrated at the fluid/air interface these invasive techniques may underestimate the true surface tension of aqueous tears in the eye. The rotational force applied to the tear sample by the spinning drop tensiometer is also likely to enhance packing of the lipid component (of meibomian origin) at the interface between the two fluids, which would result in the value of the dispersive component resembling a pure hydrocarbon, as was found in this study. Lipids secreted by the meibomian glands include hydrocarbons, wax esters, cholesterol esters and fatty acids. Lipids from the sebaceous glands in the skin around the eye may have contaminated the tear sample, although care was taken to avoid this during collection. The tears studied were collected from a person with a blocked tear duct. These results show that our Tear Model possesses similar surface chemical properties to tears. For both our Tear Model and human tears, the polar component makes up approximately 60%, and the dispersive component 40%, of the total surface tension.

The polar and dispersive forces, arising from hydrophilic and hydrophobic properties of the high molecular weight mucin glycoprotein, provide it with surfactant qualities. Surfactants tend to pack into an interface, resulting in a lowering of surface tension, and have an ability to expand into the surface as the liquid spreads and its surface area increases. This process has been proposed as being important in maintaining a coherent tear film over the cornea, giving rise to the layered structure of the tear film, illustrated in Chapter 1 (Figure 1.1). The results in Tables 3.2 and 3.3 also show that our Tear Model possesses similar surface chemical properties to tears.

3.4 Viscosities of Tear Models

For the purpose of this work, artificial tears, and those contact lens solutions designed to be instilled into the eye, are included in the category of model solutions. Viscosity is a measure of the internal resistance offered to the relative motion of different parts of the liquid. Viscosity is an important aspect of ophthalmic design, particularly when formulating drug-carrying solutions. The traditional approach to improve ocular drug retention relies upon increasing the viscosity of the drug vehicle. Studies on rabbits by Robinson and associates have established that increasing the viscosity of a solution significantly retards its elimination from the eye^{165,166}. Newtonian flow occurs when the shearing force per unit area, t , between two parallel planes of liquid in relative motion is proportional to the velocity gradient, dv/dx , between the planes:

$$t = h \, dv/dx$$

where h is the coefficient of viscosity, in units $\text{mass.length}^{-1} \text{time}^{-1}$. For the majority of pure liquids, and many solutions and dispersions, h is a well-defined quantity for a given

temperature and pressure which is independent of t and dv/dx , when flow is laminar (streamlined). For many other solutions and dispersions, particularly where the concentration or the particles are asymmetric, deviations from Newtonian flow are observed. The main causes of non-Newtonian flow are the formation of a structure throughout the system and orientation of asymmetric particles caused by the velocity gradient. Two non-Newtonian types of behaviour are called dilatant behaviour and pseudoplastic behaviour. In dilatant behaviour, the liquid shows higher viscosities as the shear rate is increased, while pseudoplastic liquids become less viscous at higher shear rates.

3.4.1 Techniques used to Measure Viscosity

Two techniques were used during this study, the concentric cylinder and the cone and plate methods. The techniques are outlined here, but further information is given in section 2.9 of Chapter 2.

A Carri-Med CS Rheometer, connected to an IBM computer and printer, was used with coaxial cylinder attachments. The solution sample is pipetted into the larger cylinder before the smaller diameter cylinder is placed within it. Cylinder 5222 and cylinder 5200 were each used, with a 1mm gap. although it was found that the 5200 cylinder being slightly smaller than the 5222 cylinder did not allow such high shear rates to be achieved. The torque was set to a level which gave maximum shear rates for the solution, although where possible it was kept at the same level for different solutions in order that the viscosity measurements would be comparable. The computer attachment generated shear stress/shear rate curves. By measuring the gradient of the shear stress

curve, the viscosity/shear rate curve could be plotted. A diagram of a concentric cylinder rheometer is shown in Figure 2.9. Maximum shear rate achieved was 2500 sec^{-1} for some samples, but mostly shear rates in the order of 1000 sec^{-1} were reached.

3.4.2 Rheology of Ophthalmic Solutions

Rheology is the science of the deformation and flow of matter. The most straightforward rheological behaviour is exhibited by Newtonian viscous fluids and Hookean elastic solids. Most materials, however, exhibit mechanical behaviour which falls between these two extremes. These are termed viscoelastic materials. Many polymer solutions exhibit varying degrees of shear thinning (pseudoplastic) behaviour. With such solutions, increasing shear rate causes the flexible polymer molecules to deform to an ellipsoid conformation and at larger shear rates the molecules will be completely aligned. The flexibility of molecules in a polymer solution gives rise to a finite viscosity at low shear rates, called the zero shear viscosity. The zero shear viscosity is dependent on molecular weight, whereas at high shear rates the viscosity is mainly determined by concentration. Most of the polymer solutions designed for ocular instillation show pseudoplastic behaviour. This means that although the solutions appear to be fairly viscous, following application they quickly become much thinner during blinking and other rapid eye movements.

Table 3.4 gives the viscosities of some artificial tears and contact lens care solutions at a low shear rate and at a high shear rate. These were measured using the cone and plate viscometer.

Table 3.4 Viscosity measurements using cone and plate viscometer.

Solution	Shear Rate, sec ⁻¹	
	500	3970
	<u>Viscosity (cP)</u>	
Liquifilm Tears	6.0	3.2
Tears Naturale	8.1	3.9
Sno Tears	18.1	8.6
Stericlens	13.1	8.3
One-Solution	17.0	7.1
Miraflow	33.5	25.2+
Boston Lens Cleaner	43.6	23.4
Vistasol	11.6	5.2
Cleaning & Soaking	5.0	2.1
Contactaclean	8.1	5.0
B & L Cleaner	30.0	16.1

Figure 3.1 shows the viscosity/shear rate curve for three artificial tear solutions, also measured with the Epprecht cone and plate viscometer.

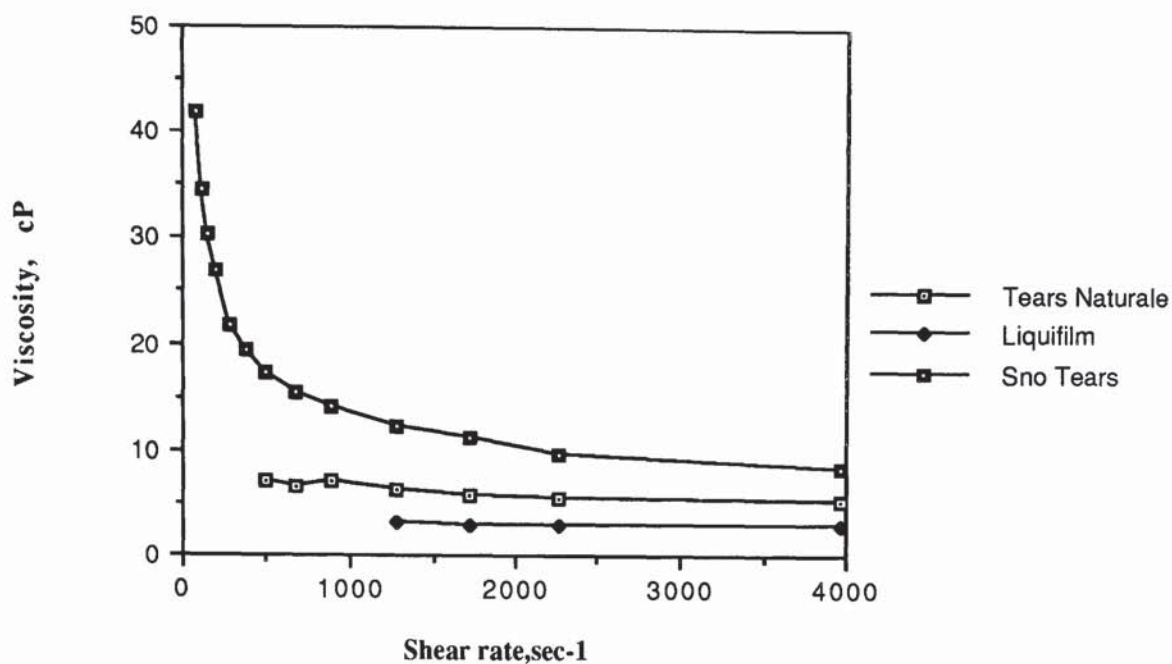


Figure 3.1 The effect of shear rate on the viscosity of some artificial tear solutions.

Viscosity measurements could not be obtained for all solutions due to instrument limitations. The cone and plate viscometer was not sufficiently sensitive to measure low viscosity liquids and solutions such as water, nor dilute polymer solutions at low shear rates. The concentric cylinder viscometer was more sensitive at low shear rates, but the limitations of this instrument were such that shear rates as high as those achieved by the cone and plate model could not be reached.

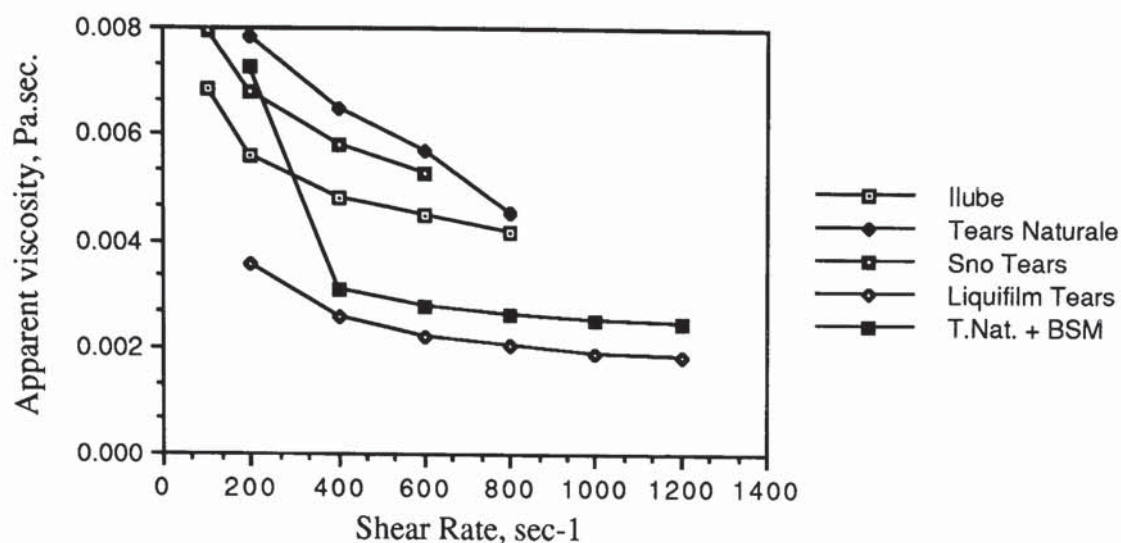


Figure 3.2 Viscosity/shear curves for some artificial tear solutions measured by Carri-med concentric cylinder viscometer.

The viscosity/shear rate curves in Figures 3.1 and 3.2 both show Liquifilm tears to be the least viscous of the artificial tear solutions tested. It also appears to approach Newtonian flow, where viscosity remains constant, at a lower shear rate than the other solutions tested. The addition of mucin to the solution with most pronounced non-Newtonian flow at the shear rates tested (Tears Naturale) appeared to have a marked stabilising effect, producing a similar viscosity curve to that of Liquifilm Tears, although slightly more viscous. This effect is shown to take place for a different tear model (formulated using foetal calf serum and saline) once shear rates have reached 500sec^{-1} in Figure 3.3, using the concentric cylinder technique.

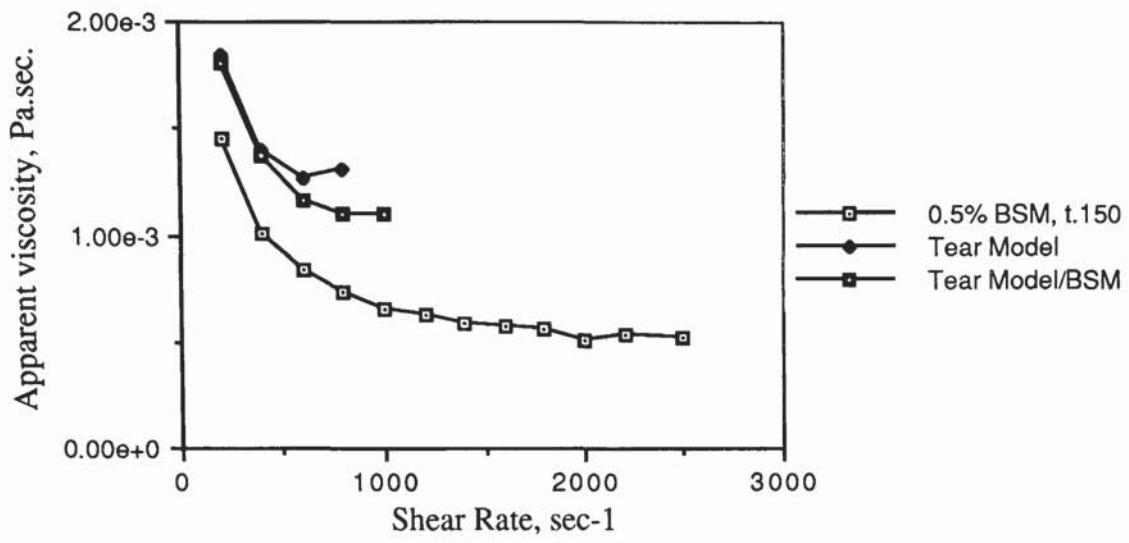
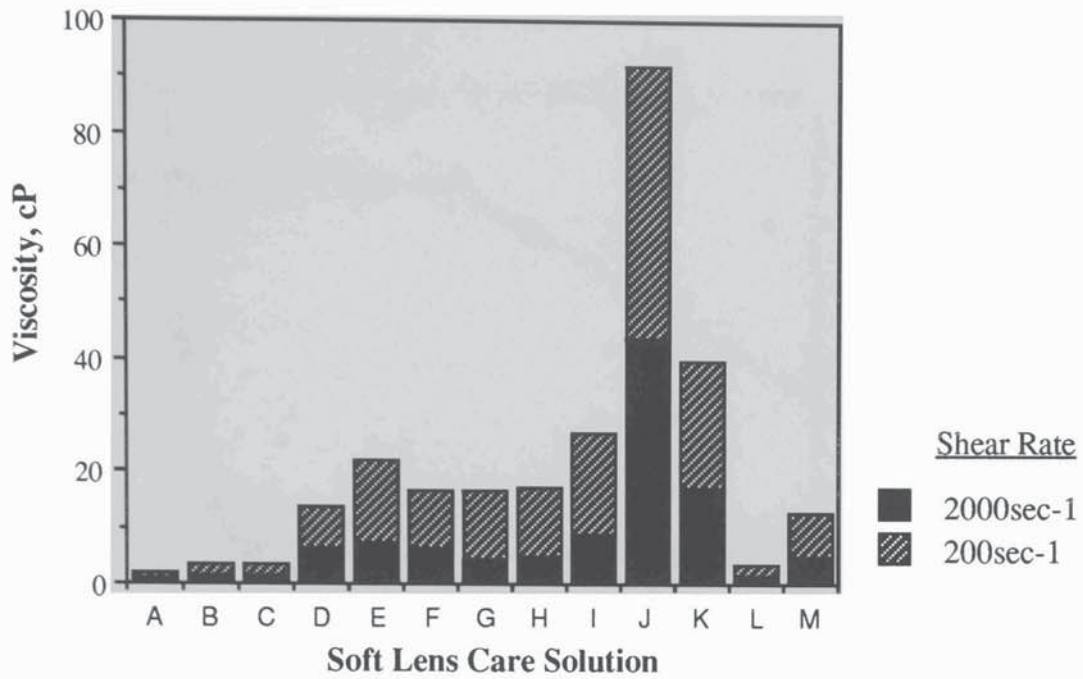


Figure 3.3 Apparent viscosity at different shear rates of 0.5% BSM, and a tear model with and without added mucin.



- | | |
|-----------------------------------|---------------------------|
| A Saline | H Preflex (Alcon) |
| B LC-65 (Allergan) | I Pliagel (Alcon) |
| C Prymclean (Smith & Nephew) | J Miraflo (Coopervision) |
| D Barnes-Hind Cleaner No.4 | K Total (Allergan) |
| E Sauflon Soft Lens Daily Cleaner | L Clean & Soak (Allergan) |
| F Hydroclean (Ciba) | M Complete Care (CIBA) |
| G Bausch & Lomb Daily Cleaner | |

Figure 3.4 The effect of shear rate on the viscosity of some soft contact lens cleaning solutions.

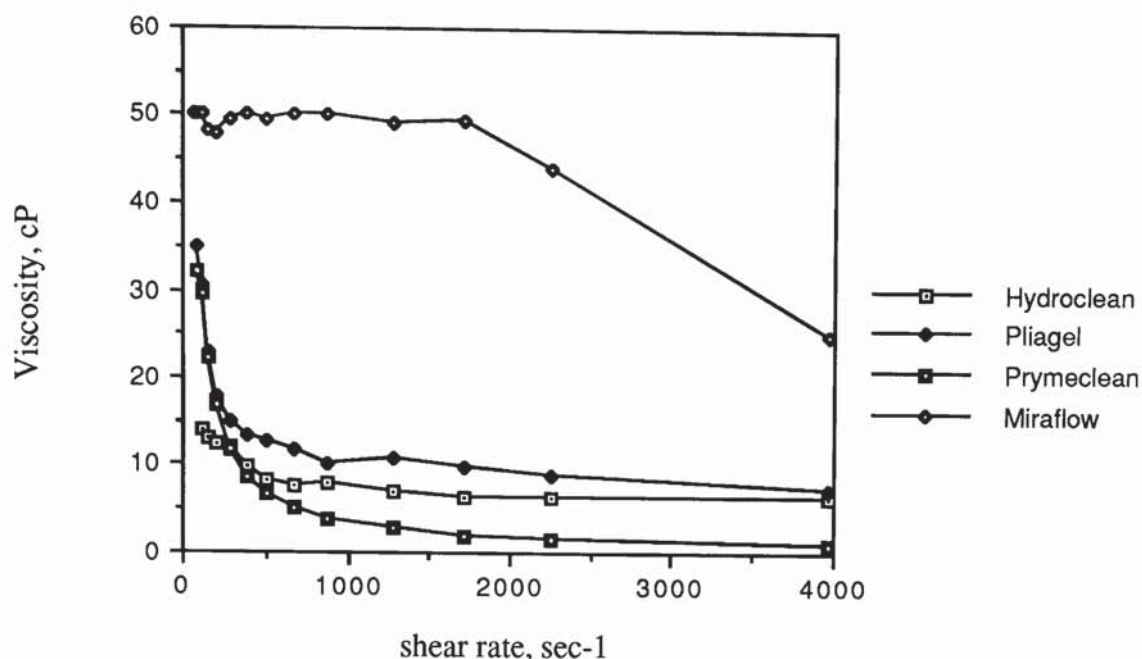
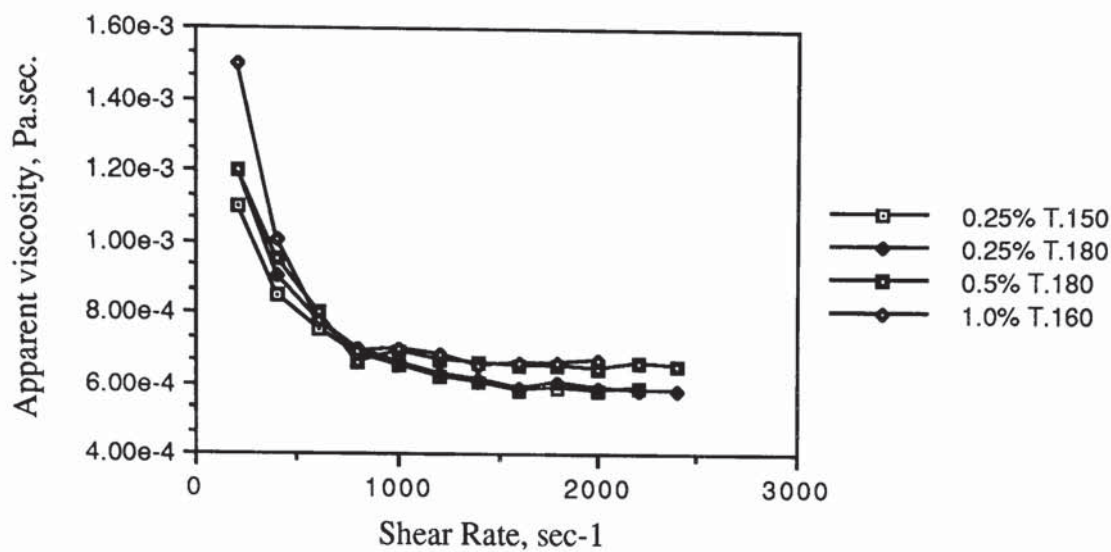


Figure 3.5 The effect of increasing shear rate on the viscosities of contact lens cleaning solutions

As Figure 3.4 illustrates, most contact lens surfactant cleaners exhibit pseudoplastic, non-Newtonian, behaviour; as shear rates increase their viscosity rapidly diminishes. This behaviour does not affect the surfactant properties of the cleaning agent, but may facilitate the rinsing of solution from the cleaned lens. Miraflo, however, exhibits essentially Newtonian flow properties until shear rates above 2000sec^{-1} are reached. Again, this does not affect its cleaning ability but its high viscosity can give the impression of greater efficacy, and it is popular with contact lens wearers and opticians. Whether its viscosity makes it more difficult to remove from a lens surface has not been established.



("T" = torque, mNm⁻¹)

Figure 3.6 Effect of concentration on HPMC viscosity at increasing shear rate.

In Figure 3.6, the viscosity of different concentrations of HPMC were measured using the Carri-Med concentric cylinder rheometer, and high shear rates were achieved. The graph shows that the rate of shear-thinning is similar for all the solutions. At high shear rates the torque makes little difference to the measured viscosity of 0.25% HPMC, and the viscosities of 0.5% and 1% HPMC converge.

3.5 Contact Angles of Wetting Solutions

A comparison of the contact angles made by a range of contact lens care solutions was made. The angles of sessile drops were measured using a goniophotometer, and a range of surfaces with different surface activities were used.

As the results in the Table 3.5 show, none of the wetting solutions or artificial tear solutions, including the Aston Tear Model, has the capacity to spontaneously wet an hydrophobic surface. Mucin also exhibits higher contact angles than would be expected from a solution believed to wet a supposedly hydrophobic epithelial cell layer. With the measured contact angles of intermediate values, 40-60°, on a typically hydrophobic surface such as polythene, the artificial tear solutions would probably wet the surface with the help of physical spreading. If the ocular surface is indeed as hydrophobic as some researchers have suggested, the blink action would fulfill the role of spreading these solutions, and mucin, over the cornea.

Table 3.5 Contact angles of contact lens solutions

Solution	Glass	Melindex	Polythene	Perspex
Stericlens	57	61	62	59
One-Solution	40	53	61	58
Vistasoak	43	58	67	63
Clean & Soak	16	47	48	52
Sno Tears	27		65	52
Liquifilm	23	57	60	58
Tears Naturale	21	46	53	53
0.5% BSM	48	48	50	65
Tear Model	40	72	79	
Water	44	74	88	79

3.6 Summary and Discussion.

Solutions designed to be instilled into the tear film include artificial tears, therapeutic eye drops, and some contact lens solutions. These are formulated on the basis of the behaviour of solutions *in vitro*, and may not perform as expected in the eye. The tear fluid is a complex solution containing many different components, and these may interact with the instilled solution. Similarly, components of the introduced solution, especially surface active ingredients, can effect the stability of the tear film and the integrity of the ocular surface. If the surfactant content of an artificial tear solution was increased as a means to improve its wetting properties, which would be reflected by its producing smaller contact angles on a surface than mucin, it could disperse the ocular mucin and lipid layer, destabilising the tear film. This would result in the dry eye symptoms recurring frequently, encouraging the user to instill more artificial tears. In time, damage to the corneal epithelial cells could occur.

Studying the interfacial and surface chemical properties of ophthalmic solutions, and making use of models which mimic the ocular environment, provides valuable information on the likely effect these solutions will have once in the tear film. By using the surface and interfacial tension values measured for tears, it was possible to calculate the polar and dispersive components contributing to its total surface tension. These values (polar: 26.1 mNm^{-1} , dispersive: 17.5 mNm^{-1}) were proportionately similar to those calculated for the Aston Tear Model (polar: 31.9 mNm^{-1} , dispersive: 22.1 mNm^{-1}). The fairly high dispersive component, 40%, reflects the lipid and surfactant content of tears.

Contact angles provide a useful *in vitro* technique which can be used to predict

how solutions may behave in the eye. A comparison of the contact angles produced at the surface of different polymers by artificial tear solutions, a mucin solution, and the Tear Model was made. None of the solutions was able to spontaneously wet an hydrophobic surface, although the mucin solution made the smallest contact angles. The artificial tear solutions tended to produce lower contact angles than the Tear Model, but similar angles to those formed by the mucin solution. When these solutions are introduced into the tear film, we can predict from these results that they would interact with the mucin layer, probably increasing its thickness and thereby relieving dry eye symptoms. Although the rheological properties of artificial tears and wetting solutions do not provide for long-term retention in the tear film, the widespread and popular use of artificial tears and contact lens wetting solutions, nevertheless, indicates that they in some way provide relief from ocular discomfort for many users. The retention and drainage of ophthalmic solutions is considered in further detail in Chapter 7.

CHAPTER 4

**Investigations of the interaction of mucin with hydrogel
surfaces.**

4.1 Introduction

The innermost layer of the tear film is rich in mucin, the ocular glycoprotein, which is secreted by conjunctival goblet cells and specialised cells of the corneal epithelium. A deeper understanding of the relationship between the tear film and the corneal surface, and the role of mucin in this relationship, is essential in order to be able to develop drug formulations which will optimise the delivery of a drug to its target. The ocular mucins are widely believed to adhere to the corneal surface, and it is this interactive capacity which is held to be of central importance to the development of drug formulations for sustained drug delivery. Current systems of ocular drug delivery are very inefficient, in terms of both the frequency of application and drug concentration required, due to the rapid loss of therapeutic agent from the tear film through reflex tearing and drainage. It is anticipated that the development of vehicles capable of adhering to the conjunctival and corneal tissue, and/or to its mucin coating, may provide a means of improving the bioavailability of ophthalmic formulations by prolonging their retention in the tear film¹⁶⁷. Increasing the ocular residence time of a drug in this way maximises the time available for topical local action and also reduces any systemic side-effects the drug may have by minimising the proportion of drug lost through drainage¹⁴⁵. In addition, the drug concentration required to achieve therapeutic levels in the target tissue would be reduced thus minimising the risk of toxic side-effects which can occur with the high dosage levels used in conventional formulations.

The interaction between a polymer and a mucin surface, leading to a net attraction, is termed mucoadhesion. The concept of mucoadhesives arose from the need to increase the gastric residence time of orally delivered drug dosage forms¹⁶⁸. Although

recently more attention has been given to ocular applications of mucoadhesives, most work in producing and evaluating polymers which adhere to mucus membranes has concentrated on their potential use in oral drug delivery systems¹⁶⁷⁻¹⁷⁰.

The aim of the work presented in this chapter was to find a mucoadhesive polymer which could be used as a model cornea. It is a widely held principle in scientific research that information obtained with "model" systems (e.g. simplified synthetic equivalents) can throw light on the behaviour of the more complex original. This chapter presents preliminary investigations into the possibility of developing a corneal surface model using hydrogel polymers such as ethyl methacrylate. When this work was started it was thought that the most important property which this artificial cornea would have would be that mucin would adhere to its surface, in accordance with the overall consensus of published reports in this field that the cornea is covered with a layer of adherent mucin. Since little work has been carried out previously in this area, however, much of the work presented in this chapter has been necessarily concerned with methodology and the assessment of different techniques for monitoring mucin.

Assuming that mucin does adhere to the cornea, then the most likely class of polymers which will in this way mimic the cornea, which is a highly hydrated hydrogel, are the synthetic hydrogels. Contact lenses provide a convenient way of looking at the effects of different chemistries on mucin adhesion. Furthermore, mucin was regularly implicated in the "mucopolysaccharide debris" on worn contact lenses during the 1970s and '80s, so it was interesting to discover whether this would be borne out experimentally: whether mucin would adhere to both clean and spoilt lens surfaces. More recently it has

been suggested that an adherent layer of mucin coating a contact lens will inhibit further spoilage by proteins or lipids, much in the same way as the mucin-coated cornea is not contaminated by other tear components. If this is the case, then the biocompatibility of a contact lens might be determined by its ability to retain mucin, as will be discussed later. In addition, the toxicity data is known for these commercial materials, so they offered a logical starting point in the search for a potential drug carrier. Other hydrogels were also assessed for their ability to interact with mucin so that all the most commonly used hydrogel monomers were covered in this study.

For this work bovine submaxillary mucins (BSM) and porcine stomach mucin (PSM) were used since these are believed to be similar to human ocular mucin which is not easily available. BSM has been analysed and found to be 59% (dry weight) carbohydrate, while PSM is 90% carbohydrate; human ocular mucin is thought to consist of three glycoproteins (GP1, GP2, GP3M) and have an overall carbohydrate content somewhere between the values for PSM and BSM¹⁷¹. Separate studies, on the other hand put the proportion of carbohydrate of human ocular mucus at 53-55% of the dry weight¹⁷² and, alternatively, as over 65%¹⁷³. Five types of oligosaccharide residue are present: L-fucose (6-deoxy - L-galactose), D-glucose (6-deoxy - L-galactose), D-galactose, N-acetyl - D - glucosamine (2- acetamido - 2-deoxy - D-glucose); N-acetyl - D-galactosamine (2-acetamido - 2-deoxy - D-galactose), and sialic acid (N-acetylneuraminic acid). Either sialic acid or fucose occurs as the terminal monosaccharide of the carbohydrate side chains, with sialic acid providing mucin with its overall negative charge. The presence of sulphate residues, in the form of ester sulphate groups borne by other sugar residues, also contributes to the negative charge, making the glycoprotein a polyelectrolyte. There are, on

average, 8-10 monosaccharide residues per oligosaccharide side chain. There is very little detailed knowledge about the protein core of the mucin glycoprotein, except that the high density of carbohydrate suggests that the polypeptide is likely to be in an extended form. The structure of mucin is, therefore, believed to be a bottle-brush arrangement, with the relatively short carbohydrate chains forming the "bristles" as they radiate from the extended protein core. When freshly produced, porcine stomach mucin is believed to exist as four sub units linked by disulphide bridges to produce a tetramer of molecular weight 2.1×10^6 . This is illustrated in Figure 4.1. However, the difficulty of purifying mucin means that the composition of mucin from any source is variable, and it is possible that samples of mucin of the same origin, but from different batches, may have slightly different physical and chemical properties. The labelling of PSM as Type I, II or III, depending on purity and physicochemical characteristics illustrates this.

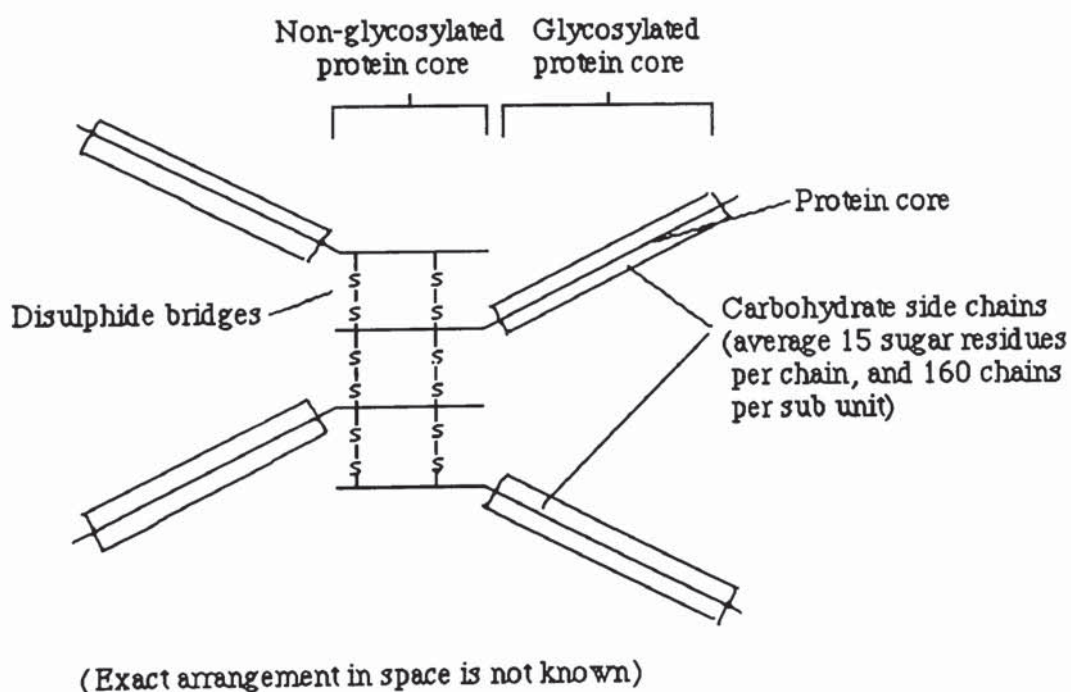


Figure 4.1 Structure of porcine stomach mucin¹⁷⁴
(molecular weight 2.1×10^6).

There is no satisfactory method, so far established, for the study of mucus glycoprotein interaction with polymeric materials. This work, therefore, presents the results of primary studies of mucin/polymer interactions and, necessarily, is concerned with assessing the value of the experimental techniques used. The techniques used in this work to investigate the interaction of these glycoproteins with a range of polymers include the use of dyes, Alcian blue and Basic yellow, which are reported to be specific for glycoproteins. The polymers studied cover a range of hydrogels with different water contents and component chemical groups, the significance of which will be discussed later. Microscopy, both phase contrast and fluorescence, and fluorophotometry were used to study the stained mucins and assess whether they adhere to these polymer surfaces. Again, it should be emphasised that the aim of this work, in addition to pinpointing mucoadhesive hydrogels, was to design a successful technique for monitoring mucin on hydrogels.

By finding a polymer with which mucin would interact in a way similar to the process occurring at the surface of the eye, it was anticipated that this polymer could be used as a model cornea. As such it could be used, in conjunction with the tear model developed at Aston, to investigate the effect of different ophthalmic formulations on tear stability. It was also anticipated that this muco-adhesive polymer could be used to develop an ophthalmic formulation which would be retained in the ocular environment for greater lengths of time than are achieved by conventional formulations, 50% of which are often lost through drainage within the first few minutes¹⁴⁵.

4.2 Investigation of Mucin Adhesion to Polymers.

Mucin has been reported to adhere equally well to all polymer surfaces, including glass. However, other studies indicate that polymer properties, particularly charge sign and functional group characteristics, play an important role. A study by Leung and Robinson (1990)¹⁵⁵ suggests, for instance, that the strength of mucin adhesion to a polymer will be increased by raising both the polymer chain segment mobility and the number of hydrophilic functional groups. These factors are related since by increasing the number of charged, water-binding groups, mucoadhesion is increased because hydrating and expanding the polymer network exposes sites available for hydrogen bond formation, as well as mobilising the polymer chains for interpenetration.

The ability of mucin to interact with a range of polymers of various composition is investigated here, to discover whether mucin does interact equally with different surfaces - hydrophobic and hydrophilic, charged and uncharged. The polymers used were chosen to encompass these different properties. A further aspect addressed by this work is the extent of mucin interaction with contact lens materials, and the relation of this to lens spoilage and tear stability in contact lens wearers. It was assumed in the early part of the last decade¹⁷⁵ that mucopolysaccharides interact preferentially with the hydroxyl groups of hydrogel materials, leading to increased spoilage by mucopolysaccharide debris and subsequent protein deposition. In general, higher water content lenses are found to spoil more rapidly and to a greater extent than lower water content lenses. However, the picture painted by Ruben was shown to be overly optimistic since there is no relationship between hydroxyl content of the lens material and spoilage. In fact, higher water content lenses contain more water and less polymer for a given surface area than do low water content

materials, and tend to have some of the hydroxyl groups replaced by groups such as vinyl pyrrolidone. Thus, research has shown that the relationship between contact lens and extent of spoilage is not determined by water content alone. In addition, different contact lenses with similar water contents can produce very different levels of protein deposit, because other factors such as the ionic character of the lens material play an important role in spoilage processes¹⁷⁶.

It has been proposed more recently, that a contact lens polymer which will support a layer of adherent mucin will be truly biocompatible, and that this layer will inhibit spoilage by other tear components, in the same way that the mucin-coated cornea is not contaminated with tear proteins or lipids. Whatever the truth behind these conflicting claims, a lens producing as little disturbance to the structured tear film as the cornea would be most compatible. Thus, in leading to a greater understanding of these conflicting aspects of hydrogel biocompatibility, this investigation makes a potential contribution to the improvement of ocular drug delivery systems and to the establishment of the necessary properties of an ideal contact lens.

A range of polyethylene oxide - containing terpolymers were also used in this work since a recent study has shown that the resistance to deposition (in an *in vitro* tear model) of poly 2-hydroxyethyl methacrylate/ethylene glycol dimethacrylate (HEMA/EGDM) (99:1) based copolymers is increased by incorporating polyethylene oxide side chains¹⁷⁹. The effect of methoxy-terminated polyether side chains (methoxy polyethylene glycol methacrylate (MPEGMA)) and hydroxy-terminated polyether side chains (polyethylene glycol 10 units ethylene oxide (PEG10EO) and hexapropoxylated

hydroxypropylmethacrylate (HHPMA)) on protein and lipid deposition on the hydrogels differs¹⁷⁹, so these are compared with respect to mucin adhesion. Preferential mucin adhesion to polyethylene oxide-modified polymers could produce greater biocompatibility and, by the preceding argument, be used to make the ideal contact lens.

The aim of this work was, firstly, to develop a suitable technique for monitoring mucin, and, secondly, to identify a suitable polymer to which mucin adheres and which, therefore, would have potential as a corneal model and drug carrier.

Alcian blue and Basic yellow dyes were used to detect mucin, being generally accepted as specific for acidic mucopolysaccharides. Preliminary investigations were carried out in order to assess the best means of staining mucin on hydrogels using these dyes.

4.3 Detection of Mucin Deposits on Polymers using Alcian Blue

This dye was chosen since it is reported to be specific for sulphated and sialo-mucins. The structure of the dye (Figures 2.5 and 2.6) and staining techniques are given in Chapter 2. Further details of the methods used are outlined where necessary.

4.3.1 Detection of Mucin Using the Alcian Blue - Periodic Acid/Schiff's (PAS) Method.

Fresh 0.5% solutions of bovine submaxillary mucin (BSM) and porcine stomach mucin (PSM) were prepared in distilled water, and smears were made on glass slides. Lillie's fixative was used as a suitable fixative, and this was made up as described

made up as described in Chapter 2.

The prepared smears were left to fix in this solution for 24 hours at room temperature. The smears were then rinsed briefly in 3% aqueous acetic acid and stained for 2 hours in 1% Alcian blue 8GX solution (made up in 3% acetic acid as described in Ch.2). After removal from the dye they were rinsed briefly in water and then in 3% acetic acid, running water and distilled water. The stained slides were then oxidised in 1% periodic acid (aqueous) at room temperature for 10 minutes and washed in running water for 5 minutes. They were next immersed in Schiff's reagent for 10 minutes, washed once more in running water for 2 minutes, and finally rinsed in 3 changes of 0.5% sodium bisulphite, 1 minute each. The stained smears were dehydrated in 70%, 90% and absolute alcohol, and cleared in xylene. A 'control' stain was also prepared using a clean glass slide. The mucin smears were examined using the light microscope. A blue, granular stain (positive) was apparent for mucin, as shown by the micrograph in Figure 4.2, while the controls remained clear.

4.3.2 Preliminary Investigations, Using the Alcian Blue-PAS Method, of Detecting Mucin on Different Hydrogel Surfaces.

Poly 2-hydroxyethyl methacrylate (HEMA) lens (38.6% water), methyl methacrylate (MMA) / vinyl pyrrolidone (VP) (74% water), and poly HEMA / VP (71% water) lenses were left to soak in 0.5% BSM type 1-S for 48 hours before staining. The same three types of lens were also left to soak in 0.5% PSM for 42 hours and stained. Prior to staining the hydrogel samples were removed from the mucin solution and air dried (without rinsing) in a dust free environment. Poly HEMA, the hydrogels hydroxypropyl acrylate/ 2-ethoxyethyl methacrylate/styrene (HPA/EEMA/ST) (60:30:10), HEMA/MMA

(70:30), and HEMA:MMA (80:20), an Aquaflex tetrafilcon lens (copolymer of HEMA, VP and MMA), a Eurothin lens (HEMA), and were used as 'controls' by staining them with Alcian blue with no prior application of mucin. Further details of these materials, including their water contents can be found in Tables 2.2 and 2.3.

For Alcian blue in the presence of the Schiff's reagent, a pale blue stain indicates periodate unreactive, Alcianophilic substances, acid mucosubstances, including hyaluronic acid and sialomucins. A purple stain indicates periodate reactive and Alcianophilic substances. The Alcian blue staining mechanism is described in more detail in Chapter 2 (section 2.4.2). The results of staining the range of hydrogel materials are shown in Table 4.1 and are illustrated by the micrograph in Figure 4.3.



Figure 4.2 Porcine stomach mucin smear (fixed on glass slide) stained with Alcian blue (x 125)

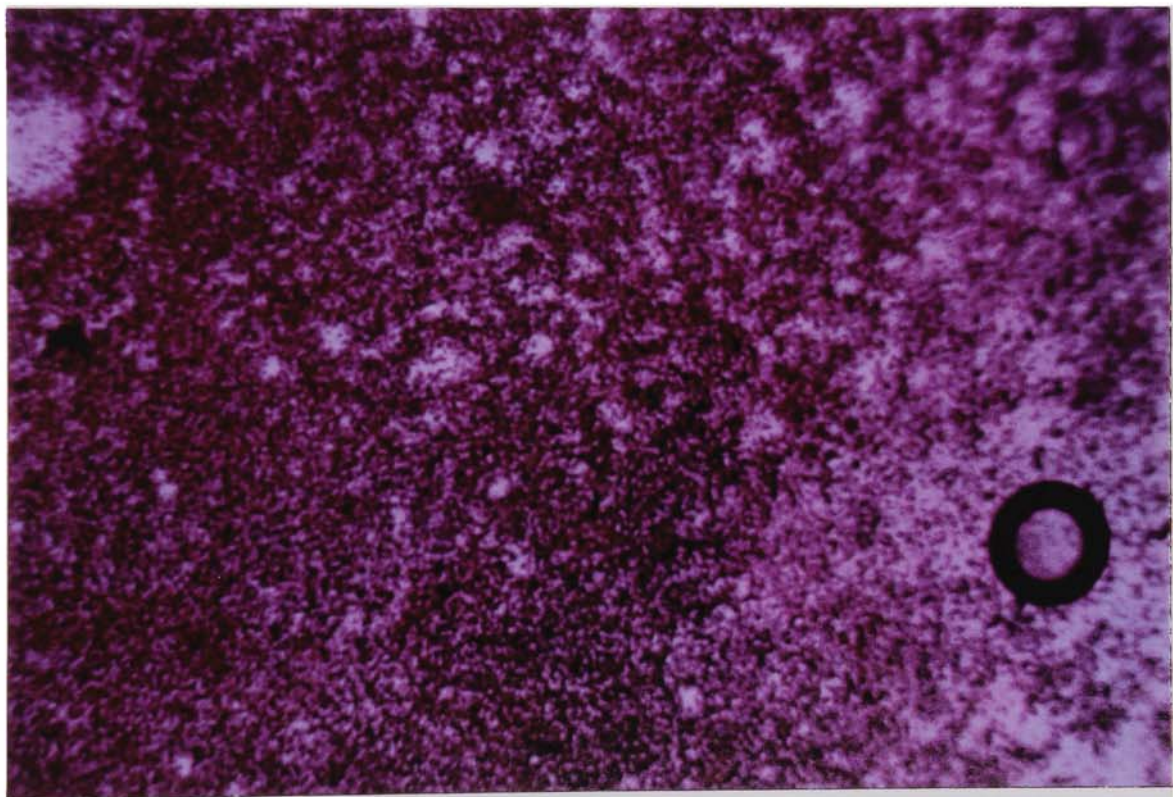


Figure 4.3 PSM fixed on a hydrogel surface, stained with Alcian blue.
(x 125)

As the table of results and Figure 4.3 show, hydrogels tend to absorb Schiff's reagent and turn pink which makes the presence of mucins more difficult to ascertain. The difference in stain colour between HEMA and MMA/VP or HEMA/VP may be due to the hydrogel matrix structure and/or its components. The hydrogels containing VP seem to take up more of the stain which probably affects the final stain colour after addition of mucin. Attempts to remove stain from hydrogel matrices involved varying the strength of the reducing agent, sodium bisulphite. Although 20% sodium hydrogen sulphate (NaHSO_3) removed much of the stain from HEMA, the rinsing and dehydration processes caused a return of the pink colour. Sodium hypochlorite solution, NaOCl , was also used and removed the pink stain from HEMA/EGDM but not entirely from HEMA. The results of this study are in agreement with a previous investigation (Klein, 1989)¹⁸⁰ which concluded that a mucin-specific PAS reaction could not be distinguished from the nonspecific colouration that develops in the hydrogel lens.

Table 4.1 Effect of Alcian blue staining on various hydrogels

<u>Material</u>	<u>No mucin</u>	<u>0.5% PSM,42h</u>	<u>0.5% BSM, 48h</u>
Poly HEMA	pink	pale blue	pale pink
MMA/VP (70:30)	pale blue	purple	pale purple
MMA/VP (80:20)	pale blue	pale purple	pale purple
HEMA/VP	pink	purple	purple
Aquaflex(HEMA/VP/MMA)	pink		
Eurothin (HEMA)	pink		
Hydron (HEMA)	pink		
HEMA/EGDM(90:10)	pink		
Melinex	no stain	blue	blue
HPA/EEMA/ST (60:30:10)	pale blue		

The Schiff's reagent appears to be the cause of hydrogel discoloration so, in the following work with Alcian blue, a different technique was used which does not include the Schiff reagent. This is described below.

4.3.3 Detection of mucin interaction with polyethylene oxide-modified hydrogels.

Recent work has shown that incorporation of polyethylene oxide side chains by copolymerisation with polyethylene oxide enhances the biocompatibility of hydrogels¹⁷⁹. It is possible that increased ocular tolerance of these materials (with less resulting

deposition of protein and lipid species) is achieved through their ability to support an adherent layer of mucin. This theory is based on the observation that the mucin-covered corneal and conjunctival epithelia do not become impaired by protein or lipid contamination. A range of these polyethylene-modified hydrogels were examined for any mucoadhesivity.

Hydrogels containing different chain lengths of methoxy polyethylene glycol methacrylate (MPEGMA) were immersed in a 0.5% mucin for 4 hours at room temperature and then for 5 days at 0-5 C. This range of PolyHEMA/EGDM (90:10) copolymers contained either 5, 10, 15, or 20% of one of the following components: MPEG200 MA, MPEG400 MA, and MPEG1000MA, making twelve samples in total. These hydrogels differ with respect to the polyethylene chain lengths incorporated into the terpolymer; MPEG200MA has 4 to 5 repeating ethylene oxide groups, MPEG400MA has 9 to 10 repeating groups, and MPEG1000MA has 22 to 23 ethylene oxide groups per monomer. Their equilibrium water contents (EWC), which range from 26.7% to 34.6%, are recorded in Chapter 2.

On removal from the mucin solution, the samples were rinsed in distilled water and immersed in 3% acetic acid for 3 minutes, then soaked for 2 hours in 1% Alcian blue solution. After dipping the samples in distilled water, they were then rinsed for 5 minutes in 3% acetic acid and, finally, in running distilled water. The stained hydrogel samples were examined using phase-contrast microscopy. This series of hydrogels did not take up the Alcian blue dye to the same extent as the HEMA and MMA/VP copolymers used in the previous test (Table 4.1), although this may be due to the omission of Schiff's reagent

from the staining technique. Very little staining was observed on any of the hydrogels examined by phase contrast microscopy, there being a slight blue stain on the 5% and 10% MPEGMA (molecular weights 200, 400 and 1000), but none on the terpolymers containing higher percentages of polyethylene. From this, it may be inferred that little or no mucin had become adsorbed onto the polymer surfaces during their incubation with the mucin solution.

Thus, while being fairly specific to mucins (it did not stain other carbohydrate-based substances tested such as hydroxyethyl cellulose and mucic acid), Alcian blue is, overall, unsuitable for detecting the presence of mucin on a hydrogel surface since many hydrogel matrices tend to absorb the stain itself. Nevertheless, the absence of staining is a true negative result, and could be used as proof that no mucin is present.

4.4 Fluorescence Studies of Mucin-Hydrogel Interactions

The azo dye Basic yellow was used to fluorescently label mucin. It was chosen because it is reported to be specific for mucopolysaccharides, causing them to fluoresce in the region 530-580nm. The method of staining is described in Chapter 2, although further details are given here. Fluorophotometry provides a semi-quantitative method of fluorescence analysis in that the height of the emission peak recorded is proportional to the concentration of fluorescent material in the path of the excitation beam. In the tables of results presented in this chapter, the level of fluorescence is expressed as a percentage. This was calculated from the spectra by measuring the maximum height above the baseline which the chart recorder will reach (i.e. height of reference peak at 360nm) and, using this as a reference point identified as 100% fluorescence, the height of each fluorescence peak is

then calculated as a percentage of this.

4.4.1 Effect of Mucin Type and Concentration on Fluorescence with Basic Yellow.

In order to stain mucin on a surface, it is sufficient in most cases to apply a few drops of 0.01% Basic yellow (BY) solution. The use of this dye enables mucin to be detected by fluorophotometry or by fluorescence microscopy. The fluorescence spectra of different concentrations of Basic yellow dye relative to mucin concentration were recorded by connecting a chart recorder to the fluorophotometer. Maximum fluorescence was found to result from a mucin to dye ratio of 3:1, containing solutions of PSM and Basic yellow, 0.5% by weight and 0.1% by weight, respectively. This solution gave 100% fluorescence (a peak height of 16cm at 530nm on the chart recorder) with the fluorospectrophotometer slit widths set at 2.0mm (emission) and 0.2mm (excitation). The excitation wavelength was 360nm. Table 4.2 shows the relative degree of fluorescence obtained with different solutions.

PSM type II showed less fluorescence activity than the type III mucin, but more than the bovine mucin. Most activity occurred with a 4:1 solution of 0.5% PSM type II and 0.1% Basic yellow, which showed 59.4% fluorescence relative to the maximum shown by the typeIII PSM solution above.

Table 4.2 Fluorescence of PSM and BSM in different aqueous environments.

<u>Solution</u>	<u>pH</u>	<u>Fluorescence (%)</u>	<u>Wavelength (nm).</u>
0.5% PSM (in water)	6.0	11.3	430
0.5% PSM (in PBS)	7.5	6.3	430
0.5% PSM(H ₂ O) + BY, (3:1)		100.0	580
0.5% PSM(PBS) + BY, (3:1)		7.2	580
0.5% BSM (in PBS)	7.5	0	580
0.5% BSM (in water)	6.0	0	580
0.5% BSM(H ₂ O) + BY, (3:1)		0	580
0.5% BSM(PBS) + BY, (3:1)		9.6	580

Values given in the table, and in all the fluorescence tables, are percentages of maximum (full scale) peak height, measured from the spectra. Figure 4.4 shows the fluorescence of different PSM concentrations stained with 0.1% Basic yellow solutions. In each case 1ml 0.1% Basic yellow was added to 3ml mucin solution.

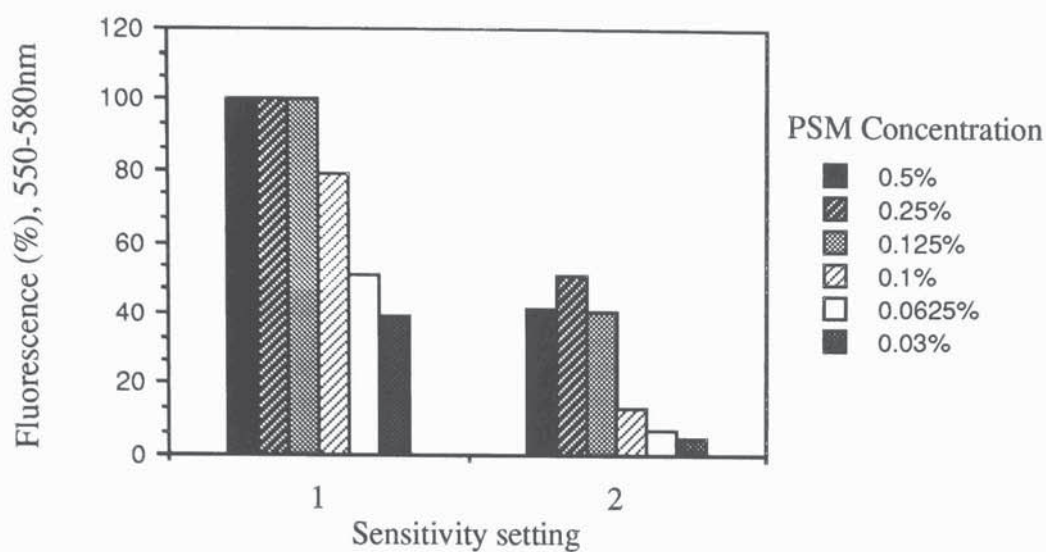


Figure 4.4 Relative fluorescence of different mucin concentrations stained with Basic yellow.

Sensitivity setting 1 refers to an excitation slit width of 0.5mm, and setting 2 to an excitation slit width of 0.2mm. For both sets of readings full scale was used and the emission slit width was set at 2.0mm.

In the following investigations mucin was tested for adhesion to a range of polymers, chiefly hydrogels. Samples of the polymers were immersed in mucin solutions, some of which were fluorescently tagged with Basic yellow. After a given time the polymer was stained with Basic yellow (if it had been in an unlabelled solution) and examined for fluorescence using the fluorospectrophotometer.

4.4.2 Mucin Adhesion to Hydrogels - Assessment of Fluorescence Spectra.

In order to investigate whether mucin adheres to the surfaces of hydrogels, such as those commonly used in the manufacture of contact lenses, samples of different hydrogels were exposed to 0.5% aqueous solutions of porcine stomach mucin (PSM) and 0.5% aqueous solutions of bovine submaxillary mucin (BSM). The mucin solutions were fluorescently labelled by adding 0.1% aqueous Basic yellow dye (BY). These fluorescent mucin solutions were prepared, using the results outlined in Table 4.2 in order to obtain maximum fluorescence. Thus, the porcine stomach mucin was dissolved in distilled water, and 0.1% Basic yellow added in the ratio 3:1 (v/v); the bovine submaxillary mucin was dissolved in phosphate buffered saline (PBS) and 0.1% Basic yellow added in the ratio 3:1.

The fluorescence results are, as usual, expressed as percentages, as measured from the spectra obtained. Measurements were taken at 530-550nm, the range at which the fluorescently-stained mucin has an emission peak. Samples were excited at 360nm.

Table 4.3 Fluorescence (%) at 530-550nm of different hydrogel types, immersed for different lengths of time in a fluorescent mucin solution, as an indication of mucin adhesion.

<u>Hydrogel</u>	Solution and length of immersion		
	PSM/BY(H ₂ O),	BSM/BY(PBS),	PSM/BY(H ₂ O),
	2 days	2 days	21 days
HEMA/MMA 70:30	10.6	4.4	3.1
HEMA/MMA 80:20			1.2
HPA/EEMA/ST 60:30:10	1.0	0	1.0
HEMA/MAA 75:25			5.0

PolyHEMA, melinex (polyethylene terephthalate) and polythene samples showed no fluorescence following immersion for 2 days in either the PSM or the BSM solutions. This infers that no mucin had become adsorbed on to the surfaces of these samples, and contradicts statements elsewhere in the literature that mucin adheres to all hydrophobic polymer surfaces³⁰.

4.4.3 Detection of Mucin Adhesion to Polyethylene Oxide-Modified Hydrogels using Fluorescence

This work is a further investigation, following on from the earlier study using Alcian blue, into the ability of mucin to adhere to hydrogels containing polyethylene oxide (PEO). It was anticipated that the use of the fluorescent dye, Basic yellow, would provide a more effective and semi-quantitative method of detecting the presence of mucin on or within a hydrogel matrix.

Poly HEMA/EGDM (90:10) plus 5%, 10%, 15% and 20% of each of MPEG 200 MA, MPEG 400 MA and MPEG 1000 MA samples were tested for mucin adhesivity. The structure of the MPEGMA monomer is illustrated in Chapter 2 (Figure 2.2). The MPEG 200 MA, MPEG 400 MA, and the MPEG 1000 MA monomers contain chain lengths of 4-5 units, 9-10 units, and 22-23 units of ethylene oxide per monomer, respectively. Samples of the hydrogels were placed in 0.5% BSM for 7 days and then stained using Basic yellow.

Table 4.4 Fluorescence (%) at 530-550nm to show the effect of MPEGMA chain length and concentration in a HEMA/EGDM(90:10) copolymer on mucin adhesion.

	MPEG MA chain length		
	MPEG200 MA	MPEG400 MA	MPEG1000 MA
<u>MPEG MA concentration (%)</u>			
5%	1.9	2.5	1.3
10%	7.0	0	5.1
15%	1.9	-	1.9
20%	1.9	0	1.9

As table 4.4 shows, very little fluorescence was measured for any of the samples, with lowest activity occurring with those samples containing higher percentages of MPEG. Mucin does not appear to interact significantly with these polymers.

To investigate the effect of water content on mucin adhesion, a series of hydrogels containing polyethylene oxide side chains of uniform length (10 units per monomer), but increasing water content were studied. The following HEMA/EGDM (99:1) hydrogels were polymerised with polyethylene glycol monomethacrylate 10 moles EO (PEG MA 10 EO):

HEMA/EGDM (99:1) + 5% PEGMA 10 EO; HEMA/EGDM (99:1) + 10% PEGMA 10 EO; HEMA/EGDM (99:1) + 15% PEGMA 10 EO; and HEMA/EGDM + 20% PEGMA 10.

This series of terpolymers, with their lower crosslinker concentration, has been found to

have a greater ocular compatibility (reduced spoilation) than the corresponding HEMA/EGDM (90:10) series. This effect, which becomes greater as the molecular weight of PEG increases, is thought to be the result of an increase in the water content of these hydrogels (37-50% for 99:1 series, compared with 26.5-35% for the 90:10 series) combined with greater chain mobility¹⁷⁹.

Table 4.5 The effect of PEGMA 10 EO concentration on equilibrium water content.

<u>Copolymer composition</u>	<u>EWC (%)</u>
HEMA:EGDM (99:1) + 5% PEGMA 10 EO	38.4
HEMA:EGDM (99:1) + 10% PEGMA 10 EO	39.6
HEMA:EGDM (99:1) + 15% PEGMA 10 EO	41.0
HEMA:EGDM (99:1) + 20% PEGMA 10 EO	42.1

The materials were carefully cleaned in a 1% Tween detergent solution, rinsed and left to soak for 24 hours in distilled water. Discs of the hydrogels were cut using a size 6 (12 mm diameter) cork borer. Two discs of each terpolymer sample were placed in a fluorescent solution of 0.5% PSM with 0.1% Basic yellow (3:1), and a third disc was placed in a 0.01% Basic yellow solution. For each terpolymer, emission spectra were recorded for one of the two hydrogel discs after 90 minutes in the fluorescent mucin solution, and for the second disc after 4 hours in the solution. Spectra were recorded for the discs in the Basic yellow dye after 4 hours.

Table 4.6 Fluorescence (%) recorded at 530-550nm to show the effect of PEGMA 10 EO concentration in a HEMA/EGDM (99:1) hydrogel on mucin adhesion.

Treatment	Concentration of PEG(10EO)MA in membrane			
	5%	10%	15%	20%
<u>Clean Hydrogel</u>	2.5	2.8	2.0	2.5
Time immersed in				
<u>PSM/BY(3:1):</u>				
90 mins	31.5	16.0	31.0	16.0
4 hours	18.5	17.0	20.5	18.0
0.01% BY:				
4 hours	24.0	15.0	22.0	13.5

Values in the table are percentage maximum fluorescence at 530nm. Fluorospectrophotometer settings: (0.3 (full scale), slit widths: emission 2.0mm and excitation 0.5mm). These results are shown graphically in Figure 4.5.

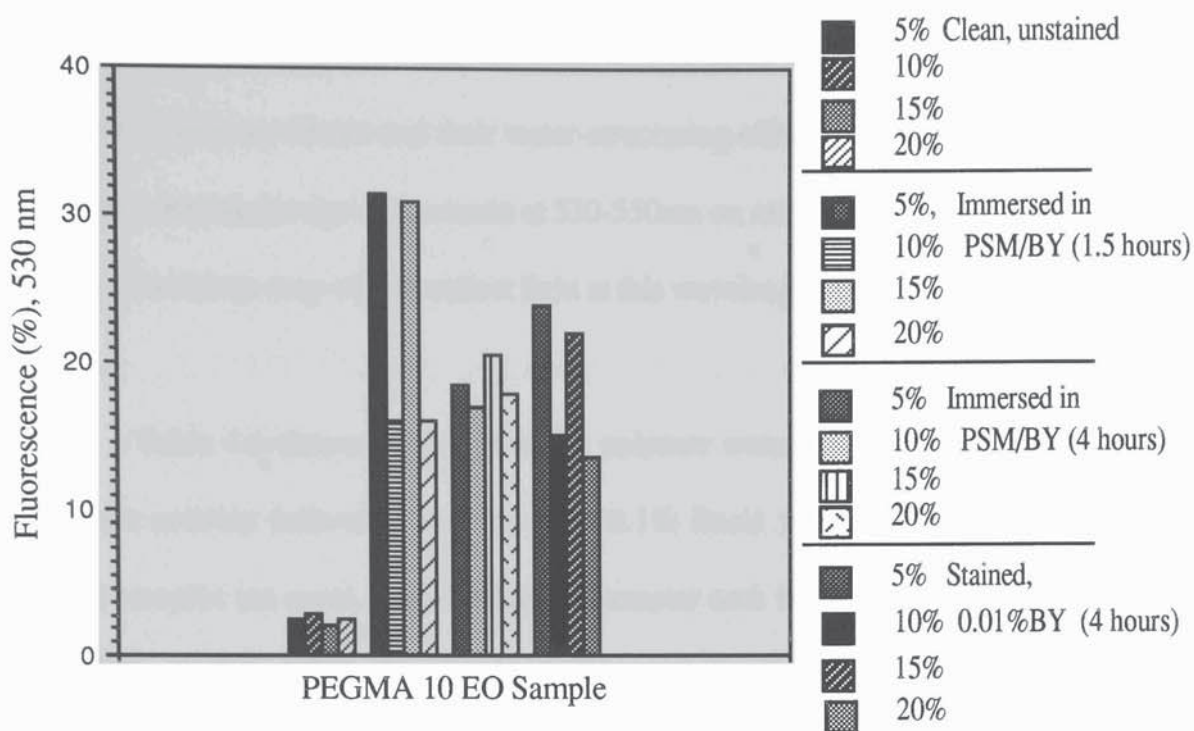


Figure 4.5 Detection of mucin adhesion to PEGMA 10 EO hydrogels using Basic yellow.

At first glance, the fluorescence results of the ethylene oxide-containing hydrogels seem to show some evidence of mucin adhesion. The hydrogels containing 5% PEG(10EO)MA and those containing 15% PEG(10EO)MA appear to be more mucoadhesive than the hydrogels containing 10% or 20% PEG(10EO)MA. This is interesting since it indicates that the relationship of mucin adhesivity to increasing water content, or decreasing polymer density, is not straightforward. However, it is apparent that the samples stained with Basic yellow solution (without mucin) also produce similar emission spectra to those obtained for the samples exposed to mucin. This, too, is surprising since the 0.01% Basic yellow solution does not fluoresce at 530-550nm when

excited at 360nm, although it does show some activity at 480nm, detected at the sensitivity setting used for this analysis. The conformation of these ethylene oxide (OCH₂CH₂) groups in the polymer chains and their water-structuring effect may provide the necessary conditions to enable the dye to fluoresce at 530-550nm or, alternatively, it could be that the polymers themselves may emit or reflect light at this wavelength.

Table 4.6 shows the effect of the polymer water content upon the degree of fluorescent activity following staining with 0.1% Basic yellow dye. For this study, hydrogel samples (as usual, cut with 12mm diameter cork borer) were left in a dust free environment to dry. When the samples had lost all the free water in their matrices by evaporation and had become reduced in size and brittle, they were immersed in 0.1% Basic yellow solution for two minutes, rinsed in distilled water, and analysed by fluorophotometry using the Aminco-Bowman fluorospectrophotometer.

Table 4.7 Fluorescence (%) at 530nm of PEGMA 10 EO hydrogels to show the effect of dehydration on fluorescent activity.

	HEMA/EGDM (99:1) containing:	
	5% PEGMA 10 EO (EWC = 38.4%)	20% PEGMA 10 EO (EWC = 42.1%)
Clean hydrogel	27.2(480nm)	30.0(480nm)
Stained with Basic yellow	16.8	11.4
Dehydrated, then stained	44.3	26.3

From the results shown in Table 4.7, it would seem that the level of fluorescent activity recorded following staining with Basic yellow solution is affected by the water content of the hydrogel. The fully hydrated hydrogel has a relatively expanded network, with the water having a structuring effect on the distribution of the polyether side groups. When the hydrated material is placed in a solution of Basic yellow, there will be an exchange of some water molecules for Basic yellow, which is small enough to be absorbed into the hydrogel matrix, eventually leading to an equilibrium dye concentration in the matrix. In the dehydrated material, on the other hand, the dye will be taken up very rapidly into the matrix, with no concurrent water exchange, so that the equilibrium dye concentration will be reached much earlier following exposure to the dye. It is possible, therefore, that the hydrated hydrogels had not reached their equilibrium dye concentration when analysed for fluorescence whereas the previously dehydrated materials had done so. In the dehydrated hydrogel, the matrix is collapsed and there is a greater density of polyether groups for a given area of hydrogel surface. Another possible explanation of the greater fluorescent activity of these samples is that it is some effect of the polyether groups which is causing this series of hydrogels to fluoresce with Basic yellow. The increase in side chain density could cause the increase in activity shown by the dehydrated polymers by binding more of the dye. Both Basic yellow solution and the hydrogels do exhibit some fluorescent activity at 480nm at this sensitivity, so the concentration of dye in the matrix and the effect of the material may be to increase and shift this peak to 530nm, with the level of fluorescence reflecting the capacity of the material to absorb the dye. Alternatively, the effect could be explained by the higher refractive index of lower water content materials. This, combined with an increase in reflectance, may be sufficient to produce the observed rise in fluorescent activity.

4.4.3.1 Mucin adhesion to PEO-modified hydrogels containing Hexapropoxylated hydroxypropyl methacrylate (HHPMA)

This study aimed to investigate whether mucin would show any specific interaction with surfaces containing HHPMA which have a lower water content than HEMA and PEGMA polymers, and 7 units of polypropylene oxide per monomer repeat unit.

The membranes used were a range of copolymers of HEMA (+ 1% EGDM) with 10%, 15%, and 20% HHPMA (wt/wt), with equilibrium water contents of 35.0%, 34.2%, and 33.6%, respectively. Four 14mm discs of each membrane were cut, washed in 1% Tween (3 hours), rinsed in several changes of distilled water, and left to soak in distilled water in order to remove any unreacted monomers.

0.5% solutions of PSM and BSM were prepared in phosphate-buffered saline.

The following staining solutions were also prepared:

0.1% Alcian blue 8GX; 0.01% Basic yellow; 0.5% PSM + 0.1% Basic yellow (3:1).

Two of each of the membrane discs were left to soak in the PSM/BY solution for varying lengths of time and tested for fluorescence in the fluorospectrophotometer. The PSM/BY solution exhibited 100% fluorescence using full scale with 0.2mm and 0.5mm (excitation) slit width settings. Table 4.8 shows the results of this study.

Table 4.8 Fluorescence (%) at 530nm of hydrogels containing HPHPMA measured to investigate the effect of length of exposure to mucin, and HPHPMA concentration, on mucin adhesion to hydrogel surfaces.

<u>Time in PSM/BY</u>	% HPHPMA					
	<u>(0.2mm slit width)</u>			<u>(0.5mm slit width)</u>		
	10	15	20	10	15	20
150 mins	1.9	2.5	2.5			
270 mins	3.1	2.5	3.8			
20 hours	2.5	-	-	21.5	18.3	22.2

From these results it appeared that time had little effect upon the degree of fluorescence. The apparent amount of adherent mucin is small. However, it was evident that by altering the slit width settings the sensitivity to fluorescent activity could be increased. A further experiment was carried out in order to investigate further the effect of method and HPHPMA concentration on apparent mucin adhesion to the polymers, the results of which are shown in Table 4.9. The fluorescence was measured using sensitivity settings of 0.3 (full scale), 2.0mm (emission slit width), and 0.5mm (excitation slit width).

At first mucin appeared to show some adhesion to the membrane samples containing 15% and 20% proportions of HPHPMA. However, as the experiment proceeded it became clear that other factors could be effecting the level of fluorescence

activity recorded. Soaking in PBS actually seemed to increase the fluorescent activity of the hydrogels containing 15% and 20% HPHPMA, so this effect was investigated further.

Table 4.9 Effect of method and HPHPMA concentration on fluorescence at 530nm.

<u>Treatment</u>	<u>10% HPHPMA</u>	<u>15% HPHPMA</u>	<u>20% HPHPMA</u>
(1) PSM/BY			
90 min. soak	19.5	22	63
(2) PSM/BY			
19 hour soak	31.5	50	38.5
(3) PSM/BY			
19hr. + PBS shake	16	14.5	12.5
(same sample as (2))			
(4) PBS			
second rinse	18	19.5	30.5
(5) PBS soak(2hr.)			
+ Shaken in			
1% Tween	18	27	25.5
(6) Digital clean			
LC-65 (30 sec)	14	15.5	11.5
(7) PBS soak			
20 hours	12.5	40.5	48.5

N.B. Procedures (2)-(7) were performed upon the same samples. Tween was found to

fluoresce, also at 530 nm. LC-65 was chosen as a surfactant cleaner since it has been found to be the most effective commercially available contact lens cleaner.

Figure 4.6 is a graphical representation of the results obtained for the hydrogels containing 5% and 15% HPHPMA. For both materials there is an increase in fluorescent activity following immersion in a fluorescently labelled solution of 0.5% PSM. This is dramatically reduced by shaking in phosphate buffered saline (PBS). Although the level remains above that of the clean hydrogel sample, it is reduced to less than the fluorescence recorded for the clean hydrogels which had been stained with Basic yellow. The increase in fluorescence following cleaning with Tween detergent may be attributed to the native fluorescence of this surfactant. Table 4.10 shows a comparison between the effect of rinsing these HPHPMA-containing samples with water and with buffered saline. There seems to be little difference in the degree of fluorescence following treatment with water and with saline, indicating that another factor is causing the observed increase in fluorescence after rinsing to take place. Furthermore, it is apparent that there is little difference between the heights of the emission peaks recorded for the hydrogels containing 5, 10 and 15% HPHPMA. This consistency in fluorescence continues even following the use of cleaning agents. Increasing the concentration of HPHPMA in the polymer matrix has the effect of reducing the equilibrium water content (EWC) of the hydrogel. HEMA/EGDM (99:1) with 5% HPHPMA, for instance, has an EWC of 37.1%, while increasing the HPHPMA content to 20% results in a hydrogel with an EWC of 33.6%¹²⁶. All the HPHPMA-containing hydrogels studied had EWCs which are lower than the EWC of polyHEMA (38%). Reducing the water content of such polymers has the effect of increasing their reflectance through the frequent presence of gas bubbles in the membrane,

and also causes an increase in the relative refractive index.

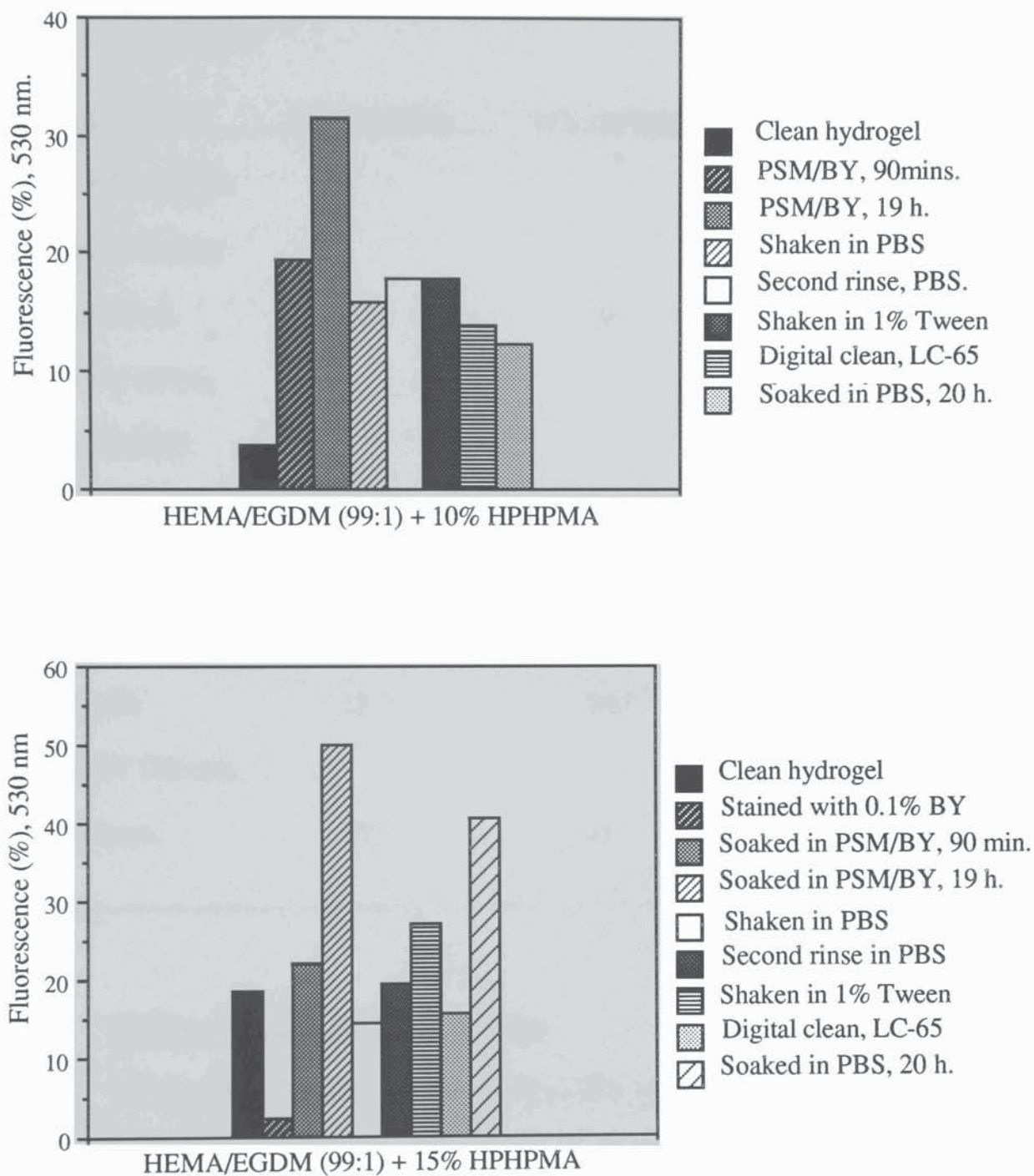


Figure 4.6 Fluorescent activity of hydrogels containing HPHPMA after exposure to mucin, and following cleaning procedures.

Table 4.10 Effect of PBS on fluorescence (%) (measured at 530nm) recorded for HPHPMA hydrogels.

<u>Treatment</u>	<u>10% HPHPMA</u>	<u>15% HPHPMA</u>	<u>20% HPHPMA</u>
(1) PSM/BY, 120min. + soak in distilled water, 20 hrs.	23	26	30
(2) PSM/BY 120min + soak in PBS, 20 hrs.	14.5	26	64.5
(3) 0.1% BY, 20hrs.		18.5	22
(4) PSM/BY 120 min., PBS rinse	28	26.5	21.5
(5) PSM/BY 120 min., water rinse.	13.5	23	35

4.5 Mucin Adhesion to Spoilt Lenses

Mucin does not appear to adhere well to any of the surfaces of the polymers studied, since no fluorescence has been recorded at 550-580nm for stained mucin. This seems to infer that these hydrogels have no significant mucoadhesivity. The following studies attempted to discover whether mucin possesses the ability to adhere to biological species, such as proteins and lipids, which it is exposed to in its native environment. A method of spoiling the surfaces of contact lenses (and other polymers) had been developed

in the laboratory, and is discussed more fully elsewhere¹⁸¹, and this was employed in the following investigations. The spoilation of hydrogels involves incubating them, for at least 48 hours on an automatic shaker, in a solution of foetal calf serum and buffered saline. This solution is spiked with particular proteins and lipids, and acts as a model tear fluid. At the end of the spoilation period, examination of the hydrogel surface reveals adsorbed and absorbed lipids and proteins. Values given in the following tables are percentages of maximum peak height, measured from the spectra obtained.

4.5.1 Porcine Stomach Mucin Adhesion

A variety of contact lenses were obtained which had been spoilt using the artificial tear solution described above. The ability of mucin to adhere to the surfaces of these spoilt lenses was studied using fluorescence analyses. Some of the lenses were immersed in a fluorescent solution of 0.5% PSM with Basic yellow, while others were immersed in a solution of PSM alone.

Table 4.11 Measurement of relative PSM adhesion to spoilt MMA/NVP contact lenses (copolymerised with weight fractions of methacrylic acid expressed as a percentage: 1%, 2%, & 5% (w/v)) by fluorophotometry, indicated by the native fluorescence of PSM at 430 nm (%).

MA content of lens	Time lens immersed in 0.5% PSM:			
	Pre-mucin	24hr.	48hr.	72hr.
1%	30.4	22.2	18.4	24.7
	14.0	20.3	23.7	
2%	20.9	18.4	19.0	
	27.0	29.0	29.1	
	36.7	19.0	12.0	22.8
5%	13.3	10.8	15.8	

Values are percentage of maximum fluorescence, measured from recorded spectra. Some of the individual lenses do appear to show an increase in fluorescence as the length of exposure to mucin increases, as shown in Figure 4.7.

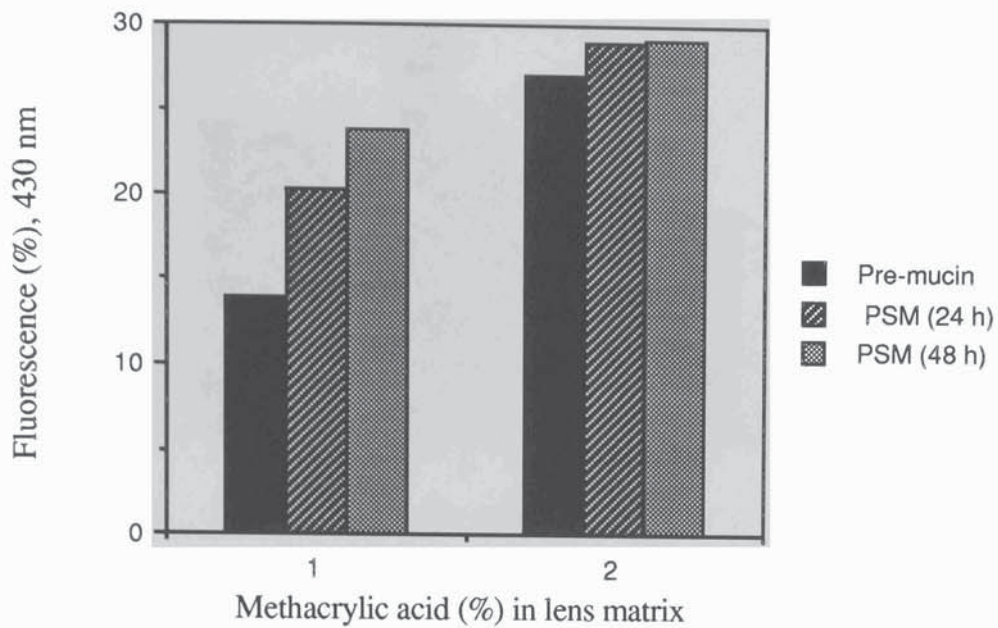


Figure 4.7 Detection of mucin adhesion to spoilt lenses using native fluorescence.

As Figure 4.7 illustrates, a slight increase in overall fluorescent activity was recorded following incubation with 0.5% porcine submaxillary mucin for some of the spoilt lenses. The spoilt lens material used for these studies is an MMA/NVP copolymer containing weight fractions of methacrylic acid expressed as a percentage. Thus, lens 1 contains 1% methacrylic acid, while lens 2 contains 2% methacrylic acid. However, by taking the average fluorescence values recorded for the same lens types, and for a MMA/NVP + 5% MA sample, a different pattern results. This is shown in Figure 4.8.

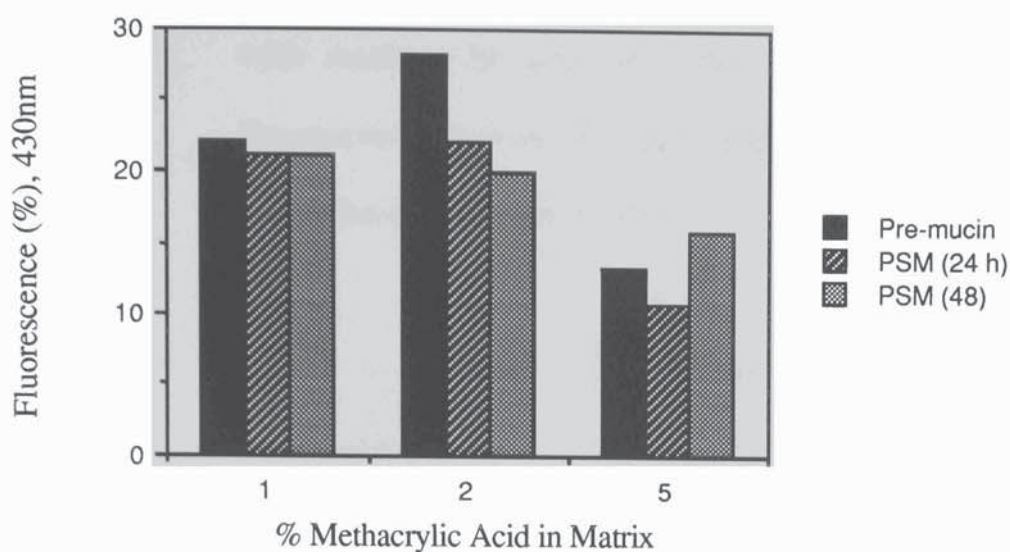


Figure 4.8 Average fluorescence recorded for spoilt MMA/NVP hydrogels containing methacrylic acid following immersion in a mucin solution.

The graphs in Figure 4.8 show that, overall, there is a reduction in fluorescence activity after 2 days in a mucin solution for the spoilt lenses containing 1% or 2% methacrylic acid in the lens matrix, and only a slight increase after 2 days for the lens containing 5% methacrylic acid. Interestingly, the greatest reduction in fluorescence is observed for the lenses which were initially more heavily deposited with protein species, as judged from the level of fluorescence at 430nm. Table 4.12 shows the results obtained from a similar study, using spoilt lenses of the same material, using Basic yellow to fluorescently label the mucin solution.

Table 4.12 Fluorescence of spoilt MMA/NVP lenses (copolymerised with methacrylic acid) at 530nm after immersing in a fluorescent solution of mucin and the dye, Basic yellow, for different lengths of time.

MA content of lens	Time lens immersed in				
	Pre-mucin (+ BY)		0.5% PSM/0.1%BY (3:1)		
			24hr.	48hr.	72hr.
1%	7.8	(3.0)	7.1	31.0	24.1
2%	7.7	(3.5)	7.1	20.3	8.2

The values in brackets are % fluorescence at 530 nm of the spoilt lens following staining with Basic yellow (BY). Clean, unspoilt lenses gave zero fluorescence at 530nm and 4-6% fluorescence at 430nm. All lenses were excited at 360nm. This information is presented more clearly in Figure 4.9.

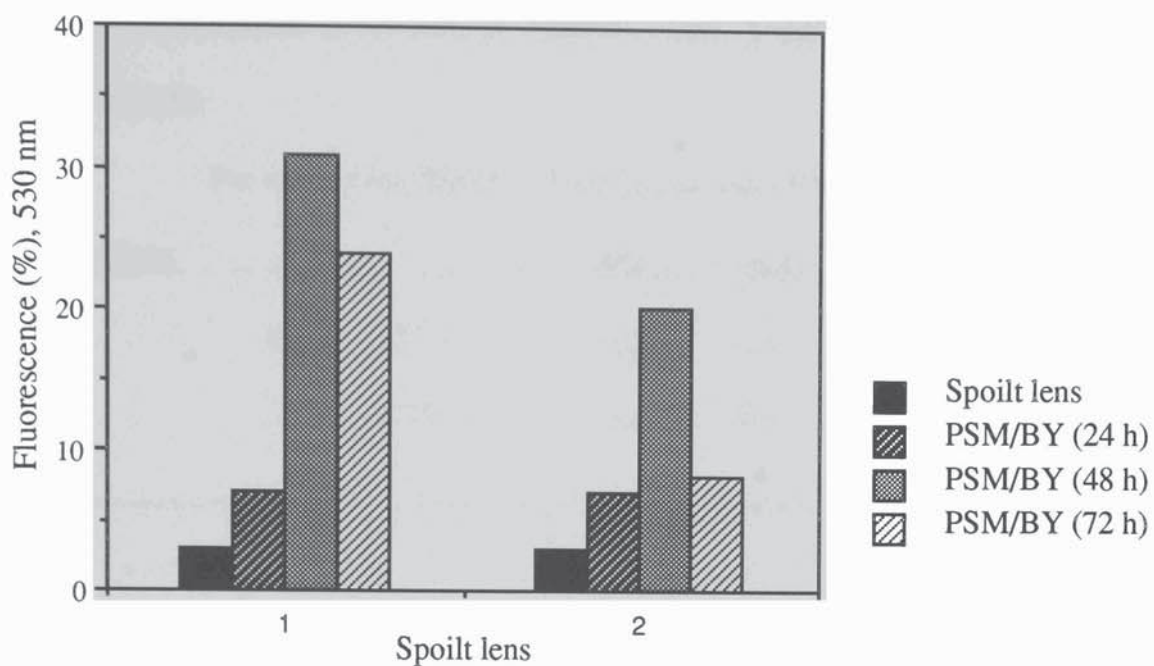


Figure 4.9 Detection of mucin adhesion to spoilt lenses using Basic yellow.

Looking at the results as a whole, the overall level of fluorescence tends to decrease with time (native fluorescence), while the amount of mucin (Basic yellow results) seems to increase up until 48 hours after immersion in the mucin solution, and then decreases.

A parallel investigation was carried out using bovine submaxillary mucin (BSM). The results are shown in Table 4.13. A general reduction in fluorescent activity over time using both native fluorescence and the Basic yellow dye is observed.

Table 4.13 Bovine submaxillary mucin adhesion to spoilt MMA/VP hydrogels containing methacrylic acid (MA):

i) Fluorescence recorded at 530nm using Basic yellow to stain mucin

MA content of lens	Pre-mucin (+0.1%BY)		Time immersed in BSM/BY	
			24hr.	48hr.
1%	32.3	(7.0)	3.2	3.8
2%	37.3	(7.6)	3.2	5.1

ii) Fluorescence recorded at 430nm using the native fluorescence of mucin:

MA content of lens	Pre-mucin	Time immersed in 0.5% BSM	
		24hr.	48hr.
1%	19.0	23.4	11.1
2%	54.4	33.5	37.0

The results shown in Tables 4.11 and 4.12, and in Figures 4.7 and 4.8, could be interpreted as showing PSM to interact with the surfaces of spoilt contact lenses, although the results using the native fluorescence of mucin indicate differently. The results for BSM, in Table 4.13, do not indicate that BSM adheres to the spoilt lenses, following a similar pattern to the results obtained for the unstained PSM study. The choice of experimental design enabled the recorded trend in fluorescence to be detected. An alternative

design could have given rise to a different trend; for example, if the lenses immersed in 0.5% BSM had been analysed for fluorescence every 48 hours, the observed increase in activity at 24 hours would not have been detected and the pattern obtained may have been a steady downward trend in fluorescence over time.

The greatest fluorescence was detected after spoilt lenses had been 48 hours in the solution of PSM and Basic yellow, with an apparent decrease after 72 hours. This decrease could be due to a number of factors. It could result from the denaturing of bound mucin causing a subsequent diminished fluorescent activity. Alternatively, there may exist an equilibrium between mucin in solution and mucin on the lens surface. Fluctuating quantities of adherent mucin may also be due to the surfactant behaviour of mucin; it is possible that it is associating with lipid and protein species at the spoilt lens surface, subsequently causing them to go into solution. Mucin has indeed been reported to interact with lysozyme and albumin in the tear fluid^{192,193}. This behaviour could be responsible for the decrease in activity at some lens surfaces, following immersion in a mucin solution, such as is shown in Figure 4.8, and would explain why the most heavily spoilt lenses exhibit the greatest fall in fluorescence over time. Finally, since a significant increase in relative fluorescence is observed only when Basic yellow has been used, it is possible that this dye is staining a component other than mucin which is present on the lens and which becomes more accessible to the dye as certain spoilage species are dislodged over time. The effect of ionic charge and acid groups on the fluorescence of Basic yellow is discussed below.

4.6 Effect of Surface Charge on Fluorescence Staining

Acuvue lenses were chosen for this study since they possess negative carboxylate groups on their surface. Sample lenses were treated with mucin, by immersing in a 0.5% solution of PSM, as for previous studies presented earlier, and tested for fluorescence activity in order to see whether the presence of the negative carboxylate group would effect the ability of a hydrogel to interact with mucin. To determine the effect of the carboxylate group on the dye, Basic yellow, one of each type of lens was placed in the acetic acid for 75 minutes, and one of each type was placed in the sodium hydrogen carbonate solution for 75 minutes. Each lens type was then rinsed with phosphate buffer and placed in 0.1% Basic yellow solution for 5 minutes. After rinsing again, the fluorescence spectra were recorded (excitation wavelength 280nm, at full scale with slit widths of 0.2 and 0.5). The results are presented in Table 4.14, together with the fluorescence data for Acuvue lenses spoilt with the Aston Tear Model (unspiked and spiked with additional protein, stained and non-stained with Basic yellow) for comparison.

Table 4.14 Effect of different treatments on the fluorescence of lenses containing carboxylate ions.

Lens Type	Treatment	Fluorescence %		
		360nm	480nm	530nm
Clean		---	39.2	0
Clean	0.5% PSM(1hr.)	---	16.0	0
Clean	0.5% PSM(24hr.)	---	43.0	0
Clean	BY stain(1hr.)	0	14.2	8.2
Spoilt (unspiked)	no stain	0	15.6	---
Spoilt (unspiked)	BY stain	0	0	12.5
Spoilt (spiked)	no stain	43.8	30.0	15.0(680nm)
Spoilt (spiked)	BY stain	5.0	0	10.0
Clean	Acid + BY	0	0	15.0, 44.0
Clean	Base + BY	0	0	17.5, 7.0

Rinsing with water rather than phosphate buffer was carried out for three clean Acuvue lenses, one of which was placed in 1% acetic acid for 60 minutes, one in 1% sodium hydrogen carbonate for 60 minutes, and the third simply rinsed in distilled water, prior to staining each with 0.1% Basic yellow for 2 minutes. Each of these lenses gave a fluorescence peak of 14.0-14.5% at 530nm.

The fluorescence results do not suggest that mucin interacts any better with the

ionic hydrogel surface than it does with the other series of polymers studied. In fact, the lenses themselves seem to fluoresce at 480 nm when using the increased sensitivity settings on the fluorophotometer. After immersion for 24 hours in a PSM solution, the level of activity recorded is only slightly raised. None of the lenses fluoresced at the same wavelength as a fluorescently-labelled mucin solution (550-580nm).

The effect of soaking the Acuvue lenses in acetic acid solution would be to convert COO^- to COOH , and effectively remove any surface anionic charge. Soaking in the sodium hydrogen carbonate solution would have the opposite effect, causing the ionisation of carboxylate groups. The results of this investigation show that the ionic Acuvue lens material does exhibit a small degree of fluorescence with the Basic yellow stain. Soaking in acidic or basic solution had little effect in most cases, although one of the lenses which had been treated with acid and then stained showed 44% fluorescence, considerably higher than the other lenses. This reading could, however, be due to contamination of the lens or cuvette. Another explanation for the fact that these etafilcon lenses fluoresce with Basic yellow is that the polymer possesses some structural, conformational or molecular similarity to the part of the mucin molecule which binds to and excites the active site of the Basic yellow compound. However, in light of the interesting result obtained after treating one of the lenses with acid, a further investigation into the effect of acidity on the fluorescence activity of the dye was carried out.

An aqueous solutions of glucuronic acid (0.5%, pH 2.5) was prepared, and its effect on fluorescence activity of 0.1% Basic yellow studied. The results are shown in Figure 4.10.

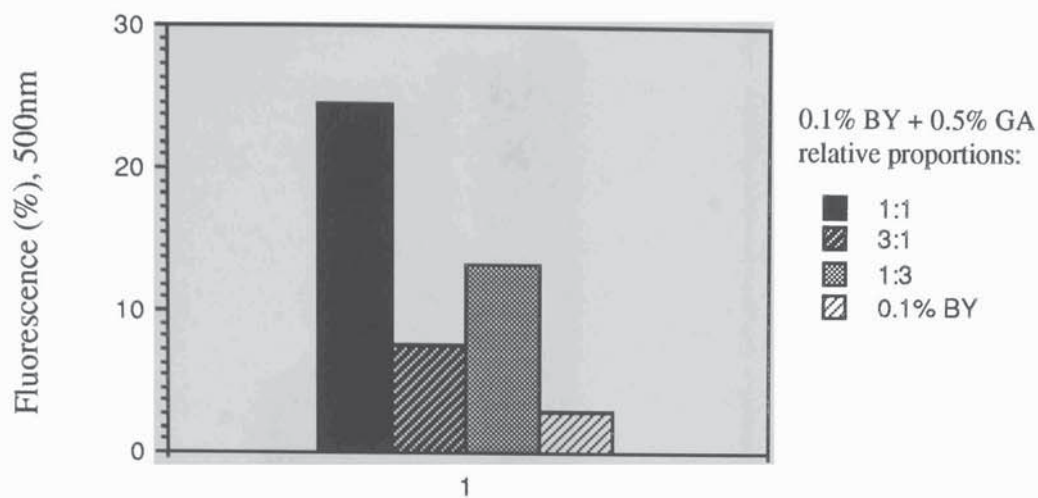


Figure 4.10 Effect of glucuronic acid on Basic yellow fluorescence

From these results, it does appear that the presence of acidic groups influences the fluorescence activity of Basic yellow. To verify this, the effect of adding a dilute base (1% NaHCO_3) was assessed. Equal proportions (5ml) of 1% glucuronic acid (GA) and 0.1% Basic yellow were added together and tested for fluorescence, as above. To this solution, approximately 2ml of 1% NaHCO_3 were added. The effect of glucuronic acid and NaHCO_3 on the fluorescence of Basic yellow is illustrated in Figures 4.9 and 4.10.

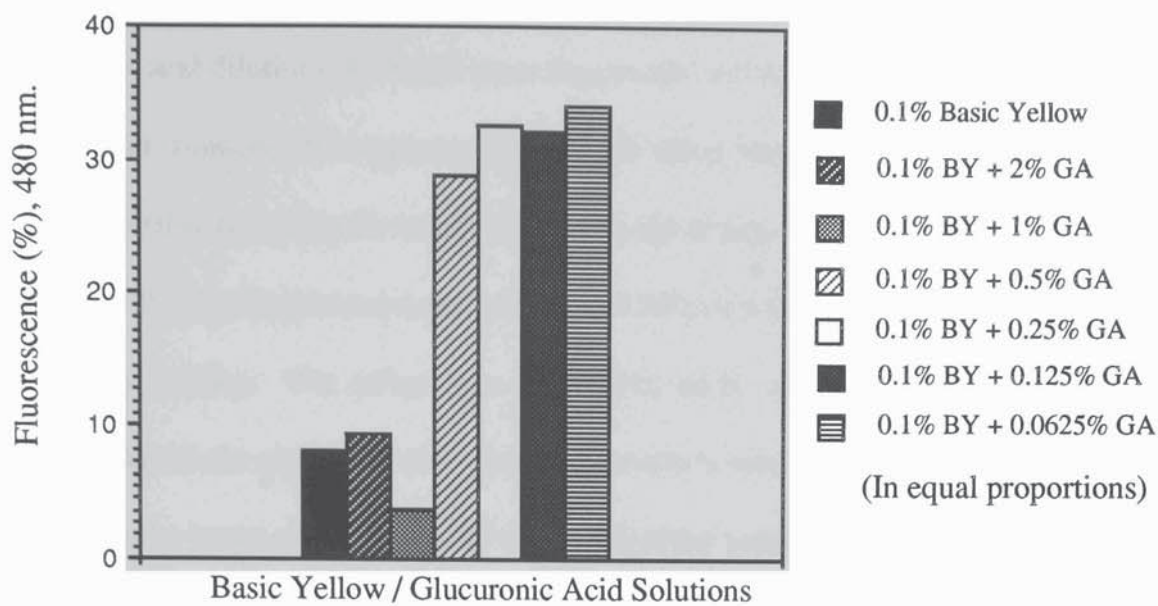


Figure 4.11 The effect of varying acid concentration on Basic yellow fluorescent activity at 480 nm.

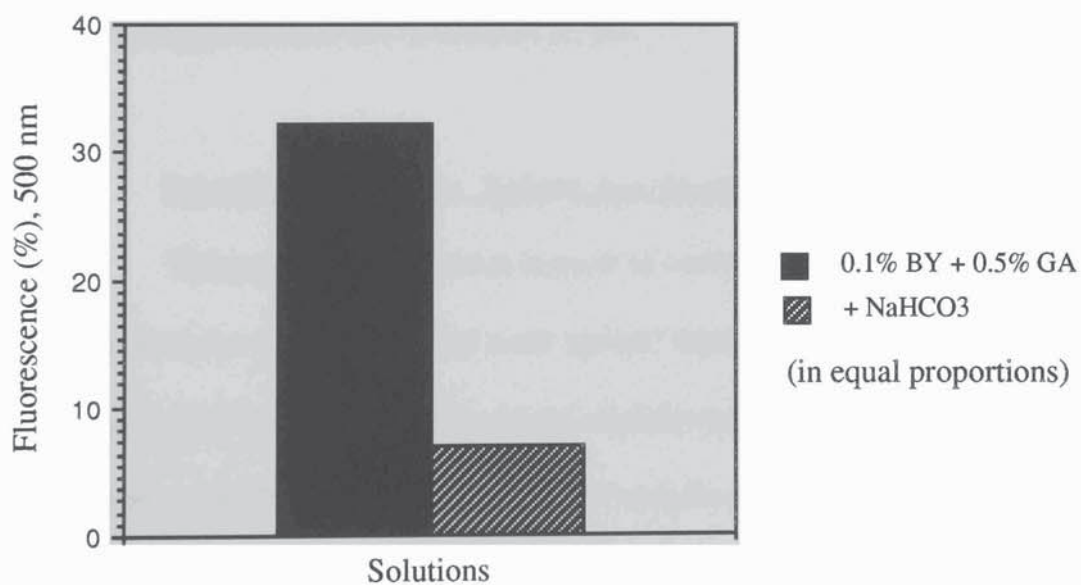


Figure 4.12 The effect of neutralizing the acid in a fluorescent Basic yellow solution.

The pH of the glucuronic acid solutions ranged from 2.1(2%) to 2.6(0.0625%). The weaker acid dilutions produced more fluorescent activity with Basic yellow than the stronger acid solutions, although the difference in effect between the 0.5 - 0.0625% acids is small, possibly reflecting the small variation in pH of these solutions. The results of this work show that the fluorescence recorded at 480-500nm when using Basic yellow can be affected by acidity. The effect does not appear to be mediated by the presence or conformation of the glucuronic acid groups themselves since the more concentrated acid solutions had a lesser effect, but rather on the pH of the solution. This is borne out by the fluorescence quenching effect of adding a dilute base (1% NaHCO₃).

Considering the results of staining the hydrogels containing polyethylene or polypropylene side chains, HPHPMA in particular, with Basic yellow, it could be that residual methacrylic acid monomer which was found to be a contaminant in this hydrogel matrix was causing the observed fluorescent activity.

4.7 Specificity of Basic Yellow for Mucin

This study was carried out in order to verify the specificity of Basic yellow for mucins. In order to do this lenses were spoiled with a range of different solutions, some of which contained glycoproteins while a lipid/protein solution was made up which did not. Clean, unspoilt, lenses were also stained with the dye. Lenses made of MMA/VP were chosen since this copolymer is non-ionic and does not possess surface carboxylate ions which may effect the staining properties. The lenses used for this study included the following: MMA/VP lenses (Vista Optics), 60% water content, 1 Permalens (HEMA/VP/methacrylates, 71% water), and 1 Permafex lens (VP/MMA/methacrylates,

74% water).

The lenses underwent *in vitro* spoilation in either the spiked tear model, the tear/mucin model (spiked tear model + 1% PSM (w/v)), or a protein/lipid solution (0.1g lysozyme, 0.1g albumin, 0.05g cholesteryl palmitate, fatty acid, 20 ml PBS). The lenses were spoiled for either 72 hours or 24 hours. The spoilation process involved placing one lens into a vial containing glass beads (approximately 1mm diameter) plus 5mls of the spoilation solution, sufficient to just cover the beads. The vials were sealed and placed on an automatic shaker for up to 72 hours. The lenses were tested for native fluorescence and then stained with Basic yellow for 2 minutes and the fluorescence spectra again recorded. The results are shown in Table 4.15 and in Figures 4.11 and 4.12. There was no difference in fluorescence spectra for the slightly higher water content Permaflex and Permalens contact lenses. The spectra were recorded at the lower sensitivity, using an excitation slit width of 0.2mm, to minimise the native fluorescence of hydrogels and Basic yellow at 480nm.

As the results show, the fluorescence peaks tend to occur in the same regions whether or not mucin is present in the spoilation model. A solution of 0.5% PSM fluoresces naturally at 430nm, and at 580nm when stained with Basic yellow, as Figure 4.15 and Appendix 1 (spectra 3 and 4) show. However, none of the spoilt lenses exhibits fluorescent activity at these wavelengths. From this it could be concluded that little (or too little to be detected) mucin is associated with the deposited material on the spoilt lenses. Lipids fluoresce within the 450-500nm range and, when lipid-spoilt lenses are stained with Basic yellow, this fluorescence peak is apparently shifted further along to 500-550nm. It

could be that fluorescence quenching causes the lack of activity at the lower 450-500nm region and the reduction in the height of the fluorescence peaks at wavelengths 360nm and 680nm. The peak occurring at 500nm for all the lenses stained with Basic yellow may be due to dye adsorption to the deposits and to absorption of the dye into the lens matrix. This theory is supported by the slightly smaller (5% in comparison with 5-10% for spoilt lenses) fluorescence peak at 500nm produced by a clean lens of the same material which is stained with Basic yellow for 2 minutes. There is no evidence to suggest that the amino acids or fatty acids present in the lens deposits exert a fluorescence-enhancing effect since addition of Basic yellow to the spoilations produces a fall in the relative fluorescence recorded at all wavelengths, although the activity at 480nm does seem to shift to 500nm.

Table 4.15 Specificity of Basic yellow for mucin: the fluorescence activity of contact lenses spoilt with different solutions.

Spoilation solution	Lens	Fluorescence wavelength	
		24 hr. spoilation	72 hr. spoilation
Protein/lipid	(1)	<u>360</u> , 469, 680	359, <u>459</u>
	(2)		371, <u>492</u> , 705
	(3)		372, <u>481</u> , 700
Protein/lipid + BY	(4)	469, 515	<u>367</u> , 528, 689
	(5)	359, <u>500</u>	
	(6)		<u>537</u>
Tear model	(7)	<u>384</u> , 481,	<u>383</u> , 466, 705
	(8)		394, <u>489</u>
Tear model + BY	(9)	<u>506-519</u>	366, <u>508</u>
	(10)		360, <u>500</u>
Tear model + PSM	(11)	384, <u>485</u>	<u>359</u> , 472, 670
	(12)		<u>369</u> 684-710
Tear model +PSM+BY	(13)	360, <u>519</u>	<u>358</u> , 517, 684
	(14)		<u>348</u> , 506, 669
	(15)		358, <u>531</u>
PSM + BY	----		<u>580</u>

The underlined wavelengths are those at which the greatest activity was recorded, as measured from the spectra.

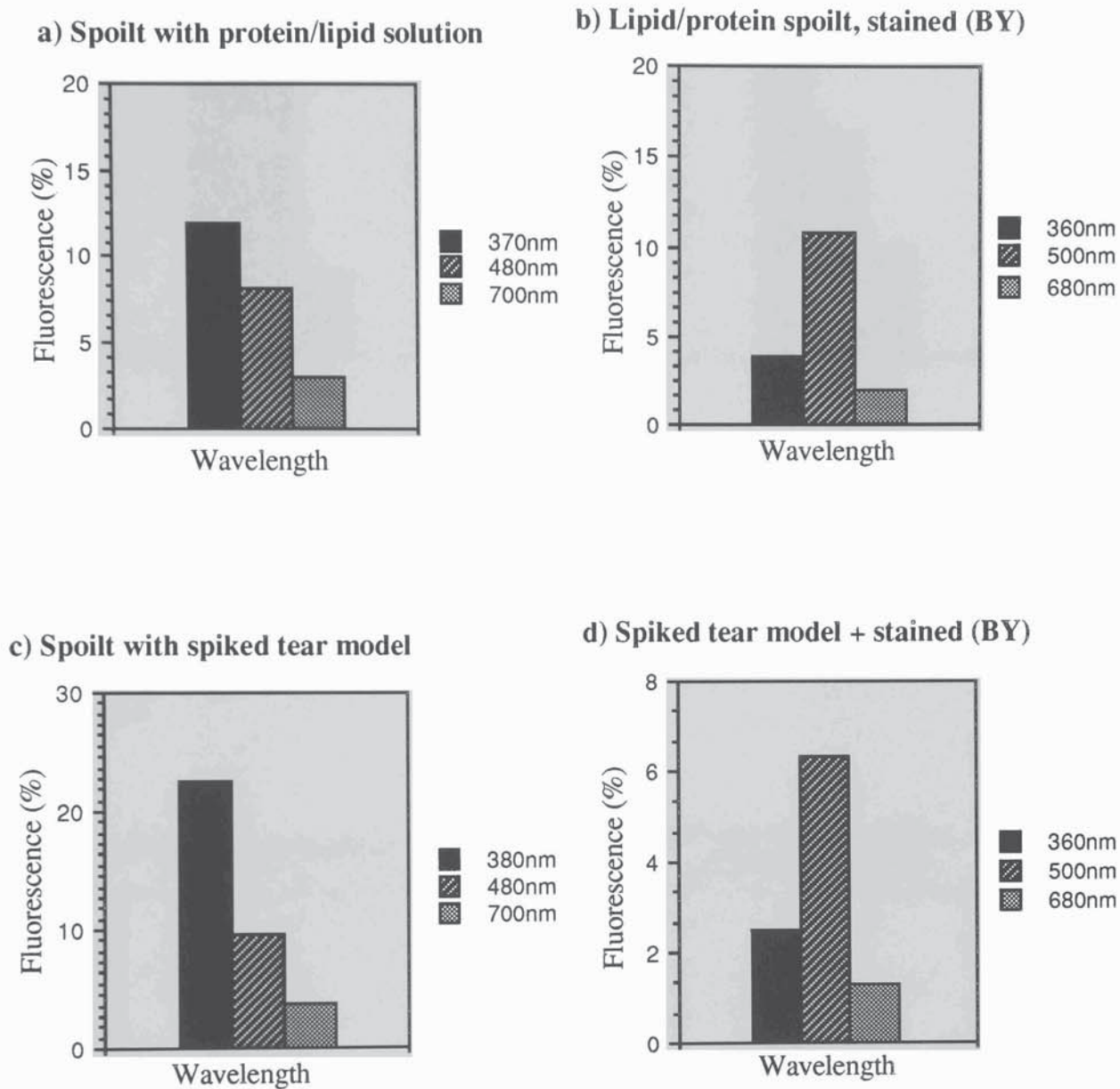


Figure 4.13 Fluorescence activity of spoiled MMA/VP lenses:

(a)-(d) spoilation solutions without added mucin.

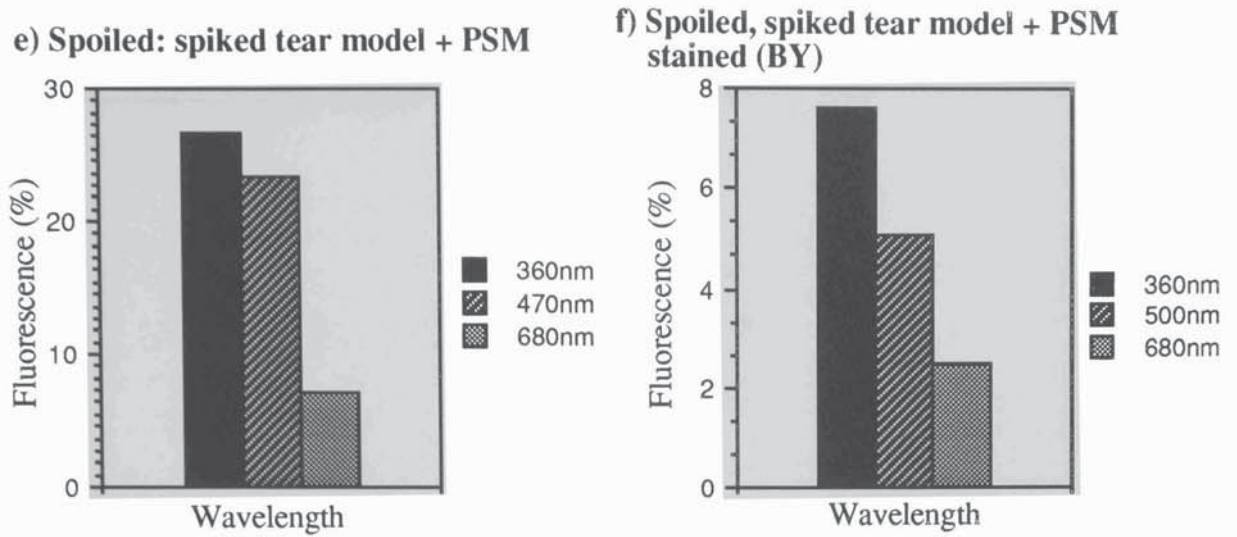


Figure 4.13 Fluorescence activity of spoiled MMA/VP lenses:

(e)-(f) spoilation solutions with added mucin

The fluorescence patterns of the solutions used to spoil the lenses are shown

Figure 4.14.

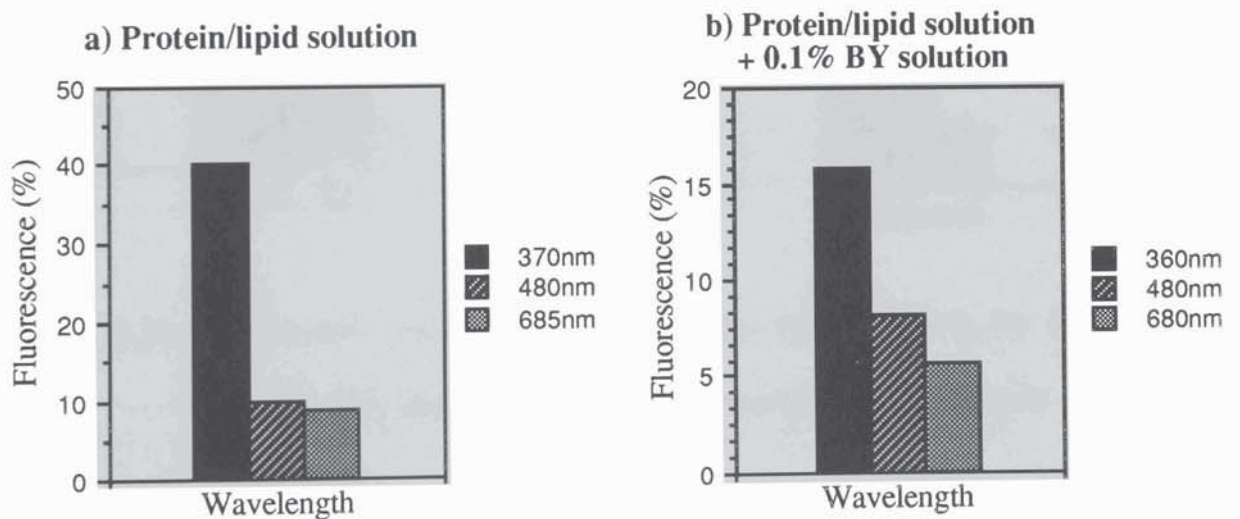


Figure 4.14 Fluorescence activity of the solutions used to spoil the lenses:

(a)-(b) protein/lipid solution.

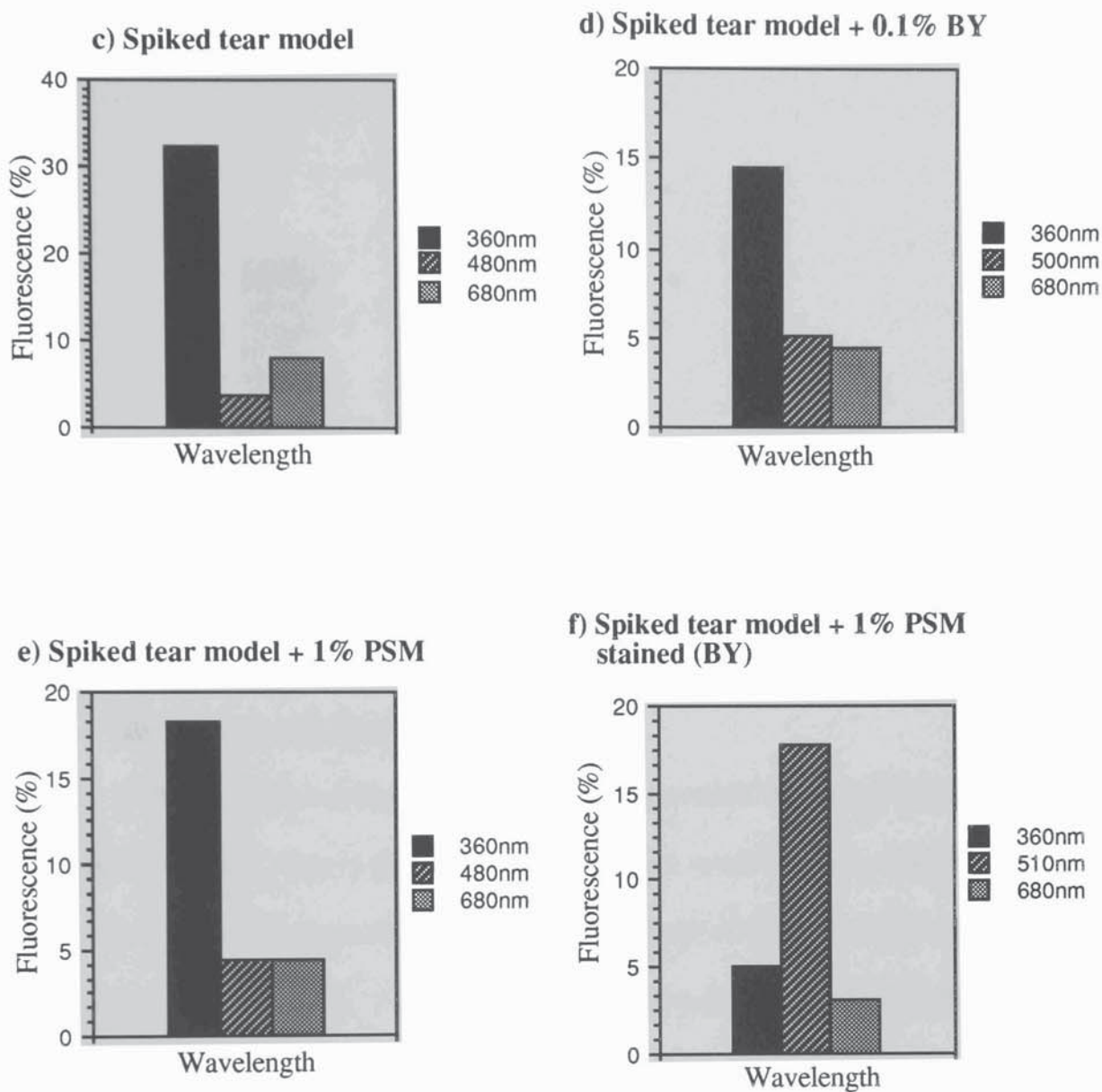


Figure 4.14 Fluorescence activity of the solutions used to spoil the lenses:

(c)-(f) spiked tear model with and without added mucin.

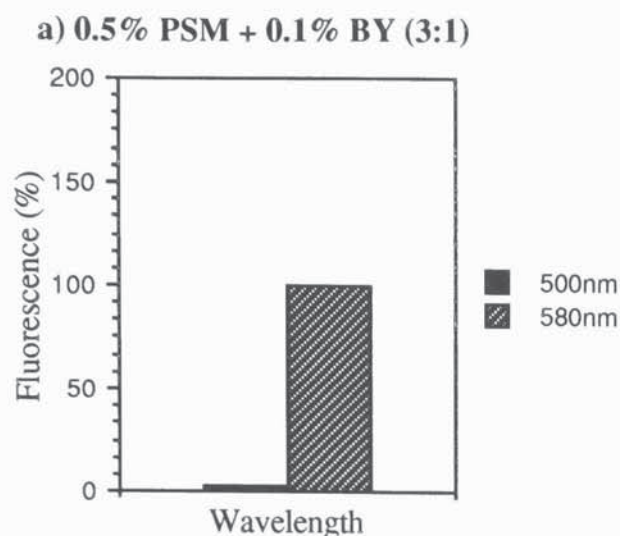


Figure 4.15 Fluorescence of porcine stomach mucin with Basic yellow.

4.8 Summary and Conclusions

A deeper understanding of the relationship between mucin and the corneal surface will be of great value in the future development of biomaterials for efficient drug delivery. It is widely believed that the inner tear film layer of mucin is adherent to the cornea, and much of the current research aimed at developing new drug formulations relies on this being the case.

Mucin-specific stains and fluorescence analyses were used in this study to investigate the adhesive capacity of mucin for polymer surfaces. Porcine stomach mucin possesses the greatest fluorescence activity when stained with the fluorescent dye Basic yellow (100% at 580nm for a 3:1 solution of 0.5% PSM and 0.1% Basic yellow, using even the low sensitivity setting). Alcian blue was also used as a specific stain for mucins, but since it was adsorbed into the polymer networks of many of the hydrogels studied, it was not suitable for these investigations.

Fluorophotometry was used to assess whether fluorescently-labelled mucin was present on the polymer surfaces, following incubation with a mucin solution and rinsing. Using the sensitivity achieved by setting the fluorophotometer slit widths to 2.0mm (emission) and 0.2mm (excitation) there was little indication of mucin adhesion to any of the polymers irrespective of hydrogel character. Poly HEMA, melinex and polythene samples showed no sign of being mucoadhesive, nor did the anionic hydrogel matrix of Acuvue lenses with their surface carboxylate (COO^-) groups. Hydrogels containing PEGMA 10 EO also gave very little sign of being mucoadhesive, with relative fluorescence peak heights, following immersion in a fluorescent mucin solution, of only 2-5% at 530-550nm.

At the higher sensitivity, achieved by increasing the excitation slit width to 0.5mm from 0.2mm, increasing the concentration of PEGMA 10 EO in the hydrogel network did seem to increase the adhesion of mucin to the surface of the polymer sample to some extent, judging from the height of the fluorescence peaks recorded, although membranes with concentrations of 5% and 15% of this ethylene oxide-containing group showed greater fluorescence than those containing 10% or 20% following immersion in a fluorescent solution of PSM. However, clean polymers also gave rise to similar fluorescence peaks following staining with Basic yellow solution. At this higher sensitivity, both hydrogels and Basic yellow produce fluorescence activity at 480nm. The Basic yellow dye molecule is small enough to be absorbed by hydrogel matrices, staining them yellow, so the combined effect of material and dye could give rise to the observed peak at 530nm. For the polyethylene- and polypropylene-containing hydrogels, this fluorescent effect tended to decrease slightly with increasing PEGMA 10 EO concentration

(increasing water content), but increase with increasing HPHPMA concentration (decreasing water content). This seems to infer that a structural effect is taking place, with more fluorescence being recorded for stained hydrogels of lower water content and less expanded polymer network. If there is a physical interaction between the dye and components of the hydrogels, the concentration of dye for a given volume of more dense hydrogel will be greater, giving rise to a greater fluorescence peak.

Another possibility, that residual methacrylic acid monomers may be causing the staining behaviour of these polyethylene-containing hydrogels, was investigated. However, rinsing with acid or base solutions prior to staining had no appreciable difference in effect on the degree of fluorescence observed, making it unlikely that a low residual concentration of MAA monomers would have any greater effect. On the other hand, glucuronic acid did produce considerable fluorescent activity with Basic yellow in certain concentrations. This activity could be quenched by addition of a basic solution, indicating that pH does effect the staining properties of Basic yellow.

Overall, the results of these investigations provide no indication that mucin is capable of adhering well to hydrogels. Although the conclusions drawn from this work appear largely negative, one significant triumph has been the optimisation of the Basic yellow staining technique. There are many variables to consider when interpreting the results of Basic yellow staining in this context, and the majority of these have been addressed during this study. Chief among these variables are the material characteristics: water content, acidity, presence, and chain length, of ethylene oxide chains (or any other particular comonomer); solution pH, time of exposure to mucin and dye, length of time of

leaching, concentration ratio of PSM and Basic yellow. Other possible variables which were not investigated include solution temperature, but this was not likely to have been important here because all studies were carried out at room temperature. Basic yellow is, despite the number of factors which require consideration, nevertheless, a very sensitive stain for mucin. Mucin concentrations of 0.1% (a solution of 3 volumes 0.125% PSM to 1 of 0.1% Basic yellow) give 100% fluorescence, and those as low as 0.03% give fluorescence peaks of almost 40%, at the higher sensitivity setting on the fluorophotometer. Although in some cases fluorescence readings of a similar value were recorded for some hydrogels at this sensitivity following immersion in a fluorescent mucin solution, when the activity of the stained material is taken into account, the value attributable to mucin is greatly reduced. Even at lower sensitivity, Basic yellow is an effective stain for mucin, producing fluorescence peaks of at least 50% for the Basic yellow-labelled PSM solutions used throughout this study. The non-detection of fluorescently-labelled mucin on hydrogels at this lower sensitivity reinforces the impression that these polymers are poorly mucoadhesive.

Since none of the polymers studied was exhibiting signs of mucin adhesivity, lenses spoilt with biological species (proteins, lipids, etc.) were tested in the same way to see whether mucin possesses a more specific adhesive capacity for such compounds. Mucin did appear to adhere to some individual spoilt hydrogel surfaces, although, taken as a whole, there was an overall reduction over time in the native fluorescence of spoilt lenses following immersion in a mucin solution. This could be attributed to the surfactant ability of mucin, which could result in the solubilisation of adsorbed proteins or lipids. Denaturing of mucin following association with the sorbed components may also occur,

causing a reduction in fluorescent activity. For up to 48 hours incubation in a fluorescent mucin solution, however, the degree of fluorescence of the sample tended to increase before falling after 72 hours. There is no indication that Basic yellow stains components other than mucin in the spoilage solutions, since adding dye to these solutions produces a fall in relative fluorescence at all wavelengths. The observed increase in fluorescence staining over time of the spoiled lenses must, therefore be due to some interaction between material and dye.

The specificity of the dye for mucin was tested by adding Basic yellow to spoilage solutions containing lipids, fatty acids and proteins. The native fluorescence peak of lipid species normally occurs at around the 460-500nm region but, following staining, this peak is quenched and a smaller degree of activity at the higher wavelengths of 500-540nm is recorded. The result of staining was, in fact, a fall in relative fluorescence at all wavelengths over the spectra obtained for the native fluorescence of these species. The presence of mucin in the solution did enhance the emission recorded at 550-580nm. For spoiled lenses, however, there appears to be some absorption of dye by lipid species, giving rise to some fluorescence activity at wavelengths close to those occurring for fluorescently stained mucin. For this reason, use of Basic yellow for situations where lipids may be present will require care to be taken when interpreting the fluorescence spectra. In fact, analysis of spectra due to native fluorescence may prove to be a superior means of differentiating different components in such cases.

The main conclusion to be drawn from this work is that mucin does not adhere well to any of the polymers studied, despite the range in characteristic included, such as water content and monomer groups. This led to the question of whether in fact there is a

specific mucin/glycocalyx adhesion occurring at the corneal surface. This is considered in Chapter 5.

CHAPTER 5

Properties of the Corneal Cell Surface

5.1 **Introduction**

The three-layered tear film structure, suggested by Wolff in 1954¹ and now widely accepted, infers that the inner layer of mucin is adherent to the surface of the corneal and conjunctival epithelial cells. The work described in this chapter was intended to investigate whether mucin has a specific capacity to bind to the surface of corneal cells since, as discussed in the previous chapter, mucin does not interact nonspecifically with polymer surfaces.

Two approaches to studying the surface properties of corneal epithelial cells were used for this work. The first method involved analysing freshly removed rabbit corneas, while the second aimed to avoid the difficulty of maintaining the integrity of isolated animal tissue in the experimental conditions by making use of cultured rabbit corneal cells (SIRC). For the cell culture work, a completely new approach, whereby confluent layers of cells are grown on the surfaces of hydrogel membranes, was used. This system was designed to exploit the ability of the corneal cell line to adhere to the hydrophilic gels which present a number of advantages over the use of tissue culture plastic. In particular, the hydrogel matrix provides a transparent, deformable, platform, which can be cut to the desired size, facilitating the spectroscopic examination of adherent cells.

The SIRC cell line was chosen because it does not produce any surface glycoproteins, so that any interaction with mucins introduced to the cell culture would be relatively simple to detect. A further advantage this established cell line has for this work is that it is relatively simple to culture and maintain.

5.2 Investigation of Mucin Adhesion to Rabbit Cornea

Freshly excised rabbit corneas were tested for mucin adhesion by immersing in a fluorescent solution of 0.5% PSM (to which had been added 0.1% Basic yellow solution, 1ml for every 3ml mucin solution). They were tested for fluorescence at 3 hours and 5 hours after immersion. As a control, part of one cornea was stained for mucin with 0.1% Basic yellow, and tested for fluorescence after 1 hour.

Immediately the corneas were obtained they were treated as follows:

- i) half of cornea (1) was placed in a 0.1% Basic yellow solution;
- ii) cornea (2) plus the other half of cornea (1) was placed in a fluorescently-tagged PSM solution (3:1, PSM/BY).

Values in the tables of results are percentages of maximum peak height at 530nm, measured from the spectra.

Table 5.1 Corneal fluorescence at 530nm

<u>Solution</u>	<u>Cornea 1</u>	<u>Cornea 2</u>
0.1% Basic yellow, 1 hr.	26.0	
PSM/BY, 3 hrs.	21.5	10.1
PSM/BY, 5 hrs.	9.5	7.6
0.1% BY, 1hr.; PBS, 4 hrs.	6.3	

As the results show, there was a progressive reduction in the fluorescence of

the rabbit corneas with time, for both the sample treated with only Basic yellow and the corneal samples treated with the fluorescent mucin solution. The corneas swelled and became gradually opaque. This denaturation and decomposition of the rabbit cornea, which occurred fairly rapidly, probably caused a reduction of fluorescence detected at the surface. The opacity would also have reduced the amount of light transmitted through the corneal samples, and this would cause less detection of any fluorescent material on the inner surface (corneal endothelia) of the samples. A further problem in interpreting the results of this study is that the fluorescent behaviour of rabbit ocular mucins in comparison with porcine stomach mucins is not known. There may be an equilibrium concentration which is approached by mucin at the corneal surface and in solution. Addition of PSM to the already disturbed system could displace some rabbit mucin from the cornea, particularly if it has a greater surfactant ability. If rabbit mucin is more fluorescent than PSM, then this could result in apparently less mucin being detected.

To avoid most of the problems associated with the work with excised rabbit corneas, for further investigations into the relationship of mucin with the ocular surface cultured corneal cell were used.

5.3 Cell Culture Work : SIRC Cells.

5.3.1 Preparation of a Confluent Cell Layer

For all the studies involving a confluent layer of cultured corneal cells, SIRC cells were grown in minimum essential medium (MEM) with Hanks' salts, 10% foetal calf serum, 2mM L-glutamine, non-essential amino acids and antibacterial and antimycotic agents. The method used is described in the Materials and Methods Chapter. Figure 5.1 is

a micrograph of a confluent cell layer.

5.4 Effect of Staining for Mucin on SIRC Cells

Glycoproteins are present at animal cell surfaces, normally occurring as glycosylated transmembrane proteins. A series of studies were carried out to discover whether the staining of cell surfaces would produce positive results with the stains for mucin. Alcian blue and Basic yellow.

5.4.1 Basic Yellow Staining of SIRC Cells

Confluent cell layers were prepared on 1cm discs of HEMA/MMA (80:20) copolymers and on clean glass cover slips. Each layer was examined under a few drops of buffered saline using phase contrast microscopy. In an attempt to stain for glycoproteins the PBS was withdrawn by pipette and replaced with a few drops of 0.01% Basic yellow, as described below.

A few drops of 0.01% Basic yellow were added to cells on a cover slip, left for 15 seconds, and rinsed with PBS. When examined under u.v. illumination of the fluorescence microscope the cell nuclei appeared to exhibit green/yellow fluorescence. Figure 5.2 is a fluorescence micrograph showing fluorescent staining of the SIRC cells.

0.5% PSM was added to a cell layer on a cover slip, left for 1 minute, then a 0.01% solution of Basic yellow was added for 15 seconds and rinsed with PBS. The result obtained was similar to the outcome of adding Basic yellow alone, with the cells themselves fluorescing.

Cells on HEMA/MMA (80:20) were treated with 0.01% Basic yellow solution for 15 seconds and rinsed with buffered saline solution. They were tested for fluorescence in the spectrofluorometer. Using slit width settings 2.0,0.5 and percentage full scale dial set at 0.3, 12.5% fluorescence was recorded. A sample disc of HEMA/HPMA (85:15) was immersed in 0.5% PSM for 1 minute and then stained with Basic yellow, 15 seconds, and rinsed with PBS. 20.5% fluorescence was recorded.

In the above experiments the SIRC cells fluoresced green/yellow following the addition of 0.01% Basic yellow solution. This is to be expected since cell membranes, internal membranes as well as the plasma membrane, possess glycoproteins. When the fluorescently-labelled mucin solution was introduced to the cell layer the entire field of view was observed to fluoresce green/yellow. Rinsing with PBS and re-examining under phase contrast showed many of the cells to have become dislodged from the hydrogel surface. The fluorescent cells appear as roughly circular, indicating that they are starting to come away from the substrate. This could result from osmotic or other environmental stress exerted by the staining solution, since a similar effect was observed with and without the presence of mucin. When Basic yellow is applied to PSM the fluorescence peak is located at about 530nm. When SIRC cells are present the peak is shifted forward to 500-510nm.

The application of Basic yellow appears to disrupt the cell layer and fewer cells are visible when a treated sample is viewed under the microscope.

5.4.2 Alcian Blue Staining of SIRC Cells

A 0.1% Alcian blue solution was made up in 0.05M sodium acetate buffer with 0.2M MgCl₂ added. Confluent SIRC cells were grown on discs of HEMA/MMA (90:10) and HEMA/ST (90:10). The effects of staining with Alcian blue are summarised in Table 5.2

Table 5.2 Effect of varying staining procedure on Alcian blue staining of corneal cells

Substrate	Time (minutes) in	Time (min.) in	Result after rinsing in PBS
	0.5% PSM	0.1% Alcian Blue	
HEMA/MMA (90:10)	5	15	The hydrogel substrate had no effect on the appearance of the SIRC cells.
	10	10	
	15	10	
	15	15	
	60	20	
HEMA/ST (90:10)	--	5	In general, the longer the time immersed in PSM or in Alcian blue, the fewer cells remained spread on the hydrogel surface.
	--	10	
	--	15	
	10	5	The cells Stained pale blue with Alcian blue.
	10	8	
	10	10	No difference in staining was observed following exposure to PSM. Thus, there was no indication that mucin adheres to the corneal cells.
	15	10	
	15	5	
HPA/EEMA/ST (60:30:10)	5	10	
	15	5	
	15	10	



Figure 5.1 A confluent SIRC cell layer

(x125)

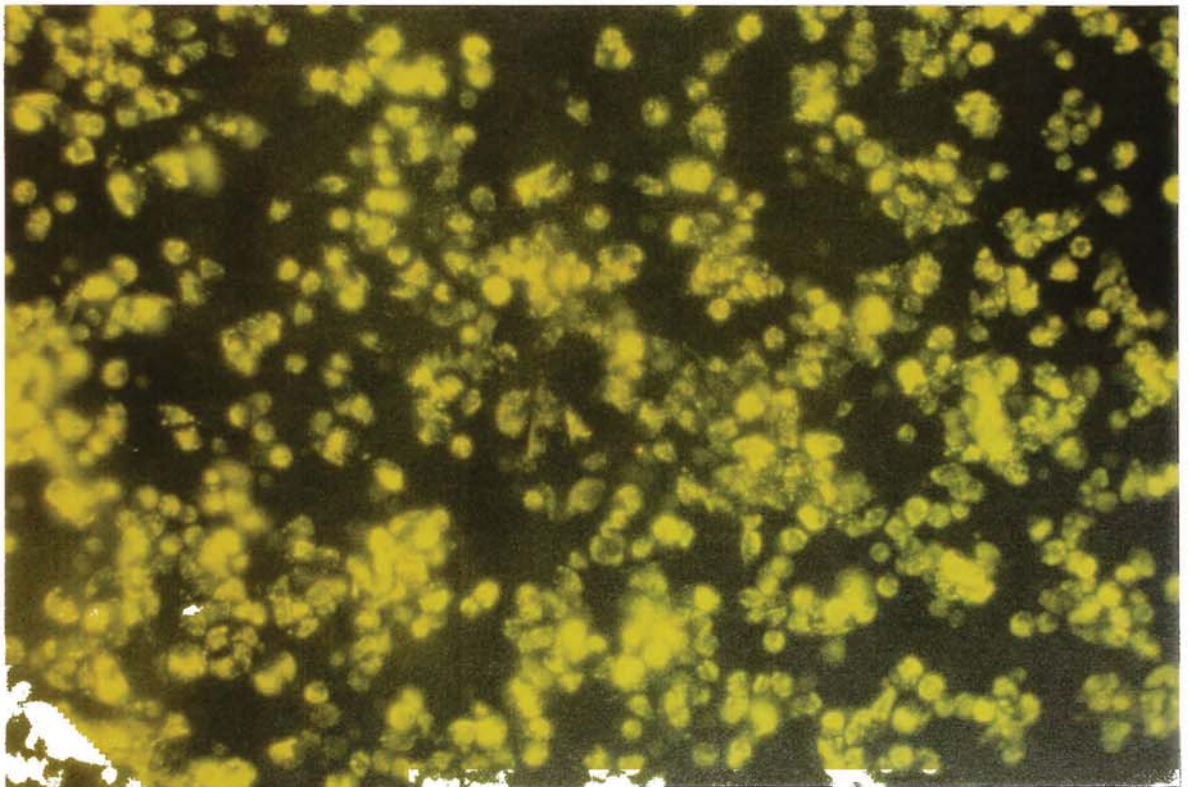


Figure 5.2 SIRC cells stained with Basic yellow (15 sec.).

(x500)

SIRC cell confluent layers were prepared as before on hydrogel membranes, and also on glass cover slips. These were examined under the microscope and photographs were taken before and after treatment. For comparison a smear of 0.5% mucin was prepared on a glass slide and stained with a few drops of the Alcian blue solution.

1. Cells on a glass cover slip were immersed in 1% Alcian blue for 30 seconds and rinsed with Dulbecco's PBS.

Slight blue staining of the cell membranes was observed.

2. Cells on HEMA/MMA (92.5:7.5) were treated as above.

A similar staining pattern was observed, indicating that substrate has little effect on the properties of the cell layer.

3. Cells on HEMA/MMA (95:5) were immersed in 0.5% PSM for 1 minute, rinsed, 1% Alcian blue added for 30 seconds and rinsed twice in PBS.

Staining, again, was not pronounced, but cell membranes had slight blue tint and some possible strands of mucin, in deeper blue, between the cells were observed.

4. A glass cover slip was immersed in BSM for 1 minute, left to air-dry in a dust-free environment, stained with Alcian blue, 30 seconds, and finally briefly rinsed with PBS. This "control" produced the characteristic blue staining of the mucin.

These studies indicate that cell substrate has little effect upon the staining properties of the cell layer, nor upon its poor mucoadhesiveness. Figure 5.3 shows the effect of staining a confluent layer of SIRC cells with Alcian blue, with and without application of mucin. When these micrographs are compared with Figure 5.4, which

shows the staining pattern of a fixed mucin smear, the apparent inability of mucin to adhere to a cell surface is made clear.

A confluent cell layer on a hydrogel membrane, HEMA/MMA (92.5:7.5), when excited at 360nm, emits energy at about 480nm. At the settings normally used on the spectrophotofluorometer (0.3,2.0,0.5) 34% fluorescence was recorded.

When Basic yellow is applied to PSM the fluorescence peak is located at about 530nm. When SIRC cells are present the peak is shifted forward to 500-510nm. The application of Basic yellow appears to disrupt the cell layer and fewer cells are visible when a treated sample is viewed under the microscope.

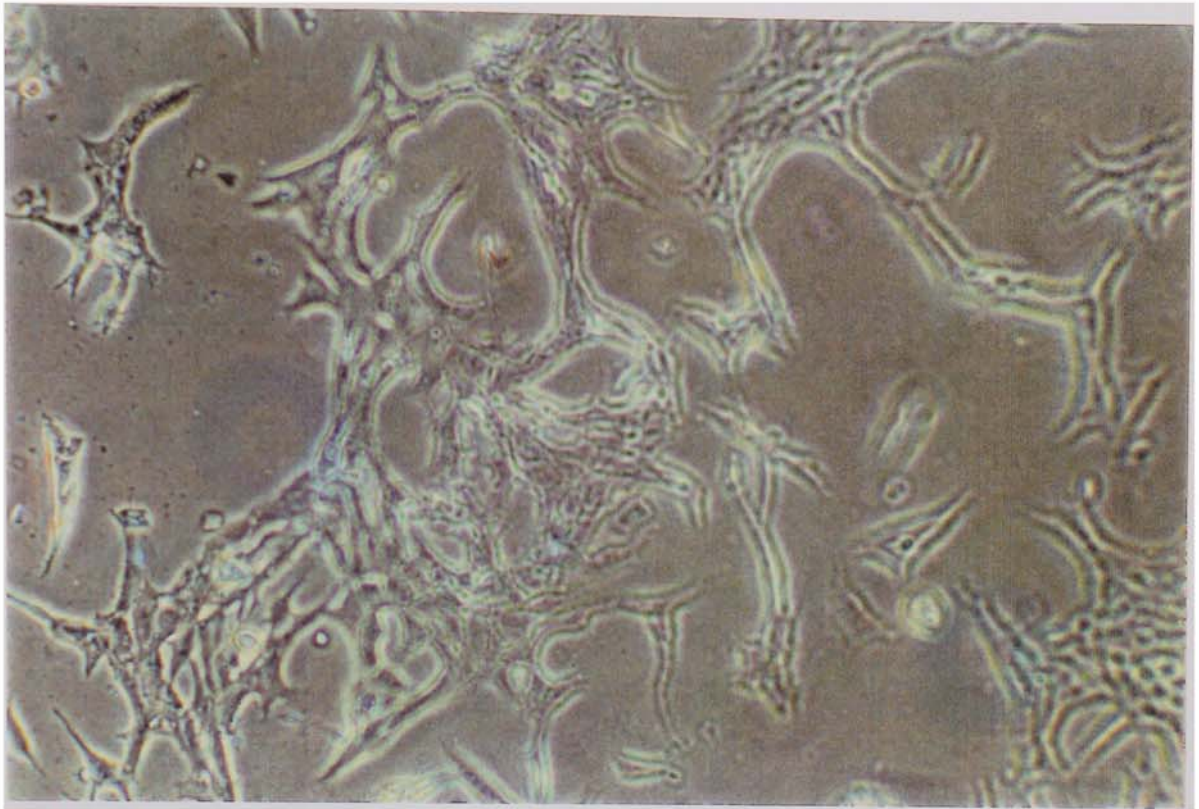


Figure 5.3(a) SIRC cells stained with Alcian blue (5 mins).

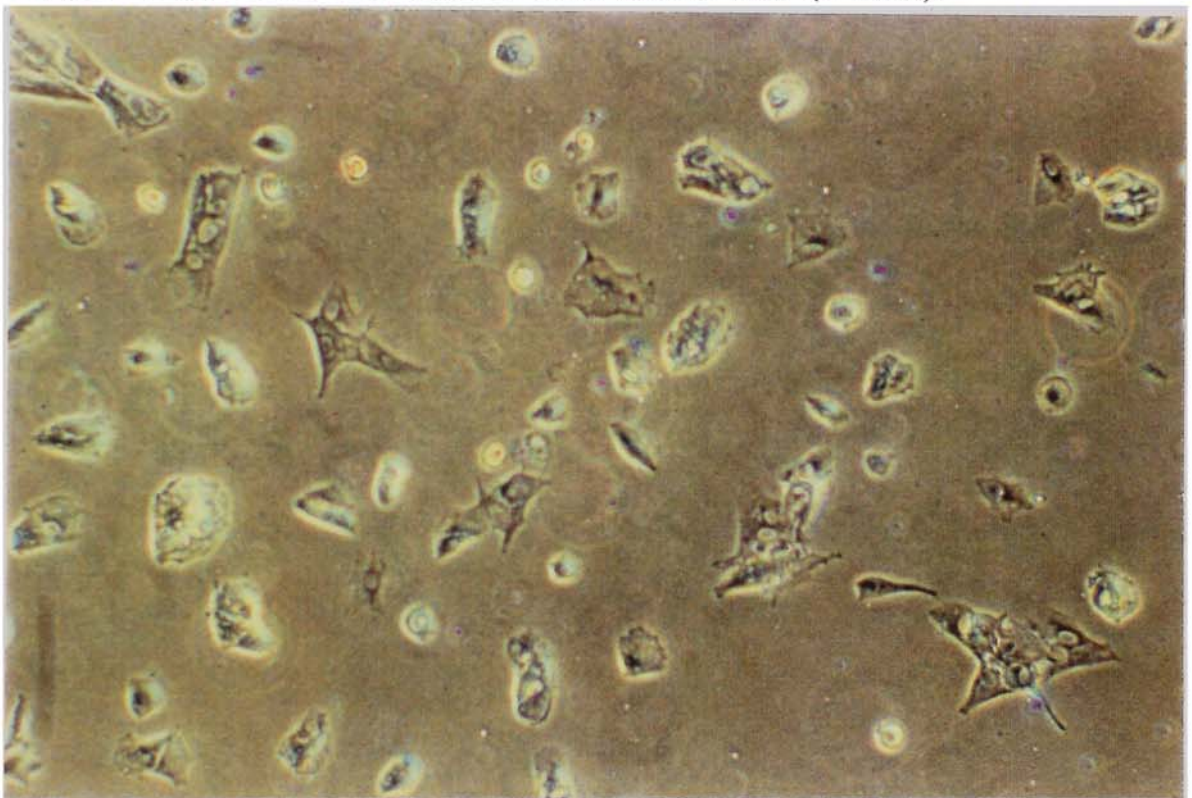


Figure 5.3(b) Effect of staining SIRC cells with Alcian blue (5 mins) following exposure to a mucin solution (15 mins) (x 500).



Figure 5.4 Mucin smear stained with Alcian blue (x 125).

5.5 Wettability of Corneal Epithelium: Contact Angle Measurements.

Confluent layers, of a range of cell densities, of SIRC cells were prepared on glass cover slips. Using a goniophotometer, measurements were made of contact angles made by air (captive bubble technique) and n-octane on the cultured cells. Glass cover slips were also coated with layers of the proteins albumin and fibronectin by incubating them in solutions of these proteins, and contact angle measurements were taken in the same way. Measurements were also made for cover slips which had been incubated with foetal calf serum.

The values obtained were used to calculate the critical surface tension of the SIRC cells, and the protein-coated surfaces. The value obtained for the corneal epithelial

cells, around 66mNm^{-1} , is much higher than the value (28mNm^{-1}) measured for excised cornea by other workers (Holly, 1971)². However, the excised cornea had been "wiped clean" prior to making sessile drop contact angle measurements. If the structure of animal cell membranes is considered, it is easy to assume that this process is likely to have destroyed the fragile glycocalyx and possibly the epithelial membranes themselves. Also, the lipids present in the tear film may well have collapsed onto the cornea, to form a Langmuir film, upon removal of the eye for analysis.

In 1935, Danielli and Davson proposed that hydrophilic proteins present in the cell membrane compensated the oily component in order to explain the low cell-aqueous interfacial tension¹⁸². This concept was later developed further and gave rise to the fluid-mosaic model of membrane structure which was proposed in 1971 by Nicholson and Singer¹⁸³. In this model, which is still widely accepted, the proteins may be thought of as icebergs floating in a sea of lipid. The proteins may be either intrinsic (or "integral") - these have hydrophobic regions that interact with the hydrophobic tails of the lipid molecules in the interior of the bilayer, and hydrophilic regions that are exposed at one or both sides of the membrane - or extrinsic ("peripheral") - these resemble water-soluble globular proteins, and may be anchored to the membrane by non-covalent interactions with transmembrane proteins, or by means of covalently attached fatty acid chains that extend into one of the monolayers. In addition to the exposed hydrophilic membrane proteins, all eukaryotic cells possess surface carbohydrate, mostly as oligosaccharide side chains covalently bound to membrane proteins (glycoproteins) and, to a lesser extent, bound to lipids (glycolipids). In total, the proportion of carbohydrate in plasma membranes varies between 2% and 10% by weight¹⁸⁴, and is normally 10-20nm thick¹⁸⁵. Figure 5.1 illustrates a typical eukaryotic

cell membrane.

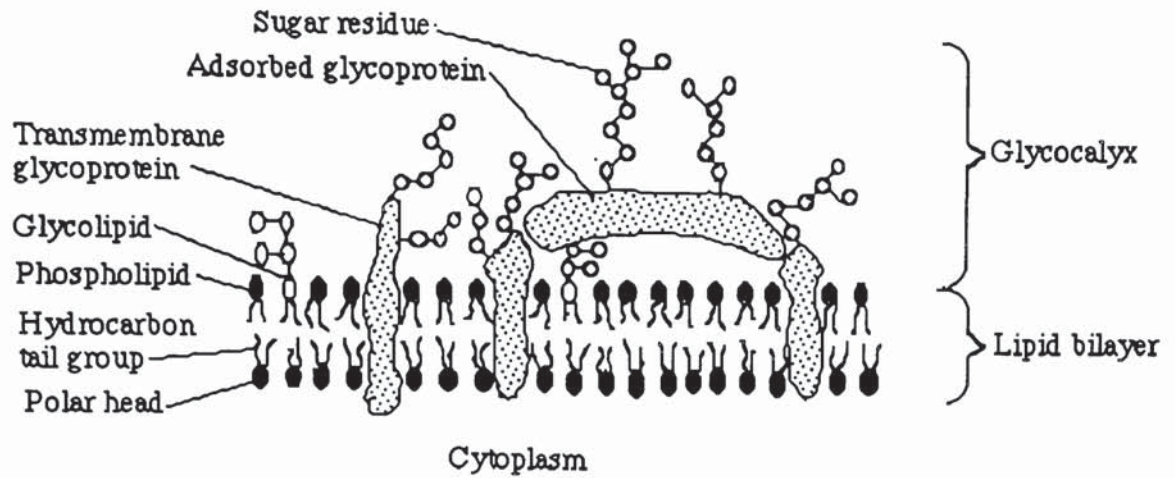


Figure 5.5 Schematic diagram of the cell coat (glycocalyx), which is made up of the oligosaccharide side chains of intrinsic membrane glycolipids and glycoproteins, as well as of adsorbed glycoproteins and proteoglycans (not shown).

The carbohydrate-rich peripheral zone of the cell membrane is referred to as the glycocalyx. The principal carbohydrates in membrane glycoproteins and glycolipids are galactose, mannose, fucose, galactosamine, glucosamine, glucose, and sialic acid. Like the sugar side chains of mucin, the terminal sugar group is normally sialic acid, and this is mainly responsible for the net negative surface charge that characterizes all eukaryotic cells. With the fragile nature of the glycocalyx in mind, it is clear that wiping the surface of the corneas in the fore-mentioned investigation is probably destroying the glycocalyx and causing the hydrophobic lipid content of the cell membranes to mask the hydrophilic domains of the surface and transmembrane proteins. In fact, as Table 5.4 illustrates, a critical surface tension as low as 28mNm^{-1} is normally expected for pure hydrocarbons and fluorohydrocarbon compounds.

Table 5.3 Surface tension components of SIRC cells, calculated from captive bubble contact angle measurement.

Surface	Air Contact Angle	n-Octane Contact Angle	γ_d mNm ⁻¹	γ_p mNm ⁻¹	Critical Surface Tension, mNm ⁻¹
SIRC 10 ⁶ cm ⁻¹	25	152	20.8	45.3	66.1
SIRC 5x10 ⁵ cm ⁻¹	25	149	22.4	44.0	66.4
SIRC 5x10 ⁴ cm ⁻¹	24	154	20.8	45.9	66.8
SIRC 10 ⁴ cm ⁻¹	25	157	19.5	46.9	66.4
SIRC 5x10 ³ cm ⁻¹	28	147	21.6	43.1	64.7
Substrate + Cell medium	25	153	20.4	45.6	66.0
Substrate + Fibronectin	23	149	23.7	43.9	67.6
Substrate + Albumin	31	150	16.8	44.3	63.1
Substrate + Serum	29	147	21.3	42.9	64.2

The critical surface tensions calculated in this study are similar for the varying cell densities and for the adsorbed proteins. This may reflect a trapped layer of water at the cell or protein surfaces. Even if this is the case in vivo, then the corneal surface itself is wettable by the tears (which have a surface tension of around 43 mNm^{-1}) without the need for an adsorbed layer of mucin.

In order to find out whether the captive bubble measurements performed give a true indication of the cell surface properties, rather than being due to an inability of the air or octane bubble to displace water from the sample surface, the same procedure was used for two hydrophobic surfaces, melinex and polythene.

Table 5.4 Surface tension components (mNm^{-1}) of melinex and polyethylene, calculated from captive bubble contact angle measurements

Polymer	Contact angle measurement		Surface tension component		Total S.T
	Air	n-Octane	Polar	Dispersive	
Melinex	71.0	78.0	8.0	36.2	44.2
Polyethylene	95.0	38.0	0.6	35.6	36.2

The results in Table 5.4 reflect the relatively hydrophobic character of polyethylene and melinex, and justify the conclusion drawn from the measurements made for corneal cells that the surface of the cells is hydrophilic in an aqueous environment.

Table 5.5 Effect of surface chemistry on critical surface tension (CST)

Surface Chemistry	C.S.T. at 20 ⁰ C
CF ₃ & -CF ₂ - (1:2)	16.2
-CF ₂ -CF ₂ -	18.5
-CF ₂ -CFH-	22
-CH ₃	22-24
-CFH-CH ₂ -	28
-CH ₂ -CH ₂ -	31
=CH-(Phenyl ring edge)	35

The value often quoted in the literature for the critical surface tension of a "clean" corneal epithelium is 28-31mNm⁻¹. As can be seen from the table of values above, such a low C.S.T. would be expected if the cell surfaces were entirely composed of lipid species. Figure 5.5 illustrates the accepted concept of animal cell membrane structure, which leads us to expect a much higher value for the C.S.T. than those measured.

Measurements made using cultured corneal cell layers in this laboratory provide a higher estimate for corneal cell critical surface tension, indicating a greater polar component, and therefore more hydrophilic nature, than suggested by the earlier figures. It could be that the method of cleaning and drying corneal samples results in the destruction of cell surface integrity and loss of the hydrophilic glycocalyx and exposed proteins.

5.6 Design of a Corneal Surface Model

The results that the work presented in this chapter, and in Chapter 4, has produced indicate that mucoadhesiveness is not an essential feature required by an effective corneal model. Rather than concentrate, therefore, on developing a polymer which will retain an adsorbed layer of mucin, perhaps the most obvious way of designing an artificial cornea is to mimic the exposed cell surface groups. It is the glycocalyx - the oligosaccharide side-chains of the cell membrane glycolipids and glycoproteins - which provide the cornea with its physico-chemical surface properties, including its critical surface tension and its negative charge.

For a corneal model which is to be used in the design of a possible drug carrier, flexibility and biocompatibility are essential. Hydrogels provide these characteristics. A reaction scheme was devised which will incorporate sugar groups into a hydrogel membrane so that the sugar chains are exposed at the surface of the membrane, and resemble the orientation of those at a cell surface. This is shown in Figure 5.6. In the Figure, stage 1 makes use of sugar acids containing terminal alpha-hydroxy acids (eg. gluconic, glucuronic, lactobionic) to synthesize cyclic esters. These form oligomeric macromers with allyl alcohol (shown), or an acrylate, as the initiator of the reaction.

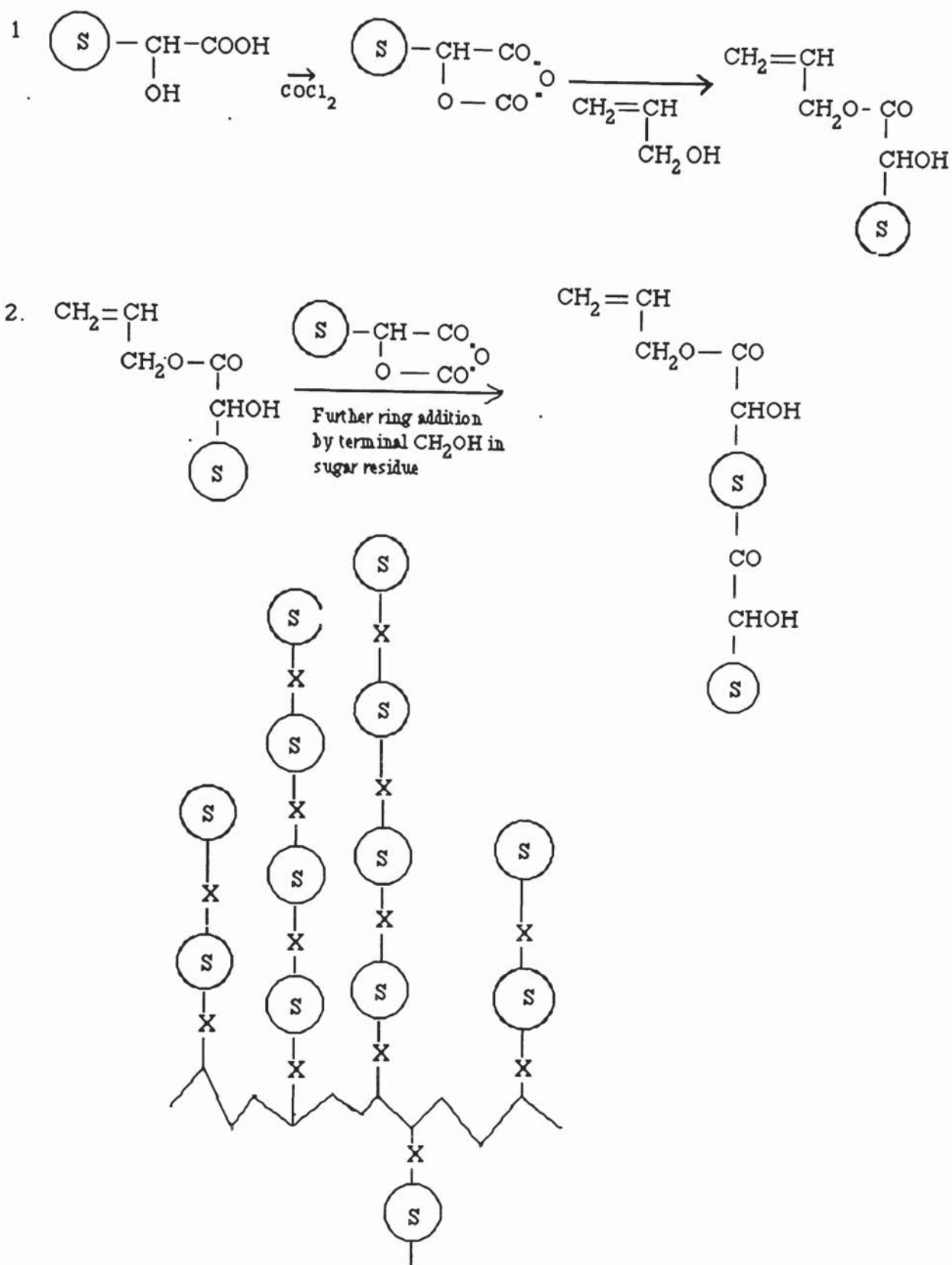


Figure 5.6 Preparation of a corneal surface model by incorporation of sugar monomers into a hydrogel matrix.

In stage 2 of the reaction scheme shown in the diagram, further sugar ring addition is achieved via the terminal CH_2OH in the sugar residue. The sugar acid monomers are then used together with conventional gel-forming monomers to form a series of models of the sugar chains at the cell surfaces.

The time limitations of this project did not allow further investigation of the properties of these cell surface models, so further work is necessary to exploit the potential they may have in the design of drug carriers. One possible problem may be that the presence of sugar groups could encourage microbial or bacterial infection by providing these opportunist organisms with a food source. A way of overcoming this eventuality would be to use L-sugars, rather than the normal D-sugar orientation, since cells cannot utilise glucose monomers in this form. By using the ester rather than the glycosidic linkages between the sugar monomers, however, it is likely that any cleavage of the sugar chains (by esterases) will be slow.

5.7 Discussion and Conclusions

The hydrated cell surface is hydrophilic and, as captive bubble contact angle measurements indicate, has a trapped surface layer of water. This results in an estimate for the critical surface tension for a corneal cell surface *in vivo* of about 66mNm^{-1} . However, earlier measurements of corneal surface tension have been much lower, (28mNm^{-1}), and infer a hydrophobic cell surface which would require an adsorbed layer of surface active glycoprotein (mucin) in order to make it wettable by tears. Our knowledge of cell membrane structure may help explain this anomaly. Simply put, cell membranes are believed to be essentially fluid phospholipid bilayers, arranged so that the polar head

groups are exposed to the cell interior and exterior while the hydrocarbon chains are oriented to the centre of the membrane, with associated integral and peripheral proteins. The integral proteins are amphipathic, with polar regions protruding from the cell surface and nonpolar regions embedded in the hydrophobic interior of the membrane. This molecular organisation creates a polar cell surface in *in vivo* conditions. At the same time, the fluid nature of the membrane means that, if exposed to the air, or to any hydrophobic environment, the polarity of the cell surface can be effectively masked by the reorientation of the lipids. Thus, when measurements of the critical surface tension of the cornea were made in air using corneas which had been wiped dry, values comparable to those of pure hydrocarbons were obtained. These low estimates of surface tension do not reflect the nature of the corneal surface in its native, aqueous environment. The estimate produced in this study using the captive bubble technique, which maintains the hydrated condition of the corneal cells, is likely to be closer to the actual critical surface tension of corneal cells *in vivo*.

A corneal surface model has been devised, using sugar acids and conventional hydrogel monomers, which mimics the glycocalyx of a cell surface. Future work will establish whether the model has potential as a drug vehicle. It may also be of value in elucidating the interactions which occur between corneal cells and the tear fluid components, by providing a flexible, easy to handle substitute for animal corneas or corneal cultures which deteriorate rapidly in non-physiological environments.

CHAPTER 6

The Interaction of Extrinsic Factors with Hydrogels

6.1 **Introduction**

It became apparent during the course of this work that hydrogels are capable of taking up dyes and retaining them in the gel matrix. Of particular interest to this project was whether the biocompatibility of ocular biomaterials (exemplified by contact lenses) could be permanently compromised by the extrinsic factors to which they are commonly exposed. Examples of extrinsic factors are skin lipids, cosmetics, convertible care solution components (e.g. sorbic acid), and sundry strongly partitioned contaminants (e.g. dyes and stains). Materials, such as cosmetics and make-up removers, enter the eye unintentionally and have been implicated in contact lens spoilage problems. Contact lens spoilage compromises the stability of the tear film and leads to further complications, including red eye, and reduces the life span and tolerance of the lens.

A recent study (Tighe et al.¹⁸⁶) has established the importance of the fingers as a source of lipid contamination of the lens surface, with the area of finger-lens contact shown being more important than length of contact time. The fatty acids present in these skin lipids possess both hydrophobic and hydrophilic components so they are able to migrate into the lens matrix, creating localised hydrophobic regions on the lens surface. This process has been implicated in the development of further contact lens spoilage, and subsequent compromise of tear film stability, by facilitating the deposition and uptake of tear lipids.

Sorbic acid is an example of a synthetic, water-soluble, non-coloured species that produces lens discoloration, particularly in conjunction with heat sterilisation and/or high environmental levels of ultraviolet (u.v.) light. The low toxicity of sorbic acid makes

it a popular choice of preservative, and its use in solutions in which the lens is soaked (such as saline and soaking solutions) can result in its uptake into the lens matrix. Lens discoloration is a greater problem with higher water-content, more polar materials used for extended wear soft contact lenses, in which the sorbic acid molecule may become concentrated¹⁸⁶.

Hydrogel lenses are capable of taking up and holding coloured species within the gel matrix. This was illustrated in Chapter 4, by the uptake of Basic yellow, but also occurs with components which may be present in some contact lens solutions. The factors responsible for this type of spoilage reaction may be classed as inherent structural features and the ability of the species to partition into the polymer matrix. For Basic yellow, an azo dye, the dye complex is sufficiently small to be taken up into a hydrogel matrix. Its hydrophobic component (it has two benzene rings) make it soluble in the hydrogel matrix, while its water-binding hydrophilic component make it water-soluble. In the case of sorbic acid, the small water-soluble molecules are only immobilised in the gel matrix when dimerisation (through thermal or photochemical activation) renders the molecules too large to be released. The formation of multiple double bonds by these reactions produces chromophores which are capable of absorbing light and give rise to colour. Many species are, similarly, more soluble in the lens matrix than they are in water, but remain undetected because they lack colour. Thus, the group of molecules that cause discoloration are those whose chemical structures contain multi-unsaturated chromophore groups. To be absorbed into the gel matrix, therefore, a molecule must possess hydrophobic organic regions to give it solubility in the polymer, and hydrophilic groups to render it water-soluble and enable it to bind to hydrophilic sites in the matrix. Molecules which possess these characteristics are

typically synthetic or vegetable dyes, many of which are commonly encountered in everyday life.

Factors which influence the uptake, retention, and colour stability of dyes in hydrogel lenses include the water content or pore size of the matrix, the presence of acidic or basic groups in the hydrogel, the size of the dye molecule, and the sensitivity to pH change of the dye molecule. A number of factors, for instance, may be responsible for the more rapid uptake of dyes, and uptake of larger dyes, by higher water content lenses. These lenses contain basic vinyl pyrrolidone (VP), which attracts dyes containing acid groups such as SO_3H or COOH which are commonly found on dye molecules with some water solubility. The acid-base attraction plays a powerful role in the partitioning process and gives the dye a high affinity for the polymer.

6.2 Contact Lens Contamination With Cosmetics

Perhaps the most frequently encountered dyes are those present in cosmetics. The application of eye make-up brings a wide range of constituents to the ocular environment. The work presented in this chapter is an investigation into the effects of cosmetics on contact lens contamination, and the ability of different cleaning systems to remove these contaminants. The implications for contact lens interaction with the tear film components are discussed.

For this study, soft contact lenses were contaminated by direct application of cosmetics and make-up removers. The three types of contamination looked for were contamination of the lens surface by pigment particles, discoloration of the matrix, and the

contamination of surface and matrix by contact transfer and migration by non-coloured components.

The cosmetics used to contaminate the lenses were all brands manufactured and supplied by Boots the Chemist. The contact lenses were polyHEMA material. Table 6.1 lists the typical constituents of mascara, eye-shadow and eye-liner.

Table 6.1 Typical constituents of eye make-up

Beeswax	Triethanol amine
Carnuba wax	Phenoxyethanol
Hydrogenated palm kernal oil	Magnesium aluminium silicate
Microcrystalline wax	Potassium octoxynol phosphate
Ozokerite	Sodium lauryl sulphate
Paraffin hydrocarbons	Methyl-para-aminobenzoic acid
Butylated hydroxyanisole	Propyl-para-aminobenzoic acid
Cetostearyl alcohol	Cyclomethicone
Stearic acid	Dimethicone
DEG stearate	Oleamide
Propylene glycol	Quaternium-15
Butylene glycol	SD alcohol-1
PVP	Various pigments
Carbopol	Water
Lecithin	

Initially, it was attempted to apply a fluorescent marker to the make-up remover solutions using Methylene Blue and Nile Blue dyes. A 1% Nile Blue solution and a 0.5% Methylene Blue solution each showed some activity with the waterproof eye make-up remover. Presence of the make-up remover on a lens could then be established using fluorospectrophotometry; the fluorescence peak was in the region 500-600nm.

Since none of the other cosmetics displayed any fluorescence, however, u.v.-spectrophotometry was used to quantify the extent to which the cosmetics spoiled the lenses prior to and after cleaning with Boots' soft lens cleaning solution. Spectrophotometry measures u.v. absorption, so any coloured substance left on the surface of a lens will be detected.

The lens to be contaminated was placed above a melinex sheet upon which had been drawn a 3mm² box. Using a fine glass pipette the make-up being tested was applied to the lens surface so that the area above the box was filled. The make-up was spread to achieve a thin film with out clumps of material. All lenses were kept in the hydrated state prior to and during analyses. To prevent any water-soluble make-up from being dispersed from the lens surface, the following technique was used: A supporting melinex "pocket" was made by sealing two pieces of melinex (approximately 5cm²) together at the edges, leaving one side free. The contact lens to be examined was inserted into the pocket and flattened to remove any air bubbles. The open side of the support was then sealed. A new melinex pocket was made for each run in the spectrophotometer.

The spectrophotometer was calibrated to zero against an empty melinex pocket.

The following procedure was used for each cosmetic:

Spectrophotometric analysis of:

- i) the clean contact lens;
- ii) the same lens plus make-up contamination;
- iii) the contaminated lens following cleaning with Boots Soft Lens Cleaning Solution.

The cleaning procedure involved digital cleaning using 2ml cleaning solution, each lens surface being gently rubbed for 20 seconds. The lenses were briefly rinsed with distilled water. The bar chart in Figure 6.1 shows the percentage of u.v. absorption by each of the cosmetics on the lens surface before and after the lens was cleaned with the Boots' soft lens cleaning solution.

The cosmetics are numbered as follows:

1. No. 7 Lashsilk Mascara
2. No. 7 Superlash Mascara
3. 17' Sensitive Eyes Mascara
4. No. 7 Pure Care Eye Colour "Tender Teal"
5. No. 7 Aqua Shadow
6. No. 7 Eye Pencil
7. Eye Make-up Remover Lotion
8. Non-Oily Remover
9. Waterproof Eye Make-up Remover

Cosmetics (3), (4), and cleaner (8) are described as suitable for contact lens wearers.

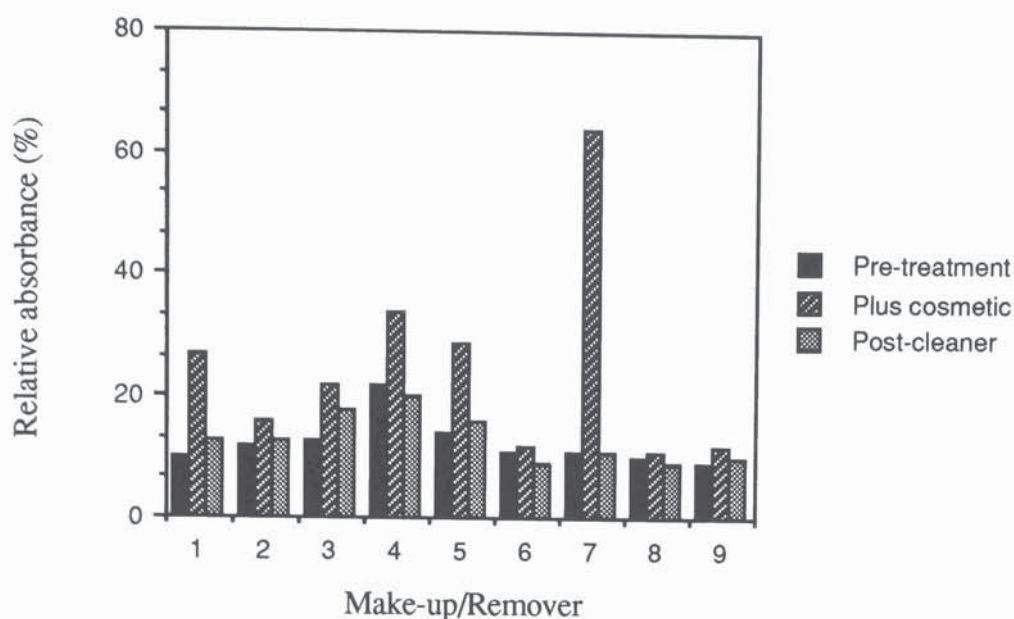


Figure 6.1 Relative absorbance of polyHEMA lenses after application of cosmetics and make-up removers, and after cleaning the treated lenses with a surfactant cleaner (1-3, mascara; 4-5, eye shadow; 6, eye pencil; 7-9, make-up remover).

The technique of u.v.-visible spectroscopy used for these analyses is only sensitive to contamination of the lens by coloured species. The most marked contamination detectable by this method arises from coloured make-ups and lotions which absorb strongly in the visible wavelengths. The contact lens cleaner appears to be fairly effective in removing most of the contamination arising from the coloured components of the cosmetics, although some residual pigments are detected for the lenses treated with mascara and one of the eyeshadows.

To establish the effects of cosmetic contamination on the surface properties of

the lenses, contact angle measurements were made on the lenses. The measurements were taken after the treated lenses had been cleaned and stored in distilled water for several weeks. Each lens was removed from its storage vial and blotted dry prior to making measurements of the water and methylene iodide contact angles. A goniophotometer was used for this work, as described in Chapter 2. The surface tension for each lens, and its polar and dispersive components, was calculated from the contact angle information.

Table 6.2 Effect of cosmetic treatment on surface tension and polarity of polyHEMA contact lenses (all lenses were cleaned before contact angles (θ) measured).

Cosmetic applied to lens	Contact Angles		Surface Tension Components		
	Water	Methylene Iodide	(mNm ⁻¹)		
	(θ)	(θ)	γ_p	γ_d	Total
PolyHEMA lens	35.0	41.0	33.2	28.8	62.0
Mascara (waterproof)	54.5	52.5	24.2	23.0	47.2
Mascara (+ "fibres")	14.0	31.0	42.8	28.6	71.4
Mascara (non-waterproof)	39.0	46.5	34.4	23.8	58.2
Eye shadow	33.0	23.0	31.2	33.5	64.7
Eye liner	48.0	43.0	26.1	27.1	53.2
Make-up remover lotion	45.5	42.5	27.9	26.9	54.8
Non-oily remover	60.5	42.5	16.3	29.8	46.1
Waterproof remover	53.0	41.0	21.5	29.0	50.5

The changes in polarity and surface tensions observed for the lenses treated with the cosmetics, when compared to a clean lens, indicate that, despite cleaning and storing in distilled water, the cosmetics and make-up removers have a residual effect on the surface chemistry of the hydrogel. In most cases the overall effect is a decrease in the total surface tension of the lens, largely due to a reduced polar component. This is explained by the fact that most of the components of cosmetics are oils and waxes, leading one to expect that any residual material would cause a decrease in the polar component of surface tension of the hydrophilic gel, and a corresponding reduction in the total surface tension of the lens. Cosmetics, particularly make-up removers, contain surfactants, and if these are absorbed and immobilised in the lens matrix, their ability to bind water may give rise to artificially low water contact angles, which would in turn reduce the magnitude of the reduction in polar component caused by the presence of the waxy components. Other colourless constituents of cosmetics, while not detected by spectroscopy, may also affect the surface chemistry of the polyHEMA matrix. The lens which had been treated with the mascara containing "fibres" shows a dramatic increase in surface polarity, so two possible explanations for this effect are either that treatment of the lens with this cosmetic has produced an artificially "rough" surface on the lens creating physical irregularities which can lower the contact angle measurements, or that certain water-binding components of the make-up have been absorbed into the lens matrix. The first explanation appears to be more likely since both water and methylene iodide contact angles on this lens have been reduced in comparison to those measured for the lens treated with the non-fibrous mascara.

6.3 **Conclusions**

Extrinsic factors play an important, and underestimated, role in contact lens spoilage processes. Probably most significant are the finger lipids and cosmetics since contact lenses are regularly brought into close contact with these sources of contamination. Spectrophotometry is an effective means of detecting the presence of coloured species on or within a hydrogel lens. Using this technique, a surfactant-based contact lens cleaner was shown to be fairly effective in removing most pigmented contaminants. However, further work has indicated that residual contamination, by non-coloured components of cosmetics or make-up removers, may affect the surface chemistry of the lens material. This has important implications for tear film stability, and the efficacy of any ocular drug delivery process in the presence of either cosmetic-contaminated lenses or, simply, the presence of the cosmetics themselves around the external eye.

CHAPTER 7

Summary and Suggestions for Further Work

7.1 Introduction

What has been made clear during this research work is that the chemistry and the physical properties of the tears impinge greatly on the level of success of any ocular therapeutic treatment. Perhaps the main reason for the low efficacies of current drug delivery systems is the poor understanding of the properties and functioning of the lacrimal system, since what happens to a substance immediately following its introduction into the tear film is not fully appreciated. This work has made some progress in unravelling some of the mystery, beginning by drawing together the widely scattered information available on the tears. As a result of the extensive literature survey, and further investigations into the properties of tears and the corneal surface, it was possible to consider rationally the reasons for the poor performance of ocular drug delivery systems and to propose a means of improving drug retention in the tear film. Furthermore, in the light of the new information gained through studies on the physico-chemical properties of tears and the cornea, a synthetic model of the corneal surface has been designed. This will facilitate the *in vitro* assessment of drug formulations in terms of their ability to interact with, and be retained by, the corneal surface.

7.2 An Overview of the Precorneal Tear Film

One of the most striking features of the tear fluid, made clear during the literature survey, is its complexity and diversity and, moreover, the need for this complexity. It is not secreted as a complete mixture from a single source, but as a discrete number of separate combinations of components, at an overall rate of about $1\text{-}2\mu\text{l min.}^{-1}$, with the secreting glands located in the vicinity of the globe and eyelids^{12,109,114,116,187}. The blinking action of the eyelids serves both to mix these

various tear components, (which vary in concentration depending on a number of factors such as age, sex, and health), and to help spread the resulting mixture over the exposed surface of the eye. During the process of blinking, which also acts as the driving force behind tear drainage, a considerable shearing force (of at least 3000 sec^{-1}) is exerted on the tear fluid. This causes a shear-thinning effect since the tear fluid is, and behaves as, a dilute solution of polymers, such as mucin, which have pseudoplastic properties. Another important feature of the tear film which has been frequently reported in the literature is its supposed 3-layered structure. This model of the tear film was first described in 1954 by Wolff¹ to consist of a surface lipid layer (100nm thick), a middle aqueous layer ($7\mu\text{m}$ thick), and an inner mucus layer ($0.02\text{-}0.05\mu\text{m}$ thick). While this view of the tear film is still generally accepted today, recent developments of new techniques have enabled non-invasive estimates of tear thickness (earlier methods involved the introduction of glass filaments or fluorescein into the tear film) to be made, and these indicate a much thicker tear film, of about $40\mu\text{m}$, with an inner layer of mucus of at least $30\mu\text{m}$ ⁶. This structural complexity needs careful consideration when designing drug delivery systems, or any solution, for the eye. For successful treatment, a therapeutic agent needs to be maintained at effective levels in the tear film while, at the same time, causing minimal disturbance to the ocular environment.

7.3 Corneal Wettability: Considering the Role of Mucin

The question surrounding the relationship between the ocular glycoprotein and the corneal surface is of considerable significance to the development of efficient drug delivery systems for the eye, and requires careful investigation. Early measurements of corneal critical surface tension (Holly and Lemp, 1970¹²⁰) indicated that it is hydrophobic,

with critical surface tension calculated as 28mNm^{-1} , and this led to the proposal that an adsorbed layer of mucin on the corneal surface is required to enable the tear fluid to spread. The reliability of these critical surface tension measurements is questionable, however, since the technique used to make them involved contact angle measurements performed on corneas removed from their usual aqueous environment. It has been established¹⁸³ that the fluidity of cell membranes allows them to mask any polar or hydrophilic groups when exposed to a hydrophobic environment such as air. This makes it likely that in-air measurements of contact angles on dry corneas will produce surface tension values which are not representative of the *in vivo* corneal surface. Furthermore, if one considers the orientation of the phospholipids and membrane proteins of living animal cells, whereby the hydrophilic groups are exposed to the usually aqueous external environment, it is unlikely that the corneal epithelial cells in contact with tear fluid will be as hydrophobic as suggested. Indeed, the contact angle measurements of hydrated corneal epithelial cells carried out for this research programme indicate a much more hydrophilic surface, with the critical surface tension estimated as around 66mNm^{-1} . A surface this hydrophilic would be wettable by tear fluid which has a surface tension measured as 43mNm^{-1} , without a need for an adsorbed layer of mucin.

7.4 Implications of the Ocular Environment for the Design of Drug Delivery Systems

Conventional administration of drugs, to the anterior surface of the eye does not exploit the physiology of the tear film and, as a consequence, is very inefficient. The reasons for the inefficiencies of current ocular drug delivery systems have been discussed earlier in this work, but principal among them are reflex tearing, blinking and lacrimal

drainage, all of which are consequences of disturbing the tear film, and all act to rapidly expel the foreign agent from the eye. Thus, both the concentration and duration of exposure of a medication in the eye are determined by the character and dynamics of the precocular tear film.

7.4.1 Retention of Ophthalmic Solutions in the Tear Film

Some work has been carried out¹⁸⁸⁻¹⁹¹ to evaluate the retention of solutions in the tear film. Tear turnover has been estimated to be about 16% per minute under normal physiological conditions, but this increases several fold upon stimulation¹³⁰, such as may arise by introducing ophthalmic solutions, particularly if they are non-isotonic and at non-physiological pH. Studies have shown that under normal circumstances the half-time of residence of an introduced solution ranges from 10 seconds to 4 minutes, depending on the degree of reflex tearing elicited¹³⁶. Further investigations have suggested that the elimination rate can be correlated to the dropsize instilled. The effect of dropsize on retention in the tear film is illustrated by Figure 7.1 which shows concentration remaining in the tear film.

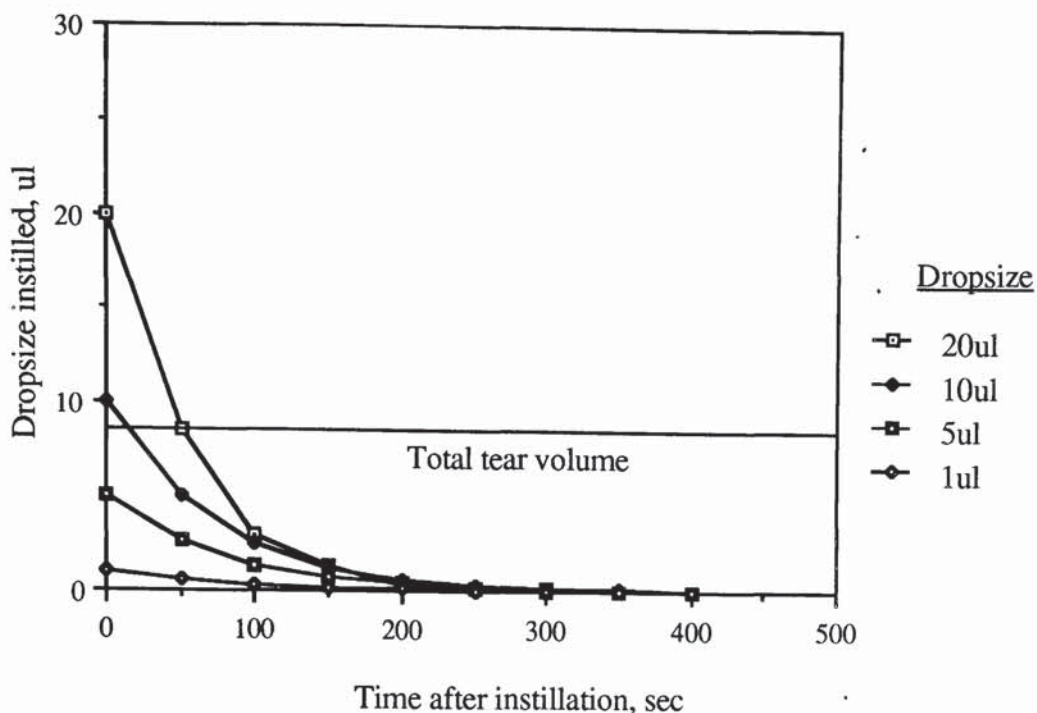


Figure 7.1 Effect of dropsize instilled on rate of elimination from the tear film.

Figure 7.1 uses data from work carried out by Ludwig and Van Ooteghem¹⁸⁸ using fluorescein instillation to show the effect of dropsize on the rate of elimination from the tear film. As the graph makes clear, the larger drops are eliminated from the tear film at a more rapid rate. No information on any difference in lacrimal secretion as a result of instillation is reported, but elsewhere in the literature lacrimal stimulation is stated to be insignificant for drop sizes below $20\mu\text{l}$ ¹³⁴. If the total tear volume remains relatively constant, at around $8.5\mu\text{l}$, the concentration of drug formulation as a percentage of total tear volume can be calculated. This is shown in Figure 7.2.

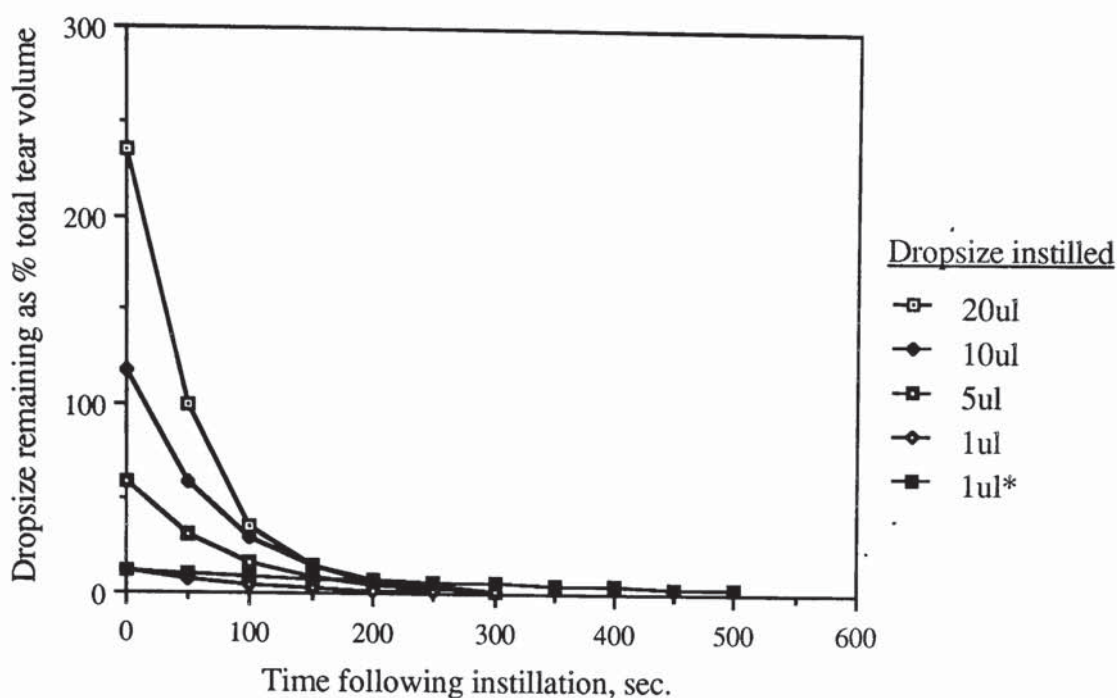


Figure 7.2 Volume of different drop sizes remaining following instillation, as a percentage of total tear volume. (Based on work published by Ludwig & Van Ooteghem¹⁸⁸ and Puffer et al.¹⁸⁹).

As Figure 7.2 illustrates, whatever size drop is instilled, the volume remaining after 200 seconds is similar and close to zero. As a result of instillation, the drug levels in the tear film will reach a maximum quickly, and then fall rapidly to a minimum, when further administration becomes necessary. If these concentration maxima and minima fall above or below the toxic level or minimum effective level, respectively, alternating periods of toxicity or inefficiency can result, and this is an important reason why there is such a need for improved ocular drug delivery systems. What is also apparent from the graph, though, is that the smaller the drop volume instilled, the more gradual is its elimination and, hence, its concentration more stable in the tear film. One inference to be drawn from this is

that drug delivery by the introduction of smaller volumes of concentrated agent may be a more effective means of treatment than are frequent applications of larger, more dilute, drops. A foreseeable problem with this approach, however, is the difficulty of delivering such small volumes to the eye. Overdosing, since the patient may not perceive the introduction of a droplet of this size, could be a particular risk. Correspondingly, if the patient assumes drop delivery has taken place when it has for some reason failed, the eye may go untreated. This is not one of the problems associated with conventional ophthalmic drug applicators which dispense droplets averaging about $50\mu\text{l}$ ⁸. A drop this size can displace most of the tear film, since the maximum volume of fluid held by the cul-de-sac is 20 to $30\mu\text{l}$, washing away the surface oils and hence reducing break-up time. As discussed above, there is no advantage in introducing a drop size this large into the tear film. Rather than reducing the frequency of application required, the more rapid rate of elimination of a large introduced volume may necessitate treatment at shorter intervals than would smaller, more concentrated, dosages.

A number of studies have been carried out which indicate that by increasing its viscosity, the retention of a solution in the tear film may be prolonged^{130,138,139,141,142,165,166,190}. In addition, there is some evidence that polymer structure can also affect the rate of its elimination. Figure 7.2 compares the retention times of two different polymer solutions of similar viscosity with those of saline and a petrolatum-mineral oil ointment.

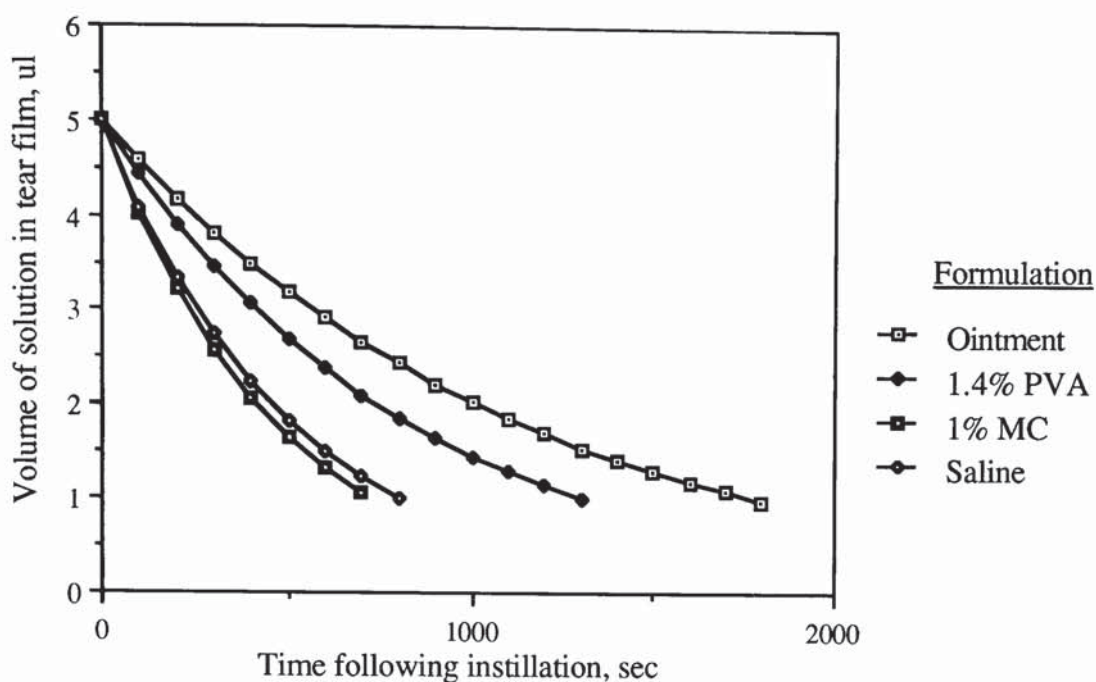


Figure 7.3 Effect of formulation on rate of elimination from tear film.
(Based on results published by Hardberger et al.¹⁹⁰).

These results indicate that oil-based ointment is retained longer on the eye than are solutions relying on viscosity for improved retention times. Unfortunately, ointments are not easy to administer in precise dosages and can cause blurring of vision. Of the two polymer solutions, both of which are commonly used in ophthalmic formulations, polyvinyl alcohol (PVA) shows the longest retention times in the tear film. Methyl cellulose (MC), however, shows little improvement on saline in terms of its ability to remain in the tear film. Since these polymer solutions are of similar viscosity, the improved performance of PVA must be due to some ability of this polymer to interact with components in the tear fluid or on the cornea. Although a 5 μ l volume of 1.4% PVA is reduced by half within 7 minutes of instillation, its relatively long retention time is exploited

by its inclusion in some artificial tear solutions. Further work is required to assess the potential of PVA as a drug vehicle.

While these studies of droplet retention have provided useful comparative results for different solutions and dropsizes, account must be taken of the techniques used. Figure 7.4 illustrates the different results obtained for the elimination rate of a 10 μ l drop using alternative monitoring methods.

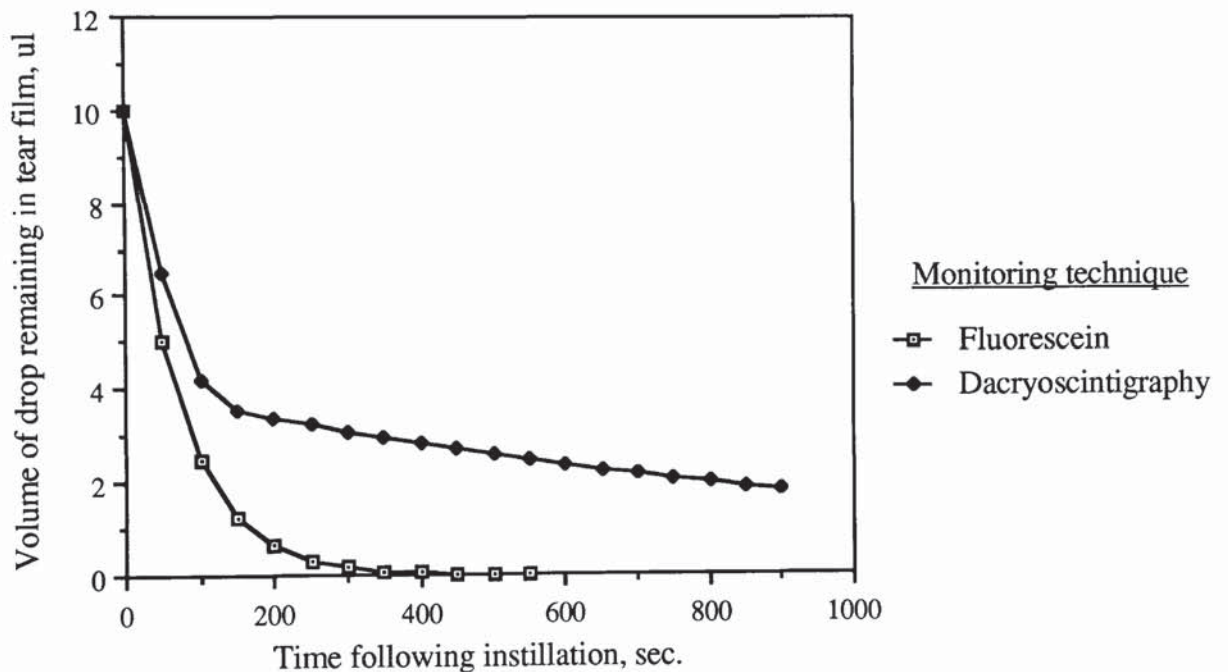


Figure 7.4 Effect of monitoring technique on recorded rate of solution elimination from the tear film.

The two most frequently used methods are dacryoscintigraphy, which involves radioactive labelling of the ophthalmic solution and subsequent measurement of radioactivity levels, and slit-lamp fluorophotometry which measures the level of

fluorescence remaining in the tear film after fluorescein instillation. Dacryoscintigraphy produces considerably higher estimates than fluorescence analysis for retention time of an instilled drop of low viscosity. One possible explanation is that the radioactive particles of pertechnetate become entrapped in the viscous mucus coating the conjunctiva and hence lead to an overestimation of solution retention. This theory is supported by the observation that sodium pertechnetate, which was used to obtain the results shown in Figure 7.3, gives a much longer estimate for saline retention than does slit-lamp fluorophotometry used to obtain the results shown in Figure 7.1. Both methods may, ofcourse, give a distorted picture of the true retention times of introduced solutions if they destabilise the tear film or irritate the ocular surface to a different extent than would the solution alone.

7.4.2 Rheological and Tensiometric Properties of Tears and Ophthalmic Solutions

Before an ideal drug delivery system can be designed, the interactive behaviour of tears with both instilled solutions and with the cornea needs to be investigated in order to assess the effect different formulations may have on corneal integrity and tear film stability. It is also essential to consider that an instilled solution will be affected by the high shear stress exerted by the eyelid and, possibly, by the chemical components of the tear fluid. The study of the interfacial and rheological properties of tears is, therefore, a complex but important part of understanding the behaviour of solutions instilled into the eye. The work carried out for this project sheds light on the physical properties of tears and a range of solutions designed for ocular instillation. In particular, estimates for the polar and dispersive components of the tear fluid were calculated, and these suggest that nonpolar components contribute as much as 40% to the total surface tension. However, these

calculations were partly based on published reports of tear surface tension values which may, in fact, be underestimates since they involved surface-invasive measurements of static tears.

The results of research using contact angle measurements have enabled predictions to be made of how solutions may behave when introduced into the tear film. When artificial tear solutions are instilled, we can predict from comparative ability of these solutions and a mucin solution to wet different surfaces, and by comparing the interfacial tensions they exhibit against an oily phase with those measured for mucin, whether they would interact with the mucin layer in the tear film. The general conclusion for most of the artificial tear formulations studied is that they would act by increasing the thickness of the fluid over the eye, thereby temporarily relieving dry eye symptoms. However, contact angle measurements may give rise to misleading interpretations of the ability of an artificial tear solution to perform as a surrogate tear due to the surfactant content of these solutions (preservatives often present in these solutions, such as benzalkonium chloride, are powerful surfactants). If the surfactant concentration is increased in an attempt to improve what are perceived to be "wetting" properties, reflected by the production of smaller contact angles on a surface than mucin, the surfactants could actually disperse the ocular mucin and lipid layer, thereby destabilising the tear film. Ultimately the epithelial cell membranes would be destroyed.

7.5 Adhesive Properties of Mucin: Pertinence to Ophthalmic Design

Mucin-specific stains and fluorescence analyses were used in this study to investigate the ability of mucin to adhere to polymer surfaces, encompassing a wide spectrum of hydrogel groups, including neutral, ionic and polyethylene-modified hydrogels. One of the triumphs of this work has been the development and assessment of the Basic yellow dye and fluorophotometry as a mucin-monitoring technique. Basic yellow is a very sensitive stain for mucin. Optimum mucin/dye concentration ratios for maximum fluorescence have been established, and the specificity of the dye for mucin evaluated. As discussed above, there was little indication of mucin adhesion to any of the polymers irrespective of hydrogel character. In support of this, there was no detection of fluorescently-labelled mucin at all on hydrogels using lower photometer sensitivity, despite the fact that even at lower sensitivity, Basic yellow is an effective stain for mucin, producing fluorescence peaks of at least 50% for the Basic yellow-labelled PSM solutions used throughout this study. The absence of fluorescent activity anywhere approaching the level which would be expected if mucin were adherent to the polymers studied further underlines the conclusion, arising from this work as a whole, that mucin does not adhere to the corneal surface.

A number of recent efforts to improve ocular drug delivery have concentrated on the development of mucoadhesive polymers as drug vehicles. The ultimate success of these systems in practice will rely on the adherence of the drug/polymer complex to the mucus layer coating the surface of the eye and its consequent retention in the precorneal tear film. This is one reason why it is essential to investigate the nature of the interaction between mucin and the corneal and conjunctival epithelia, and to establish the turnover rate

of mucin in the tear film. This research project has gone some way in the former area, obtaining no evidence that mucin possesses any specific binding capacity for corneal epithelial cells. Further insight into the properties of mucin were also obtained by investigating its relationship with a variety of hydrogel polymers. These studies found no indication of any significant mucin adhesion on the surfaces of these polymers. Since hydrogels possess similar physical properties to the hydrated cornea, it could be argued that if mucin does not adhere to these polymers it is unlikely to do so to the cornea. Other studies^{28,129}, discussed in Chapter 1, suggest that mucin is not adherent to the cornea but is present as layer loosely associated with the corneal glycocalyx extending from the microvilli. Dilly¹²⁹ suggests a thicker layer of mucin occurs over the conjunctiva as an interconnected network of strands, attached at point of secretion and to various anchor sites on conjunctival cell surfaces. Further, a rapid turnover of mucin is indicated by the removal of any strands over the cornea, formed during a blink, by their adhesion to the mucous network of the undersurface of the upper lid during the same blink¹²⁹. Also, if the rigidity of the corneal surface is considered, together with the short-chain sugar groups of the glycocalyx presented at its surface, it is unlikely to provide favourable conditions for binding mucin which has a massive and flexible "bottle-brush" structure much more likely to form interactions with the extensive binding sites present on other mucin molecules.

By collating all this information, the picture developing is of a relatively transient retention of mucin at the corneal surface but greater mucin association with the conjunctiva. This is a further point of pertinence to the design of drug delivery systems. The outcome of incorporating drugs into mucoadhesive vehicles may be that they are retained for extended periods, when compared with conventional drug delivery systems.

However, if these become located preferentially over the conjunctiva, rather than the cornea, much of the drug released could be lost to the systemic circulation since the conjunctiva is vascularised. This effect would lead to some of the very problems associated with conventional ocular therapy which new systems hope to avoid: namely, the difficulty of optimising drug concentration, and risks of systemic side effects.

7.6 New Strategies for the Design of Ocular Drug Formulations

In order to avoid the problems associated with mucoadhesive drug carriers, it may be better to develop a drug formulation which will interact with the corneal cell surface rather than with mucin. Amongst the several empirical principles of polymer chemistry is that similar polar, but uncharged, structures tend to interact with each other. The results of work presented here, for example, infer that mucin glycoproteins associate more readily themselves than with other hydrophilic polymers. Suggestions arising from this work, and from reports in the literature, that there is relatively little mucus over the cornea support this proposal.

If one considers the physical properties of the cornea in comparison with those of other mucous membranes, there are a number of striking differences which could explain why the mucoadhesive strategy is an unsatisfactory approach for ophthalmic drug delivery. The gastrointestinal tract, for example, where mucoadhesives have been used with more success for sustained drug delivery systems, has a number of properties which would make it much more receptive to mucoadhesives: particularly the very large surface area, with a high density of villi, which is in constant motion, together with the large quantities of mucus present. In addition, the mucoadhesive properties of polymers such as

poly(acrylic acid) are thought to be enhanced by the acidity of the gastric environment which increases the capacity for hydrogen bonding between polymer and mucin to occur. The cornea, on the other hand, has a much higher rigidity modulus with a comparatively small surface area and only a thin film of mucus, which is constantly removed and replaced with each blink, over its surface. The opportunities for mucoadhesion are at the outset very small, and the efficient turnover of mucin limits the potential retention of mucoadhesives. This may explain the low efficacies of ophthalmic solutions designed to be retained by the eye which rely on mucin look-alikes, such as hydroxypropyl methyl cellulose, for their performance in this respect.

During the course of this research, a novel polymer was designed which incorporates sugar groups into a hydrogel matrix in order to resemble the corneal glycocalyx. A synthetic model of the corneal surface may prove effective in adhering to the cornea when introduced into the tear film in the form of a linear soluble polymer. This, and other polymer design strategies, has been undertaken as a result of the conclusions drawn from the work described herein. In particular, it would be advantageous if a synthetic glycoprotein, designed to possess similar structural and physical properties to the natural polymers, were developed. Readily available mucins of animal origin, such as porcine and bovine gastric mucins, cannot be sufficiently purified to be used clinically for the alleviation of mucus deficiencies in man, and alternative glycoprotein replacements such as hyaluronic acid, which is used intraocularly, are too expensive to be used in cases where relatively large quantities would be required, such as in preparations for the digestive system.

7.7 Concluding Summary

In conclusion, at the outset of this research programme the task in hand was an apparently straight forward case of pinpointing the types of hydrogels which possess the characteristics considered to be desirable for new drug delivery systems. The most important characteristic was initially presumed to be mucoadhesivity since the cornea was reported to be coated with an adsorbed layer of mucus. However, as has become clear during the course of these studies, in reality the requirements for optimizing drug retention in the tear film are not so simple. This work has made a number of important contributions to this area of research. Firstly, an extensive review of published reports on the tear film has helped to bring together information that was previously widely scattered but of importance when considering the development of improved ophthalmic drug delivery systems. Second, in doing so, it has drawn attention to the incomplete level of understanding of the tear film and its relationship with the ocular surface. For instance, by critically examining the information available, the great variability in reported tear component concentrations is shown to be a probable result of differences in the sensitivity of the analytical techniques used, and may be reduced by considering component ratios rather than absolute concentrations. A third achievement has been the development of fluorescence spectroscopy as an analytical technique for detecting mucin both in solution and on hydrogel surfaces. As a side issue, the potential role of extrinsic factors, such as cosmetics, in contact lens spoilage was evaluated using spectroscopy since any processes which compromise tear film stability may be relevant to our overall understanding of the ocular environment. Fourthly, as a result of this work, and work involving cultured corneal cells, the widely held view of an adherent layer of mucin over the corneal surface is challenged.

The final message from this work is that in order to achieve successful extended ocular drug delivery through retention of the active ingredient in the tear film, a drug carrier may need to structurally resemble the corneal surface. With this in mind, a corneal model has been designed which bears exposed structural groups, similar to those occurring in the glycocalyx of the corneal epithelial cells.

7.8 Suggestions for Further Work

The values reported for tear fluid surface tension are likely to be underestimates since the measurements were made using invasive techniques on a static tear sample which will have a disproportionate concentration of surface active components, such as fatty acids, at its surface. It is difficult to foresee how it would be possible to avoid this problem, but a better method may be the measurement of tear surface tension by non-invasive means, such as the pulsating bubble method, and future work should include this technique.

Subsequent work could be carried out to develop further the use of the Basic yellow dye for mucin. A particular feature of the Basic yellow dye molecule which has been addressed during this work is that it is sufficiently small to be absorbed by hydrogel matrices, staining them yellow and resulting in some hydrogels exhibiting fluorescence activity at a similar wavelength to stained mucin. The cause of this behaviour is not yet clear and requires further investigation. It would be interesting to assess the effect of treating materials exposed to fluorescently-labelled mucin with acetyl cysteine. Acetyl cysteine would remove any mucin from the surface of the material, so that any difference in fluorescence before and after treatment could be attributable to mucin.

A technique which may prove a useful tool for detecting the presence of mucin, and other components, on polymer or cell surfaces is Fourier Transform - Infra-Red (FTIR) spectroscopy. Every chemical group reflects infra-red light at a characteristic wavelength. FTIR spectroscopy is a technique that can be used to obtain a spectrum which is the digital representation of the amount of infra-red energy transmitted through, or reflected by, a sample over a specified frequency range. The peaks obtained on such a spectrum can therefore be related to the chemical composition of the sample being analysed. The results of preliminary work, shown in Appendix 2, carried out for this project show that it is possible to differentiate between cells and substrate from the spectra obtained for a confluent cell layer grown on a hydrogel membrane. The same technique could theoretically be used to evaluate the ability of the model cornea to adhere to cell surfaces; confluent layers of cells exposed to samples of the novel polymer could be rinsed in saline and then analysed by FTIR. The characteristic peaks produced by the polymer would indicate its presence on the cell layer.

Further work is needed to investigate the properties of the proposed corneal surface model and to assess its potential in the field of ophthalmic formulation. In addition, new linear polymers incorporating oligosaccharide groups as short side chains, such as poly(itaconic acid), should be made. The conclusions drawn in this work about the nature of the corneal surface suggest that polymers of this type might possess the necessary properties to enable them to adhere to the cornea, and this corneal adhesivity could initially be studied using the corneal model. These novel linear polymers may provide the basis for the design of a branching, mucin look-alike with potential for use as a replacement in cases of deficiency of mucus glycoproteins.

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APPENDICES

APPENDIX 1

Fluorescence Spectra

APPENDIX 1

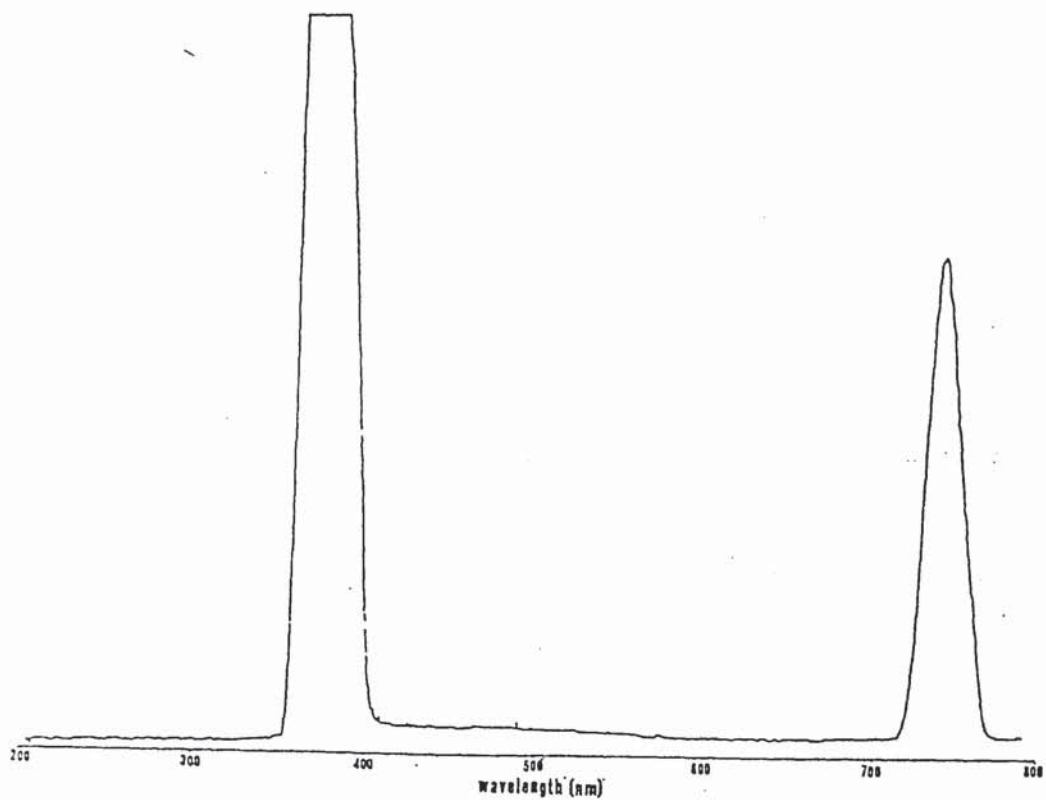
Fluorescence Spectra

An Aminco - Bowman fluorospectrophotometer (SPF - 125STM) was used to obtain fluorescence spectra. This machine, which consists of an optical unit and a xenon lamp power supply, automatically scans the excitation or emission wavelengths from 200 to 800 nanometers (nm). The fluorohotometer was connected to an X-Y chart recorder which traced the spectra.

For the following emission spectra, an excitation slit width of 2mm was used since this gives optimum stability, and the emission slit width was set at 0.5mm. The "percent full scale" multiplier switch was set to 0.3 for full-scale recordings.

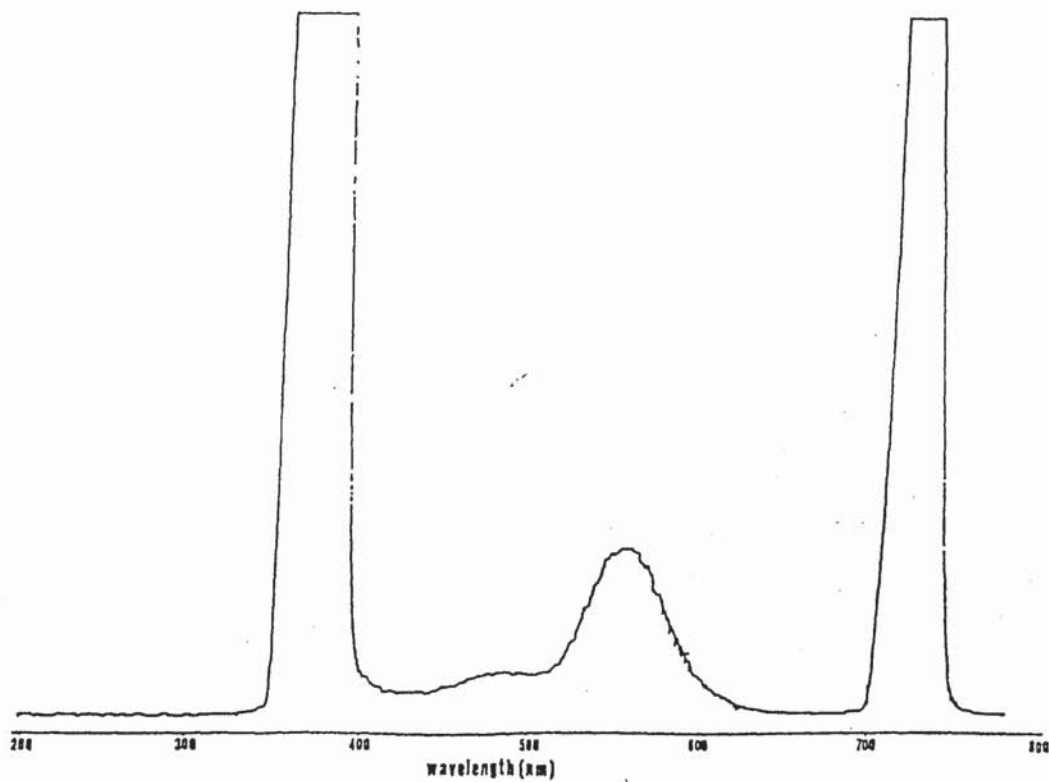
1.

0.5% BSM (type 1-S) in PBS



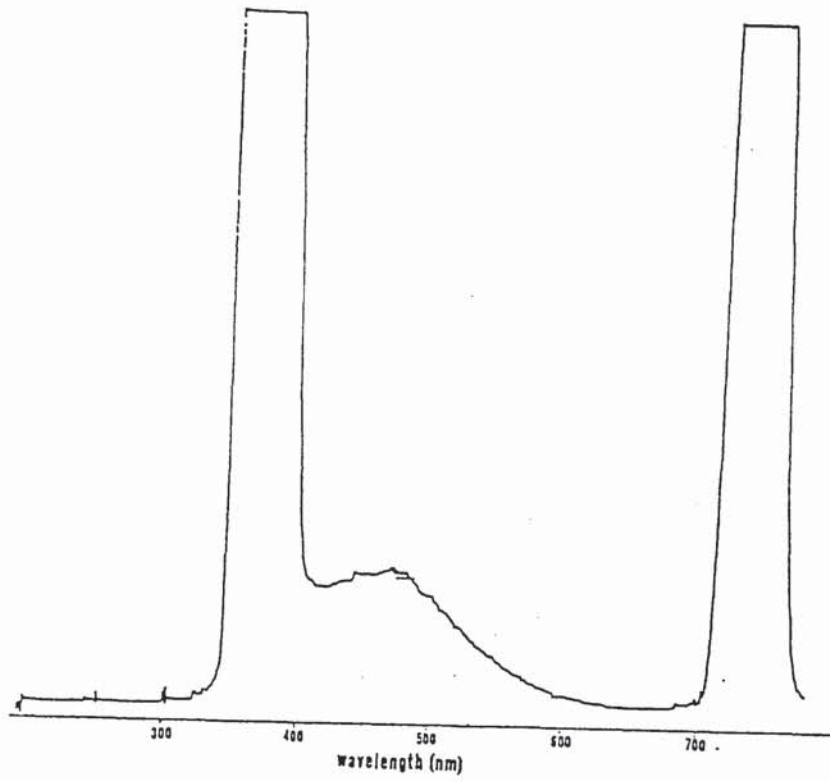
2.

0.5% BSM (in PBS) + 0.1% Basic Yellow (3:1)



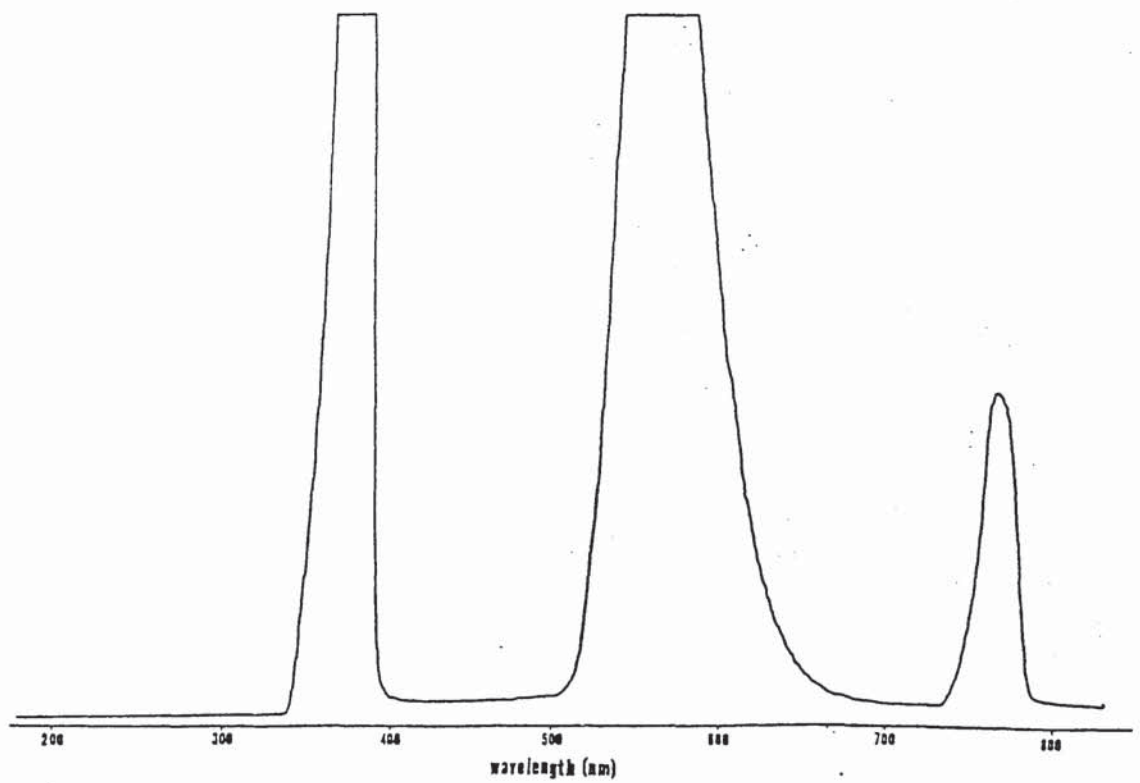
3.

0.5% PSM (In H₂O)

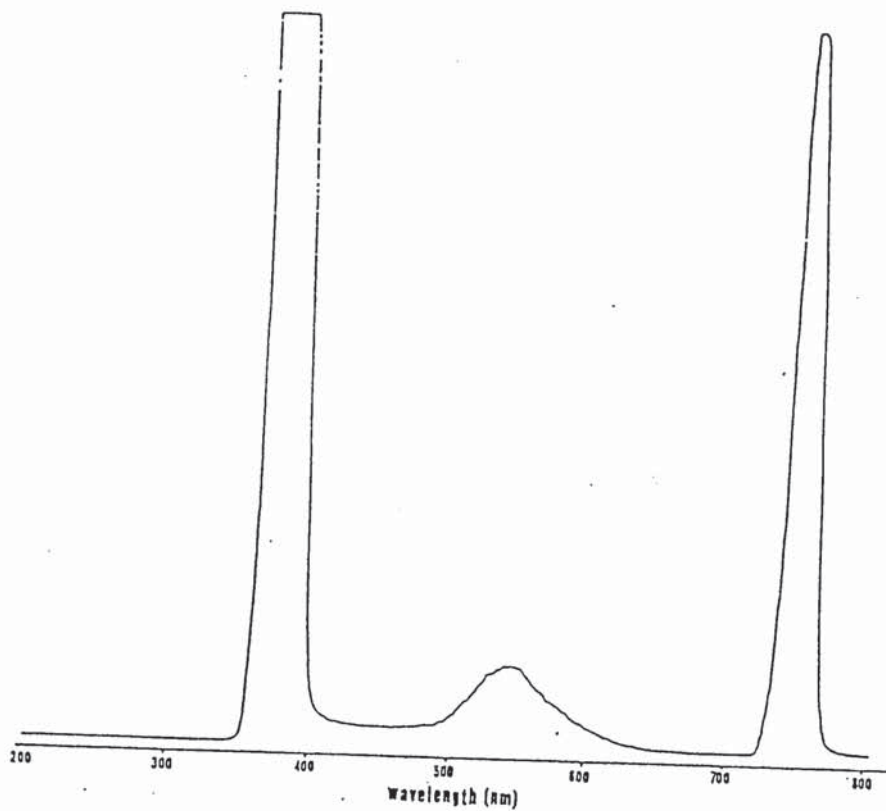


4.

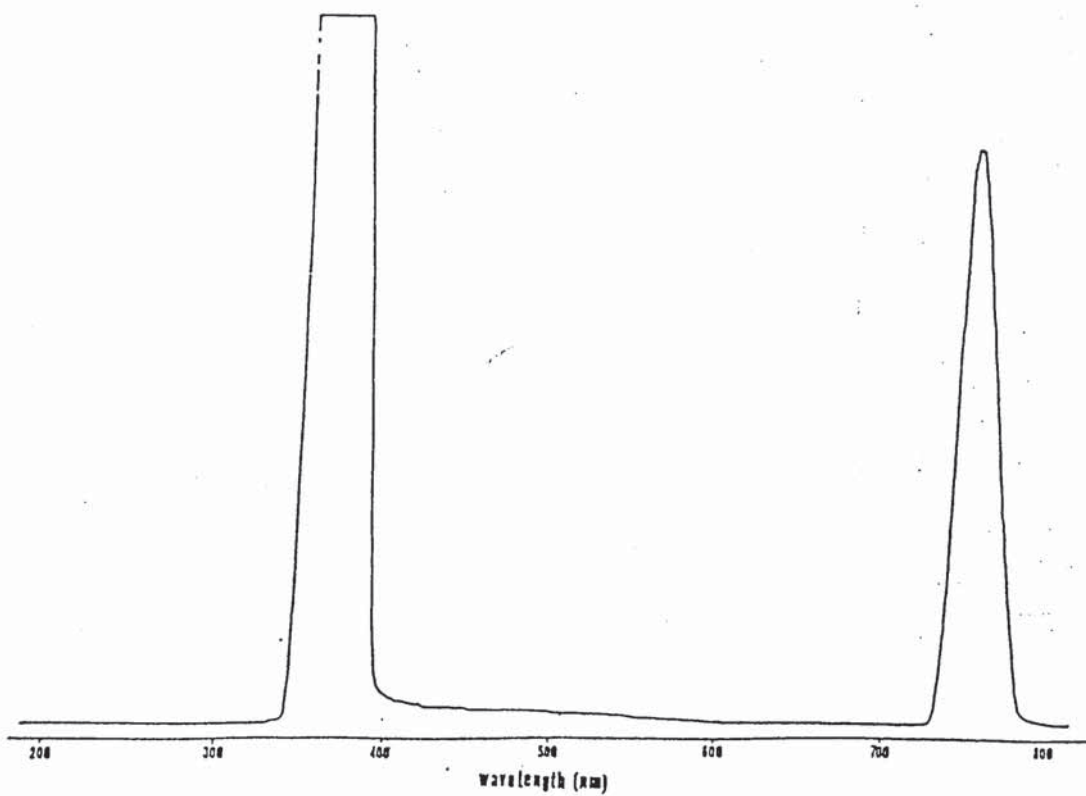
0.5% PSM / 0.1% Basic Yellow (3:1)



5. HEMA/MMA (70:30) + PSM/Basic Yellow (48hrs in solution)

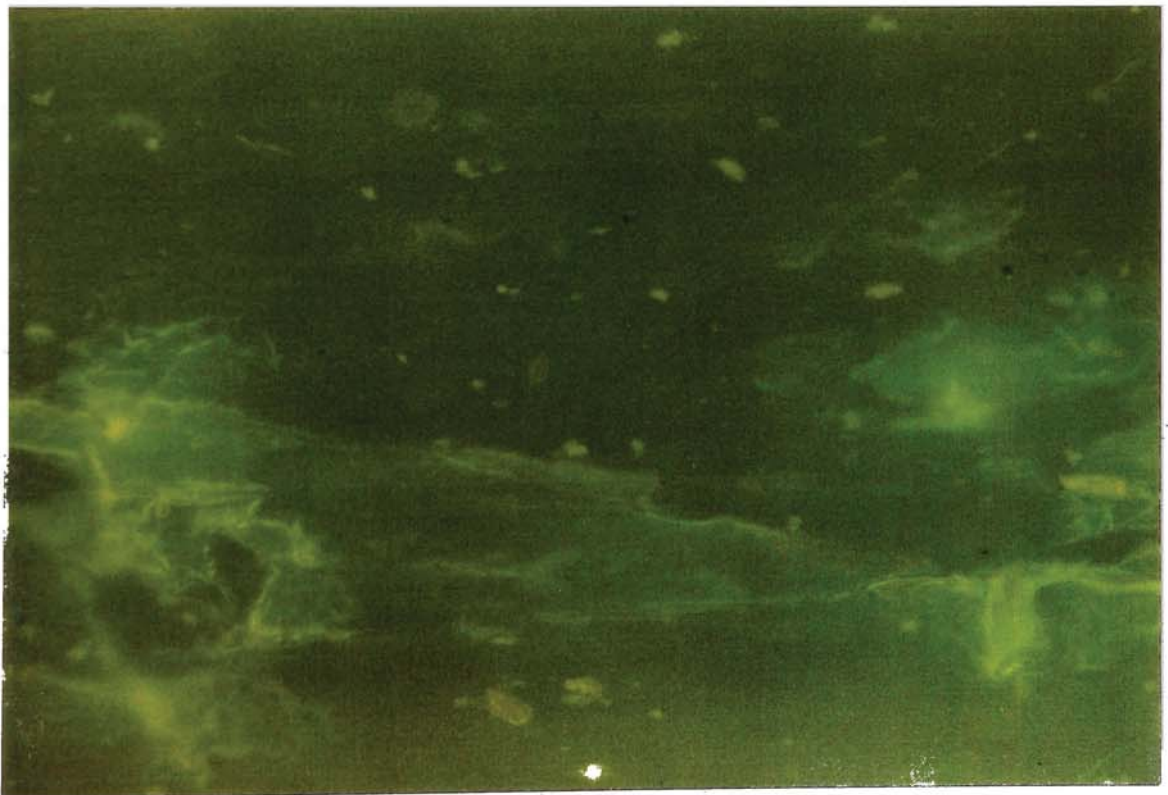
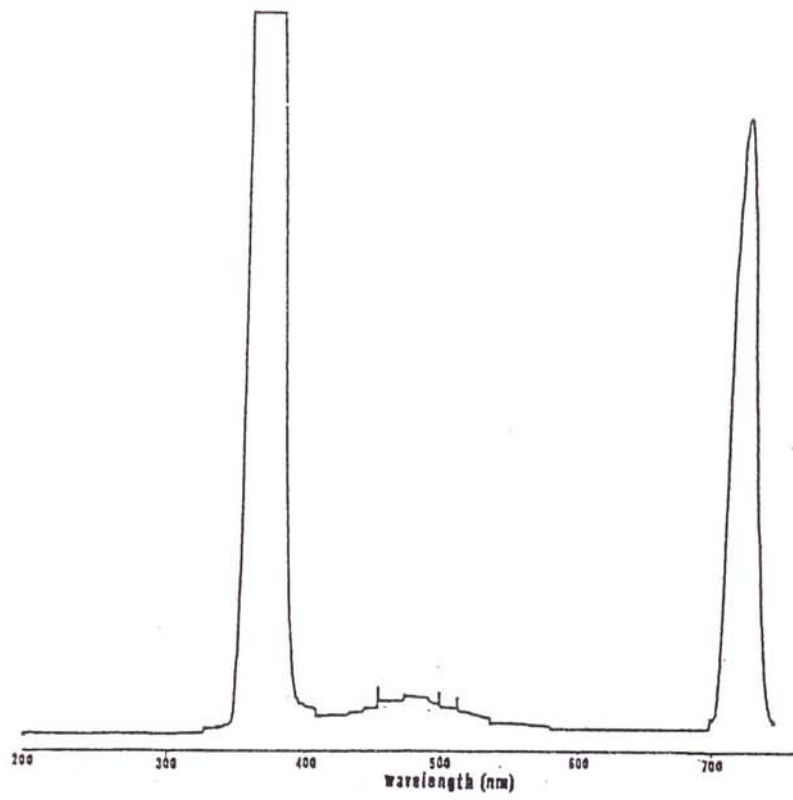


6. HEMA/MMA (70:30) + 0.5% PSM/0.1% Basic Yellow Rinsed with PBS



7.

0.1% Basic Yellow



Fluorescence micrograph of a porcine mucine smear to show its native fluorescence (x 125)

APPENDIX 2

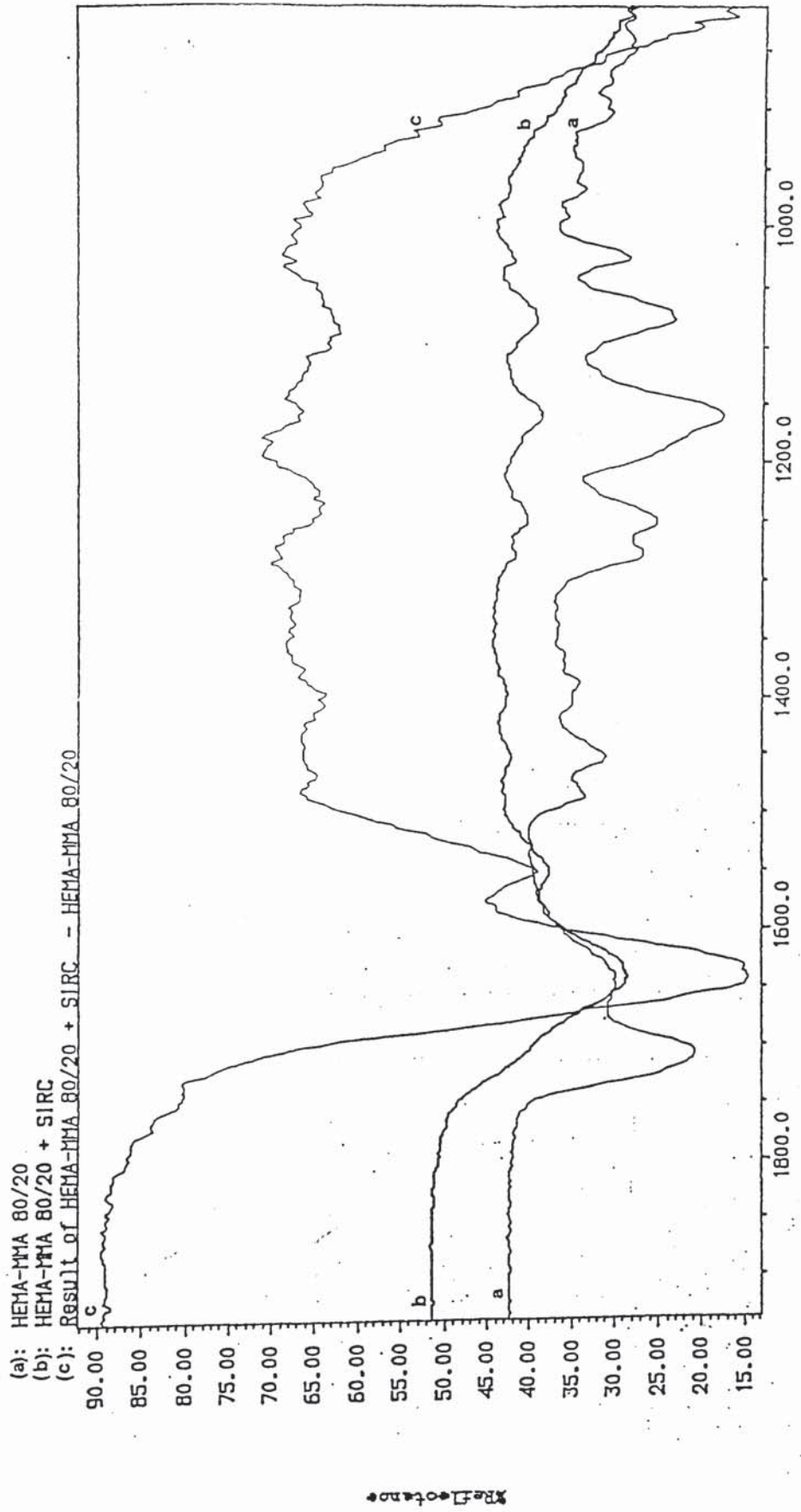
Fourier Transform - Infra-Red (FTIR) spectroscopy

APPENDIX 2

Fourier Transform - Infra-Red (FTIR) spectroscopy

Figure 1 is a typical spectrum obtained for a layer of SIRC cells grown on a HEMA/MMA (80:20) hydrogel membrane. As illustrated, it is possible to subtract the contribution to the spectrum attributable to the hydrogel in order to produce a spectrum of the cells themselves. Reflectance peaks are located at wavelengths correlating to the following chemical groups: C-C; C-O; C-N; C-H; O-H; N-H; C=C; C=O; C=N. In addition, peaks corresponding to the presence of primary, secondary and tertiary alcohols ($\text{CH}_2\text{-OH}$, CH-OH , C-OH), and secondary and tertiary amines ($\text{CH}_2\text{-NH-CH}_2$, CH-NH-CH , $(\text{CH}_2)_3\text{N}$), occur. Thus, the infra-red spectrum reflects the lipid and protein composition of the cell membranes. Although not developed further here, due to time limitations, FTIR-spectroscopy has the potential to be a useful method of detecting the presence of glycoproteins on cell surfaces. By attaching a marker, such as basic yellow, to mucin in solution, which would produce a characteristic spectrum, it should theoretically be possible to differentiate between cell membrane glycoproteins and any adherent mucin adsorbed onto the cell surfaces. In the same way, the mucoadhesive properties of polymers could be investigated. The height of the peaks could be used to estimate the amount of mucin present.

HEMA/MMA SIRC-clean
Result of HEMA-MMA 80/20 + SIRC - HEMA-MMA 80/20



Wavenumber (cm-1)