JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 1087– 1092

Polyurethanes as potential substrates for sub-retinal retinal pigment epithelial cell transplantation

R. L. WILLIAMS[1,2,*], Y. KRISHNA[2], S. DIXON[3], A. HARIDAS[1], I. GRIERSON[2], C. SHERIDAN[2]
[1]Clinical Engineering and [2]Ophthalmology, School of Clinical Sciences, University of Liverpool, L69 3GA
E-mail: rlw@liv.ac.uk
[3]Biomer Technology Ltd, Tudor Road, Manor Park, Runcorn, Cheshire, WA7 1SY, UK

Transplantation of cultured retinal pigment epithelial (RPE) cells under the failing macular is a potential treatment for age related macular degeneration. An important step in the development of this procedure is the identification of a suitable membrane on which to grow and transplant the cells. This paper evaluates the potential of using polyurethanes in this application since they possess several of the required properties, such as, flexibility, robustness, biostability and good biocompatiblilty although their hydrophobicity can limit cell adhesion. Three commercially available polyether urethanes (Pellethane®, Tecoflex® and Zytar®) were evaluated in terms of their wettability using dynamic contact angle analysis and their ability to support a monolayer of functioning RPE cells (ARPE-19) . Furthermore Pellethane® and Tecoflex® were treated with a simple air plasma treatment and analysed as above. In the "as received condition" only a few RPE cells attached to the Pellethane® and Tecoflex® and remained clumped. RPE cells grew to confluence on the Zytar® substrate by 7 days without further surface modification. Air gas plasma treatment of both Pellethane® and Tecoflex® increased the wettability of the surfaces and this resulted in the growth of a monolayer of well-spread RPE cells on both materials. Morphologically these cells grew with a normal 'cobblestone' phenotype. These results demonstrate the potential of these polyurethanes for this application.

1. Introduction

Age Related Macular Degeneration (ARMD) is the most common form of blindness in the Western World and as yet there is no effective treatment either to arrest the visual deterioration or to restore lost vision. Although the pathology of ARMD is not completely understood it is known that deficiencies in the support tissue of the macula, particularly the retinal pigment epithelium (RPE), are pivotal to the development of the disease. As a result, transplantation of fresh cultured RPE under the failing macula represents a reasonable way ahead for a potential treatment for ARMD [1].

Two of the major problems that exist include long-term rejection of homologous RPE and the identification of a pliable and biologically optimal substrate for transplantation. The substrate needs to be both sufficiently pliable and robust in very thin films for handling and implantation, be able to be manufactured as a porous structure to allow transport of nutrients and waste, be biostable and have an excellent biocompatibility and sustain a monolayer of functioning RPE cells. Polyurethanes are a class of material that fulfils many of these criteria. Polyurethanes are block copolymers with a hard and a soft segment and the properties can be manipulated by changing the ratio and chemistry of each of these components [2]. In this way the material can vary from a hard engineering polymer to a soft elastomer. The biostability of polyurethanes has been extensively reported in the literature [3]. Several polyether urethanes have been evaluated for *in vivo* use but many have been found to be susceptible to oxidative degradation, in particular, in stressed environments [4]. Poly carbonate urethanes and polyurethanes containing silicones have been shown to have good biostability [5]. Thus the mechanical properties and biostability of polyurethanes could be designed to be suitable for this application. They can be cast into thin films which are robust and can be manufactured by electrostatic

*Author to whom all correspondence should be addressed.

spinning to create a porous substrate [6]. One of the disadvantages of polyurethanes is that they tend to have a hydrophobic surface and therefore are not well suited for growing a monolayer of well adhered and spread cells. The choice and ratio of the hard and soft components can be manipulated to reduce the hydrophobicity or it is possible to modify the surface properties of the polyurethane without influencing the bulk mechanical properties of the film to overcome this problem [7].

In the present study we have evaluated three commercially available polyether urethanes [1 aliphatic, 2 aromatic] to test out the hypothesis that polyurethanes can be used to establish a monolayer of RPE cells that may be suitable for subretinal transplantation.

2. Materials

Three commercially available polyurethanes were used: Pellethane® grade 2363 80AE (kindly donated by Dow Benelux N.V.), Tecoflex® (Thermedics, Inc) and Zytar® (Z1A1) (Biomer Technology Ltd.). The Pellethane® was provide in sheets approximately 1mm thick and was used in this form. The Tecoflex® was dissolved in dimethylacetamide and methyl ethyl ketone and cast against a glass plate at 35–40 °C for 3 h and then placed in a vacuum oven for a further 2 h to produce a film approximately 200 μm thick. The Zytar® was provided as a film approximately 100 μm thick and was used in this form. All materials were cleaned in 100% ethanol and then allowed to dry.

3. Methods

3.1. Cell adhesion

The quantitative analysis in this study was performed using human ARPE19 cells, an established but non-immortalised human RPE cell line obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in a 1:1 vol/vol mixture of Dulbecco's modified Eagle's and Ham's F12 medium (Gibco) containing 3 mM L-glutamine (Gibco), 10% fetal bovine serum (Gibco), and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin sulfate, (Gibco).

Cells were removed from their flasks using 0.1% trypsin and 0.04% EDTA, then mixed with complete medium and centrifuged for 10 min at 800 rpm. After the supernatant was discarded, the cell pellet was resuspended in fetal bovine serum (FBS). Cell numbers and viability were determined by trypan blue exclusion in a Haemocytometer under the ×25 lens of a light microscope.

Samples were incubated with the ARPE-19 cell line for various time periods up to 7 days. Tissue culture polystyrene (TCPS) was used as a control. After each time period the media was aspirated and the substrates were initially rinsed in PBS (3 × 5 min) then fixed in 10% neutral buffered formalin for 10 min. Fixed cells were stained with Alexa-488 Phalliodin (Molecular Probes) for 30 min and/or counterstained with Propidium Iodide (Sigma) for 10 min and mounted in fluorescent mounting medium (Dako). The cell number was determined using a light microscope (Polyvar, Reichert-Jung). For the cell adhesion assay the average number of cells on the control surface after one hour was set to 100% and the average number of cells on each substrate was presented as a percentage of the control. For the quantification of cell number over longer time periods the data are presented as the average number of cells per field of view (45998 μm^2) ±1 standard deviation (SD) ($n = 6$ for Tecoflex® and Pellethane®; $n = 15$ for Zytar®).

3.2. Dynamic contact angle measurements

The surface wettability of Pellethane® and Zytar® (dip coated glass coverslips) were evaluated using dynamic contact angle analysis[8] using a Camtel CDCA 100 (Camtel Ltd. UK). The samples were dried, attached to the sample clip and lowered into distilled water at a rate of 0.06 mms^{-1}. The advancing and receding contact angles were calculated from the hysteresis curve obtained. The number of samples was 4 for Pellethane® and 3 for Zytar®.

3.3. Gas plasma treatment

Samples of Pellethane® and Tecoflex® were air plasma treated in an Emitech K1050 X Plasma Asher (Emitech Ltd., England) for 2 min at a power of 80 W and an air flow of 12 ml min^{-1}. At the end of the treatment time the samples were removed from the plasma chamber and placed directly into sterile distilled water at room temperature. They were stored in this environment for at least 24 h and then evaluated using dynamic contact angle analysis as above. All treated samples were subsequently re assessed following the cell adhesion and cell number methods detailed above.

3.4. Assessment of monolayer function—phagocytosis of photoreceptor outer segments (POS)

Fresh bovine retinas were isolated from eyes obtained from a local abbatoir. Approximately 20 retinas were homogenised by manual agitation in 0.73 M sucrose solution, filtered through a 100 μm nylon mesh under far-red illumination, layered on top of a discontinuous sucrose density gradient (0.8, 1.0, 1.2 M) and centrifuged (Beckman L8-70) at 60,000 g (22,000 rpm) at 4 °C for 1 h. The purified POS were harvested from the interface between 0.8 M and 1.0 M sucrose solutions, placed in PBS and pelleted at 27,000 g (12,000 rpm) for 20 min at 4 °C. Resuspended POS concentration was given in μg/ml using a Micro BCA protein estimation assay kit. Haemocytometer counts revealed that 50 μg (POS protein/ml contained approximately 2×10^7 POS/ml [9]. POS were labelled in the dark with a pH sensitive dual fluorophore 5-(and-6)-carboxy SNAFL-2, succinimidyl ester (Molecular Probes) as per the manufacture's protocol. Microscopic analysis of the POS protein-SNAFL conjugate showed outer segment discs. The labelled POS were stored at −80 °C. ARPE-19 cells were seeded on air gas plasma treated Tecoflex® and control TCPS and incubated as above. At Day 7, when cells were known to have reached confluence, each of the samples were rinsed with fresh media and challenged with 10 μg/ml of POS; then incubated for

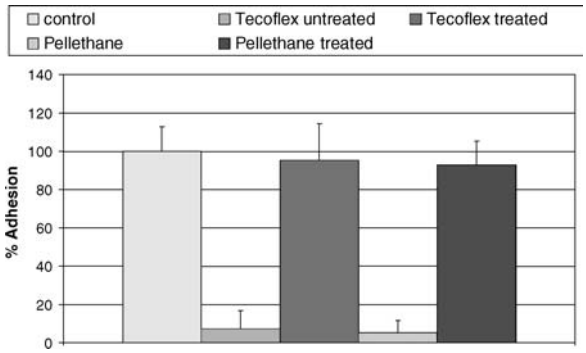

Figure 1 The data presented are the mean number of cells (nuclear counts) per field attached to each surface after 1 h as a percentage of the control ±1SD. This histogram shows that the untreated polyurethane substrates supported very low levels of ARPE-19 cell adhesion but air plasma treated polyurethane supported cell adhesion levels that were similar to control levels.

3 and 24 h. At each time-point, samples were rinsed five times with PBS, mounted in glycerol at pH 9 and immediately visualised using fluorescence microscopy, with excitation at 488 nm and emission at >530 nm. Ten fields of view were photographed per treatment and control and the number of attached (which fluoresced red) and internalised POS (which fluoresced green), were counted. The mean number of attached and internalised POS per field of view (±1 SD) were presented in a histogram.

4. Results

4.1. Cell culture

After one hour untreated Pellethane® and Tecoflex® substrates supported very low levels of ARPE-19 cell adhesion as a percentage of the TCPS control (7.3% ± 9.3 for Tecoflex® and 5.3% ± 6.3 for Pellethane®), which were statistically significant ($P < 0.001$) (Fig. 1). The numbers of ARPE-19 cells populating the untreated Tecoflex® and Pellethane® substrates did not significantly increase during 5 days incubation (Fig. 2).

The number of ARPE-19 cells attached to the Zytar® surface over the 7 day period are presented in Fig. 2. After 1 day there were 13.8 (±11.8) cells adhered per field of view (45998 μm^2) on the Zytar® samples and 19.0 (±2.7) on the control surface. Cell number on the Zytar® was significantly lower ($p = 0.05$) than on control for all time-points. However, after 4 and 7 days the number of cells attached to the Zytar® surface had increased to 51.7 ± 23.4 and 95.9±22.6 respectively. The morphology of the cells at day 7 on Zytar®, as shown by actin staining (Fig. 5), suggests that the cells are well spread and have reached confluence by this time.

Subsequent air plasma treatment of the Pellethane® and Tecoflex® surfaces significantly increased the number of ARPE-19 cells adhered, as compared to untreated surfaces (95.0% ± 19.3 for Tecoflex® and 92.7%±12.6 for Pellethane®; $P < 0.001$). The cell numbers reached TCPS control levels following one hour incubation (see Fig. 1). Similarly the number of cells adhered to the air plasma treated surfaces increased rapidly (Fig. 3). By day 2 the number of cells adhered to the treated Tecoflex® and Pellethane® were 87.2 ± 17.8 and 90.5 ± 10.8 per field of view, respectively. By 3 days these values had risen to 118 cells for both materials and this remained approximately constant for day 4 and 5. These values were significantly different to untreated substrates at all time points ($p < 0.001$).

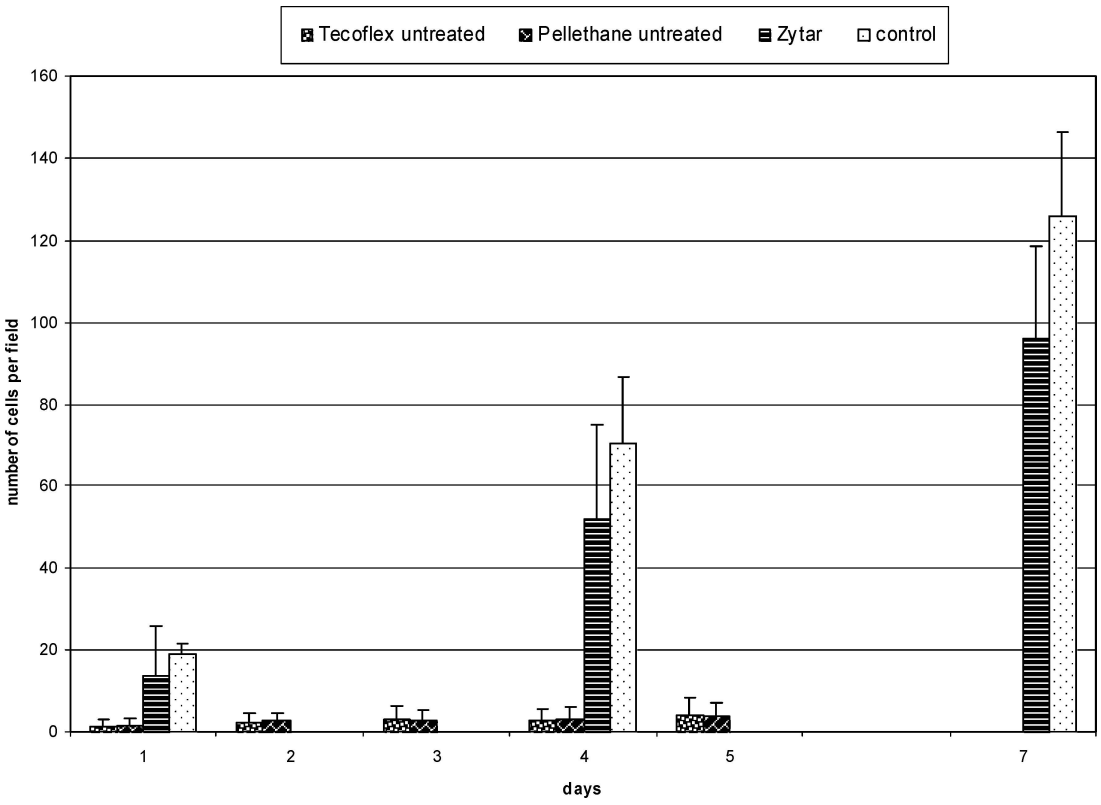

Figure 2 Histogram illustrating the cell number with number of days on the untreated Tecoflex®, untreated Pellethane®, Zytar® and control (TCPS). Low cell numbers were seen on the untreated polyurethanes over all time points, whilst an increasing sigmoid trend was seen on zytar and control. Data presented as mean cell number (nuclear counts) per field ±1 SD.

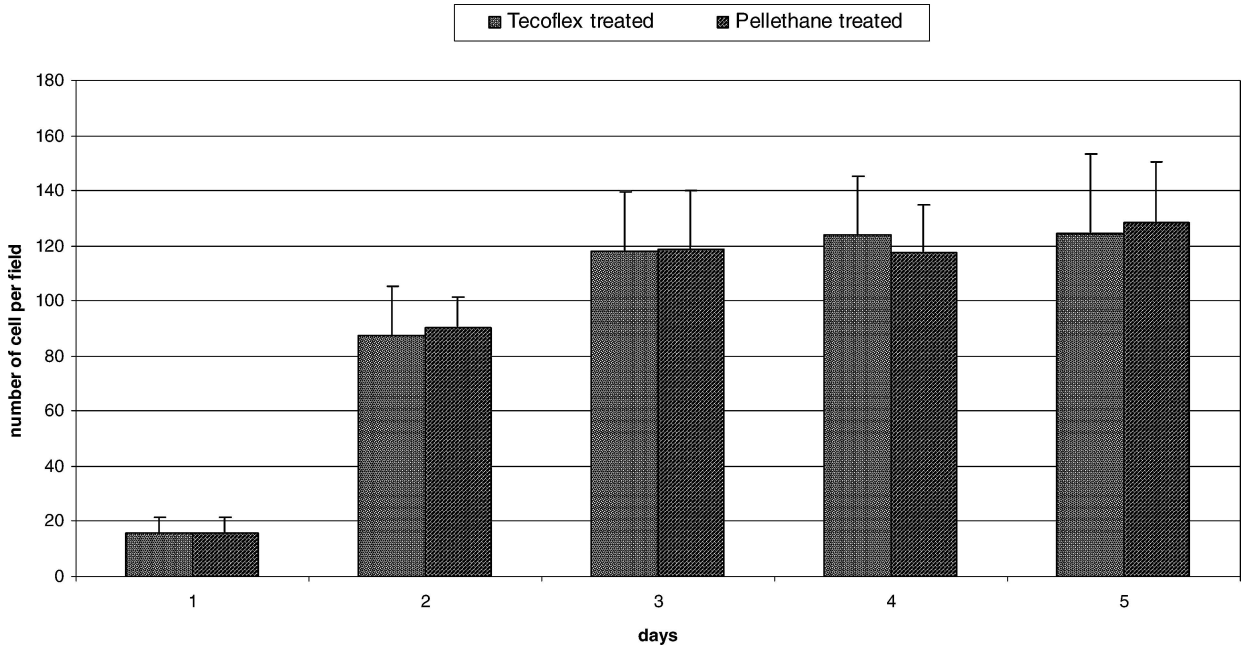

Figure 3 Histogram showing cell number versus number of days on the air plasma treated Tecoflex® and Pellethane®. An increasing sigmoid trend was seen. Data presented as mean cell number (nuclear counts) per field ±1 SD.

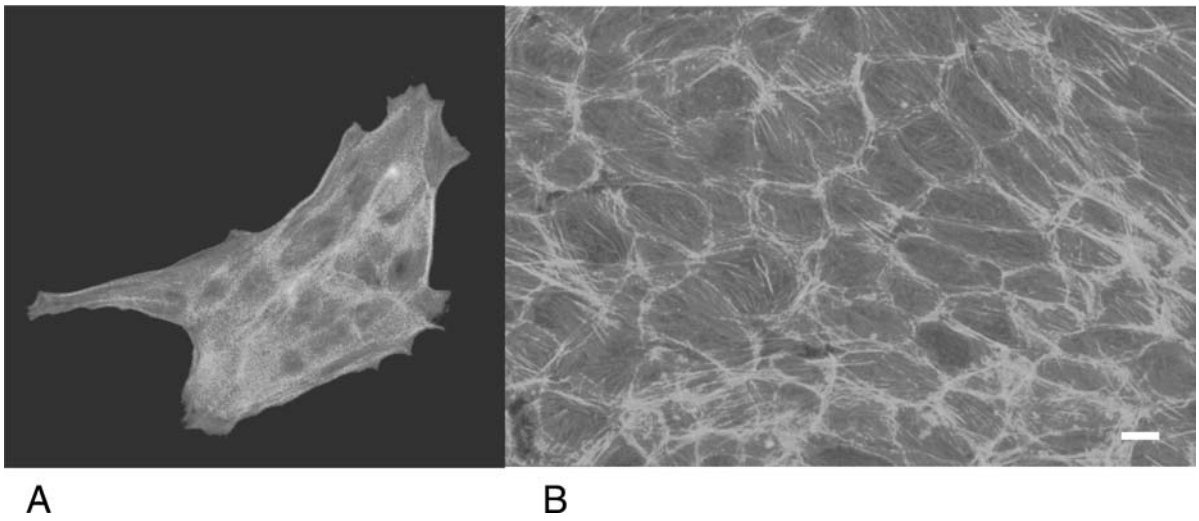

Figure 4 Representative photomicrographs of ARPE-19 cells stained with Alexa-488 Phalliodin on both untreated (A) and treated (B) Pellethane® and Tecoflex® after 5 days. (A) On the untreated substrates the cells that did adhere to the surface formed clumps (×40) whereas in (B) the cells on the air plasma treated surfaces were well spread and appeared to form a normal cell monolayer. Scale bar = 10 μm.

Morphological assessment of cells by phase contrast microscopy and fluorescent microscopy demonstrated that by day 5 on the untreated substrates the cells that did adhere to the surface were not well spread and appeared to clump (Fig. 4(a)) whereas the cells on the air plasma treated surfaces were well spread and appeared to form a normal 'cobblestone' pattern as the cells reached confluence (Fig. 4(b)).

4.2. Dynamic contact angle measurements

The advancing and receding contact angles for the Zytar® and the untreated and air plasma treated Pellethane® are presented in Table I. It can clearly be seen that the air plasma treatment followed by the post treatment storage in water reduced both the advancing and receding contact angles of the Pellethane® indicating an increase in the wettability of the polyurethane surface ($P < 0.001$). The advancing and receding contact angle for Zytar® fell between the untreated and treated Pellethane® results.

4.3. Phagocytosis of POS

The functional behaviour of the attached ARPE-19 cells was evaluated using a phagocytosis assay. The data

TABLE I Dynamic contact angle data for untreated and air plasma treated Pellethane® ($n = 4$) and Zytar® ($n = 3$) presented as the mean ± standard deviation

	Advancing angle/°	Receding angle/°
Zytar®	80.4 ± 2.8	59.7 ± 3.0
Untreated Pellethane®	93.1 ± 1.8	70.9 ± 2.0
Air plasma treated Pellethane®	61.6 ± 1.3	34.5 ± 3.1

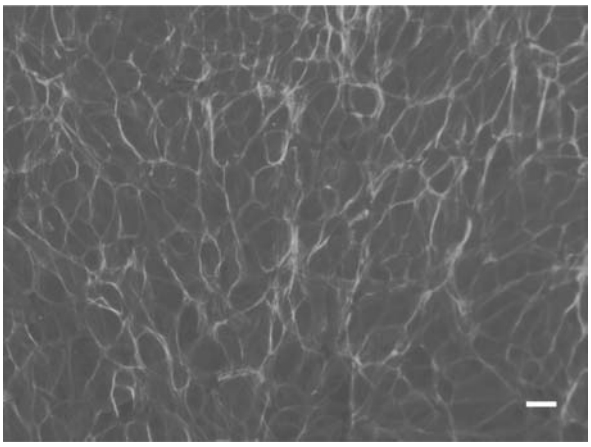

Figure 5 Representative photomicrograph of ARPE-19 cells stained with Alexa-488 Phalloidin on Zytar® after 7 days showing well spread cells with a normal 'cobblestone' morphology (×10). Scale bar = 40 μm.

presented in Fig. 6 show that the cells attached to the air plasma treated Tecoflex® after 7 days were able to phagocytose the fluorescently labelled POS in a time dependent fashion and in a similar manner to the cell adhered to the control TCPS surface. At 3 h a mean of 46.5 (±7.3) POS were attached to the cell membranes and a mean of 19.7 (±3.5) POS were internalised on the Tecoflex® whereas 65 (±9.2) were attached to the cells adhered to the control and 21.2 (±3.6) were internalised. By 24 h the mean attached and internalised POS were very similar for both substrates ranging between 24.4 and 28.6 ($p = 0.05$ for internalised for both time-points).

5. Discussion

Age-Related Macular Degeneration (ARMD) is by far the commonest cause of blindness in the United Kingdom and represents a massive clinical and financial burden locally and nationally [10]. We consider that transplantation of fresh cultured retinal pigment epithelial cells under the failing macular is a plausible route to design a new treatment for ARMD. There are two major problems that need to be addressed to develop a suitable process that could be clinically successful. The first relates to the source of RPE cells and the second relates to the design of a suitable substrate on which to grow the cells and use as a vehicle to transplant the cells into the subretinal space.

The key to healthy RPE cells is attachment to a healthy Bruch's membrane. RPE have been shown not to repopulate experimentally damaged Bruch's membrane [11, 12] and therefore our approach is to develop a substrate on which we can culture a functioning monolayer of cells *in vitro*. The RPE monolayer can subsequently be transplanted subretinally together with the underlying substrate which will then also protect the fresh RPE cells from the diseased tissue. To date a number of substrates have been studied such as cryoprecipitated membranes, anterior lens capsule, cadaver Bruch's membrane, Descemet's membrane, synthetic biodegradable polymer films and collagen type I [1]. Most of these materials are either biological, and thus variable and difficult to handle or degradable, having the potential to cause adverse tissue responses as well as dispersion of the RPE monolayer.

All three polyurethanes satisfied a number of the criteria for the substrate but it was not known, until the present study, how their surface properties could influence RPE cell behaviour. It is known that the cellular response to a material is controlled by its surface properties via an adsorbed protein layer. For untreated Pellethane® and Tecoflex® only a few RPE cells attached to the surface after one hour incubation and the number of cells adhered remained low over the following 5 day time period. Furthermore morphological examination of these cells showed that they remained clumped. On the Zytar® surface, however, although the number of cells attached after 1 day was lower than on

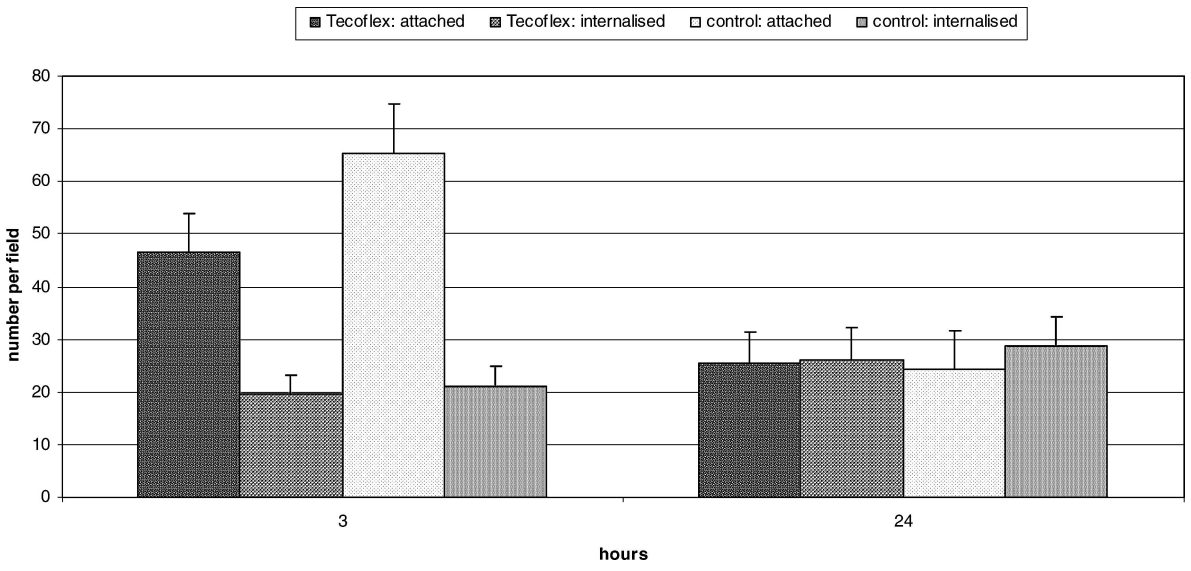

Figure 6 Histogram illustrating the mean number of photoreceptor outer segments (POS) per field attached and internalised by ARPE-19 cells grown on air plasma treated Tecoflex® and control (TCPS). At 3 h most of the POS were attached to the cell membranes but by 24 h there were approximately equal numbers of internalised POS as attached. Bars: ±1 SD.

the control surface the numbers increased over the following 7 days. The morphological analysis suggested that by the 7 day time point the cells had reached confluence. The Pellethane® had higher advancing and receding contact angles than the Zytar®. Therefore we can suggest that the surface of both Pellethane® and Tecoflex® were too hydrophobic to provide an appropriate surface for RPE monolayer formation. The lower advancing and receding contact angles on Zytar®, however, promoted RPE cells adhesion and allowed a monolayer to form within 7 days which appeared to have a normal 'cobblestone' morphology. This suggests that increasing the hydrophilicity allows proteins to interact with the surface such that they form an appropriate surface for RPE cells.

We have shown that the surface properties of commonly used biomaterials can be modified using simple gas plasmas and followed by a post treatment reaction in distilled water. The modifications produced have varying effects on different cell types.[7, 13–15] Changes in the surface chemistry will control the characteristics of adsorbed proteins and so influence the cellular interactions in terms of adhesion and activation. [16–18] We hypothesised that, if the cells did not adhere to the polyurethane "as received," it was possible to modify the surface of materials such that the particular chemistry produced would control the development of a protein layer that could be optimised for the required cells.

This study shows that an air plasma treatment, followed by incubation in distilled water of Pellethane® and Tecoflex® significantly enhanced the adhesion and monolayer formation of ARPE-19 cells. Dynamic contact angle analysis of the Pellethane® demonstrated that the air plasma treatment increased the hydrophilicity of the polyurethane. Previous work with other gas plasmas [7, 13–15] has shown that the increase in hydrophilicity correlates with the incorporation of polar functional groups at the surface. In this study the air plasma treated Pellethane® was more hydrophilic than the Zytar® although both surfaces supported monolayer formation. Furthermore it is essential to establish the functional behaviour of the cell monolayer and one way to do this is to ensure that they are capable of phagocytosis. The results demonstrated that the monolayer of RPE cells on plasma treated Tecoflex® was indeed able to phagocytose photoreceptor outer segments and the time dependence of the phagocytosis process was similar for the Tecoflex® and the control TCPS surfaces. In our further work we aim to find a surface on which proteins will adsorb to form an appropriate basement membrane for adhesion and monolayer formation of functioning RPE cells. The present study demonstrates that careful choice of polyurethane or surface modification can enhance the RPE response.

Acknowledgments

Work supported by Dunhill Medical Trust, Fight for Sight, technical assistance from Faye Barker and Biomer Technology Ltd. We would also like to thank Mrs Deborah Rice for isolating and supplying the photoreceptor outer segments.

References

1. C. SHERIDAN, R. WILLIAMS and I. GRIERSON, *Graefe's Arch. Clin. Exp. Ophthal.* **242** (2004) 68.
2. R. J. ZDRAHALA and I. J. ZDRAHALA, *J. Biomater. Appl.* **14** (1999) 67.
3. A. EDWARDS, R. J. CARSON and M. SZYCHER *et al.*, *J. Biomater. Appl.* **13** (1998) 23.
4. D. J. MARTIN, L. A. POOLE WARREN and P. A. GUNATILLAKE *et al.*, *Biomaterials* **22** (2001) 973.
5. D. J. MARTIN, L. A. POOLE WARREN and P. A. GUNATILLAKE, *et al.*, *ibid.* **21** (2000) 1021.
6. T. V. HOW and D. ANNIS, *J. Biomed. Mater. Res.*, **21** (1987) 1093.
7. D. J. WILSON, N. P. RHODES and R. L. WILLIAMS, *Biomaterials* **24** (2003) 5069.
8. J. ANDRADE, in "Surface and Interfacial Aspects of Biomedical Polymers, Surface Chemistry and Physics," (Plenum Press, New York, 1985) vol. 1.
9. G. T. HIGGINS, H. J. WANG, P. DOCKERY, P. E. CLEARY and H. P. REDMOND, *Invest. Ophthalmol. Vis. Sci.* **44** (2003) 1775.
10. J. BONASTRE, C. LE PEN, P. ANDERSON, A. GANZ, P. BERTO and G. BERDEAUX, *Eur. J. Health. Econ.* **3**(2) (2002) 94.
11. C. SHIRAGAMI, T. MATSUO and F. SHIRAGA *et al.*, *Br. J. Ophthalmol.* **82** (1998) 1056.
12. T. H. TEZEL, H. J. KAPLAN and L. V. DEL PRIORE *Invest. Ophthalmol. Vis. Sci.* **40** (1999) 467.
13. D. J. WILSON, R. L. WILLIAMS and R. C. POND, *Surf. Interf. Anal.* **31** (2001) 385.
14. *Idem.*, *ibid.* **31** (2001) 397.
15. R. L. WILLIAMS, D. J. WILSON and N. P. RHODES *Biomaterials* **25** (2004) 4659.
16. F. R. PU, R. L. WILLIAMS and T. MARKKULA *et al.*, *J. Mater. Sci. Mater. in Med. Special Issue* **12** (2001) 970.
17. F. R. PU, R. L. WILLIAMS and T. MARKKULA *et al.*, *Biomaterials* **23** (2002) 2411.
18. *Idem.*, *ibid.* **23** (2002) 4705.

Received 30 June
and accepted 18 July 2005