J Mater Sci: Mater Med (2007) 18:329–338
DOI 10.1007/s10856-006-0697-2

Plasma polymer coated surfaces for serum-free culture of limbal epithelium for ocular surface disease

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Received: 9 June 2006 / Accepted: 25 September 2006
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Abstract The potential use of plasma polymer coatings as substrates for serum-free expansion of limbal epithelial cells was investigated. Preliminary studies using a human corneal epithelial cell line showed that acrylic acid-coated surfaces performed better than allyl amine and allyl alcohol coated surfaces in terms of cell metabolic activity and confluence as assessed using the MTT assay. Subsequently, the proliferation and maturity of primary human limbal epithelial cells in co-culture with growth arrested 3T3 fibroblasts on a range of acrylic acid plasma coated surfaces, octadiene plasma coated surfaces and tissue culture plastic was investigated using MTT and cytokeratin 3 immunostaining. The cells performed better in the presence of serum on all surfaces. However, the acrylic acid coated surfaces successfully sustained a serum-free fibroblast/epithelial cell co-culture. The metabolic activity of the epithelial cells was superior on the acrylic acid coated surfaces than on tissue culture plastic in serum-free conditions and their levels of differentiation were not significantly higher than in the presence of serum. These results suggest that these surfaces can be used successfully for the serum-free expansion of human limbal epithelial cells.

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1 Introduction

The corneal epithelium exists as a rapidly regenerating stratified squamous epithelium. Under both normal conditions as well as following injury, the maintenance of the corneal epithelial cell mass is achieved by a distinct population of unipotent stem cells (SC) believed to reside in the basal epithelium of the corneoscleral limbus [1–4].

Numerous patients suffering from conditions such as chemical burns, Steven Johnson syndrome, or aniridia experience great discomfort and visual loss due to limbal SC failure. As a result of a partial or total depletion of limbal epithelial SC to those patients, the neighboring conjunctival epithelium migrates over the surface of the cornea causing vascularisation, pain and ultimately blindness.

Rapid laboratory expansion and transplantation of human limbal epithelial SC can be a valuable aid in the treatment of those patients. So far, the gold standard of *in vitro* limbal epithelial cell expansion requires the presence of a growth arrested fibroblast feeder layer and serum supplemented culture medium [5].

This method was based upon the original protocol by Rheinwald and Green for the expansion of human epidermal keratinocytes [6].

With growing concerns about the transmission of bovine spongiform encephalitis (BSE) and animal viruses, it would be preferable to culture cells for human transplantation under animal-free conditions. This has so far not been achieved.

Plasma polymerization technique has been successfully used to improve the biocompatibility of medical of biomedical materials. Surface functionalisation by plasma polymer coating has been used both to promote and to reduce protein adsorption.

For example, Shen et al. have used plasma deposition of tetraethylene glycol dimethyl ether (tetraglyme)

to reduce fibrinogen adsorption on a poly (fluorinated ethylene-propylene (FEP) surface and thus improving its blood compatibility [7]. In a different study, Hollander et al. have used a low-pressure oxygen plasma to coat polyethylene surfaces with polyammonium which resulted in significant reduction of E-Coli and Microccocus luteus growth on those surfaces giving them antibacterial properties [8].

Alternatively, as a result of improved protein adsorption, the cell adhesion and confluence on polymers can increase. Barry et al., have successfully deposited allylamin throughout the porous network of a three-dimensional poly(D,L-lactic acid) (P(DL)LA) scaffold which resulted to increased fibroblast attachment and proliferation [9].

Plasma polymer coated surfaces have been successfully used as dermal epithelial cell culture substrates in serum-free conditions [10–12]. Using plasma polymer technology, CellTran Ltd. has developed myskin^{TM}, a human keratinocyte culture substrate that acts both as a cell carrier and a wound dressing. This technology has been used in the clinic since 2004 for the treatment of burns and chronic ulcers [10].

The aim of this project was to assess a range of chemically defined plasma polymer coated surfaces as substrates for serum-free culture of primary human limbal epithelial cells co-cultured with 3T3 fibroblasts and to determine conditions for serum-free culture of these cells for subsequent clinical use. It is also necessary to ensure that the expanded limbal epithelial cells, apart from exhibiting good morphology and confluence also remain undifferentiated. A substrate enhancing cell proliferation under serum-free conditions would mean a significant step towards a safer method for limbal epithelial SC delivery in the clinic.

2 Materials and methods

2.1 Plasma polymer coating

Monomers were obtained from Sigma-Aldrich (Gillingham, UK) and were degassed by several freeze-pump-thaw cycles prior to use. 1, 7-Octadiene (OD) [$H_2C{=}CH(CH_2)_4CH{=}CH_2$] was used to create a hydrocarbon surface, acrylic acid (AAc) [$H_2C{=}CHC({=}O)OH$], allyl alcohol (AAl) [$CH_2{=}CHCH_2OH$] and allyl amine (AAm) [$CH_2{=}CHCH_2NH_2$] were used to produce films containing carboxylic acid, hydroxyl and amine functional groups respectively. Plasma polymer coated surfaces were produced using a cylindrical glass reactor, of 10 cm internal diameter and 50 cm in length, as previously described [14]. The ends were closed by stainless steel flanges, sealed with viton o-rings. Vacuum was obtained via a two stage rotary vane pump, and a liquid nitrogen cold trap was used to trap volatile components. Pressure in the reactor was monitored using a Pirani, and the base pressure in the reactor was less than 5.0×10^{-3} (which reduced to less than 1.0×10^{-3} with nitrogen in the trap) prior to each polymerisation.

Plasma was initiated and sustained using a radio-frequency (RF) (13.56 MHz) power supply (Co-axial Power Systems Ltd., Sussex, UK), coupled to the plasma via an impedance matching unit and externally wound turns of insulated copper wire. Monomers were placed in round-bottomed flasks, and a needle valve was used to control the flow rate through the reactor. Plasma was sustained for 15 min to ensure sufficient thickness of the polymer coating. For each monomer, a range of plasma processing conditions was used, including depositions with flow rates of 2 & 10 sccm and at 1, 5 & 10W RF power. Samples for coating were either 24-well cell culture trays, or 13 mm glass coverslips, including a coverslip to be used for XPS analysis.

2.2 Surface characterisation

Plasma polymer surface coatings were characterised by X-ray photoelectron spectroscopy (XPS) analysis using a Kratos Axis Ultra XPS instrument, with a monochromated Aluminium X-ray source at energy of 1486.69 eV. For each sample, a survey scan of the binding energy range 0–1200 eV at pass energy of 160 eV was obtained to identify and quantify elements present, and a high-resolution scan (pass energy of 20eV) of the C1s elemental core level was obtained to enable peak-fitting of the chemical functional groups.

2.3 Human corneal epithelial cell (HCEC) line attachment and growth

Surfaces were initially assessed for cell attachment and growth using a HCEC line (LGC Prochem, Middlesex, UK). The HCEC line was formed from a primary culture of corneal epithelium transfected with the Rous sarcoma virus. Cells were cultured in Epilife Medium (Cascade Biologics, Nottinghamshire, UK), with a human corneal growth supplement (HCGS).

A pre-coated protein layer of rat tail collagen I (0.03 mg/ml in acetic acid), fibronectin (0.01 mg/ml) and bovine serum albumin (0.01 mg/ml) was used to provide a positive control for cell growth, whilst a plasma polymerised OD hydrocarbon surface was used as a negative control.

Cells were seeded onto surfaces at a density of approximately 3.0×10^4 cells/cm^2. Attachment was assessed by measuring cell viability after 24 h, using a 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-eluted stain assay (MTT-ESTA, Sigma). Cells were washed

with 0.5 ml of Phosphate Buffered Saline (PBS) solution and then incubated with MTT at 0.5 mg/ml in PBS for 40 min. Three hundred microlitres of acidified isopropanol, (Sigma) per well were used to elute the stain. A 150 μl volume was then transferred to a 96 well plate. The absorbance was read at 540 nm using a Zafire spectrophotometric plate reader. Cell viability at 48 h gave an indication of cell growth on the surfaces.

2.4 Maintenance of 3T3 mouse fibroblasts for use as a feeder layer

3T3/J2 mouse fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM Gibco) supplemented with 10% Adult Bovine Serum (Gibco) and 1% penicillin/streptomycin (Gibco). Culture medium was changed three times a week and the cultures were passaged upon reaching 60–70% confluence at a ratio of 1:8. The cultures were kept at 37°C and 5% CO_2.

The fibroblasts were growth arrested in fibroblast culture medium with added mitomycin C (Sigma) at a concentration of 4 μg/ml.

2.5 Primary human limbal epithelial cell isolation and culture

Human limbal epithelial (HLE) cells were cultured in Corneal Epithelial Culture Medium (CECM) consisting of DMEM F12 (1:1) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (Gibco), 0.1 nM cholera toxin (Sigma), 5 μg/ml human recombinant insulin (Sigma), 0.05 mM hydrocortisone (Sigma) and 10 ng/ml epidermal growth factor (Invitrogen). Culture medium was changed three times a week and the cultures were passaged upon reaching 70–80% confluence at a ratio of 1: 3.

HLE cells were isolated from research consented corneas supplied by the Moorfields Eye bank, London. Corneas or limbal rims were cut into four segments and immersed in a 1.2 U/ml dispase II solution (Roche) for 2 h at 37°C or overnight at 4°C. After dispase treatment, the tissue pieces were transferred into a 35 mm Petri dish. With the epithelial side uppermost, the epithelial cells were gently scraped from the limbus area using a pair of fine pointed forceps. The cells were collected using 5 ml CECM. The cell suspension was pipetted up and down on the tissue segments to disperse the cells. The cell suspension was then placed with another 5 ml CECM into a T-75 tissue culture flask (Nunc) containing growth arrested 3T3 mouse fibroblasts plated at cell density of 2.4×10^4 cells/cm^2. Viable epithelial cells developed colonies in 2–3 days. The fibroblasts had been growth arrested by treatment for 2 hours in Dulbecco's Modified Eagle Medium (DMEM Gibco) supplemented with 10% Adult Bovine Serum (Gibco), 1% penicillin/streptomycin and 4 ng/ml mitomycin C (Sigma). The cultures were incobated at 37°C and 5% CO_2 in air.

2.6 Cell morphology and metabolic activity

For investigation of 3T3 fibroblast cell attachment and metabolic activity cells were plated at a seeding density of 10^4 cells/cm^2 into a 24 well plate containing the plasma polymerised surfaces. Cell viability was assessed using the MTT-ESTA assay.

HLE cells were plated on growth arrested 3T3 fibroblast feeder layers at seeding densities of 1.5×10^4 cells/cm^2 and 2.4×10^4 cells/cm^2 respectively and cultured in 1 ml culture medium per well. Both serum- supplemented and serum-free culture media were compared. Before conducting the MTT assay the remaining fibroblast feeder cells were removed using 5% EDTA solution (Sigma) for 2 min followed by gentle tapping.

Subsequently, the MTT assay was carried out as described above. The cell morphology in all cases was monitored using a Nikon inverted phase contrast microscope.

2.7 Immunocytochemistry

Human limbal epithelial cells were cultured in the same manner as described above in 24 well plates (Nunc).

On day 6, the culture medium was removed; the cells were washed three times with PBS, fixed for 15 min at room temperature in 4% (wt/vol) paraformaldehyde, and treated with 20% (wt/vol) sucrose before storage at −20°C. For immunocytochemistry a Vectastain® ABC-AP kit (Vector Laboratories, Burlingame, CA) was used. The blocking reagent was added to the cells in Tris-Buffered Saline (TBS) for 30 min followed by the mouse monoclonal antibody for cytokeratin 3 (1:100 diluted in blocking reagent; Chemicon International) or blocking reagent only (negative control) for 1 h. The remaining protocol was followed according to the manufacturing instructions. The biotin-labeled rabbit anti-mouse bridging antibody (1:300 dilution in TBS) was added for 45 min, and the cells were left in a moist chamber for 45 min followed by a further 45 min in streptavidin-avidin-alkaline phosphatase complex (both reagents were part of the Vectastain ABC-AP kit). Red AP substrate (Vector, Burlingame, CA) was added until a sufficient color reaction had occurred (less than 30 min). The cells were washed briefly to remove the red substrate, counterstained with DAPI (1:500, Sigma) and mounted between a microscope slide and coverslip using MOWIOL 99 (Calbiochem). All incubations were performed at room temperature, and each step was interspersed with three TBS rinses.

Table 1 Summary of XPS C1s peak fitting of the plasma polymer surfaces

Monomer	Plasma conditions	% C(=O)OH	% C–OH	% C–NH_2
Acrylic acid	1 W / 2 sccm	10.9	14.0	–
	1 W/10 sccm	20.4	6.7	–
	5 W/2 sccm	9.2	15.9	–
	5 W/10 sccm	13.8	9.6	–
	10 W/2 sccm	8.6	16.6	–
	10 W/10 sccm	9.4	14.9	–
Allyl alcohol	1 W / 2 sccm	2.2	14.8	–
	1 W/10 sccm	1.9	31.4	–
	5 W/2 sccm	2.4	14.7	–
	5 W/10 sccm	1.4	20.7	–
	10 W/2 sccm	2.6	14.6	–
	10 W/10 sccm	1.7	13.9	–
Allyl amine	1 W / 2 sccm	–	–	17.9
	1 W/10 sccm	–	–	19.9
	5 W/2 sccm	–	–	18.1
	5 W/10 sccm	–	–	19.3
	10 W/2 sccm	–	–	19.9
	10 W/10 sccm	–	–	18.5
1,7-Octadiene	5 W / 2 sccm	–	10.6	–

3 Results

3.1 Surface analysis

Peak fitting of the C1s spectra was performed using a number of component peaks. For OD, three component peaks were fitted: the hydrocarbon peak (C–C, C–H) at 285.0 eV, hydroxyl (C–OH) and carbonyl (C=O) at 287.9 eV. The presence of oxygen containing functional groups is a result of the plasma polymerisation process where, generally, up to 6% atomic oxygen is incorporated into the OD plasma polymer [11]. For AAc and AAl, the above peaks plus a carboxyl peak (C(=O)OH) at 289.3 and a β-shifted carbon peak associated with the carboxyl group (C–C(=O)OH) at 285.7eV were included. The area of the β-shifted peak was constrained to be of equal area to the carboxyl peak for curve fitting purposes. AAm was peak fitted using the hydrocarbon peak, plus amine (C–NH_2) at 286.3eV, amide (NC=O) at 287.9 eV and nitrile (C≡N) at 286.7 eV). A summary of the XPS analyses is shown in Table 1.

It can be seen from Table 1 that, for AAc, there is greater carboxylic acid retention at lower plasma powers, as expected [15]. For a flow rate of 2sccm, retention varies from 10.9% at 1 W to 8.6% at 10 W. The effect of increasing the flow rate is to increase acid retention, to a maximum of 20.4% for 1 W/10 sccm. In addition, the fragmentation products hydroxyl and carbonyl (not shown) are reduced. For AAl, a small carboxyl component is present due to fragmentation and recombination of bonds in the plasma. This appears to be reduced at the higher flow rate. The hydroxyl component does not vary significantly with RF power at 2 sccm, but increasing the flow rate increases the hydroxyl retention for all powers except 10 W, which is not significantly affected. Hydroxyl retention is a maximum of 31.4% at 1 W/10 sccm. Amine retention of AAm plasma polymers does not appear to vary significantly with RF power or monomer flow rate.

4 Cell morphology and proliferation

4.1 HCEC culture

In the initial screening of each of the surfaces available, HCEC growth on a range of acid, alcohol and amine surfaces was measured after 24 and 48 h by MTT assay (Fig. 1). It can immediately be seen that the AAc surfaces produce better levels of HCEC attachment and growth than the other plasma polymer surfaces. Attachment and growth is similar to the positive control. AAl and AAm surfaces are seen to produce low levels of attachment, with relatively low levels of growth after 48 h.

Based on this preliminary work, the AAc surfaces were examined more closely by looking at long term growth over a period of five days (120 h). For this experiment, a seeding density of 3.0×10^3 cells/cm^2 was used, and the cells were given a media change after 48 h. Cell viability was assessed by MTT-ESTA assay after 48 and 120 h.

Figure 2 shows that initial attachment on all the AAc surfaces is similar after 48 h, and is significantly greater than the OD control. Attachment to the pre-coated protein layer was initially slightly greater than the AAc surfaces and after 120 h showed slightly more metabolic activity than all the AAc surfaces except for the 1 W/2 sccm surface. Hence acrylic acid coated surfaces were selected for further study using primary limbal epithelial cells.

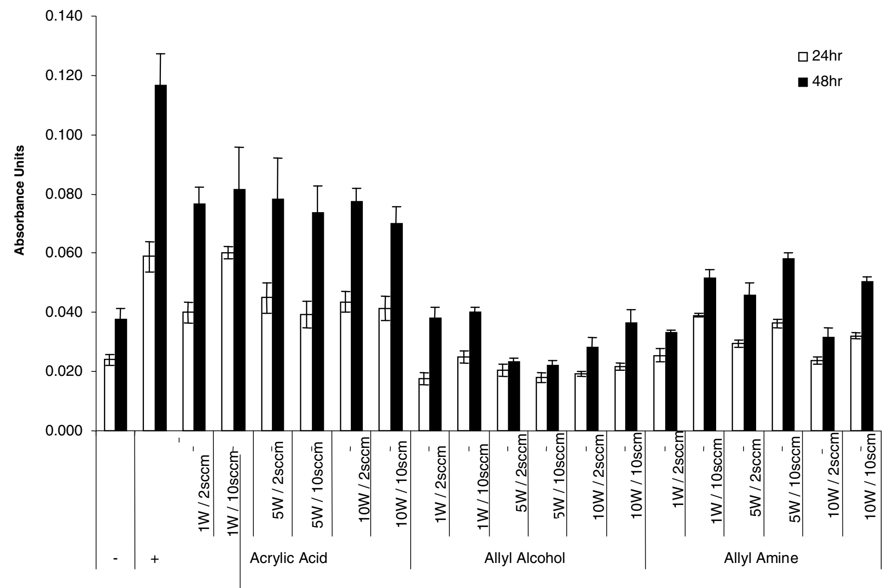

Fig. 1 Attachment (24 h) and growth (48 h) of HCECs on a range of plasma polymer surfaces. The negative control is a 5 W OD plasma polymer, whilst the positive is a pre-coated layer of collagen I, fibronectin and BSA

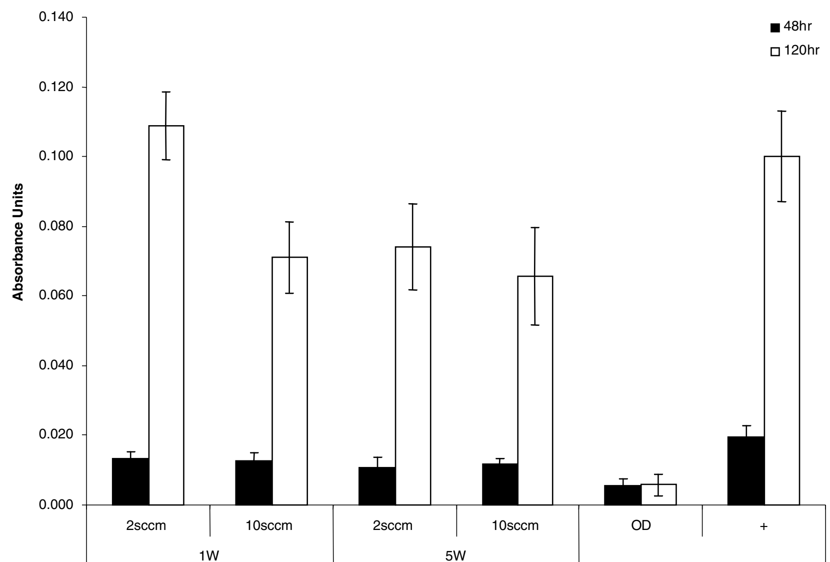

Fig. 2 Attachment and long-term growth of HCECs on a range of acrylic acid plasma polymer surfaces

4.2 Fibroblast culture on acrylic acid coated surfaces

The proliferation of 3T3 mouse fibroblasts was assessed using the MTT assay after 6 days in culture under serum-supplemented and serum-free conditions (Fig. 3). Cell morphology was monitored using light microscopy (Fig. 4). Cells performed well on all acrylic acid coated surfaces tested. Cell metabolic activity indicating proliferation was higher in the presence of serum compared to serum-free conditions. This is supported by light microscopy data. Cells cultured under serum supplemented conditions exhibited a typical fibroblastic morphology and higher cell confluence whereas serum-free cultures were less confluent and cells showed a highly elongated stressed morphology.

4.3 HLE in co-culture with growth arrested fibroblasts on acrylic acid coated surfaces

The acrylic acid surfaces were used as substrates for HLE in co-cultures with growth arrested 3T3 fibroblasts acting as a feeder layer. The experiments were carried out in both serum-supplemented and serum-free conditions. The growth arrested fibroblasts were seeded in serum-containing DMEM and left to attach on the surfaces for 4 h. Thereafter, the medium was removed and the HLE cells were plated on the feeder layers in either CECM supplemented with serum (serum +) or serum-free (serum −). By 2 days, the HLE cells had formed colonies of small tightly packed cells. HLE cells exhibited good cell confluence and proliferation on all

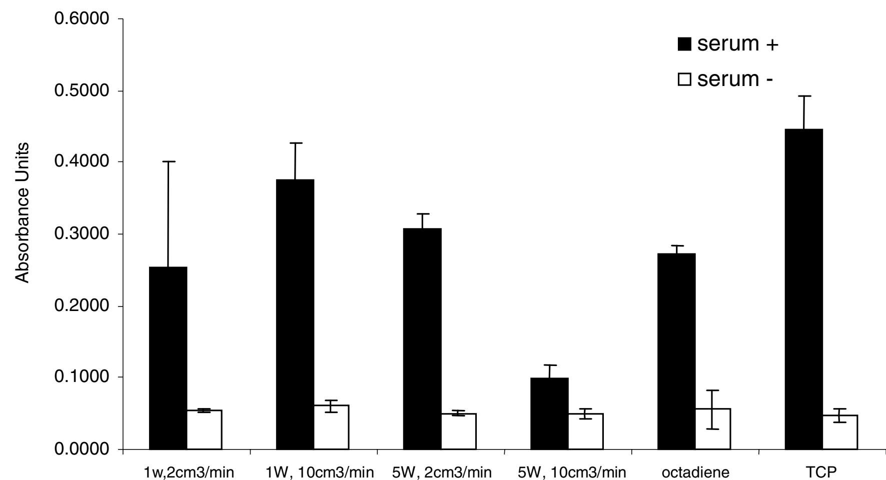

Fig. 3 3T3 mouse fibroblast metabolic activity after 6 days of culture on acrylic acid coated surfaces and tissue culture plastic. Results represent the means ± standard error of triplicate wells of cells

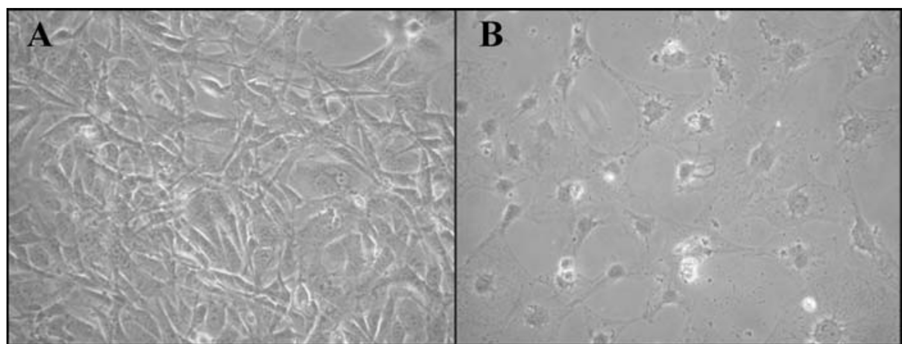

Fig. 4 3T3 fibroblast morphology on an acrylic acid plasma coated surface (1 W, 2 cm^3/min) in (A) serum supplemented and (B) serum-free culture (6 days)

surfaces tested including 1,7-octadiene and tissue culture plastic in the presence of serum. By the sixth day HLE had formed a confluent sheet on all surfaces. The epithelial cells did not show signs of differentiation such as cell enlargement to a great extent. The cell morphology and cell confluence were similar to 1,7-octadiene and tissue culture plastic (Fig. 5). Under serum-free conditions, the tissue-culture plastic substrate was inferior to all the plasma copolymer surfaces, in terms of cell confluence (Fig. 5) whereas there was no significant difference in cell confluence observed between any of the surfaces.

These results are quantified by MTT assay data (Fig. 6). Serum free HLE cultures had lower metabolic activity values compared to serum supplemented cultures. Serum free co-culture on tissue culture plastic had lower metabolic activity values than any other surface. This suggests that surface chemistry as well as the presence of the feeder layer contributed to the HLE cell proliferation and the maintenance of their undifferentiated morphology as determined by light microscopy.

4.4 Immunocytochemistry

Cytokeratin 3 was used to assess the epithelial cell maturity and differentiation. Fibroblasts were firstly removed with 5% EDTA solution (Sigma) before immunocytochemical staining. Figure 7 illustrates cytokeratin-labeled HLE cultures. Cytokeratin 3 production was associated with enlarging cells. The fluorescence microscopy data did not reveal any differences in the number of cytokeratin 3-positive (differentiated) cells between any of the surfaces studied after 6 days in culture both in serum-free and serum-supplemented cultures using either 3T3 mouse fibroblasts or HLF (Fig. 7).

5 Discussion

The aim of this study was to establish the possibility of using a plasma polymer coated surface as a substrate for serum-free culture and expansion of human limbal epithelial cells while limiting as far as possible epithelial cell differentiation. A preliminary study using a human corneal epithelial cell line cultured in a serum-free system containing a defined supplement. The Human Corneal Epithelial Cell line (HCEC) was used as a preliminary screening tool owing to its sensitivity to the culture surfacesand its relative similarity to HLE as a means of selecting the most promising substrate for detailed analysis with primary HLE cultured from cadaveric donor tissue of limited supply.

The MTT assay results of this study showed that the acrylic acid plasma polymer coated surface was preferable to allyl

Fig. 5 HLE cell and 3T3 fibroblast co-cultures on plasma polymer surfaces fabricated at different power and flow rate conditions or tissue culture plastic (TCP), cultured for 6 days in serum supplemented or serum-free conditions

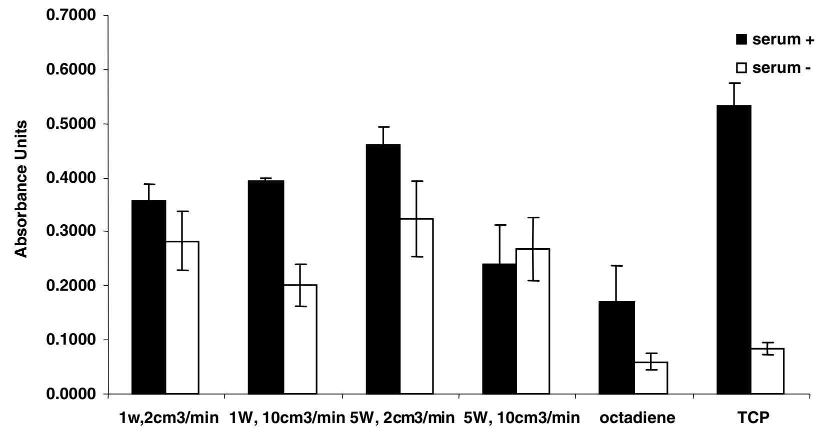

Fig. 6 HLE cells metabolic activity after 6 days of culture on acrylic acid coated surfaces and tissue culture plastic. Results represent the means ± standard error of triplicate wells of cells

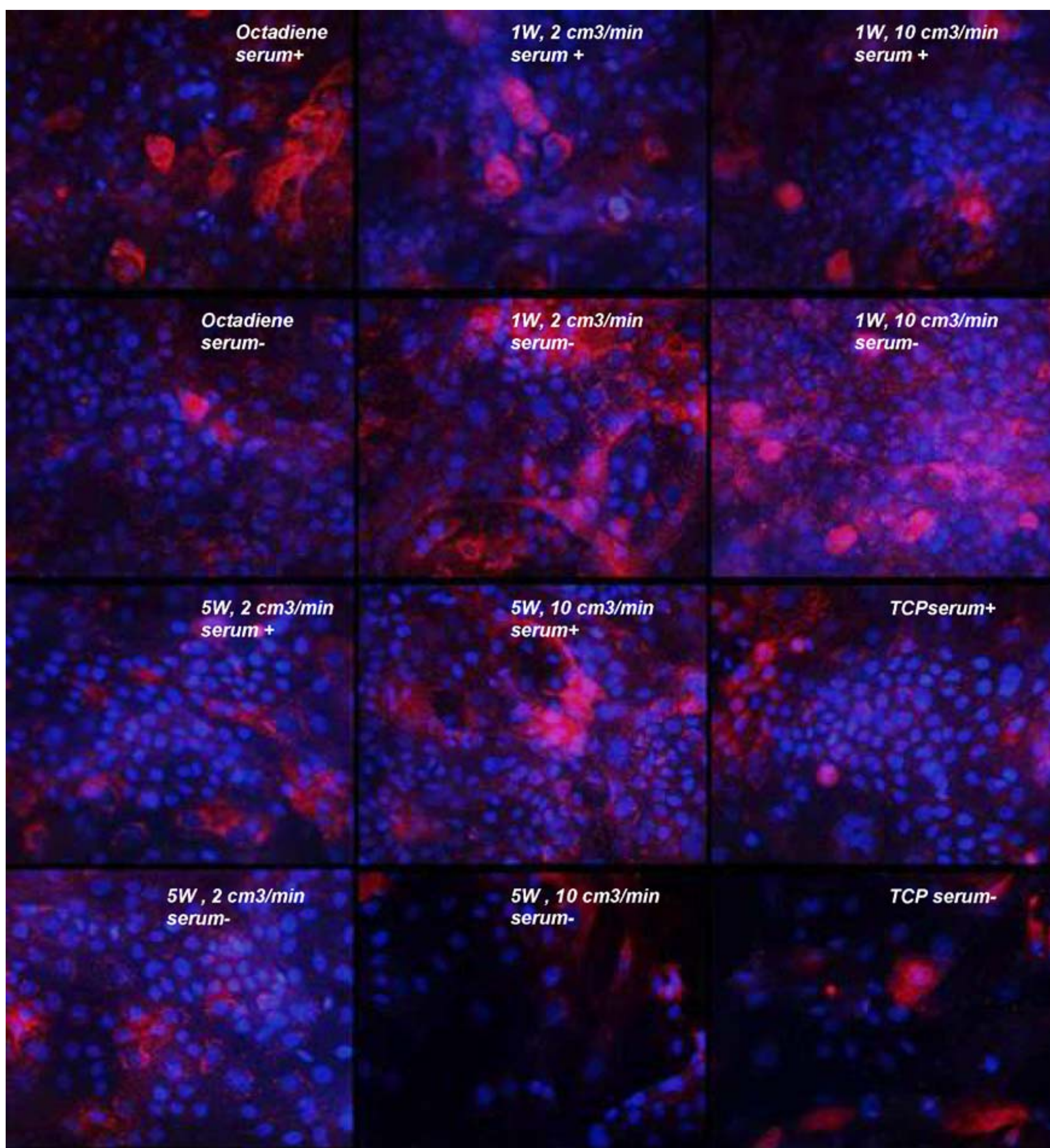

Fig. 7 Cytokeratin 3 labeled HLE cells that have been co-cultured with 3T3 fibroblasts on plasma polymer surfaces fabricated at different power and flow rate conditions or tissue culture plastic (TCP) in serum supplemented or serum-free conditions. Fibroblasts were removed prior to staining

alcohol and allyl amine plasma polymer coated surfaces. As has been previously reported, acid terminated surfaces promote cell attachment and proliferation [11, 12, 16]. These surfaces may have increased protein absorption when serum (or in the same respect a defined supplement) is present. Serum contains adhesive proteins such as fibronectin and vitronectin which contain the Arg-Gly-Asp (RGD) sequence and have been found to enhance cell focal attachment and improve cell proliferation and confluence [17].

Although it is possible that the HCEC cell line may behave differently to primary cells, subsequently the acrylic acid coated surfaces were shown to be suitable substrates for the culture of human limbal epithelial cells in the presence of growth arrested fibroblasts both in serum supplemented and in serum-free conditions, the latter being a significant finding in this study. Moreover, when serum was not present the HLE cells responded better on the plasma coated surfaces than on the tissue culture plastic. Currently, the expansion of HLE for transplantation in serum supplemented conditions on tissue culture plastic in the presence of growth arrested fibroblast feeder cells is considered to be the gold standard [4, 5, 21]. A growing concern throughout European countries currently is that BSE cannot be detected effectively using an *in vitro* diagnostic method. For that reason, a defined, serum-free

alternative would be a preferred approach. This study is an encouraging advance towards that aim.

Other serum-free studies have been attempted but did not lead to a successful and reproducible method that would eliminate the need for the presence of serum in the culture system without adding other animal derived products such Bovine Pituitary Extract (BPE) [18, 19]. In this study the levels of differentiation in serum-free cultures were similar to existing methodologies whilst, at the same time cell proliferation was improved compared to tissue culture plastic.

The low levels of cell differentiation found in serum-free cultures, as indicated by cytokeratin 3 immunostaining, was encouraging as this is a requirement for limbal epithelial cell expansion. During limbal epithelial stem cell expansion for clinical application, it is imperative to prevent as many stem cells as possible from differentiating in culture in order to ensure optimum functionality post-transplantation.

Although human limbal epithelial cells proliferated on acrylic acid surfaces in the presence of 3T3 fibroblast feeder cells and serum, their performance on those surfaces was better than tissue culture plastic under serum-free conditions as illustrated using on the light microscopy images (Fig. 5) and quantified by MTT assay (Fig. 6). This highlights the importance of the surface chemistry of the acrylic acid surfaces in promoting epithelial cell attachment and proliferation. Moreover, the serum-free human limbal epithelial cell cultures exhibited characteristic cobblestone morphology and the levels of differentiation were similar to the gold standard conditions of culture (with FCS on tissue culture plastic). When cultured separately, non growth arrested fibroblasts exhibited good confluence on all surfaces in the presence of serum whereas in serum-free cultures significantly lower cell attachment and proliferation were observed. The MTT assay showed no significant effect of the percentage of –COOH groups on the fibroblasts metabolic activity confirming the results of an earlier study for human dermal fibroblasts [12]. However, although it appears that the fibroblasts did not proliferate well in serum-free conditions, they were presumably able to provide all the necessary growth factors and to produce sufficient extracellular matrix proteins to sustain the epithelial cells in serum-free conditions. The synergistic effect of the feeder layer in combination with the surface chemistry of the acrylic acid surfaces provides a promising serum-free culture protocol for human limbal epithelial cells.

Acid terminated surfaces [20] and specifically acrylic acid coated substrates [12] have been used successfully for expansion of epithelial cells in serum-free conditions. It has been postulated that the high energy of this polymer is responsible for the success of this system as a keratinocye culture substrate [9]. In this study, the percentage of the –COOH groups as quantified using XPS analysis did not have a significant effect on limbal epithelial cell proliferation. In a previous investigation, increase in the percentage of –COOH groups on the surface of an acrylic acid/1,7-octadiene plasma copolymer coating enhanced keratinocyte attachment and proliferation [9]. However, it has to be noted that the percentage of –COOH on the surface of the latter was much lower (in a range of approximately 1% up to 9%) than in this work where it ranged from 9 to 20%. This indicated that epithelial cells might be sensitive to changes in the acid content up to approximately 9–10% and after that limit the increase of the percentage of carboxyl groups on the substrate surface does not affect the levels of cell proliferation.

The prospect of serum-free expansion of human limbal epithelial cells on a plasma polymer coated surface is exciting. A serum-free culture system utilizing a defined synthetic surface could lead to a xenobiotic-free alternative to current protocols for expanding limbal epithelial stem cells which currently require bovine serum and often use amniotic membrane as a substrate. The next step to develop this system towards a clinical application will be to examine the potential of growth arrested primary human limbal fibroblasts to act as a feeder layer and the ability of limbal epithelial cells to transfer to an *in-vitro* wound model.

6 Conclusions

Plasma polymer coated surfaces have been found to successfully sustain serum-free cultures of HLE. This suggests that these materials could be useful substrates for serum-free *in vitro* expansion and transplantation of human limbal epithelial SC to treat conditions including alkali burn, aniridia and Stevens Johnson Syndrome.

Acknowledgment The authors gratefully acknowledge the UK Government's Biotechnology and Biological Sciences Research Council (BBSRC) for their support and funding under the Small Business Research Initiative (SBRI) scheme and also the Special Trustees of Moorfields Eye Hospital.

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