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Corneal epithelialisation on surface-modified hydrogel implants

Artificial cornea

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Abstract The objective was to investigate corneal re-epithelialisation of surface-modified polymethacrylate hydrogel implants in order to evaluate them as potential materials for an artificial cornea. Polymethacrylate hydrogels were modified with amines and then coated with different extracellular matrix proteins (collagen I, IV, laminin and fibronectin). The modified hydrogels were surgically implanted into bovine corneas maintained in a 3-D culture system for 5 days. The epithelial growth across the implant surface was evaluated using fluorescent, light and electron microscopy. Full epithelialisation was achieved on 1,4-diaminobutane-modified hydrogels after coating with collagen IV. Hydrogels modified with 1,4-diaminobutane but without further coating only showed partial re-epithelialisation. Hydrogels modified with other amines (1,2-diaminoethane or 1,3-diaminopropane) showed only partial re-epithelialisation; further coating with extracellular matrix proteins improved epithelialisation of these surfaces but did not result in complete re-epithelialisation. Evaluation of the corneas implanted with the 1,4-diaminobutane-modified hydrogels coated with collagen IV showed that the artificial corneas remain clear, integrate well and become covered by a healthy stratified epithelium. In conclusion the 1,4-diaminobutane surface-modified hydrogel coated with collagen IV supported the growth of a stable stratified epithelium. With further refinement this hydrogel has the potential to be used clinically for an artificial cornea.

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1 Introduction

Worldwide more than 10 million individuals have their vision compromised because of corneal disease, which often occurs in the younger, economically important population. Although corneal transplantation can often be performed successfully, some patients affected by specific conditions such as chemical burns, ocular cicatricial pemphigoid, SJS or recurrent graft failure have a poor prognosis. In addition, in many parts of the world, particularly in Asia, the demand for corneas greatly exceeds supply. This has prompted many groups to try to develop an artificial cornea or keratoprostheses from synthetic materials. Ideally these materials need to be biocompatible, transparent, permeable to nutrients and growth factors, integrate well with the stroma and have anterior surface characteristics that permit epithelialisation and survival of corneal epithelial cells. It is this last requirement which has proven to be most difficult to achieve.

The development of artificial corneas has had a long history and their clinical use has been associated with problems such as extrusion, necrosis of adjacent tissue and inflammation [1]. Materials including PMMA and silicone

have been used for the optical core with varying results [2–4]. A significant problem with these materials is the lack of stable re-epithelialisation. Synthetic onlays have been tested; some encouraging results have been obtained using perfluoropolyether onlays [5, 6]. It is generally accepted that hydrogels are the most promising of materials for a full thickness artificial cornea [7, 8]. They can be transparent, hydrated and biocompatible; with the correct mechanical properties, they can be made porous to allow the passage of nutrients and growth factors. The current commercially available keratoprostheses of Alphacor™ (Chirila KPro) are hydrogel-based and have a lower incidence of the complications traditionally associated with keratoprostheses. This notwithstanding, it has been acknowledged that the long-term stability of such keratoprostheses would be improved if they could also support stable epithelial growth on their surface [9, 10].

Several groups have attempted to modify the surface of hydrogels to encourage re-epithelialisation with some success [11, 12] and this approach holds great potential for the development of a fully re-epithelialised artificial cornea. Our previous work has demonstrated that modification of the hydrogel surface by alkyl α–ω diamines with carbon chain lengths of between 3 and 6 can greatly increase the growth of cultured corneal epithelial cells on hydrogel surfaces [13].

In this study, we have evaluated the re-epithelialisation of amine-modified polymethacrylate hydrogels with or without further coating by extracellular matrix proteins. These materials were surgically implanted into bovine corneas maintained in a sophisticated air/liquid organ culture system [14] over a period of 5 days.

2 Materials and methods

2.1 Materials

The composition of hydrogel used in this study was: glycerol monomethacrylate (pbw) 75, lauryl methacrylate (pbw) 12, EGDMA (pbw) 8, glycidyl methacrylate (pbw) 5. The hydrogel was made by photopolymerization and it was amine-modified by soaking in ethanolic solutions containing 1,2-diaminoethane, 1,3-diaminopropane, or 1,4-diaminobutane according to previous descriptions [13]. The monomers were dissolved in IPA along with 2-hydroxy-2-methylpropiophenone (1wt% of the monomers). The mixtures were added to a polymerization mould and irradiated with 200 arc 400 W mercury discharge lamp at a distance of 10 cm in a Dimax model Bondbox on a rotating table for 40 s on each side. The polymerization mould consisted of two 4 mm thick glass sheets covered with 100 mm PET film (hifi films pmx727, no slip) attached by the minimum amount of 3 M spray-mount adhesive. The plates were separated with a rectangular 500 mm PTFE spacer. Amine functional polymers (10 g) were produced by soaking freshly prepared polymers in ethanolic solutions containing the required amine (250 cm^3, 5 vol%) in large excess for 24 h at room temperature. The equilibrium water content (EWC) of modified hydrogels was between 45 and 54 wt% [13].

2.2 Extracellular matrix molecule coating

Amine-modified hydrogels membranes were trephined by using a 3-mm trephine punch and coated with collagen I, IV, fibronectin, or Matrigel (laminin) according to the manufacturers' guidelines. Briefly, hydrogels were covered with either collagen I or IV (0.5 mg/ml; Sigma, UK) at 4°C overnight or Matrigel (BD Biosciences, UK; 1:3 in DMEM) at room temperature for 1 h, or 0.05 mg/ml fibronectin (Sigma, UK) at 37°C for 2 h. After which, the hydrogels were washed twice with PBS before use.

2.3 Implantation of hydrogels into the corneal organ culture model

Bovine eyes were obtained from freshly slaughtered animals. A trephine was used to make a superficial keratotomy groove approximately 200–300 μm deep centrally. Using a freehand technique, the base of the keratotomy groove was expanded approximately 0.5 mm towards the limbus creating an interlamellar pocket within the stroma. Following wounding, the wound bed was swabbed and rinsed with sterile PBS to remove any loose cellular debris. The 3 mm diameter amine-modified hydrogel implants (200 μm in thickness) with or without extracellular protein coating was tucked into the expanded pocket of each cornea concealing the implant edge within the pocket. Following implantation, the corneoscleral button was excised from the eyeball and cultured on a culture chamber for 5 days as previously described [14]. Corneas cultured without a hydrogel implant were used as controls.

2.4 Fluorescein staining

To monitor the extent of re-epithelialisation, preservative-free sodium fluorescein eye drops (2% w/v Minims, Chauvin Pharmaceuticals Ltd, Essex, UK) were applied to the corneal wound site. After washing with saline, exposed regions of the hydrogel implant retained the fluorescein stain. Digital images were taken every 12 h.

2.5 Light and transmission electron microscopy

Specimens were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) and post-fixed in 2% aqueous

osmium tetroxide. They were washed in PBS before passage through a graded ethanol series and then embedded in epoxy resin. Semithin (0.5 μm) sections were collected on glass slides and stained with toluidine blue before examination under a light microscope. Ultrathin (70 nm) sections were collected on copper grids and stained with 1% vanadyl sulphate, 1% phosphotungstic acid, and Reynolds lead citrate before examination with a transmission electron microscope (JEM 1010; JEOL, Tokyo, Japan).

2.6 Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide. They were washed again in PBS before passage through an alcohol series. After two 20-min changes of 100% ethanol, the samples were transferred to hexamethyldisilazane for 10 min and air dried. Samples were then mounted and sputter-coated with gold before examination on a scanning electron microscope (JSM 5600; JEOL).

2.7 Immunolabelling

Specimens were fixed in 2.5% glutaraldehyde, washed in PBS before passage through a graded ethanol series and then embedded in LR white resin. Semithin (0.5 μm) sections were collected on glass slides, and then incubated for 20 min in 1.0% Triton X-100 in PBS. The sections were rinsed thrice in PBS, incubated in cytokeratin CK3/12 primary antibody (AE5, 1 in 100 in PBS) (Progen Biotechnik, Heidelberg, Germany) overnight at 4°C. Sections were then washed thrice in PBS. After which, sections were incubated with goat anti-mouse secondary antibody conjugated with FITC at a concentration 1:150 in PBS for 2 h, washed with PBS and mounted with medium (Hydromount, National Diagnostics, Atlanta, GA). Substitution of primary antibody with same species serum (mouse) served as a negative control. The sections were visualised under a DMIRE2 confocal microscope (SB2-AOBS). Cells exhibiting fluorescence under a FITC filter were considered to have stained positive for the antibody.

3 Results

3.1 Fluorescein staining

The epithelialisation for implanted corneas was monitored over 5 days. Full epithelialisation was only achieved for the hydrogel modified with 1,4-diaminobutane and coated with collagen IV. Sodium fluorescein stained the whole implant area immediately after insertion (Fig. 1a, a1). At 14 h post-implantation there was still no obvious epithelial migration (Fig. 1b, b1). After this latent phase, the epithelial cells started to cover the implant area. At 36 h, the majority of the wounded area was already covered with epithelial cells (Fig. 1c, c1). By 48 h, a complete coverage of the wounded area was achieved (Fig. 1d, d1). After 5 days, the samples were collected and, histological and immunohistochemical examinations were performed.

Hydrogels modified with 1,4-diaminobutane but without further coating with extracellular matrix molecules only showed partial re-epithelialisation. Hydrogels modified with amines including 1, 2-diaminoethane, or 1,3-diaminopropane showed only partial re-epithelialisation, and further coating with extracellular matrix proteins improved re-epithelialisation of these surfaces but did not result in complete re-epithelialisation.

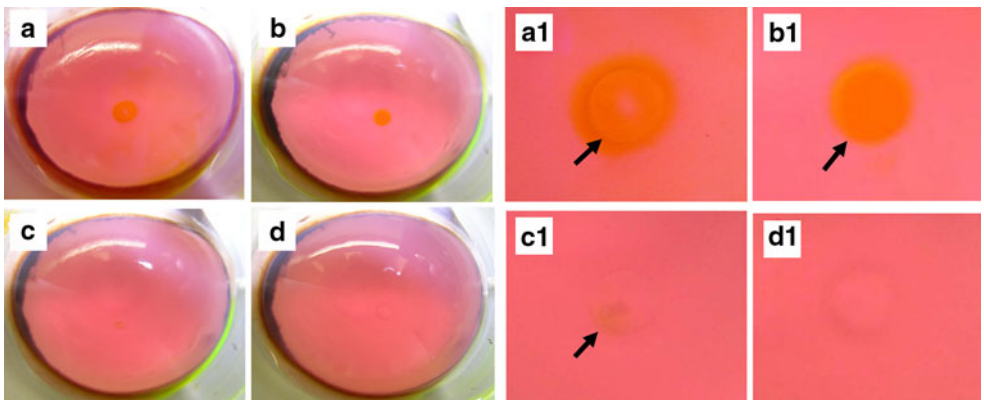

Fig. 1 Photographs of corneal epithelialisation in a representative fluorescein-stained cornea with implantation of 3-mm diameter hydrogel implant at selected time points. **a–d** shows the whole cornea and **a1–d1** shows enlarged images of the implant region (*arrows*). **a**, **a1** At time zero. **b**, **b1** 14 h post-implantation. **c**, **c1** 36 h post-implantation. **d**, **d1** 48 h post-implantation; by 48 h, the hydrogel surface was fully covered with corneal epithelium and the cornea remained clear

3.2 Light microscopy

The epithelium of the 1,4-diaminobutane-modified and collagen IV coated hydrogel implants was examined with a light microscope and compared with the central region of a control bovine cornea. The 5-day implants showed a healthy, stratified sheet of epithelium on the anterior surface (Fig. 2a). In appearance it was very similar to that of bovine corneal epithelium from the control (Fig. 2b). The epithelial tissue was firmly attached to the hydrogel surface and displayed good overall integrity. Cells forming the basal layer were in direct contact with the hydrogel surface. The thickness of the epithelial layer on the implant was similar to that of the control (Fig. 2a, b).

3.3 Electron microscopy

Transmission electron microscope examination of the interaction between the epithelial cells and the hydrogel shows the basal surface of the epithelial cells to be in continuous contact with the hydrogel polymer (Fig. 3a). The overlying epithelial cells were healthy with numerous desmosomal junctions evident between them (Fig. 3b). The basal region of the epithelial cell often exhibited what appeared to be hemidesmosomal attachments to the underlying hydrogel polymer (Fig. 3c), very similar to the hemidesmosomal junctions of epithelial cells of the control corneal samples (Fig. 3d).

Examination of the apical surface of epithelial cells grown on an implant by scanning electron microscopy showed a continuous layer of epithelial cells (Fig. 4a). The cells were stratified and appeared healthy (Fig. 4b). Higher magnification showed the cells to be tightly opposed to each with the typical polygonal appearance (Fig. 4c). Transmission electron microscopy shows these to be healthy with no sign of necrosis or apoptosis (Fig. 4d).

Transmission electron microscope examination of the corneal stroma in the region of the hydrogel implant showed that the corneal stroma appeared normal (Fig. 5a). Also that the stromal collagen fibrils are in direct contact with the sides of the hydrogel implant appearing to adhere to it (Fig. 5b). Even at high magnification there is no sign of oedema in the corneal stroma next to the implant (Fig. 5c). High magnification images of the hydrogel polymer shows that it has pores present within in it that are about 100 nm in size (Fig. 5d).

3.4 Immunolabelling

Immunohistochemical analysis from 5-day cultured corneas showed that cytokeratin 3/12 was present in epithelial cells grown on the surface of an implant (Fig. 6a). Substitution of the primary antibody with a non-specific primary resulted in no staining, illustrating the specificity of this antibody for cytokeratin 3/12 (Fig. 6b).

4 Discussion

The development of an artificial cornea or keratoprosthesis would facilitate the treatment of many patients with challenging conditions and overcome the shortage of donor tissue. The development of suitable biomaterials is a prerequisite for achieving these goals. The ideal material should be transparent, biocompatible and allow the growth of a continuous confluent sheet of epithelium on its anterior surface. Some of the most promising results for re-epithelialisation have come from a perfluoropolyether-based polymer [6, 15]. However, there are limitations; the perfluoropolyether-based polymer has a tendency to deposit lipids and is relatively hydrophobic [16] compared to the cornea.

We have based our approach on a polymethacrylate-based hydrogel and the hydrogel surface has been further functionalized with primary amines which our previous work has shown to enhance cell adhesion in vitro [13]. We

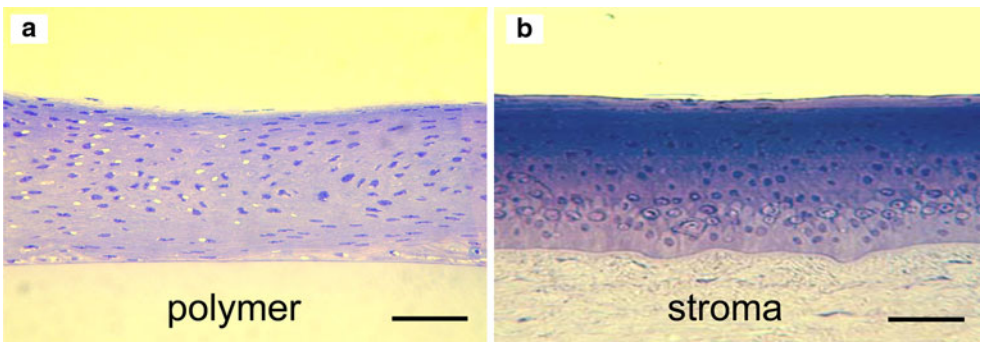

Fig. 2 **a** Epithelial cell sheet on the surface of the hydrogel implant after implantation for 5 days. The epithelium was healthy, stratified and firmly attached to the hydrogel surface. It has a similar thickness to the control cornea. **b** Epithelium from control cornea without hydrogel implantation. *Scale bars* 100 μm

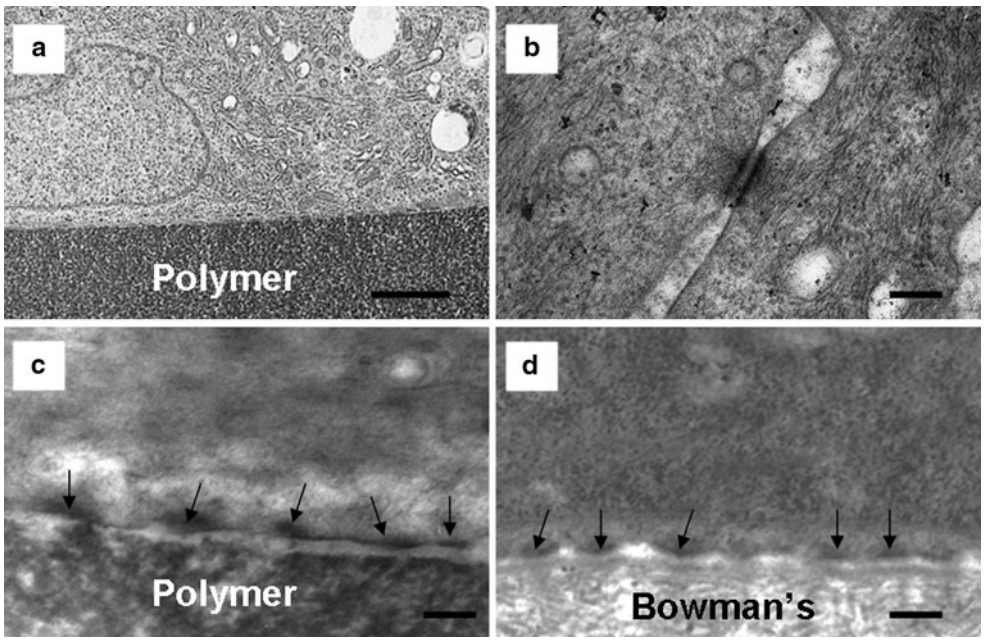

Fig. 3 Transmission electron microscope images of corneal re-epithelialisation on the hydrogel implant. **a** Shows the basal region of an epithelial cell which has migrated onto the hydrogel polymer. **b** Shows a desmosomal junction between adjacent epithelial cells on the hydrogel polymer. **c** Shows the basal region of an epithelial cell attaching to the hydrogel polymer via hemidesmosomal junctions (*arrows*). **d** Shows the basal region of a normal epithelial cell attaching to Bowman's membrane via hemidesmosomal junctions (*arrows*). *Scale bars* **a** 1 μm, **b** 200 nm, **c** 100 nm, **d** 100 nm

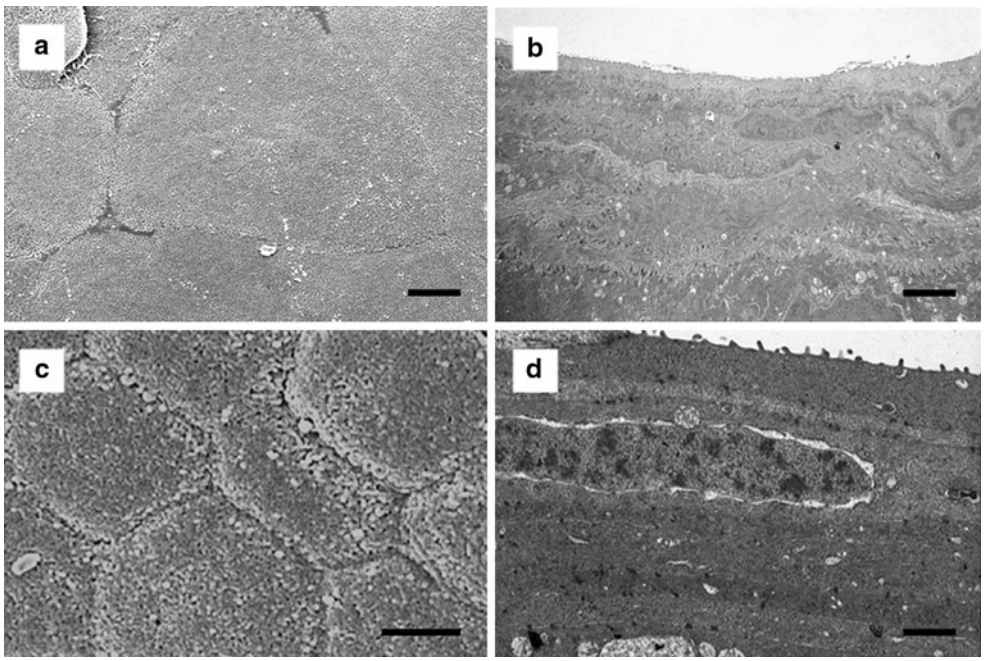

Fig. 4 Scanning (**a**, **c**) and transmission (**b**, **d**) electron microscope images of corneal re-epithelialisation on the hydrogel implant. **a**, **b** A continuous layer of flat polygonal epithelial cells has migrated over the implant. These cells are healthy and stratified with no evidence of any abnormalities. **c**, **d** Higher magnification shows that the epithelial cells are polygonal in shape with surface microvilli, there is no evidence of necrosis or apoptosis. *Scale bars* **a** 10 μm, **b** 10 μm, **c** 10 μm, **d** 2 μm

evaluated these hydrogels using a 3-D corneal organ culture system [14]. Bovine cornea was used as it is similar to human in particular its limbal stem cell distribution. [17–20]. Our culture system has previously been shown to maintain the cornea in good condition for several weeks and replicates epithelial wound healing in vivo [14]. Initial

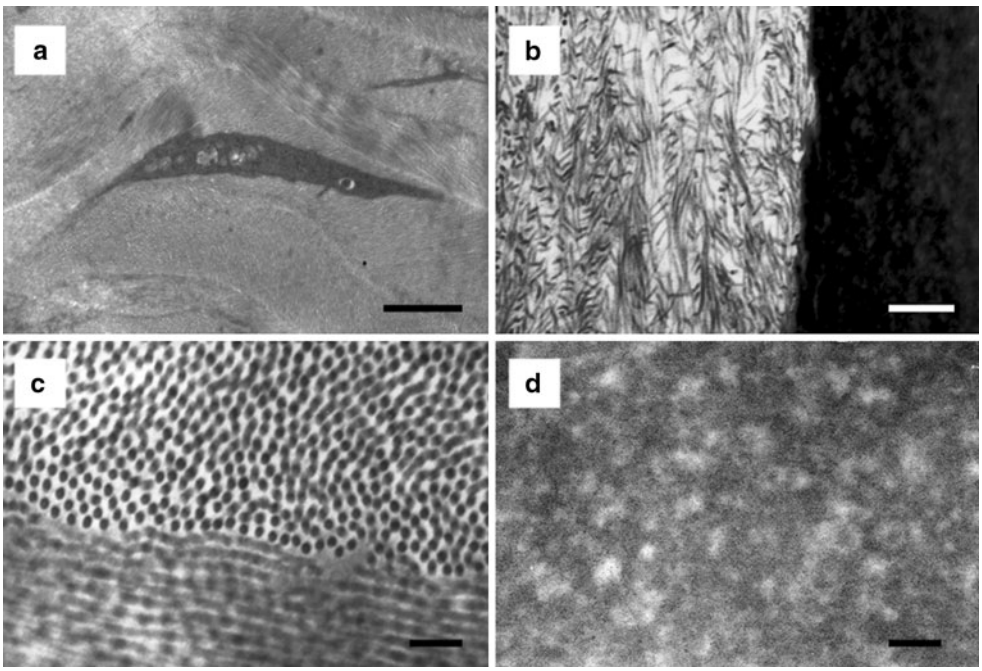

Fig. 5 Transmission electron microscope images of the corneal stroma and the hydrogel implant. **a** Shows that the corneal stroma and keratocytes adjacent to the implant appear normal. **b** Shows that stromal collagen fibrils are in direct contact with the sides of the hydrogel implant. **c** Shows a high magnification image of the corneal stroma; note that there is no sign of oedema. **d** Shows a high magnification image of the hydrogel polymer, note the pores present which are less than 100 nm in size. *Scale bars* **a** 2 μm, **b** 1 μm, **c** 100 nm, **d** 100 nm

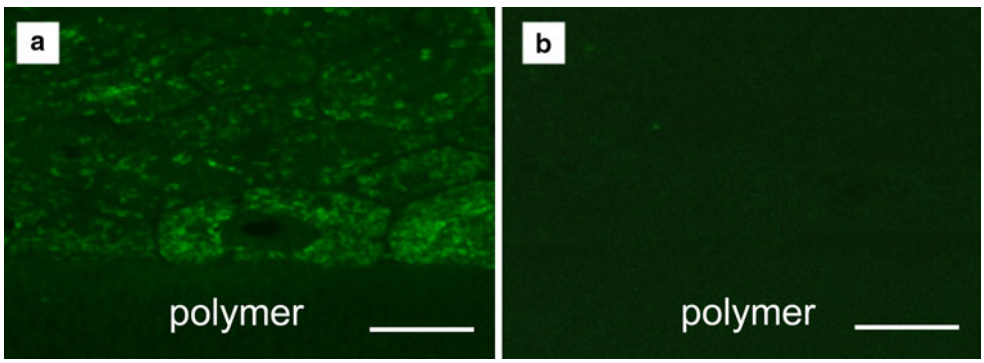

Fig. 6 **a** Immunohistochemical labelling (CK3/12) of epithelial cells grown on the surface of the hydrogel implant. The labelling is shown as green fluorescence. **b** Negative control. *Scale bars* 10 μm

studies showed that compete re-epithelialisation of the implant did not occur with amine modification alone, but this was achieved after further surface coating with collagen IV.

Collagen IV and laminin are major basement membrane components of the corneal epithelium [21]. Collagen I is the main component of the corneal stroma [22, 23]. Fibronectin is synthesised in the wound area after acute injury [22]. Coating of synthetic surfaces with extracellular matrix proteins has been demonstrated in previous studies to enhance corneal epithelial cell attachment and growth [13, 24, 25]. All of these extracellular matrix molecule coatings evaluated on our amine-modified hydrogel resulted in some improvement in the degree of re-epithelialisation but, the only coating which resulted in full re-epithelialisation was collagen IV on 1,4-diaminobutane-modified hydrogel.

Type IV collagen, is a common component of basement membranes. In addition to its structural role, type IV collagen is implicated in the adhesion and migration of corneal epithelial cells. Thus, it is not surprising that it promotes re-epithelialisation of the hydrogel implant. A previous study also reported that type IV collagen enhanced re-epithelialisation of implants [26] although

even better results were found with type I collagen. This study was carried out in cats and without amine modification, so the two studies cannot be directly compared.

Of critical importance is that the hydrogel should be transparent with a similar refractive index to that of cornea, that it should not be rigid but be flexible like the normal cornea. Finally, it should be permeable to quite large molecules such as growth factors. This last requirement is one of the most challenging. Recent work has shown that in order for epithelial cells to survive and thrive they must be able to interact via soluble factors, including growth factors, with underlying fibroblast cells. Glucose and insulin also have roles maintaining the survival of the corneal epithelium [13, 27]. This is why feeder fibroblast cells are necessary when carrying out ex vivo expansion of epithelial cells [28]. Thus, the hydrogel polymer must be porous, but because the polymer must be transparent these pores must not be larger than 200 nm otherwise they will start to scatter light. Our polymer has of pores around 100 nm in diameter, which allows the passage of growth factors, but which are not large enough to scatter visible light. The pores are almost certainly generated as a result of phase separation processes taking place during the polymerization, as the monomer/polymer mixture shrinks in the presence of non-reactive solvent (2-propanol). It has been shown that corneal implants with this range of pore sizes are ideal as they do not impede the corneal physiological communication [29, 30] but at the same time are not large enough to scatter light and make the implants opaque.

In this study, we observed that at 14 h post-implantation, there was no obvious migration of epithelial cells. After this latent phase, epithelial growth began inward from the wound periphery. At 36 h, the majority of the implant surface was covered by epithelial cells. At 48 h, a full coverage with epithelial cells was achieved. This largely follows the normal wound healing process although the latent phase is longer [31, 32]. Full epithelial thickness was achieved within 5 days, but only for the implant with a 1,4-diaminobutane-modified surface coated with collagen IV.

Ultrastructural data showed the epithelium covering the coated implant shared many similarities to the normal bovine corneas. There were microvilli on the apical surface of epithelium grown on the implant. Desmosomes were evident between the adjacent cells which provided some integrity to the tissue. Ultrastructural examination of the interface between the base of the epithelial cells and the hydrogel polymer revealed hemidesmosome structures very similar to those on the base of normal epithelial cells.

Our immunohistochemical results showed that cells covering the implant were strongly positive for CK3/12, confirming that the newly migrated and regenerated cells had a corneal epithelial cell phenotype.

5 Conclusions

In summary, the results of our study demonstrate that a 1,4-diaminobutane-modified polymethacrylate hydrogel coated with collagen IV has potential for development of a synthetic artificial cornea in that it supports the re-epithelialisation of a wounded cornea by adjacent corneal epithelial cells.

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