Acta Biomaterialia 10 (2014) 3029–3037

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Amine functional hydrogels as selective substrates for corneal epithelialization

Enas Hassan [a], Pallavi Deshpande [a], Frederik Claeyssens [a], Stephen Rimmer [b], Sheila MacNeil [a,*]

[a] Department of Materials Science & Engineering, The Kroto Research Institute, University of Sheffield, Broad Lane, Sheffield, S3 7HQ, UK
[b] Polymer and Biomaterials Chemistry Laboratory, Chemistry Department, University of Sheffield, Sheffield, S3 7HF, UK

ARTICLE INFO

Article history:
Received 13 November 2013
Received in revised form 3 February 2014
Accepted 24 February 2014
Available online 4 March 2014

Keywords:
Amine
Limbal epithelial cells
Synthetic hydrogels
Cornea

ABSTRACT

The aim of this study was to develop a synthetic hydrogel to act as a corneal substitute capable of selectively supporting the adhesion and proliferation of limbal epithelial cells (LECs) while inhibiting growth of limbal fibroblasts. Deficiency of LECs causes conjunctival epithelial cells to move over the cornea, producing a thick scar pannus. Unilateral defects can be treated using LEC cultured from the unaffected eye, transplanting them to the affected cornea after scar tissue is removed. The underlying wound bed is often damaged, however, hence the need to develop a corneal inlay to aid in corneal re-epithelialization. Transparent epoxy-functional polymethacrylate networks were synthesized using a combination of glycerol monomethacrylate, ethylene glycol dimethacrylate, lauryl methacrylate and glycidyl methacrylate that produced two different bulk hydrogel compositions with different equilibrium water contents (EWCs): Base 1 and Base 2, EWC = 55% and 35%, respectively. Two sets of amine-functional hydrogels were produced following reaction of the epoxide groups with excesses of either ammonia, 1,2-diamino ethane, 1,3-diamino propane, 1,4-diamino butane or 1,6-diamino hexane. Neither series of hydrogels supported the proliferation of limbal fibroblasts irrespective of amine functionalization but they both supported the adhesion and proliferation of limbal epithelial cells, particularly when functionalized with 1,4-diamino butane. With Base 1 hydrogels (less so with Base 2) a vigorous epithelial outgrowth was seen from small limbal explants and a confluent epithelial layer was achieved in vitro within 6 days. The data support the development of hydrogels specific for epithelial formation.

1. Introduction

Corneal tissue engineering approaches traditionally begin with the development of a biocompatible substrate that supports the adhesion, proliferation and maintenance of a cell population. Synthetic polymer hydrogels can be useful for such purposes and unlike biological materials can be tuned to exhibit specific bulk and interfacial properties in order to support cells. Hydrogels in their swollen state allow diffusion of biomolecules through their networks and the low interfacial tension reduces immunogenicity [1–3]. This makes them an interesting candidate for use as a corneal inlay. However, hydrated networks are usually resistant to protein adsorption and hence are not cell-adhesive. As a result these types of hydrogel have previously been used as keratoprosthetics [4–6] or as cell delivery substrates after modification by methods such as plasma polymerization [7]. For tissue engineering applications hydrogels can be modified to allow cell adhesion and proliferation while still exploiting the hydrogels' ability to allow diffusion of oxygen, water and glucose. Some common strategies employed to achieve this are: (i) modification with hydrophobic segments [8,9]; (ii) modification with cell-adhesive peptides [10–13]; (iii) modification with matrix proteins [14,15]; and (iv) surface modification with acid groups [16,17] and other moieties. We have previously reported that modification with alkyl amines can provide good substrata for corneal epithelial cells when co-cultured with bovine keratocytes [18,19]. Another study indicated that modifying hydrogels with 1,6-diamino hexane (1,6 DAH) can encourage A549 epithelial cells to grow up to and over their surface much better than unmodified gels [20]. The clinical efficacy of inlay materials will depend on the following criteria: they must be transparent, non-toxic, biostable and must have interfacial properties that can allow continued adhesion and proliferation of corneal epithelial cells [21–23]. A stable epithelium over a synthetic implant is reputed to contribute to its survival [5,24,25] as the epithelium will help prevent entry of pathogens and fluid loss. However, non-colonizable inlays such as Alphacor™ are still considered as treatments in extreme cases [26,27]. To achieve

* Corresponding author.
E-mail address: s.macneil@sheffield.ac.uk (S. MacNeil).

http://dx.doi.org/10.1016/j.actbio.2014.02.043
1742-7061/

epithelialization, some of the above-mentioned strategies may be used but, despite extensive studies in this field, there has been limited success with inlay devices. Some common yet complex issues have been highlighted. Collagen-modified surfaces have been shown to be prone to collagenase activity over time [28], and methods used to increase protein adsorption, such as adding hydrophobicity or other proteins, may facilitate non-specific protein binding which has been thought to be the molecular event that triggers calcification [29,30]. Corneal inflammation may also be an issue if proteins such as fibronectin are used with implants as it affects natural wound-healing processes such as fibroblast migration, [4] wound contraction and macrophage chemotaxis [31]. In general, most bulk hydrogel materials used for corneal regeneration have had moderate to high water contents to allow easy diffusion of nutrients and have used polymers such as poly(ethylene glycol)/poly(acrylic acid) (PEG/PAA) [32], poly(hydroxyethyl acrylate) (PHEA) [25] or poly(hydroxyethyl methacrylate) (PHEMA) [33]. However, each of these systems requires proteins such as fibronectin, collagen or other peptides to facilitate cell adhesion and proliferation. Also, modification with these matrix proteins inherently permits, to varying degrees, non-specific adhesion of cells. Our group has previously shown that amine-modified polymethacrylate hydrogels can support epithelialization both in mono-culture and co-culture with corneal stromal cells [18,19], or if coated with collagen IV [15] or VII [34]. A small increase in epithelial cell proliferation was observed in co-culture and some epithelialization could also be achieved in mono-culture [18–20]. It was evident from these earlier studies that alkyl amine functionality could provide excellent substrata for epithelial cells. In contrast, aminated surfaces using plasma polymerization methods do not provide good substrates for general cell adhesion, while acid functionalization [17] can improve cell adhesion. Clearly, these studies suggest that both bulk and surface properties influence cell behaviour. These early studies indicate that optimization of the alkyl-aminated hydrogels could provide a material that supports a stable epithelium. Moreover, it is proposed that the selected material can be used to culture an epithelium with minimal stromal cell contamination directly from limbal explants. It is important to establish a corneal epithelial barrier quickly to minimize the risk of infections and further complications. This is an issue if the limbal epithelial stem cell (LESC) population responsible for corneal regeneration is destroyed due to injury or disease. The use of small limbal explants may contain the LESCs as well as the architectural microenvironments required for their survival. Regeneration of a corneal epithelium from limbal explants has recently been demonstrated in the clinic using small corneal explants fibrin-glued to a biological substrate, the amniotic membrane [35,36]. This concept has also recently been shown by our group to regenerate an epithelium on an ex vivo rabbit corneal model using a synthetic, rapidly degradable poly(D,L-lactide-*co*-glycolide) (PLGA) membrane developed as an alternative to the use of donor human amniotic membrane [37]. The current study goes beyond this to ask whether synthetic biomaterials can be developed to selectively promote epithelial development. In looking at cell responses to these materials this study also examines the influence of bulk vs. surface properties of these materials on corneal epithelial and stromal cell adhesion, migration and proliferation.

2. Experimental methods

2.1. Statistics

Statistical analysis using one-way ANOVA was performed using Origin Pro 8 and carried out between functional groups of the same base. All data are expressed as mean ± standard error of the mean (SEM), with $P < 0.05$ considered significant.

2.2. Cell culture and cell isolation

Rabbit eyes were obtained from Hook Farm, Hampshire, UK. They were received in chilled phosphate-buffered saline (PBS) within 24 h of extraction. The eyes were first cleaned from excess tissue and then immediately disinfected using 3% Vidine for 2 min and washed in PBS. Eyes were then transferred to a class II tissue culture cabinet and immersed in 1.5% Videne for 1 min before being washed in PBS.

The limbal regions were isolated and the remaining tissue discarded. The limbal explants were cut into 3–4 sections and placed in a solution of Dispase II (Roche) (2.5 mg ml^{-1} in serum-free Dulbecco's Modified Eagle's Medium (DMEM) + Glutamax (Life Technologies, UK)) and placed in an incubator for 1 h at 37°C and 5% CO_2. Explants were subsequently transferred into a dish of fresh DMEM + Glutamax media and cells were scraped from the epithelial surface using a pair of blunt forceps; the resulting cell suspension was centrifuged at 200*g* for 5 min and resuspended in the epithelial culture medium, henceforth referred to as RLE media (1:1 ratio of DMEM + Glutamax and Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 10 ng ml^{-1} epidermal growth factor, 5 μg ml^{-1} insulin, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B) and seeded into a T25 flask containing 3T3 cells whose growth had been arrested by gamma irradiation. The remaining de-epithelialized tissue explants were placed into T25 flasks and left in the incubator to adhere before adding 10% DMEM (DMEM plus 10% FCS, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2×10^{-3} mol l^{-1} glutamine and 0.625 μg ml^{-1} amphotericin B). Explants were cultured until rabbit limbal fibroblast (rLF) outgrowth reached 80% confluence, then passaged and used as required. Rabbit limbal epithelial cells (rLECs) were not used beyond passage 2. In the case of fibroblasts, explants were removed and adhered cells passaged and used between passages 3 and 6 when required. For immunohistochemistry controls, central corneal epithelial cells were obtained by trephination of the central cornea using an 8 mm biopsy punch and then isolated using precisely the same method described above to extract epithelial cells from the limbal region.

2.3. Hydrogel synthesis

The monomers lauryl methacrylate (LMA), glycerol monomethacrylate (GMMA), ethylene glycol dimethacrylate (EGDMA) and glycidyl methacrylate (GME) in varying ratios (see Table 1; total 9 g) were dissolved in 4 ml of isopropanol (IPA). The mixture was purged of oxygen by bubbling nitrogen through the solution for ~20 min. Just before curing, 90 mg (1 wt.% monomers) of 2-hydroxy-2-methylpropiophenone (HMPP) was added and the total mixture degassed for a further 2 min. The solution was added to a polymerization mould (see Fig. 1), which stops oxygen entering the system, and irradiated with a 2" arc 400 W mercury discharge lamp at a distance of 10 cm in a Dymax Bond box curing chamber on a rotating table for 3 min, 90 s on each side. The polymerization

Table 1
Monomer percentage ratios made up to 9 g in 4 ml IPA.

Hydrogel	Glycerol monomethacrylate GMMA	Glycidyl methacrylate GME	Ethylene glycol dimethacrylate EGDMA	Lauryl methacrylate LMA
Base 1	80	5	3	12
Base 2	75	5	8	12