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Human corneal endothelial cell sheets for transplantation: Thermo-responsive cell culture carriers to meet cell-specific requirements

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ABSTRACT

Corneal endothelial diseases lead to severe vision impairment, motivating the transplantation of donor corneae or corneal endothelial lamellae, which is, however, impeded by endothelial cell loss during processing. Therefore, one prioritized aim in corneal tissue engineering is the generation of transplantable human corneal endothelial cell (HCEC) layers. Thermo-responsive cell culture carriers are widely used for non-enzymatic harvest of cell sheets. The current study presents a novel thermo-responsive carrier based on simultaneous electron beam immobilization and cross-linking of poly(vinyl methyl ether) (PVME) on polymeric surfaces, which allows one to adjust layer thickness, stiffness, switching amplitude and functionalization with bioactive molecules to meet cell type specific requirements. The efficacy of this approach for HCEC, which require elaborate cell culture conditions and are strongly adherent to the substratum, is demonstrated. The developed method may pave the way to tissue engineering of corneal endothelium and significantly improve therapeutic options.

1. Introduction

Human corneal endothelial cells (HCEC) form a squamous, monolayered epithelium on the posterior side of the cornea, commonly termed the corneal endothelium to distinguish it from the multilayered anterior epithelium of the cornea. They maintain corneal transparency by pumping ions and excess water from the corneal stroma into the anterior chamber [1]. HCEC are arrested in G1-phase of the cell cycle and show almost no regenerative capacity in vivo. The corneal endothelium experiences a physiological cell loss with age, which is increased by corneal disease, injury or surgical trauma. Cell loss from over 3000 cells per mm^2 at birth to below a threshold of ~500 cells per mm^2 dramatically compromises the overall endothelial pumping capacity [2] and results in vision impairment or blinding of the affected eye due to corneal edema and opacification. Full thickness transplantation (keratoplasty) of long-term cultured (30–37 °C) or short-term cold-stored (4 °C) donor corneas is the gold standard therapeutic option. The endothelial cell density and morphology serve as sensitive and also the only biomarkers to evaluate the quality of a donor cornea. Recently, lamellar endothelial keratoplasty has come into focus and has been investigated as an alternative strategy [3]. Lamellae are only composed of the corneal endothelium, its basal lamina (Descemet's membrane) and a thin portion of the underlying corneal stroma, and are ~100–200 μm thick [4,5]. Both transplantation techniques require donor tissue with high endothelial cell densities, a demand which is difficult to meet by present organ culture or preservation techniques. A loss of ~20% of donor tissue due to endothelial cell loss is common in European eye banks [6]. Manual or laser-aided cutting of thin lamellae from donor corneas exerts stretching or thermal forces on the endothelium, so that endothelial cell loss occurring during graft preparation and processing of the tissue remains a major issue. However, only a thin graft allows for a faster recovery of visual acuity with minimal alteration of corneal refraction [4]. Therefore, visual outcome is inversely dependent on the thickness of the graft.

Hence, a variety of tissue engineering strategies for corneal endothelium replacement have been investigated, and many efforts aim to establish methods for in vitro cultivation and generation of transplantable HCEC sheets [7]. However, HCEC are very sensitive to culturing procedures [8,9] and are also exceedingly adhesive to the cultivation substrate. The use of stimuli responsive polymer (SRP) surfaces [10] may facilitate harvesting of HCEC,

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together with their extracellular matrix (ECM) as an intact cell sheet of extreme thinness, an important characteristic with respect to visual outcome after endothelial transplantation in patients. SRP with a thermally stimulated volume phase transition, such as poly(*N*-isopropylacrylamide) (PNiPAAm) [11] and NiPAAm containing copolymers [12], can be prepared on solid surfaces by different techniques [13]. On immersion in aqueous media, the abrupt change in solubility at the phase transition temperature leads to reversible swelling and collapsing of the immobilized SRP. This effect was used to design switchable cell culture carriers that allow enzyme-free harvest of adherent cells or entire cell sheets in general [14] or HCEC sheets in particular [15,16].

However, SRP-based cell culture carriers require a balance of cell adhesion to achieve optimal cell growth and effective cell detachment upon stimulation. Compared with the previous work of Sumide et al. [15] and Lai et al. [16], the current study aims to achieve better understanding of this balance and the respective material parameters where HCEC are employed as a highly relevant example. Cell adhesion, a prerequisite for proper cell and tissue functionality [17], is secured for HCEC by an ECM consisting of laminin, collagen type IV and type VIII and proteoglycans [18]. While adhesion of cells can be facilitated by biofunctionalizing the culture substrate with proteins, detachment of confluent cell sheets often remains a challenge, especially for anchorage-dependent cells such as HCEC, which can be strongly adherent [19,20]. The present study therefore investigated how the properties of a thermo-responsive carrier that determine cell adhesion and stimulated detachment can be tuned to meet the requirements of highly fastidious cells such as HCEC.

Previously explored approaches to modulating the characteristics of switchable cell culture carriers include variation in the layer thickness [21], stiffness, degree of swelling and absolute switching amplitude [22], terminal functionalization of SRP chains [23] or inclusion of poly(ethylene glycol) units [24]. Furthermore, immobilization of proteins and peptides [25] was shown to promote initial cell adhesion and proliferation.

In the current study, a novel set of cell culture carriers based on poly(vinyl methyl ether) (PVME; exhibiting a distinct phase transition temperature close to cell cultivation temperature [26]) was prepared by simultaneous electron beam cross-linking and grafting of solid polymer films onto polystyrene surfaces followed by biomolecular functionalization [27]. The resulting material platform is demonstrated to be highly tunable and therefore suitable to support cultivation and effective enzyme-free harvest of HCEC sheets.

2. Materials and methods

2.1. Preparation and biomolecular functionalization of cell culture carriers

To improve the applicability of analytical techniques, SRP layers were prepared on polystyrene thin films on different supports, instead of in standard cell culture dishes. Polystyrene thin films (PS, type 148H; BASF, Ludwigshafen, Germany) ~30 nm thick were applied by spin coating (solution 1% w/w in toluene, 2000 rpm, 1500 rpm s^{-1}, 30 s) onto microscopy cover slips (20 mm in diameter; Menzel Gläser, Braunschweig, Germany) or silicon wafers (15×20 mm^2). PS surfaces were treated with air plasma to obtain appropriate wetting behavior (Harrick Plasma Cleaner PDC-002, 1 min). Subsequently, blends of PVME (TCI Europe, Zwijndrecht, Belgium) and the alternating copolymer of vinyl methyl ether and maleic acid (PVMEMA; Sigma-Aldrich, Munich, Germany) were prepared by spin coating (solution 2% w/w or 10% w/w in methanol, 2000 rpm, 1500 rpm s^{-1}, 30 s) on the PS surface (referred to as samples with thin or thick SRP layers). A copolymer content of 1% w/w or 10% w/w led to samples with a different functional group density (referred to as samples with low or high content of binding sites for immobilization of proteins/peptides).

Electron beam irradiation with 150 keV, corresponding to a penetration depth in polymeric materials of ~200 μm, was carried out using the ADU low-energy electron facility (Advanced Electron Beams, Wilmington, USA) under a nitrogen atmosphere at room temperature (RT). The samples were irradiated with an absorbed dose of 258 or 774 kGy (referred to as samples with a low or high degree of cross-linking). The dose was applied stepwise in order to reduce the temperature increase during electron beam treatment. Finally, samples were rinsed in deionized water and ethanol to remove unbound material.

The dry thickness of PVME-blend-PVMEMA layers was determined by ellipsometry (SE400adv; Sentech Instruments, Berlin, Germany) on coated silicon wafers. Swelling behavior was characterized by spectroscopic ellipsometry (M-2000VI, J.A. Woollam Co., Lincoln, USA) as reported in a previous study [27]. Briefly, coated silicon wafers were placed in a liquid media cell (angle of incidence 68°) filled with deionized water (pH 6.5). A computer-controlled heating device was used for temperature variation with a rate of 1 K min^{-1}. To calculate the thickness and optical properties of the swollen SRP films, fit procedures based on Cauchy multilayer models and an effective medium approximation were applied to the ellipsometric data. The swelling degree was calculated as $Q = d_T/d_{dry}$, where d_T corresponds to the swollen film thickness at a given temperature, and d_{dry} to the dry film thickness.

Mechanical properties of PVME-blend-PVMEMA layers on microscopy cover slips were determined by nanoindentation experiments using atomic force microscopy (AFM; NanoWizard II, JPK Instruments, Berlin, Germany) mounted on an inverted optical microscope (Observer D.1, Zeiss, Jena, Germany). Samples were placed in Petri dishes (diameter 35 mm). Measurements were performed in phosphate buffered saline without Mg^{2+}/Ca^{2+} (PBS w/o Mg^{2+}/Ca^{2+}; Biochrom AG, Berlin, Germany) at 25 and 37 °C controlled by a PetriDishHeater (JPK Instruments). A v-shaped cantilever with a nominal spring constant of 0.07 N m^{-1} (MLCT, Bruker AFM Probes, Camarillo, USA) was used. Cantilevers were calibrated using the equipartition theorem [28]. Force–distance curves were acquired up to 1 nN contact force and 2 μm s^{-1} approach/retract velocity. The Young's modulus was extracted from approach force–distance curves using the Herz model (corrected for the use of a quadratic pyramid as intender) provided by AFM data processing software (JPK Instruments).

For cell culture experiments, samples were sterilized by incubation in 0.02% v/v ProClin®300 Preservative for Diagnostic Reagents (Sigma-Aldrich) in PBS w/o Mg^{2+}/Ca^{2+} over night at RT. ProClin®300 residuals were removed by rinsing in PBS w/o Mg^{2+}/Ca^{2+} over night at RT. To allow for covalent protein/peptide immobilization, maleic acid groups were converted into anhydride moieties in a thermal annealing step (90 °C, overnight) [29]. Subsequently, samples were cooled to RT and immediately incubated in a solution of either 10 μg ml^{-1} laminin (LN; Sigma-Aldrich) and 10 mg ml^{-1} chondroitin-6-sulfate (CS; Sigma-Aldrich) in Medium 199 (Biochrom AG) or 50 μg ml^{-1} cyclo(arginine-glycine-aspartic acid-D-tyrosine-lysine) (cRGD; Peptides International, Louisville, USA) in PBS w/o Mg^{2+}/Ca^{2+} for 2 h at 37 °C under sterile conditions. Fig. 1 summarizes the preparation, biochemical functionalization and structural constitution of the novel cell culture carrier.

A hydrophilic polystyrene surface (air plasma treated PS) similar to commercial tissue culture polystyrene (TCP) and a thermo-responsive cell culture system based on the statistical copolymer of *N*-isopropylacrylamide and diethyleneglycol methacrylate (poly(NiPAAm-*co*-DEGMA)) [30] developed in a previous study [31] were used as controls. Briefly, a thin film of