Strategies for faster detachment of corneal cell sheet using micropatterned thermoresponsive matrices†

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1. Introduction

Penetrating keratoplasty is considered as the gold standard treatment for corneal transplantation. However, newer procedures such as selective tectonic lamellar keratoplasty are currently being used, which selectively replace only the diseased or damaged layers of the cornea while retaining the healthy parts.1 In practice, this technique is utilized by dissecting a single donor cornea into several segments and subsequently transplanting them into multiple patients to treat mild corneal dysfunctions. For high-risk transplantation procedures, tectonic lamellar keratoplasty has been reported2 by combining the corneal transplant with a decellularized cornea; this association has showed prolonged longevity of transplants with minimal inflammation. To eliminate the dependency on corneal grafts, a promising alternative to replace selective portions of the cornea is to use tissue engineering. However, the complex 3D architecture of corneal stroma makes it a huge challenge to simulate a functionally alike tissue in the laboratory, in terms of both transparency and mechanical strength.3 Though cells cultured on patterned and stacked polymeric films3,4 have been reported as early steps towards developing corneal stromal equivalents, a strategy to

The development of transplantable cell sheets of functional keratocytes embedded within an aligned collagen type I matrix is a viable approach for constructing a bioequivalent of corneal stroma. Thermoresponsive materials based on poly(N-isopropylacrylamide) (PolyNIPA) have been utilized to recover carrier-free corneal cell sheets by inducing temperature changes. In this study, we employed direct-write assembly (DWA) to develop microperiodic parallel patterns of silk–PolyNIPA and gelatin–PolyNIPA. Semi-interpenetrating networks of PolyNIPA hybrids (with silk/gelatin) exhibited temperature-responsive nature and thereby have potential use in cell sheet engineering. Silk–PolyNIPA and gelatin–PolyNIPA hybrids demonstrated a hydrophobic surface at 37 °C (i.e. above their lower critical solution temperature) with a contact angle of 59.1 ± 0.3° and 55.7 ± 3°, respectively, whereas the surface roughness of silk–PolyNIPA was double that of gelatin–PolyNIPA. The reduction of temperature to 20 °C resulted in a decrease in the value of surface roughness and water contact angle for both hybrids. All four parallel patterned substrates guided corneal cell alignment along the direction of the patterns. Collagen type-I and aggrecan gene expression was higher when the cells were grown over the gelatin–PolyNIPA matrix after 3 weeks of culture when compared to silk–PolyNIPA. In addition, a significantly higher metabolic activity as well as enhanced vinculin expression of keratocytes on the gelatin–PolyNIPA matrix indicated the improved cytocompatibility compared to the silk, gelatin and silk–PolyNIPA matrices. Interestingly, the detachment of keratocytes cell sheet was achieved from the silk–PolyNIPA and gelatin–PolyNIPA planar films only within 10 min and 30 min, respectively, but the patterns could not yield intact sheet recovery. Hence, we conclude that while gelatin–PolyNIPA hybrids with parallel patterns fabricated using DWA will benefit from the application of cellular alignment, some optimization in the pattern parameters may be required for rapid sheet recovery from such substrates. Understanding the keratocytes responses to such hybrid biomaterials suggests viable options to develop a corneal stromal bioequivalent.
develop a carrier-free cell sheet with a highly ordered arrangement of extracellular matrix (ECM) and cells that can be directly transplanted in the corneal tissue would be highly desirable.\(^5,6\)

To this end, pioneering work has been performed by Okano and co-workers on temperature-responsive substrates to develop carrier-free, stratified cell sheets that form rapid and stable attachment to the host eye.\(^5,6\) PolyNIPA, above its lower critical solution temperature (LCST) (i.e. > 32 °C), demonstrates hydrophobic nature, which favours cell adherence and proliferation. However, when the temperature falls <32 °C, the polymer surface becomes hydrated, eventually leading to the detachment of cells as a single intact sheet without disrupting the cell–cell junctions.\(^5\)

However, PolyNIPA has certain limitations such as toxicity, slow deswelling rate, poor biocompatibility and inadequate mechanical properties,\(^9,10\) which demand the development of mixed hybrids of the polymer with specific proteins such as silk\(^10,11\) or gelatin.\(^12,13\) In an attempt to improve the characteristic properties of PolyNIPA, the incorporation of 30 wt% silk fibroin resulted in the faster deswelling rate for silk–PolyNIPA\(^14\) by improving the viscoelastic properties of the blend, as suggested by the increased values of storage modulus (\(G^\prime\)) and viscous modulus (\(G^\prime\)).\(^15\) Gelatin–PolyNIPA hydrogels exhibited surfaces with rougher topography and increased micro-mechanical strength, which provide strong anchorage for cell traction forces, resulting in cell spreading on the surface.\(^12\)

In the field of cell sheet engineering, the rapid recovery of cell sheets is highly desirable as it would not only aid in maintaining a functionally viable cell sheet by lowering the incubation time at lower temperatures but would also alleviate patient burden in clinical setups. Although PolyNIPA is widely used in cell sheet engineering, cell sheet recovery on the PolyNIPA surface is relatively slow.\(^14\) To facilitate rapid recovery, numerous methods, such as surface initiated radical polymerization, covalent tethering of end group polymer grafting, plasma induced polymerization, and electron beam irradiation polymerization,\(^14,15\) have been employed to prepare PolyNIPA-based hybrids; for example, the grafting of hydrophilic polycrylamide chains on PolyNIPA by electron beam irradiation resulted in the hydration of the PolyNIPA chains, which in turn led to faster cell-sheet detachment at 20 °C when compared to a pure PolyNIPA surface.\(^14\) The topography of the substrate plays a crucial role in dictating the cellular alignment of the cornea.\(^5,16,17\) Phu and co-workers cultured rabbit-derived corneal fibroblasts on electropun collagen I scaffolds and measured the optical transparency and expression of alpha-smooth muscle actin (\(\alpha\)-SMA; stress fibers developed in hazy cornea).\(^18\) Quantitative evidence revealed that there was 28% ± 5% downregulation in the expression of \(\alpha\)-SMA when corneal fibroblasts were grown over aligned fibrous matrix compared to the cells over unaligned collagen fibers, with a significant reduction in scattered light. Wu and co-workers showed that corneal stromal stem cells cultured on aligned poly(ester urethane) urea matrices (60–70 \(\mu\)m thick) secreted a multilayered collagen lamellae with orthogonally oriented fibrils under the influence of FGF-2 and TGF\(\beta\), mimicking the pattern of transparent corneal stroma.\(^19\) Moreover, the parallel orientation of patterns aids in cellular alignment.\(^20\) Crabb and co-workers cultured stromal fibroblasts on a 2 \(\mu\)m microgrooved collagen surface and observed that the cellular alignment was parallel to the direction of the grooved pattern.\(^21\) Interestingly, the fraction of transmittance (0.90 ± 0.02 and 0.97 ± 0.02 at 400 and 700 nm, respectively) for the cell-seeded microgrooved surfaces was comparable to that of the native cornea. In another study, transwell membranes with parallel grooves (200–300 nm spacing) and 0.43 \(\mu\)m pores on the surface oriented the collagen type-I secreted by the cells.\(^20\) Therefore, it is important to optimize the width and depth of the nano-topography and underlying matrix composition that best stimulates the ECM production and orientation of cultured cells.\(^20,21\) Furthermore, patterned silk substrates coupled with an RGD sequence and the surface roughness of 162.6 ± 2.0 nm demonstrated adherence and orientation of human corneal cells along the grooves. Differential gene expression of selected keratocyte markers was substantially upregulated with minimal levels of \(\alpha\)-SMA expression when compared to silk substrates without RGD sequences.\(^16\)

Various methods for pattern fabrication have been employed lately. The photolithographic technique was used to fabricate striped polycrylamide patterns (5, 10 and 50 \(\mu\m\)m wide) on a thermoresponsive PolyNIPA surface showed rapid detachment of adhered NIH-3T3 fibroblasts on the patterned surface over the non-patterned PolyNIPA.\(^22\) A double polymeric nanolayer of PolyNIPA and hydrophilic polycrylamide were deposited on tissue culture polystyrene using electron beam irradiation.\(^14\) In their set-up, the basal polycrylamide component promoted the hydration of the upper PolyNIPA layer, subsequently inducing a rapid detachment of bovine carotid endothelial cells. A dual surface modification technique employed electron beam induction and site-specific biomolecular insertion, which yielded a net-like artifact in addition to a continuous monolayer construct.\(^7\) In another study, 3D cell laden patterning was investigated using photolithography on micropatterned gelatin methacrylate hydrogels.\(^23\) The technique demonstrated a rapid proliferation of NIH-3T3 fibroblasts and their reorganization in 3D. Quantitative evidence revealed that by varying the width of the patterned microgrooves from 50 \(\mu\)m to 200 \(\mu\)m, the percentage of aligned cells dropped from 64% ± 8% to 31% ± 8%, whereas the mean nuclear shape index increased from 0.807 ± 0.02 to 0.917 ± 0.02, respectively, with no significant difference noticed between the 200 \(\mu\)m microgrooves and the unpatterned surface. Nevertheless, all reported techniques of pattern fabrication using thermoresponsive polymers are based on the use of either photolithography or electron beam radiation or both, which are complex, time consuming, costly and restrict the design parameters of the matrix. Therefore, a relatively simple technique, which can enable the production of customized thermoresponsive polymeric patterns, is required.

Taken together, there is a need to impart topographical guidance to corneal cells as well as precisely optimize the surface chemistry to facilitate the rapid recovery of oriented corneal stromal cell sheets. In this study, we have evaluated the alignment and recovery of corneal stromal cell sheets on parallel patterns of thermo-responsive PolyNIPA hybrids (silk–PolyNIPA and gelatin–PolyNIPA)
developed using a direct-write technique. Direct-write assembly (DWA) has been previously used for developing 2D parallel or microperiodic 3D patterns from concentrated colloidal ink to soft hydrogels. The hypothesis was that the incorporation of the two proteins i.e. silk or gelatin in PolyNIPA would improve the surface chemistry of the hybrids over pure PolyNIPA by impacting cellular behavior and rapidly yielding an intact, functionally active corneal stromal cell sheet. For this, hybrid patterns were analyzed for cell viability and differential gene expression for selected corneal stromal matrix specific genes. We also compared the PolyNIPA hybrid patterns with planar films with regards to the alignment and time of cell sheet recovery for corneal cells over the two surfaces.

2. Materials and methods

2.1 Materials

* Bombyx mori* cocoons were kindly provided by the Central Silk Technological Research Institute (Central Silk Board), Bangalore, Ministry of Textiles, Government of India. Gelatin powder (type B, average Mw 40–50 kDa, pl 4.7–5.2) and poly(N-isopropylacrylamide) (Cat. No. 535311-10G, Mw 19 000–30 000, m.p. 96 °C) were purchased from Sigma-Aldrich, India.

2.2 Methods

2.2.1 Silk ink. Direct-write ink composed of a 20%–25% (w/v) aqueous silk fibroin solution was prepared as described earlier. Briefly, small pieces of *Bombyx mori* cocoons were boiled in 0.02 M Na2CO3 for 30 min, followed by thorough rinsing with distilled water to extract the glue-like sericin proteins. Following degumming, the extracted silk fibroin was dissolved in 9.3 M LiBr (Sisco Research Laboratories Pvt Ltd, India) at 60 °C for 4 h, yielding a 20% (w/v) silk fibroin solution. The silk–LiBr solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (MWCO 3500, Pierce) at RT for 2 days. Subsequently, this silk fibroin aqueous solution (6–7 wt%) was again dialyzed against poly(ethylene glycol) (6000 g mol–1, Fisher Scientific, India) solution at RT using Slide-a-Lyzer dialysis cassettes (MWCO 3500). After 12 h, the concentrated silk fibroin solution (20%–25% (w/v)) was carefully removed using a syringe to avoid excessive shearing. The resultant solution was stored at 4 °C before use.

2.2.1.2 Gelatin ink. Gelatin ink was prepared by swelling gelatin in deionized water at RT followed by dissolution at 37 °C under continuous stirring using a magnetic stirrer, in order to obtain a 20% (w/v) gelatin ink for DWA. This was stabilized by chemical crosslinking with 6 mM 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDC) (Cat. No. 054886, Sisco Research Laboratories Pvt Ltd, India) and 2.4 mM N-hydroxysuccinimide (NHS) (Cat. No. 084718, Sisco Research Laboratories Pvt Ltd, India).

2.2.1.3 PolyNIPA concentration. Because a concentration of >10 wt% of PolyNIPA exhibits a non-flowing gel behaviour at temperatures exceeding 32 °C, a 5% concentration was used to form a hybrid gel with the biopolymer (silk or gelatin).

2.2.1.4 Silk–PolyNIPA blend ink. To prepare stock solutions, a suspension of 20% (w/v) silk fibroin with 5% (w/v) PolyNIPA (diluted in deionized water) was mixed overnight at RT on a magnetic stirrer at desirable volume fractions, as listed in Table 1. The resultant suspensions were further cross-linked by the addition of bis-acrylamide (Sigma-Aldrich; 2.7 wt% of PolyNIPA) along with N,N′,N″,N″-tetramethylthelylenediamine (TEMED; Sigma-Aldrich; 10 µl) and ammonium persulphate (APS; Sigma 10 mg) (catalyst and free radical generator).

2.2.1.5 Gelatin–PolyNIPA blend ink. In order to produce a homogenous printable gel, gelatin was partially crosslinked by reducing the concentration of EDC to 3 mM and NHS to 1.2 mM. To prepare stock solutions, a suspension of 20% (w/v) partially cross-linked gelatin with 5% (w/v) PolyNIPA (diluted in deionized water) was mixed overnight at RT on a magnetic stirrer at desirable volume percentages (vol%), as listed in Table 1. The resultant suspensions were further cross-linked by the addition of bis-acrylamide (Sigma-Aldrich; 2.7 wt% of PolyNIPA) along with TEMED (Sigma-Aldrich; 10 µl) and APS (Sigma; 10 mg).

The vol% of PolyNIPA varied from 10% to 30% and that of the corresponding biopolymer (silk or gelatin) from 90% to 70% (Table 1). The hybrids have been denoted by the vol% of PolyNIPA component in the silk and gelatin hybrids.

2.2.2 Differential scanning calorimetry (DSC). DSC was performed using TA instruments Q 200 (USA). 8–10 mg of both dry and wet samples of PolyNIPA hybrids (with silk or gelatin) (n = 3) were placed in an aluminium pan and heated over a temperature range of 0–45 °C at a ramp rate of 10 °C min–1. For wet samples, hermetic pans filled with water swollen PolyNIPA hybrids were taken and subsequently sealed to avoid any vaporization and water loss during heating. Pure PolyNIPA was used as a control (n = 3).

2.2.3 Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). ATR-FTIR spectra of PolyNIPA, silk, gelatin, and silk and gelatin–PolyNIPA hybrids (n = 3) were obtained using an Alpha-P spectroscope (Bruker, USA). For each measurement, 50 scans were coded in the spectral range of 4000–500 cm–1 at a data acquisition rate of 4 cm–1 per point in absorbance mode followed by spectral analysis for relative comparison. Deconvolution and curve fitting was conducted on the average spectra using automated peakfitting/deconvolution version 4.1 software (SeaSolve software inc., State, USA).

### Table 1 PolyNIPA hybrids at varying volume percentages

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2.2.4 Static water contact angle. The sessile drop method was used to measure the static water contact angles on all the four polymer films. Contact angles were recorded using a goniometer (Ramé-Hart Instrument Co., Netcong, NJ, USA) at 20 °C and 37 °C. For each measurement, the sample was positioned onto the stage top and a drop of deionized water was placed on the sample followed by image capture. The contact angle was measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.2.5 Rheology. The rheological properties of silk fibroin (20 wt%), gelatin (20 wt%) and the PolyNIPA hybrids (with a PolyNIPA concentration ranging from 10–30 mol% and silk or gelatin concentrations from 70–90 mol%) (Table 1) were examined using an Anton Paar Modular Compact Rheometer (MCR 302, USA). Measurements were taken using a cone and plate (25 mm diameter) with a configuration tilt at 1° and the distance between the two plates adjusted to 0.052 mm. The environmental cuff was used in all experiments. Temperature dependent viscosity was monitored by the temperature sweep in the range of 15 °C to 60 °C. To evaluate the flow behaviour, the viscosity of all the above-mentioned solutions was measured at different shear rates (0.1 to 100 s⁻¹) by selecting 10 points in each decade in rate-controlled mode. Thereafter, the dynamic frequency-sweep test at a constant strain (0.01) was recorded. Preliminary experiments (data not shown) demonstrated that the rheological properties were independent of the applied strain (0.01) in this range. The dynamic elastic modulus (G') and viscous modulus (G'') were also measured in oscillatory mode. Each test was carried out in triplicate.

2.2.6 Atomic force microscopy (AFM). AFM was performed in contact mode at 20 °C and 37 °C, using silicon nitride with a Digital Instruments Nanoscope. The surface of the polymers (silk, gelatin, silk–PolyNIPA and gelatin–PolyNIPA films; n = 3) was imaged. The RMS (root mean square) of surface roughness was calculated from three different areas of 10 μm × 10 μm dimensions.

2.2.7 Patterns and films

2.2.7.1 Direct-write assembly for patterns. 2D patterns of the polymers (silk, gelatin, silk–PolyNIPA hybrid and gelatin–PolyNIPA hybrids) were fabricated using a three-axis micro-positioning stage (Fiber Align, Aerotech Inc., Pittsburgh, USA) controlled by a customized software (3D Inks, Stillwater, OK, USA). The concentrated polymer solution was housed in a syringe (EFD Inc., East Providence, RI, USA) that was mounted on the x-y-z stage. The polymer ink was extruded through precision micropipet stainless steel tip with a 60 μm inner diameter (Suzhou Lanbo Needle Co. Ltd, China) onto a stationary glass substrate under an applied pressure of 15–30 psi at a constant deposition speed of 1 mm s⁻¹ over a thin layer of same polymer. Silk was deposited in a methanol reservoir, whereas gelatin was cross-linked chemically using 12 mM EDC and 4.8 mM NHS for 3 h at RT. To stabilize the gelatin–PolyNIPA patterns, the samples were subjected to UV light for 10 h, whereas the silk–PolyNIPA patterns were stabilized using 80% methanol.

2.2.7.2 Films. To generate silk–PolyNIPA and gelatin–PolyNIPA in the form of planar films, 50 μl of 30% PolyNIPA and 70% silk or gelatin were pipetted over 10 × 10 mm² glass slides and allowed to settle at RT. To stabilize the gelatin–PolyNIPA films, the samples were subjected to UV light for 10 h, whereas silk–PolyNIPA films were stabilized using 80% methanol.

2.2.8 Cell culture

2.2.8.1 Explant culture. Goat cornea was isolated from the ocular globe by removing the sclera, soft connective tissue and limbal rings with approval from ethical committee of IIT Delhi. After separating the three layers carefully, the stromal layer was cut into small pieces of approximately 2–3 mm and washed extensively with PBS supplemented with antibiotics (100 U ml⁻¹ penicillin streptomycin (Lonza), 50 μg ml⁻¹ gentamycin sulfate (Himedia) and 100 μg ml⁻¹ amphotericin B (Himedia)). After thoroughly rinsing with PBS, the fragmented tissue pieces were carefully lifted using sterile forceps and incubated at the bottom of a T25 tissue culture flask for 7 days in DMEM (Cellclone, Cat. No. CC3004.05L). The freshly isolated keratocytes were expanded in DMEM with 10% fetal bovine serum (FBS; Biological Industries, Cat. No. 04-121-1A). For serial passaging, cells were washed with PBS and dissociated using 0.25% trypsin (v/v) (Lonza) diluted in PBS. Standard culture conditions of 5% CO₂ with 95% humidity and 37 °C were maintained.

2.2.8.2 Cell seeding on patterns. Prior to cell seeding, gelatin, silk, silk–PolyNIPA and gelatin–PolyNIPA patterns (n = 3) were incubated in 50 μl of DMEM for 2 h. Following this, the pre-wetted polymer patterns were placed in 12 well plates using sterile forceps and seeded with 5 × 10⁴ goat corneal stromal cells for 1 h to permit cell attachment, before adding 1 ml of complete medium, DMEM (Cellclone, Cat. No. CC3004.05L) with 10% FBS (Biological Industries, Cat. No. 04-121-1A). The well plates were incubated for 21 days in 5% CO₂ and 95% humidity at 37 °C.

2.2.9 Scanning electron microscope (SEM). The cellular morphology of gelatin, silk, silk–PolyNIPA and gelatin–PolyNIPA was observed using SEM (Model EVO 50, Zeiss, UK) under high vacuum. For the procedure, the cell-seeded patterns were removed from the culture, washed with PBS and fixed in 10% formalin (Merck, India) for 4 h. Post-fixation, the samples were dehydrated using an alcohol gradient, vacuum dried and coated with gold using a gold sputter coater (EMITECH K550X, UK) set at 25 mA. For the procedure, the cell-seeded patterns were removed from the culture, washed with PBS and fixed in 10% formalin (Merck, India) for 4 h. Post-fixation, the samples were dehydrated using an alcohol gradient, vacuum dried and coated with gold using a gold sputter coater (EMITECH K550X, UK) set at 25 mA for 1 min to form a 15–20 nm thick coating. Images were captured at an accelerating voltage of 20 kV to monitor the cell alignment and morphology.

2.2.10 MTT assay. The metabolic activity of cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Gelatin, silk, silk–PolyNIPA and gelatin–PolyNIPA patterns (n = 3) were harvested after 2 and 7 days of culture, rinsed with PBS and were incubated in a 9:1 ratio of standard culture media and 5 mg ml⁻¹ MTT (MilliporeCT0-A, USA) for 3 h at 37 °C. The MTT treated patterns were solubilized in dimethyl sulphoxide (DMSO, Merck, India) and the optical density was measured using an ELISA reader (BIORAD iMark Microplate reader, USA) at 560 nm.
2.2.11 Quantitative real-time polymerase chain reaction (RT-PCR). Total mRNA was isolated from cell-seeded patterns using an Rneasy mini kit (Qiagen). The RNA concentration and purity was measured with a Nanodrop spectrophotometer (Thermo Scientific). cDNA of extracted RNA was synthesized with a first strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed with a SYBR Green Master Mix and Rotor gene Q thermocycler (Qiagen). The assay-on-demand primers GAPDH (Cat. No. QT00079247), Collagen I (COL I; Cat. No. QT00037793) and Aggrecan (ACAN; Cat. No. QT00001365) were used. The analysis was carried out with Rotor gene Q software and the relative expression levels were calculated using GAPDH as a control. For each time point, the samples (silk, gelatin, silk–PolyNIPA, gelatin–PolyNIPA) were analyzed in triplicate.

2.2.12 Confocal microscopy. All four patterns of corneal stromal cells were stained for actin and vinculin on day 7. Briefly, specimens were fixed in 10% formaldehyde (Merck, India) for 4 h, washed extensively with PBS, blocked with 10% (v/v) bovine serum albumin (diluted in PBS) for 30 min at RT and stained with vinculin-FITC conjugate (Sigma, Cat. No. V9264) for 6 h at RT. For actin cytoskeleton staining, the specimens were incubated with rhodamine phalloidin (Sigma, Cat. No. P1951) for 30 min at RT, followed by fluorescent secondary antibody staining using Alexafluor 546 (Cat. No. A11003, Millipore, MA, USA). DAPI (4’,6’-diamidino-2-phenylindole) was used as the nuclear stain. After three consecutive washes with PBS, the stained specimens were visualized using a confocal microscope (Leica TCS SP5) and images were captured using Leica software application suite (LAS V3.8).

2.2.13 Cell attachment/detachment assay. The effect of temperature on cell sheet detachment was investigated in both the films and patterns of silk–PolyNIPA and gelatin–PolyNIPA after 21 days in culture. For the experiment, cells were seeded at the density of $5 \times 10^4$ cells per cm$^2$ and incubated in complete medium for 21 days under standard culture conditions. To initiate detachment, the polymers were incubated at 4°C. Each sample was used in triplicate for the assay. Images were captured using a light microscope at 4 randomly chosen regions for each sample.

2.2.14 Statistical analysis. Statistical comparisons were conducted by a Student’s $t$-test. Results with $p \leq 0.05$ were considered to be statistically significant. The data pertaining to the MTT assay, contact angle and surface roughness were expressed as the mean value ± standard deviation.

3. Results

3.1 Phase transition temperature of PolyNIPA

Dried samples of pure PolyNIPA and PolyNIPA hybrids (with silk/gelatin) did not exhibit any peak for phase transition in the range of 10°C–45°C (Fig. 1a and b). However, in wet conditions, LCST was observed in the case of pure PolyNIPA at 34°C, which indicates that (Fig. 1c and d) PolyNIPA underwent a phase transition only in the aqueous form and not under dry conditions.

![Fig. 1](https://example.com/fig1.png) Differential scanning calorimetry analysis of (a) dried PolyNIPA and silk–PolyNIPA hybrids, (b) dried PolyNIPA and gelatin–PolyNIPA hybrids, (c) water swollen PolyNIPA and silk–PolyNIPA hybrids, and (d) water swollen PolyNIPA and gelatin–PolyNIPA hybrids. Abbreviation: gel–PolyNIPA: gelatin–PolyNIPA.
In the hybrids, the phase transition peak appeared only in hybrids having 30 vol% PolyNIPA content. The DSC curve for silk–PolyNIPA (30%) (Fig. 1c) showed a phase transition at 34 °C. The released exothermic energy was 0.558 J g$^{-1}$ in the silk–PolyNIPA hybrids and 2.649 J g$^{-1}$ in the case of pure PolyNIPA. We hypothesize that this substantial difference in the exothermic energies of the two could be a result of the presence of silk biomolecules in PolyNIPA, which may have enhanced its hydrophobicity at 34 °C, hence decreasing the exothermic energy from 2.649 J g$^{-1}$ to 0.558 J g$^{-1}$. The DSC of water swollen gelatin–PolyNIPA (30%) (Fig. 1d) exhibited an LCST at 27.61 °C, due to the formation of hydrophobic hydration layer at lower temperatures. Gelatin formed a partial triple helix at low temperature and the water molecules were entrapped within the network made the surroundings hydrophobic, which further lowered the LCST of PolyNIPA from 34.18 °C to 27.61 °C (Fig. 1d). Thus, we can conclude that 30% PolyNIPA present in the globular form at RT formed a stable association with gelatin.

3.2 ATR-FTIR

ATR-FTIR absorbance spectra of the hybrid samples and Fourier transform self-deconvolution in the amide I region (1600–1700 cm$^{-1}$) were studied (Fig. 2a and b) in order to identify the type of bond formation in the hybrids. The broadening of the amide I peak along with its shift to a lower wavenumber was observed in hybrids with 30% PolyNIPA (Fig. 2) indicating a higher intermolecular interaction in such hybrids. In the deconvoluted spectra of the silk–PolyNIPA hybrids (Fig. 2f, h and j), peaks for β-sheet crystals at 1620 cm$^{-1}$ and 1612 cm$^{-1}$ became more predominant with higher volume fractions of PolyNIPA (20% and 30%). In silk–PolyNIPA with 20% volume fraction of PolyNIPA, the peaks appearing at 1678 cm$^{-1}$ and 1688 cm$^{-1}$ indicated the presence of β-turns. This appeared to be a transition stage, which was further converted into β-sheets upon increasing the PolyNIPA volume fraction to 30%, as indicated by the appearance of peaks at 1666, 1626, 1631 cm$^{-1}$. In gelatin–PolyNIPA (Fig. 2g, i and k), with increasing volume fractions of PolyNIPA, a steep increase in 1956 cm$^{-1}$ could be observed. In the deconvoluted spectra, the absence of the 1606 cm$^{-1}$ peak in pure PolyNIPA (Fig. 2d) and gelatin–PolyNIPA (Fig. 2g and k) (containing 10%, 30% PolyNIPA content) indicated a predominant hydrophobic globular state of PolyNIPA.

3.3 Rheology

In order to determine the optimal composition of the polymer ink and temperature, at which the solution becomes printable, rheometry tests (oscillatory sweep, temperature sweep and frequency sweep) were conducted on all four polymers. The change in viscosity in pure gelatin and the gelatin–PolyNIPA hybrids was studied in relation to the change in temperature (Fig. 3a). A temperature range of 15 °C–50 °C was selected as it entails to both, the phase transition temperature of PolyNIPA (32 °C) and sol–gel transition temperature of gelatin (35 °C to 45 °C). Pure gelatin solution of 20% (w/v) exhibited a temperature dependent viscosity. Temperature sweep analysis showed conformational changes in gelatin involving a transition from random coil to triple helix conformation, which first appeared at around 30 °C, and subsequently converted the gel form into a solution. A sudden drop in viscosity was observed at around 30 °C indicating the conformational transition and water adsorption of the gelatin polymer chains, which lasted till 45 °C, and thereafter, the viscosity seemed to have stabilized.

A similar trend was observed in all gelatin–PolyNIPA blends up to 32 °C, which is the phase transition temperature (LCST) of PolyNIPA. However, as the temperature rose beyond 32 °C, a sharp increase in the viscosity was observed in gelatin–PolyNIPA containing a higher PolyNIPA content (20%, 30%), which was not applicable in the case of gelatin–PolyNIPA containing 10% PolyNIPA. This increase in viscosity noticed for higher concentrations of PolyNIPA was perhaps governed by the enhanced interactions between the hydrophobic segments of PolyNIPA. In addition, the reduced orientation of water molecules around the hydrophobic groups of PolyNIPA with an increase in temperature might increase its viscosity. The minimal increase in viscosity levels in the case of gelatin–PolyNIPA (10%) beyond 32 °C indicated that this particular concentration was not sufficient for exhibiting the typical character of PolyNIPA in the mixed hybrids.

The higher storage modulus ($G'$) noticed in the gelatin–PolyNIPA hybrids, when compared to the loss modulus ($G''$), indicated a stronger interaction between PolyNIPA and gelatin (Fig. 3c). PolyNIPA being completely entrapped within the gelatin polymer chains demonstrated a reduced hydrophobicity on the surface at 20 °C. On the other hand, storage modulus of gelatin–PolyNIPA decreased with an increase in the PolyNIPA volume fraction (20%, 30%). In addition, the water content of the hybrid hydrogels increased with increasing volume fraction of PolyNIPA that may further result in the loss of storage and elastic modulus. The high water content separates the polymer chains from each other, which leads to the formation of a loosely bonded network that can decrease the modulus of the hybrid hydrogel (with 30% volume fraction of PolyNIPA) in comparison to pure gelatin. Gelatin and gelatin–PolyNIPA hybrids exhibited a decrease in viscosity with increasing shear rate (Fig. 3b). This shear thinning behaviour observed was apparently an outcome of some breakage in the physical links at higher shear rates resulting from an increase in enthalpy. This change in energy level also altered the gelatin conformation from triple helix to a random coiled structure.

The temperature sweep of the silk–PolyNIPA hybrids exhibited an increase in viscosity at around 32 °C (Fig. 3b), whereas in pure silk, no such increase in viscosity was observed, which could be due to hydrophilic–hydrophobic transition at the LCST of PolyNIPA.

The frequency sweep curve (Fig. 3d) depicted that $G'$ exceeds $G''$ in pure silk as well as in silk–PolyNIPA hybrids. Furthermore, $G'$ appeared independent of the angular frequency in all compositions of the silk–PolyNIPA hybrids and pure silk. This indicated the viscoelastic behaviour of silk and the silk–PolyNIPA hybrid hydrogels.

The moduli of the hybrids decreased with a higher content of PolyNIPA volume fractions (10% to 30%), whereas pure silk
had the highest storage modulus. This could be due to the β-sheet content of silk fibroin, which helps to impart viscoelasticity to the silk–PolyNIPA hybrids.\textsuperscript{11} In addition to the β-sheet crystal content, the water content of the silk–PolyNIPA hybrids can also control the storage and loss modulus. As already mentioned, the water content increased with an increasing volume fraction of PolyNIPA, which might decrease the storage and loss modulus of the silk–PolyNIPA hybrids due to the loosely bonded network formed as a result of the presence of water molecules in-between.

Fig. 2 ATR-FTIR spectra of (a) silk, PolyNIPA and silk–PolyNIPA hybrids, (b) gelatin, PolyNIPA and gel–PolyNIPA hybrids, (c)–(k) deconvoluted ATR-FTIR spectra (1600–1700 cm\textsuperscript{-1}) of PolyNIPA, silk, gelatin and various hybrids. Abbreviation: gel–PolyNIPA: gelatin–PolyNIPA.
The flow curve of pure silk (Fig. 3f) and silk–PolyNIPA (30%) depicted a slight decrease in viscosity with increasing shear rate, and this shear thinning behaviour might be due to molecular extension of the polymer chains. The drop in viscosity with shear rate could be due to the loosening of molecular entanglement, which aligns the polymeric chains along applied shear stress, thus decreasing the viscosity of the gel with the application of stress. The silk–PolyNIPA (10%, 20%) hybrids exhibited almost Newtonian flow, which could be due to a resistance caused by the molecular entanglement of the silk and PolyNIPA chains.

### 3.4 Contact angle measurement

The contact angle of silk, gelatin, silk–PolyNIPA and gelatin–PolyNIPA was measured at two different temperatures (20 °C and 37 °C; Table 2) using a goniometer (Fig. 4). At 20 °C, the contact angle of gelatin was found to be 61° ± 0.1°, whereas in gelatin–PolyNIPA (30%), the contact angle decreased to 29° ± 1°. A similar decline in the contact angle value of silk–PolyNIPA (30° ± 2°) was observed over pure silk (70° ± 0.3°). Thus, the hydrophilicity of the samples increased with the addition of PolyNIPA at 20 °C (<LCST of PolyNIPA). A logical assumption could be that because more polar amide groups of PolyNIPA face the water phase at 20 °C, this causes an increase in the hydrophilicity of the hybrids over the pure protein (silk/gelatin) substrates. At 37 °C (>LCST of PolyNIPA), the water contact angle of gelatin and silk was found to be 52° ± 0.3° and 71° ± 0.25°, respectively, whereas gelatin–PolyNIPA and silk–PolyNIPA exhibited 55° ± 0.3° and 59° ± 0.37°, respectively. While the contact angle in gelatin–PolyNIPA increased (from that of pure gelatin) due to the hydrophobicity exhibited by PolyNIPA at elevated temperatures (LCST > 32 °C), this was not observed in the case of silk as the contact angle is already high due to β-crystallization of the silk protein as a result of processing with 80% methanol.
3.5 AFM analysis

AFM was used to evaluate the surface roughness of the four polymer surfaces at two different temperatures, 20°C and 37°C (Table 3). The results demonstrated a substantial increase ($p < 0.05$) in the surface roughness of gelatin–PolyNIPA from 2.117 to 19.558 nm with increase in temperature (from 20 to 37°C). Similarly, the surface roughness of silk–PolyNIPA escalated from 15.816 (20°C, see ES1, Fig. S1) to 37.361 nm at 37°C (Fig. 5). A minimal increase in surface roughness value ($p > 0.05$) was observed in pure Gelatin: 6.134 nm (20°C) to 9.506 nm (37°C). Similarly, the surface roughness of pure silk increased slightly from 71.012 to 73.018 nm with increase in temperature (from 20 to 37°C). Pure silk appeared to be the roughest amongst all the samples.

### Table 3 Surface roughness of the polymers

<table>
<thead>
<tr>
<th>Surface type</th>
<th>Surface roughness (in nm)</th>
<th>Water contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20°C (&lt;LCST)</td>
<td>37°C (&gt;LCST)</td>
</tr>
<tr>
<td>Gelatin–PolyNIPA (30%)</td>
<td>6.134</td>
<td>9.506</td>
</tr>
<tr>
<td>Gelatin</td>
<td>2.117</td>
<td>19.558</td>
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<tr>
<td>Silk</td>
<td>71.012</td>
<td>73.018</td>
</tr>
<tr>
<td>Silk–PolyNIPA (30%)</td>
<td>15.816</td>
<td>37.361</td>
</tr>
</tbody>
</table>

3.6 Cellular alignment and morphology

The cellular alignment and morphology of the seeded constructs were observed via SEM (Fig. 6b–e). The corneal stromal cells were organized in a parallel fashion along all the patterns (silk, gelatin, silk–PolyNIPA and gelatin–PolyNIPA). The cell morphology appeared more irregular on pure proteins when compared to the hybrids (Fig. 7b). The computed values for the cell aspect ratio (Fig. 7a) were considerably lower for silk (7.1 ± 6.2) and gelatin (7.2 ± 4.4). On the other hand, corneal cells cultured on the silk–PolyNIPA and gelatin–PolyNIPA hybrids demonstrated spindle shaped morphology with an aspect ratio of 10.5 ± 7.3 and 8.2 ± 5.4, respectively.

3.7 Confocal microscopy

The expression of focal adhesion points and cytoskeletal organization have been monitored to generate insights on the cellular responses to the underlying matrices using confocal microscopy (Fig. 6f–i). The typical spindle shaped cell morphology on the PolyNIPA hybrids was confirmed by actin staining. Differences in the hydrophobicity of the materials seem to influence cell attachment as revealed by vinculin staining. On qualitative examination, the corneal stromal cells seemed to adhere well onto the gelatin–PolyNIPA surfaces with intense actin and vinculin expression (Fig. 6i). A discrete vinculin accumulation indicated the formation of focal adhesions localized around the nucleus (white arrow) as well as peripheral stress fibers (yellow arrows). In contrast, the cells exhibited a moderate vinculin expression in silk–PolyNIPA (Fig. 6f), whereas only a faint signal was observed in cells seeded on the pure silk and gelatin patterns (Fig. 6g and h). A moderate adhesion of corneal cells was noticed on the silk surface which might be due to the absence of cell adhesion motifs (such as RGD) on Bombyx mori silk.16

3.8 MTT assay

The metabolic activity of corneal stromal cells cultured on all the polymers (silk, silk–PolyNIPA, gelatin and gelatin–PolyNIPA) was investigated (Fig. 7c). Corneal stromal cells cultured on gelatin–PolyNIPA showed the highest cellular activity ($p < 0.05$), which was ~1.5 times, 3.4 times and 4.6 times higher than silk–PolyNIPA on day 3, 5 and 7, respectively. However, the metabolic activity of cells seeded on pure gelatin was approximately twice that of pure silk and 2.5 times over silk–PolyNIPA at day 7. Amongst the four polymers, silk–PolyNIPA ($p > 0.05$) revealed lowest metabolic activity at day 7.

3.9 Quantitative RT-PCR analysis

Differential gene expression of COL-I and ACAN synthesized by cultured corneal cells at different time points on the polymer surfaces was evaluated using RT-PCR (Fig. 7d and e). The relative fold change of COL-I on gelatin–PolyNIPA and silk–PolyNIPA with respect to the polystyrene surface was found to be minimal, i.e. 1.2 and 1.8, respectively, after 7 days of culture. Similarly, for silk and gelatin surfaces, the upregulation of COL-I expression corresponded to 0.5 and 2.3 fold after 7 days (Fig. 7d). However, after 21 days of culture, the expression of COL-I was significantly upregulated in all polymers except pure gelatin corresponding...
to a 5.5 fold increase in gelatin–PolyNIPA, 2.26 fold in silk–PolyNIPA and 1.36 fold in pure silk and minimal expression was observed in gelatin (0.434 fold). Minimal expression of ACAN was observed on all the other polymers after 7 days of culture, whereas after 21 days, a 13 fold increase on gelatin–PolyNIPA and 7.8 fold change on pure silk was observed (Fig. 7e).

3.10 Cell attachment/detachment

The rate of cell sheet recovery was monitored for films only (Fig. 8c–f), as intact sheets could not be recovered from the patterned surfaces (Fig. 8a and b). At 21 days, 85% confluence was achieved over both the silk–PolyNIPA and gelatin–PolyNIPA hybrids upon macroscopic examination of the surface. To initiate cell detachment, culture plates were incubated at 4 °C and the time measurements for each of the hybrids were recorded. Temperatures lower than the LCST of PolyNIPA could induce cell detachment by passive hydration of the PolyNIPA chains. Therefore, in the current study, the cell detachment process was carried out at 4 °C as the cell metabolic activities get arrested at this temperature with minimal cytoskeleton movements, thus controlling the cell shape.30

With an increase in the level of hydrophilicity of the substrate, the surface roughness (Fig. 5) declined synergistically with the contact angle (Fig. 4), which initiated cell detachment. The detachment procedure was observed to be initiated at the periphery of the samples and extended towards the centre.

In silk–PolyNIPA (Fig. 8c and d), almost 40% of the corneal cell sheet was seen to detach around the periphery after 5 min of incubation at 4 °C. This rapid detachment of cell sheet in silk–PolyNIPA led to the simultaneous rolling of the cell sheet while detaching from the surface, eventually leading to complete folding of the sheet from the detached surface within 10 min. In the case of gelatin–PolyNIPA (Fig. 8e and f), after 10 min of incubation, about 20% of the cell sheet was removed from the surface. This detachment was again initiated from the periphery of the surface. After 20 min, nearly 40% of the cells detached, which further extended towards the centre of the surface. The entire cell sheet was removed from the gelatin–PolyNIPA surface within 30–40 min. The overall mechanism of sheet removal upon macroscopic examination showed that the silk–PolyNIPA films resulted in a rolled up sheet, whereas a more intact, planar cell sheet was recovered from gelatin–PolyNIPA films.

4. Discussion

Interpenetrating networks of PolyNIPA hybrids with silk10,11 and gelatin12,13 have been previously reported, but their potential as
suitable patterns fabricated using DWA for corneal cell sheet recovery has not yet been determined. In the current study, parallel patterns fabricated via DWA using silk- and gelatin–PolyNIPA hybrids, with thermo-responsive characteristics (Fig. 1) along with tailorable viscoelastic properties (Fig. 3) have been attempted for the first time. Based on the physical and biological characterization of the hybrid patterns (silk–PolyNIPA and gelatin–PolyNIPA), it was found that both hybrids exhibited thermo-responsive behavior, an essential feature to allow cell sheet detachment. As satisfactory recovery rate of the corneal cell sheets was achieved when the same hybrids were tested as films (Fig. 8), the hybrids were expected to perform well as parallel patterns. In contrast to other complicated and expensive techniques for fabricating patterned films,7,8,22,23 DWA allows relatively simple adjustment of pattern parameters over a wide range.24-27 In the present study, the intent was to control the alignment, morphology and ultimately the recovery of corneal stromal cells through patterning of polymer surfaces, where the role of temperature-responsive PolyNIPA (30 vol% as validated by DSC, Fig. 1) was to recover the cell sheets, a potential technique for cell sheet engineering. Previously reported techniques used to control the cellular alignment over thermo-responsive patterns involve mechanical and electrical stimulations,31 whereas our current study introduced a simple, inexpensive and time-saving technique to create customized materials with predefined aligned microarchitecture eliminating the need for any kind of external stimuli or mask fabrication.

While both the patterned hybrids (silk–PolyNIPA and gelatin–PolyNIPA) guided the cellular alignment and morphology, gelatin–PolyNIPA demonstrated an increased metabolic activity and gene expression in seeded goat corneal cells. In terms of cell sheet recovery, an intact cell sheet could not be harvested from either of the patterned hybrids, but when the same hybrids (with similar polymer composition) were fabricated in the form of planar films, rapid detachment was seen. This may be attributed to the pattern dimensions at the microscale level, which may require further optimization.

4.1 Material properties

For DWA, the aim was towards creating a printable gel by varying the composition of gelatin and silk (90–70 vol%) with PolyNIPA (10–30 vol%) to produce a homogenous viscoelastic gel. Here, the DSC results determined that 30% PolyNIPA with 70% protein (silk/gelatin) is a critical composition ratio for showing LCST volume transition (Fig. 1) and to form semi-interpenetrating networks (Fig. 2), while still maintaining the thermo-responsive character of pure PolyNIPA even in hybrid form. The surface chemistry, hydrophobicity (measured by static water contact angle) and surface roughness decreased significantly in the two PolyNIPA hybrids as the temperature was lowered from 37 °C to 20 °C. This was a function of PolyNIPA as the polymer changes its conformation to a globular state at temperatures below its LCST, which enhances the cellular detachment from the surface, which is a characteristic property of the polymer exploited in cell sheet engineering.7-9,32 On the other hand, the increased roughness and hydrophobicity of the PolyNIPA hybrids at 37 °C (> LCST) facilitated cell attachment.31 Amongst the two hybrids, a comparatively lower contact angle (Table 2) and surface roughness (Table 3) indicated a relatively lower hydrophobicity of gelatin–PolyNIPA over silk–PolyNIPA. The primary reasons can be two fold: (i) the gelatin polymer is hydrophilic in nature28 and therefore demonstrates a lower contact angle (52° ± 0.3°) and surface roughness (9.506 nm), under cell culture conditions, and (ii) in practice, silk is stabilized by methanol treatment, which induces β-sheet crystallization in the structure.27 The resultant silk structure becomes hydrophobic in nature (water contact angle of 71° ± 0.25° and surface roughness of 73.01 nm), which contributes towards the increased hydrophobicity of the silk–PolyNIPA hybrid. The presence of β-sheets in silk was also confirmed using FTIR spectroscopy (Fig. 2).

4.2 Cellular interactions

The cellular alignment of goat corneal stromal cells was achieved on all four patterns (gelatin, silk, gelatin–PolyNIPA, ...)
and silk–PolyNIPA, Fig. 6). The present results are in accordance with the previous reports, where directed contact guidance of patterned substrates, such as silicon wafers with polyurethane patterns, silk fibroin, nanopatterned poly(acrylic acid) and poly(allylamine hydrochloride) with polydimethylsiloxane, have influenced the alignment of cultured corneal cells, highlighting the importance of topographical cues in directing cell behaviour in vitro. Along with topology, the surface chemistry of the underlying matrix was seen to considerably affect the morphology of seeded corneal cells. A spindle shaped morphology, typical of resident keratocytes in native cornea, was achieved only on the PolyNIPA hybrids (Fig. 7b) and not on the pure silk and gelatin patterns. Cell spreading is usually an outcome of stress fibers, which force the cells to elongate as a result of the balanced cytoskeleton forces. Therefore, it might be that the PolyNIPA hybrids provide lesser chemical constraints as a result of hybridizing with proteins (i.e. silk or gelatin), which ultimately leads to their improved cytocompatibility. Furthermore, discrete patches of vinculin expression in gelatin–PolyNIPA patterns (Fig. 6i) indicated the presence of large focal adhesions, which are known to play a crucial role in cell anchorage. We hypothesize that this strong cell-material interaction in gelatin–PolyNIPA attributed towards the increased cell metabolic activity of stromal cells (Fig. 7c) and their subsequent differentiation into ECM-specific genes expression (Fig. 7d and e) when compared to silk–PolyNIPA, pure silk or gelatin. It is already known that vinculin, a cytoskeletal protein located in the foci of cell-substrate attachment, responds to favourable substrates by increasing the cell-traction forces between ECM-associated integrins and cytoskeleton. This unique mechanosensing induced by vinculin

Fig. 7 Cell compatibility of corneal cells cultured on polymers. (a) Box & whisker plot showing the cell aspect ratios, (b) light micrographs of cell seeded polymers (scale bar 100 μm), (c) MTT assay, and (d, e) RT-PCR analysis. (d) COL-1 and (e) ACAN. Abbreviation: gel–PolyNIPA: gelatin–PolyNIPA.
is a key regulator in assisting cell shape and migration via cytoskeletal contractile mechanisms.\textsuperscript{36,39} As a matter of fact, the level of vinculin in native keratocytes increases only in the case of corneal injuries and subsequently subsides with the regeneration of the healthy tissue. This transient increase of vinculin by stromal keratocytes is an auto-immune mechanism used to physically heal the wound by escalating cell migration into the wounded region.\textsuperscript{40} In context of our study, it indicates that on day 7, a part of the gelatin–PolyNIPA seeded stromal keratocytes were in active motility (as indicated by the prevalence of focal adhesions around the peripheral stress fibers), resembling the transient reparative state of native cornea, in an attempt to align the themselves and ultimately synthesize an organised stromal matrix. This phenomenon was not observed in other patterns, which expressed moderate to negligible expression of vinculin mainly concentrated around the nuclear region.

Though gelatin–PolyNIPA presented a lower hydrophobicity and surface roughness than silk–PolyNIPA (Tables 2 and 3), the reason for its increased cytocompatibility and gene expression can be attributed to the gelatin component. Gelatin has naturally incorporated RGD peptide sequences, which are known to facilitate cell adhesion,\textsuperscript{41} ultimately forming a more aligned and homogenously distributed collagen fibrils,\textsuperscript{16} which were absent in the case of \textit{Bombyx mori} obtained pure silk patterns.\textsuperscript{16} Intact cell sheets of goat corneal stromal cells were successfully fabricated on the hybrids, both in patterns as well as the planar films. Due to the thermoresponsive character of the hybrids, confluent cell sheets readily detached themselves at 4 °C, maintaining the cell–cell junctions. This was a result of the hydration of the PolyNIPA surface at lower temperatures (<LCST), marked by a significant decrease in the water contact angle, which subsequently affected the cell–material interactions.\textsuperscript{33} Cells usually adhere onto the surfaces that present a temperate wettability with a water contact angle in the range of 40°–60°,\textsuperscript{33} which was precisely the case in gelatin–PolyNIPA (52° ± 0.3°) and silk–PolyNIPA (59° ± 0.37°) under cell culture conditions. A drop in the contact angle (Table 2) and surface roughness (Table 3) from 37 °C to 20 °C loosened the cell adhesion points, leading to the gradual detachment of cell sheet. As a result, intact corneal stromal cell sheets were recovered from the hybrid films of gelatin–PolyNIPA (Fig. 8e and f) and silk–PolyNIPA (Fig. 8c and d) in 30 min and 10 min, respectively. The relatively rapid recovery on silk–PolyNIPA may be attributed to poor cell adhesion on silk\textsuperscript{16} when compared to gelatin.\textsuperscript{41}

Overall, the time taken for cell sheet recovery in both the hybrid films employed in our study is significantly lower when compared to the commercially available Upcell\textsuperscript{38} surfaces, where approximately 3 h\textsuperscript{10} and 64.7 min\textsuperscript{22} were reported to completely harvest the cell sheet of human mesenchymal stem cells and NIH-3T3 fibroblasts, respectively. Furthermore, intact cell sheets (in the cm range) could not be harvested from either of the hybrid patterns in our study (Fig. 8a and b). We used 60 µm patterns, which exceed the optimal size range reported for intact cell sheet recovery.\textsuperscript{22} Previous studies have reported that intact fibroblast sheets could only be recovered on narrow polyacrylamide patterns in the range from 5 µm (13.7 min) and 10 µm (21.3 min), indicating the relevance of pattern sizes on the rate control of cell sheet recovery.\textsuperscript{22} On the contrary, incomplete recovery was achieved on 50 µm polyacrylamide patterns, which is close to the pattern sizes we developed. Other studies supporting this hypothesis include the work carried out by Aubin and co-workers,\textsuperscript{23} where the proliferation of NIH-3T3 fibroblasts achieved on 50 µm polyacrylamide patterns, which is close to the pattern sizes we developed. Other studies supporting this hypothesis include the work carried out by Aubin and co-workers,\textsuperscript{23} where the proliferation of NIH-3T3 fibroblasts decreased as the width of patterned microgrooves was changed from 50 µm to 200 µm. Moreover, the percentage of aligned cells dropped from 64% ± 8% to 31% ± 8% on 50 µm and 200 µm patterns, respectively, with no significant differences noticed between 200 µm microgrooves and the unpatterned surface. These studies suggest that by narrowing the pattern dimensions down to 5–20 µm, which can be successfully achieved using DWA,\textsuperscript{22} we can possibly attain rapid and intact sheet recovery of corneal stromal cell sheets from gelatin–PolyNIPA surfaces. Taken together, gelatin–PolyNIPA patterns have undisputably demonstrated optimal surface features in corneal cell sheet development when compared to the other three matrices. However, some optimization in the pattern dimensions would be required for achieving rapid recovery of intact cell sheets for developing a corneal stromal bioequivalent.

Shortage of donated healthy corneal tissue has encouraged efforts to fabricate bioengineered human corneal equivalents. Therefore, the evaluation of the role of the patterned topographical cues and thermoresponsive surface chemistry of underlying matrix on keratocyte response might prove critical for the repairing a corneal wound and tectonic defects caused by infection,
superficial chemical burns, and corneal dystrophy. Furthermore, this strategy can be used as an in vitro model system for assessing ophthalmic drug delivery and pharmaceutical efficacy studies. Moreover, this study also indicates that the patterned protein–PolyNIPA hybrid films could be utilized for engineering other oriented tissue structures using cells of cardiac, muscular and neuronal origin, beyond only corneal stromal tissue engineering.

5. Conclusions

A simple and time-saving method of patterning, i.e. direct-write assembly, was employed to develop parallel patterns of thermoresponsive hybrids. Both silk–PolyNIPA and gelatin–PolyNIPA guided corneal cell adherence and alignment along the patterns. Because the cells demonstrated enhanced metabolic activity and increased ECM gene expression on the patterned gelatin–PolyNIPA surfaces, this finding may be utilized for cell sheet engineering to produce carrier-free transplants for cornea. In addition, the thermoresponsive hybrid films permit the rapid recovery of corneal cell sheets by altering their surface characteristics with changing temperature, which could not be achieved in the patterns. Therefore, we can conclude that direct-write technique for patterning gelatin–PolyNIPA hybrids is an effective tissue engineering strategy for the recovery of transplantable corneal cell sheets. However, future studies need to focus on the pattern parameters for executing a rapid recovery of aligned cell sheets.

Acknowledgements

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References