

## Accepted Manuscript

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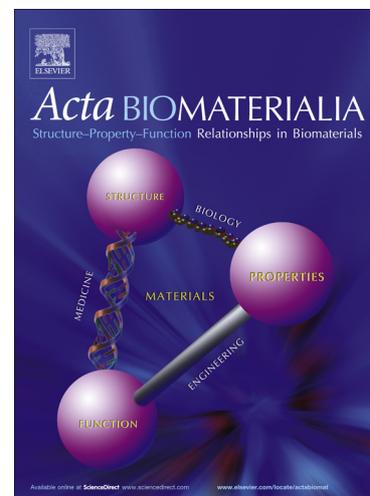
PII: S1742-7061(14)00486-3  
DOI: <http://dx.doi.org/10.1016/j.actbio.2014.10.035>  
Reference: ACTBIO 3452

To appear in: *Acta Biomaterialia*

Received Date: 2 May 2014  
Revised Date: 8 October 2014  
Accepted Date: 24 October 2014

Please cite this article as: Mirazul Islam, M., Cėpla, V., He, C., Edin, J., Rakickas, T., Kobuch, K., Ruželė, Ž., Bruce Jackson, W., Rafat, M., Lohmann, C.P., Valiokas, R., Griffith, M., Functional Fabrication of Recombinant Human Collagen-Phosphorylcholine Hydrogels for Regenerative Medicine Applications, *Acta Biomaterialia* (2014), doi: <http://dx.doi.org/10.1016/j.actbio.2014.10.035>

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**Functional Fabrication of Recombinant Human Collagen-Phosphorylcholine  
Hydrogels for Regenerative Medicine Applications**

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**Abstract**

The implant-host interface is a critical element in guiding tissue or organ regeneration. We previously developed hydrogels comprising interpenetrating networks of recombinant human collagen type III and 2-methacryloyloxyethyl phosphorylcholine (RHCIII-MPC) as substitutes of the corneal extracellular matrix that promote endogenous regeneration of corneal tissue. To render them functional for clinical application, we have now optimized their composition and thereby enhanced their mechanical properties. We have demonstrated that such optimized RHCIII-MPC hydrogels are suitable for precision femtosecond laser cutting to produce complementing implants and host surgical beds for subsequent tissue welding. This avoids the tissue damage and inflammation associated with manual surgical techniques, thereby leading to more efficient healing. Although we previously demonstrated in clinical testing that RHCIII-based implants stimulated cornea regeneration in patients, the rate of epithelial cell coverage of the implants needs improvement, e.g. modification of the implant surface. We now show that our 500  $\mu\text{m}$  thick RHCIII-MPC constructs comprising over 85% water, are suitable for microcontact printing with fibronectin. The resulting fibronectin micropatterns promote cell adhesion, as compared to the bare RHCIII-MPC hydrogel. Interestingly, a pattern of 30  $\mu\text{m}$  wide fibronectin stripes enhanced cell attachment and showed highest mitotic rates, an effect that potentially can be utilized for faster integration of the implant. We have therefore shown that laboratory-produced mimics of naturally occurring collagen and phospholipids can be fabricated into robust hydrogels that can be laser profiled and patterned to enhance their potential function as artificial substitutes of donor human corneas.

**Keywords:** hydrogel, cornea, collagen, fibronectin, laser ablation, surface modification

## 1. Introduction

Biointeractive scaffolds designed to mimic the extracellular matrix (ECM) of the body are becoming the scaffolds of choice in regenerative medicine. The interface between implant and host is now recognized as a critical element in guiding the repair and regeneration of target tissues or organs. Biologically inert materials developed as implants for the cornea and other tissue systems can be rendered biointeractive by a range of microfabrication techniques. Micromoulding, microfluidics, lithographic patterning and similar processes have already employed for introduction of grooves, channels or other topographical/cell adhesion-promoting features [1-5], or for grafting of interactive peptides [6], growth factors and other bioactives to promote cell attachment and growth [2, 7-9]. To date, biomaterials comprising naturally-derived and mimics of ECM components and hybrids of natural and synthetic polymers have also been developed as implants that serve as regeneration scaffold and templates by providing instructive cues to endogenous cells to affect regeneration. A range of biomimetic materials fabricated as hydrogels, membranes, meshes and other structures have now been developed as tissue models and as scaffolds or templates for regenerative medicine. An extensive review of the latest biomimetic and bioinspired materials and their application in engineering different tissues and organs has been collected by Jabbari et al. in the Handbook of Biomimetics and Bioinspiration series [10].

In the cornea, the bulk of the corneal extracellular matrix comprises type I collagen. However, our team had shown that recombinantly produced human type I and type III collagen behaved similarly as implants in mini-pigs over 12 months [11]. More importantly, we showed in a clinical study that carbodiimide crosslinked recombinant human collagen type III (RHCIII) hydrogels as corneal implants promoted epithelial and stromal cell, and nerve regeneration [12]. Despite the successful regeneration, compression of the soft biomaterials by the sutures used in the surgery, as well as a sub-optimal fit into the wound bed delayed epithelial coverage of the implant by endogenous in-growing epithelial cells, leading to localized implant thinning and fibrosis in some patients [12]. Hence, robust implants that could be grafted with more precision and without

suturing, and that could encourage more rapid cell coverage to circumvent infection would be a vast improvement for future clinical application. Indeed, others have shown that post-fabrication modification is often needed to ensure a seamless host-graft interface and/or modulate cell behaviour to obtain the desired clinical behaviour [13, 14].

We have previously fabricated RHCIII implants that were reinforced by introduction of a second network of 2-methacryloyloxyethyl phosphorylcholine (MPC) to form interpenetrating networks of RHCIII-MPC [15] and showed in a rabbit alkali burn cornea model of severe pathology that these hydrogels stimulated regeneration while repelling unwanted invasion of blood vessels into the implants [16]. In this study, we increased the mechanical strength of these hydrogels to render them suitable for post-fabrication modification to circumvent the problems of the previous generations of RHCIII and RHCIII-MPC implants. We evaluated the use of ultra-high speed femtosecond lasers developed for ophthalmic surgery to profile RHCIII-MPC hydrogels for surgical grafting. These lasers have been shown to produce cuts within 10  $\mu\text{m}$  deviation from the target [17]. Creating matched cuts in the implant and surgical bed would allow precise lock-and-key fitting of the implant into host cornea enabling suture-free and hence compression-free retention. For surface modification to enhance cell attachment and rapid implant coverage, a range of established methods exist. These range from the creation of aligned collagen fibrils by molecular crowding [18] to various lithographic methods. However, most of these were developed for chips and other solid supports [19] and not soft hydrogels that comprise over 85 % water. Here, we tested the feasibility of microcontact printing ( $\mu\text{CP}$ ) [20] as a means for introduction of surface patterning onto fully hydrated, 500  $\mu\text{m}$ -thick hydrogels. Taking advantage of the improved mechanical stability of the RHCIII-MPC hydrogel, we printed and analysed micropatterns that used human fibronectin (FN) as “ink”. FN is a cell-interactive protein in the ECM that promotes adhesion and migration of corneal epithelial cells during wound healing [21, 22] and has been successfully tested therapeutically for the repair of persistent epithelial defects (PEDs) of human cornea [23]. We quantitatively evaluated the effect of the printed FN micropatterns on the growth of corneal epithelial cells.

## 2. Materials and Methods

### 2.1 Materials

All inorganic salts and basic chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO and Steinheim, Germany), Carl Roth GmbH (Karlsruhe, Germany) or Merck KGaA (Darmstadt, Germany) unless otherwise stated. Research grade RHCIII, produced in yeast (*Pishia pistoris*), was purchased from 3H Biomedical (Uppsala, Sweden) and Fibrogen (San Francisco, CA). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid Monohydrate (MES), Poly(ethylene glycol) diacrylate (PEGDA,  $M_n = 575$ ), Ammonium persulphate (APS) and N,N,N,N-tetramethylethylenediamine (TEMED) were obtained from Sigma-Aldrich (MO, USA). 2-methacryloyloxyethylphosphorylcholine (MPC) was obtained from Biocompatibles (UK) and Paramount Fine Chemicals Co. Ltd. (Dalian, China).

### 2.2. Recombinant human collagen-phosphorylcholine hydrogels

RHCIII-MPC hydrogels were produced as we previously published [15] but with a range of different starting concentrations of RHCIII to select an optimal formulation for laser profiling and  $\mu$ CP. Briefly, a 450-500 mg of RHCIII solution of one of a range of starting concentrations (13.7 (w/w)%, 18 (w/w)% or 20 (w/w)%) was buffered with 150  $\mu$ l of 0.625 M MES buffer within a syringe mixing system in an ice-water bath. 200  $\mu$ l MPC solution in 0.625 M MES was added into the mixing system. The MPC:RHCIII (w/w) ratios used with either 1:4, 1:2 or 1:1. PEGDA was then added by a microsyringe (PEGDA:MPC (w/w) = 1:3). Calculated volumes of 4% (w/v) APS solution in MES and 2% (v/v) TEMED solution in MES were added sequentially (APS/MPC (w/w) = 0.015:1, APS:TEMED (w/w) 1:0.77). After thoroughly mixing, calculated amounts of NHS (10% (w/v) in MES) and EDC (5% (w/v) in MES) solutions were added and the reactants were thoroughly mixed at 0 °C (EDC:RHCIII-NH<sub>2</sub> (mol:mol) = 0.3 – 1.5:1, EDC:NHS (mol:mol) = 1:1).

The final mixed solution was immediately cast into cornea-shaped moulds (12 mm diameter, 500  $\mu\text{m}$  thick) or between two glass plates with 500  $\mu\text{m}$  spacers. The hydrogels were cured overnight at 100% humidity under nitrogen at room temperature. The cornea-shaped implants were cured for an additional 5 h at 37  $^{\circ}\text{C}$ . After demoulding, they were washed thoroughly with 10 mM phosphate buffered saline (PBS) and then stored in PBS containing 1% chloroform to maintain sterility.

### 2.3. Characterization of hydrogels

All samples were tested in triplicate. The water content of RHCIII-MPC hydrogels was determined by weighing samples that were blotted dry to remove surface liquid to obtain the wet weight ( $W_0$ ). These pre-weighed hydrogels were then dried at room temperature under vacuum to constant weight, which is the dry weight ( $W$ ). The equilibrated water content of hydrogels ( $W_t\%$ ) was obtained according to the following equation:  $W_t\% = (W_0 - W)/W_0 \times 100\%$ .

Optical properties of the resulting hydrogels were characterised by obtaining the refractive indices (RIs) of 500  $\mu\text{m}$  flat, fully hydrated hydrogels equilibrated in PBS using an Abbe refractometer (Model C10, VEE GEE Scientific Inc., Kirkland, Washington) at 21  $^{\circ}\text{C}$  with bromonaphthalene as the calibration agent. Light transmission and back-scattering measurements were carried out at 21 $^{\circ}\text{C}$  on a custom-built instrument equipped with a quartz halogen lamp for white light measurements as previously reported [24]. The percentage light transmission through the hydrogels was compared to the open beam intensity. The amount of backscattered light (%) from each sample was measured by the circular array of eight built-in photodiodes, angled at 30 degrees off axis.

The mechanical properties (tensile strength, moduli and elongation at break) were determined using an Instron mechanical universal tester (Model 3342, Instron, Canton, MA) equipped with a 0.01 kN load cell and Instron Series IX/S software. Flat hydrogels (12 mm  $\times$  5 mm rectangular strips, 0.44 mm thick) were measured. The gauge length of each specimen tested was 5 mm. The crosshead speed was 10 mm  $\text{min}^{-1}$  and the sampling rate was 10 points  $\text{s}^{-1}$ . Implants

were not pre-stressed. Measurements were taken at room temperature.

The morphology of the RHCIII-MPC hydrogels compared to human corneas and RHCIII hydrogels alone was examined using scanning electron microscopy (SEM). All samples were lyophilized to minimize the shrinkage of the human cornea and collagen scaffolds due to the vacuum applied during freeze-drying and SEM imaging. Briefly, PBS-equilibrated samples of each construct or human eye bank corneas were frozen over night at  $-80\text{ }^{\circ}\text{C}$  and then placed in the drying chamber of a lyophilizer at a condenser temperature of  $-40\text{ }^{\circ}\text{C}$  and vacuum pressure of 0.8 Torr and dried for 7 hrs. The scaffolds did not collapse through the process while there was some degree of shrinkage for all test samples, e.g. 40% for human cornea and 50% for the biosynthetic collagen hydrogels due to the applied vacuum. The samples were then cut using a sharp surgical knife at a vertical angle to minimize the changes in the scaffolds' structure due to the cut and attached onto metal holders using conductive double-sided tape, and sputter coated with a gold layer for 60 seconds at 0.1 bar vacuum pressure (Cressington Sputter Coater 108) prior to SEM examination. SEM micrographs were taken at 25 kV at various magnifications on a scanning electron microscope (Model S-2250N, Hitachi, Japan). Comparisons were made against human eye bank corneas prepared in the same way.

#### *2.4. Precision laser cutting of the hydrogels and tissue welding*

Laser trephination (or cutting) of three cornea-shaped RHCIII-MPC hydrogels (12 mm in diameter and 500  $\mu\text{m}$  thick) was carried out using a femtosecond laser (IntraLase™ FS Laser, Abott Medical Optics, Abbott Park, Illinois, USA). The laser was set to cut out a top-hat shaped implant from the hydrogel (one of the more common configurations for laser-assisted tissue trephination). The cutting parameters used were 6.0 mm diameter of the anterior side cut, 8.0 mm diameter of the posterior side cut, and a depth of 250  $\mu\text{m}$  for the lamellar cut. The energy for the ring lamellar cut, anterior side cut and posterior side cut were 2.80  $\mu\text{J}$ , 1.50  $\mu\text{J}$  and 1.50  $\mu\text{J}$ , respectively.

For implant/tissue welding, matched complementary cuts were also made on excised porcine

corneas (obtained from an abattoir) and RHCIII-MPC implants using the femtosecond laser (Wave Light GmbH Erlangen, Germany). RHC-MPCIII implants inserted into the matched donor cornea bed were welded together using standard riboflavin/dextran crosslinking techniques (1% riboflavin, 365 nm, 3 mW/cm<sup>2</sup>, 30 min.) [25] using a MLase UV crosslinking device (MLase AG, Germering, Germany).

### 2.5. Microcontact printing on hydrogels

RHCIII-MPC hydrogels containing 18% RHCIII, with RHCIII:MPC = 2:1 and EDC:RHCIII-NH<sub>2</sub> (mol:mol) = 0.4:1 were used for all  $\mu$ CP. Flat-cast hydrogels were cut into approximately 2 cm  $\times$  2 cm  $\times$  500  $\mu$ m pieces and washed with 0.1 M PBS, pH 5.7 and dried under a nitrogen gas (N<sub>2</sub>) stream prior to use. Free native carboxyl groups from the hydrogel surface were activated by immersing the samples in 2 ml of the PBS (pH 5.7) solution containing 10 mM EDC and 2.5 mM NHS for 15 min. All samples were rinsed for 30 s in fresh PBS buffer and dried with N<sub>2</sub> gas just before printing.

Square 1.5 cm  $\times$  1.5 cm poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI, USA) stamps were prepared using a previously published protocol [26]. To examine the attachment and proliferation of single human corneal epithelial cells, a stamp that comprised 30  $\mu$ m-wide protruding stripes separated by 60  $\mu$ m spaces was designed. To examine the attachment and spread of multiple cells, 200  $\mu$ m-wide stripes, separated by 200  $\mu$ m spaces were tested. Prior to printing, the PDMS stamps were thoroughly rinsed in ethanol, dried under a stream of nitrogen gas and treated with oxygen plasma (20 W power) for 30 s in plasma dry cleaner (Femto, Diener Electronic GmbH, Ebhausen, Germany). The stamps were then pre-loaded with “ink” comprising a 30  $\mu$ L solution of 0.5 mg/ml human fibronectin (FN) (Yo Proteins AB, Huddinge, Sweden) with 2  $\mu$ g/ml bovine serum albumin conjugated with fluorescent Texas Red (BSA-TR<sup>®</sup>; Invitrogen, Eugene, OR, USA) in 0.1 M PBS buffer, pH 8.0 for visualisation of the patterns. For cell seeding experiments, BSA-TR was omitted to avoid any unwanted nonspecific interactions between BSA-TR and cells.

After 10 min. of incubation with “ink” at room temperature, the stamps were rinsed in water for 20 s and dried for 1 min. under a N<sub>2</sub> stream.

The activated RHCIII-MPC samples were placed in a Petri dish and then the upper surfaces were dried carefully with the N<sub>2</sub> gas in preparation for  $\mu$ CP. The bottom corners of the hydrogel samples were left in contact with water to prevent complete dehydration and therefore undesired deformation of the hydrogels. Surfaces then were printed by applying the PDMS stamp onto the hydrogel surface. All printing was performed manually without any load, using the nitrogen stream to get the stamp into contact with the hydrogel surface. The stamp was left on for five minutes and then removed using tweezers. The patterned hydrogel surface was incubated with a solution containing 10 mM (PEG)<sub>3</sub>NH<sub>2</sub> (Molecular Biosciences, Boulder, CO, USA), in PBS, pH 8.0 for 40 min. to de-activate all remaining unreacted carboxyl groups. The surfaces were then carefully washed several times with fresh PBS, pH 7.3 and stored in this buffer solution prior to further use.

## 2.6. Characterization of printed hydrogels

To characterise the reproducibility of the  $\mu$ CP patterns, the optically transparent patterned hydrogels were imaged using an Olympus BX51 upright microscope (Olympus, Tokyo, Japan) equipped with a 10x, NA 0.3 water immersion objective and a Peltier-cooled Fview II CCD camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Fluorescence images of fibronectin-BSA-TR<sup>®</sup> patterns were acquired and analysed using analySIS software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

The presence of FN in the micropatterns was confirmed by using a primary rabbit polyclonal antibody against FN (Abcam, Cambridge, UK), followed by a secondary antibody, donkey anti-rabbit IgG, conjugated with quantum dots Qdot655 (H+L) (Life Technologies, USA).

To investigate the effect of  $\mu$ CP on the micro- and nano-topography of fully hydrated RHCIII-MPC constructs, atomic force microscopy (AFM) measurements were carried out with the samples fully immersed in 0.1 M PBS buffer, pH 7.3 using a NanoWizard<sup>®</sup> 3 AFM microscope

(JPK Instruments AG, Berlin, Germany) mounted onto an Olympus IX81 (Olympus, Tokyo, Japan) inverted optical microscope to allow for combined optical and AFM imaging of the same surface. Sample topography images were obtained in contact mode using SNL-10 (Bruker, Billerica, MA, USA) probes. Regions of interest for AFM analysis were selected with fluorescence microscopy to include the printed and unprinted zones of the surface within one large area ( $70 \times 70 \mu\text{m}^2$ ) scan. Several different size scans were made. Images obtained were processed and the surface roughness was calculated using the region analysis tool within the AFM JPK data processing software.

### 2.7. Cytotoxicity evaluation

All samples with  $30 \mu\text{m}$  and  $200 \mu\text{m}$  patterned stripes, and control samples comprising deactivated and unmodified hydrogels, were placed into 24-well plates. Immortalized human corneal epithelial cells (HCECs) from a cell line [27] were seeded onto the surface of the materials at a density of  $2 \times 10^4$  cells per well. The HCECs were maintained in Keratinocyte Serum-Free Medium (KSFM; Life Technologies, Invitrogen, Paisley, UK) containing  $50 \mu\text{g/ml}$  bovine pituitary extract and  $5 \text{ ng/ml}$  epidermal growth factor for 48 hours in a humidified  $37^\circ\text{C}$  incubator. Cell viability was measured by using a Live/Dead staining kit (Life Technologies, Invitrogen, Paisley, UK), where cells were double-stained by calceinacetoxymethyl (Calcein AM) and ethidium homodimer-1 (EthD-1). The live cells displayed green fluorescence while the dead cells fluoresced red.

### 2.8. Cell attachment and proliferation

Green fluorescence protein (GFP) transfected HCECs were employed to facilitate cell counts. A stable GFP-HCEC cell line was established by transfection of HCEC with a vector containing a puromycin resistant gene together with GFP, using the Lipofectamine<sup>®</sup> 2000 Transfection Reagent (Life Technologies, California, USA). Selection of puromycin resistant cells with  $2 \mu\text{g/ml}$  of puromycin added to the medium was performed to obtain stable GFP-expressing

lines.

To examine proliferation rates on the different surfaces, 6 mm hydrogel discs with 30  $\mu\text{m}$  and 200  $\mu\text{m}$  FN striped patterns, and PEG-NH<sub>2</sub> deactivated and unmodified samples, were placed within 96 well plates to fit snugly. Five thousand GFP-HCECs were seeded into each well. GFP-HCECs were also seeded onto the tissue culture plate as a control for overall cell health. The cells were maintained in KSFM within a humidified 37 °C incubator with 5 % CO<sub>2</sub>. Photomicrographs of the cells were taken at 2, 6, 24, 48 and 96 hours, using a fluorescence microscope (AxioVert A1, Carl Zeiss, Göttingen, Germany). Counts were made by sampling three different 1290 x 965  $\mu\text{m}^2$  areas on each hydrogel surface.

## 2.9. Immunohistochemistry

GFP-HCECs were cultured on hydrogels for 4 days until when the cells reached confluence/near confluence on the patterns. Each sample was washed in 10 mM PBS, pH 7.4 and then fixed in 4 % paraformaldehyde in PBS for 20 min. For permeabilization and blocking of unspecific binding, samples were incubated in PBS containing 0.25 % Triton X-100 for 10 min. and 5 % foetal calf serum (FCS) in PBST (0.05 % Tween-20 in PBS) (FCS/PBST) for 1 hour, respectively. Samples were then incubated overnight at 4 °C with the following primary antibodies diluted in FCS/PBST: anti-proliferating cell protein Ki67 antibody (1:600) (Sigma-Aldrich, MO, USA), anti-focal adhesion kinase (FAK) antibody (1:500) (Abcam, Cambridge, UK) and anti-integrin beta 1 (integrin  $\beta$ 1) antibody (1:200) (Abcam). After rinsing, the samples were exposed to the secondary antibody, Alexa Fluor-594 (1:400) (Invitrogen, Oregon, USA) at room temperature for 1 hour in the dark for visualisation. After another rinse, the samples were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (PromoKine, Heidelberg, Germany) for 1 min. as nuclear counterstain. All samples were given a final rinse prior to mounting and examination under a confocal microscope (LSM700, Carl Zeiss, Göttingen, Germany). A total area of 50000  $\mu\text{m}^2$  per sample was used for all the counts.

### 2.10. Statistical Analyses

Statistical analyses were performed using general linear models that relate responses to linear combinations of predictor variables. These p value tests were followed by a Tukey test in cases where significance was found. The analyses were performed using Minitab<sup>®</sup> software (Minitab Ltd., Coventry, UK). Ratios of cell populations are shown in Fig. 5 as percentages and therefore not portrayed with standard deviations. The ratio data have skewed distributions that are close to zero and one. Hence, these data were log transformed before analysis to better comply with the assumptions of general linear models. Intervals for feature sizes are  $\pm 1$  standard deviation.

## 3. Results

### 3.1. RHCIII-MPC hydrogels

A range of adjustments in hydrogel formulations were tested for producing a mechanically reinforced material suitable for post-fabrication of the implants. These included a starting RHCIII content of 13.7%, 18% and 20%; different RHC:MPC ratios and EDC:RHCIII-NH<sub>2</sub> ratios. The resulting optical and mechanical properties of the hydrogels are given in Appendix A. The resulting enhancement in properties of RHCIII-MPC hydrogels after optimization of the above parameters as compared to our previously published formulation [15] is shown in Table 1. In both 13.7% and 18% RHCIII-MPC hydrogels, we found that a 2:1, RHCIII:MPC ratio with 0.4 EDC:RHCIII-NH<sub>2</sub> gave the optimal formulations.

Increasing of the collagen content from 13.7% to 18% decreased the water content from 90.1 $\pm$ 2.4% to approximately 86% (85.5 $\pm$ 0.2% for RHCIII-MPC and 86.0 $\pm$ 0.2% for RHCIII only). Hence, it turned out that the MPC content did not make much of a difference. Additionally, the EDC:RHCIII-NH<sub>2</sub> ratio showed no obvious influence on the RI, while the RI increased with

increasing MPC content within the gels.

The measured transmittance of white light decreased with the increased RHCIII concentration. Compared to the native cornea (transmittance: ~87%, backscatter: ~3%), however, all the RHCIII-MPC gels showed a higher transmittance and lower backscatter. The RHCIII only gels, on the other hand, exhibited a transmittance comparable to the native cornea (approx. 87%). All the cornea substitutes displayed a slightly lower RI of 1.35, which is that of water, compared to native cornea (1.37~1.38 [28]).

The effect of the material composition on mechanical properties is summarized in Fig. A2 (Appendix A). In general, increasing the solids content of the hydrogels from 13.7% to 18% increased the tensile strength of RHCIII-MPC hydrogels from  $1.29\pm 0.31$  MPa to  $2.12\pm 0.18$  MPa (Table 1). The tensile strengths of the 18% RHCIII only hydrogels were comparable to the 18% RHCIII-MPC hydrogels at  $2.37\pm 0.4$  MPa. However, RHCIII only hydrogels were stiffer (an elastic modulus of  $15.33\pm 2.67$  MPa) and less elastic (elongation at break of  $28.21\pm 2.01\%$ ) than corresponding RHCIII-MPC hydrogels (lower elastic modulus of  $9.46\pm 3.58$  MPa; higher elongation at break of  $33.34\pm 3.87\%$ ).

The 18% RHCIII-MPC hydrogels were more thermodynamically stable ( $T_d = 57.6$  °C) than both the 18% RHCIII only ( $T_d = 53.1$  °C) or 13.7% RHCIII-MPC hydrogels ( $T_d = 54.1$  °C). However, none were as stable as the native corneas ( $T_d = 65.1$  °C) (Table 1).

SEM imaging of cross-section of samples was performed for structural comparison of the produced RHCIII-MPC and RHCIII only hydrogels with native human corneas. A distinct lamellar structure could be seen for both human and biosynthetic corneas (Figs 1A-C). The lamellar structure was observed throughout the entire cross-section of all samples while it was more homogeneous for the human cornea and better representative of the whole structure than that of the biosynthetic ones. In addition, the RHCIII-MPC hydrogels comprised lamellae-like layers interconnected by some tiny fiber-like structures (Fig. 1B), that better mimics the structure of the human cornea (Fig. 1A) than the RHCIII crosslinked by EDC/NHS which had large lamellae

without any inter-lamellar connections (Fig. 1C). However, the inner morphology of the biosynthetic corneas differed from their native counterpart in the number of lamella layers and the spacing between layers. For example, the lamella-like layers in the biosynthetic corneas were thicker and their number was lower compared to lamellas of the native cornea. In addition, the spacing between the ordered lattice structures of the native cornea was smaller (~ 15 microns) than that of the biosynthetic corneas (~ 150 microns). The lamella spacing for the human cornea had a wide size distribution ranging from 2 to 40 microns. Overall, despite some similarities between the biosynthetic corneas and their natural counterparts, the human cornea has a much more complex microstructure with higher degree of interconnectivity, as expected.

### 3.2. Precision laser profiling and tissue welding

The optimized hydrogels were sufficiently robust to allow complete cutting of “top-hat” implants using the femtosecond laser. The resulting mean dimensions of the cuts were: anterior diameter of the 5.9 mm, posterior diameter of 8.1 mm, and depth of 260  $\mu\text{m}$  for the lamellar cut (Fig. 2A). For example, RHCIII-MPC hydrogels containing 18% RHCIII, with RHCIII:MPC = 2:1 and EDC:RHCIII-NH<sub>2</sub> (mol:mol) = 0.4 : 1 (designated RHCIII-18/MPC(2/1)-E0.4), which had shown good mechanical properties, cut well with the laser. As shown in Fig. 2B, top-hat shaped gels with precisely cut edges could be obtained. However, hydrogels with the lower 13.7% RHCIII content (but otherwise with same proportions of MPC and EDC crosslinker), fell apart during cutting (data not shown).

Laser cut implants were successfully fitted into matched laser cut surgical beds (Fig. 2C). After riboflavin/UV-crosslinking, the implants remained permanently adhered to the corneal bed (Fig. 2D) and sub-microscopical physical fibre-like connections were present between the implant and adjacent cornea (Fig. 2E) when viewed by SEM. In contrast, implants detached completely in controls without UV radiation.

### 3.3. Overall quality of the microcontact printed patterns

The most stable RHCIII-18/MPC(2/1)-E0.4 hydrogels which lent themselves well to laser profiling were also optimal for  $\mu$ CP (process diagramed in Fig. 3A). Fig. 3B, C show typical resulting striped FN patterns as visualized by staining with an anti-fibronectin antibody. Analysis of the fluorescence micrographs of the hydrogel samples confirmed good replication of the original patterns of the 30 and 200  $\mu$ m stripes defined in the photolithographic mask production step (not shown). On the PDMS stamp, the width of the narrow stripes was  $26.8 \pm 0.5 \mu\text{m}$  (based on bright-field microscopy), while that of the printed FN stripes was  $27.1 \pm 0.8 \mu\text{m}$ . The thick stripes on the PDMS stamps and the resulting FN patterns had widths of  $190.6 \pm 1.7$  and  $191.7 \pm 2.6 \mu\text{m}$ , respectively. The good reproducibility indicated that uncontrolled FN ink diffusion on partially dried collagen hydrogel samples was minimal. On the samples printed with 30  $\mu$ m stripe pattern, the printed area occupied approx. 37 % of the sample area, and approx. 32 % of the total hydrogel surface was patterned and defect-free. On the 200  $\mu$ m striped samples, the printed sample area occupied 40 % of the sample and approximately 26 % of the total hydrogel surface was printed and free from printing defects. Also, FN ink distribution on the entire patterned surface was satisfactorily homogeneous.

### 3.4. Atomic force microscopy

The effect of  $\mu$ CP on the micro- and nanotopography of fully hydrated RHCIII-MPC hydrogels was determined by AFM analysis. The original, unmodified collagen hydrogel surfaces appeared uneven, with irregularly arranged surface fibrils visible (Fig. 4A). The root mean square (RMS) of surface roughness as measured within a representative  $2 \times 2 \mu\text{m}^2$  scan area was 3.5 nm. The regions of interest (ROIs) for AFM scans on the chemically activated and microcontact printed samples were identified by simultaneous fluorescence microscopy imaging. The AFM tip was positioned in the ROIs that were patterned with FN and BSA-TR<sup>®</sup> mixture. In topography images of the hydrogel surface after  $\mu$ CP and/or deactivation, the collagen fibrils appeared to be partially

aligned (Fig. 4B), in contrast to the unmodified surface (Fig. 4A). However, the fine details of the collagen fibrils could not be resolved. There was no distinct overall height difference between the majority of the printed and adjacent unprinted area, either due to the high initial surface roughness and/or due to the ability to print a very thin, possibly monolayer thickness of FN. The edges of the stripes, however, were slightly corrugated by the printing and served to delineate the stripe boundaries. The RMS surface roughness of the FN-printed and unprinted regions on this particular sample shown in Fig. 4B, was very similar, at 1.3 nm and 1.4 nm, respectively. The AFM analysis therefore showed that the  $\mu$ CP resulted only in a very small change in the surface topology.

### *3.5. Effect of patterning on cytotoxicity, cell adhesion and proliferation*

Printing with PDMS stamps can result in a slight contamination of the sample surface with loose PDMS particles [26, 29], but these can be removed by subsequent sample deactivation and washing steps. Live/dead staining performed indeed showed that there were no cytotoxic effects from the patterning. The numbers of dead cells (stained red) were negligible:0 in most of the samples, 1-2 cells in a few others (Fig. 5A-D).

Analysis of the proliferation of HCECs on the different surfaces over time showed obvious differences between the patterned and non-patterned samples (Fig. 5E). The only two types where the growth rate could not be told apart ( $P>0.05$ ) were the unmodified and deactivated RHCIII-MPC surfaces. The total number of cells counted per a defined ROI was highest on the hydrogels patterned with 200  $\mu$ m stripes. However, when taking into account that the total FN-coated surface area per ROI was two times less on the 30  $\mu$ m pattern than on the 200  $\mu$ m pattern, the results show that the thinner stripes yielded approx. 26% more cells after normalization of the surface areas.

### *3.6. Immunohistochemistry and cell behaviour*

Fig. 6 shows the behaviour of cells on the different surfaces. The integrin  $\beta$ 1 family, which is involved binding ECM macromolecules including collagen and fibronectin, was expressed by

HCECs growing on all groups. The proportion of integrin  $\beta$ 1-expressing cells in the deactivated surfaces, 30 and 200  $\mu$ m patterns, respectively, were significantly different from those growing on the unmodified RHCIII-MPC hydrogel surfaces (Fig. 6A), with the highest expression on the 30  $\mu$ m stripes, although not that significantly different from the 200  $\mu$ m stripes and deactivated surfaces ( $P > 0.05$ ).

Immunohistochemical localization of FAK-positive cells showed that significantly more cells were growing on the 30  $\mu$ m striped patterns that were positively stained ( $P \leq 0.05$ ) than those of the other groups (Fig. 6B).

Staining for the Ki67 protein associated with cell proliferation showed that the samples with 30  $\mu$ m striped patterns supported a significantly larger proportion ( $P \leq 0.05$ ) of positively stained cells than the other groups (Fig. 6C).

#### 4. Discussion

Hydrated interpenetrating networks of RHCIII-MPC hybrid hydrogels were prepared by simultaneous crosslinking of RHCIII with EDC/NHS, and radical polymerization of MPC monomer with APS/TEMED as the initiator system and the PEG-DA as the macromolecular crosslinker. Biphasic responses were found in hydrogels with increases in RHCIII starting concentration and MPC content. In particular, the increased MPC content, ensuing in RHCIII:MPC molar ratios of 1:1, resulted in weak, brittle hydrogels. This could be due to a high MPC concentration that might have hindered the intertangement and crosslinking of the RHCIII microfibrils. The increased RHCIII concentration from 18 to 20 % that resulted in overall weaker gels could be due to the increased in viscosity of the collagenous mixture making it difficult to properly crosslink the very viscous RHCIII. The higher solids content would have therefore made the hydrogel stiffer but not stronger. An increase in EDC concentration relative to RHCIII-NH<sub>2</sub> content beyond 0.4 did not enhance the strength of the hydrogels. The combination of 18 % RHCIII with a ratio of

RHCIII:MPC of 2:1 and molar equivalent of 0.4 EDC (RHCIII-18/MPC(2/1)-E0.4) resulted in strong, stable and optically clear hydrogels. Although they comprised over 85 % water, these hydrogels were nevertheless sufficiently robust for subsequent post-fabrication modifications, i.e. laser cuts and  $\mu$ CP.

SEM analysis shows that both RHCIII only and RHCIII-MPC hydrogels had a lamellar arrangement of collagen fibrils. Addition of the second network resulted in additional fibrous structures that connected the lamellae in RHCIII-MPC hydrogels. These tiny interlamellar structures were only seen in RHCIII-MPC hydrogels and were likely the result of combined long-range and short-range crosslinkers that resulted in an interpenetrating network. The interlamellar connecting structure was not observed in RHCIII only scaffolds. Still, the interconnected multilayer structure was simpler and sparser than that of the native corneas, which had a larger number of lamellar and many more interlamellar connections. The driving force behind the formation of the lamellar structures are unclear. The most plausible explanation has been put forward by Ruberti's group [18, 30]. At the high collagen concentrations used to fabricate implants and under the confined environment of the moulds used, molecular crowding as described by Ruberti et al. is present. Prior to moulding, the collagen is mixed very thoroughly using a syringe mixing system [31], which generates a lot of shear force. Finally during the moulding, the mixed collagen is compressed with a mould with a final compression cum shearing action.

Femtosecond lasers are now being used in surgery, particularly ophthalmic refractive surgery, as alternative to conventional mechanical cutting tools. They have ultra-short,  $10^{-15}$  second pulses that can cut out three-dimensional shapes within the cornea by producing multiple adjacent minute spots of explosions in it. The RHCIII-18/MPC(2/1)-E0.4 hydrogels, like human corneal tissue, lent themselves to being precisely cut by the femtosecond laser, as shown by the resulting well-defined cuts. On the contrary, the laser pulses broke up weaker hydrogels. This shows that RHCIII-MPC hydrogels were sufficiently robust to maintain the integrity during the laser cutting, which is quite important for the grafting applications without sutures. We also showed that in the

excised porcine corneas, the implants could be precisely fitted and welded together with the host cornea. This opens up the possibility of future sutureless implantation, or the use of fewer sutures.

Microcontact printing has been used as a method to transfer proteins and other biological macromolecules onto solid substrates without the loss of their biological activity [32, 33]. However, most of the literature on  $\mu$ CP applications typically describes patterning on hard substrates such as glass, silicon, metals or robust and elastic polymers (e.g. polystyrene, PMMA (polymethylmethacrylate), PDMS) [32-34]. In this study, we showed that  $\mu$ CP could be used to introduce reproducible functional protein patterns onto the surfaces of thick, highly hydrated, largely collagenous matrices. The reproducibility of the patterns was good with a standard deviation of 0.8  $\mu$ m for the 30  $\mu$ m pattern and 2.6  $\mu$ m for the 200  $\mu$ m pattern. It should be noted that the RHCIII-MPC hydrogels developed in this study for  $\mu$ CP had a tensile strength of  $2.15 \pm 0.14$  MPa and Young's modulus of  $10.53 \pm 3.25$  MPa, which is comparable to 2.24 MPa tensile strength and Young's modulus of 0.360-0.870 MPa for PDMS [35], which is a common stamp material used for  $\mu$ CP. However, unlike PDMS, which is hydrophobic, the RHCIII-MPC hydrogels comprised  $85.5 \pm 0.2$  % water.

Fibronectin is a large glycoprotein that is present in the basement membrane of the normal corneal epithelium. Its elaboration is increased during wound healing and the macromolecule appears at wound sites where it serves as a temporary matrix for cell migration [36]. The fibronectin molecule and its bioactive peptide derivatives Arg-Gly-Asp (RGD) and Pro-His-Ser-Arg-Asn (PHSRN) have been reported to enhance healing of corneal epithelial wounds in rabbit models [21, 22] and in clinical trials [23]. Fibronectin enhances wound healing through its interactions with  $\beta$ 1 integrins. Corneal epithelial cells express  $\beta$ 1 integrins and migrate over the newly laid down FN. After healing is complete, both integrin and FN expression is diminished [36].

Cell attachment to FN triggers integrin-mediated signalling. Previously,  $\beta$ 1 integrin-FAK signalling has been shown to initiate proliferation of metastatic lung cancer cells [37] and other cells, and in this case, HCECs. In our hydrogels, there was a trend towards the aligned cells on

30  $\mu\text{m}$  FN stripes having the highest expression of  $\beta 1$  integrin, FAK and Ki67, showing that the width of the patterned FN stripes were able to influence cell behaviour. Thus, although the total number of the cells counted per a ROI was highest on the 200  $\mu\text{m}$  stripe pattern, proliferation as demonstrated by the expression Ki67, a cellular marker for proliferation, was highest in the 30  $\mu\text{m}$  stripes. We have therefore shown that despite the ability of the unmodified RHCIII-MPC hydrogels to support corneal epithelial cells, the attractiveness of the hydrogel as a substrate was enhanced by the transfer of FN onto the hydrogel surface. Micropatterning has been previously used to manipulate cell growth and differentiation, and nanoscale topography has been shown to affect corneal epithelial cell migration [38]. In the present study, however, there was little change in pitch and surface roughness of printed and unprinted areas were similar. The manipulation of cell adhesion has been found to affect mitosis [39]. In our study, the 30  $\mu\text{m}$  stripes are approximately the diameter of a corneal epithelial cell, so each cell will only have two neighbours along the FN stripe. On the 200  $\mu\text{m}$  stripes, each cell is surrounded by neighbouring cells. It would appear that a combination of differential adhesion afforded by the FN, and presence/absence of neighbouring cells affected the proliferation of corneal epithelial cells on our surfaces. Whether or not the micropatterning with an array of thin stripes that promoted the highest proliferation rates will do the same *in vivo* is yet to be determined by micropatterning on cornea shaped hydrogels for animal studies.

## 5. Conclusion

We have shown that biomimetic hydrogels comprising interpenetrating networks of synthetically produced human collagen and phosphorylcholine can attain mechanical properties that are suitable for post-fabrication modification using laser cutting and  $\mu\text{CP}$ . Use of the femtosecond laser produced highly precise cuts on the RHCIII-MPC gels that would be suitable for implantation. Microcontact printing of FN was able to further enhance cell adhesion and growth on the 500  $\mu\text{m}$

thick, already biocompatible hydrogels that comprised over 85% water.

## 6. Acknowledgements

We thank Dr. Chyan-Jang Lee for establishing the GFP-HCEC cell line used for this study; Dr. Sadhana Kulkani and David Priest, University of Ottawa Eye Inst. for assistance with the laser cutting study; Ms. Kimberley Merrett for assistance in characterization of the hydrogels; and Dr. Joanne M. Hackett for assistance with cell culture/biocompatibility studies, during optimization of the RHCIII-MPC hydrogels. We thank Johannes Junger and Michael Baumann, MLase AG for help with the UV crosslinking, and the Medical Devices Bureau, Health Canada for use of the SEM system. We gratefully acknowledge funding from an NSERC-CIHR Canada Collaborative Health Research Project grant (MG) and subsequent funding for an EU Nanomedicine ERAnet project “I-CARE” to MG, RV and MLase AG through the Swedish Research Council, Science Council of Lithuania, and VDI Germany, respectively.

## References

- [1] Fink J, They M, Azioune A, Dupont R, Chatelain F, Bornens M, et al. Comparative study and improvement of current cell micro-patterning techniques. *Lab Chip* 2007; 7:672-80.
- [2] Chung BG, Lee KH, Khademhosseini A, Lee SH. Microfluidic fabrication of microengineered hydrogels and their application in tissue engineering. *Lab Chip* 2012;12:45-59.
- [3] Thevenot P, Hu W, Tang L. Surface chemistry influences implant biocompatibility. *Curr Top Med Chem* 2008;8:270-80.
- [4] Jeon HJ, Simon CG, Kim GH. A mini-review: Cell response to microscale, nanoscale, and hierarchical patterning of surface structure. *J Biomed Mater Res Part B* 2014;00B:000-000.
- [5] Singh RK, Seliktar D, Putnam AJ. Capillary morphogenesis in PEG-collagen hydrogels.

Biomaterials 2013;34:9331-40.

[6] Hynd MR, Frampton JP, Dowell-Mesfin N, Turner JN, Shain W. Directed cell growth on protein-functionalized hydrogel surfaces. *J Neurosci Methods* 2007;162:255-63.

[7] Klenkler BJ, Griffith M, Becerril C, West-Mays JA, Sheardown H. EGF-grafted PDMS surfaces in artificial cornea applications. *Biomaterials* 2005;26:7286-96.

[8] Lim JY, Donahue HJ. Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning. *Tiss Eng* 2007; 13:1879-1891

[9] Coq N, van Bommel T, Hikmet RA, Stapert HR, Dittmer WU. Self-supporting hydrogel stamps for the microcontact printing of proteins. *Langmuir* 2007;23:5154-60.

[10] Jabbari E, Kim DH, Lee LP, Ghaemmaghami A, Khademhosseini A, editors. *Biologically-Driven Engineering of Materials, Processes, Devices, and Systems. Handbook of Biomimetics and Bioinspiration*. Singapore: World Scientific Series in Nanoscience and Nanotechnology: Volumes 9; 2014.

[11] Merrett K, Fagerholm P, McLaughlin CR, Dravida S, Lagali N, Shinozaki N, et al. Tissue-engineered recombinant human collagen-based corneal substitutes for implantation: performance of type I versus type III collagen. *Invest Ophthalmol Vis Sci* 2008;49:3887-94.

[12] Fagerholm P, Lagali NS, Merrett K, Jackson WB, Munger R, Liu Y, et al. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study. *Sci Transl Med* 2010;2:46ra61.

[13] Dirk SM, Lin P, Buerger S, Cicotte KN, Reece GP, Hedberg-Dirk E. Biomimetic approaches to peripheral neuroprosthetic interfaces. In: Jabbari E, Kim DH, Lee LP, Ghaemmaghami A, Khademhosseini A, editors. *Handbook of Biomimetics and Bioinspiration*. Singapore: World Scientific Publishing; 2014. p. 121-51.

[14] Kim K, Hong S, Lee H. Mussel-inspired adhesive biomaterials. In: Jabbari E, Kim DH, Lee LP, Ghaemmaghami A, Khademhosseini A, editors. *Handbook of Biomimetics and Bioinspiration*. Singapore: World Scientific Publishing; 2014. p. 273-91.

- [15] Liu W, Deng C, McLaughlin CR, Fagerholm P, Lagali NS, Heyne B, et al. Collagen-phosphorylcholine interpenetrating network hydrogels as corneal substitutes. *Biomaterials* 2009;30:1551-9.
- [16] Hackett JM, Lagali N, Merrett K, Edelhauser H, Sun Y, Gan L, et al. Biosynthetic corneal implants for replacement of pathologic corneal tissue: performance in a controlled rabbit alkali burn model. *Invest Ophthalmol Vis Sci*. 2011;52:651-7.
- [17] Holzer MP, Rabsilber TM, Auffarth GU. Femtosecond Laser-Assisted Corneal Flap Cuts: Morphology, Accuracy, and Histopathology. *Invest Ophthalmol Vis Sci* 2006;47:2828-31.
- [18] Saeidi N, Karmelek KP, Paten JA, Zareian R, DiMasi E, Ruberti JW. Molecular crowding of collagen: a pathway to produce highly-organized collagenous structures. *Biomaterials* 2012;33:7366-74.
- [19] Lan S, Veiseh M, Zhang M. Surface modification of silicon and gold-patterned silicon surfaces for improved biocompatibility and cell patterning selectivity. *Biosens Bioelectron* 2005;20:1697-708.
- [20] Qin D, Xia Y, Whitesides GM. Soft lithography for micro- and nanoscale patterning. *Nature Protocols*. 2010;5:491-502.
- [21] Fujikawa LS, Foster CS, Harrist TJ, Lanigan JM, Colvin RB. Fibronectin in healing rabbit corneal wounds. *Lab Invest* 1981;45:120-9.
- [22] Nishida T, Nakagawa S, Nishibayashi C, Tanaka H, Manabe R. Fibronectin enhancement of corneal epithelial wound healing of rabbits in vivo. *Arch Ophthalmol* 1984;102:455-6.
- [23] Yamada N, Morishige N, Yanai R, Morita Y, Kimura K, Chikama T, et al. Open clinical study of eye drops containing the fibronectin-derived peptide PHSRN for treatment of persistent corneal epithelial defects. *Cornea* 2012;31:1408-13.
- [24] Priest D, Munger R. A new instrument for monitoring the optical properties of corneas. *Invest Ophthalmol Vis Sci* 1998;39:S352.
- [25] Meek KM, Hayes S. Corneal cross-linking--a review. *Ophthalmic Physiol Opt*. 2013;33:78-93.

- [26] Zhou Y, Valiokas R, Liedberg B. Structural characterization of microcontact printed arrays of hexa(ethylene glycol)-terminated alkanethiols on gold. *Langmuir* 2004;20:6206-15.
- [27] Araki-Sasaki K, Ohashi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* 1995;36:614-21.
- [28] Patel S, Marshall J, Fitzke FW, 3rd. Refractive index of the human corneal epithelium and stroma. *J Refract Surg* 1995;11:100-5.
- [29] Graham DJ, Price DD, Ratner BD. Solution Assembled and Microcontact Printed Monolayers of Dodecanethiol on Gold: A Multivariate Exploration of Chemistry and Contamination. *Langmuir* 2002;18:1518-27.
- [30] Saeidi N, Sander EA, Zareian R, Ruberti JW. Production of highly aligned collagen lamellae by combining shear force and thin film confinement. *Acta Biomater* 2011;7:2437-47.
- [31] Liu Y, Gan L, Carlsson DJ, Fagerholm P, Lagali N, Watsky MA, et al. A simple, cross-linked collagen tissue substitute for corneal implantation. *Invest Ophthalmol Vis Sci* 2006;47:1869-75.
- [32] Bernard A, Delamarche E, Schmid H, Michel B, Bosshard HR, Biebuyck H. Printing patterns of proteins. *Langmuir* 1998;14:2225-9.
- [33] Bernard A, Renault JP, Michel B, Bosshard HR, Delamarche E. Microcontact printing of proteins. *Adv Mater* 2000;12:1067-70.
- [34] Fukuda J, Sakai Y, Nakazawa K. Novel hepatocyte culture system developed using microfabrication and collagen/polyethylene glycol microcontact printing. *Biomaterials* 2006;27:1061-70.
- [35] PDMS (polydimethylsiloxane), 6.777J/2.751J Material Property Database. Online. 2014. Available from URL: <http://www.mit.edu/~6.777/matprops/pdms.htm>.
- [36] Murakami J, Nishida T, Otori T. Coordinated appearance of beta 1 integrins and fibronectin during corneal wound healing. *J Lab Clin Med* 1992;120:86-93.
- [37] Shibue T, Weinberg RA. Integrin beta1-focal adhesion kinase signaling directs the proliferation

of metastatic cancer cells disseminated in the lungs. *Proc Natl Acad Sci USA* 2009;106:10290-5.

[38] Diehl KA, Foley JD, Nealey PF and Murphy CJ. Nanoscale topography modulates cornea epithelial cell migration. *J Biomed Mater Res A* 2005; 75A: 603-611.

[39] Théry M. Micropatterning as a tool to decipher cell morphogenesis and functions. *J Cell Sci* 2010; 123:4201-4213.

[40] Maurice DM. In: Davson H, editor. *The eye*. New York: Academic Press Inc;1962. p. 296.

[41] van den Berg TJ, Tan KE. Light transmittance of the human cornea from 320 to 700 nm for different ages. *Vision Res* 1994;34:1453-6.

[42] Beems EM, Van Best JA. Light transmission of the cornea in whole human eyes. *Exp Eye Res* 1990;50:393-5.

[43] Zeng Y, Yang J, Huang K, Lee Z, Lee X. A comparison of biomechanical properties between human and porcine cornea. *J Biomechan* 2001;34:533-7.

[44] Crabb RA, Chau EP, Evans MC, Barocas VH, Hubel A. Biomechanical and microstructural characteristics of a collagen film-based corneal stroma equivalent. *Tissue Eng* 2006;12:1565-75.

[45] Jue B, Maurice DM. The mechanical properties of the rabbit and human cornea. *J Biomechan* 1986;19:847-53.

## Tables

**Table 1.** Comparison of key properties of 18% RHCIII-MPC (RHCIII:MPC (w/w)= 2:1; EDC:RHCIII-NH<sub>2</sub> (mol:mol)=0.4:1) hydrogels with a previous 13.7% RHCIII-MPC (RHCIII:MPC (w/w)= 2:1; EDC:RHCIII-NH<sub>2</sub> (mol:mol)=0.3:1) hydrogels formulation grafted into alkali burn rabbit cornea and 18% RHCIII only (EDC:RHCIII-NH<sub>2</sub> (mol:mol)=0.4:1) collagen hydrogels. Human cornea properties from the literature serve as a benchmark.

<b>Properties</b>	<b>13.7% RHCIII-MPC*</b>	<b>18% RHCIII-MPC</b>	<b>RHCIII only</b>	<b>Human cornea</b>
Water content (%)	90.1±2.4	85.5±0.2	86.0±0.2	78 [40]
<b><i>Optical Properties</i></b>				
Refractive index	1.35	1.36	1.35	1.37-1.38[28]
Transmission (%)	98.3±1.5	91.6±0.46	87.4±0.9	87 [41]
Backscatter (%)	1.8±0.0	1.9±0.2	1.3±0.4	3 [42]
<b><i>Mechanical Properties</i></b>				
Tensile strength (MPa)	1.29±0.31	2.12±0.18	2.37±0.4	3.81±0.4 [43]
Elongation at break (%)	37.89±10.31	33.34±3.87	28.21±2.01	
Elastic modulus (MPa)	5.26±1.5	9.46±3.58	15.33±2.67	3-13 [44, 45]
<b><i>Thermodynamic Properties</i></b>				
Td (°C)	54.1	57.6	53.1	65.1[11]

\* Data from [15]

### Figure Captions

**Fig. 1.** SEM micrographs of cross-sections of (A) human cornea, showing the lamellar structure of the collagenous ECM with inter-lamellar connections (arrows). Scale bar, 10  $\mu\text{m}$ . (B) 18% RHCIII-MPC(2/1) hydrogel crosslinked with EDC (RHCIII:MPC (w:w) = 2:1 and EDC:RHCIII-NH<sub>2</sub> (mol:mol) = 0.4 : 1) showing lamellae and inter-lamellar connections, and (C) 18% RHCIII only hydrogel crosslinked with EDC (EDC:RHCIII-NH<sub>2</sub> (mol:mol) = 0.4 : 1) depicting the lamellar structure without the inter-lamellar connections. Scale bars, 20  $\mu\text{m}$ .

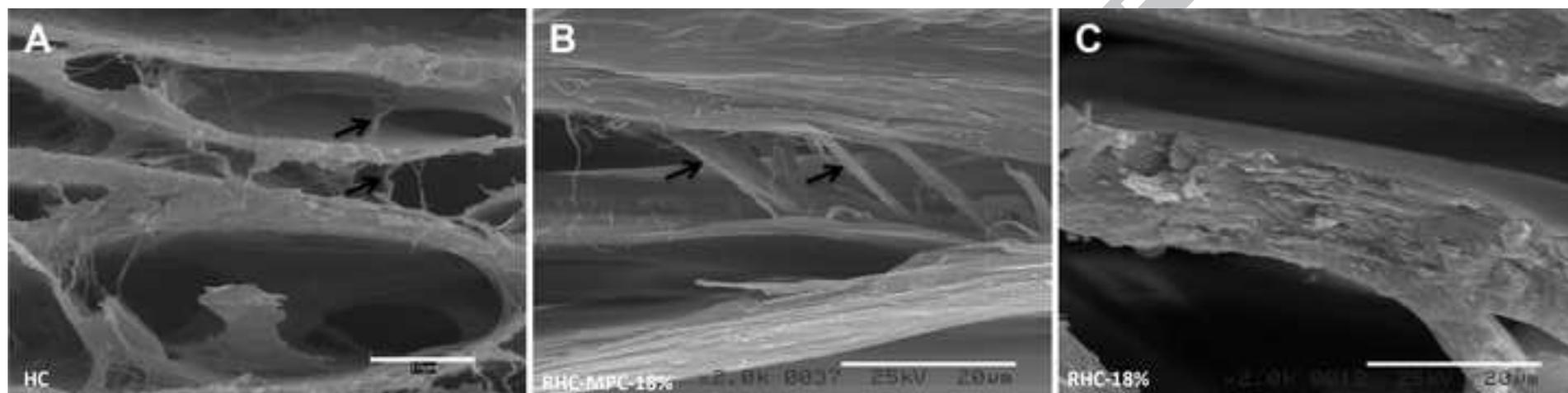
**Fig. 2.** A) RHCIII-MPC implant after cutting with a femtosecond laser, showing the dimensions of the cuts. Red arrow: anterior diameter of 5.9 mm; green arrow: posterior diameter of 8.1 mm; yellow arrow: anterior lamellar cut of 260  $\mu\text{m}$ ; white arrow: remaining posterior depth of 240  $\mu\text{m}$ . B) Laser-cut implant forming a top-hat shaped hydrogel. The precisely cut edges are indicated by arrows. Scale bar, 2mm. C) Cut implant after tissue welding to a matched laser-cut surgical bed in an excised pig cornea by UV crosslinking. The apposed edges are arrowed. Scale bar, 4 mm. D) Cornea (co) and implant (i) as viewed under polarized light showing the very tight apposition between the implant and host tissue after tissue welding (arrows). Scale bar, 50  $\mu\text{m}$ . E) SEM image of the “welded” implant-cornea interface showing the presence of small fibrous crosslinks (arrows) established between the two. Scale bar, 6  $\mu\text{m}$ .

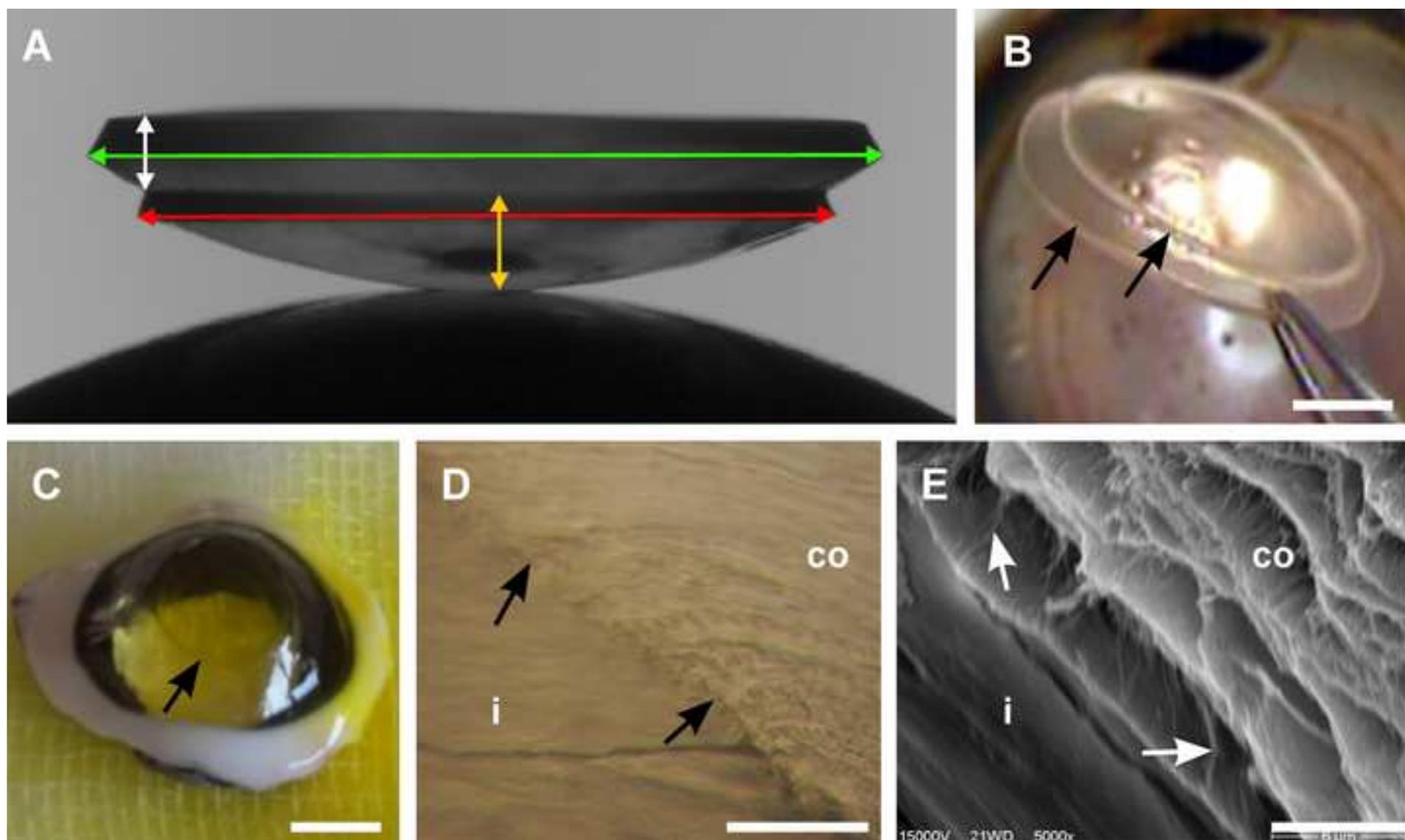
**Fig. 3.** A) Schematic of the microcontact printing procedure- including free collagen hydrogel carboxyl group activation, “ink” printing and passivation steps. Fluorescence microscopical images of the resulting patterned B) 30  $\mu\text{m}$  and C) 200  $\mu\text{m}$  stripes on the surface of RHCIII-MPC hydrogels, visualized by staining with an anti-fibronectin antibody.

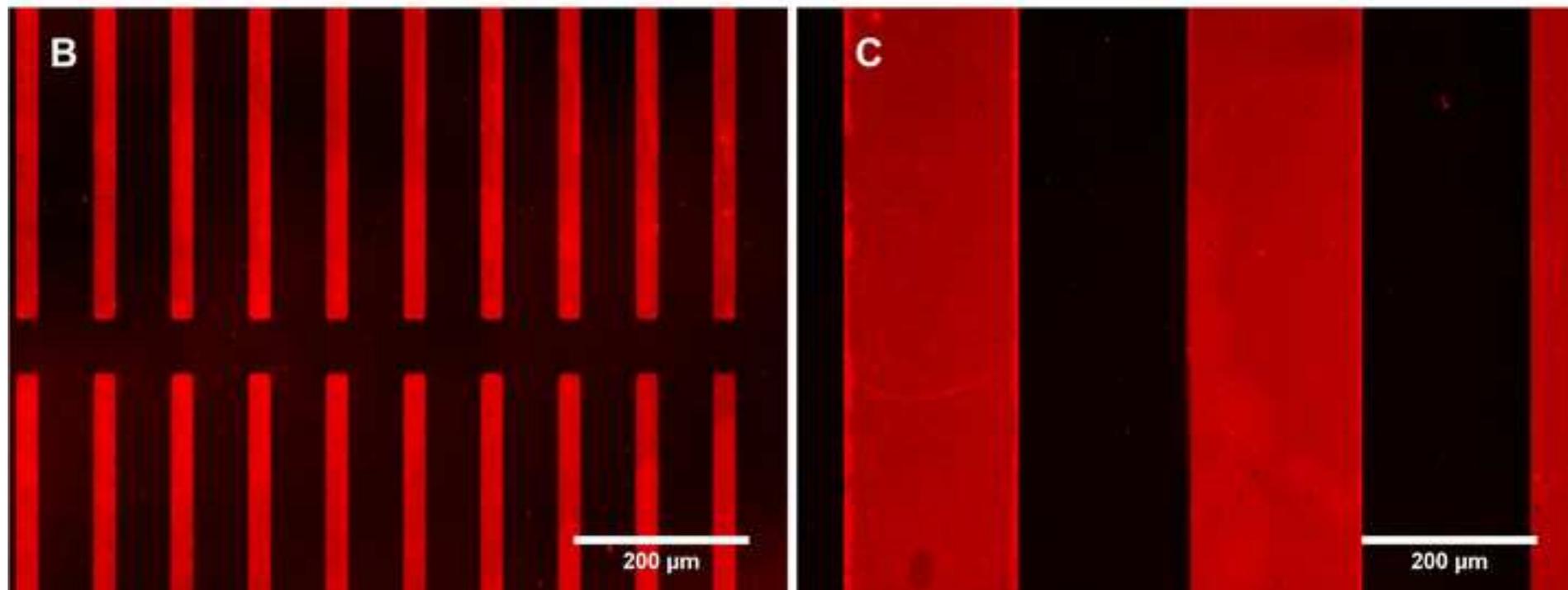
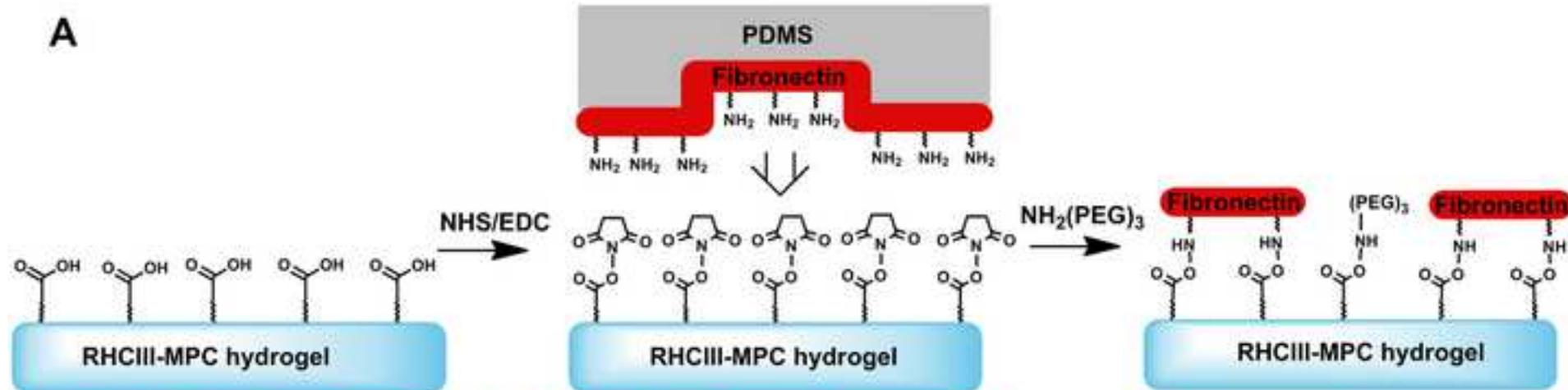
**Fig. 4.** Contact mode AFM topography images of the RHCIII-MPC hydrogel surface before and after the microcontact printing. A) Unmodified hydrogel, showing a surface that comprised uneven, irregularly arranged fibrils. Inset, 10  $\mu\text{m}$  scan image. B) Printed hydrogel surface, overlaid with a fluorescence microscopy image of a printed fibronectin stripe. Surface collagen fibrils that were visible appeared to be partially aligned.

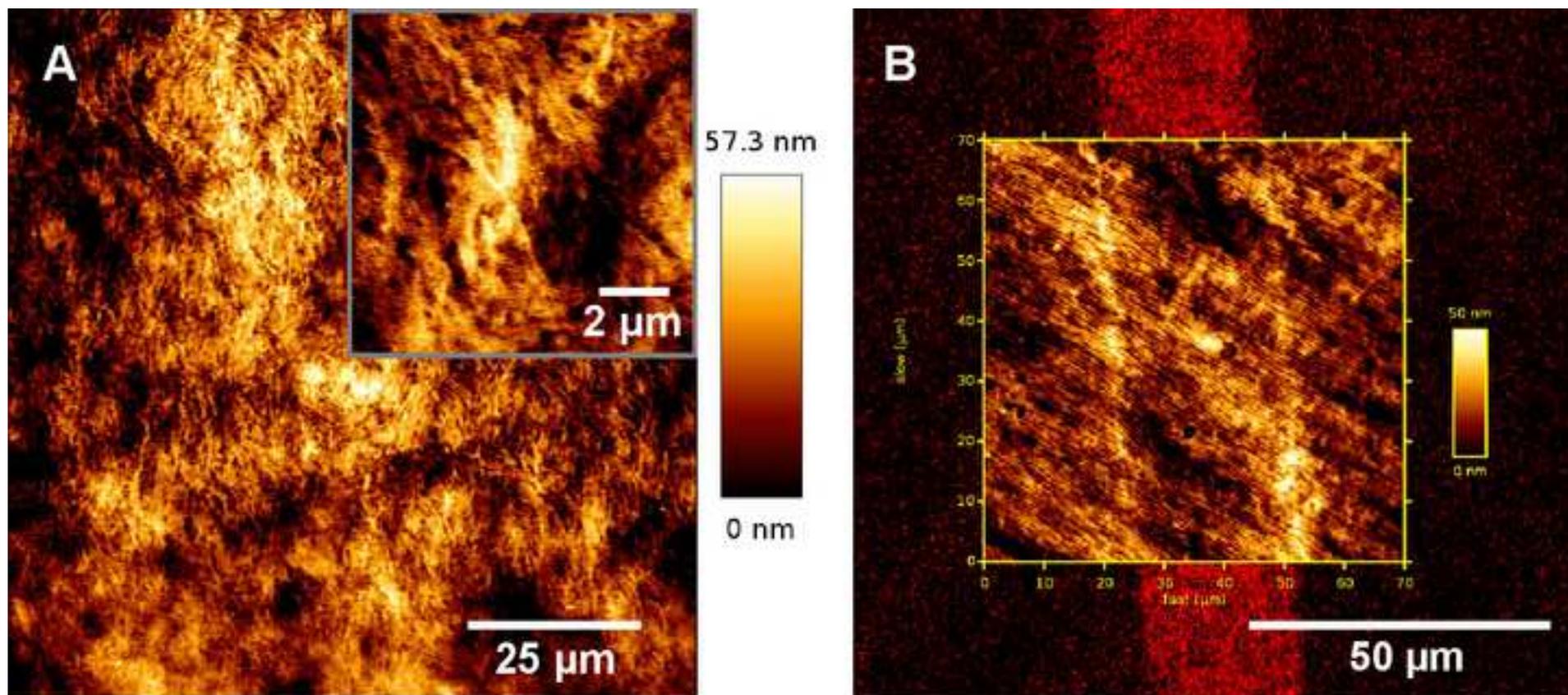
**Fig. 5.** Cell growth on RHCIII-MPC hydrogel surfaces that were A) unmodified, B) deactivated, or patterned with C) 30  $\mu\text{m}$  and D) 200  $\mu\text{m}$  lines. Live/Dead staining shows live cells as green while dead cells fluoresce red. Scale bars, 50  $\mu\text{m}$ . The proliferation rate of cells on the different surfaces is shown in (E).

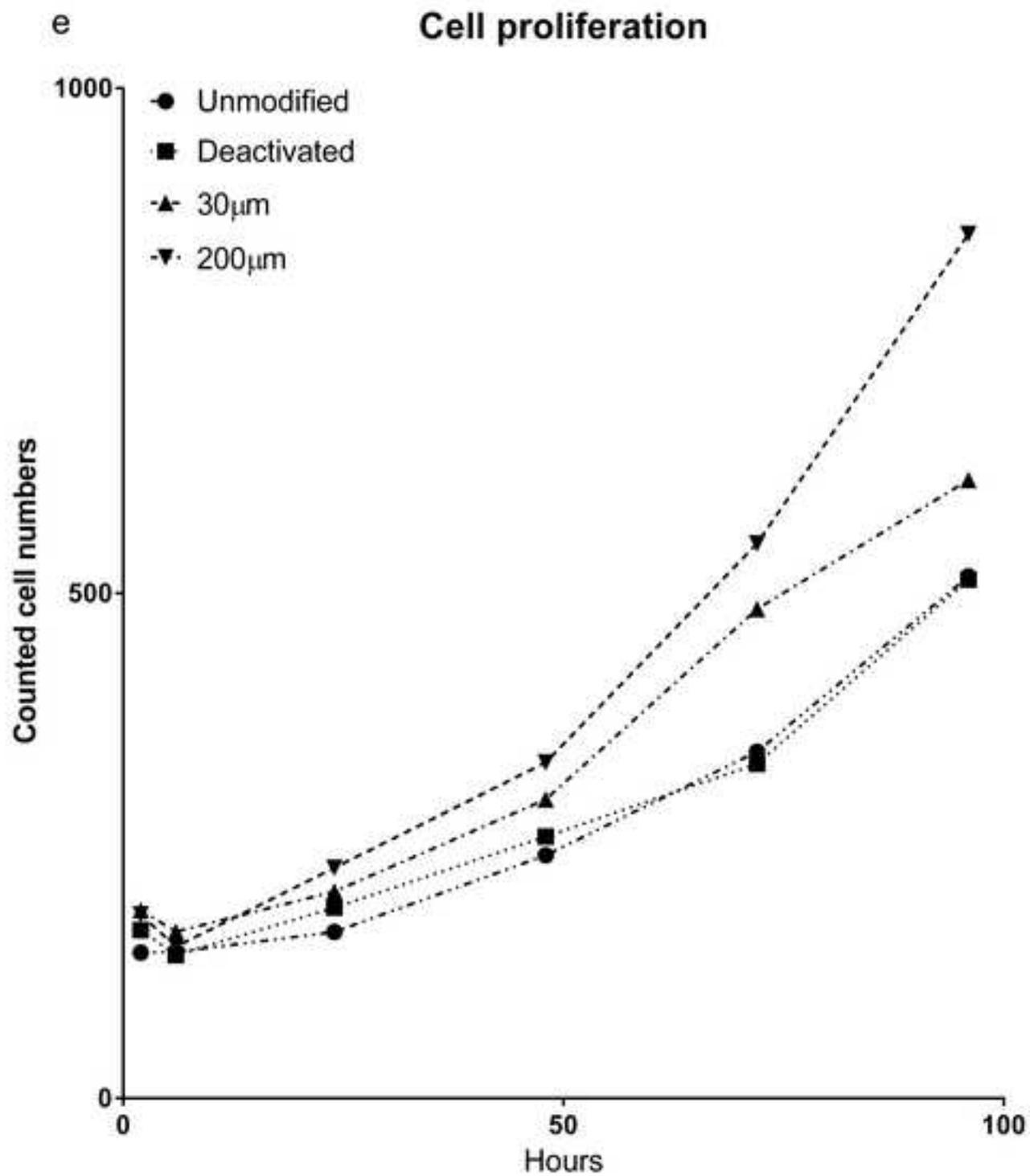
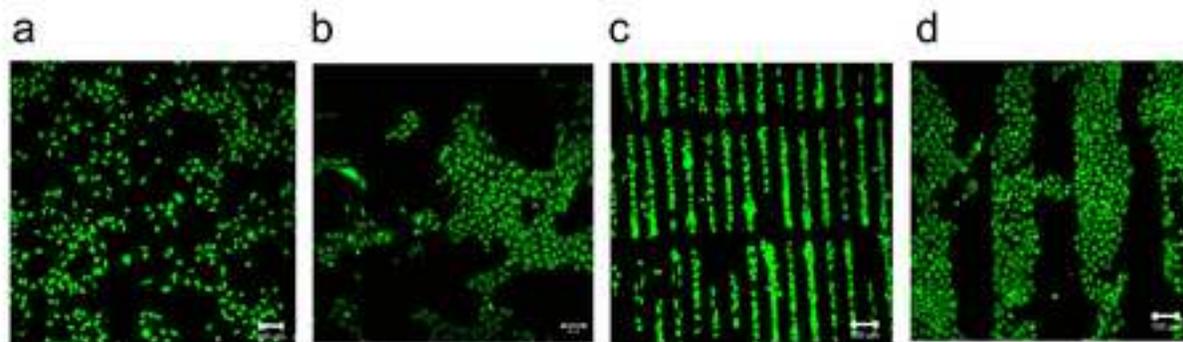
**Fig. 6.** Immunostaining for A) integrin  $\beta 1$ , B) focal adhesion kinase (FAK) and C) Ki67. The micrographs show cells on the hydrogel surfaces, from top left- deactivated, top right- unmodified, bottom left – patterned 30  $\mu\text{m}$  stripes and bottom right – patterned 200  $\mu\text{m}$  stripes. Scale bars, 50  $\mu\text{m}$ . The bar graphs show the ratios between the total cells count and cells stained for D) integrin  $\beta 1$ , E) FAK and F) Ki67. Bars with similar symbols (\* or +) are not significantly different from each other ( $P > 0.05$  by Tukey test). In contrast, bars with dissimilar symbols are significantly different ( $P \leq 0.05$ ) from each other.

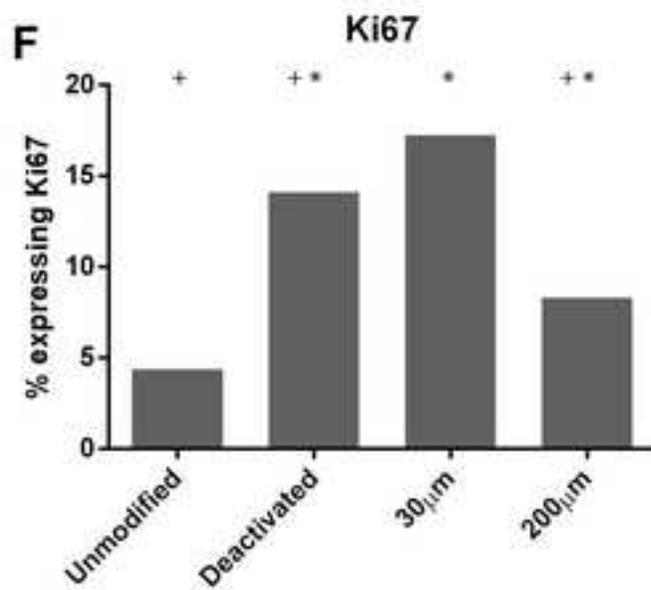
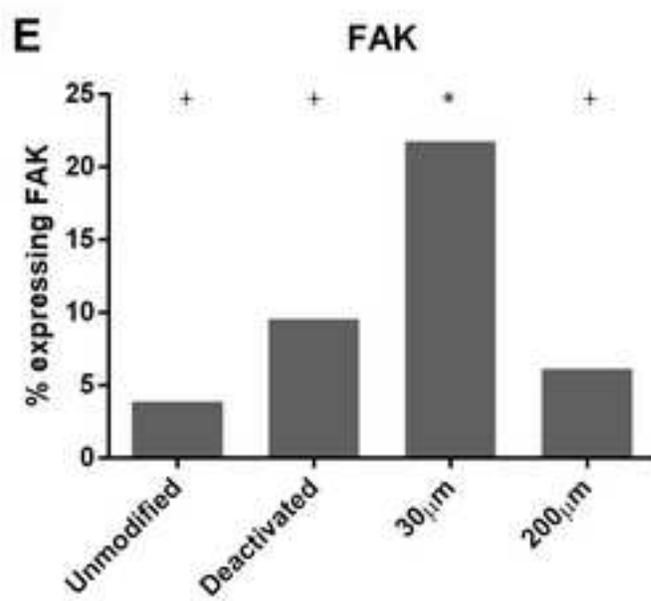
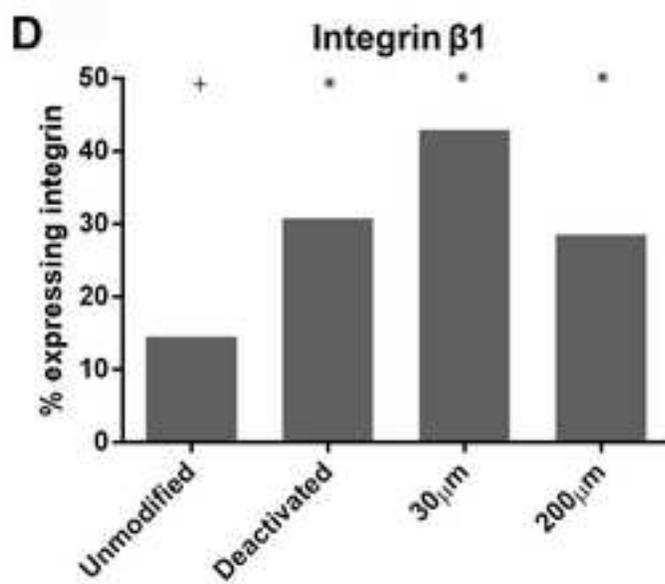
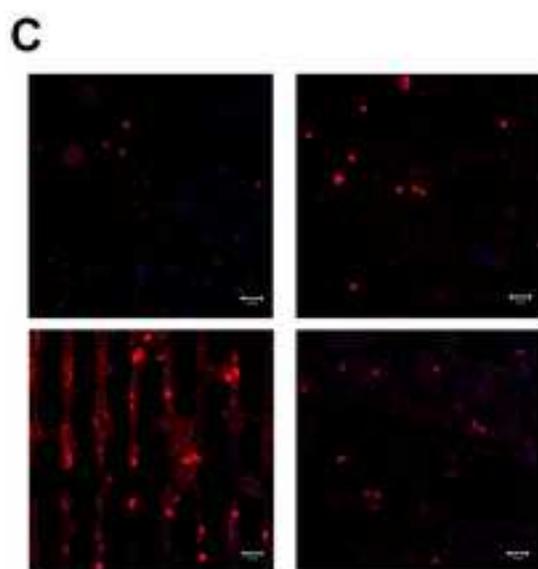
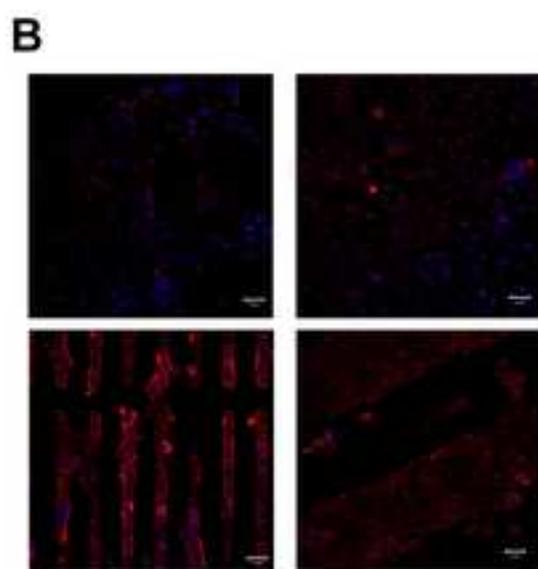
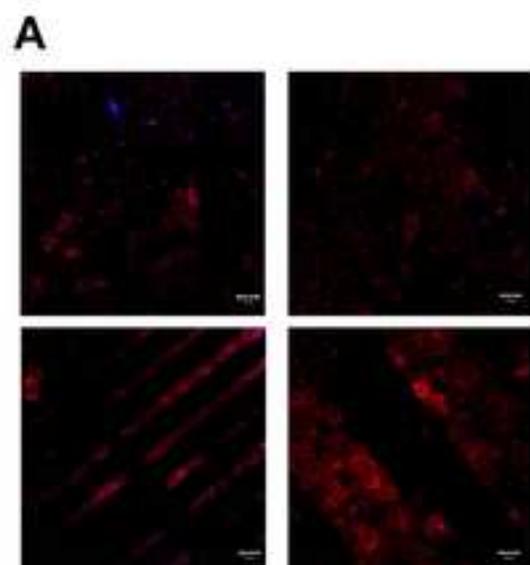


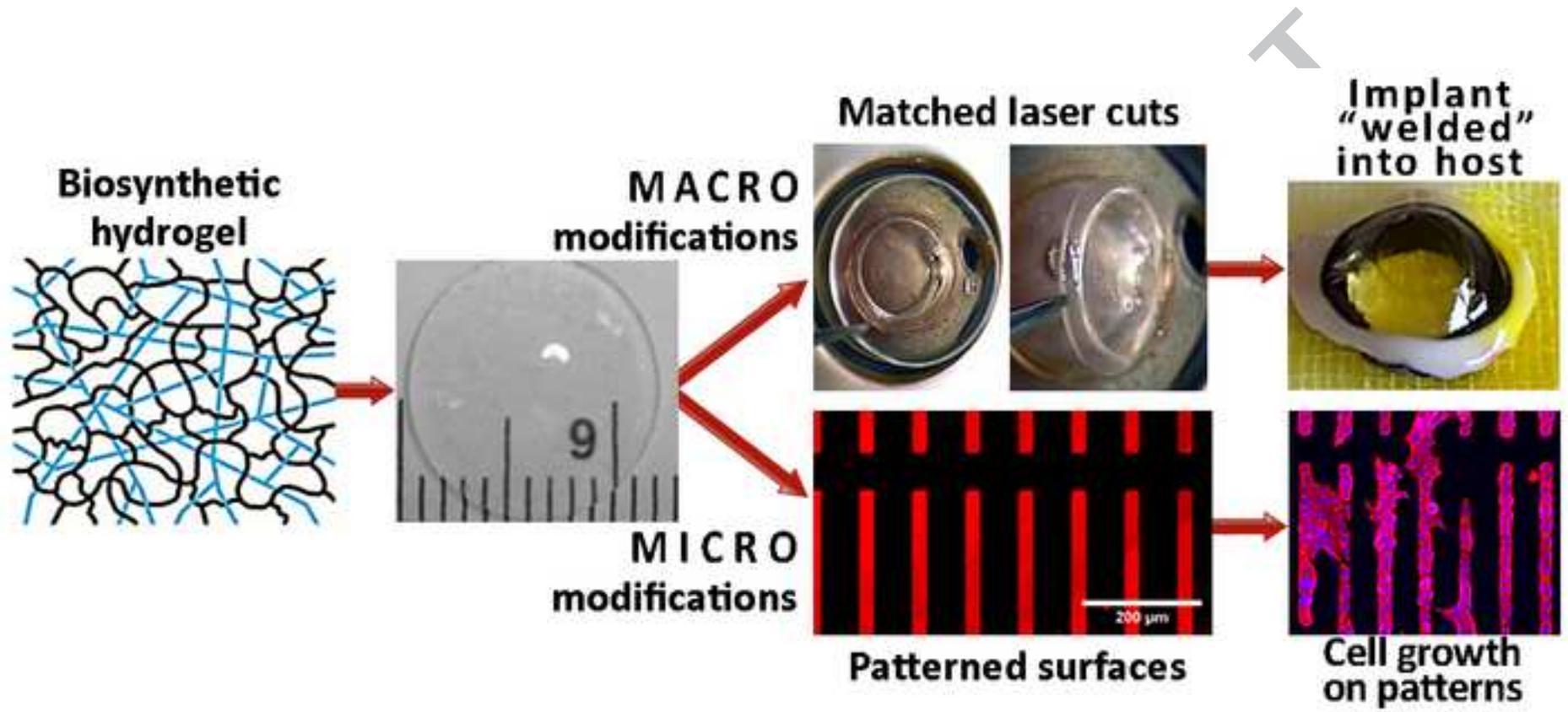












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