

# Artificial Polymeric Scaffolds as Extracellular Matrix Substitutes for Autologous Conjunctival Goblet Cell Expansion

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**PURPOSE.** We fabricated and investigated polymeric scaffolds that can substitute for the conjunctival extracellular matrix to provide a substrate for autologous expansion of human conjunctival goblet cells in culture.

**METHODS.** We fabricated two hydrogels and two silk films: (1) recombinant human collagen (RHC) hydrogel, (2) recombinant human collagen 2-methacryloylxyethyl phosphorylcholine (RHC-MPC) hydrogel, (3) arginine-glycine-aspartic acid (RGD) modified silk, and (4) poly-D-lysine (PDL) coated silk, and four electrospun scaffolds: (1) collagen, (2) poly(acrylic acid) (PAA), (3) poly(caprolactone) (PCL), and (4) poly(vinyl alcohol) (PVA). Coverslips and polyethylene terephthalate (PET) were used for comparison. Human conjunctival explants were cultured on scaffolds for 9 to 15 days. Cell viability, outgrowth area, and the percentage of cells expressing markers for stratified squamous epithelial cells (cytokeratin 4) and goblet cells (cytokeratin 7) were determined.

**RESULTS.** Most of cells grown on all scaffolds were viable except for PCL in which only 3.6 ± 2.2% of the cells were viable. No cells attached to PVA scaffold. The outgrowth was greatest on PDL-silk and PET. Outgrowth was smallest on PCL. All cells were CK7-positive on RHC-MPC while 84.7 ± 6.9% of cells expressed CK7 on PDL-silk. For PCL, 87.10 ± 3.17% of cells were CK7-positive compared to PET where 67.10 ± 12.08% of cells were CK7-positive cells.

**CONCLUSIONS.** Biopolymer substrates in the form of hydrogels and silk films provided for better adherence, proliferation, and differentiation than the electrospun scaffolds and could be used for conjunctival goblet cell expansion for eventual transplantation once undifferentiated and stratified squamous cells are included. Useful polymer scaffold design characteristics have emerged from this study.

**Keywords:** polymeric scaffolds, goblet cells, cell growth

The conjunctiva is the mucous membrane that, along with the cornea, comprises the ocular surface. It is comprised of layers of nonkeratinized stratified epithelial cells that are separated from the underlying stroma by a basement membrane.<sup>1</sup> Goblet cells, which are interspersed among the epithelial cells,<sup>2</sup> are critical to ocular surface integrity because they secrete protective mucins.<sup>3</sup> A healthy conjunctiva is essential to ocular comfort, the maintenance of a clear cornea, and successful outcomes in glaucoma and ocular surface surgery.<sup>1,4-6</sup>

In conditions, such as Stevens-Johnson syndrome, mucous membrane pemphigoid, and chemical or thermal burn injuries, severe conjunctival scarring can occur. Surgical reconstruction of the ocular surface is necessary to avoid loss of conjunctival and limbal epithelial stem cells, which is associated with vision loss, ocular discomfort, and pain. While techniques for homologous corneal transplantation, and autologous and allogenic limbal epithelial stem cell transplantation are well-established,<sup>7,8</sup> their success depends on a healthy conjunctiva. However, autologous

transplantation of either the conjunctiva,<sup>9</sup> buccal membrane,<sup>10</sup> or nasal mucosa<sup>11</sup> is associated with morbidity at the donor site and cosmetically blemishing results. Additionally, only a limited amount of donor tissue can be harvested.

Allogeneic transplantation of human amniotic membrane to replace the conjunctiva, with or without an ex vivo expanded autologous conjunctival epithelial cell layer, has demonstrated some success.<sup>12-15</sup> Transplantation of human amniotic membrane induces rapid epithelialization, and reduces inflammation, vascularization, and fibrosis, but carries risks of disease transmission. There also is biologic variability in donor tissue, limited mechanical strength, and insufficient growth of goblet cells, all of which are critical to ocular surface integrity. Therefore, an optimal source of conjunctival donor tissue for transplantation is lacking.

To address the need for a better substitute for the extracellular matrix (ECM) and ex vivo expansion of cells, natural and synthetic polymeric scaffolds in the form of hydrogels, silk films, and electrospun meshes were examined and compared.<sup>16</sup> Natural or bioinspired ECMs substrates, such as collagen and silk fibroin, have been studied extensively for repair of the cornea.<sup>16,17</sup> Synthetic scaffolds, such as PCL, PCL blends, and poly(lactic-co-glycolic acid), also were studied for transplantation of conjunctival cells,<sup>2</sup> limbal epithelial stem cells,<sup>18-20</sup> and skin cells.<sup>21</sup> However, only limited numbers of conjunctival goblet cells were cultured on these scaffolds.

An optimal ECM substitute should confer mechanical stability and elasticity to the conjunctival construct. These substrates also should provide a scaffold for the cell-cell and cell-matrix interactions that are essential to cell adhesion, migration, proliferation, differentiation, and maintenance of conjunctival squamous epithelial and goblet cells. Importantly, substrates also must support stem cells as these cells are vital to the long-term viability of the transplants for a variety of tissues.<sup>22-24</sup> In addition, the substrate must be sufficiently porous to allow for free diffusion of glucose, proteins, and ions, as well as in-growth of regenerating nerves and blood vessels to penetrate through the membrane.

To meet the need of conjunctival ECM substitutes, we fabricated two hydrogels, two silk films, and four electrospun polymers to investigate their use as substrates for autologous ex vivo conjunctival stratified squamous and goblet epithelial cell expansion. The biopolymers were recombinant human collagen (RHC) hydrogel, recombinant human collagen-2-methacryloyloxyethyl phosphorylcholine (RHC-MPC) hydrogel, poly-D-lysine (PDL) coated silk, and arginine-glycine-aspartic acid (RGD) modified silk. The electrospun polymers were scaffolds made of collagen, poly(acrylic acid) (PAA), poly( $\epsilon$ -caprolactone) (PCL), and poly(vinyl alcohol) (PVA). We hypothesized that a polymeric scaffold that approximates the mechanical properties of the native conjunctiva will induce optimum growth and cellular viability of conjunctival goblet, stratified squamous, and undifferentiated epithelial cells. As goblet cells are difficult to grow in culture and the lack of goblet cells is an important limitation to current conjunctival reconstructive procedures, our first objective was to develop a suitable ECM and a cell culture protocol that was optimized for goblet cell growth. The amount of stem cells in an explant is a second important limitation; thus, upon identification of suitable substrates for goblet cells we then will use the optimal scaffolds to focus on undifferentiated and stem cells.

## METHODS

### Materials

RPMP-1640 culture medium, L-glutamine, penicillin-streptomycin, nonessential amino acids (NEAA), and sodium pyruvate were obtained from Lonza (Walkersville, MD, USA); fetal

bovine serum from Hyclone Laboratories (Logan, UT, USA); monoclonal antibodies against cytokeratin 7 (CK7; Clone RCK105) and cytokeratin 4 (CK4) were from MP Biomedicals (Solon, OH) or Abcam (Cambridge, MA, USA); normal mouse IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and Cy3-conjugated donkey anti-mouse antibody from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Calcein-acetoxymethyl ester (CAM) and ethidium homodimer-1 (EH-1) were obtained as a part of a live/dead kit from Invitrogen (Carlsbad, CA, USA). All other chemicals used for cell culture were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Recombinant human collagen type III was purchased from Fibrogen (San Francisco, CA, USA). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid monohydrate (MES), poly(ethylene glycol) diacrylate (PEGDA), ammonium persulfate (APS), and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Sigma-Aldrich Corp. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was obtained from Paramount Fine Chemicals Co. Ltd. (Dalian, China). *Bombyx mori* silkworm cocoons were purchased from Tajima Shoji Co. (Yokohama, Japan). Arginine-glycine-aspartic acid solution was purchased from Bachem America, Inc. (Torrance, CA, USA). Polyethylene terephthalate (PET) membranes were obtained from cell culture inserts from BD Labware (Franklin Lakes, NJ, USA) and Corning Incorporated (Corning, NY, USA) and were used as a control substrate. The PET membranes were tissue culture treated, had a surface area of 0.3 cm<sup>2</sup>, and pore size of 0.4  $\mu$ m. All polymers (PAA, PCL, and PVA) were purchased from Sigma-Aldrich Corp. All other reagents for the fabrication of scaffolds were purchased from Fisher Scientific (Pittsburgh, PA, USA) and used as received without further purification.

### Preparation of RHC and RHC-MPC Hydrogels

Recombinant human collagen and RHC-MPC based hydrogels were fabricated following a previously published protocol.<sup>25</sup> Briefly, for both types of hydrogels, 500 mg of 18% (wt/wt) RHC was buffered with 0.625 M MES buffer and mixed with NHS and EDC solution to crosslink the collagen. For RHC hydrogel, an excess of MES was added to the final mixing buffer to equalize the dilution factor to the RHC-MPC hydrogel final solution. For RHC-MPC hydrogel, MPC and its coreactant were added before adding EDC solution. The collagen:MPC ratio was 2:1 (wt/wt) and the MPC:PEGDA ratio was 3:1 (wt/wt). Ammonium persulfate (4% wt/vol) and TEMED (2% wt/vol) in MES were added in collagen solution. The ratio of MPC:APS was 1:0.03 (wt/wt) and the ratio of APS:TEMED was 1:0.77 (wt/wt). Calculated volumes of NHS (10% wt/vol) and EDC (5% wt/vol) in MES were added. The molar equivalent ratio of RHC-NH<sub>2</sub>:EDC was 1:0.4 and EDC:NHS was 1:1. The final mixed solution was immediately dispensed between two glass slides with a 100  $\mu$ m (for RHC alone) and 250  $\mu$ m (for RHC-MPC) thick spacer. After demolding, hydrogels were washed thoroughly with PBS and stored in 1% chloroform in PBS to maintain sterility.

### Preparation of Silk Solution

Silk solution was prepared as described previously.<sup>26</sup> *Bombyx mori* silkworm cocoons were cut into small pieces and boiled in 0.02M Na<sub>2</sub>CO<sub>3</sub> for 30 minutes. After three rinses in ultrapure water, extracted fibroin fibers were dried at room temperature overnight. Purified silk was dissolved in a concentrated solution of 9.3 M lithium bromide solution for at least 4 hours at 60°C. The solution was dialyzed against water (MWCO 3500; Pierce, Inc., Woburn, MA, USA) for 48 hours. After centrifugation

gation to remove impurities, the fibroin solution obtained from one batch of 5 g of cocoons was approximately 50 ml at 7% to 8% (wt/vol) and stored at 4°C.

### Preparation of Silk Films

Silk films were prepared using polydimethylsiloxane (PDMS) replica mold, as described previously.<sup>27</sup> Two percent aqueous silk solution was cast on squared PDMS substrates (1 cm<sup>2</sup>) and allowed to dry overnight to generate the films. Once dry, the films were easily detached from the PDMS substrate due to its hydrophobicity. Then, the silk films were water-annealed in a water-filled desiccator for 2 hours.<sup>28</sup> Silk films were coated with 0.1 mg/ml PDL solution for 15 minutes. Arginine-glycine-aspartic acid modified silk films were prepared by presoaking the film in MES buffer (100 mM borate, 150 mM and NaCl, pH 6.5; Pierce, Inc.) for 30 minutes.<sup>29</sup> The silk was activated by incubation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl)/NHS solution for 30 minutes at room temperature. This allows for the -COOH groups from the aspartic and glutamic acid residues in the silk to form stable and amine-reactive NHS-esters on the silk film surface. The activated silk films then were washed with MES buffer twice before incubation in RGD solution (Bachen America, Inc., Torrance, CA, USA) at room temperature for 2 hours. All silk films were sanitized with 70% EtOH for 30 minutes and washed and stored in PBS before use.

### Preparation of Electrospun Scaffolds

Collagen scaffolds were prepared using an in situ crosslinking method as reported previously.<sup>30</sup> EDC and NHS were dissolved in ethanol at a ratio of 200 and 400 mM, respectively. PBS (×20) then was added at a ratio of 50:50 vol/vol. Semed S collagen (Kensley Nash Corporation, Exton, PA, USA) then was added to the PBS/ethanol mixture to produce a collagen solution of 16 wt%. The collagen solution was stirred at room temperature for 10 minutes until all collagen had dissolved. The collagen solution was electrospun using a constant flow rate of 0.5 mL/hour using a syringe pump. The positive voltage lead was attached to the polymer needle with 22 kV. The fibers were collected onto a rotating grounded collecting drum with an air gap distance of 12 cm. Fibers were collected at room temperature in a chamber with a relative humidity between 25% and 35%. Electrospinning continued until the average scaffold thickness was 60 μm as measured by a micrometer. This was the minimum size that allowed for removal from the drum in one piece. The scaffold was placed into a desiccator set at 43% for 3 days to allow the in situ cross-linking process to complete.

Poly(acrylic acid) (average  $M_w$  450 kg/mol) was dissolved in ethanol at room temperature and stirred overnight to produce a 4 wt% solution. Anhydrous (EG) was added to the solution at a concentration of 16 wt% relative to PAA to facilitate crosslinking. Sulfuric acid (1 M) was added to the PAA/EG solution immediately before electrospinning at a concentration of 50 μl/ml. The PAA solution was loaded into a 5 mL plastic syringe equipped with a blunted 18-gauge needle. The polymer solution was electrospun using a constant flow rate of 0.8 ml/h using a syringe pump. The positive voltage lead was attached to the polymer needle with 15 kV. The fibers were collected onto a rotating grounded collecting drum with an air gap distance of 22 cm under ambient conditions. Electrospinning proceeded until the average scaffold thickness measured 40 μm by a micrometer. The resulting scaffold was placed in an oven at 130°C in vacuo for 30 minutes to crosslink the scaffold. The scaffold was removed from the oven and allowed to cool.<sup>31</sup>

Poly(caprolactone) (average  $M_n$  80 kg/mol) was dissolved in chloroform at room temperature with stirring overnight to produce a 10 wt% solution. The PCL solution was loaded into a 5 mL plastic syringe equipped with a blunted 18-gauge needle. The polymer solution was delivered using a constant flow rate of 1.0 mL/h using a syringe pump. The positive voltage lead was attached to the polymer needle with 12 kV. The fibers were collected onto a rotating grounded collecting drum with an air gap distance of 12 cm under ambient conditions.<sup>32</sup> Electrospinning continued until the average scaffold thickness measured 40 μm by a micrometer.

Poly(vinyl alcohol) (99+% hydrolyzed; average  $M_w$  130 kg/mol) was dissolved in distilled water at 90°C in a round-bottom flask fitted with a condenser column for approximately 6 hours with constant stirring to produce a 10 wt% solution. The solution was allowed to cool to room temperature before adding Triton X-100 in a concentration of 2.5% (wt/wt) PVA. The mixture was allowed to stir for an additional hour to assure homogeneity. The PVA solution was loaded into a 5 mL plastic syringe equipped with a blunted 18-gauge needle. The polymer solution was electrospun using a constant flow rate of 1.5 ml/h using a syringe pump. The positive voltage lead was attached to the polymer needle with 20 kV. The fibers were collected onto a rotating grounded collecting drum with an air gap distance of 15 cm under ambient conditions. Electrospinning proceeded until the average scaffold thickness measured 40 μm by a micrometer. The resulting scaffold was rendered water-insoluble by complete submersion in methanol for 24 hours. The scaffold was removed from methanol and allowed to dry at ambient conditions overnight.<sup>33</sup>

All scaffolds were examined by scanning electron microscopy (SEM) using a JEOL JSM-6510 LV after deposition of a 5 nm layer of gold using a sputter coater. Average fiber diameter and distribution were measured using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA).

### Cell Culture

Human conjunctival fornices were obtained from the Eversight (Ann Arbor, MI, USA) and Saving Sight (Kansas City, MO, USA). Donor age ranged from 13 to 73 years and the number of days from death to culture ranged from 2 to 9 days. The donor tissue was stored in Optisol medium at 4°C. This study was in strict accordance with the tenets of the Declaration of Helsinki.

Hydrogel and silk scaffolds were cut into pieces approximately 10 × 10 mm while electrospun scaffolds were cut into pieces of approximately 8 × 8 mm. Glass coverslips and PET membranes, excised from the cell culture inserts, were used as positive controls. The scaffolds were sterilized by incubation in ethanol overnight at room temperature, rinsed in PBS, and equilibrated in culture medium for at least 1 hour. After meticulous removal of connective and fatty tissue from the donor conjunctiva under a dissecting microscope, the epithelial sheet was divided into explants. Explant sizes are listed in the Table. A piece of scaffold and medium was added to each well of a six-well culture dish. The cell culture medium consisted of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/mL penicillin-streptomycin, 87 μM NEEA solution, 870 μM sodium pyruvate, and 8.7 mM HEPES. One explant was placed, epithelial side down, in the center of each scaffold. The culture dishes contained just enough medium (0.5 ml) to cover the bottom of the dish, preventing explant flotation and detachment. This method replicated the physiologic conditions of the ocular surface where nutrition is provided by the underlying stroma and a thin liquid tear film covers the cells. The cells were refed every 2 to 3 days and incubated under routine culture conditions of 95% air and 5% CO<sub>2</sub> at 37°C for

TABLE. Average Explant Size

Scaffold Type	Average Explant Size, mm <sup>2</sup>
Coverslip	1.9 ± 0.7
RHC	2.4 ± 0.5
RHC-MPC	1.7 ± 0.3
RGD silk	1.5 ± 0.3
PDL silk	1.0 ± 0.2
PAA	1.5 ± 0.1
PCL	1.3 ± 0.1
PET	1.2 ± 0.2

approximately 2 weeks. During the first 3 to 4 days of culture, only part of the culture media was removed and new media added, half in the morning and half in the evening, to minimize large volume changes that might have caused explant detachment. When cells were refed with more media than the initial 0.5 ml, the hydrogels and scaffolds with cultured cells floated and remained at the air-liquid interface throughout the culture period.

### Viability Assay

The live/dead kit used CAM, which converted to green fluorescent calcein in the cytoplasm of live cells. Ethidium homodimer-1 produced red fluorescence after binding to nucleic acids, but was only able to penetrate damaged cell membranes of dead cells. Using serial dilution, the optimal concentrations for the assay were determined to be 2 μM CAM and 2 μM EH-1.

After 9 to 15 days of cell culture, the scaffolds were washed for 5 minutes in sterile PBS, and incubated in a solution containing CAM, EH-1, and PBS for 30 minutes in room temperature. The scaffolds then were mounted on microscope slides using mounting media containing 100 mM Tris (pH 8.5) containing 25% glycerol, 10% polyvinyl alcohol, 2.5% 1,4-diazabicyclo-[2.2.2]-octane, and 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained at ×400 magnification using confocal laser scanning microscopy (Leica TSC-SP2; Leica, Mannheim, Germany). For the hydrogels and silk scaffolds, the number of live cells (green), dead cells (red), and total number of cells (DAPI) were counted in a masked fashion using Image J 1.48v (NIH) from 3 separate experiments. For electrospun scaffolds, because the cells expanded vertically and horizontally throughout the scaffolds, confocal z-series were obtained, and Image Pro Plus v.7 (Media Cybernetics, Rockville, MD, USA) was used to count the cells based on fluorescence intensity and cell size from 4 separate experiments. The software user was blinded to the representation of the green and red cells. Cells cultured on glass coverslips and PET were used as positive controls.

### Outgrowth

After 9 (biopolymers) or 15 (synthetic polymers) days of culture, the scaffolds were rinsed in PBS, fixed with 100% methanol for 30 minutes, and then rinsed again in PBS. The outgrowth on biopolymers was visualized by bright field microscopy and quantified by Image J 1.48v. Because of the opaque nature of the electrospun scaffolds, these scaffolds were placed on slides with mounting media containing 0.1% DAPI. The DAPI-stained nuclei were visualized by fluorescence microscopy (Eclipse E 800; Nikon, Tokyo, Japan) at a magnification of ×20 to ×40. Outgrowth and explant size were quantified using Spot Advanced Plus software (Diagnostic Instruments, Sterling Heights, MI, USA). Cells cultured on glass coverslips and PET were used as positive controls. Fold growth

was calculated as outgrowth area divided by explant area from 3 separate experiments for coverslips, PET, RHC-MPC, 4 separate experiments for RHC, RGD-silk, PDL-silk, PCL, and from 6 separate experiments for PAA.

### Immunocytochemistry

The expression of CK7- and CK4-positive cells was used to determine if substrate modulation would affect the phenotype of the cultured cells. Cytokeratin-7 is a goblet cell-specific keratin, and CK4 is specific for stratified squamous nongoblet epithelial cells.<sup>34,35</sup> Scaffolds with methanol-fixed cells were rinsed in PBS and incubated in blocking buffer containing 2% BSA and 0.2% Triton X-100 in PBS for 1 to 2 hours. The scaffolds were incubated with primary antibodies against CK7 and CK4, both diluted to 1:100 with 2% BSA, overnight at 4°C in a humidified chamber. The scaffolds then were incubated with the secondary antibody, anti-Cy3-conjugated donkey anti-mouse IgG diluted 1:300, for 1 hour at room temperature. The scaffolds were mounted on slides using mounting media containing 0.1% DAPI. Normal mouse IgG diluted 1:100 was used as the negative control. Cells cultured on glass coverslips and PET were used as positive controls. The cells were viewed using a laser scanning confocal microscope (TSC-SP5; Leica) at ×400 magnification. Because the cells grew on top of each other and through the full thickness of the electrospun scaffolds, it was not possible to count the cells using a single two-dimensional image. For all scaffolds, confocal z-series were obtained at the 3, 6, 9, and 12 o'clock areas equidistant between the explant and outgrowth edge. Cell nuclei were detected using DAPI.

ImageJ 1.48v was used to quantify the total number of cell nuclei in biopolymers, while Image Pro Plus v.7 was used to quantify the total number of cells in electrospun scaffolds. The number of colocalized cells was divided by the total number of cells as indicated by DAPI to obtain the percentage of CK7- and CK4-positive cells, respectively. Data were obtained from 1 experiment for PAA; 3 separate experiments for coverslips, RHC, RHC-MPC, RGD-silk, PDL-silk; and 4 separate experiments for PCL and PET.

### Statistical Analysis

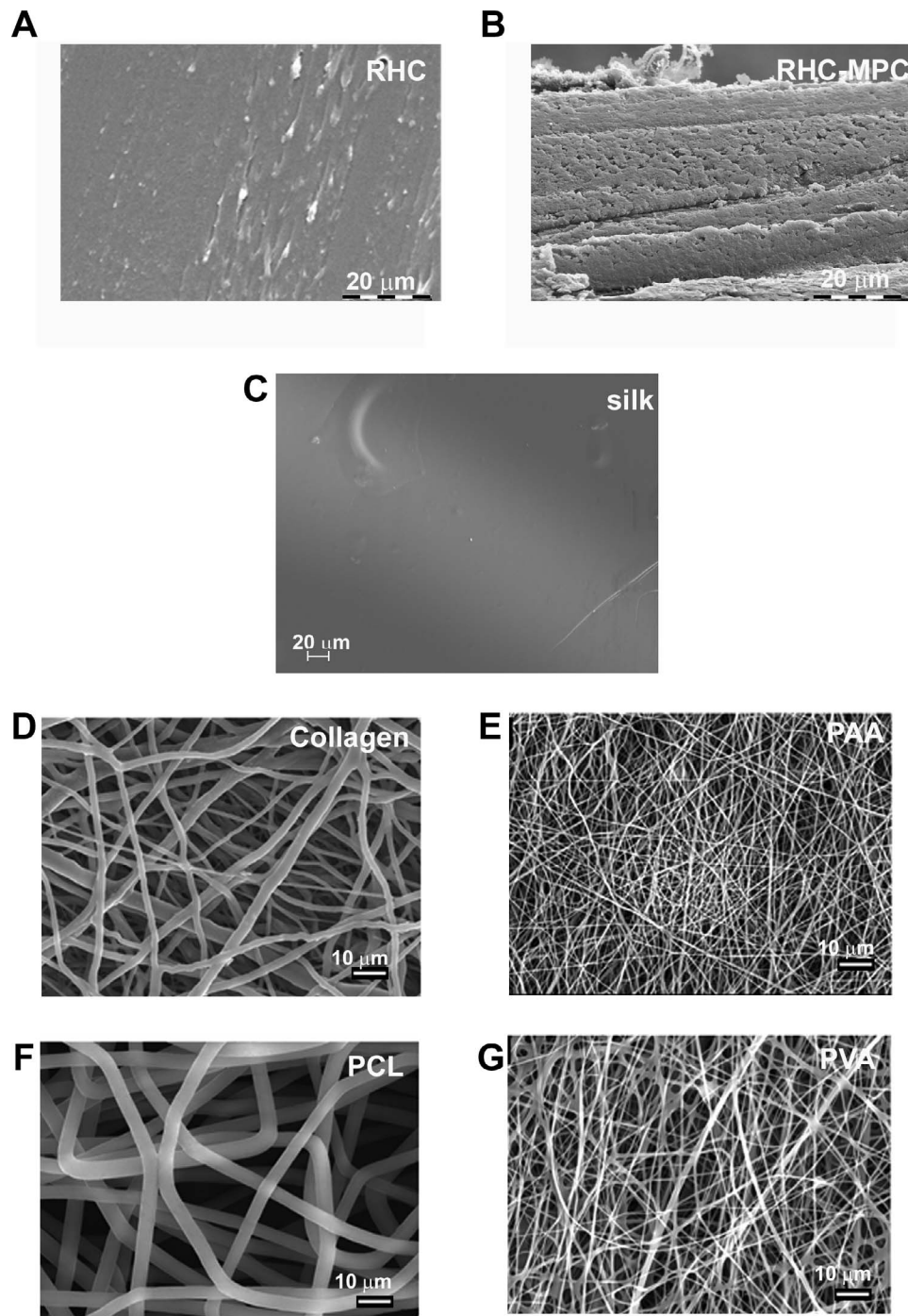
Data are expressed as mean ± SEM. Data were analyzed by Student's *t*-test, and *P* ≤ 0.05 was considered statistically significant.

## RESULTS

### Characteristics of Scaffolds

Polymer scaffolds were imaged using SEM to assure consistent fiber morphology that was devoid of defects (Fig. 1). Both hydrogels have a lamellar arrangement of collagen fibrils, similar to human cornea. There were no morphologic differences between the RGD- and PDL-coated silk.

Fiber diameters for electrospun scaffolds were consistent with literature results, with the exception of cross-linked collagen, which showed higher than reported average fiber diameters of 0.42 ± 0.11 μm<sup>31</sup> (Fig. 2). This was most likely due to the scaffolds being partially hydrated and swollen, increasing the fiber diameter. The collagen scaffold also had a wide distribution of fiber diameters, caused by partial fusion of fibers during the cross-linking process. The PAA fiber had an average diameter of 0.39 ± 0.10 μm. Poly(caprolactone) exhibited the largest average fiber diameters of 4.74 ± 0.58 μm, which is consistent with literature values.<sup>32</sup> This is principally the result of the relatively low conductivity of



**FIGURE 1.** Scanning electron microscopy of scaffolds. Scanning electron microscopy of (A) RHC hydrogel, (B) RHC-MPC hydrogel, (C) silk, (D) in-situ crosslinked collagen scaffold, (E) PAA scaffold, (F) PCL scaffold, (G) PVA scaffold. Scale bars are indicated.

PCL/chloroform solutions compared to ethanol, ethanol/PBS, or water used for electrospinning the other polymers. The average fiber diameter of PVA was  $0.84 \pm 0.32 \mu\text{m}$ .

### Effect of Scaffolds on Cellular Viability

Human goblet cells were grown on biopolymers and synthetic polymers for either 9 (biopolymers) or 15 (electrospun scaffolds) days and the viability of the cells on the scaffolds was determined using a commercial live/dead kit. PET was used as the positive control for electrospun scaffolds as these inserts are specifically designed for optimal cell growth.

However, these inserts are not suitable for transplantation. Glass coverslips were used as a second type of positive control as these are transparent, and we have experience growing and identifying cells on this material. Most of the cells grown on the coverslips, biopolymers, silk, PAA, and PET were stained with calcein indicating that the cells were viable (Fig. 3A). In contrast, there were very few viable cells on PCL (Fig. 3A). The percentages of live cells on coverslips, RHC, RHC-MPC, RGD-silk, and PDL-silk were  $98.6\% \pm 0.7\%$ ,  $99.8\% \pm 0.2\%$ ,  $99.7\% \pm 0.3\%$ ,  $100\% \pm 0.0\%$ , and  $100\% \pm 0.0\%$ , respectively (Fig. 3B). There were no significant differences of the number of live

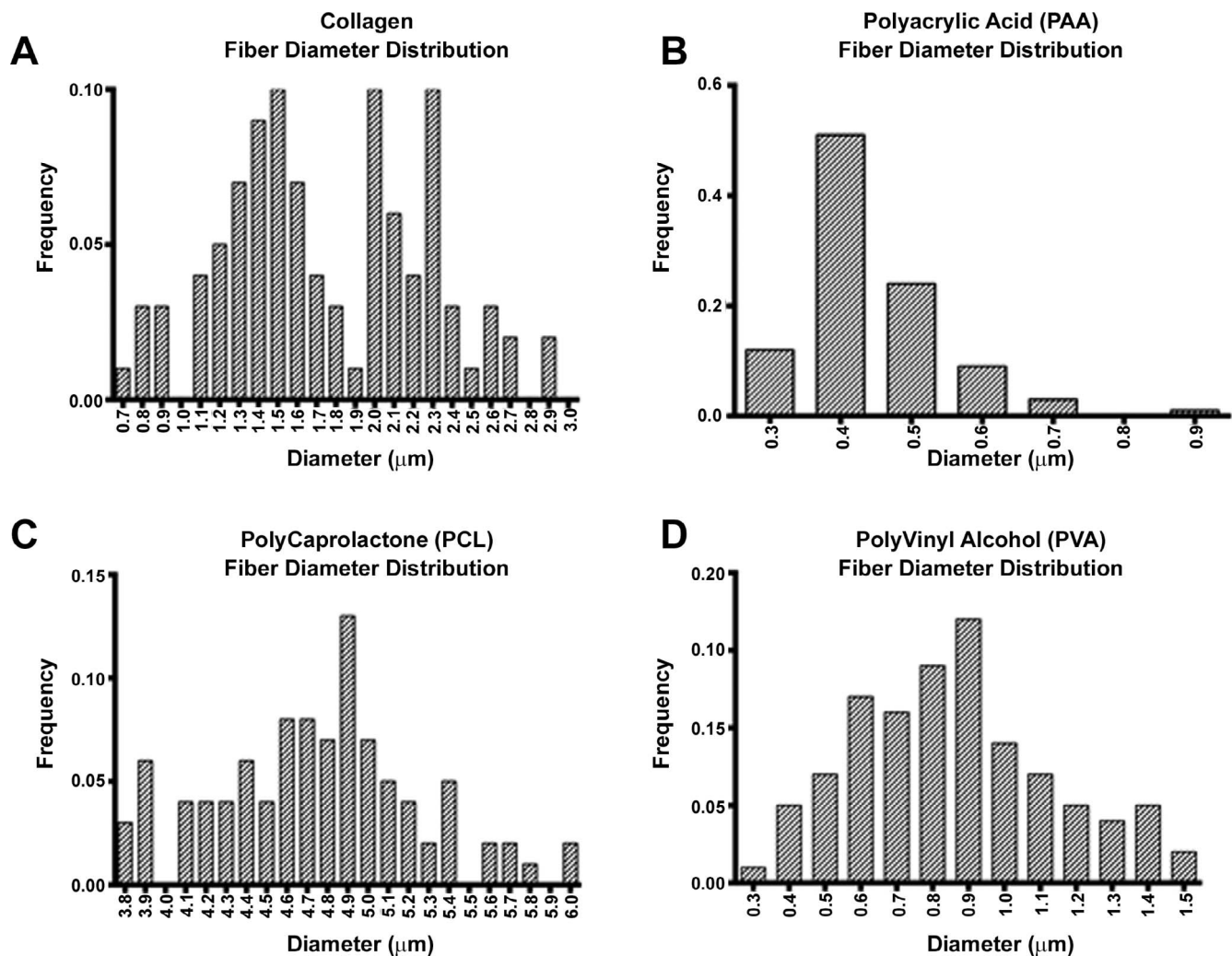


FIGURE 2. Diameter and frequency of fibers in electrospun scaffolds. (A) In-situ crosslinked collagen scaffold. (B) Poly(acrylic acid) scaffold. (C) Polycaprolactone scaffold. (D) polyvinyl alcohol.

cells between these scaffolds. The percentages of live cells on PAA, PCL, and PET were  $94.7\% \pm 1.6\%$ ,  $3.6\% \pm 2.2\%$ , and  $92.9\% \pm 5.4\%$ , respectively (Fig. 3B). Cell viability on PCL was significantly lower than on either PAA or PET ( $P < 0.05$ ) while viability between PAA and PET was not significantly different from each other (Fig. 3B). Due to difficulty handling the electrospun collagen, only one culture on this scaffold was cultured successfully and analyzed. The viability on this scaffold was 94.2% live cells (data not shown). All explants detached from PVA during culture, and no cells were observed on the scaffold.

### Effect of Scaffolds on Cell Growth

The sizes of explants placed on the biopolymers ranged from 0.6–3.5 mm<sup>2</sup> with an average explant size of  $1.8 \pm 0.4$  mm<sup>2</sup>. The average explant size for each scaffold is shown in the Table. There were no significant differences between any of the explant sizes on any of the materials used.

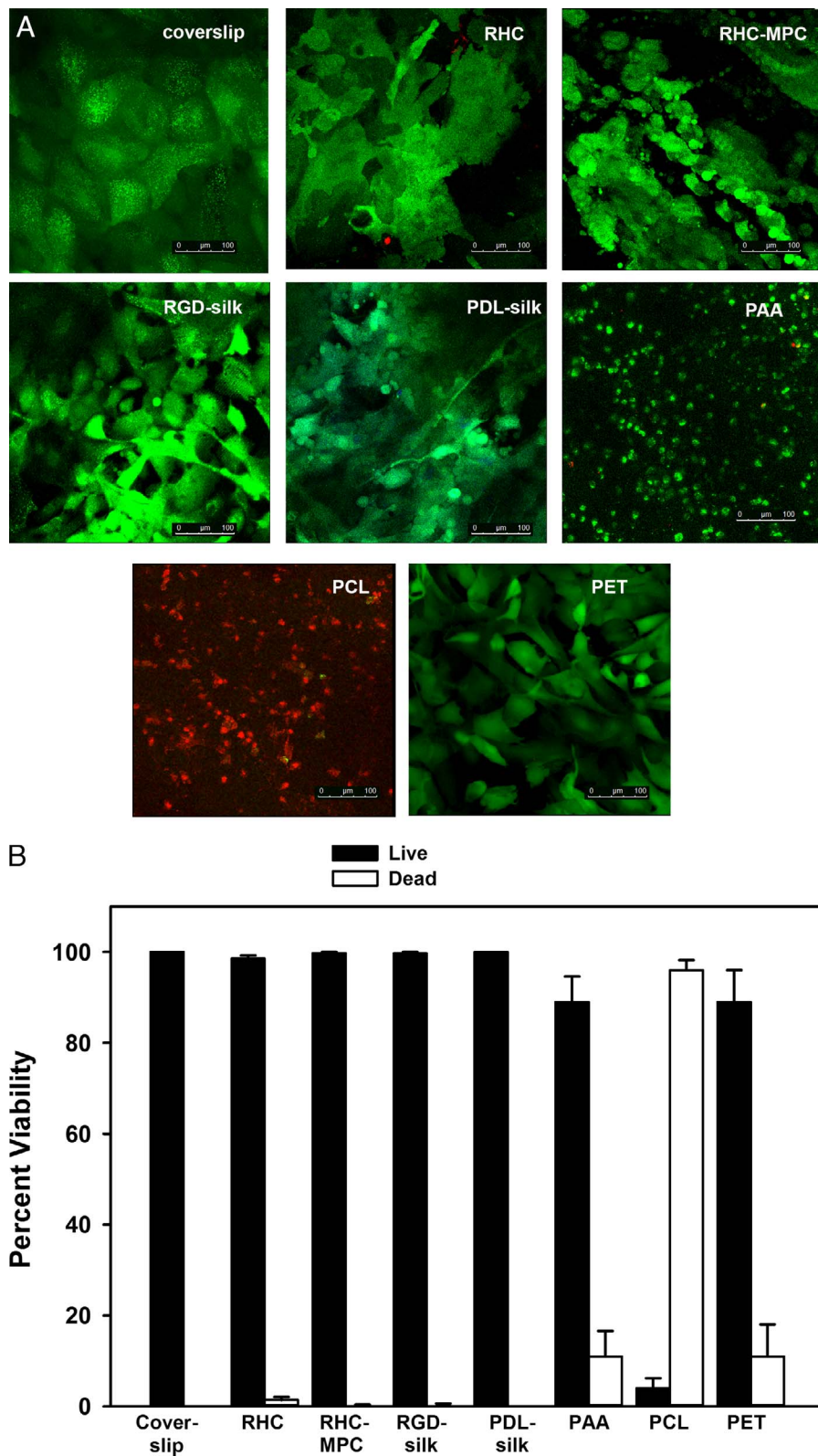
Outgrowth of primary cultures on coverslips and the four biopolymers was determined by light microscopy and is shown in Figure 4A. Due to opaque nature of the electrospun scaffolds the outgrowth was measured by fluorescent microscopy using the nuclear stain DAPI and is shown in Figure 4B. The outgrowth from cells grown on coverslips was  $23.7 \pm 5.7$ -fold (Fig. 4C). This is compared to  $19.2 \pm 5.7$ -,  $50.3 \pm 14.0$ -,  $22.4 \pm 1.5$ -, and

$27.8 \pm 10.2$ -fold growth on RHC, RHC-MPC, RGD-silk, and PDL-silk, respectively (Fig. 4C). There were no significant differences between growth on different biopolymers.

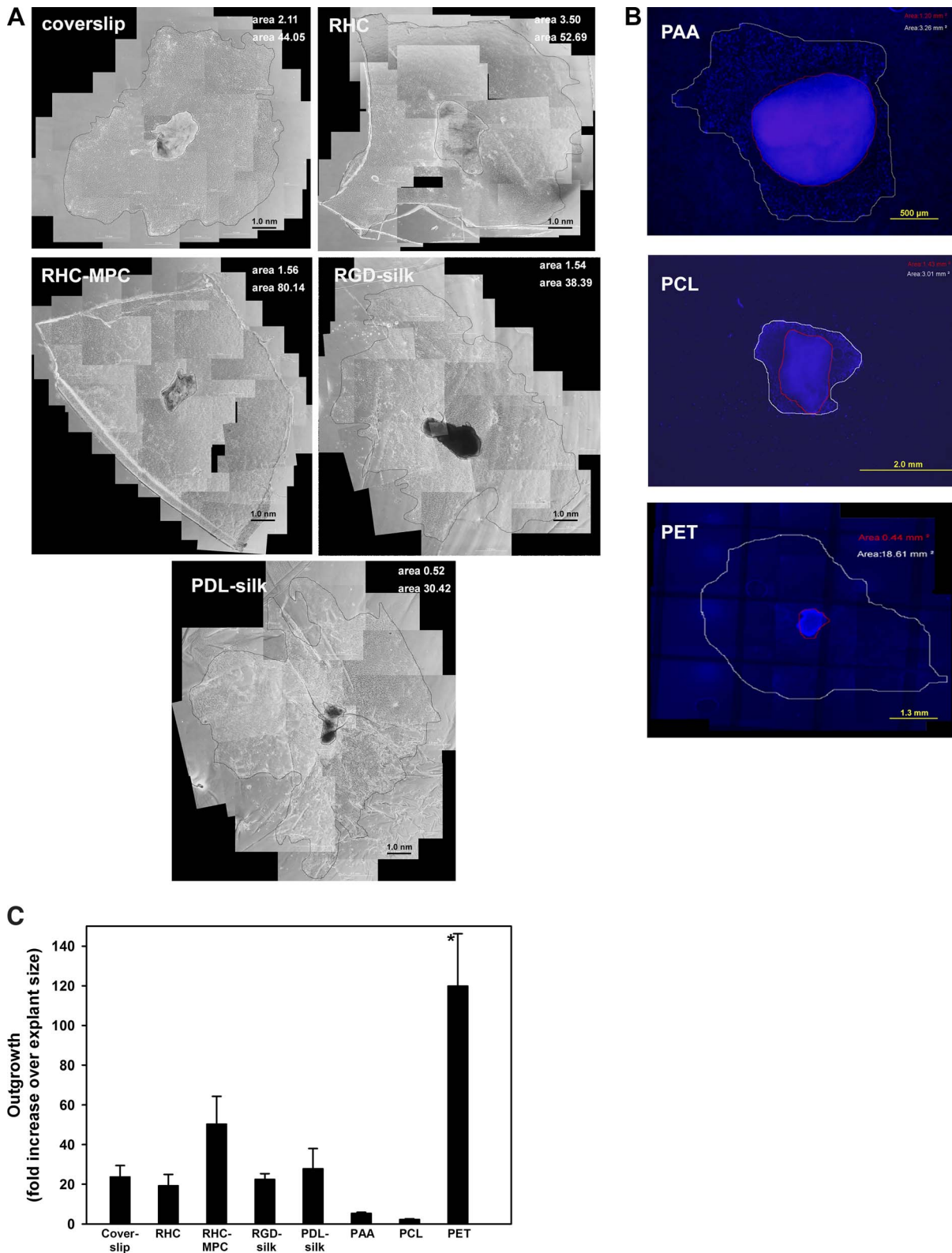
For electrospun scaffolds, the outgrowth on was  $4.3 \pm 2.4$ - and  $2.3 \pm 0.3$ -fold growth on PAA and PCL, respectively (Fig. 4C). The fold growth on PET, the positive control, was  $119.9 \pm 26.3$ -fold. The fold growth on PET was significantly greater compared to the growth on PAA and PCL ( $P < 0.05$ ). All explants on PVA detached during culture and no DAPI staining was visualized on the scaffolds. Only one culture on collagen could be cultured successfully and had a 3.7-fold outgrowth. One culture on PET grew to confluence, so that fold outgrowth was at least 168.8, but probably would have been larger if the membrane had been larger. This value was included in the mean fold outgrowth on PET, because excluding it would cause the reported mean fold outgrowth on PET to be erroneously low.

### Effect of Scaffold on Cell Type

To determine the type of cell that grew on scaffold, immunofluorescence studies were performed using CK7, a specific marker for conjunctival goblet cells, and CK4, a marker for stratified squamous cells of the conjunctiva.<sup>3,35,36</sup> Our culture conditions successfully favored the growth of CK7-positive cells over CK4-positive cells on all scaffolds (Figs. 5A, 5B). In comparing coverslips, a positive control, and biopoly-

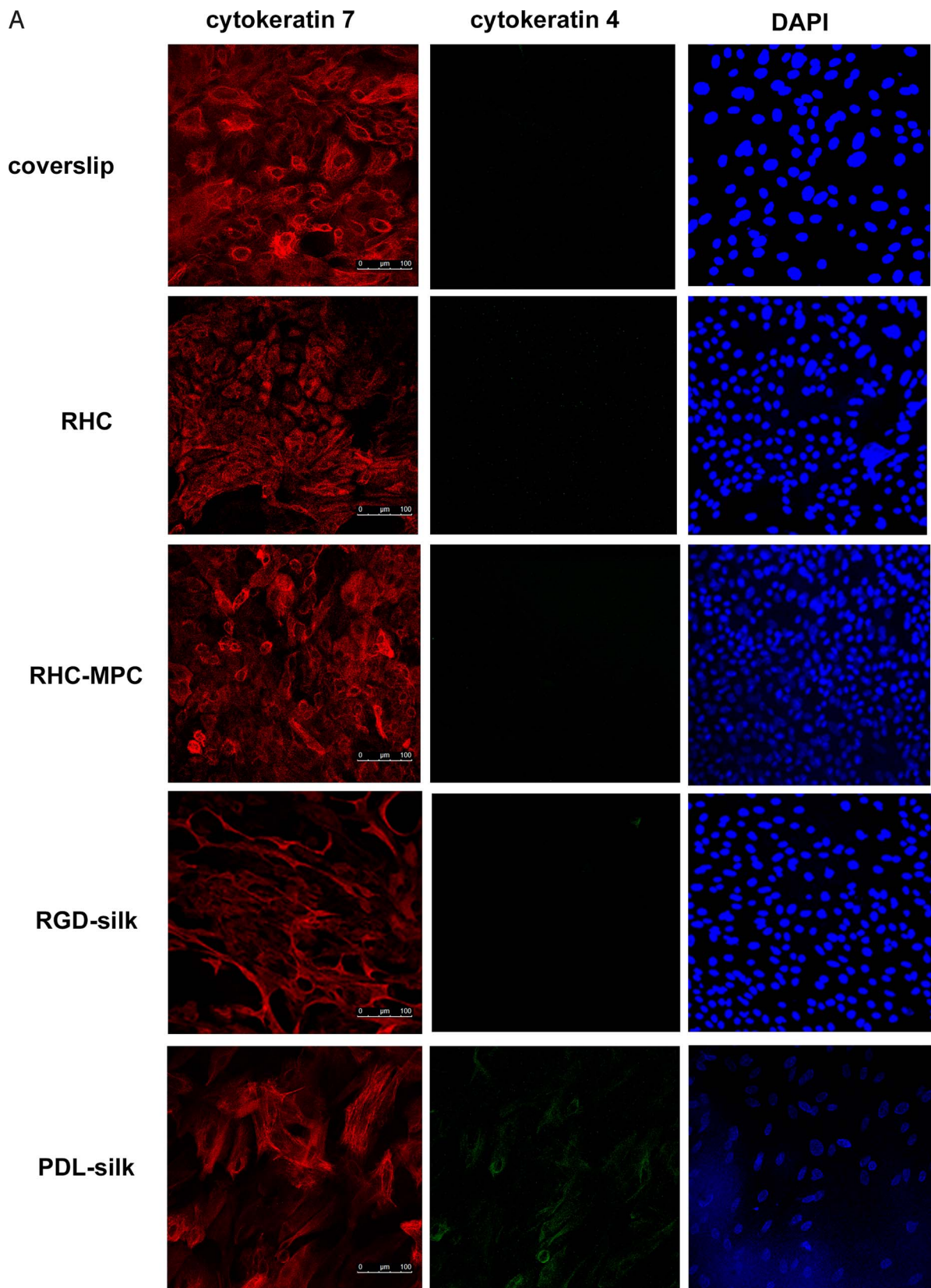


**FIGURE 3.** Viability of cells grown on hydrogels, silk films, and electrospun scaffolds. Confocal laser scanning micrographs of primary cultures of conjunctival goblet cells after viability staining are shown in (A). Live cells are calcein-positive (*green*) while dead cells are ethidium homodimer-1-positive (*red*). Total number of *green* and *red* cells were counted and percentages of each are shown in (B). Micrographs are representative from 3 (coverslip, RHC hydrogel, RHC-MPC hydrogel, RGD modified silk, PDL coated silk, and PET) or 4 (PAA and PCL) independent experiments. Data are mean  $\pm$  SEM.



**FIGURE 4.** Growth of cells on hydrogels, silk films, and electrospun scaffolds. Outgrowth of primary cultures on scaffolds were determined. Cells grown biopolymers were visualized by bright field microscopy (A). Area of outgrowth as indicated by *black line* was determined. Cells grown electrospun scaffolds were visualized by fluorescence microscopy using DAPI-stained nuclei (B). Area of outgrowth as indicated by *white line* was determined. Area of outgrowth was calculated by NIH Image (biopolymers) or Spot Advanced Plus software (electrospun scaffolds) and are shown in (C). Micrographs are representative from an individual experiment of 3 (coverslips, RHC-MPC hydrogel and PET), 4 (RHC hydrogel, RGD modified silk, PDL-coated silk, and PCL), and 6 (PAA). Mean of fold outgrowth for each scaffold is shown in (C). Data are mean  $\pm$  SEM. \*Significant difference from PAA and PCL.





**FIGURE 5.** Identification of cell types on hydrogels, silk films, and electrospun scaffolds. Confocal micrographs of primary cultures of conjunctival goblet cells grown on biopolymers (**A**) after incubation with antibodies directed against CK7 (*left column* shown in red) and CK4 (*middle column* shown in green). Nuclei of cells were identified using DAPI and are shown in *right column*. Confocal micrographs of primary cultures of conjunctival goblet cells grown on electrospun scaffolds (**B**) after incubation with antibodies directed against CK7 with DAPI (*left two columns*) or CK4 with DAPI (*right two columns*). Percentage of cells expressing CK7 and CK4 were calculated and for each scaffold is shown in (C). Micrographs are representative of 1 (PAA), 3 (coverslip, RHC hydrogel, RHC-MPC hydrogel, RGD modified silk, and PDL-coated silk) or 4 (PCL and PET) independent experiments. Data are mean  $\pm$  SEM.

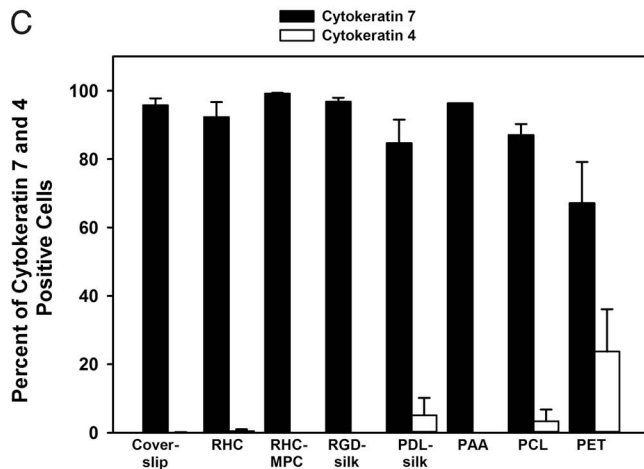
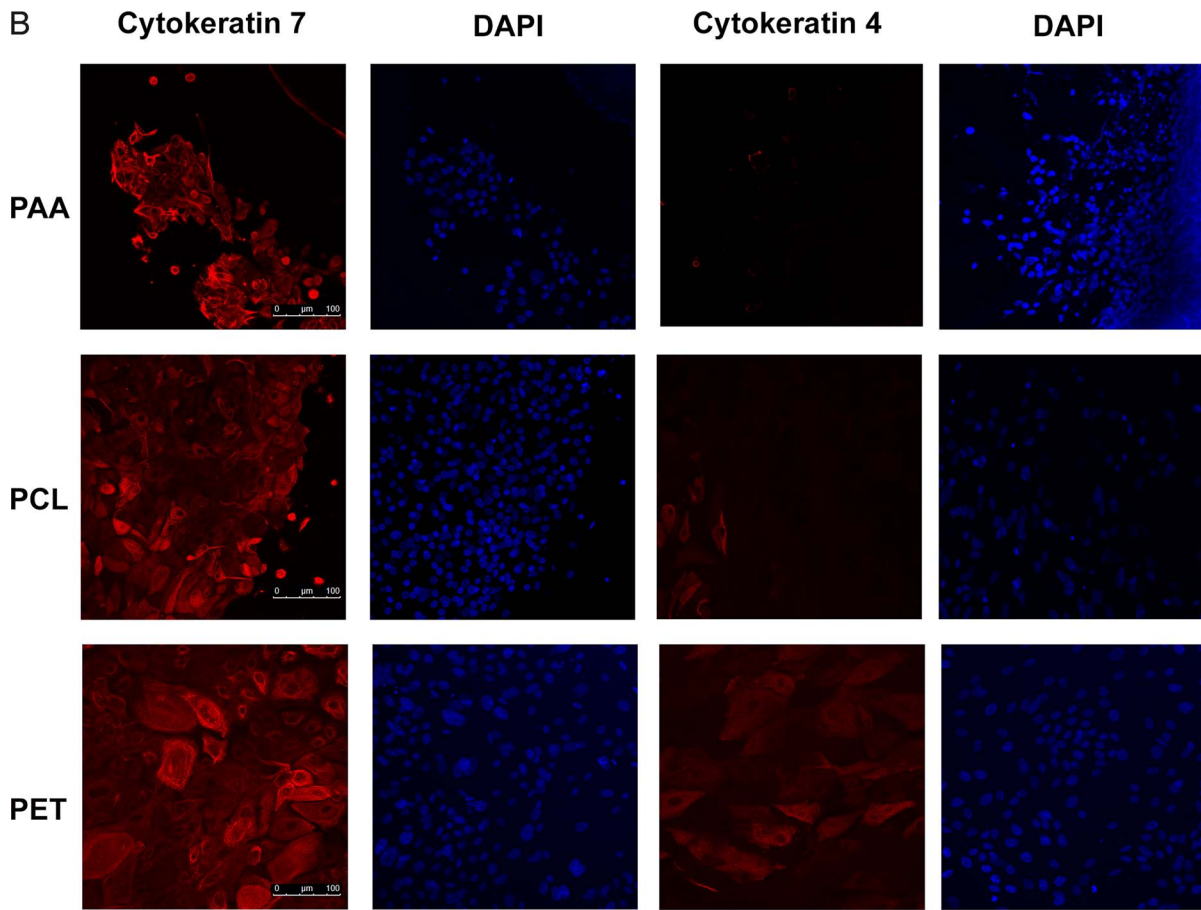


FIGURE 5. Continued.

mers, the percentage of cells expressing CK7 were  $95.8\% \pm 2.0\%$ ,  $92.3\% \pm 4.4\%$ ,  $99.2\% \pm 0.3\%$ ,  $96.8\% \pm 1.2\%$ , and  $84.7\% \pm 6.9\%$  on coverslips, RHC, RHC-MPC, RGD-silk, and PDL-silk, respectively (Fig. 5C). Only one culture could successfully be processed on PAA on which 96.4% of the cells from this culture were CK7-positive and none was CK4-positive (Figs. 5B, 5C). On PCL, the percentage of CK7-positive cells was  $87.1\% \pm 3.2\%$ , while on PET, a positive control, the percentage of CK7-positive cells was  $67.1\% \pm 12.1\%$ . There were no significant differences of the number of either CK7- or CK4-positive cells between the different scaffolds. Due to the lack of cellular attachment on PVA and because the collagen scaffolds were

too fragile to be processed, no immunofluorescent studies were performed with these scaffolds.

No immunofluorescence was detected when normal mouse IgG was used as negative control for CK7 and CK4 on all scaffolds (data not shown).

### DISCUSSION

Conjunctival stratified squamous cells<sup>37-39</sup> and goblet cells<sup>3,40</sup> can be cultured on glass and plastic, but ex vivo expansion of conjunctival cells, especially goblet cells, for autologous

transplantation still is in its infancy due to the lack of a suitable scaffold. Artificial polymeric scaffolds can be designed to overcome many problems associated with the use of human amniotic membrane or autologous tissue for conjunctival reconstruction.<sup>41,42</sup> To address this problem, we tested two types of polymers as scaffolds for the growth of conjunctival goblet cells, biopolymers made from natural materials and synthetic electrospun scaffolds. Both types have advantages and disadvantages. Biopolymers do not usually provoke immune responses and can integrate with surrounding tissues more readily, and degradation products are generally less toxic compared to synthetic scaffolds. However, biopolymers also can have variable mechanical strengths, structure, and degradation rates.<sup>16</sup> Synthetic polymers can be advantageous due to their known chemical composition, mechanical strength, and ability to control the properties of the scaffold.<sup>16</sup> In this study, we used fibrillary, porous electrospun scaffolds, which replicated the structure of normal conjunctival stroma. This would allow for nutrition and regenerating nerves and blood vessels to penetrate the scaffold.

Four different biopolymers were tested as hydrogels and films to support human conjunctival cell, particularly goblet cell, growth. Conjunctival explants attached and cells grew equally well on all four biopolymers tested, RHC hydrogel, RHC-MPC hydrogel, PDL, and RGD-modified silk. In addition, the majority of cells on these scaffolds were CK7-positive, indicative of goblet cells. There were no significant differences between these scaffolds on any of the parameters measured in this study. Collagen hydrogels and silk have been successfully used to culture corneal epithelium, mesenchymal stem cells, and muscle.<sup>16,43,44</sup>

In addition to the biopolymers, four different electrospun materials, collagen, PVA, PAA, and PCL, also were used. The collagen scaffold was extremely soft and fragile limiting its use. Reinforcement of the collagen scaffold with either 50% collagen/50% polylactic-co-glycolic acid, or cysteine cross-linking was insufficient to stabilize the collagen membrane (data not shown). Conjunctival tissue explants would not attach to PVA scaffolds at all, and experiments were discontinued on this scaffold as well. Poly(acrylic acid) supported attachment and limited proliferation of human conjunctival goblet cells that remained viable after 2 weeks in culture. However, PAA also was very soft and difficult to handle and, thus, only one culture on PAA was available to identify the type of cell on the scaffold. Poly(caprolactone) had the best mechanical properties of the four membranes. The PCL yielded an average of 84% CK7-positive cells, indicating a goblet cell density much higher than previously studied scaffolds.<sup>45</sup> However, viability and outgrowth were low on PCL. Use of a more hydrophobic PCL and fibronectin dipcoating of the PCL, as used previously,<sup>2,46</sup> had no effect on conjunctival cell growth (data not shown). We also used plasma treated PCL to replicate the tissue culture treatment that is used on commercial PET membranes, and in other studies,<sup>47,48</sup> but this also did not enhance outgrowth in our experiments (data not shown). Thus, while goblet cells could be cultured in high densities on PAA and PCL, none of the synthetic electrospun scaffolds consistently yielded high cell viability and outgrowth compared to PET, the positive control.

There are several possible explanations for the poor outcome with the synthetic electrospun scaffolds. A negative electrical charge appears to be an important factor for cellular adhesion, possibly due to increased hydrophilicity.<sup>49</sup> The addition of ECM components to the scaffold also is important for cellular adhesion and proliferation.<sup>50-52</sup> While collagen carries a negative surface charge and has ECM components, its mechanical weakness limited its use in our studies. Poly(acrylic acid) is negatively charged, but contains no ECM components,

and PCL is electrically neutral and also does not have any ECM components. It also is possible that the porous surface of the scaffolds prevented conjunctival goblet cells from attaching, migrating, and proliferating. It is possible that a continuous surface that better mimics the basal lamina of the conjunctiva, such as the hydrogels and silk films used in this study, may be better suited to culturing conjunctival goblet cells.

Human conjunctival explants were used to replicate the clinical conditions where autologous conjunctival tissue would be used for ex vivo expansion and subsequent transplantation to the eye. Donor tissue from the conjunctival fornices was used as this area contains a high density of conjunctival progenitor cells,<sup>53-58</sup> which are critical to the success of a transplant. However, our culture medium was optimized for fully differentiated goblet cells because the presence of goblet cells is the hallmark of a mature conjunctiva.<sup>3</sup> Goblet cells have not been propagated successfully on any scaffolds studied to date. To obtain more stratified squamous cells and undifferentiated cells, a different culture medium could be used as well as altering supplements, such as serum or calcium concentration.<sup>2,59</sup> We plan to continue this study to include undifferentiated, stem, and stratified squamous cells in addition to the goblet cells. It is critical for the constructs to contain these other cell types, especially the undifferentiated and stem cells, before these constructs can be used for transplantation. In addition, we cultured cells at the air-liquid interface, which better mimics the physiologic condition at the ocular surface.<sup>39,50,56,59</sup>

In conclusion, we demonstrated that polymers synthesized from collagen hydrogels or modified silk film allowed for attachment and growth of conjunctival goblet cells. Therefore, these biopolymers could provide scaffold needed for eventual conjunctival transplantation. While the electrospun scaffolds chosen were not optimal, their use in this study served as a useful guide for the design of synthetic substrates for attachment and growth of conjunctival goblet cells. We hypothesized that the collagen hydrogels and modified silk films will be permissive for the growth of undifferentiated or stem cells that would produce the goblet and stratified squamous cells of transplanted conjunctival tissue, the next step in our project.

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