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Journal	Journal of Biomedical Materials Research. Part B, Applied biomaterials, 81(1): 201-206
URL	http://hdl.handle.net/10130/505
Right	This is a preprint of an article published in Uchino Y, Shimmura S, Miyashita H, Taguchi T, Kobayashi H, Shimazaki J, Tanaka J, Tsubota K. Amniotic membrane immobilized poly(vinyl alcohol) hybrid polymer as an artificial cornea scaffold that supports a stratified and differentiated corneal epithelium. J Biomed Mater Res B Appl Biomater. 2007 Apr; 81(1): 201-6.

Amniotic membrane immobilized poly (vinyl alcohol) hybrid polymer as an artificial cornea scaffold that supports a stratified and differentiated corneal epithelium

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Commercial relationship disclosure: Patent Pending S.S, H.M, T.T, H.K and K.T.

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Key words: cornea, epithelium, keratoprosthesis, poly (vinyl alcohol), amniotic membrane

ABSTRACT

Polyvinyl alcohol (PVA) is a biocompatible, transparent hydrogel with physical strength that makes it promising as a material for an artificial cornea. In our previous study, type I collagen was immobilized onto PVA (PVA-COL) as a possible artificial cornea scaffold that can sustain a functional corneal epithelium. The cellular adhesiveness of PVA *in vitro* was improved by collagen immobilization, however, stable epithelialization was not achieved *in vivo*. In order to improve epithelialization *in vivo*, we created an amniotic membrane (AM) -immobilized polyvinyl alcohol hydrogel (PVA-AM) for use as an artificial cornea material. AM was attached to PVA-COL using a tissue adhesive consisting of collagen and citric acid derivative (CAD) as a crosslinker. Rabbit corneal epithelial cells were air-lift cultured with 3T3 feeder fibroblasts to form a stratified epithelial layer on PVA-AM. The rabbit corneal epithelial cells formed 3 to 5 layers of keratin-3-positive epithelium on PVA-AM. Occludin-positive cells were observed lining the superficial epithelium, the gap-junctional protein connexin43-positive cells was localized to the cell membrane of the basal epithelium, while both collagen IV were observed in the basement membrane. Epithelialization over implanted PVA-AM was complete within 2 weeks, with little inflammation or opacification of the hydrogel. Corneal epithelialization on PVA-AM in rabbit corneas improved over PVA-COL, suggesting the possibility of using PVA-AM as a biocompatible hybrid material for keratoprosthesis.

Key words: artificial cornea, amniotic membrane, polymer, collagen, epithelial cells

Corneal transplantation, or keratoplasty, is one of the most successful forms of transplantation performed using allogenic tissue. However, many countries suffer from a shortage of donor corneas, and corneal disease accounts for millions of blind patients worldwide. The development of a stable keratoprosthesis (KPros) may be a solution towards solving this problem. Several models of Kpros are already in clinical use [1-5], and 2 models are currently approved by the FDA [2,6]. The stability of Kpros was greatly enhanced by the design of a porous skirt that allowed the integration of host corneal tissue into prosthetic peripheral region [7].

One candidate material for a biocompatible artificial cornea is poly (vinyl alcohol) (PVA). Low temperature-crystallized PVA is transparent, and has mechanical properties suitable for Kpros [8]. Although pure PVA shows low cell affinity, we have shown that covalent immobilization of collagen type I to the surface of PVA (PVA-COL) combined with air-lift organotypic culture techniques support a fully stratified corneal epithelium, which show similar physiological and morphological characteristics with the native cornea [9]. However, in vivo stability was not achieved due to the lack of basement membrane components required for a stable epithelium.

Attempts to improve in vivo stability include hybrid polymers that combine both artificial polymers and biological molecules to design corneal scaffolds that can support cell growth [10]. These polymers allow for regeneration of cornea nerves which are crucial in maintenance of an intact ocular surface following surgery. However, biodegradable materials alone are also unstable in the presence of inflammation, which may cause degradation of polymers by proteases [11]. In an attempt to take advantage of both the stability of artificial polymers with the biocompatibility of natural materials, we designed a hybrid polymer composed of a PVA hydrogel with amniotic membrane

(AM). Human AM tissue is currently in clinical use for reconstructing the ocular surface [12-14], and has proven to be useful as a biological carrier for epithelial sheet transplants [15]. Here we show that a PVA based keratoprosthesis prototype with an AM surface (PVA-AM) allows for a fully stratified epithelium with intact basement membrane components that was not achieved with PVA-COL.

MATERIALS AND METHODS

Immobilization of amniotic membrane onto collagen-PVA

PVA-COL was prepared as described previously [16,17]. Briefly, PVA powder was dissolved in a dimethyl sulfoxide (DMSO)-water (80-20) mixed solvent. The resulting viscous solution was placed into a mold with silicone rubber spacers (200 µm thick) between two glass plates, and then left to stand at -20°C for 24 h for setting a gel. The PVA hydrogel was dehydrated for further surface modification. Isocyanate groups were first introduced onto the surface by using hexamethylene dissocyantate (HMDI). The activated PVA was immersed in type I collagen solution (porcine skin, 0.5 mg/ml, Nitta Gelatin Co Ltd, Osaka, Japan) to immobilize the collagen on the surface of the PVA. PVA-COL was then washed successively with phosphate buffered saline (PBS) using an ultrasonic cleaner for 10 min in order to remove the absorbed collagen, and sterilized by UV irradiation. Amount of collagen immobilized was determined by the bicinchoninic acid (BCA) protein assay by measuring the absorbance (562 nm) with a multi-plate reader, GENios, TECAN Japan Co. Ltd, Japan. From the result, it was found that approximately 0.5 $\mu g/cm^2$ of collagen was covalently immobilized on the surface.

Human AM were obtained as previously described after written informed

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consents were obtained[13]. Briefly, AM from cesarean sections were processed and stored in 15 % DMSO in saline buffer at -30 °C for several weeks. AM were thawed and the spongy layer was surgically removed. AM were then treated with 10 % ammonia water in PBS at room temperature (RT) for 15 min, and AM epithelium was removed from by cell scraper. After washing in PBS, AM were cut into segments of approximately 2 cm x 2 cm, air-dried and stored in -30 °C until use.

Immobilization of AM onto resulting PVA-COL was performed by the following procedure. First, AM swollen in PBS was placed on a glass plate. PVA-COL was then fixed on the AM using a tissue adhesive consisting of collagen and citric acid derivative (CAD) as a crosslinker[18]. The concentration of alkali-treated collagen and CAD was fixed at 30 w/w% and 100 mM, respectively. The reaction was continued for 24 hrs at 4°C. The AM immobilized PVA hydrogels (PVA-AM) were then washed with excess PBS in order to remove DMSO in the tissue adhesive. PVA-AM were arranged into a disk of 10 mm in diameter for use in cell culture experiments.

Rabbit corneal epithelium on PVA-AM

Air-lift cell culture

PVA-AM and PVA-COL were placed on a culture insert (Transwell clear, Corning, Corning, NY) in 6 well plates, which mitomycin C-treated 3T3 feeder cells (2.4-5x 10^4 / cm^2) were seeded on the bottom. Rabbit limbal segments, which contain corneal epithelial stem cells, were treated with Dispase II (1 U / ml, 37 °C, 60 min, Roche Diagnostics GmbH, Mannheim, Germany) to separate epithelium from stroma, and subsequently treated with trypsin EDTA (0.05%, 37°C, 30 min). Dissociated cells were poured into the cloning ring (10^5 cells / cm^2), which was placed on the PVA-AM

(n=6) and PVA-COL polymer (n=14). After cells reached confluency, the volume of

the medium was reduced until the epithelial cells were placed at the air-liquid interface.

The culture medium used was a 1:1 mixture of Dulbecco's modified Eagle medium

and Ham's F12 medium (DMEM/F12, Gibco BRL, Rockville, MD) containing 15 %

fetal bovine serum, and supplemented with insulin (5 ng/ml, Sigma-Aldrich, St. Louis,

MO), cholera toxin (0.1 µg/ml, EMD Biosciences, San Diego, CA), human

recombinant epidermal growth factor (10 ng/ml, Gibco BRL) dimethyl sulfoxide

(0.5 %, Sigma-Aldrich), penicillin (0.7 mg/ml, Wako Pure Chemical Industries, Osaka,

Japan), and streptomycin (1.39 mg/ml, Wako). The culture medium is a modified

version of the SHEM medium originally reported by Jumblatt et al [19].

Immunohistochemistry

Immunohistochemistry against kerain3 and collagen IV were done using

paraffin sections according to the AMeX method [20]. Samples were fixed in acetone at

4 °C overnight, immersed in methyl benzoate at RT for 1 h (x2), and with xylene at RT

for 1 h (x2). After incubation in 60 °C paraffin for 1.5 h (x2) and over night once,

samples were embedded in paraffin. AMeX paraffin sections were dewaxed, immerse in

acetone to remove xylene, and rinsed in PBS. Sections were treated with protease XXV

(Neomarkers, Lab Vision corporation, Fremont, CA) at 37 °C for 5 to 10 min for the

immnostaining of collagen type IV. For immunohistochemistry against other proteins,

samples were embedded in OCT compound and frozen in liquid nitrogen.

After washing, sections were blocked with PBS containing 10% normal donkey

serum and 1% BSA, and treated with anti-keratin 3 (AE5, 0.2 µg/ml, Progen Biotechnik

GmbH, Heidelberg, German), anti-occludin (clone OC-3F10, 10 µg/ml, Zymed

laboratories Inc, South San Francisco, CA), anti-collagen type IV (clone IV-3A9, 10

µg/ml, Daiichi Fine Chemical Co Ltd, Toyama, Japan), and anti-connexin43 (1.0 µg/ml,

Chemicon International Inc, Temecula, CA) mouse monoclonal antibodies at 4 °C over

night. After washing, sections were treated with FITC-conjugated donkey anti-mouse

IgG antibody (28 µg/ml, Jackson ImmnoResearch Laboratories Inc, West Grove, PA) or

Cy3-conjugated donkey anti-mouse IgG antibody (Chemicon), and counterstained with

6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 µg/ml, Dojindo Laboratories,

Kumamoto, Japan). After mounting coverslip, fluorescence was detected by the Zeiss

Axioscop 2. Filter sets used were by Carl Zeiss 488001-0000-000 (Excitation filter

BP365/12, dichroic mirror FT395, barrier filter LP397) for DAPI, 488009-0000-000

(BP470/40, FT510, LP520) for FITC, and 488015-0000-000 (BP546/12, FT580,

LP590) for Cy3.

Intracorneal implants

PVA-AM (n=10) and PVA-COL (n=3) were transplanted into the eyes of

Japanese white rabbits (female, 3 kg body weight, Shiraishi Experimental Animal

Breeding Farm, Tokyo, Japan) one by one according to the ARVO statement for the

Use of Animals in Ophthalmology and Vision Research. Recipient animals were

anesthetized with 4 ml intramuscular ketamine and xylazine (1:7 mixture) and topical

xylocain.

A pocket was made in rabbit corneal stroma from the limbus and a segment of

cornea over the pockets was excised to create a window. PVA-AM and PVA-COL was

implanted into the pocket, and epithelialization over the PVA-AM and PVA-COL was

examined. All animals received topical antibiotics (levofloxacin) and steroids

(betamethasone) twice a day following surgery. Transplanted eyes were followed by slit lamp examination At 2, 3, 5 weeks after transplantation, epithelialization on the PVA-AM, after which animals were sacrificed for histology. The recipient cornea was fixed with 4 % paraformaldehyde in PBS at 4 °C overnight, and embedded in paraffin. Four to 6 micrometer sections were stained with hematoxylin and eosin (HE).

RESULTS

Rabbit corneal epithelium on PVA-AM

Air-lift cell culture

Stratified rabbit corneal epithelial cells were cultured on PVA-AM and PVA-COL using mitomycin-C treated 3T3 feeder cells (Fig. 1 A and B). A fully stratified epithelium was successfully engineered using PVA-AM, however, defects in the epithelial sheet were observed in approximately half of the PVA-COL polymers (Table.1).

Immunohistochemistry

Collagen type IV, a basement membrane component, was positive in cultured rabbit corneal epithelium on PVA-AM (Fig. 2A) and normal limbus of rabbit (Fig. 2B), but was negative in epithelial sheets cultivated on PVA-COL (fig. 2C). The anti-keratin 3 (K3) antibody AE5, a differentiation marker of the corneal epithelium, stained suprabasal layers on PVA-AM (Fig.3A) similar to PVA-COL (Fig.3B) and normal rabbit corneal epithelium (Fig.3C). Tight junction associated protein occludin was observed in the uppermost layer of the epithelium on PVA-AM (Fig. 3D). Normal rabbit control for occludin is shown in Fig.3E. PVA-AM multilayered corneal

epithelium expressed the gap-junctional protein connexin43, which was localized to the cell membrane of the basal epithelium (Fig. 3F) likewise normal rabbit corneal epithelium (Fig. 3G).

Intracorneal implants

PVA-AM and PVA-COL were transplanted into the rabbit stroma, and animals were sacrificed after 5 weeks. All 3 eyes transplanted with PVA-COL lost epithelium by 2 weeks (Fig.4A). On the other hand, of the 10 rabbits transplanted with PVA-AM, 5 eyes had partial epithelialization and 5 eyes had complete epithelialization at 2weeks (Fig.4B). Fluorescein exclusion stains showed that the epithelium was intact, and that the fluorescent dye did not diffuse into the stroma. Typical HE staining of PVA-COL at 2 weeks shows a defect in the stratified epithelium (Fig. 4C), while epithelialization on PVA-AM was intact (Fig. 4D). Rabbits were followed for up to 5 weeks, at which point rabbits with inflammation deveolped epithelial defects due to disintegration of the AM.

DISCUSSION

We have shown in our previous report that surface modified PVA (PVA-COL) can support a fully stratified corneal epithelium that express appropriate differentiation markers, as well as form a functional barrier at the apical junction [9]. However, cross-linking with type I collagen was not sufficient to form a normal basement membrane complex to allow firm attachment of epithelial cells *in vivo*. This also led to incomplete differentiation of cells in different layers of the epithelium. In the present study, we crosslinked human AM to PVA in order to resolve the shortages observed in

the PVA-COL prototype. As expected, PVA-AM supported a stratified epithelial layer, with a more differentiated morphology observed in each layer of stratification (Fig. 1). While the epithelial cells on PVA-COL were irregular in size and shape, cells supported by PVA-AM consisted of cuboid basal cells and flattened intermediate and superficial cells similar to the natural cornea. The proper orientation and polarity of cells is further evident by the maintenance of a basement membrane observed by anti type IV collagen immunohistochemistry (Fig. 2). The AM has its own proper epithelial cells in vivo, therefore, the basement membrane observed in our study may be of donor origin. However, it is clear that the epithelium overlying the AM is normal in morphology, suggesting that the basal cells are capable of producing additional basement membrane components as required.

The expression of the differentiation markers K3 and connexin was also observed in PVA-AM in a pattern very similar to the natural cornea. K3 is mainly observed in the superficial layers, while connexin 43 shows a reciprocal pattern of staining in the basal epithelial layer. The tight junctional protein, occludin, was also observed lining the superficial surface of the epithelial cell sheet. A functional tight junction following surgery is vital in protecting the ocular surface against invading organisms. The use of AM in ocular surface surgery was introduced by Kim et. al. [12], and has since proven to be useful in several aspects of reconstruction of the corneal structure [13]. Recent trends in regenerative medicine using tissue engineering techniques led to the clinical use of cultured epithelial sheet transplants using both carrier-free and AM carriers [15]. Our own clinical experience with cultivated AM sheets has produced clinical results comparable to a more conventional technique of corneal epithelial stem cell transplantation [21]. In this aspect, using AM as an artificial cornea components

poses few ethical or medical issues.

An AM epithelial sheet alone is too thin to serve as an artificial cornea. As shown in Fig.1, the AM-epithelium complex is less than 10% of the entire corneal thickness, which is approximately 500 µm in humans. The PVA-AM hybrid polymer can compensate for the thinness of AM alone, while maintaining a transparent medium. The presence of PVA beneath the epithelium does not hinder the transportation of glucose from the anterior chamber of the eye [9]. Furthermore, the additional thickness and material property of PVA will facilitate handling of the polymer during surgery. In our transplantation experiments, we found PVA-AM easy to maneuver and transplant to the cornea. Epithelium following surgery was intact for a longer period of time compared to the PVA-COL model reported previously. An intact epithelium that excludes fluorescein dye was observed for over 4 weeks following surgery. However, we found that some of the transplanted eyes developed epithelial defect towards the end of the observation period. Loss of AM was observed in eyes with epithelial defects. We have also experienced the disintegration of AM in some clinical cases with inflammation (unpublished observation).

Stabilizing the AM component of PVA-AM is still an issue remaining to be resolved. However, both *in vitro* differentiation of epithelial cells and *in vivo* stability was greatly enhanced compared to PVA-COL polymers. The PVA-AM hybrid polymer benefits from both the natural basement membrane components of AM tissue and the transparency and durability of the artificial PVA component. Improvements in cross-linking procedures and the use of anti-proteolytic therapy may improve in vivo results to a more clinically durable standard.

Acknowledgement

	Complete corneal Epithelium in culture	Multilayered corneal epithelium
PVA-AM	6/6　(100%)	3/6　(50%)
PVA-COL	6/14　(42.8%)	4/12　(33.3%)

Table. 1 Air lift cultured corneal epithelium on PVA-AM and PVA-COL

FIGURE LEGEND

Figure 1. HE staining of air-lift cultured stratified corneal epithelium.

Rabbit corneal epithelium on PVA-AM (A, enlarged in C) and on PVA-COL (B,

enlarged in D). Epithelium on PVA-AM shows a more normal cellular polarity

compared with epithelial cells on PVA-COL. Straight arrow: epithelium, dotted arrow:

amniotic membrane. Scale bar=100 µm in A and B, 25 µm in C, D.

Figure 2. Expression of the basal membrane component collagen type IV.

Rabbit corneal epithelium cultivated on PVA-AM (A) and normal rabbit cornea (B)

were positive for collagen type IV (red), while epithelium cultivated on PVA-COL (C)

was negative. (Blue=nuclear staining by DAPI). Scale bar =100 µm

Figure 3. Rabbit corneal epithelium on PVA-AM expressed differential markers.

Corneal epithelium-specific keratin 3 (K3) was expressed in epithelial cells cultivated

on PVA-AM (A) as well as on PVA-COL (B). (C) Normal rabbit cornea. Corneal

epithelium on PVA-AM expressed the tight junctional protein occludin (D) (arrow),

similar to normal rabbit cornea (E). PVA-AM multilayered corneal epithelium also

expressed the gap-junctional protein connexin43, which was localized to the cell

membrane of the basal epithelium (F) similar to normal rabbit corneal epithelium (G).

Scale bar=50 µm

Figure 4. Corneal epithelialization on transplanted PVA-AM and PVA-COL.

Corneal epithelium on PVA-COL (A) sloughed off by 2 weeks after transplantation.

However, epithelium on PVA-AM was intact after 2 weeks (B). HE staining of

PVA-COL at 2 weeks shows a defect in the stratified epithelium (C, arrow), HE

staining of PVA-AM at 2 weeks shows a healthy stratified epithelium (D). The

intra-lamellar space is an artifact of tissue fixation. (Arrow: epithelium, dotted arrow:

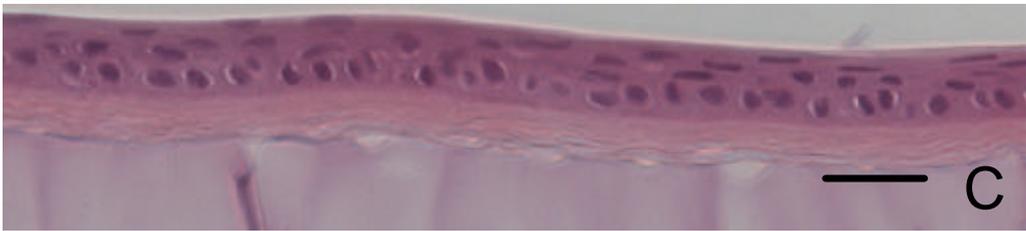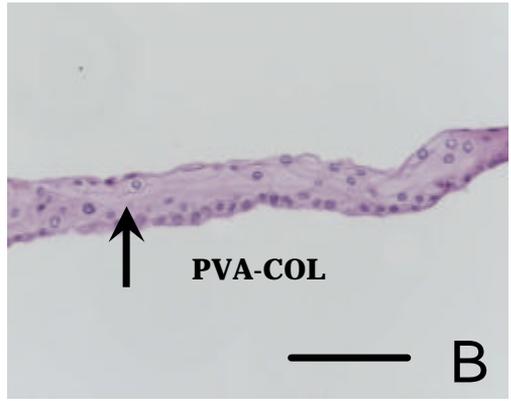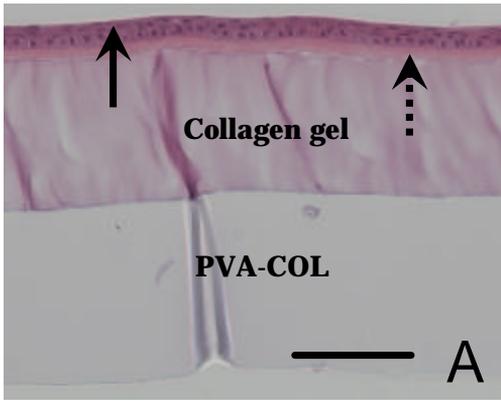amniotic membrane, open arrow: PVA-COL, curved arrow: collagen-gel). Scale
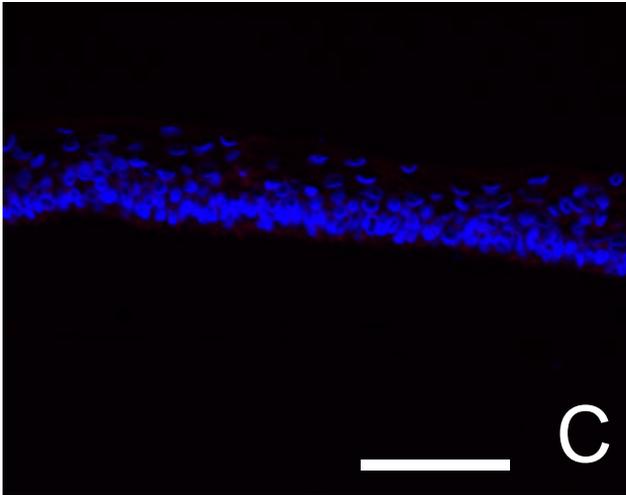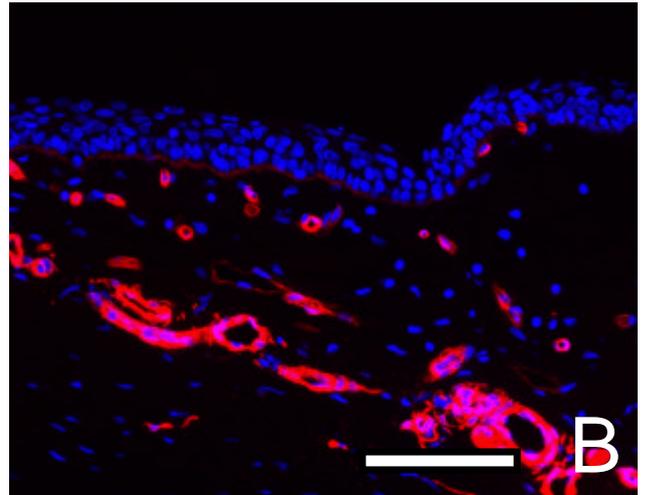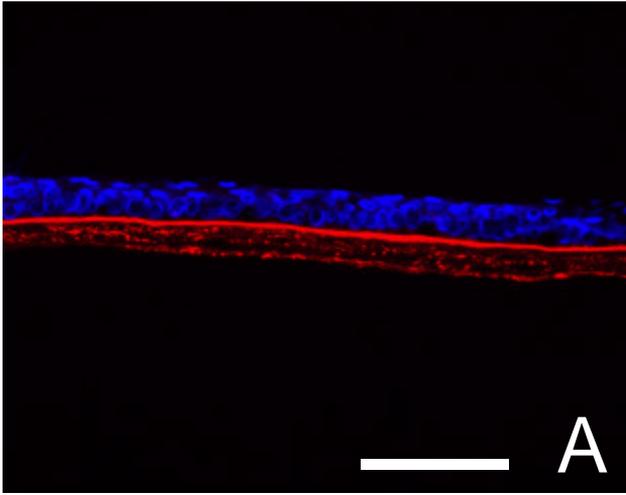
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A

Collagen gel

PVA-COL

B

PVA-COL

C

D

PVA-AM	rabbit
PVA-COL	