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Fabrication of a corneal model composed of corneal epithelial and endothelial cells via a collagen vitrigel membrane functioned as an acellular stroma and its application to the corneal permeability test of chemicals

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A list of nonstandard abbreviations:

AS, acellular-stroma; BM, Bowman's membrane; CEpi, corneal epithelium; CEpi-AS, corneal epithelium-acellular stroma; CEpi-AS-Endo, corneal epithelium-acellular stroma-endothelium; CEpi-Endo, corneal epithelium-endothelium; CVM, collagen vitrigel membrane; CXM, collagen xerogel membrane; DF-medium, 1:1 mixture medium of Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5 µg/ml recombinant human insulin, 10 ng/ml recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 units/ml penicillin and 100 µg/ml streptomycin; D-medium, Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 100 units/ml penicillin and 100 µg/ml streptomycin; FD, FITC-dextran; HBSS, Hank's balanced salt solution; O.C.T., Optimal Cutting Temperature;  $P_{app}$ , permeability coefficient; PBS, phosphate buffered saline; PET, polyethylene terephthalate; TEER-epi, trans-epithelial electrical resistance; TEER-end, Trans-endothelial electrical resistance

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## Abstract

A collagen vitrigel membrane (CVM) we developed can function as both a scaffold for cells and a pathway for chemicals. To extrapolate the corneal permeability of chemicals *in vivo*, we proposed six corneal models using the CVM. Thin and thick CVMs were utilized as models for Bowman's membrane (BM) and an acellular-stroma (AS), respectively. Models for a corneal epithelium (CEpi), a corneal epithelium-acellular stroma (CEpi-AS), a corneal epithelium-endothelium (CEpi-Endo) and a corneal epithelium-acellular stroma-endothelium (CEpi-AS-Endo) were fabricated by culturing corneal epithelial cells and/or corneal endothelial cells on the surface of CVMs. Subsequently, the permeability coefficient ( $P_{app}$ ) value of each model was calculated using five chemicals with different molecular radii; cyanocobalamin and four FITC-dextran (FD-4, FD-10, FD-20 and FD-40). The slopes of  $P_{app}$  versus molecular radii of those chemicals in the both BM and AS models were almost similar to data using an excised rabbit corneal stroma. The ratios of  $P_{app}$  values in models for BM, CEpi and CEpi-Endo against those in data using an excised rabbit cornea were calculated as 75.4, 6.4 and 4.5-folds for FD-4 and 38.7, 10.0 and 4.2-folds for FD-10, respectively. Similarly, those in models for AS, CEpi-AS and CEpi-AS-Endo were calculated as 26.1, 2.5 and 0.6-folds for FD-4 and 26.1, 1.5 and 0.6-folds for FD-10, respectively. These results suggest that the CEpi-AS-Endo model with both the barrier function of corneal cell layers and the diffusion capacity of chemicals in thick CVM is most appropriate for extrapolating the corneal permeability of chemicals *in vivo*.

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## Introduction

Corneal permeation studies required for ophthalmic drug development are generally performed by the test methods utilizing laboratory animals *in vivo* or *ex vivo* (Hahne et al., 2012; Dave et al., 2015). However, animal experiments have disadvantages such as ethical issues on the sacrifice of life, high costs, poor reproducibility and the questionable extrapolation of animal results to humans (Acheampong et al., 2002; Reichl and Becker, 2008; Baranowski et al., 2014; Scott et al., 2010; Pescina et al., 2015). To overcome these issues on animal experiments, a novel corneal model in cell culture systems *in vitro* appropriate for extrapolating the corneal permeability of chemicals *in vivo* is required for promoting the efficient development of eye drops.

Human cornea is made of three different tissues. Corneal epithelium, stroma and endothelium in the order from the outside is formed by about 6 corneal epithelial cell layers with barrier function, keratocytes scattered in high density-collagen fibrillar layers in approximately 500  $\mu\text{m}$  thick with diffusional inhibition and an endothelial cell monolayer with barrier function, respectively. The corneal permeability of chemicals is mainly dependent upon the barrier function of corneal epithelium and is collaterally regulated by the diffusion rate of corneal stroma and the barrier function of corneal endothelium (Reichl and Becker, 2008). Therefore, the single models of corneal epithelium (CEpi) and the combined models of corneal epithelium-stroma-endothelium in cell culture systems have been developed by utilizing various scaffolds. CEpi models exhibited multi-layer architecture like CEpi *in vivo* and revealed the formation of epithelial barrier function with rising trans-epithelial electrical resistance (TEER-epi) values. However, the CEpi

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models lack the effect of corneal stroma and endothelium. From this viewpoint, several corneal models composed of not only epithelium but also stroma and endothelium have been developed by utilizing three different types of corneal cells and unique scaffolds functioned as an artificial corneal stroma, e.g. crosslinked collagen-chondroitin sulfate (Griffith et al., 1999), an acellular corneal matrix derived from porcine (Xu et al., 2008) and a type I collagen gel (Reichl et al., 2004). Such corneal models *in vitro* well mimicked cornea *in vivo* in morphological and physiological features. Especially, the corneal model using the collagen gel showed the similar behavior with excised porcine cornea in the permeability of model drugs such as pilocarpine hydrochloride, befunolol hydrochloride, and hydrocortisone. However, none of the corneal models *in vitro* have been in widespread use as pharmaceutical routine works. One of the reasons is the limitation of producing same models due to disadvantages of previous scaffolds such as mechanical weakness, variation of animal-derived materials, complex preparation, etc. Therefore, the novel scaffold that allows the brief and reproducible fabrication of a corneal model is necessary for promoting the efficient development of eye drops.

A collagen vitrigel membrane (CVM) is a stable gel produced by rehydration after vitrification of a traditional collagen gel, and consequently it is formed of high density collagen fibrils comparable to connective tissues *in vivo*. The CVM is tough and transparent, and also bioactive chemicals with various molecular weight can penetrate it. Therefore, it is easily handled with forceps and functions as a scaffold to reconstruct useful tissues for the studies on regenerative medicine, drug developments and alternatives to experimental animals, etc. (Takezawa et al., 2004, 2007a, 2007b, 2007c). Moreover, we developed a

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mass fabrication technology of not only a thin CVM but also a thick one (Takezawa et al., 2012). We established a fabrication method of a CEpi model utilizing an air-liquid interface culture system. The culture system facilitates the induction of layering corneal epithelial cells cultured on the CVM scaffold that was prepared on a polyethylene terephthalate (PET) membrane of a Millicell chamber. (Takezawa et al., 2008, 2011). However, this model is unsuitable for immunohistological analyses by freeze sectioning due to the existence of hard PET membrane. Therefore, we developed a new chamber merely accompanying a CVM without the PET membrane and established its mass production process (Takezawa et al., 2012).

From a viewpoint of developing an ideal scaffold functioned as an artificial corneal stroma, the CVM has several advantages such as mechanical toughness, reproducibility and brief preparation. Moreover, the CVM is composed of 10 to 25% collagen fibrils equivalent to corneal stroma (Takezawa et al., 2007c; Reichl et al., 2008). In this study, we aimed to define an optimum corneal model involving the CVM scaffold appropriate for extrapolating the corneal permeability of chemicals *in vivo*. The CVM scaffolds with two different thicknesses of about 20  $\mu\text{m}$  as a replacement of Bowman's membrane and about 450  $\mu\text{m}$  as an acellular corneal stroma were designed, and subsequently six corneal models composed of merely a thin CVM, CEpi on a thin CVM, corneal epithelium-endothelium (CEpi-Endo) via a thin CVM, merely a thick CVM, CEpi on a thick CVM, and CEpi-Endo via a thick CVM were fabricated (Fig. 1). Then, the permeability coefficient of each model was calculated by using five model chemicals with different molecular radii and was compared with that of excised rabbit cornea.

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## Material and methods

**Antibodies and Reagents.** Rabbit polyclonal antibodies for ZO-1, occludin and connexin-43 were purchased from Thermo fisher scientific (Grand Island, NY), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Sigma-Aldrich (St Louis, MO, USA), respectively. Mouse monoclonal antibodies for cytokeratin 3, MUC1 and Na<sup>+</sup>-K<sup>+</sup> ATPase were purchased from Progen Biotechnik GmbH (Heidelberg, Germany), Sanbio B.V. (Uden, Netherlands) and Merck-Millipore (Darmstadt, Germany), respectively. A goat Alexa Fluor 555-conjugated secondary antibody for rabbit IgG and a goat Alexa Fluor 488-conjugated secondary antibody for mouse IgG were purchased from Thermo fisher scientific. Hoechst33342 was purchased from Dojindo Laboratories (Kumamoto, Japan). Normal goat serum was purchased from Sigma-Aldrich. Tissue-Tek Optimal Cutting Temperature (O.C.T.) Compound was purchased from Sakura Finetek Japan (Tokyo, Japan). Cyanocobalamin and FITC-dextran (fluorescein isothiocyanate labeled dextran with average molecular weight 4, 10, 20 and 40 kDa: FD-4, FD-10, FD-20 and FD-40) were purchased from Sigma-Aldrich.

**Preparation of CVM chambers.** A chamber (ad-MED Vitrigel<sup>TM</sup>) with a collagen xerogel membrane (CXM) containing 0.05 mg type-I collagen per 1.0 cm<sup>2</sup> was purchased from Kanto chemical Co., Inc. (Tokyo, Japan) in which CXM chambers were manufactured in accordance with our previous report (Takezawa et al., 2012). The CXM chamber was set in the well of a 12-well plate. Then, the CXM was



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immersed in a culture medium by pouring 1.5 ml outside and 0.5 ml inside the chamber in the well for 10 min to convert the xerogel into a vitrigel immediately before use, resulting the preparation of a chamber with a thin CVM of about 20  $\mu\text{m}$ -thickness. Here, the thin CVM of chamber can be used as a Bowman's membrane (BM) model because of the similarity of architecture and components. Regarding the chamber with a thick CVM of about 450  $\mu\text{m}$ -thickness (i.e. an acellular-stroma (AS) model), we custom-ordered a chamber with a CXM containing 1.125 mg type-I collagen per 1.0  $\text{cm}^2$  to Kanto chemical Co., Inc. in the similar procedure of ad-MED Vitrigel<sup>TM</sup>.

**Culture of HCE-T cells and BCD C/D-1b cells.** A SV40-immortalized human corneal epithelial cell strain (HCE-T cells; RCB #2280) was obtained from RIKEN BioResource Center (Tsukuba, Japan) (Araki-Sasaki et al., 1995). The cells were cultured and maintained in a 1:1 mixture medium of Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 supplemented with 5% heat-inactivated fetal bovine serum, 5  $\mu\text{g}/\text{ml}$  recombinant human insulin, 10  $\text{ng}/\text{ml}$  recombinant human epidermal growth factor, 0.5% dimethyl sulfoxide, 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (DF-medium) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air.

An immortalized bovine corneal endothelial cell line (BCD C/D-1b cells: ATCC CRL-2048) was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured and maintained in a Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (D-medium) at 37°C in a

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humidified atmosphere of 5% CO<sub>2</sub> in air.

#### **Isolation and characterization of an endothelial cell-like clone from BCD C/D-1b cells. BCD**

C/D-1b cells suspended in the D-medium were seeded and cultured in a 96-well cell culture plate at a density of 0.5cells/well. Several endothelial cell-like clones were morphologically selected by phase-contrast microscopy. Each clone was expanded to about  $1 \times 10^7$  cells and preserved in liquid nitrogen. One clone representing excellent proliferative performance was subjected to the following experiment. The clonal cells recovered from liquid nitrogen were defined as passage 1 and were sub-cultured until passage 28 to estimate their morphological stability.

To confirm the properties essential for the fabrication of a corneal endothelial layer, the endothelial cell-like clone was cultured in the chamber with a thin CVM and subjected to the time-dependent analyses for cell morphology, protein expression and barrier function. The clonal cells suspended in 0.5 ml of the D-medium at a density of  $2.0 \times 10^5$  cells/ml were seeded onto the thin CVM of chamber pre-set in a well of a 12-well plate with 1.5 ml of the fresh medium in the well and cultured for 7 days. The media were changed at day 1, 4 and 7. The cell morphology and immuno-histology for ZO-1 and Na<sup>+</sup>-K<sup>+</sup> ATPase protein expressions were observed at day 1, 3 and 7 by a phase-contrast microscope (TE300; Nikon, Tokyo, Japan) and a laser scanning confocal microscope (FV1000; Olympus, Tokyo, Japan), respectively. Also, the barrier function was analyzed by measuring the electrical resistance values of each CVM chamber before ( $R_{\text{blank}}$ ) and after ( $R_{\text{sample}}$ ) culturing the cells at day 0 (2 hours), 1, 2, 3, 4 and 7 using an electrical

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resistance meter (Kanto Chemical). Trans-endothelial electrical resistance (TEER-end) value was calculated by using the following formula:  $TEER\text{-end} = (R_{\text{sample}} - R_{\text{blank}}) \times \text{effective surface area}$

Here, the effective surface area of CVM chamber was 1.0 cm<sup>2</sup>.

**Fabrication of six corneal models.** We prepared six corneal models as shown in Fig. 1. The preparation of BM model and AS model were described above. Corneal epithelium (CEpi) model, corneal epithelium-endothelium (CEpi-Endo) model, corneal epithelium-acellular stroma (CEpi-AS) model and corneal epithelium-acellular stroma-endothelium (CEpi-AS-Endo) model were fabricated as follows.

In the fabrication of CEpi model, HCE-T cells suspended in 0.5 ml of the DF-medium at a density of  $1.2 \times 10^5$  cells/ml were seeded onto the thin CVM (i.e. a BM model) of chamber pre-set in a well of a 12-well plate with 1.5ml of the fresh medium in the well and the cells were cultured for 2 days. Subsequently, the exterior medium of chamber was changed and the interior medium of it was removed to start the additional culture under the air-liquid interface for 4 days. The exterior medium of chamber was changed on the third day in the additional culture. Also, the fabrication of CEpi-AS model was achieved by using the thick CVM (i.e. an AS model) of chamber in the above cell seeding process.

In the fabrication of CEpi-Endo model, HCE-T cells were first cultured for 3 days in the same procedure for fabricating the CEpi model. Subsequently, the CVM chamber was transferred to a well of 6-well plate and flipped upside down in order to make a reverse-side compartment for cell culture by fixing a plastic

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cylinder with an appropriate size (e.g. inner-outer diameter: 11-16 mm; length: 8.5 mm) onto the bottom of CVM chamber. The endothelial cell-like clonal cells derived from BCD C/D-1b cells suspended in 0.5 ml of the D-medium at a density of  $2.0 \times 10^5$  cells/ml were seeded into the reverse-side compartment. The clonal cells were cultured for 2 hours to induce the sufficient adhesion to the CVM, resulting the co-culture with HCE-T cells via the CVM. Subsequently, the endothelial cell medium was removed and the cylinder was detached from the CVM chamber. Then, the CVM chamber was set in a well of a 12-well plate with 1.5ml of the DF-medium and both cells were co-cultured for additional 3 days under air-liquid interface. Also, the fabrication of CEpi-AS-Endo model was achieved by using the thick CVM (i.e. an AS model) of chamber in the above cell seeding process.

All models were subjected to histology and permeability examinations.

**Histology and immunohistology.** For clarifying morphological characteristics, corneal endothelial layers in a CVM chamber at day 1, 3 and 7 and three corneal models (CEpi-Endo model, CEpi-AS model and CEpi-AS-Endo model) except for the CEpi model as previously reported (Yamaguchi et al., 2013) were isolated from the plastic cylinder of the CVM chamber using an appropriate disposable biopsy punch and fixed for 5 min in methanol kept on ice immediately after sufficiently chilling it at  $-45^{\circ}\text{C}$ .

The fixed corneal endothelial layers were washed with phosphate buffered saline (PBS) three times. Subsequently, they were incubated with PBS containing 1% normal goat serum for 30 min to block non-specific adsorption of antibodies. Then, the first antibodies against ZO-1 or  $\text{Na}^+\text{-K}^+$  ATPase prepared

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in PBS containing 1% normal goat serum at a concentration of 5 µg/ml were applied and incubated for 16 hours at 4°C, followed by washing them with PBS three times. The secondary antibodies against rabbit IgG or mouse IgG prepared in PBS containing 1% normal goat serum at a concentration of 4 µg/ml were applied and incubated for 3 hours at room temperature, followed by washing them with PBS three times. Subsequently, cell nuclei were counterstained with Hoechst33342. Stained cells were observed by a laser scanning confocal microscope (FV1000; Olympus, Tokyo, Japan).

The fixed corneal models were embedded in an O.C.T. Compound after removing the excessive methanol around them with an absorbent paper towel, frozen in liquid nitrogen, and stored at -80°C. The samples were vertically cut into cross-sections with a thickness of 5 µm against the CVM using a cryostat (CM3050S; Leica Microsystems, Wetzlar, Germany). The frozen sections spread on a glass-slide were dried out for 60 min at room temperature. For histology, the sections were immersed in water for 5 min to remove the O.C.T. Compound, stained with hematoxylin and eosin, and observed by a light microscope (E600; Nikon, Tokyo, Japan). For immunohistology, the sections derived from CEpi-AS model were subjected to immunostaining using the first antibodies against ZO-1, occludin, connexin-43, cytokeratin 3, or MUC1 and counterstaining using Hoechst33342 in a similar procedure mentioned above.

**Calculation of permeability coefficient.** All 6 corneal models were subjected to the permeability test using the following 5 test chemicals; cyanocobalamin, FD-4, FD-10, FD-20 and FD-40 representing approximate 8.5, 14, 23, 33 and 45 Å in Stokes' radius, respectively. Also, a commercially available

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Millicell hanging cell culture insert possessing a multi-porous PET membrane with a pore size of 1.0  $\mu\text{m}$  (MCRP12H48; Merck Millipore, MA, USA) were subjected to the permeability test as a control chamber with a common scaffold for fabricating corneal models. Each corneal model and Millicell control were set in the well of 12-well plate, and subsequently 1.8 and 0.5 ml of Hank's balanced salt solution (HBSS) were poured in the outside and inside of chamber, respectively. After incubating the plate for 10 min at 37 °C, the inside HBSS of each chamber was changed to the test chemical solution, a same volume of HBSS containing 10  $\mu\text{g/ml}$  cyanocobalamin, 5  $\mu\text{g/ml}$  FD-4, 5  $\mu\text{g/ml}$  FD-10, 5  $\mu\text{g/ml}$  FD-20 or 5  $\mu\text{g/ml}$  FD-40. To quantitatively analyze the time-dependent penetration of test chemicals from the inside to the outside of chamber, 100  $\mu\text{l}$  of HBSS in the outside of chamber was sampled and the same volume of fresh HBSS was returned there at every 10 min from the starting point immediately after adding test chemical solution to the ending point on the elapsed time of 180 min. The concentration of cyanocobalamin or FDs in each sample was analyzed by measuring the absorbance at 365 nm using an absorbance multi plate reader (VERSA max; Molecular Devices, Sunnyvale, CA) or the fluorescent intensity at 490 nm (excitation) and 520 nm (emission) using a fluorescence multi plate reader (SPECTRAMAX GEMINI XS; Molecular Devices), respectively.

The permeability coefficients of test chemicals in each model were calculated using the following formula:

$$P_{\text{app}} = dQ/dT \times 1/A \times 1/C_0$$

Here,  $P_{\text{app}}$ ,  $dQ/dT$ ,  $A$  and  $C_0$  represent the permeability coefficient (cm/s), the flux ( $\mu\text{g/s}$ ), the surface area

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of each model (cm<sup>2</sup>) and the initial chemical concentration (μg/mL), respectively.

The  $P_{app}$  values of FD-4 and FD-10 in excised male rabbit corneas are reported as  $0.056 \times 10^{-6}$  and  $0.031 \times 10^{-6}$  cm/s, respectively (Sasaki et al., 1995). The ratios of  $P_{app}$  values for FD-4 and FD-10 in all 6 corneal models against those in the report using excised rabbit corneas were calculated by dividing each  $P_{app}$  value of the former by that of the latter. Here, the ratio of 1.0 represents an ideal model equivalent for excised rabbit cornea. Next, the contribution of epithelium and endothelium in 6 corneal models were analyzed by the concept for reduction degree when the  $P_{app}$  values of CEpi (or CEpi-AS) models were compared with those of BM (or AS) models and when the  $P_{app}$  values of CEpi-Endo (or CEpi-AS-Endo) models were compared with those of CEpi (or CEpi-AS) models, respectively. Here, the reduction degree for epithelium was calculated using the following formula:

$$\text{Reduction degree for epithelium (\%)} = \{1 - (P_{app} \text{ of CEpi model}) / (P_{app} \text{ of BM model})\} \times 100 \text{ or } \{1 - (P_{app} \text{ of CEpi-AS model}) / (P_{app} \text{ of AS model})\} \times 100$$

Similarly, the reduction degree for endothelium was calculated using the following formula:

$$\text{Reduction degree for endothelium (\%)} = \{1 - (P_{app} \text{ of CEpi-Endo model}) / (P_{app} \text{ of CEpi model})\} \times 100 \text{ or } \{1 - (P_{app} \text{ of CEpi-AS-Endo model}) / (P_{app} \text{ of CEpi-AS model})\} \times 100$$

## Results

### **Isolation and characterization of an endothelial cell-like clone from BCD C/D-1b cells.** BCD

C/D-1b cells before cloning grew and formed monolayer in a culture dish. However, the individual

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cellular shapes were not uniform polygon but different varieties such as small round, cobblestone, spindle and hypertrophy. Also, some interstices were observed especially around the fibroblast-like cells even in the confluent stage (Fig. 2A). Several endothelial cell-like clones showing the morphology of cobblestone were successfully isolated from the BCD C/D-1b cells by the limiting dilution method. One clone representing excellent proliferative performance was subjected to the following experiment. The endothelial cell-like clonal cells at passage 1 formed a confluent monolayer and the individual cellular shapes represented cobblestone (Fig. 2B). Also, the clonal cells at passage 28 showed the same morphological properties as that at passage 1 (Fig. 2C), suggesting that the clonal cells could maintain the morphological stability for the following experimental period.

The clonal cells cultured in a CVM chamber for 1 day formed a confluent monolayer with loose cell-to-cell communications in which the individual cellular shapes represented non-uniform cobblestone (Fig. 3A). The cells expressed  $\text{Na}^+\text{-K}^+$  ATPase that is active transporter known as a marker of corneal endothelium (Fig. 3D), however they rarely did ZO-1 that is tight junction-associated protein (Fig. 3G). Meanwhile, the cells on day 3 fabricated a confluent monolayer with tight cell-to-cell communications in which the individual cellular shapes represented hexagon and they well expressed not only  $\text{Na}^+\text{-K}^+$  ATPase but also ZO-1 (Fig. 3B, 3E and 3H). Subsequently, the cells on day 7 showed the more uniform thickness of a corneal endothelial layer with expressions of  $\text{Na}^+\text{-K}^+$  ATPase and ZO-1 (Fig. 3C, 3F and 3I).

Also, the TEER-end value of the clonal cells cultured in a CVM chamber increased for initial 3 days with significant differences, and subsequently it showed the nearly constant without significant differences



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until day 7 (Fig. 4).

These data suggest that a corneal endothelial layer with barrier function was fabricated by culturing the clonal cells in a CVM chamber for 3 days.

**Histological and immunohistological characterization of corneal models.** Histological and immunohistological characteristics of four corneal models (CEpi model, CEpi-AS model, CEpi-Endo model, and CEpi-AS-Endo model) were compared.

As previously reported (Yamaguchi et al., 2013), the CEpi model on a thin CVM possessed about six layers of HCE-T cells with barrier function showing the TEER-epi value of about  $60 \Omega \cdot \text{cm}^2$  and resembled human corneal epithelium in the expression pattern of five corneal epithelium-associated proteins, ZO-1, occluding, connexin-43, cytokeratin 3 and MUC1. Also, the CEpi-AS model possessed about six layers of HCE-T cells on a thick CVM (Fig. 5A). The uppermost layer was covered with extremely flattened cells and the other layers were mostly composed of squamous cells (Fig. 5B). Regarding the five corneal epithelium-associated proteins, ZO-1 and occludin that are tight junction-associated proteins were abundantly expressed in the lateral and basal surfaces of cells in the superficial layer in comparison to the other layers (Fig. 5C, D). Connexin-43 consisting gap junctions and cytokeratin 3 that is a type II cytokeratin in corneal epithelium were expressed in the membrane and cytoplasm of cells in all layers, respectively (Fig. 5E, F). MUC1 that is a cell membrane-spanning mucin was merely expressed in the apical surface of cells in the superficial layer (Fig. 5G). These data demonstrated that the epithelial

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region of CEpi-AS model as same as the CEpi model resembled HCE *in vivo*.

The CEpi-Endo model and the CEpi-AS-Endo model exhibited the architectures composed of about six cell layers of HCE-T cells and monolayer of endothelial cell-like clonal cells derived from BCD C/D-1b cells via thin and thick CVMs, respectively (Fig. 6). The histological data demonstrated that the CEpi-AS-Endo model well reflected the vertical structure of human cornea *in vivo*.

**Permeability coefficient of test chemicals in corneal models.** The  $P_{app}$  values of cyanocobalamin, FD-4, FD-10, FD-20 and FD-40 in the six corneal models were calculated as shown in Table 1. The  $P_{app}$  values of cyanocobalamin in CEpi-Endo, CEpi-AS and CEpi-AS-Endo models cannot be calculated because its concentration in each sample was lower than the detection limit.

In the comparison of acellular models with a common scaffold for fabricating corneal models, the slopes of permeability coefficients versus molecular radii of test chemicals in BM model, AS model and commercially available multi-porous PET membrane were -2.64, -2.04 and -0.84, respectively (Fig. 7). These results reveal that the  $P_{app}$  value corresponding for each test chemical in the both models exponentially decreases as its molecular radius increases although that in the multi-porous PET membrane slightly does. In addition, the  $P_{app}$  values of cyanocobalamin, FD-4, FD-10 and FD-20 in the AS model were significantly low in comparison to that in the BM model, suggesting that the permeability coefficient of test chemicals was regulated by the thickness of CVM used for each model.

In the comparison of culture models with an excised rabbit cornea, the  $P_{app}$  values of FD-4 and FD-10 in

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the models were reduced in order of CEpi model, CEpi-Endo model, CEpi-AS model and CEpi-AS-Endo model. Also, the ratios of  $P_{app}$  values in models for BM, CEpi and CEpi-Endo against those in data using an excised rabbit cornea were calculated as 75.4, 6.4 and 4.5-folds for FD-4 and 38.7, 10.0 and 4.2-folds for FD-10, respectively. Similarly, those in models for AS, CEpi-AS and CEpi-AS-Endo were calculated as 26.1, 2.5 and 0.6-folds for FD-4 and 26.1, 1.5 and 0.6-folds for FD-10, respectively. Meanwhile, the effect of epithelium and endothelium on the reduction degree of  $P_{app}$  values was 90.4 % and 76.4% for FD-4 and 94.3% and 58.7% for FD-10, respectively. In particular, there was no significant difference between the  $P_{app}$  value of CEpi-AS-Endo model and that of excised rabbit cornea (Fig. 8). These results reveal that the architecture composed of not only both epithelium and endothelium but also acellular stroma is essential for fabricating a culture model equivalent to the excised rabbit cornea.

## Discussion

The corneal permeability of drugs *in vivo* is mainly dependent upon the barrier function of epithelial cell layers on Bowman's membrane and is collaterally regulated by the diffusion capacity in stromal matrix and the barrier function of an endothelial layer on Descemet's membrane (Reichl et al., 2008). Here, the main component of Bowman's membrane, stromal matrix and Descemet's membrane is a network of high density collagen fibrils. In the current study, we focused on the network of high density collagen fibrils functioned as not only a scaffold for cells but also a pathway for drugs and newly prepared a thick CVM equivalent to acellular stroma in addition to a previous thin CVM equivalent to Bowman's membrane.

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Based on this concept, six corneal models possessing matrix-dependent diffusion capacity and/or cell layer(s)-dependent barrier function were fabricated by utilizing thin and thick CVMs, corneal epithelial cells and endothelial cells; BM model, CEpi model as previously described (Yamaguchi et al., 2013), CEpi-Endo model, AS model, CEpi-AS model and CEpi-AS-Endo model. Subsequently, an optimal corneal model *in vitro* useful for extrapolating the corneal permeability was determined.

The CVM can play a role of not only a bifacial scaffold in three-dimensional cell culture but also a pathway for proteins with a wide range of molecular weight (Takezawa et al., 2007a, 2007b). From this viewpoint, we proposed six corneal models using chambers accompanying thin and thick CVMs (Fig.1). The CVM potential as a scaffold for fabricating four culture models was examined as follows. First, we confirmed that the thin CVM provided an appropriate scaffold for corneal endothelial cell-like clonal cells and consequently that a corneal endothelium with barrier function and specific protein expressions was well reconstructed by culturing the clonal cells on it for at least 3 days (Fig. 2-4). Next, we fabricated 4 culture models as follows. A CEpi model with barrier function and specific protein expressions was prepared by culturing HCE-T cells on the thin CVM of chamber for 6 days (data not shown) as previously reported.<sup>21</sup> According to this fabrication procedure, a CEpi-AS model was prepared by culturing HCE-T cells on the thick CVM of chamber for 6 days. Subsequently, the model was confirmed to exhibit both epithelial barrier function (data not shown) and specific protein expressions (Fig. 5). Also, a CEpi-Endo model and a CEpi-AS-Endo model were prepared by culturing HCE-T cells in the thin and thick CVM chambers for 2 days and subsequently co-culturing corneal endothelial cell-like clonal cells on the opposite

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surface of CVMs for 4 days, respectively (Fig. 6). These data suggest that the CVM chambers in comparison to the traditional scaffolds are appropriate for mass-production with high reproducibility and can provide an easy handling in cell culture manipulation and a short-term fabrication of not only tissue-type but also organ-type culture models.

The CVM potential as a pathway for penetrating five test chemicals was examined as follows. We calculated the  $P_{app}$  values in both acellular models and culture models from the viewpoint of the diffusion capacity in a network of high density collagen fibrils and the barrier function of cell layer(s), respectively (Table 1). First, in the acellular models, the slopes of permeability coefficients versus molecular radii of test chemicals in BM model, AS model and commercially available multi-porous PET membrane were investigated (Fig. 7). Meanwhile, the slope value in the excised rabbit corneal stroma was calculated as -2.52 from the previous report describing the  $P_{app}$  values of four test chemicals; phenylephrine, acebutolol, hemoglobin and albumin representing approximate 4.0, 5.0, 31 and 35 Å in Stokes' radius, respectively. (Prausnitz and Noonan., 1998). This work demonstrated that the  $P_{app}$  values decrease as the molecular radii increase in the excised rabbit corneal stroma. Also, our current data revealed that the  $P_{app}$  values decrease as the molecular radii increase in both a BM model and an AS model, and consequently their slope values were -2.64 and -2.04, respectively. Meanwhile, the slope of a multi-porous PET membrane was -0.84. This calculation demonstrated that the slope in the excised rabbit corneal stroma almost matched both a BM model and an AS model although it was far from that of a multi-porous PET membrane. These findings suggest that acellular models utilizing CVM chambers can be utilized as

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alternatives to the excised rabbit corneal stroma due to their similarity in the diffusion rate of chemicals.

Next, in the culture models, we compared the  $P_{app}$  values of four culture models with that of an excised rabbit cornea to clarify the contribution of barrier function based on cell layers. Consequently, the ratios of  $P_{app}$  values of FD-4 in a BM model, a CEpi model and a CEpi-Endo model against an excised rabbit cornea were calculated as 75.4-fold, 6.4-fold, and 4.5-fold, respectively. Also, those of FD-10 were calculated as 38.7-fold, 10.0-fold, and 4.2-fold, respectively. These calculations mean that the effect of epithelium and endothelium on the reduction degree of  $P_{app}$  values is 91.5 % and 30.6% for FD-4 and 74.2% and 58.1% for FD-10, respectively. Similarly, those of FD-4 in an AS model, a CEpi-AS model and a CEpi-AS-Endo model against an excised rabbit cornea were calculated 26.1-fold, 2.5-fold and 0.6-fold, respectively. Also, those of FD-10 were calculated as 26.1-fold, 1.5-fold and 0.6-fold, respectively. Meanwhile, the effect of epithelium and endothelium on the reduction degree of  $P_{app}$  values is in the range of 74.2-94.3% and 30.6-76.4%, respectively. This suggests that both epithelial and endothelial cell layers contribute to the regulation of the permeability coefficient although the former effect is larger than the latter. Moreover, the  $P_{app}$  values of FD-4 and FD-10 in not a CEpi-Endo model but a CEpi-AS-Endo model were very close to those of an excised rabbit cornea (Fig. 8). These data suggest that not only the barrier function of both epithelial and endothelial cell layers but also the diffusion capacity in a network of high density collagen fibrils is indispensable to fabricate corneal models *in vitro* appropriate for extrapolating the corneal permeability of chemicals *in vivo*. The advantages of our CEpi-AS-Endo models utilizing CVM scaffold are that the fabrication period is short and the handling in

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permeability assay is easy in comparison to the traditional *in vitro* corneal models utilizing fragile scaffold (Reichl et al., 2004; Xu et al., 2008; Rönkkö et al., 2016). To investigate the feasibility of our CEpi-AS-Endo models in comparison to the traditional models *in vitro*, however, further studies using candidate chemicals for ophthalmic drugs are essential in not only corneal permeability test but also metabolic assay.

In this study, we fabricated a novel corneal model utilizing corneal epithelial cells, endothelial cells and a CVM functioned as both a bifacial scaffold and a pathway for chemicals, and subsequently the permeability of test chemicals with different molecular size were analyzed to evaluate the correlation with the data on an excised rabbit cornea. Consequently, we succeeded in developing a new technology for extrapolating the corneal permeability of test chemicals. Therefore, we hope that this study would provide a new concept for fabricating an artificial cornea as a research tool useful for the development of ophthalmic drugs.

#### Authorship Contributions

Participated in research design: Takezawa.

Conducted experiments: Yamaguchi.

Performed data analysis: Yamaguchi and Takezawa.

Wrote or contributed to the writing of the manuscript: Yamaguchi and Takezawa.

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#### Footnotes

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## Figure Legends

FIG. 1. Schematic illustration of six corneal models using chambers with thin and thick CVM scaffolds.

Bowman's membrane model (A), corneal epithelium model (B), corneal epithelium-endothelium model (C), acellular stroma model (D), corneal epithelium-acellular stroma model (E), and corneal epithelium-acellular stroma-endothelium model (F). Black lines represent a plastic cylinders and hangers of a CVM chamber.

FIG. 2. Phase-contrast microscopic observations of BCD C/D-1b cells. BCD C/D-1b cells before cloning (A), endothelial cell-like clonal cells at passage number 1 (B) and 28 (C) after cloning were cultured in a tissue culture plate. Scale bars represent 50  $\mu$ m.

FIG. 3. Microscopic observations of a corneal endothelial layer in a CVM camber. The endothelial cell-like clonal cells from BCD C/D-1b cells were cultured for 1 day (A, D and G), 3 days (B, E and H), and 7 days (C, F and I). The clonal cells were observed with a phase-contrast microscope (A-C), and were stained with antibodies for Na<sup>+</sup>-K<sup>+</sup> ATPase (D-F) and ZO-1 (G-I). Nuclei of cells were counterstained with Hoechst 33342 (D-I). Scale bars represent 50  $\mu$ m.

FIG. 4. Time-dependent change of TEER-end values of a corneal endothelial layer in a CVM camber.

Each value represents the mean  $\pm$  SD (n=3). \*P<0.05.

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FIG. 5. Microscopic observations of a corneal epithelium-acellular stroma model. Cross-sections of the model were stained with hematoxylin and eosin (A, B) and were also stained with antibodies for ZO-1 (C), occludin (D), connexin-43 (E), cytokeratin 3 (F) and MUC1(G). Nuclei of cells were counterstained with Hoechst 33342 (C-G). Scale bars represent 50  $\mu$ m.

FIG. 6. Microscopic observations of corneal models composed of corneal epithelial cells and endothelial cells via a CVM. Cross-sections of the corneal epithelium-endothelium model (A) and the corneal epithelium-acellular stroma-endothelium model (B) were stained with hematoxylin and eosin. Scale bars represent 50  $\mu$ m.

FIG. 7. Comparison among the permeability coefficient of a Bowman's membrane (BM) model (A), that of an acellular stroma (AS) model (B) and that of a commercially available multi-porous PET membrane (C). Each permeability coefficient was tested using Cyanocobalamin, FD4, FD10, FD20 and FD40. Dashed lines represent slopes calculated by the least-square. Each values represents the mean  $\pm$  SD (n=3).  
\*P<0.05.

FIG. 8. Comparison between the permeability coefficient of FD-4 (A) and FD-10 (B) in each corneal model reconstructed in CVM chamber described in Table 1 and that in excised rabbit cornea. Each value

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represents the mean value  $\pm$  SD (n=3). \*P<0.1, \*\* P<0.05, \*\*\* P<0.01, \*\*\*\* P<0.001.



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TABLE 1

Permeability coefficient of six corneal models

Type of model		Permeability coefficient <sup>a</sup> ( $\times 10^{-6}$ cm/s)				
		Cyanocobalamin	FD-4	FD-10	FD-20	FD-40
Acellular model	BM	$9.38 \pm 0.47$	$4.22 \pm 0.18$	$1.20 \pm 0.31$	$0.46 \pm 0.06$	$0.12 \pm 0.002$
	AS	$4.45 \pm 0.32$	$1.46 \pm 0.14$	$0.81 \pm 0.11$	$0.24 \pm 0.03$	$0.17 \pm 0.03$
Culture model	CEpi	$0.82 \pm 0.097$	$0.36 \pm 0.01$	$0.31 \pm 0.02$	$0.36 \pm 0.06$	$0.054 \pm 0.01$
	CEpi-Endo	Not detected	$0.25 \pm 0.04$	$0.13 \pm 0.02$	$0.084 \pm 0.01$	$0.050 \pm 0.01$
	CEpi-AS	Not detected	$0.14 \pm 0.06$	$0.046 \pm 0.07$	$0.009 \pm 0.002$	$0.017 \pm 0.009$
	CEpi-AS-Endo	Not detected	$0.033 \pm 0.02$	$0.019 \pm 0.01$	$0.016 \pm 0.005$	$0.033 \pm 0.028$

<sup>a</sup> Each value represents mean value  $\pm$  SD of three independent experiments.

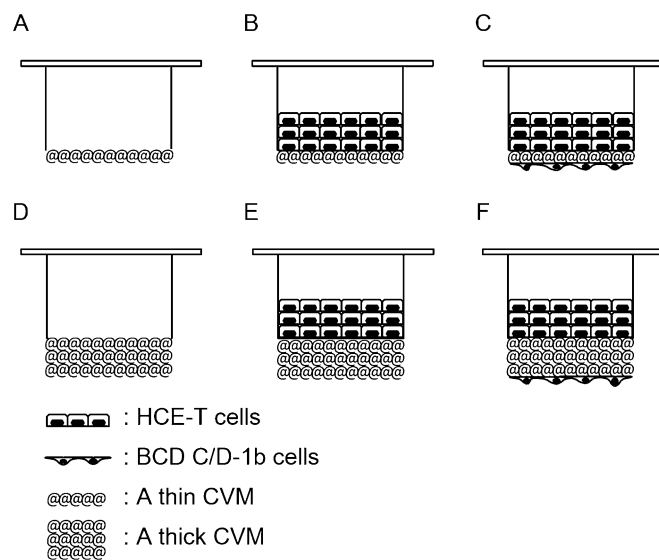


Figure 1.

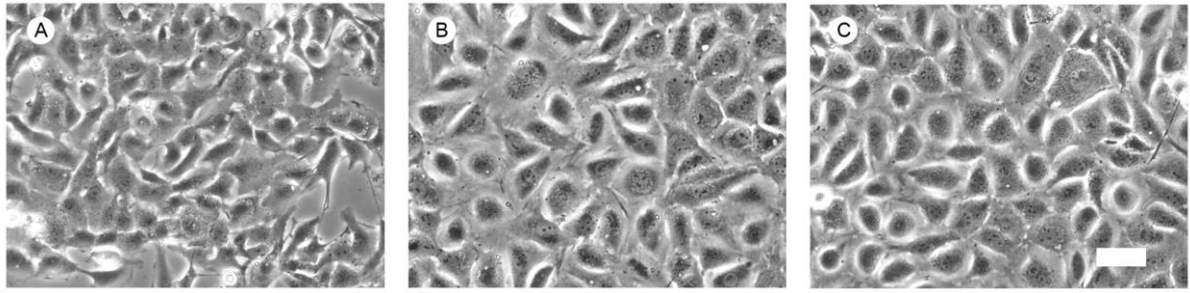


Figure 2.

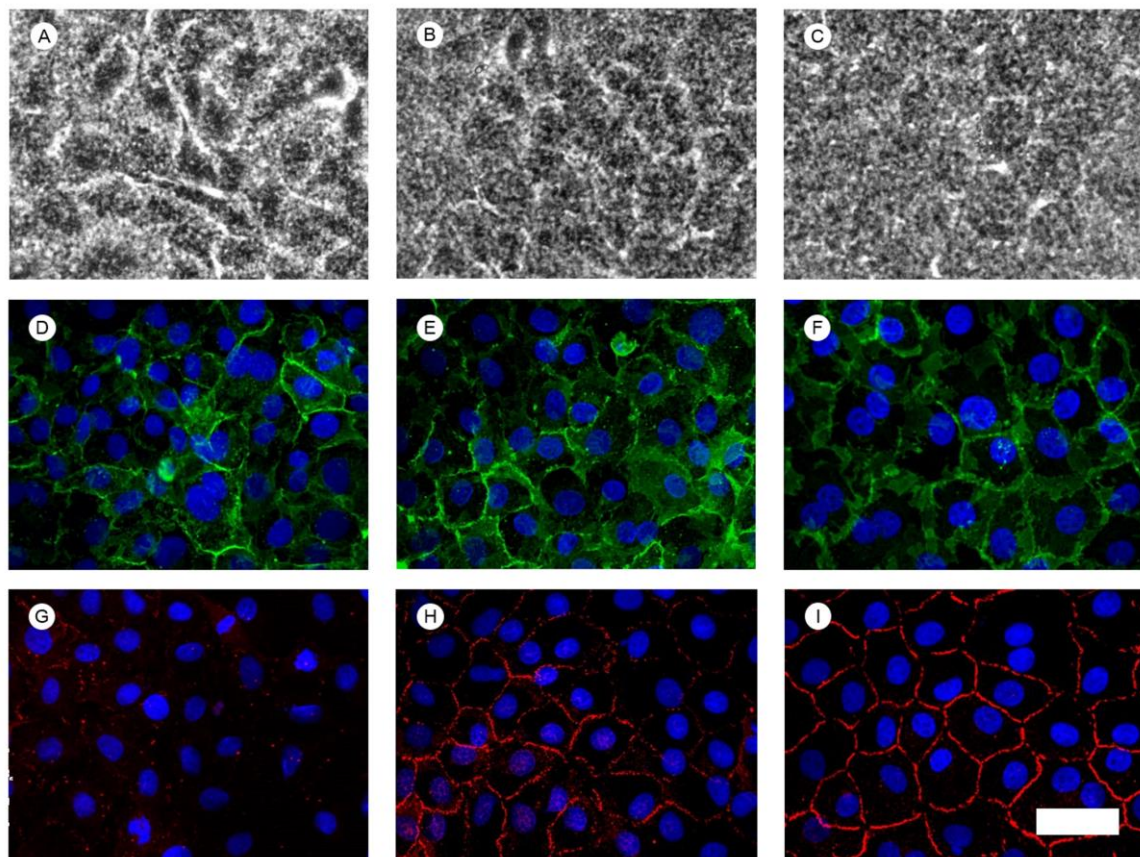


Figure 3.

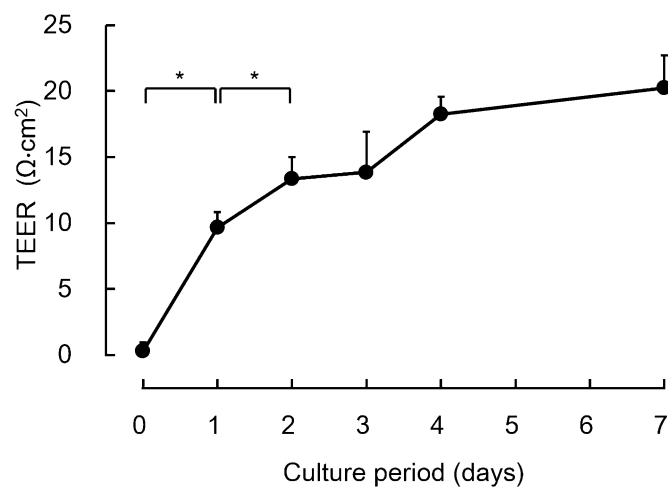


Figure 4.

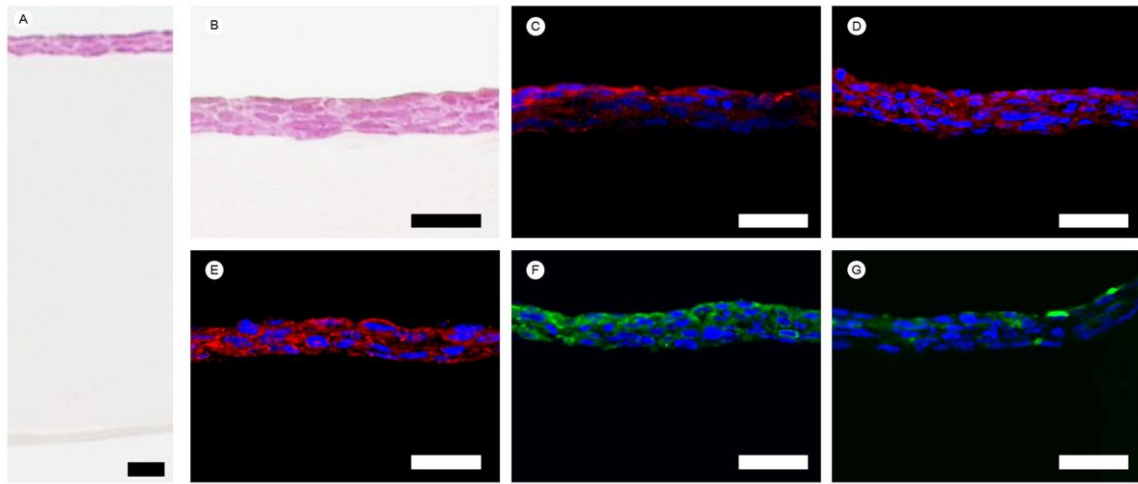


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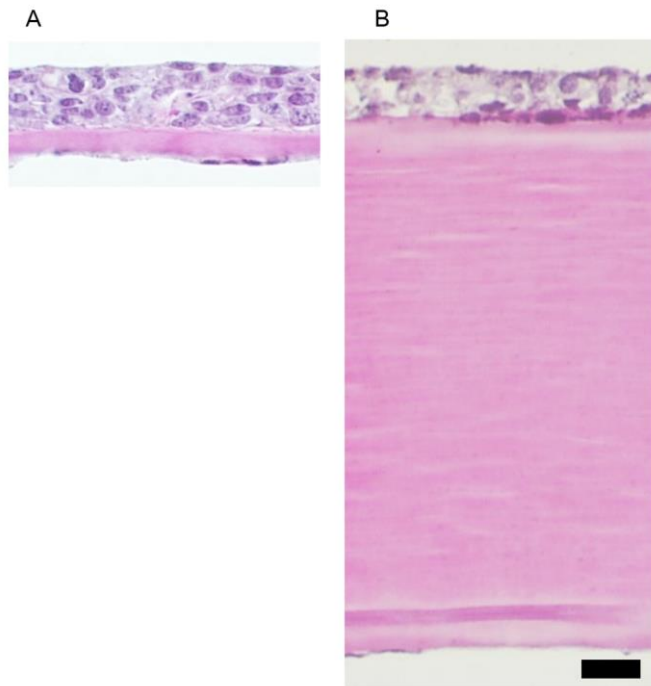


Figure 6.

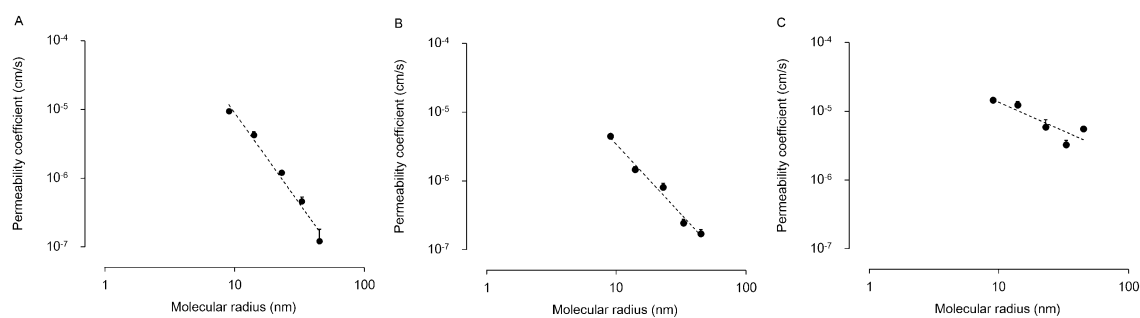


Figure 7.



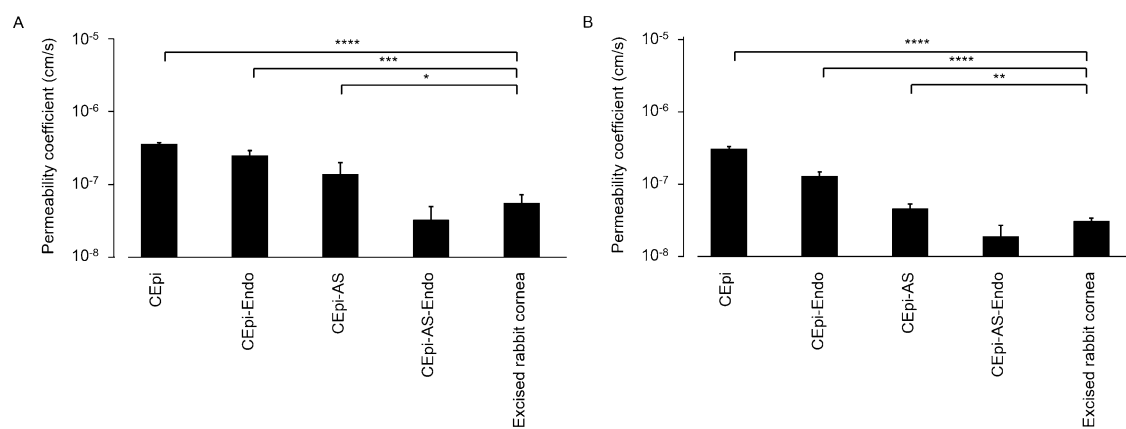


Figure 8.