Electronic Supplementary Material (ESI) for Journal of Materials Chemistry

Supplementary Information

Synthesis, characterization and ocular biocompatibility of potential keratoprosthetic hydrogels based on photopolymerized poly(2-hydroxyethyl methacrylate)-*co*-poly(acrylic acid)

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Water content measurements

Table S1 Summary of the results of water content for each hydrogel[a]

Sample code	Incubation time (h)				
	0.5	1	2	4	6
#0-4-3	18.6 ± 2.9%	24.3 ± 1.7%[b]	31.4 ± 3.6%[b]	37.5 ± 2.7%	40.3 ± 4.0%
#1-4-3	52.7 ± 2.1%	69.1 ± 2.2%[b]	71.5 ± 1.9%	74.9 ± 3.0%	76.8 ± 2.1%
#2-4-3	59.4 ± 1.8%	73.7 ± 2.6%[b]	76.5 ± 1.7%	80.1 ± 2.4%	83.1 ± 1.8%
#1-2-3	56.1 ± 1.6%	72.2 ± 1.8%[b]	75.8 ± 2.5%	79.6 ± 1.9%	81.1 ± 1.5%
#1-4-3	52.7 ± 2.1%	69.1 ± 2.2%[b]	71.5 ± 1.9%	74.9 ± 3.0%	76.8 ± 2.1%
#1-6-3	46.8 ± 1.5%	53.6 ± 1.9%[b]	65.9 ± 2.4%[b]	70.3 ± 2.2%	72.9 ± 1.6%
#1-4-1	57.4 ± 2.2%	73.5 ± 1.4%[b]	74.2 ± 2.6%	77.1 ± 2.8%	80.6 ± 0.9%
#1-4-3	52.7 ± 2.1%	69.1 ± 2.2%[b]	71.5 ± 1.9%	74.9 ± 3.0%	76.8 ± 2.1%
#1-4-5	44.3 ± 2.7%	53.4 ± 2.1%[b]	62.7 ± 1.8%[b]	66.8 ± 2.4%	69.2 ± 2.3%

[a] $n = 6$ for each group.

[b] Significant difference as compared with the value at the previous time point within each group ($p < 0.05$).

Fourier transform infrared spectroscopy

Table S2 Summary of the results of peak intensity for each hydrogel

Sample code	Wavenumber (cm^{-1})						
	1076	1161	1560	1646	1706	2952	3396
#0-4-3	0.174	0.246	0.031	0.107	0.183	0.055	0.207
#1-4-3	0.154	0.205	0.098	0.119	0.179	0.049	0.200
#2-4-3	0.128	0.160	0.131	0.123	0.174	0.043	0.196
#1-2-3	0.140	0.178	0.096	0.128	0.161	0.046	0.200
#1-4-3	0.154	0.205	0.098	0.119	0.179	0.049	0.200
#1-6-3	0.157	0.231	0.099	0.110	0.195	0.050	0.196
#1-4-1	0.133	0.200	0.100	0.146	0.175	0.053	0.199
#1-4-3	0.154	0.205	0.098	0.119	0.179	0.049	0.200
#1-4-5	0.173	0.202	0.097	0.090	0.176	0.051	0.197

Analysis of cross-linked structure

Experimental

The cross-linked structure of the resulted hydrogel such as degree of cross-link and average molecular weight of polymer chains between two consecutive junctions was analyzed according to the method reported previously [V. Charulatha and A. Rajaram, *J. Biomed. Mater. Res.*, 1997, **36**, 478-486.]. After immersion in deionized water for 6 h at 25°C (i.e., the testing temperature), the hydrogel membranes (20 mm × 10 mm) were mounted between two clamps of an Instron Mini 44 universal testing machine (Canton, MA, USA). The lower clamp was then adjusted downward until the sample was just in tension and the unstressed length was noted. Following determination of mechanical properties, the specimens were removed from the clamps and blotted with tissue paper, and the density was determined by the specific gravity bottle method. A graph of σ against (α-α^{-2}) would be a straight line with the slope giving $RT\rho V^{1/3}/M_c$, where σ = the force per unit area of the swollen unstretched sample; α = extension ratio; R = gas constant; T = absolute temperature; ρ = density of sample; V = volume fraction; and M_c = average molecular weight of the chains between cross-links. The number of cross-links per unit mass would be given by $(2M_c)^{-1}$.

Table S3 Summary of the results of M_c and and $(2M_c)^{-1}$ for each hydrogel[a]

Sample code	M_c	$(2M_c)^{-1}$
#0-4-3	5359	9.33
#1-4-3	6329	7.90
#2-4-3	10041	4.98
#1-2-3	7850	6.37
#1-4-3	6329	7.90
#1-6-3	5803	8.62
#1-4-1	7745	6.46
#1-4-3	6329	7.90
#1-4-5	4502	11.11

[a] M_c = average molecular weight of the chains between cross-links, $(2M_c)^{-1}$ = the number of cross-links per unit mass.

In vitro biocompatibility studies

Experimental

A single extract of the test article was prepared using regular growth medium containing MEM, 10% FBS, and 1% A/A solution. The extracts were obtained by incubation of the sterilized materials with culture medium at 37°C for 24 h with an extraction ratio of 0.2 g/ml. Each test extract was then placed onto SIRC cell cultures with a seeding density of 5×10^4 cells/well, and maintained at 37°C in the presence of 5% CO_2 for 48 h. The cells in regular growth medium without contacting material samples served as control groups (Ctrl).

Cell morphology was observed by phase-contrast microscopy (Nikon, Melville, NY, USA). Furthermore, cell growth was estimated using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation MTS Assay, as described in previous section. All experiments were performed in quadruplicate.

Cell viability was determined using a membrane integrity assay, the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) which contains calcein AM and ethidium homodimer-1 (EthD-1). It depends on the intracellular esterase activity to identify the living cells, which cleaves the calcein AM to produce a green fluorescence. In dead cells, EthD-1 can easily pass through the damaged cell membranes to bind to the nucleic acids, yielding a red fluorescence. After washing three times with PBS, the cultures were stained with a working solution consisting of 2 µl of EthD-1, 1 ml of PBS, and 0.5 µl of calcein AM. Under fluorescence microscopy (Axiovert 200M; Carl Zeiss, Oberkochen, Germany), the live and dead cells in three fields were counted at 100× magnification. All experiments were performed in triplicate, and the viability of the SIRC cell cultures was expressed as the average ratio of live cells to the total number of cells in these nine different areas.

Pro-inflammatory cytokine interleukin-6 (IL-6) expression was detected at messenger RNA (mRNA) levels. Total RNA was isolated from SIRC cells with TRIzol reagent according to the manufacturer's procedure. Reverse transcription of the extracted RNA (1 µg) was performed using ImProm-II (Promega) and Oligo$(dT)_{15}$ primers (Promega). The primers used to amplify the rabbit IL-6 complementary DNA (cDNA) were 5'-AAGAAAACACCAGGGTCAGCAT-3' (sense) and 5'-CTTGAGGGTGGCTTCTTCATTC-3' (antisense), and those used to amplify the internal control cDNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were 5'-TTGCCCTCAATGACCACTTTG-3' (sense) and 5'-TTACTCCTTGGAGGCCATGTG-3' (antisense). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on a Light-Cycler instrument (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions with FastStart DNA Master SYBR Green I reagent (Roche Diagnostics). Each sample was determined in triplicate and the results for IL-6 were normalized to the level of GAPDH mRNA.

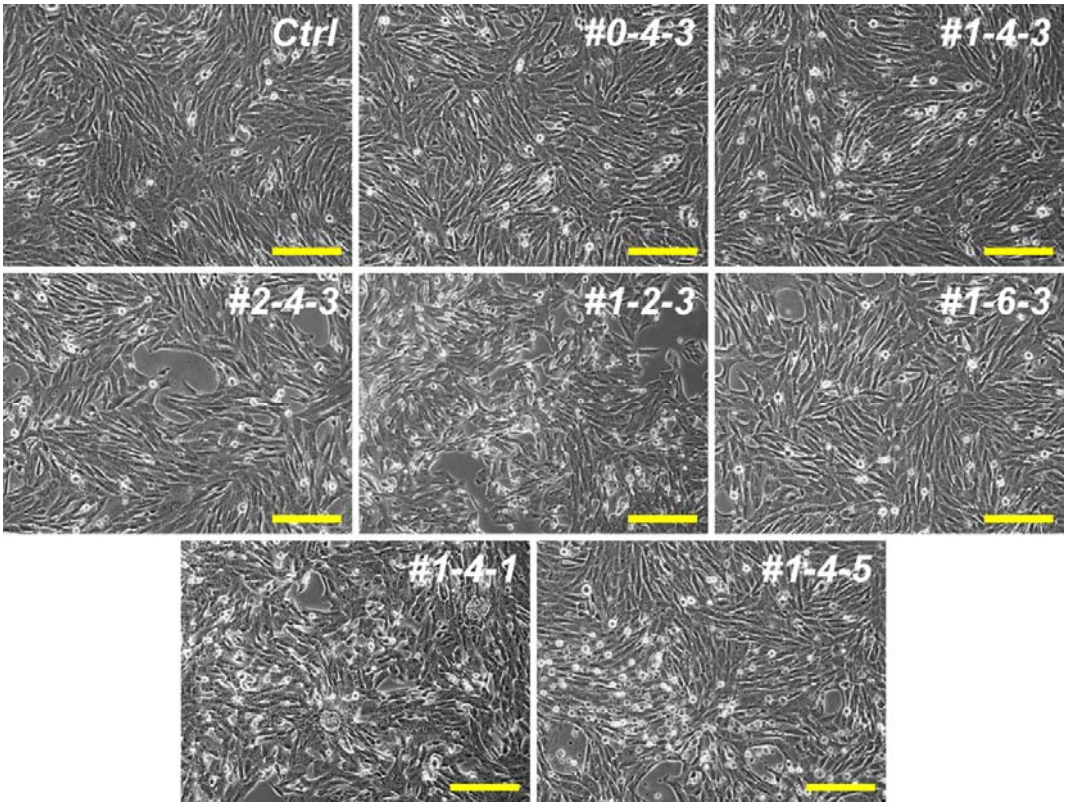

Fig. S1 Phase-contrast micrographs of SIRC cell cultures incubated for 2 days at 37°C with extract medium conditioned with various photopolymerized membranes. Ctrl: without materials. Scale bars: 100 µm.

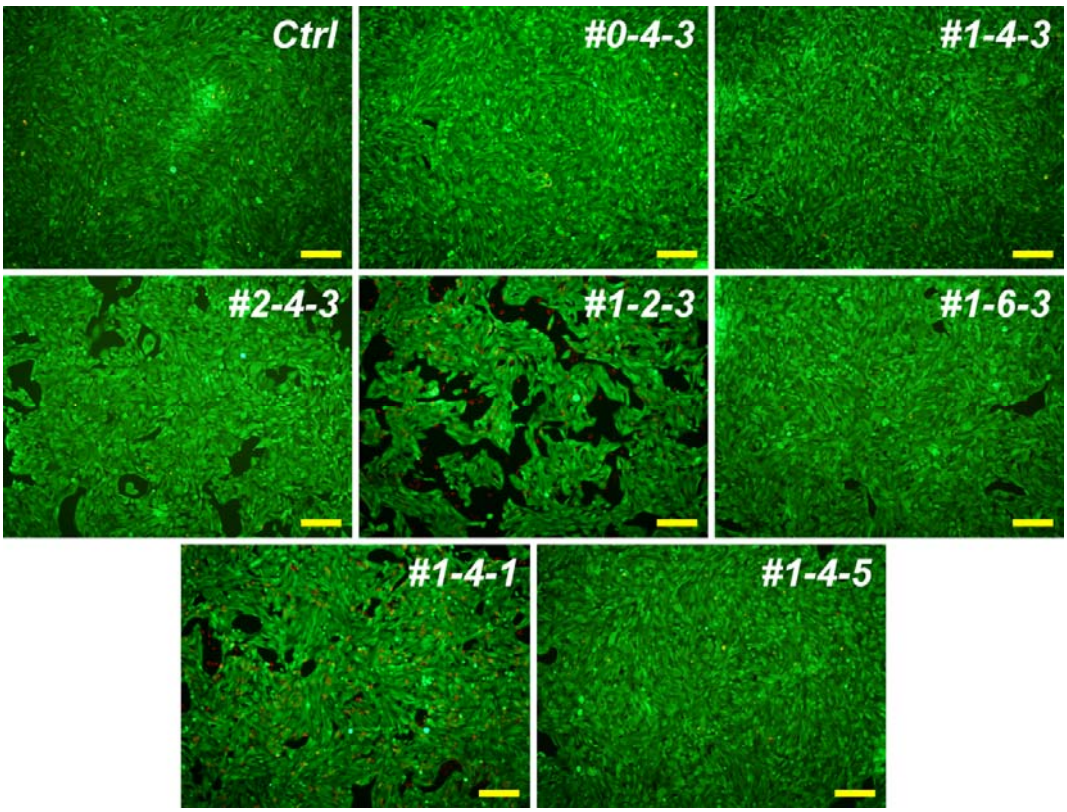

Fig. S2 Cell viability of SIRC cell cultures was determined by staining with Live/Dead Viability/Cytotoxicity Kit in which the live cells fluoresce green and the dead cells fluoresce red. Fluorescence images of cells after incubation for 2 days at 37°C with extract medium conditioned with various photopolymerized membranes. Ctrl: without materials. Scale bars: 100 μm.

In vivo biocompatibility studies

Experimental

The rabbits were anesthetized intramuscularly with 2.5 mg/kg body weight of tiletamine hydrochloride/zolazepam hydrochloride mixture (Zoletil; Virbac, Carros, France) and 1 mg/kg body weight of xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany), and topically with two drops of 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon-Couvreur, Puurs, Belgium). Surgical operation was performed in the single eye of animals, with the normal fellow eye. After disinfection and sterile draping of the operation site, the pupil was dilated with one drop of 1% atropine sulfate ophthalmic solution (Oasis, Taipei, Taiwan, ROC), and a lid speculum was placed. Under the surgical microscope (Carl Zeiss, Oberkochen, Germany), the cornea was penetrated near the limbus by using a slit knife. Then, the corneal/limbal incision was enlarged to 7.5 mm with corneal scissors to allow the insertion of an implant in the anterior chamber. The incision site was finally closed with 10-0 nylon sutures.

Ophthalmic evaluations were performed immediately after surgery, and daily for 4 weeks, and thereafter twice weekly for 24 weeks. At each study point, the morphology of anterior segment of the eye was observed by slit-lamp biomicroscopy (Topcon Optical, Tokyo, Japan); the corneal endothelial cell density was measured by specular microscopy (Topcon Optical); the intraocular pressure (IOP) and central corneal thickness (CCT) were respectively determined using a Schiotz tonometer (AMANN Ophthalmic Instruments, Liptingen, Germany) and an ultrasonic pachymeter (DGH Technology, Exton, PA, USA), according to our previously described procedure. During clinical assessment, eight parameters were recorded from rabbit eyes and were numerically graded on an increasing severity scale of 0-4 (Table S4). The means of the ocular scores at each follow-up time point were quantitatively calculated to be the sum of the scores for each group, divided by the total number of eyes in that group.

Table S4 Ocular grading system used for ophthalmic evaluations

Parameter	Ocular score				
	0	1	2	3	4
Aqueous flare	Normal	Mild	Moderate	Severe	N/A[c]
Anterior chamber fibrin	None	Mild	Moderate	Severe	N/A[c]
Corneal cloudiness severity	Normal	Mild	Moderate	Severe	N/A[c]
Corneal neovascularization	None	Mild	< 180°	180°-360°	N/A[c]
Iris neovascularization	None	Mild	< 180°	180°-360°	N/A[c]
Lens opacity	None	Mild	Moderate	Severe	N/A[c]
IOP measurement[a] (mmHg)	< 2.0	2.0-5.0	5.1-10.0	10.1-20.0	> 20.0
CCT measurement[b] (μm)	< 30.0	30.0-100.0	100.1-250.0	250.1-500.0	> 500.0

[a] Data are represented as the absolute IOP value calculated by subtracting the IOP of normal eyes from the IOP of treated eyes.
[b] Data are represented as the absolute CCT value calculated by subtracting the CCT of normal eyes from the CCT of treated eyes.
[c] Not applicable, because the biological responses were too severe to be observed.