Injectable Hydrogel Based on Gellan Gum/Silk Sericin for Application as a Retinal Pigment Epithelium Cell Carrier

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ABSTRACT: The damage to retinal pigment epithelium (RPE) cells can lead to vision loss and permanent blindness. Therefore, an effective therapeutic strategy has emerged to replace damaged cells through RPE cell delivery. In this study, we fabricated injectable gellan gum (GG)/silk sericin (SS) hydrogels as a cell carrier by blending GG and SS. To determine the appropriate concentration of SS for human RPE ARPE-19, 0, 0.05, 0.1, and 0.5% (w/v) of SS solution were blended in 1% (w/v) GG solution (GG/SS 0%, GG/SS 0.05%, GG/SS 0.1%, and GG/SS 0.5%, respectively). The physical and chemical properties were measured through Fourier-transform infrared spectroscopy, scanning electron microscopy, mass swelling, and weight loss. Also, viscosity, injection force, and compressive tests were used to evaluate mechanical characteristics. Cell proliferation and differentiation of ARPE-19 were evaluated using quantitative dsDNA analysis and real-time polymerase chain reaction, respectively. The addition of SS gave GG/SS hydrogels a compressive strength similar to that of natural RPE tissue, which may well support the growth of RPE and enhance cell proliferation and differentiation. In particular, the GG/SS 0.5% hydrogel showed the most similar compressive strength (about 10 kPa) and exhibited the highest gene expression related to ARPE-19 cell proliferation. These results indicate that GG/SS 0.5% hydrogels can be a promising biomaterial for cell delivery in retina tissue engineering.

1. INTRODUCTION

Retinal pigment epithelial (RPE) cells, the outermost layer of the retina, play a major role in the maintenance of retinal homeostasis and visual function. Because RPE cells are unable to regenerate damaged cells, dysfunction or degeneration of RPE cells can cause various retinal diseases related to vision impairment and lead to permanent loss of visual function. Particularly, age-related macular degeneration is currently the leading cause of blindness in developed countries. As a treatment for these diseases, transplantation of RPE cells into the subretinal space emerged as a potential alternative to replace damaged RPE cells. However, direct injection of the RPE cell suspension is accompanied by a low percentage of cell attachment and survival and fibrosis leading to significant cell death. Also, the considerable therapeutic effect of cell transplantation has not yet been confirmed.

To overcome these limitations, different biomaterials such as decellularized tissue, hyaluronic acid, alginate, polyethylene glycol, and so on and various types of scaffolds have been studied to promote retinal tissue formation in addition to integration into host tissues. Among various scaffolds, injectable hydrogels composed of natural polymers have attracted much attention as a promising cell delivery system because of their unique characteristics such as excellent biocompatibility and a three-dimensional (3D) network similar to that of natural soft tissue. In addition, injectability can fill irregular tissue defects and uniformly distribute encapsulated cells with a minimally invasive procedure.

Gellan gum (GG) is a nature-derived anionic polysaccharide with repeating units consisting of 1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-α-L-rhamnose. GG has been widely used for tissue engineering due to heat and acid stability, excellent biocompatibility, biodegradability, non-toxicity, tunable mechanical properties, and thermosensitive gelling characteristics. Because of these properties, it has also been extensively utilized for the development of ocular drug delivery systems. GG can physically be cross-linked by cations. Specifically, divalent cations (Mg²⁺ and Ca²⁺) provide direct bridges between pairs of double helices, and monovalent cations (Na⁺ and K⁺) induce aggregation by reducing electrostatic repulsions between carboxyl groups.
GG’s high gelation temperature, however, makes it difficult to homogeneously disperse GG at the target site during injection, and it is difficult to encapsulate cells at physiological temperature (~37.5 °C). In addition, the low cell affinity of GG inhibits cell survival and growth.21–23

Silk sericin (SS) is a glue-like glycoprotein that envelops fibroin fibers together to form silk cocoons.24 SS mainly consists of hydrophilic amino acids with polar side chains, especially serine and aspartic acid, thus enabling easy cross-linking and blending with other polymers and promoting cell attachment.25 SS is also known to have diverse bioactivities, such as antioxidant, antibacterial, and anticoagulating activity, moisturizing activity, promotion of cell growth, and differentiation of mammalian cells.26,27 Thus, SS has been widely used in soft tissue engineering such as in cornea and skin owing to these outstanding characteristics in biomedical applications.28–31 It has also been reported that sericin enhanced the proliferation of RPE cells and had a significant effect on the maturation of RPE cells by activating the NF-κB pathway.32 Earlier reports claimed that SS was responsible for an immune response. In recent studies, the immune response of SS proved to be dependent on physical association with fibroin–silk fibers. Therefore, SS itself has no immunogenicity.25,33 However, SS forms fragile scaffolds that are not suitable for biomedical applications.34,35 and the use of a cross-linking agent such as glutaraldehyde and genipin, the main fabricating method of SS hydrogels, can cause toxicity.36 To fabricate the stable SS hydrogels, cross-linking and blending with other substances such as poly(vinyl alcohol),37 chitosan,38 and gelatin39 are often investigated.

Therefore, we prepared GG/SS hydrogels that can complement the insufficient cell affinity of GG and the poor mechanical properties of SS. The purpose of this work was to fabricate GG/SS scaffolds with appropriate mechanical strength and bioactivity for retinal tissue engineering. To evaluate the optimized content of SS for retinal regeneration, the 3D GG/SS scaffolds were prepared by adding SS solution into a fixed concentration of GG solution. Subsequently, they have investigated both characterization and in vitro for their potential in tissue engineering applications.

2. MATERIALS AND METHODS

2.1. Silk Sericin Extraction. SS was extracted from Bombyx mori cocoons according to a previously described method (Figure 1).39,40 Briefly, 20 g of silk cocoons was cut into small pieces and autoclaved in deionized water (1 g of cocoons/50 mL of water) at 120 °C for 30 min. After autoclaving, the SS and fibroin mixed solution was filtrated through a Whatman filter (Advantec, Japan) to remove fibroin. Then, the SS solution was dialyzed using a dialysis bag (14,000 MWCO, Sigma-Aldrich, USA) against deionized water at room temperature for 24 h. Finally, the dialysate was lyophilized (~80 °C, 005 mTorr) for 7 days, and SS powder was stored at ~4 °C until use.

2.2. Preparation of GG/SS Hydrogels. Four hydrogel solutions of different GG/SS weight ratios were prepared. Briefly, 100 mg of low-acyl GG (GG; Gelzan CM, Sigma-Aldrich, USA) was dissolved in 9 mL of distilled water under constant stirring for 1 h at 90 °C. The temperature was then lowered to 70 °C, and 0.01% (w/v) of calcium chloride (CaCl₂, Sigma-Aldrich, USA) as a cross-linking agent was homogeneously mixed in the GG solution for 20 min. The different amounts of SS powder (0.5, 1, and 5%) were completely dissolved in 1 mL of distilled water at 70 °C. The prepared SS solution was added in the GG solution to fabricate total 1% (w/v) GG with 0, 0.05, 0.1, and 0.5% (w/v) SS, and the blended solution was stirred for 20 min. The samples were specified as GG/SS 0%, GG/SS 0.05%, GG/SS 0.1%, and GG/SS 0.5%, respectively. The sample named GG/SS 0% was added with distilled water instead of the SS solution. 7 mL of the prepared hydrogel solutions was poured into Petri dishes (50 mm × 10 mm, SPL Life Sciences Co., Ltd., South Korea) and solidified at room temperature for 10 min. After gelation was completed, cylindrical-shaped hydrogels with a diameter of 6 mm and a height of approximately 3 mm were prepared using a biopsy punch (Kai Medical Biopsy Punch, Japan).

2.3. Fourier-Transform Infrared Spectroscopy. The composition variation of the fabricated hydrogels was analyzed using an attenuated total reflectance-Fourier-transform infrared spectrometer (PerkinElmer, Boston, MA, USA) in the range of...
400–4000 cm\(^{-1}\). All hydrogels were frozen at \(-80^\circ\text{C}\) overnight and further lyophilized for the measurement.

### 2.4. Scanning Electron Microscopy

The morphological evaluation of the hydrogels was performed by scanning electron microscopy (SEM) (Bio-LV SEM, Japan, HITACHI). Before SEM imaging, the hydrogels were sequentially stored at 4, \(-20\), and \(-80^\circ\text{C}\) overnight and lyophilized (\(-80^\circ\text{C}, 005\text{ mTorr}\)) for 48 h. The dried samples were cut with single-edge blades (Dorco Living Vina Co Ltd., South Korea) and coated using a plasma sputter (Model SC500k, Emscope, UK). The pore diameters were measured using ImageJ software (Java-based image software, LOCI, University of Wisconsin).

### 2.5. Physicochemical Studies

#### 2.5.1. Mass Swelling Ratio

The prepared scaffolds were immersed in phosphate buffered saline (PBS, pH 7, Gibco) at 37 °C for 24 h. The wet weight of the samples was measured (\(W_w\)) after the initial removal of the redundant PBS and lyophilized (\(-80^\circ\text{C}, 005\text{ mTorr}\)) for 48 h. The mass of the dried samples was recorded (\(W_d\)), and the mass swelling ratio was determined using the following equation.

\[
\text{mass swelling ratio} = \frac{W_w}{W_d}
\]

#### 2.5.2. Weight Loss (%)

The weight loss (%) of the hydrogels was measured on days 7, 14, 21, and 28. The prepared scaffolds were immersed in PBS at 37 °C for 24 h. The initial weights of the samples (\(W_i\)) were recorded. Then, the hydrogels were immersed in 1 mL of PBS at 37 °C. At the specific time points, the weight of the hydrogels (\(W_w\)) was recorded. PBS was exchanged every 3 days. The weight loss (%) was calculated using the following equation.

\[
\text{weight loss (\%)} = \frac{W_i - W_w}{W_i} \times 100(\%)
\]

### 2.6. Mechanical Characterization

#### 2.6.1. Rheological Measurements

The viscosity and gelation temperature of prepared hydrogel solutions were measured using a viscometer (AMETEK Brookfield, USA). A water circulation bath (VCB 500, Emscope, UK) was used for this study. The fabricated cell-laden hydrogels were punched with a 6 mm biopsy punch (Kai Medical, HI, USA) to obtain 6 mm-diameter discs with 4 mm height and transferred to a 24-well plate. The cell culture medium was added, and the hydrogels were incubated at 37 °C in a 5% CO\(_2\) incubator. The medium was changed every 3 days.

#### 2.6.2. Injectability Test

The injectability of the hydrogel was investigated by slightly modifying the method reported in the previous study with a slight modification. First, 2.6 mL of the fabricated hydrogel solutions was molded into a silicone mold. The cells were seeded (2, 8, 2 \times 10^3 cells/well) on 96-well plates and were cultured for 24 h at 37 °C in a water bath for 24 h. Autoclaved latex was chopped into pieces for the positive control (2.5 cm^2/mL) and incubated at 37 °C in a water bath for 24 h. After the incubation period, the extracted supernatant was filtered through a 0.45 μm pore size filter (Millex Syringe Filters, Merck Millipore, Darmstadt, Germany) to remove impurities. Before the experiments, all of the materials were autoclaved for use. Cultured ARPE-19 cells were trypsinized using a 0.5% trypsin solution (Gibco, USA). The culture medium was removed, and 10 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldapetrazolium bromide (MTT; thiazolyl blue, 5 mg/mL in PBS, Amresco, TX, USA) solution was added to each well for 2 h at 37 °C in a 5% CO\(_2\) incubator. Treatments were removed, and 100 μL of dimethyl sulfoxide (DMSO, Samchun Chemical, South Korea) was added to dissolve the formazan crystal. The absorbance was measured at 570 nm with a microplate reader (Synergy MX, Biotek, Vernusky, VT, USA). All the groups were normalized with the negative control (RPMI medium) to calculate cell viability (%).

#### 2.6.3. Compression Test

Mechanical characterization was studied using the texture analyzer (FTC, Sterling, Virginia, USA) with a 10 N load cell. Cylindrical samples with 8 mm diameter and 3 mm height were immersed in PBS at 37 °C for 24 h before the compression test. The stress–strain curve of the samples was drawn, and the initial elastic modulus was calculated at 5–10% strain.

### 2.7. In Vitro Study

#### 2.7.1. Hydrogel 3D Cell Culture

Human RPE ARPE-19 (ATCC CRL-2302, ATCC) was cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM F-12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic–antimycotic (100×) (Anti-Anti, Gibco) at 37 °C under a humidified atmosphere of 95% air and 5% CO\(_2\). The cell culture medium was changed every 3 days, and the cells were subcultured until they reached a sufficient level for seeding in the hydrogels. The hydrogel solutions were prepared as described in Section 2.2 and were filtered through a 0.45 μm pore size filter (Millex Syringe Filters, Merck Millipore, Darmstadt, Germany) to remove impurities. Before the experiments, all of the materials were autoclaved for use. Cultured ARPE-19 cells were trypsinized using a 0.5% trypsin solution (Gibco, USA). The cell pellet with a density of 1 × 10^5 cells/mL was mixed with the hydrogel solution at 37 °C, and the mixture was poured into Petri dishes. The cell-laden hydrogel solutions were solidified for 10 min at room temperature. The fabricated cell-laden hydrogels were punched with a 6 mm biopsy punch (Kai Medical, HI, USA) to obtain 6 mm-diameter discs with 4 mm height and transferred to a 24-well plate. The cell culture medium was added, and the hydrogels were incubated at 37 °C in a 5% CO\(_2\) incubator. The medium was changed every 3 days.

#### 2.7.2. Cytotoxicity Assay

The cytotoxicity of the GG/SS hydrogels was evaluated by the extraction test reported in the previous study with a slight modification. First, 2.6 mL of the fabricated hydrogel solutions was molded into a silicone mold of 100 μL and solidified at room temperature for 10 min. The prepared hydrogels were immersed in 10 mL of Roswell Park Memorial Institute (RPMI) medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (PS, Gibco, USA) at 37 °C in a water bath for 24 h. Autoclaved latex was chopped into pieces for the positive control (2.5 cm^2/mL) and incubated at 37 °C in a water bath for 24 h. After the incubation period, the extracted supernatant was filtered through a 0.45 μm pore size filter. A NIH/3T3 mouse embryo fibroblast (National Institute of Health, KCLB21658) obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) was used for this study. The culture medium was changed every 3 days, and the cells were seeded (n = 8, 2 × 10^3 cells/well) on 96-well plates and were cultured for 48 h at 37 °C with 5% CO\(_2\). The culture medium was removed after 48 h and replaced with the extraction fluid and stored under standard culture conditions (5% CO\(_2\) and 37 °C) for 1, 2, and 3 days. At the specific times, the supernatant was removed, and 90 μL of cell culture medium and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldapetrazolium bromide (MTT; thiazolyl blue, 5 mg/mL in PBS, Amresco, TX, USA) solution was added to each well for 2 h at 37 °C in a 5% CO\(_2\) incubator. Treatments were removed, and 100 μL of dimethyl sulfoxide (DMSO, Samchun Chemical, South Korea) was added to dissolve the formazan crystal. The absorbance was measured at 570 nm with a microplate reader (Synergy MX, Biotek, Vernusky, VT, USA). All the groups were normalized with the negative control (RPMI medium) to calculate cell viability (%).

#### 2.7.3. dsDNA Content Analysis

The dsDNA content of the hydrogels was measured using the Quant-iT PicoGreen reagent (Life Technologies, USA) according to the manu-
Scaffolds were cultured for 3 and 28 days. At specific time points, the samples were washed with PBS and stored in a deep freezer. To induce cell lysis, the hydrogels were thawed at room temperature, and 1X TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was used adding a glass tissue grinder (Wheaton, USA) for homogenization. The solutions were transferred into a 96-well black plate (Cell Culture Plate, SPL Life Sciences Co., Ltd., South Korea), and the Quant-iT PicoGreen reagent was added to the samples in a 1:1 ratio. The samples were stored at room temperature for 5 min, and the dsDNA content quantification was measured with a microplate reader (BioTek Instruments, Inc., USA) at an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. The concentration was measured using a BioSpec trophotometer (Eppendorf, USA), and cDNA was synthesized with a Superscript First-Strand Synthesis System (Invitrogen, CA, USA) to identify the level of significance among different samples. The differences were considered significant at *p < 0.05 ( *), **p < 0.01 (**) , and ***p < 0.001 (***).

### 3. RESULTS AND DISCUSSION

#### 3.1. FT-IR Analysis

Fourier-transform infrared (FT-IR) analysis was performed to confirm the chemical structures of GG, SS, and GG/SS matrices in the wavenumber range of 4000–400 cm$^{-1}$ (Figure 2). The pure SS exhibited characteristic peaks at 3276 cm$^{-1}$ which correspond to the overlapping of N–H and O–H stretching vibrations. Also, SS had specific peaks at 1619 and 1516 cm$^{-1}$ representing amide I related to the β-sheet structure and amide II (C$\_\text{O}$ stretching, N–H bending) bands, along with an amide III (C–N stretching) peak at 1240 cm$^{-1}$. As the SS content increases, the bands related to amide I shifted from 1619 to 1605 cm$^{-1}$, suggesting a structure transition from the β-sheet to aggregated strands. However, in the FT-IR spectra of the GG/SS 0.5% scaffold, the bands related to amide I shifted from 1619 to 1644 cm$^{-1}$, demonstrating a random coil conformation of SS formed.

#### 3.2. Morphology

The pore size is a crucial property for a scaffold because it affects cell adhesion and proliferation, supply of nutrients and oxygen, and removal of metabolic byproducts. Table 1. Primer Sequences Used in RT-PCR

| primer | primer sequence |
|--------|-----------------|
| GAPDH | F: 5'-GGC ACA GTG AAG GTT CAG AAT G-3' |
| R: 5'-ATG GTG GTG AAG ACC CCA GTA-3' |
| CRALBP | F: 5'-CGT GGC GGA GGG GGT GCA AG-3' |
| R: 5'-GGT GCA GGG GAC AGC CTC TG-3' |
| RPE65 | F: 5'-CGT ATG GAC TGG GTT GTC GTA ATC-3' |
| R: 5'-CTG GTT GAG AAA CAA AGA TGG-3' |
| RHODO | F: 5'-TCA TCA TGG TCA TCG CTG TTC TC-3' |
| R: 5'-CAT GAA GAT GGG ACC GAA GT-3' |
| MITF | F: 5'-AGC TTC CCA TGT CCA AAC CAG-3' |
| R: 5'-TTC ATA CTT GGG CAC TCG CTC T-3' |
| NPR-A | F: 5'-AGA AGG AGA ACC TGA CCG ACC G-3' |
| R: 5'-ACG ATT CTG GAA TTC ATG ATA CTC-3' |
| COL I | F: 5'-CTG ACT GGA AGA AGG CAG AGT AC-3' |
| R: 5'-CCA TGT CGC AGA AGA CCA TGA-3' |

Figure 2. FT-IR spectra of SS, GG, and GG/SS hydrogel powder.

**Table 1. Primer Sequences Used in RT-PCR**

**Figure 2.** FT-IR spectra of SS, GG, and GG/SS hydrogel powder.
The porous microstructure of GG/SS scaffolds was measured by SEM images (Figure 3A). The pore diameters of the GG/SS scaffolds with 0, 0.05, and 0.1% SS were in the range of 150–200 μm with mean sizes 141.52 ± 37.38, 171.24 ± 40.87, and 205.70 ± 52.52 μm, respectively. Also, the pore sizes in GG/SS 0.5% were mainly in the range of 200–250 μm with a mean size of 223.21 ± 56.95 μm (Figure 3B). As a result, the higher SS content showed increased pore sizes. The pore size depends on the size of the ice crystals formed during freezing. Therefore, due to the hydrophilicity of SS, a high concentration of SS formed larger water droplets during the freezing process, and it seemed that large pores were introduced due to the slow temperature change. In addition, it was reported that ARPE-19 enhanced cell attachment on larger-diameter fibers (approximately 1300 nm) in a previous study, and macropores (>100 μm) play an important role in cell distribution, migration, and neovascularization. Therefore, the GG/SS hydrogels can be suggested as a matrix for supporting the growth of RPE. However, the pore sizes of the freeze-dried hydrogels are slightly different from the pore sizes of the swollen hydrogels. In order to confirm the exact microstructure of the hydrogels, it is also recommended to observe the images of the swollen hydrogels. 

3.3. Physicochemical and Mechanical Analysis. The mass swelling ratio of hydrogels is an important characteristic that determines the degradation rate and mechanical and viscoelastic properties. To investigate the swelling behavior according to SS contents, hydrogels with different GG/SS ratios were measured. The swelling behavior depends on their structural properties such as the interaction between solvents, cross-link density, and hydrophilicity. Thus, due to the

Figure 3. SEM images of scaffolds: (A) porous microstructure of GG/SS scaffolds and (B) pore size distribution for four samples.

Figure 4. Physicochemical and mechanical characterization of the GG/SS scaffolds: (A) mass swelling ratio (%), (B) weight loss (%), (C) viscosity, (D) injection force, and (E) compressive stress–strain curve [the values are mean ± SD (n = 3), p < 0.05(*), p < 0.01(**), and p < 0.001(***)].
hydrophilicity of SS, it was expected that the mass swelling ratio would increase as the content of SS increased. However, the mass swelling ratio in scaffolds decreased from 54.53 to 45.02% with the increase in SS contents (Figure 4A). These results suggested that other structural characteristics of SS had a greater effect on the swelling behavior. The pure GG showed a high weight loss rate compared to the SS-content scaffolds for 28 days (Figure 4B). As the SS concentration of gel increased from 0.05 to 0.5%, the corresponding weight loss ratios decreased, and especially GG/SS 0.5% showed the lowest weight loss rate in all hydrogels. On day 28, the weight loss of 0.5% of GG/SS was 11%, which is 5% lower than that of pure GG \( (p < 0.001) \). Therefore, the low weight loss rate of the SS-containing scaffolds is believed to be related to the low mass swelling.

In biomaterial implantation, the mechanical properties of the implanted material should be similar to those of the target tissue to promote optimal cell activity and transplant integration.\(^6\) Gelation temperature according to different SS concentrations was measured by a sharp increase in viscosity (Figure 4C).\(^57\) The GG/SS hydrogels with the concentration of SS in a range of 0–0.1% showed no significant difference in the gelation temperature. However, when the content of SS was increased to 0.5%, the gelation temperature was changed from 32 to 37 °C. Although the GG/SS 0.3 scaffold had the gelation temperature which was higher than that of other scaffolds, it showed proper gelation temperature (<37 °C) for cell encapsulation.\(^8\) Following previous studies, the SS gelation was due to the transformation of the random coil of SS molecules into the \( \beta \)-sheet structure.\(^59,60\) In this study, however, the GG/SS hydrogel was formed without change of the SS secondary structure, indicating that gel network formation of GG using \( \text{CaCl}_2 \) contributed greatly to the gelation.\(^61\) Injectable ability is a significant parameter that confirms the usability of the hydrogel.\(^62\) Therefore, the injection force of four hydrogels was evaluated (Figure 4D). The injection force first increased to approximately 1 mm displacement, which was then kept constant. Plateau values were about 1.5, 1.5, 1.8, and 2.8 N for the scaffolds GG/SS 0%, GG/SS 0.05%, GG/SS 0.1%, and GG/SS 0.5%, respectively. As with the gelation temperature, there was a significant difference in the GG/SS 0.5% hydrogel. Compressive strength was evaluated to confirm the hydrogel’s ability to withstand intraocular pressure under compression.\(^63\) Previous studies have demonstrated that the compressive modulus of the native retina is about 10–20 kPa.\(^64\) The compressive stress–strain curves of the fabricated hydrogels were presented (Figure 4E). There was no noticeable change until the content of SS was increased from 0 to 0.1%. However, the compressive stress of GG/SS 0.5% decreased from 38 to 20 kPa compared to that of GG/SS 0%, and it was broken at about 24% strain. SS has poor mechanical stability due to its high content of random coils. Therefore, in previous experiments, the strength of the gel was enhanced by increasing the proportions of the \( \beta \)-sheet via bending, synthesis, and cross-linking agents, such as ethanol, genipin, and glutaraldehyde.\(^27,65,66\) These results suggest that the random coils of SS decrease the strength of the GG/SS hydrogel.

Figure 5. Cytocompatibility assessment: (A) cytotoxicity, (B) dsDNA quantification, and (C) live/dead staining. Live cells were stained in green, and dead cells were stained in red [the values are mean ± SD (\( n = 3 \)), \( p < 0.05(*), p < 0.01(**), \) and \( p < 0.001(***) \)].
3.4. In Vitro Study. 3.4.1. Cytocompatibility Test. The cytotoxicity study for GG/SS hydrogels was performed on NIH/3T3 cells by MTT assay. The GG/SS hydrogels exhibited a higher cell proliferation rate compared to the pure GG hydrogel (Figure 5A). On day 1, the GG/SS 0% showed the highest cell viability at 93% in all hydrogels. In the following 2 days, however, the hydrogels containing SS showed a much higher proliferation rate of about 10% or more than that of the GG/SS 0% hydrogels. Finally, on day 3, the GG/SS 0.5% showed the highest cell viability among these hydrogels. In previous studies, non-cytotoxic effects of SS have been demonstrated using several types of cells. \(^{47-50}\) Therefore, it is expected that GG/SS 0.5% supported longer-term cell proliferation than pure GG/SS 0% owing to the cell-adhesive capability of SS. We quantified the amount of dsDNA on day 3 and day 28 to characterize the effects of SS on cell proliferation (Figure 5B). The experiment results followed the same tendency as MTT assay data. As expected, ARPE-19 cells were able to proliferate in hydrogels including SS, \(^{67}\) showing a higher quantity of dsDNA compared to that in GG/SS 0% hydrogels \(p < 0.001^{(* * *)}\). Due to weight loss of hydrogels, all hydrogels showed a decrease of about 50% in the dsDNA content on day 28 compared to that on day 3. However, the GG/SS 0.5% hydrogel still showed the highest cell proliferation among hydrogels. In general, cell loss due to the weight loss in the hydrogels is unavoidable, and the gel should be gradually removed from the site of action after its purpose has been achieved.\(^{9,71}\) As seen from the live/dead staining, ARPE-19 cells gradually spread and grew over time in all scaffolds, as indicated by green fluorescence (Figure 5C). However, dead cells were relatively increased in GG/SS 0% hydrogels compared to those in other hydrogels on 28 days of culture. The mechanism of how SS enhances cell proliferation has not yet been explained. However, as shown in previous studies, SS may enable cell–cell aggregation, suggesting that these cell–cell interactions could induce cell proliferation.\(^{72}\) Therefore, it is expected that the addition of SS improves the limited cell attachment ability of GG,\(^ {73}\) promoting the proliferation and growth of ARPE-19 cells.

3.4.2. Gene Expression Analysis. To determine the gene expression level of ARPE-19 cells encapsulated in hydrogels, RT-PCR was performed with RPE-specific markers such as CRALBP, RPE65, RHODO, MITF, NPR-A, and COL I (Figure 6). CRALBP is a retinoid-binding protein abundantly expressed in RPE and Müller glia and thought to be involved in the retinal visual cycle.\(^ {74}\) RPE65 is the isomerase enzyme in RPE which is involved in the visual (retinoid) cycle, allowing optical pigments in photoreceptors, maintaining sight, and absorbing photons.\(^ {75}\) RHODO is the light receptor in rod photoreceptor cells of the retina associated with visual phototransduction.\(^ {76}\) MITF is essential for melanin production in melanocytes and regulates apoptosis through cell-cycle progression genes.\(^ {77,78}\) NPR-A controls gene expression related to RPE cell proliferation and sub-retinal fluid uptake.\(^ {79}\) COL I is a major component of retinal tissue involved in extracellular matrix formation.\(^ {80}\) Compared to GG/SS 0%, GG/SS 0.1% and GG/SS 0.5% displayed remarkably higher RPE-65 gene expression \(p < 0.01^{(***)}\). Except for the RPE-65 gene, there was no significant difference in gene expression between the hydrogels; however, significant differences were found when \(p\)-values of GG/SS 0.05% and GG/SS 0.5% were calculated. In the four genes (CRALBP, RHODO, MITF, and NPR-A), GG/SS 0.05% had a \(p\)-value more than twice that of GG/SS 0.5%, and there was a 10-fold difference in COL expression. Therefore, the addition of SS is considered to play a positive role in ARPE-19 cell maturation, and in particular, GG/SS 0.5% hydrogels are thought to enhance the differentiation of RPE cells.

4. CONCLUSIONS

Injectable GG/SS hydrogels were fabricated by blending various concentrations of SS to find the proper content of SS for retinal regeneration. An increase in the SS content resulted in a decrease in mass swelling and weight loss of the GG/SS hydrogels and an increase in the gelation temperature and injection force. However, it gave mechanical strength like that of retinal tissue and showed improvements in cytocompatibility and cell differentiation. In particular, the GG/SS 0.5% hydrogel had the most similar compressive strength to native retinal tissue (about 10 kPa) and exhibited more cell proliferation and differentiation ability for the ARPE-19 cells than other hydrogels. Consequently, the introduction of SS...
enhanced the bioactivity of the GG/SS hydrogels and made GG/SS 0.5% hydrogels a promising biomaterial for retinal tissue engineering.

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S.I.K. and G.Y.J. contributed equally to this work. All authors contributed to this manuscript and have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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