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Evaluation of Saponin Loaded Gellan Gum Hydrogel Scaffold for Cartilage Regeneration

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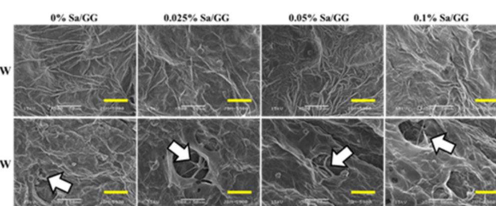
Abstract: Several concentrations of saponin (Sa), which is water-soluble, were fabricated well with gellan gum (GG) solution. Sa, found in many plants like clematis and ginseng, has long been used in traditional chinese medicine to treat joint diseases including osteoarthritis. Also it has its ability of anti-inflammatory, antioxidant and anti-cancer. GG hydrogel is suitable as a cell encapsulating agent in the field of cartilage regeneration because of its easy processing and biocompatibility. In this study, GG hydrogel scaffolds with different concentration of Sa were conducted for analysis by SEM, FT-IR, compressive strength, water uptake, degradation rate, MTT assay, mRNA expression. It was observed that 0.025 wt% Sa/GG hydrogel scaffold shows good morphology, cell proliferation and mRNA expression results. The composite material supports cell growth covered with extracellular matrix (ECM) with maintaining its function. As a result, incorporation of Sa loaded with GG hydrogel scaffolds had positive result up to 0.025 wt% in cartilage regeneration.



Keywords: gellan gum, saponin, hydrogel, scaffold, cartilage regeneration.

1. Introduction

Osteoarthritis (OA) is a common disease in the daily life and is a chronic disease. OA is caused by cartilage abrasion, meniscal injury, and synovitis, and is accompanied by severe pain.[1,2] As the population ages, the number of osteoarthritis patients is increasing and the cost is also increasing.[3-6] Therefore, replacement or implantable techniques are required for damaged cartilage tissue.[7] Tissue engineering aims at maintaining and improving the function of living tissue by implanting a substitute material in living tissue.[8-11] Tissue engineering therapy is as follows. The scaffold is made and the tissue cells are seeded to produce an artificial tissue having physical and functional characteristics similar to the actual tissue. And transplant it into the body to replace damaged tissue or help regenerate it.[12] Because transplantation into the body, the scaffold should be biocompatible and capable of cell attachment and proliferation to the scaffold.[13-16] In this study, gellan gum (GG) was used as a scaffold. GG has a linear anion polysaccharide structure containing tetrasaccharide and can form thermally reversible gels. The polysaccharide, such as GG, has good biocompatibility, no toxicity, easy controlling and processing, therefore it is widely used as biomaterials.[17] It is demonstrated that GG-based matrices could maintain high cell viability and proper functionalities, replaceable of the actual cartilage. In cartilage tissue engineering, GG hydrogels got good cyto-compatiblity and ability of maintaining chondrocyte phenotype and enhancing bio-ability of cartilaginous ECM.[18,19] Many plants containing saponins (Sa), such as clemertis and ginseng, are known to have anti-inflammatory and cartilage protective effects. Sa, a component material, has been used to treat joint diseases such as OA, having anti-inflammatory, antioxidant and anticancer properties. It is indicated that Sa is capable of ameliorating a cartilage loss and erosion in OA, by reducing ECM degradation and chondrocyte injury to protect cartilage.[20-26] In this study, Sa/GG hydrogel scaffolds were prepared by mixing Sa in the ratio of GG. The mechanical properties of the Sa/GG hydrogel scaffolds and cell proliferation and function expression on the Sa/GG hydrogel scaffolds were evaluated.

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2. Experimental

2.1. Reagents and materials

Gelzan™ CM (Sigma-Aldrich, molecular weight : 1,000,000 g/mole, G1910, USA) and Sa (Sigma-Aldrich, USA) are used for experiment. All reagents used in this experiment were of high-performance liquid chromatography (HPLC) grade.

2.2. Fabrication of Sa/GG hydrogels

0.7 wt% GG solution is prepared by heating distilled water (DW) up to 90 °C and then adding 0.7 g GG powder in 100 mL

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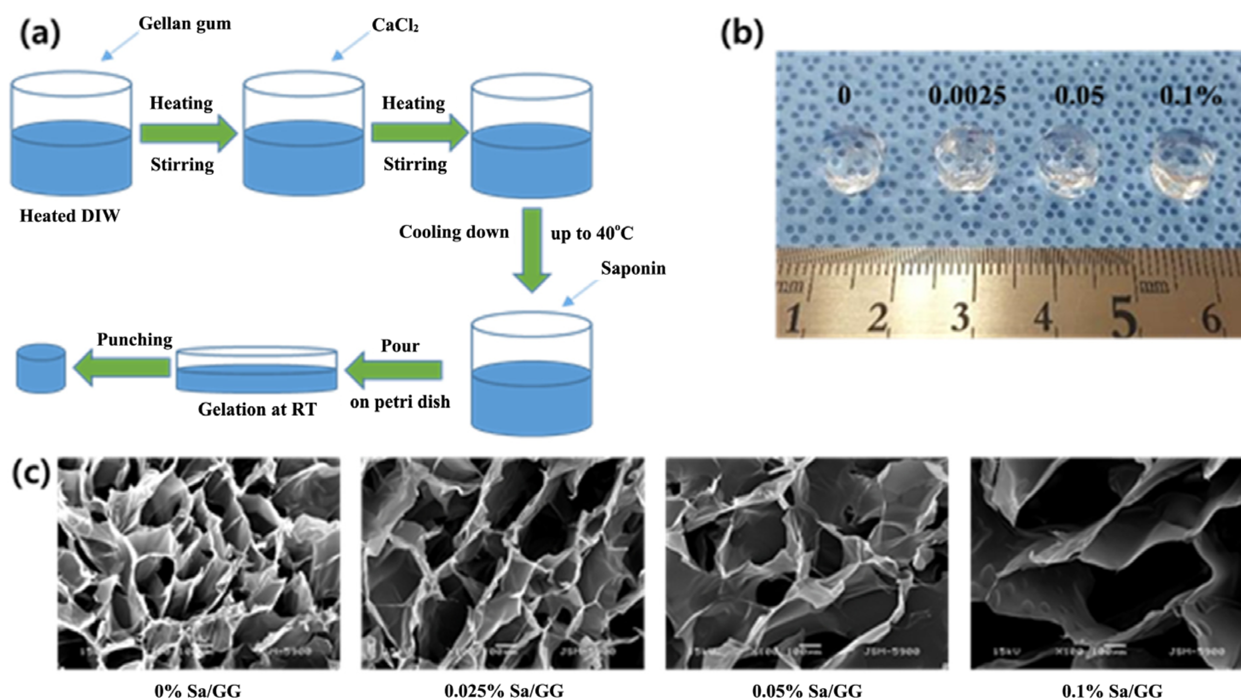

Figure 1. (a) Schematic illustration of fabrication of Sa/GG hydrogels. (b) Gross images and (c) SEM images of Sa/GG hydrogels (Magnification with × 100, scale bar=100 μm).

DW and stirring until it is perfectly dissolved. After that, 0.03 wt% $CaCl_2$ are added for crosslinking. When GG solution is cooled to 40 °C, Sa was added 0, 0.025, 0.05, and 0.1 % w/w in GG solution and dissolved evenly. This was placed in a petri dish and hardened at room temperature. A Sa/GG hydrogel with a diameter of 8 mm and a height of 4 mm was prepared using a biopsy punch of hardened GG (Figure 1(a), 1(b)).

2.3. Morphology of Sa/GG hydrogels

Scanning electron microscope (SEM, Hitachi Co., Model S-2250N, Japan) was conducted to observe the pore size of Sa/GG hydrogels. Sa/GG hydrogels were stored in refrigerator and freezing for 3 h each, quenched at -80 °C for 24 h in deep-freezer, and freeze-dried for 2 days to form sponge structures They were platinum-coated using a plasma sputter (Emscope, Model SC500K, UK) under an argon gas, and SEM was performed.

2.4. Fourier transform infrared spectroscopy (FTIR) spectrum

To confirm the chemical change of Sa/GG hydrogel, it was analyzed using FTIR spectrometer at the wavelength range of 400-4000 cm^{-1} (FTIR, GX, Perkin Elmer, USA). Sa/GG hydrogel for FTIR measurement was stored for 3 h at 4 °C and for 3 h at -20 °C in a refrigerator, quenched at -80 °C for 24 h, and freeze-dried for 24 h to make it a sponge structure.

2.5. Measurement of compressive strength

The compressive strength of the Sa/GG hydrogel was measured using a texture analyzer (TMS-Pro, Food Technology Corporation, Sterling, USA) to check mechanical properties of all Sa/GG hydrogels. The setting of the texture analyzer was as follows: measurement distance=2 mm, test speed=20 mm/sec, and compressive force=1 N.

2.7. Measurement of water uptake

The water uptake of each Sa/GG hydrogel was measured by following equation:

$$\text{Water uptake (\%)}=\frac{(W_w-W_d)}{W_d}\times 100 \quad (1)$$

Sa/GG hydrogels were fabricated and then stored at 60 °C for 3 h in the oven and then dried Sa/GG hydrogels were measured by electronic scale to take W_d. After that, they were put in PBS 1X for 1 week at 37 °C and weighed the PBS 1X-absorbed Sa/GG hydrogels (W_w).

2.8. Measurement of degradation rate

The degradation rate of each Sa/GG hydrogel was measured by following equation:

$$\text{Degradation rate (\%)}=\frac{(W_d-W_r)}{W_d}\times 100 \quad (2)$$

After measuring W_w, the PBS 1X was removed and wet Sa/GG hydrogels were freeze-dried for 24 h. The freeze-dried Sa/GG hydrogels were measured to take dried weight (W_r).

2.9. Isolation and culture of chondrocytes

Chondrocytes were separated from the legs of two-week-old female New Zealand white rabbits (Damul Sci., Korea). The legs

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were washed 3 times with PBS 1X (pH 7.4, Gibco, USA), and the knee joints of the rabbits were incised and cartilages were sliced thinly. Sliced cartilages were placed in a conical tube and centrifuged at 1200 rpm and 4 °C for 3 min. After centrifugation, the supernatant was removed and the chondrocytes were mixed with 10 mL of Dulbecco's modified eagle medium (DMEM/F-12, Gibco, USA). And then 1% wt% collagenase A (Roche, Indianapolis, USA) was added and cultured at 37 °C under 5% CO_2. After 1 day, the supernatant was removed after centrifugation at 1,200 rpm and 4 °C for 3 min. A culture medium containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% wt% penicillin (Gibco, USA) was added, pipetted and dispensed into a cell culture dish (Eppendorf AG, Germany). The culture medium was changed once every 3 days.

2.10. Cell adhesion and morphology

Chondrocytes were seeded and cultured for 3 weeks and then fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA). After cooling for 1 day, they were dehydrated in 50, 60, 70, 80, 90 and 100% aqueous ethanol solution for 30 minutes. After quenching at -80 °C for 1 day, it was lyophilized for 1 day to form a sponge. They were coated by platinum under argon gas using a plasma sputter (Emscope SC500K, UK) and then get images using FE-SEM (SUPRA 40V, Carl Zeiss, Germany).

2.11. Cell proliferation assay

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed to analyze cell proliferation. Rabbit chondrocytes were seeded on each scaffolds and cultured with the above culture (n=3). 1 mL of fresh culture medium and 100 μL of MTT solution (5 mg/mL stock in PBS, Sigma-Aldrich, USA) were added at 1 day, 1, 2 and 3weeks and incubated at 37 °C and 5% CO_2 for 4 h. After purple crystals were formed, 1 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) solution was added to dissolve the crystals. 100 μL of each solution was dispensed into 96-well plates and absorbance was measured at 570 nm using Synergy Mx monochromator-based multimode microplate reader (Biotek instuments, Inc., USA).

2.12. mRNA expression

RT-PCR was performed to confirm the expression of specific mRNAs of chondrocytes seeded on Sa/GG hydrogel scaffolds. On 3 weeks after cell seeding, the culture medium is removed and 1 mL of Trizol (Invitrogen Life Technologies Co., Groningen, Netherlands) is added to the Sa/GG hydrogel scaffold and pipetted. Then, the solution was transported to tube, and 0.2 mL of chloroform (Sigma-Aldrich, USA) was added and shaken, and centrifuged (12,000 rpm, 4 °C) for 15 min to isolate mRNA. The supernatant which were transported to new tube were mixed with 0.5 mL isopropanol (Sigma Aldrich, USA) for 24 h at 4 °C. A pellet, separated RNAs, was ligated with Oligo (dT) 12-18 primer (Invitrogen™, 5×first strand buffer (Invitrogen™), dNTP (dGTP, dATP, dTTP, dCTP, Gibco, USA), RNase inhibitor (Invitrogen™, Superscript™ RNase H (Invitrogen™) and DNase/RNase free water (Gibco, USA) were added and reverse transcribed with cDNA through an Authorized Thermal cycler (TP600, Takara Bio Inc., Japan). GAPDH, Type I collagen and Type II collagen, aggrecan PCR were performed on the reverse transcribed cDNA. The gene markers were amplified as follows: GAPDH, Type I collagen and Type II collagen, aggrecan. After PCR, the amplified DNA was electrophoresed on a 1.5% (w/v) agarose gel containing ethidium bromide (EtBr, Sigma Aldrich, USA) at 100 V, visualized by SYBR Green fluorescence (SYBRTM Green I Nucleic Acid Gel Stain, Cambrex, UK) at 360 nm. The expression levels of GAPDH, Type I collagen, and Type II collagen, aggrecan mRNA bands were quantified by Image J program.

2.13. Statistical analysis

Statistical analysis was performed statistically with Student's t-test when the P-value was less than 0.05, and all experiments were conducted 3 times (n=3).

3. Results and discussion

3.1. SEM

It was observed that inside of Sa/GG hydrogels by cross-sectioning them using SEM. The cross section of the scaffold was cut and the pore size of the inside was observed as SEM. As the content of Sa is higher, the pore size of Sa/GG hydrogels are bigger and the structures are lighter (Figure 1(c)). It is considered that the effect of pore size was affected by the foaming action when the Sa was dissolved in the GG solution. . In other words, as the amount of Sa increases, the pore size increases during lyophilization due to an increase in the foaming action.

3.2. FTIR spectrum

The analysis results of the components of the Sa/GG hydrogels are as follows. (Figure 2(a)) It was found that the main peak of GG, O-H and the C=O peak of the ester bond, appeared around 3358 cm^{-1} and around 1635 cm^{-1}. The Sa shows an O-H peak at 3354 cm^{-1}, a C-C peak at 2935 cm^{-1}, and a C=C peak at 1633-1662 cm^{-1}. The characteristic peaks of GG were observed in all scaffolds, and the characteristic peaks of Sa were also found in 0.025, 0.05, and 0.1 wt% Sa/GG hydrogel scaffolds. Therefore, it was found that all scaffolds have the uniqueness of the constituent materials. The above-mentioned main peak values are well shown, and it can be confirmed that each component has unique properties and there is no chemical change.

3.3. Compressive strength of Sa/GG hydrogels

The compressive strength of each Sa/GG hydrogel was measured for mechanical properties (Figure 2(b)). The compressive strength values of the GG hydrogel scaffolds without Sa were 1.4±0.14 N and the compressive strength values of the 0.025, 0.05 and 0.1 wt% Sa/GG hydrogel scaffolds were 1.27±0.25, 1.22±0.22, and 0.93±0.06 N value. It was observed that the higher the content of Sa, the lower the compressive strength.

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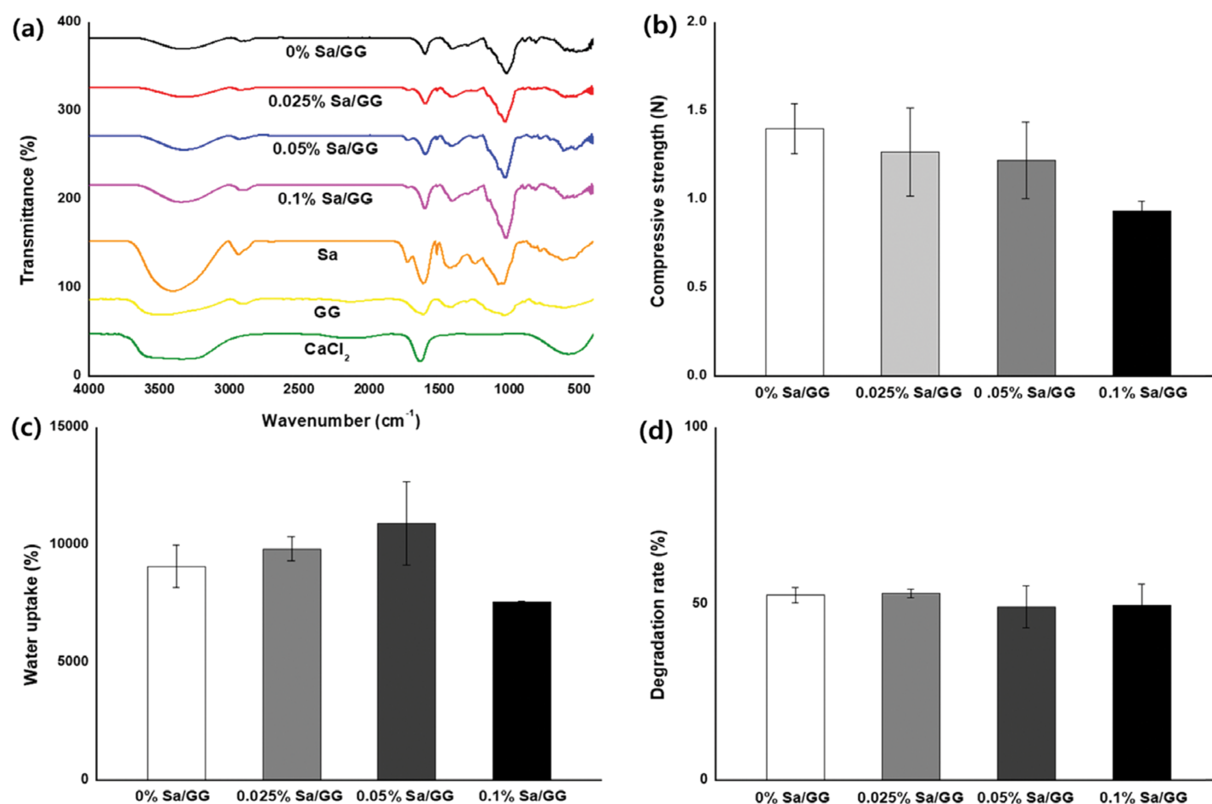

Figure 2. (a) Fourier transform infrared (FT-IR) spectra of Sa/GG. (b) Compressive strength, (c) water uptake, and (d) degradation rate of Sa/GG on 1 week.

3.4. Water uptake of Sa/GG hydrogels

The measurement of water uptake was performed to determine the water content of the Sa/GG hydrogels. The values were 9086.53±893.70, 9832.95±506.22, 10909.34±1773.84, and 7579.16 ±29.46% in order and 0.05 wt%, was the highest. The higher the water content, the higher the adhesion and adaptability of the cells. Therefore, it affects cell proliferation positively. Also, it was observed that the water uptake of Sa/GG hydrogels got higher as increasing of porosity (Figure 2(c)). The bigger pore size as content of Sa increases got higher wettability, but 0.1 wt% Sa/GG had weak strength to maintain the structure in water. So, it is supposed that 0.1 wt% Sa/GG got a lower water uptake steeply and 0.05 wt% Sa/GG were the highest.

3.5. Degradation rate of Sa/GG hydrogels

The degree of degradation for 1 week at 37 °C is measured and quantified as follows. It was showed the values of similar values 50.47±2.14, 53.01±1.18, 49.13±5.89, and 49.65±5.93% respec-

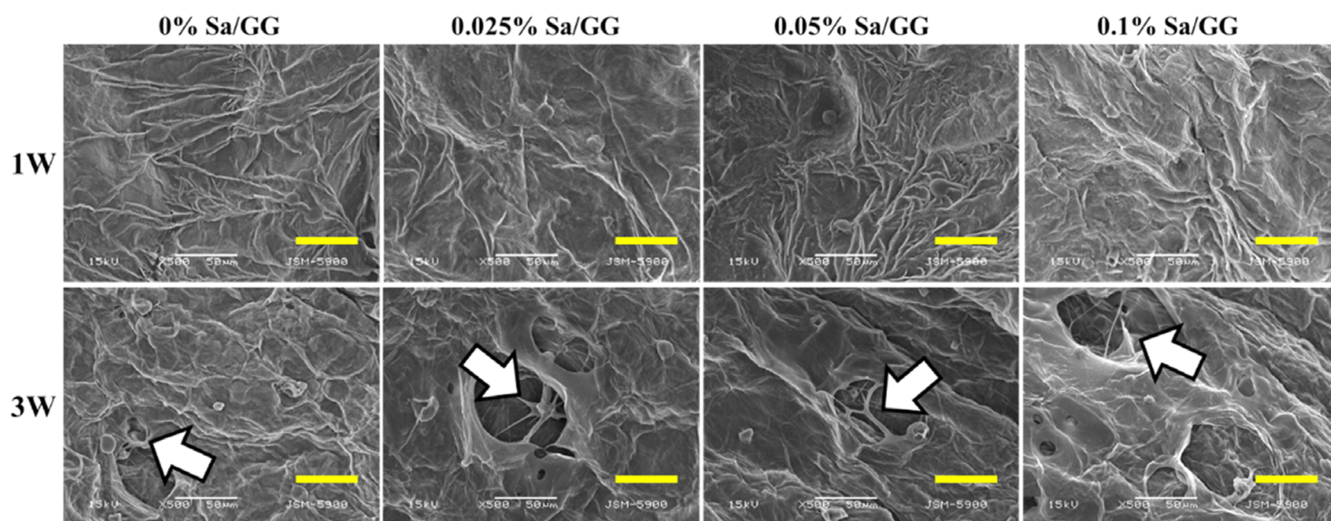

Figure 3. FE-SEM images of Sa/GG hydrogels on 1 and 3 weeks (Magnification with × 500, scale bar=50 μm, and arrow=the formation of ECM from chondrocytes on 3 weeks).

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tively and seems very similar as a whole. It was found that the Sa content did not significantly affect the degradation rate of the Sa/GG hydrogels (Figure 2(d)).

3.6. Cell adhesion and morphology

Chondrocytes were seeded on Sa/GG hydrogels and fixed, frozen, dehydrated and lyophilized on 3 weeks, and then FE-SEM images were taken (Figure 3). All lyophilized hydrogels showed a similar rough surface morphology, and chondrocytes, having round shapes and 5~10 μm size, were attached on the surface of Sa/GG hydrogels. It is showed that amount of chondrocytes increases and ECM of chondrocytes is formed as times goes by (arrow). It is indicated that chondrocytes on the surface of Sa/GG hydrogels were proliferated. The cell growth was very good when the content of Sa was 0.025 wt% as compared with the others.

3.7. MTT analysis

Chondrocytes were seeded on a Sa/GG hydrogels and MTT assay was performed on 1 day, 1, 2, and 3 weeks (Figure 4). On 1 day after seeding, similar results were obtained on all scaffolds, and the cell proliferation rate increased on 1, 2 and 3 weeks. Among them, 0.025 wt% Sa/GG hydrogels showed higher cell proliferation than the others. As a result, it can be seen that Sa has a positive effect on cartilage cells. However, the more the Sa was contained, such as 0.05 and 0.1 wt% Sa / GG hydrogels, the cell proliferation rate decreased. This is due to the fact that the toxic component of Sa is acting.[27,28] So when it contains a proper amount of Sa, it has positive effect.

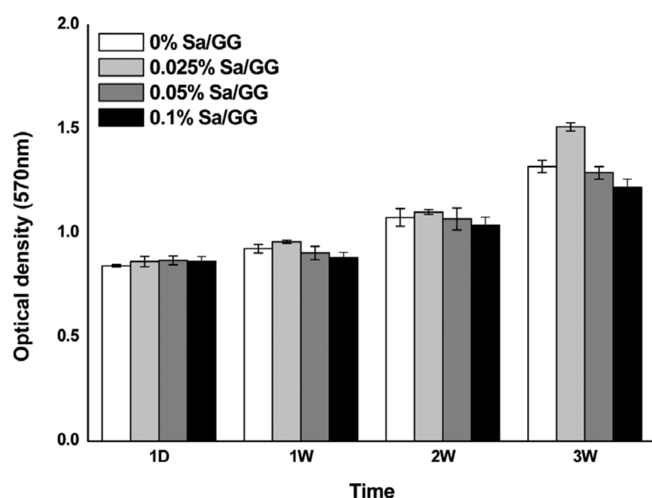

Figure 4. Cell viability result of Sa/GG by MTT assay on 1 day, 1, 2, and 3 weeks.

3.8. mRNA expression

Chondrocytes were seeded on Sa/GG hydrogels and RNA was isolated on 3 weeks and RT-PCR was performed. GAPDH which is house-keeping gene, Type I collagen, Type II collagen and aggrecan which are specific cartilage related gene were used as a primer, and the amount of cartilage characteristic gene expressed in chondrocytes analyzed through electrophoresis (Figure 5(a)). Type I and II collagen and aggrecan were normalized based on GAPDH, and the results are shown. GAPDH were all expressed, and 0.025 wt% Sa/GG hydrogel was most highly expressed in Type II collagen and aggrecan, respectively. In Type I collagen expression, it is showed that 0.025 wt% Sa/GG hydrogels got a higher value, however, no significant difference with others. *In vitro* cultured differentiating chondrocytes may produce not only hyaline cartilage matrix but fibrous cartilage as well, which normally is rich in Type I collagen fibers related with hypertrophic chondrocytes. Monitoring of the amount of Type I collagen expression, the values are much lower about 2 times than those of Type II collagen expression, related with hyaline cartilage. In addition, since the content of Sa was higher than 0.025 wt%, the degree of expression was lowered (Figure 5(b)). It is consistent with previous MTT analysis and SEM observations. However, Type II collagen and aggrecan expression of 0.05 and 0.1 wt% Sa/GG hydrogels are higher than control, because Sa helps chondrogenic differentiation as a therapeutic agent although having a little of toxicity.[29]

4. Conclusions

In this study, GG hydrogel scaffolds were prepared by mixing Sa with various proportions having anti-inflammatory, antioxidant and anticancer properties. FTIR and compressive strength were performed to evaluate the physical properties of Sa/GG hydrogels and SEM was performed to know pore sizes of them. As a result, the higher the content of Sa was, the lower the com-

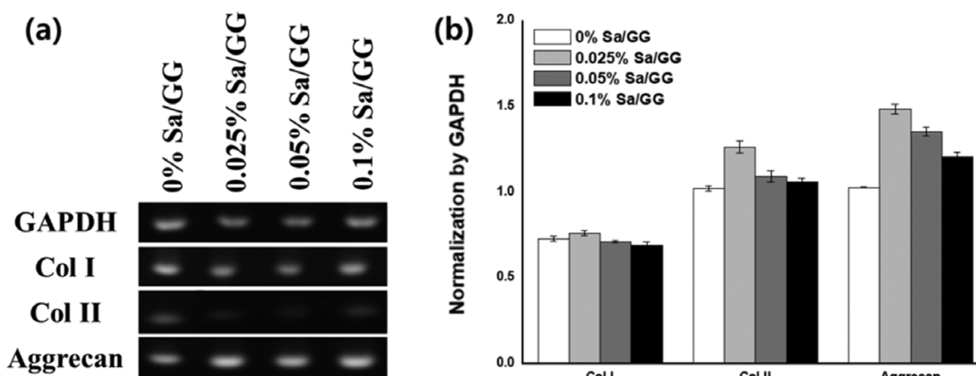

Figure 5. (a) Gel images and (b) mRNA expression of Sa/GG on 3 weeks conducted by RT-PCR and electrophoresis.

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pressive strength and bigger the pore sizes and water uptake. And no significant difference of degradation of them. As a result of FTIR result, it was shown that the characteristics of the constituent material were not lost during the fabrication process. Chondrocytes were seeded on Sa/GG hydrogels and cell adhesion, viability and proliferation ability were evaluated. SEM, fluorescence images and MTT assay showed that the cell adhesion and propagation power were maintained at the highest level on the 0.025 wt% Sa/GG hydrogel scaffold and the cells were grown with the ECM covered. mRNA expression was the highest in the measurement, and thereafter, it tended to decrease with increasing Sa content. *In vitro* results suggest that 0.025 wt% Sa/GG hydrogel scaffold is the best and it can be applied for a cartilage implant.

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