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Hybrid scaffold composed of hydrogel/3D-framework and its application as a dopamine delivery system

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ABSTRACT

Cell-based drug delivery systems (DDSs) have been increasingly exploited because cells can be utilized as a continuous drug delivering system to produce therapeutic molecules over a more extended period compared to the simple drug carriers. Although hydrogels have many advantages for this application, their mechanical properties are generally not desirable to structurally protect implanted cells. Here, we present a three-dimensional (3D) hybrid scaffold with a combination of a 3D framework and a hydrogel to enhance the mechanical properties without chemically altering the transport properties of the hydrogel. Based on the 3D Ormocomp scaffold (framework) fabricated by projection-based microstereolithography with defined parameters, we developed a 3D hybrid scaffold by injection of the mixture of cells and the alginate gel into the internal space of the framework. This hybrid scaffold showed the improved mechanical strength and the framework in the scaffold played the role of an adhesion site for the encapsulated cells during the culture period. Additionally, we confirmed its protection of exogenous human cells from acute immune rejection in a mouse model. Eventually, we demonstrated the feasibility of applying this hybrid scaffold to the treatment of Parkinson's disease as a cell-based DDS. Dopamine released from the 3D hybrid scaffolds encapsulating dopamine-secreting cells for 8 weeks suggested its clinical applicability. Further study on its long-term efficacy is necessary for the clinical applicability of this 3D hybrid scaffold for the treatment of Parkinson's disease.

1. Introduction

Even though various types of drug delivery systems (DDSs) have been studied, the major hurdle of these systems is low therapeutic efficacy resulting from the undesired loss of carried molecular activities [1,2]. Therefore, cell-based DDSs have been increasingly exploited because cells can be utilized as a continuous drug delivering system to produce therapeutic molecules over a more extended period of time compared to simple drug carriers [1]. Until now, various cell types (e.g., stem cells, progenitor cells, and genetically engineered cells) have been tested as a cell source according to their applications [3–5]. However, these cells directly injected into the body showed poor therapeutic efficacies for many reasons, such as immune rejection and decreased cell viability [1,6].

To solve these problems, a strategy for protecting cells after implantation is critical for the successful application of cell-based DDSs. One of the methods that has been extensively studied is the encapsulation of cells using biomaterials [7]. Hydrogels, such as alginate and hyaluronic acid, have been used for cell encapsulation, acting as permeable membranes to hinder immune rejection and providing the three-dimensional (3D) micro-environment to cells [8]. This micro-environment is structurally more similar to the extracellular matrices of real tissues than to other polymeric forms [9,10]. Although these hydrogels have many advantages, their mechanical properties are generally not desirable to structurally protect implanted cells [6]. Mechanical stiffness can be increased by controlling the hydrogel parameters, such as the hydrogel concentration, cross-link density, and distance between the cross-links [11,12]. However, these approaches can alter the pore size of hydrogels, which influences transport properties in hydrogels.

To overcome this, we present a new 3D hybrid scaffold that can block immune rejection as well as maintain higher mechanical strength by a combination of alginate gel and a 3D framework. Alginate gel has

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been reported to be a representative hydrogel for encapsulation of cells to protect them from immunogenicity and improve cell survival [13]. Ormocomp has been considered as a framework material because it is biocompatible and non-biodegradable [14]. Moreover, its photocurable property could provide an opportunity for the use of precise fabrication techniques using light sources, such as UV lamps or many types of lasers [15,16]. After the evaluation of the basic characteristics of the developed hybrid scaffold using these materials, we apply it to releasing dopamine for Parkinson's disease as a cell-based DDS.

Parkinson's disease is a neurodegenerative disorder resulting from the death of dopaminergic neurons in the substantia nigra (SN) [17,18]. Progression caused by this degeneration leads to motor symptoms such as tremor and postural instability [19]. Currently Parkinson's disease patients have been treated with various pharmacological approaches such as levodopa and surgical approaches such as deep brain stimulation. While these therapies show high performance in reducing the symptoms, their effectiveness is only temporary [20]. Another therapeutic approach using cell-based DDSs has been studied, in which dopamine supplied from exogenous cells implanted into the striatum could substitute for lost dopaminergic neurons for extended periods [21,22]. For this application, we present the preliminary results of releasing dopamine from our hybrid cell-encapsulating scaffolds.

2. Materials and methods

2.1. Fabrication of the hybrid scaffold

The 3D hybrid scaffold was fabricated by combining conventional alginate gel and a 3D framework. The alginate gel can block the host immune cells from access, while necessary oxygen and nutrients can penetrate through the gel, as illustrated in Fig. 1. Additionally, we expected that the rigid 3D framework could protect the encapsulated cells and the alginate gel from external forces.

2.1.1. Fabrication of 3D scaffolds as a framework

The scaffold was designed to have small external dimensions in order to be inserted through a neurosurgical tube as well as sufficient internal volume to carry a large number of cells per scaffold. The scaffold we designed is illustrated in Fig. 2(a). Before fabrication, the oxygen concentration inside the hybrid scaffold, a combination of 1×10^4 cells in 2% alginate gel and the 3D Ormocomp scaffold as a framework, was predicted using commercial software (COMSOL, version 3.5, Burlington, MA, USA) [23]. The Michaelis–Menten constant and maximum oxygen uptake rate were 1.547 μmol/L and 7.9×10^{-11} μmol/cell/s, respectively [24]. Other parameters were obtained from previous reports

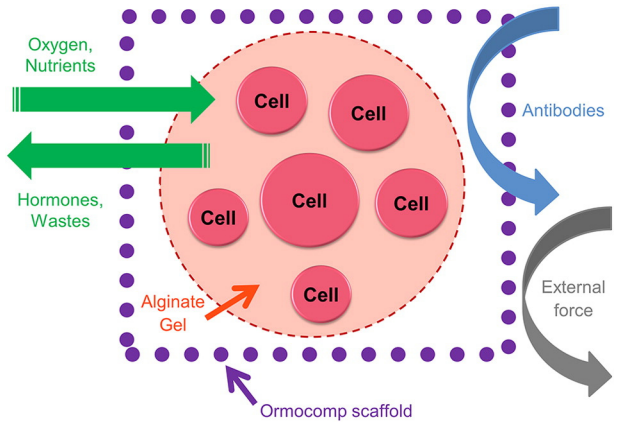

Fig. 1. Concept of the hybrid scaffold for non-autologous cell implantation. Tiny pores in alginate gel permit oxygen, nutrients, hormones, and wastes to pass through, while antibodies of the host are blocked. The 3D Ormocomp scaffold as a framework can protect cells from external forces.

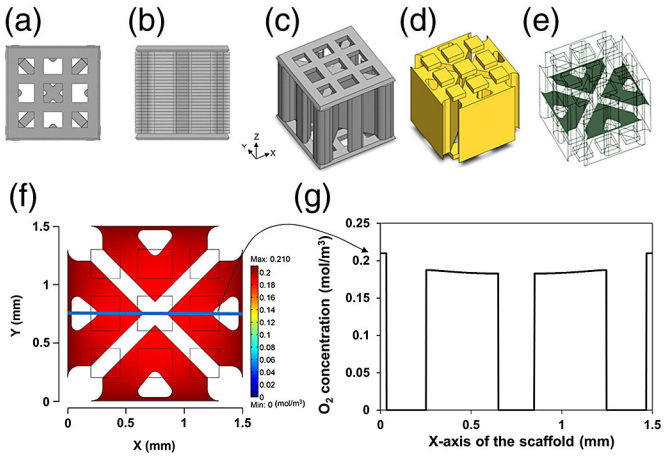

Fig. 2. Design of the Ormocomp scaffold as a framework. The designed scaffold is illustrated from a top (a), side (b), and isotropic view (c). For finite element method (FEM) analysis, the void volume of the scaffold filled with 1×10^4 cells in 2% alginate gel (d) was generated and analyzed in the cross section (green) of the gel perpendicular to the z-axis at z = 0.75 mm, which is half of the height (e). (f) The oxygen concentration distribution of the cross section at z = 0.75 mm was illustrated. (g) The oxygen concentration along the x-axis (blue line of Fig. 2(e)) at y = 0.75 was plotted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[25,26]. Fig. 2(b) illustrates that the oxygen concentration in the internal space of the framework was higher than general hypoxic conditions. The minimum concentration was 0.183 mol/m^3. This calculated data suggests that the framework we designed did not inhibit the necessary oxygen diffusion through the alginate gel.

Ormocomp (Micro Resist Technology GmbH, Berlin, Germany) was used for framework fabrication because it was non-biodegradable and biocompatible as well as photocurable. The Ormocomp scaffolds were fabricated by rapid prototyping technology based on our projection-based microstereolithography (pMSTL) system [27]. Briefly, pMSTL solidified liquid Ormocomp with illuminated two-dimensional (2D) UV images for various illumination times. Then, the 3D scaffolds were fabricated by stacking 2D solidified patterns using our customized software [28]. After finishing the fabrication process using pMSTL, uncured Ormocomp was washed away using ethanol. Finally, the fabricated 3D Ormocomp scaffolds were exposed to UV light for 24 h to improve the mechanical strength of the frameworks (post-curing).

2.1.2. Preparation of alginate gel and RGD-modified alginate gel

Two types of cross-linking methods, ionic cross-linking and cell cross-linking using RGD peptide (Calbiocam, Darmstadt, Germany), were used to observe the differences in the cell viability. Calcium chloride ($CaCl_2$) solution was used for ionic cross-linking [29]. For cell cross-linking, RGD modified alginate solution was reacted based on the carboxyl group in alginate and the terminal NH_2-group of RGD peptide [30–32]. This modified alginate was dissolved in a 0.1 M MES buffer (Sigma-Aldrich, St. Louis, MO, USA) to obtain 4% (w/v) alginate solution. RGD was added to the alginate solution in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich) and N-hydroxysulfosuccinimide (sulfo-NHS, Sigma-Aldrich). The solution was sterilized through a 0.22-μm filter.

2.1.3. Cell culture and cell encapsulation in alginate gel

For evaluation of the basic characteristics of the hybrid scaffold, human adipose-derived stem cells (ASCs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. ASCs of 1×10^4 cells/scaffold were suspended in a solution of medium and 4% alginate gel (50:50). This mixture was solidified using previously mentioned cross-linking methods. After 20 min,

the solidified samples were washed with PBS 3 times. The samples were incubated in normal growth media.

2.2. Analysis of morphological and mechanical characteristics of the hybrid scaffold

The surface morphology was observed using a scanning electron microscope (SEM) (Hitachi SU-6600; Hitachi, Tokyo, Japan) at 15 kV. Before SEM analysis, the samples were freeze-dried and platinum-coated using a sputter-coater. We measured mechanical strength using a single-column Instron 3340 mechanical testing system (Instron, Norwood, MA, USA). To compare the degradation of the alginate gel and RGD-modified alginate gel with or without cells, the weight was measured during an 8-week period (n = 5 per group). The alginate gel was dried under vacuum at room temperature and weighed using an electronic scale.

2.3. Analysis of the encapsulated-cell viability

To visualize the cells encapsulated in the 3D hybrid scaffold, cell viability was determined by the Live/Dead Viability/Cytotoxicity Kit (Molecular Probe, Eugene, OR, USA) during culture for 8 weeks. The samples were stained with calcein AM for live cells (green) and ethidium homodimer (EthD-1) for dead cell nuclei (red). Confocal images were reconstructed based on each 2D image using a confocal microscope (LSM; Carl Zeiss, Wetzlar, Germany).

2.4. Evaluation of immunerejection caused by the hybrid scaffold

Seven days after encapsulation, samples were implanted subcutaneously in 8-week-old female C57/BL6 mice. The experimental groups are described in Table 1. The animal test was performed according to the protocol approved by the Animal Care and Use Committee of POSTECH (2011-01-0013). Samples were harvested 1 week after implantation. The mice were sacrificed and blood samples were collected for mononuclear cell isolation. For the flow cytometry analysis, mononuclear cells were extracted by Histopaque (Sigma-Aldrich) density gradient centrifugation and stained with anti-CD3 coupled to allophycocyanin (APC, eBioscience, San Diego, CA, USA) [33]. Flow cytometry was performed with a FACSCalibur (BD Bioscience) and the data analyzed with Cell Quest software (BD Pharmingen, San Jose, CA, USA).

For immunostaining, the samples were fixed in 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, the samples were prepared on 4 μm sections using a cryostat (Leica, Wetzlar, Germany). Immunostaining analysis of the immune response was performed using anti-CD4 and anti-CD8 (eBioscience) and a FluoView 1000 confocal microscope (Olympus, Melville, NY, USA).

2.5. Dopamine releasing cell culture and measurement of dopamine release

SN4741 cells (Clonal SN-derived dopamine neuronal progenitor cell line) were used as dopamine-secreting cells, which proliferate progressively at 33 °C and secrete dopamine with diminished growth properties at a temperature of 37 °C or higher [34]. These cells were cultured for growing in RF medium (DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 3% D-glucose, and 1% penicillin/streptomycin) at 33 °C in a humidified atmosphere of 5% CO_2. After obtaining a sufficient number of cells, 1×10^4 cells in 2% alginate gel per structure were cultured onto the 96-well plate in growth media at 37 °C. The medium was replaced every 2–3 days and harvested every week for 8 weeks. The harvested supernatant was frozen using liquid nitrogen and stored at −80 °C until ELISA was performed. The amount of released dopamine was quantified by Dopamine Research ELISA (LDN, Nordhorn, Germany).

Table 1
Description of the experimental groups used for the acute immune rejection test *in vivo*.

Group	Description	Cross-linking
Group 1	Blank—no implantation	×
Group 2	Only Ormocomp scaffold	×
Group 3	Ormocomp scaffold + cells	×
Group 4	Alginate gel + cells	$CaCl_2$
Group 5	Hybrid scaffold (Ormocomp scaffold + alginate gel)	$CaCl_2$
Group 6	Hybrid scaffold + cells	$CaCl_2$
Group 7	Hybrid scaffold + cells	RGD

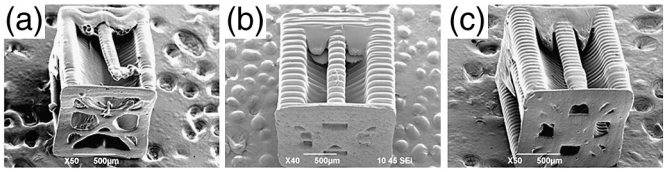

Fig. 3. Ormocomp scaffold fabrication results according to the illumination time of (a) 10 s, (b) 20 s, and (c) 30 s. The insufficient illumination time yielded incomplete or unstable Ormocomp scaffolds (a). (c) Too long an illumination time caused larger over-cured regions.

3. Results

3.1. Fabrication and characterization of the 3D Ormocomp scaffold as a framework

Illumination time was one of the key factors in determining the shape and resolution of the Ormocomp scaffold fabricated by pMSTL. Therefore, we evaluated the effect of the illumination time on the Ormocomp scaffold fabrication. Fig. 3 illustrates that the illumination time from 10 s to 30 s influenced the shape of the scaffold under the defined light intensity. Too short an illumination time resulted in incomplete scaffolds, while large undesired over-cured parts were observed in the 30-s group. Based on this result, we selected 20 s for the next step. The final dimensions of the fabricated 3D Ormocomp scaffolds were 1.5 mm × 1.5 mm × 1.5 mm.

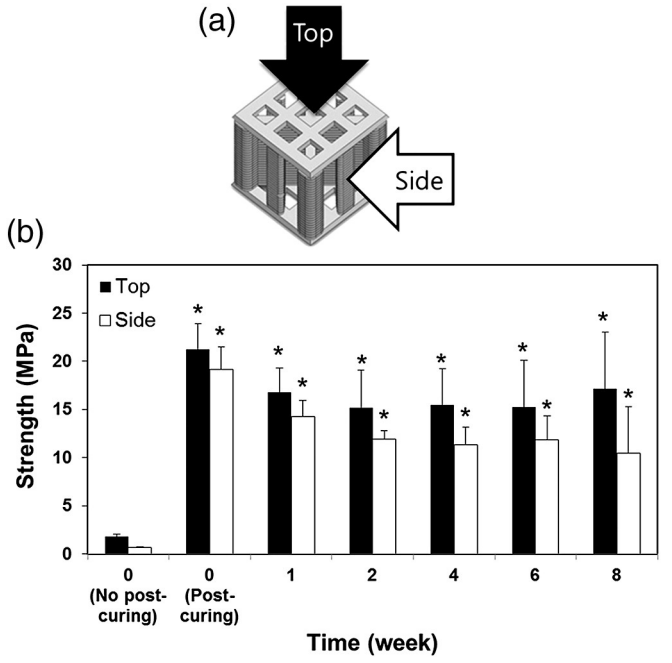

Fig. 4. Measurement of the compressive strength of the fabricated Ormocomp scaffold. (a) Forces were applied from the top and side directions. (b) All samples were post-cured except the No-post-curing group. The strength remained at over 10 MPa after post-curing for 8 weeks.

The post-curing process for the photocurable polymeric structures could generally enhance the mechanical strength. As shown in Fig. 4, the mechanical strength of the fabricated Ormocomp scaffold was influenced by the post-curing process. The force to measure the mechanical strength was applied along either the top direction or side direction (Fig. 4a). We observed that post-curing using UV after fabrication increased the mechanical strength considerably compared to the group without post-curing. Although the strength was slightly lower after 1 week, it was maintained at over 10 MPa for 8 weeks. The architecture of the scaffold seemed to cause a slight difference between the strength of the side and the top, even though it was statistically insignificant.

3.2. Characteristics of the hybrid scaffold

After the fabrication of the hybrid scaffold composed of the 3D Ormocomp scaffold as a framework, the internal space of the scaffold was filled with a mixture of alginate solution and suspended ASCs. Fig. 5 (week 1) shows that the ASCs were encapsulated (green spot) in the internal space of the scaffold. These encapsulated ASCs attached to and spread on the surface of the Ormocomp scaffold inside the gel over time. This phenomenon occurred earlier in the RGD group than the No-RGD (ionic cross-linking) group. Additionally, the intensity of the stained area in the RGD group was higher than that of the No-RGD group, especially at week 6.

The two cross-linking types (with or without RGD) did not seem to yield noticeable differences in the alginate gel degradation in the scaffold during the 8 weeks, as shown in Fig. 6. Although the weight ratio levels varied occasionally in the groups that include cells, it is safe to say that both types of alginate gels did not obviously degrade over the 8-week period. We inferred that some cells that were initially located beyond the distance within reach from the framework might not have survived at week 4 after proliferation and migration for 2 weeks. During this process, RGD crosslinking seemed to provide a more suitable environment for the cells to proliferate than crosslinking without RGD.

3.3. Evaluation of acute immune rejection in vivo

The fundamental role of this hybrid scaffold was to protect non-autologous cells from the immune rejection of the host by the extremely small pore size of the alginate gel [35]. We evaluated the feasibility of hybrid scaffolds to protect human ASCs from the mouse immune system. Six groups were tested as shown in Table 1. Fig. 7 shows that CD3 expression increased when human ASCs were exposed to mouse tissue without the alginate gel (group 3) compared to the other groups. Even though the expression level of the other groups including cells was slightly higher than that of the groups without exogenous cells, the expression level in group 3 was much higher than that of the other groups. The slightly higher CD3 expression detected in groups in which we used alginate gel (groups 4, 5, 6, and 7) indicates that samples might have been contaminated by endotoxin. Compared to the alginate encapsulating cells (group 4) with hybrid scaffolds (groups 5, 6, and 7), we confirmed that the main role of the alginate gel was to protect the encapsulated cells from immune rejection, and that its role was not affected by the mechanical strength increased by a framework. Moreover, we observed that CD4 and CD8 were highly expressed in group 3 compared to the other groups in Fig. 8. The difference between No-RGD and RGD was not recognizable.

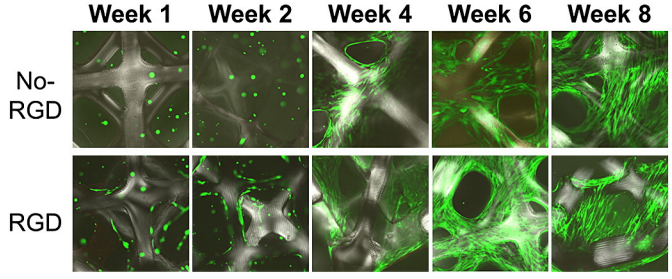

Fig. 5. Confocal images of the Live&Dead assay. Encapsulated human ASCs were observed for 8 weeks. Cell proliferation in the RGD group seemed to be higher than in the No-RGD group. Original magnification, 100×.

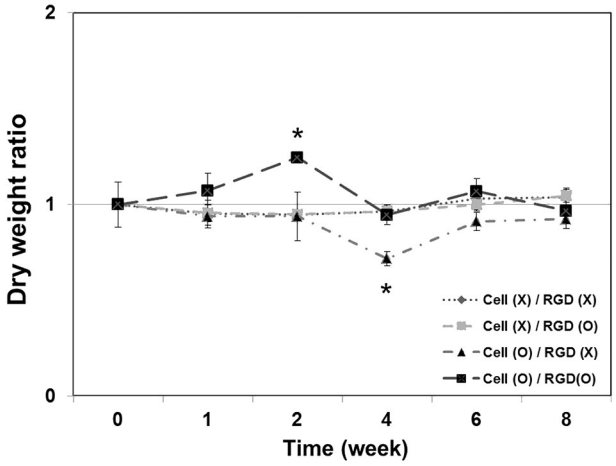

Fig. 6. Degradation of the alginate gel of the hybrid scaffold. The dry weight of the alginate gel was measured for 8 weeks. Regardless of the cross-linking type, the alginate gel of the hybrid scaffold did not degrade over the 8-week period.

3.4. Dopamine secretion test with SN4741

The feasibility of the hybrid scaffold for the treatment of Parkinson's disease was demonstrated using dopamine-secreting cells (SN4741). After the SN4741 cells were encapsulated in the hybrid scaffolds and alginate gels, the amount of dopamine released from the hybrid scaffolds and alginate gels was measured for 8 weeks using ELISA. We used both No-RGD and RGD methods. Fig. 9 indicates that dopamine was released from both the hybrid scaffolds and the alginate gels encapsulating

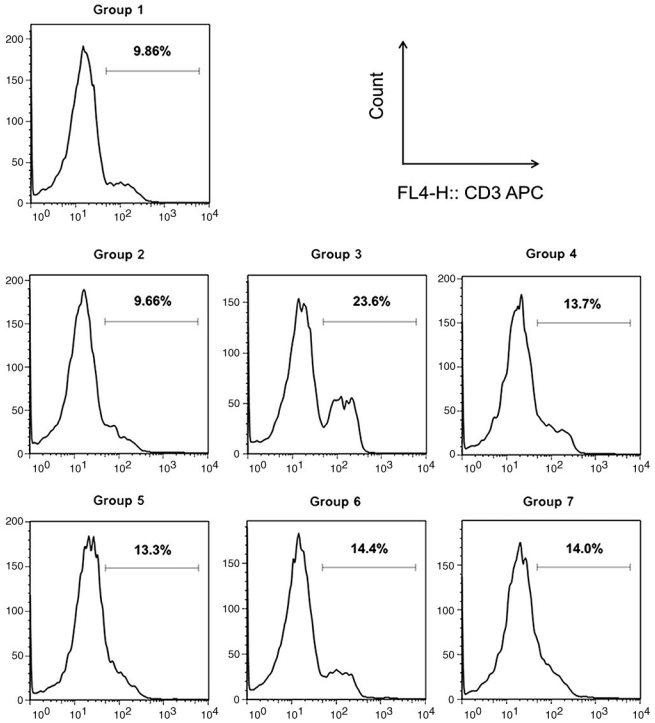

Fig. 7. CD3 expression measured using flow cytometry. CD3 was highly expressed when the cells were not encapsulated using the alginate gel (Group 3).

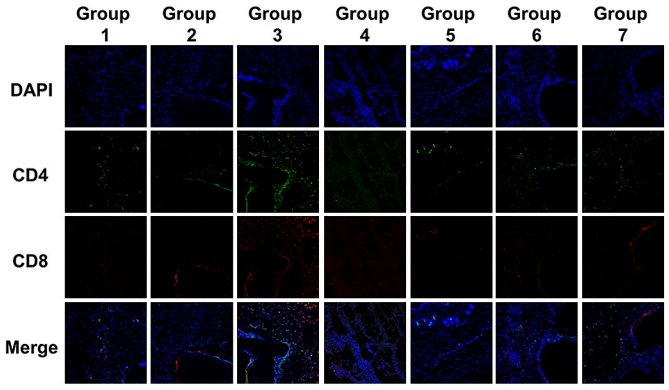

Fig. 8. Immunostaining images at day 7 after implantation. CD4 and CD8 were detected as markers of acute immune rejection. Higher expression of CD4 and CD8 was observed in.

dopamine-secreting cells over time (up to 8 weeks). The dopamine concentration became constant after 6 weeks. This also means that the cells were alive during this period. We found that a higher amount of dopamine was secreted when hybrid scaffolds were used, compared to alginate gels alone. We think that this is because an adhesion site (the Ormocomp framework) was provided to the cells rather than because the mechanical properties improved. Meanwhile, the RGD group showed a higher dopamine concentration compared to the No-RGD group. These data suggest the utility of the hybrid scaffold for treating Parkinson's disease as it releases a high concentration of dopamine.

4. Discussion

Here we presented a new 3D hybrid scaffold that was mechanically improved by utilizing a 3D Ormocomp scaffold as a framework and alginate gel for cell-based DDS applications. Basically, we had in mind the possibility of using exogenous cells from either other species or people in place of neurons that had lost their functions. The potential clinical application of this cell-based DDS was verified based on dopamine released from the 3D hybrid scaffold encapsulating dopamine-secreting cells for Parkinson's disease.

As mentioned earlier, one of the disadvantages of alginate gel is its weak mechanical properties [6,36]. Although many approaches have been investigated to improve the mechanical properties of alginate gels [6], we have suggested using a combination of the alginate gel and a 3D framework with a much higher mechanical strength (over 12 MPa) than general alginate gels (approximately 150 kPa) [37,38]. This combination could provide the better mechanical properties of the composite without altering the pore size of the gel matrices, which is related to its molecular diffusion properties [39,40].

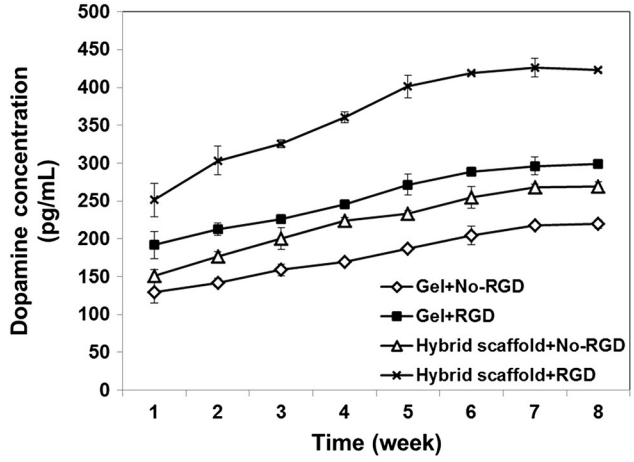

Fig. 9. Measurement of the non-cumulative dopamine released from the 3D hybrid scaffold and the alginate gel encapsulating SN4741 cells for 8 weeks. The amount of released dopamine gradually increased over the 8 weeks and reached the highest concentration at week 7. A higher concentration of dopamine was released when hybrid scaffolds were used compared to alginate gels alone, possibly because an adhesion site (the Ormocomp framework) was provided to the cells. RGD cross-linking increased the dopamine concentration compared to that of the No-RGD group.

3D hybrid scaffolds could protect encapsulated exogenous cells from the immune system of the hosts and allow the encapsulated cells to survive. Cell viability *in vitro* data suggested that encapsulated human cells could survive for 8 weeks with the necessary transport of nutrition, oxygen, and waste through the alginate gel. Acute immune rejection did not violently occur *in vivo* when the xenogenic human cells in the hybrid scaffold were implanted in the mouse subcutaneous tissue, whereas CD3, CD4, and CD8 levels were increased without gel encapsulation. The reason is that the pore size of the alginate gel used was smaller than antibodies, such as IgG [35]. Although exogenous cells implanted in the brain can be partly protected from the immune system of the host [41], xenotransplantation generally requires immunesuppression [42]. For clinical trials, the chronic rejection that could be caused by the hybrid scaffolds should be evaluated.

This represents an advantage of our hybrid scaffold over normal 3D scaffolds made of a single material [43]. Even though we could not provide information on the initial cell attachment, the use of an alginate gel can lead to lower cell loss during the cell loading process compared with direct cell seeding on the scaffolds [44]. In addition, Ashton et al. attempted to incorporate poly(lactide-co-glycolide) microspheres loaded with alginate lyase into an alginate gel in order to control gel degradation during cell culture [45]. This was a different type of hybrid scaffold and could be applied to cell-based DDSs with degradable polymer microspheres loaded with drugs that would be necessary initially. However, for the long-term use of cell-based DDSs, the required structural stability is not provided if polymer degradation occurs; furthermore, biodegradable microspheres could not function as the supporting framework. Moreover, poly(lactide-co-glycolide) could trigger acute inflammatory responses after implantation [46].

Among the possible applications, this hybrid scaffold could be used to act as dopamine delivery system for the treatment of Parkinson's disease. This disease requires long-term therapy because inserting scaffolds into the mid-brain region is difficult. This is why, from a mechanical standpoint, the scaffolds are required to be stable over a long period. Although there is a concern that inactive scaffolds should be removed from the brain after the treatment period because the framework is not biodegradable, this point should be considered after determining the “safe” treatment period, the number of necessary scaffolds, and the amount of space available in the brain. Another reason for applying our hybrid scaffolds to treat Parkinson's disease is that the molecular weight of dopamine renders it capable of diffusing through the pores of the alginate gel. Dopamine could easily diffuse through the pores of the alginate gel because the molecular weight of dopamine (approximately 153 Da) is much lower than that of permeable dextran (20 to 70 kDa) [6,47,48]. Our data on the release of dopamine supported that the developed hybrid scaffold could be utilized as a cell-based DDS for the treatment of Parkinson's disease. For *in vivo* tests, the number of hybrid scaffold encapsulating dopamine-secreting cells should be determined according to the correlation between the amount of dopamine released from the scaffold and the necessary dopamine concentration.

Moreover, we compared two cross-linking methods, such as ionic cross-linking using calcium chloride and cell cross-linking incorporated with RGD peptide [35,49]. Our data indicated that the cross-linking type did not influence degradation properties over the course of 8 weeks nor blocking acute immune rejection. The encapsulated cells attached to and spread on the Ormocomp scaffold over time regardless of the cross-linking type, although the process was slightly faster with RGD. However, the cross-linking type affected the dopamine release; in fact, the amount of dopamine released from the hybrid scaffolds encapsulating cells in the RGD group was almost two times higher than that of the No-RGD group. Although the mechanisms of the enhanced function of

releasing dopamine were not identified here, this result corresponded to previous research reporting improvement of heart function by using RGD-modified alginate gel [32].

Although cell-based treatment for Parkinson's disease has been studied using various types of dopamine-secreting cells [41], the clinical efficacy of cell injection without encapsulation has remained limited so far [50]. Our preliminary data on the 3D hybrid scaffold suggest it to be a more promising approach as a dopamine delivery system for Parkinson's disease. Further studies will be necessary to evaluate the function of 3D hybrid scaffolds over a longer period of time. Such data will be able to guarantee the stability of the amount of dopamine released from the hybrid scaffolds. Additionally, this should be verified using appropriate animal models *in vivo*, which should be able to provide a clue to determining the necessary amount of cells needed in the scaffolds.

5. Conclusion

We presented a 3D hybrid scaffold with a combination of a 3D framework and the hydrogel to enhance mechanical properties without altering hydrogel parameters, which influences transport properties. Based on the dopamine released from the 3D hybrid scaffold encapsulating dopamine-secreting cells, we demonstrated its possibility as a cell-based DDS for treatment of Parkinson's disease. In addition to the further studies on its function for an extended period, this 3D hybrid scaffold could be utilized for various applications.

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