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Fabrication of uniformly cell-laden porous scaffolds using a gas-in-liquid templating technique

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Design of porous scaffolds in tissue engineering field was challenging. Uniform immobilization of cells in the scaffolds with high porosity was essential for homogeneous tissue formation. The present study was aimed at fabricating uniformly cell-laden porous scaffolds with porosity >74% using the gas-in-liquid foam templating technique. To this end, we used gelatin, microbial transglutaminase and argon gas as a scaffold material, cross-linker of the protein and porogen of scaffold, respectively. We confirmed that a porosity of >74% could be achieved by increasing the gas volume delivered to a gelatin solution. Pore size in the scaffold could be controlled by stirring speed, stirring time and the pore size of the filter through which the gas passed. The foaming technique enabled us to uniformly immobilize a human hepatoblastoma cell line in scaffold. Engraftment efficiency of the cell line entrapped within the scaffold in nude mice was higher than that of cells in free-form. These results showed that the uniformly cell-laden porous scaffolds were promising for tissue engineering.

[**Key words:** Scaffold; Gas foaming; Gelatin; Alginate; Tissue engineering]

The objective of tissue engineering is to develop biological substitutes for damaged tissues but the design of scaffolds in this field is challenging. Requirements of scaffolds are to promote cellular adhesion, proliferation, and differentiation and to enhance engraftment of transplanted cells at targeted sites. In a general procedure to fabricate the biological substitutes, cells are first seeded in scaffolds at low density and transplanted into bodies. Penetration of blood vessels into the scaffolds is then promoted to supply sufficient oxygen and nutrients to the cells, resulting in cell proliferation in the scaffolds and following creation of the biological substitutes with high cell density (1). Scaffolds are classified into two types: porous sponges and non-porous hydrogels. The former are superior to the latter with regard to accelerated permeability of oxygen/nutrients and cell proliferation because of their porous structure (2). Especially, enhancement of permeability of oxygen and nutrients are important to keep cells alive until blood vessels penetrate into the scaffolds.

Uniform immobilization of cells in the porous scaffolds is essential for homogeneous tissue formation. Many methods for fabricating porous scaffolds have been described, such as solvent casting/particulate leaching (3–5), foam templating (6–9), and freeze-drying techniques (10,11). The conventional scaffolds are prepared using toxic compounds (e.g. organic solvents and chemical cross-linkers) or under harsh conditions (e.g., high temperature and drying). Therefore, cells are immobilized in the scaffolds after removing the toxic compounds or after the harsh process. It is quite difficult to uniformly immobilize cells within the pre-formed scaffolds by the commonly-used cell seeding method in which the scaffolds are overlaid with cell suspensions (a large proportion of the cells locate on the surface of the scaffolds in this method). Some researchers succeeded in uniform cell immobilization within porous scaffolds by incorporating cells within the scaffold matrix during the scaffold preparation process (2,12–15). Among the reports, a soft sacrificial particle leaching technique is the most likely candidate for achieving high porosity (2,12,14,15). The typical procedure of the technique for preparing porous scaffolds is as follows. Gelatin gel beads as pore templates are first prepared and then, added to a sodium alginate solution containing cells (2). The alginate solution was then formed into a gel by immersion into a calcium chloride solution. Finally, pores were formed by melting the gelatin beads by warming to 37°C. Because this process can proceed under mild conditions without using toxic chemicals, cells can be incorporated within the scaffold matrix during the scaffold preparation process, resulting in uniform cell immobilization in scaffolds. However, the maximum porosity is limited to 74% (random close-packing limit), which is accomplished when the beads are packed in their most compact arrangement. Higher porosity is needed to accelerate permeation of oxygen and nutrients, particularly for the regeneration of tissues with high oxygen and nutrient demands, such as the liver (2).

In this study, we focused on preparing uniformly cell-laden porous scaffolds with a porosity >74% by the gas-in-liquid foam templating technique. The technique developed by Barbetta et al. (16,17) is able to achieve a high porosity using a non-toxic gas. In

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the previous reports, the above authors did not achieve uniform cell immobilization in their scaffolds because they used toxic chemical cross-linkers or adopted a drying process. To prepare uniformly cell-laden scaffolds by the technique, we used gelatin as a scaffold material and microbial transglutaminase (MTG) to cross-link it. Gelatin is commonly used in the tissue engineering field because of its good cellular adhesion, biocompatibility and biodegradability (18). For use as a scaffold material, covalent cross-linking of gelatin is essential to improve its thermal stability. MTG catalyzes an acyl transfer reaction between the γ-carboxyamide groups of glutamine residues in proteins and primary amines (e.g., the amino groups of lysine residues) (19). Gelatin molecules can be covalently cross-linked in an aqueous environment by the enzyme without damaging mammalian cells (20). Thus, the combination of gelatin and MTG would enable us to uniformly immobilize viable cells in the porous scaffolds prepared by the gas-in-liquid foam templating technique.

We first attempted control of porosity and pore size in scaffolds prepared by the gas-in-liquid foam templating technique. Subsequently, we confirmed that viable cells could be uniformly immobilized into the scaffolds. Finally, to obtain basic knowledge concerning compatibility of mammalian cells with the scaffolds, the functions of the cells in the scaffolds was examined *in vivo*.

MATERIALS AND METHODS

Materials Porcine gelatin (type A) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). MTG and sodium alginate with a molecular weight of 70,000 Da (61% guluronic acid residues, Kimica I-1G) were kindly donated by Ajinomoto Co., Inc. (Tokyo, Japan) and Kimica Co. (Tokyo, Japan), respectively. Male nude mice (BALB/cA Jcl-nu, 6 weeks old) were obtained from Kyudo Co. Ltd. (Saga, Japan). A human hepatoblastoma cell line (HepG2, RCB1648) was obtained from Riken Cell Bank (Tsukuba, Japan). The cells (passage number: 35–40) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

Preparation of foam A cylindrical glass apparatus (diameter 37 mm) equipped with a porous glass septum (pore sizes 5–10 or 20–30 μm, Vidtec, Fukuoka, Japan) placed at its base was used for the preparation of foam (Fig. 1). Gelatin (12% (w/v)) and sodium alginate (0.5% (w/v)) were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate buffered-saline (PBS(−), pH 7.4). The solution (7 ml) was poured into the glass apparatus, thermostatically controlled at 40°C. Argon gas was delivered to the polymer solution through the glass septum, resulting in the generation of gas bubbles in the solution. The gas flow rate was adjusted to 5 ml/min using a syringe pump (gas volume delivered to the solution: 10.5, 21.0 or 36.8 ml). To maintain foam homogeneity during gas insufflation, the foam was stirred using an overhead impeller designed to avoid the incorporation of air (stirring rate: 100 or 250 rpm). Immediately after its formation, the foam was transferred to plastic vials (diameter 13 mm, height 3 mm, volume 0.4 ml) and incubated at 4°C for gelation of the foam through thermally-induced physical cross-linking of gelatin. After removal from the vials, the foam was immersed in a 65 units/ml MTG solution containing 100 mM $CaCl_2$ and 10 mM HEPES (pH 7.4) for 30 min at room temperature to cross-link the gelatin by MTG and the alginate by Ca^{2+}. After low vacuum (-7.5×10^4 Pa)/aeration cycles repeated five times to infiltrate the MTG solution into the foam, it was incubated in the solution for 120 min to allow further cross-linking. We confirmed negligible shrinkage of the foam during the cross-linking. Detailed experimental conditions are given in Table 1.

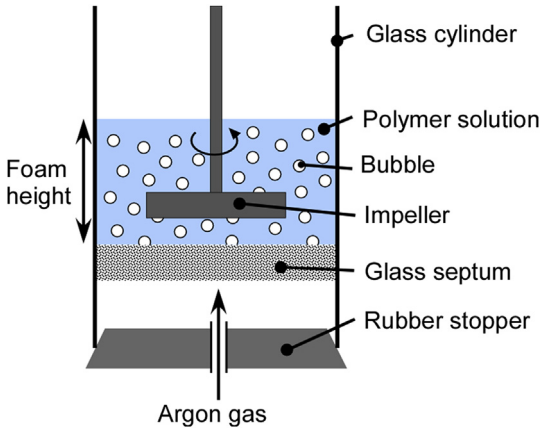

FIG. 1. Schematic depiction of foaming process.

TABLE 1. Experimental conditions.

Condition	Theoretical porosity (%)	Actual porosity (%)	Volume of delivered gas (ml)	Pore size of glass septum (μm)	Stirring time (s)	Stirring rate (rpm)
A	60	57 ± 6	10.5	5–10	126	100
B	75	73 ± 1	21.0	5–10	252	100
C	84	85 ± 1	36.8	5–10	442	100
D	60	–	10.5	5–10	442	100
E	60	–	10.5	5–10	442	250
F	84	–	36.8	20–30	442	100

The disk-shaped foam (0.4 ml) was then freeze-dried and the structure was observed using scanning electron microscopy (SEM, SS-550, Shimadzu, Kyoto, Japan). Pore size was determined from the area of more than 95 randomly selected pores using the free software package Image J (NIH, Bethesda, MD, USA) under the expedient assumption that the pore cross-sections were exact circles. Theoretical and actual porosities were determined by the following equations:

$$\text{Theoretical porosity (\%)} = \text{gas volume} \times 100/(\text{gas volume} + \text{polymer solution volume}) \quad (1)$$

$$\text{Actual porosity (\%)} = (1 - \text{polymer solution volume}/\text{foam height} \times \text{cross-sectional area of cylindrical glass apparatus}) \times 100 \quad (2)$$

where the polymer solution volume and cross-sectional area of the cylindrical glass apparatus were 7 ml and 10.8 cm^2, respectively.

Cell damage by stirring and low vacuum/aeration cycles Foam containing HepG2 was prepared using a 12% (w/v) gelatin and 0.5% (w/v) sodium alginate solution, suspending HepG2 cells at a density of 1.0×10^6 cells/ml, according to the procedure described above (pore size of glass septum 5–10 μm; gas volume 36.8 ml; stirring rate 250 rpm; and stirring time 442 s). The stirring condition was the severest for cells in all foam preparation conditions. After gelation of the disk-shaped foam by cooling at 4°C, the low vacuum (-7.5×10^4 Pa)/aeration cycle in PBS (−) was repeated five times at 15°C. The 10 disk-shaped foams (total volume: 4 ml) were then dissolved in warm PBS (−) (37°C) and the viability of the cells released in the buffer was examined by Trypan blue exclusion using a hemacytometer. This experiment was performed in triplicate.

Cell damage by gelatin cross-linking process HepG2 cells were seeded on a 96-well culture dish at a cell density of 2.0×10^4 cells/well (0.1 ml medium/well). After cultivation for 24 h at 37°C, each well was rinsed with PBS (−) and then 0.1 ml of MTG aqueous solution (65 units/ml MTG, 100 mM $CaCl_2$, 10 mM HEPES and 1 g/L glucose in PBS(−)) was added to the wells. After 3 h of incubation at room temperature, aliquots were removed and the cells were rinsed twice with cell culture media. Subsequently, culture media containing 10% (v/v) WST-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) were added to each well and incubated for 1 h at 37°C. Absorbance of the medium at 450 nm was measured using a spectrophotometer. Control cells were treated with an aqueous solution containing no MTG (100 mM $CaCl_2$, 10 mM HEPES, 1 g/L glucose in PBS(−)). Viability of MTG-treated HepG2 cells was calculated from the measured absorbances of MTG-treated and -untreated cells.

Preparation of cell-laden foam HepG2 cells were suspended in 12% (w/v) gelatin and 0.5% (w/v) sodium alginate solution at a cell density of 1.5×10^6 cells/ml. Disk-shaped cell-laden foam was prepared using the protocol described above (condition C in Table 1) and then, cross-linked by MTG and Ca^{2+}. To determine the distribution of cells in the foam, 3 foams were fixed in 10% (w/v) formaldehyde immediately after foam preparation, embedded in paraffin, sectioned (3 cross-sections per scaffold) and stained with hematoxylin and eosin. The cross-section (2 mm wide) was divided into 3 parts (upper side, middle and lower side). All cells in the 3 parts were manually counted. From the cell number, we determined the distribution of cells in the scaffold. This experiment was performed in triplicate.

In vivo evaluation of albumin productivity of HepG2 cells in foam HepG2 cell-laden foam (volume 0.4 ml; cell density 1.0×10^5 cells/foam) was prepared under condition C shown in Table 1. The foam was cultured in 1 ml DMEM with 10% FBS for 24 h and rinsed with saline. The foam was subcutaneously transplanted into male nude mice under anesthesia with pentobarbital. One week after transplantation, blood was obtained from postcava under anesthesia with pentobarbital. Plasma samples were obtained by centrifugation of the blood samples. The amount of human albumin produced by transplanted HepG2 cell in the samples was determined using a human albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). In a second condition, HepG2 cells suspended in saline (0.4 ml, 1.0×10^5 cells/0.4 ml saline) were subcutaneously injected into mice (control group). All