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Three-dimensional hepatic lobule-like tissue constructs using cell-microcapsule technology

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ABSTRACT

The proper functioning of the liver and tissues containing hepatocytes greatly depends upon the intricate organization of the cells. Consequently, controlling the shape of three-dimensional (3D) cellular constructs is an important issue for *in vitro* applications of fabricated artificial livers. However, the precise control of tissue shape at the microscale cannot be achieved with various commonly used 3D tissue-engineered building units, such as spheroids. Here, we present the fabrication of hepatic lobule-shaped microtissue (HLSM) containing rat liver (RLC-18) cells. By using cell-microcapsule technology, RLC-18 cells were encapsulated in the core region of poly-L-lysine-alginate microcapsules. After 14 days of long-term cultivation, RLC-18 cells self-assembled into HLSM, and the cells fully occupied the microcapsule. By monitoring the cell number and albumin secretion during culture and characterizing the dimensions of the fabricated tissue, we demonstrated that the HLSM showed higher hepatic function as compared with normal cell spheroids. We also showcased the assembly of these microtissues into a 3D four-layered hepatic lobule model by a facile micromanipulation method. Our technology for fabricating 3D multilayer hepatic lobule-like, biofunctional tissue enables the precise control of tissue shape in three dimensions. Furthermore, these constructs can serve as tissue-engineered building blocks for larger organs and cellular implants in clinical treatment.

1. Introduction

In vitro biofabrication of multicellular aggregates has been used as a compromise between conventional two-dimensional (2D) cultures and complex artificial organs in pharmacological assays [1] and toxicological studies [2]. Until now, the cell-laden modules used to build three-dimensional (3D) cellular constructs have employed the form of cell spheroids [3], cell microfibers [4], and cell sheets [5]. Among these, the fabrication of alginate-poly-L-lysine (PLL)-alginate (APA) microcapsules as a typical cell microcapsule was first established in 1980 by F. Lim and has been widely used for cell entrapment and drug delivery for >30 years [6–11]. The architecture of APA microcapsules includes a central core of dissolved Ca^{2+}-cross-linked alginate gel that provides an aqueous microenvironment and an alginate-PLL complex shell that acts like a semi-membrane. This semi-membrane permits the passage of low-molecular-weight substances, such as glucose and oxygen, to the core and passage of metabolic products from the core while retaining the microencapsulated islets or drugs within the microcapsule. Thus, the APA microcapsules provide a soft and "liquid-like"-system platform in which cells can form high-cell-density aggregation following long-term culture. Additionally, the PLL-alginate-complex shell can be further dissolved by treatment with a 1.6% sodium citrate solution to recover the encapsulated cells or release the drugs [12]. However, because of the gelation mechanism of the calcium-alginate hydrogel (calcium ions cross-linked with alginate), it is difficult to prepare APA microcapsules with a specific shape and size [13]. This disadvantage limits the utilization of the APA microcapsules for broader applications in tissue engineering as a bio-scaffold.

Electrodeposition methods were recently established [14–16] and are used to deposit a calcium-alginate gel film at specific electrodes addressed with a predesigned shape and size. Many research groups immobilized cells, bacteria, and other biocomponents within the alginate gel for cell-cell signaling and cell-culture studies

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based on the electrodeposition method [17,18]. The mechanism of electrodeposition involves releasing calcium ions from the region of the microelectrode surface via electrolysis (H^+ is released from the anode and reacts with $CaCO_3$ particles to release calcium ions). Therefore, these calcium ions can immediately react with the alginate near the electrode to form an alginate-hydrogel film. Simultaneously, the biocomponents are immobilized within the gel film for further applications. The advantages of the electrodeposition method include: (1) high biocomponent-immobilization resolution (~20 μm) [14] and (2) the use of simple equipment to apply potentials. Despite these advantages, major challenges related to 3D-cell-model fabrication based on electrodeposition remain. Few of these approaches have been adopted for 3D-cell-tissue construction for the following reasons: (1) most fabrication is restricted to a 2D surface used as a biofilm due to the principle of electrodeposition; (2) there is an acidic area generated near the anode electrode, resulting in the release of H^+ ions that might affect the viability of immobilized cells; and (3) cells embedded within the calcium-alginate film exhibit a low-proliferation rate due to lack of extracellular matrix (ECM) and room.

Therefore, it is critical to establish a new approach for integrating APA-microcapsule modules with the deposited shape-controlled alginate hydrogels. From this perspective, we proposed a novel method for fabricating 3D-multilayer hepatic lobule-like tissue constructs based on electrodeposition and microcapsule techniques. The design of a microelectrode device that was previously used for preparing microtissue in sphere, cuboid, and rod shapes [19] was modified to obtain suitable dimensions for preparing hepatic lobule-shaped microtissue (HLSM). The patterned microelectrode was fabricated by photolithography and is capable of simultaneous formation of alginate-gel film *in situ* with a hepatic lobule-shape based on the electrodeposition. These cell-containing gel films were then detached from the substrate and treated with PLL and sodium citrate solution to form 3D microcapsules. Cells encapsulated within the microcapsules eventually fully occupied all spaces to achieve HLSM within 2 weeks.

In addition to the HLSM-fabrication details, which can be found in our previous report [20], the current work is novel based on the comparison of the morphology, growth, and functionality of the RLC-18 HLSM with that of RLC-18 cell spheroids, which are commonly used as cell models. Additionally, a 3D four-layered hepatic lobule-like tissue was assembled by establishing a simple micromanipulation system. This study aimed to clarify that the biofunction of the HLSM having a shape similar to native tissue may differ quantitatively from the normal cell spheroid. Furthermore, our method provided a suitable platform for 3D-cell-model fabrication for potential *in vitro* applications of artificial-liver fabrication. The complete research map is shown in Fig. 1.

The contributions of this work include: (1) the combined method of cell-microcapsule technology for generating 3D microtissue to mimic the shape of the hepatic lobule, (2) quantitative comparison of microtissue with a hepatic lobule shape and spheroids based on albumin secretion and cell number during long-term culture, and (3) utilization of a simple micromanipulator system to assemble the single microtissue into 3D multilayer hepatic lobule-like tissue. Therefore, the proposed method provides a suitable platform for constructing a 3D cell model using cell-microcapsule technology. This platform has great potential to stimulate new uses of microcapsule technology in various applications, such as tissue engineering.

2. Materials and methods

2.1. Materials and solution preparation

We used sodium alginate (medium viscosity, A2033), sodium citrate tribasic dihydrate (S4641), PLL hydrobromide (molecular weight: 30,000–70,000), fluorine-doped tin oxide (FTO)-coated glass slides (surface resistivity: ~7 Ω/sq, 735,140; Sigma-Aldrich, St. Louis, MO, USA), and HEPES (346-01373; Wako Pure Chemical Industries, Osaka, Japan). Calcium carbonate ($CaCO_3$; 0.97 μm, #2300) were kindly supplied from Sankyo-Seifun, Ltd (Niimi, Japan). A photoresistor (AZ5214-E) was purchased from AZ Electronic Material GmbH (Merck KGaA, Darmstadt, Germany). Cell counting kit-8 (CCK8) was purchased from Dojindo Ltd (Tokyo, Japan). Rat albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (ERA3201-1) was purchased from Assaypro Inc (St. Charles, MO, USA). A quantitation urea kit (DIUR-100) was purchased from BioAssay Systems (Hayward, CA, USA). The water used to prepare the solution was deionized with a Millipore Direct-Q3 water purification system (Millipore, Worcester, MA, USA).

2.1.1. Chemical solution preparation

The deposition solution was prepared by dissolving 1% (w/v) alginate sodium in solution containing NaCl (126 mM), KCl (2.7 mM), $Na_2HPO_4{\cdot}12H_2O$ (8.1 mM), KH_2PO_4 (1.47 mM), and HEPES (21 mM). The pH was adjusted to 7.3 by adding 0.5 M NaOH solution. $CaCO_3$ (0.5%, w/v) was uniformly dispersed in the solution using a magnetic stirrer for 24 h.

The HEPES buffer solution was prepared by dissolving HEPES (5 g/L) in solution containing NaCl (8 g/L), KCl (0.37 g/L), Na_2HPO_4 (1.076 g/L), and glucose (1 g/L). The pH was adjusted to 7.3 by adding 0.5 M NaOH solution.

Calcium chloride (1.1%) solution was prepared by dissolving 0.55 g of $CaCl_2$ (anhydrous) in 50 mL of distilled water.

PLL solution was prepared by dissolving PLL hydrobromide (25 mg) in 50 mL of 0.9% (w/v) NaCl solution (0.05% PLL solution).

Sodium citrate solution was prepared by dissolving sodium citrate tribasic dihydrate (1.62 g) in 100 mL of 0.45% (w/v) NaCl solution (55 mM sodium citrate solution).

Cell-viability test solution consisted of a mixture of 0.8 μL calcein AM (1 mg/mL; Wako), 2.8 μL propidium iodide (1 mg/mL; Wako), and 1 mL HEPES buffer solution.

2.2. Fabrication of micropatterned electrodes

The photolithographic technique was used to construct the electrodevice in this experiment as previously described [19]. Briefly, FTO glass slides (2.5 cm × 5 cm) were sonicated with isopropyl alcohol and deionized water. The photoresistor (AZ 5214E) was coated on the surface of the FTO glass to a thickness of 1.4 μm.

The patterned electrode was fabricated using a micropattern generator (μPG 101; Heidelberg Instruments, Heidelberg, Germany) based on the AutoCAD design of microelectrodes. In this study, micropatterns of hepatic lobules and circular shapes were prepared as shown in Fig. S1 to fabricate the HLSM and cell spheroids for comparison. The outer diameter of the hepatic lobule pattern was 1.8334 mm and the inner diameter was 0.4 mm. The diameter of the circular pattern was 1 mm. To quantitatively analyze the morphology, growth, and functionality of the RLC-18 HLSM as compared with those of the cell spheroid, the designed microelectrodes for HLSM and the spheroid needed to have the same initial area as shown in Table 1.

2.3. Cell culture

Rat liver (RLC-18) cells were cultured for experiments with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU penicillin, and 100 μg/mL streptomycin in a 10 cm tissue culture dish at 37 °C in a humidified 5% CO_2 incubator. Upon reaching 90% confluence, cells were detached using trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) and gently pipetted