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Regular Article

Fabrication of cell-benign inverse opal hydrogels for three-dimensional cell culture

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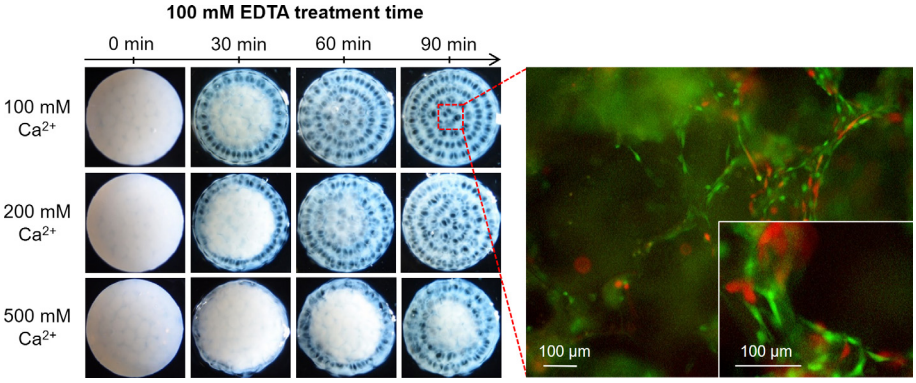
GRAPHICAL ABSTRACT



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ABSTRACT

Inverse opal hydrogels (IOHs) for cell culture were fabricated and optimized using calcium-crosslinked alginate microbeads as sacrificial template and gelatin as a matrix. In contrast to traditional three-dimensional (3D) scaffolds, the gelatin IOHs allowed the utilization of both the macropore surface and inner matrix for cell co-culture. In order to remove templates efficiently for the construction of 3D interconnected macropores and to maintain high cell viability during the template removal process using EDTA solution, various factors in fabrication, including alginate viscosity, alginate concentration, alginate microbeads size, crosslinking calcium concentration, and gelatin network density were investigated. Low viscosity alginate, lower crosslinking calcium ion concentration, and lower concentration of alginate and gelatin were found to obtain high viability of cells encapsulated in the gelatin matrix after removal of the alginate template by EDTA treatment by allowing rapid dissociation and diffusion of alginate polymers. Based on the optimized fabrication conditions, gelatin IOHs showed good potential as a cell co-culture system, applicable to tissue engineering and cancer research.

1. Introduction

The cells grown in three-dimensional (3D) microenvironments more closely mimic natural tissues and organs than cells grown in traditional two-dimensional (2D) plates. Therefore, generating artificial 3D cellular microenvironments is important for *in vitro*

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cell culture and its applications to a variety of biomedical studies, including drug screening, stem cell research, and tissue engineering [1–6]. Generally, high porosity with uniform macropores and homogeneously interconnected architectures are required for enhanced adhesion and migration of cells within the scaffold, and effective mass exchange of oxygen, nutrients, and metabolite wastes for proliferation of cells [7,8]. To fulfill these requirements, several strategies have been employed to generate 3D microenvironments based on biomaterials through gas foaming, salt leaching, freeze drying, and electrospinning [9–14]. However, most of these technologies have limitations related to uniform pore structure, size, and shape as well as poor connectivity [15,16]. All previous methods have difficulties in the generation of uniform macropores with precise control of pore size and shape due to the uncontrolled morphology of porogens, such as gas bubbles, salt crystals, and ice crystals. In addition, due to use of high pressure of gases, organic solvents, and freeze-drying processes, cells cannot be encapsulated within the matrix of macroporous biomaterials prepared in conventional methods.

In order to overcome the challenges associated with uniform pore structure and interconnectivity, inverse opal structures based on sacrificial templates have been exploited to modulate 3D architecture with uniform pores [17–19]. The reported inverse opal structures for cell culture can be categorized into two types depending on the physical state of the composing matrix. The first type is the typical inverse opal scaffold composed of a dense matrix, such as silica, polyacrylamide, chitosan, poly(ethylene glycol), or poly(lactic-*co*-glycolic acid), prepared by removing the sacrificial colloidal templates, such as silica, polystyrene, and poly (methyl methacrylate), using organic solvent or highly acidic solution [20–28]. These inverse opal scaffolds provide only the surface of macroporous structures for cell adhesion and cannot encapsulate the cells inside the matrix because of the use of toxic solvents to remove the opal templates.

The second type of inverse opal structure recently reported is composed of hydrogels (e.g., gelatin), which can encapsulate the cells [29]. In contrast to the previous approaches for fabrication of inverse opal scaffolds, the second type of structure utilizes calcium-crosslinked alginate microbeads as a sacrificial opal template that can be removed using the calcium-chelating agent ethylenediaminetetraacetic acid (EDTA), and gelatin is employed as a component of the scaffold matrix. In this structure, both the macropore surface and the inner matrix can be utilized for cell culture; this may allow the structures to mimic natural tissues and organs or facilitate investigation of biological processes, such as paracrine signaling in stem cell niches or cancer-stroma interactions. However, fabrication of this type of scaffold has not yet been optimized to achieve efficient removal of templates for the construction of 3D interconnected macropores and to maintain high cell viability during template removal. Longer treatment in EDTA solution is beneficial for complete removal of alginate microbeads but can be harmful to the cells encapsulated in the gelatin matrix.

In this report, various factors, including alginate viscosity, alginate concentration, alginate size, calcium concentration for preparation of alginate microbeads, EDTA treatment time, and gelatin concentration, were controlled to achieve high cell viability and successful generation of a 3D inverse opal structure in order to establish a 3D co-culture system (Fig. 1). Low viscosity alginate, lower crosslinking calcium ion concentration, and lower concentration of alginate and gelatin were found to obtain high viability of cells encapsulated in the gelatin matrix after removal of the alginate template by EDTA treatment by allowing rapid dissociation and diffusion of alginate polymers. Based on the optimized fabrication conditions, gelatin IOHs showed good potential as a cell co-culture system, applicable to tissue engineering and cancer research.

2. Experimental section

2.1. Materials

Gelatin (type A, ~300 g bloom from porcine skin), methacrylic anhydride (MA), 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropio phenone (I2959), alginic acid sodium salt from brown algae (medium viscosity alginate, MVA and low viscosity alginate, LVA), calcium chloride dehydrate (⩾99%), ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified eagle's medium (DMEM), and alpha minimum essential medium (Alpha MEM) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Live/Dead assay kit was purchased from Molecular probe (Eugene, OR, USA). OxiSelect™ cellular UV-induced DNA damage staining kit was purchased from Cell biolabs (San Diego, CA, USA).

2.2. Synthesis of gelatin methacrylamide (GelMA)

GelMA was synthesized as previously described [30]. First, 10 wt% gelatin solution was prepared by dissolving 1 g of gelatin powder in Dulbecco's phosphate-buffed saline (DPBS, pH 7.4) at 50–60 °C. One milliliter of MA was added to the gelatin solution at a rate of 0.25 mL/min, and the resulting solution reacted for 1 h under vigorous stirring. Subsequently, the reaction mixture was diluted with distilled water to terminate the reaction, precipitated twice in a large excess of ethanol at 4 °C, and dialyzed in distilled water using dialysis tubing with a 12–14-kDa cutoff for 5–7 days at 40 °C for purification. After dialysis, the resulting GelMA was freeze-dried for 1 week and stored at −80 °C until use.

2.3. Fabrication of gelatin hydrogels

10 wt% GelMA solution with 0.3 wt% I2959 was poured into custom-designed acryl mold (d: 15 mm, h: 5 mm) and photocured by UV irradiation for 10 min. The resulting gelatin hydrogels were separated from the mold and washed with alpha MEM medium at 37 °C for 20 min to remove the photoinitiator and by-products. To seed cells on the surface of gelatin hydrogel, MC3T3 cells (ATCC, VA, USA) were seeded on surface of gelatin hydrogel at a concentration of 1.0×10^6 cells/mL, and incubated at 37 °C.

2.4. Preparation of calcium-crosslinked alginate microbeads

Alginate microbeads were prepared using the electrostatic droplet extrusion method, as previously described [31]. Alginate solutions with different concentrations (1.5–4 wt%, depending on alginate type) were prepared by dissolving sodium alginate into distilled water and slowly rotating for 12 h. The sodium alginate solution was dropped into a 100-mM calcium dichloride solution using a syringe pump (Pump 11 Series, Harvard Apparatus) through a blunt needle (24G) under 12 kV using a high voltage power supply (230-30R, Bertan). According to the electrostatic potential difference between the needle and the calcium solution, small alginate drops were produced and dropped into the calcium solution. The alginate droplets were immediately crosslinked with calcium ions to form alginate microbeads. The resulting alginate microbeads were sterilized by immersion in 70 wt% ethanol for 1 h, washing with PBS five times, and dispersion in sterile PBS until use. The size of calcium-crosslinked alginate microbeads was measured with optical microscope (Primo Vert, Carl Zeiss). To evaluate Young's modulus of alginate hydrogels prepared based on LVA in calcium solution with different concentration, cylindrical alginate hydrogels were prepared. First, 3 wt% LVA was poured into a mold (diameter 10 mm, height 5 mm) and carefully incubated at 50, 100, 200, or 500 mM calcium solution for 3 h. The resulting