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Regeneration of neurite-like cells from induced pluripotent stem cells in self-assembled hyaluronic acid-gelatin microhydrogel

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ABSTRACT

Hybrid microhydrogel comprising hyaluronic acid (HA) and gelatin (Gel) was self-assembled with repeating units and used to culture induced pluripotent stem (iPS) cells for neuronal production. HA and Gel were conjugated with methacrylic anhydride (MA), photocrosslinked to fabricate singlet particles, organized for cell-laden HAMA-GelMA constructs, and employed to differentiate iPS cells. We found that an increase in the microhydrogel concentration from 6% to 10% (w/v) reduced the swelling ratio of self-assembled HAMA-GelMA scaffolds and enhanced the encapsulation efficiency of iPS cells. In addition, an increase in the weight percentage of HAMA promoted the swelling ratio of self-assembled HAMA-GelMA scaffolds and decreased the encapsulation efficiency of iPS cells. Immunochemical staining and flow cytometry demonstrated that the order in the ability to differentiate iPS cells toward neural lineage was HAMA-GelMA construct < construct with CDPGYIGSR < construct with CSRARKQAASIKVAVSADR < construct with the two defined peptides. The current self-assembled HAMA-GelMA constructs with neurite-inductive factor can be of potential to duplicate an engineered tissue from iPS cells to reframe nervous systems.

1. Introduction

Photocrosslinkable biopolymers, such as gelatin (Gel), hyaluronic acid (HA), collagen, chitosan, dextran, and chondroitin sulfate, have been developed as an important category of materials for a variety of applications in biotechnology [1]. Among these natural biomaterials, Gel is one of the most frequently encountered substrates. This is mainly because Gel has an excellent biodegradability, is free from adverse responses in immunology, and can improve cell adhesion via specific binding of its Arg-Gly-Asp (RGD) sequence to cell membrane $\alpha_v\beta_3$ integrin [2]. Concerning the photoreactivity, Gel using its lysine side chain could conjugate with methacrylic anhydride (MA) [3]. It has been found that the photocrosslinked Gel has a high swelling ratio (8–21 times), a high viability of fibroblast NIH 3T3 cells under an ultraviolet (UV, about 80%), and an obvious cell elongation and mutual cell interaction for two days [4]. 10% Gel without the assistance of transforming growth factor-β1 could also maintain the phenotype of aortic valvular interstitial cells for seven weeks [5]. In addition to Gel, HA is a kind of glycosaminoglycans, has an ability to promote cell propagation, and can reduce chronic inflammation during wound healing. The photopolymerization traits of HA hydrogels could be acquired by a conjugation with glycidyl methacrylate [6]. A reaction between hydroxyl group of 2-acetamide-2-deoxy-D-glucose or D-glucuronic acid and MA could also confer the capability of photocrosslinking on HA [7]. Moreover, HA has been widely employed as a supporting matrix for cell culture in tissue engineering. For example, engineered scaffolds were manufactured with HA, chitosan, and alginate for articular cartilage [8]. Photocrosslinked HA could also stimulate a generation of elastin in a culture of valvular interstitial cells and advantage the fabrication of artificial heart valve [9]. In a study on self-renewal and differentiation, the quantity of HA significantly increased after cultivation of human embryonic stem cells (ESCs) in endothelial growth medium containing vascular endothelial growth factor [10]. An entrapment of neurons and Schwann cells in HA and collagen favored the growth of Schwann cells, improved a secretion of nerve growth factor (NGF) and brain-derived neurotrophic factor, and intensely advanced the neurite outgrowth [11].

Functional organs are often composed of repeated elements such as nerves in spinal cord, nephrons in kidney, hepatic lobules in liver, and islets in pancreas. Thus, from the viewpoint of biomedical engineering, a material manufactured by a top-down technique may be inadequate to reconstruct human tissue. Instead, a formation of scaffolding matrix with repeated architecture units can be a practical strategy to regenerate physiologically active cells.

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Nomenclature

E_e	entrapment efficiency of iPS cells in HAMA-GelMA microhydrogel (%)
P_{HAMA}	weight percentage of HAMA in HAMA-GelMA microhydrogel (%)
P_{SSEA-1}	percentage of SSEA-1-positive cells in total cells (%)
$P_{\beta III\ tubulin}$	percentage of βIII tubulin-positive cells in total cells (%)
$P_{\beta III\ tubulin+SSEA-1}$	percentage of βIII tubulin- and SSEA-1-positive cells in total cells (%)
R_s	swelling ratio of HAMA-GelMA microhydrogel (%)

Abbreviations

ESC	embryonic stem cell
Gel	gelatin
GelMA	gelatin conjugated with methacrylic anhydride
HA	hyaluronic acid
HAMA	hyaluronic acid conjugated with methacrylic anhydride
H&E	hematoxylin and erosin
iPS	induced pluripotent stem
MA	methacrylic anhydride
MSC	mesenchymal stem cell
SSEA-1	stage-specific embryonic surface antigen-1

Therefore, for a better answer to this crucial issue, the bottom-up technique was evolved through organizing elementary building blocks. In a study on cell-laden biomaterials, microscale hydrogels with a specific pattern of alternating checkerboard were prepared by a micromolding method [12]. In addition, a macroscopic hierarchy of three-dimensional (3D) hydrogel was arranged from microparticles by hydrophilic attraction [13]. Microscale hydrogels could be also augmented to yield an aggregate of 3 cm in carbon tetrachloride [14]. Hence, with defined cell type and cell quantity, flocculated microhydrogel can be feasible to mimic the human tissue structure for biomedical purpose.

The aim of this study was to develop hybrid microhydrogel composed of self-assembled MA-conjugated HA (HAMA) and MA-conjugated Gel (GelMA) to produce neurite-like cells from induced pluripotent stem (iPS) cells. The neuronal differentiation of iPS cells in hydrogels played an important role in the treatment for injured nervous tissue. One of the advantages of the hydrogel system to culture iPS cells is the amelioration of the nonhomogeneous cell dispersion in traditional scaffolds [15]. In addition, the nervous tissue is very soft, in general, and hydrogel of low mechanical strength can be appropriate to repair the injured nerves, especially in the central nervous system [16]. We examined the swelling ratio and structure of self-assembled HAMA-GelMA microhydrogel and analyzed the encapsulation efficiency of iPS cells in the resultant constructs. In addition to the self-assembled HAMA-GelMA microhydrogel, CDPGYIGSR, CSRARKQAASIKVAVSADR, and NGF were used as active neurite-inductive components to guide the differentiation of iPS cells toward neurons.

2. Materials and methods

2.1. Preparation of HAMA-GelMA microhydrogel

HA (sodium form, Sigma–Aldrich, St. Louis, MO) of 0.2 g was dissolved in ultrapure water (Barnstead, Dubuque, IA) of 20 ml at 200 rpm for 20 min. This solution of 1% (w/v) HA was added with 5 N sodium hydroxide (NaOH; Sigma–Aldrich) solution of 0.8 ml and reacted with MA (Sigma–Aldrich) of 1 ml at 400 rpm for 2 h. The resultant solution was stored at 4 °C for 24 h and mixed with 95% ethanol (Taiwan Sugar, Tainan, Taiwan) to precipitate HAMA. HAMA sediments were washed with 95% ethanol, placed at 40 °C for 3 h, dissolved in ultrapure water of 20 ml, frozen in a low-temperature freezer (Frigidaire, Augusta, GA) at −20 °C for 24 h, and freeze dried using a lyophilizer (Eyela, Tokyo, Japan) at 2–4 torr and −80 °C for 24 h. The produced white HAMA powder was preserved at −20 °C. In addition, Gel (Sigma–Aldrich) of 10 g was dissolved in ultrapure water of 90 ml at 200 rpm and 60 °C for 40 min. This solution of 10% (w/v) Gel was reacted with MA of 2.5 ml at 700 rpm and 50 °C for 2 h. The resultant solution was dialyzed with cellulose bag (Spectrum Laboratories, Rancho Dominguez, CA) of 12 kDa against ultrapure water of 1 l as a buffer medium at 40 °C for 1 week with a daily replacement of ultrapure water. After dialysis, GelMA in the cellulose bag was frozen at −20 °C for 24 h and freeze dried using the lyophilizer for 24 h. The produced white GelMA powder was preserved at −20 °C.

Ocadecyltrichlorosilane (OTS; Sigma–Aldrich) of 0.25 ml was mixed with heptane (Sigma–Aldrich) of 4.75 ml. The OTS solution of 0.5 ml was uniformly dispread on cover slide of 18 mm × 18 mm and the same solution of 2.5 ml was used for cover slide of 76 mm × 22 mm. The OTS-coated cover slides were placed at room temperature for 30 min and at 80 °C for 2 h, washed with 95% ethanol, and sterilized with 75% ethanol. The HAMA-to-GelMA ratio was fixed at 0:10 and 1:9 for 10% (w/v) HAMA-GelMA microhydrogel, at 2:8 and 3:7 for 8% (w/v) HAMA-GelMA microhydrogel, and at 4:6 and 5:5 for 6% (w/v) HAMA-GelMA microhydrogel. HAMA and GelMA microhydrogel was mixed with 2% 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959; I2959; Ciba Chemicals, Perth, Western Australia) in complete darkness for 1 h. The mixed hydrogel of 20 µl was loaded on the OTS-coated cover slides to form a spherical drop, placed in a space between two cover slides of 150 µm with a photomask on the top of the drop, and exposed to UV in a distance of 1.5 cm at 365 nm and 4.5 mW/cm^2 for 1 min. The photomask was designed with the following defined pattern: regular squares of 400 µm × 400 µm with linear distance of 1 mm between any two adjacent squares. The photomask was fabricated on a polyethylene terephthalate sheet and printed out with silver bromide at a density of 10,000 dpi for nontransparent area (Taiwan Kong King Co., Taoyuan, Taiwan). Fig. 1(A) shows the experimental arrangement for the photocrosslinking of HAMA-GelMA microhydrogel. Fig. 1(B) shows the reaction mechanism for synthesizing HAMA-GelMA microhydrogel. After the reaction, the photomask was removed. The formed HAMA-GelMA microhydrogel particles were washed with ultrapure water at 37 °C to eliminate the uncrosslinked mixed hydrogel. To graft peptides, HAMA-GelMA microhydrogel was activated with Dulbecco's phosphate buffered saline (DPBS, Sigma–Aldrich) containing 4-(N-maleimidomethyl)cyclohexane-1-carboxylic-acid-3-sulfo-N- hydroxysuccinimide ester (Sigma–Aldrich) solution with a concentration of 10 µg/ml at 25 °C for 2 h. The activated HAMA-GelMA microhydrogel was washed with DPBS, dehydrated at 40 °C for 12 h, and injected with DPBS containing CDPGYIGSR (Sigma–Aldrich) and CSRARKQAASIKVAVSADR (Sigma–Aldrich) solution with a concentration of 10 µg/ml at at 4 °C for 12 h. The peptide-modified HAMA-GelMA microhydrogel was washed with DPBS and dehydrated at 25 °C for 12 h. To determine the amount of peptide being grafted, the microhydrogel particles were decomposed in sodium hydroxide (Sigma–Aldrich) of 0.1 N. The dissolved solution of 150 µl in a 96-well MicroWell plate (polystyrene, Nalge Nunc, Rochester, NY) was incubated with Coomassie protein assay reagent (Thermo Fisher, Waltham, MA) of 150 µl. The quantity of peptide was evaluated using an enzyme-linked immunosorbent assay spectrophotometer (Bio-tek, Winooski, VT) at 595 nm. The

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