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Short communication

A polyethylene glycol-based hydrogel as macroporous scaffold for tumorsphere formation of glioblastoma multiforme

Yoonjee Oh [a,1], Junghwa Cha [a,1], Seok-Gu Kang [b], Pilnam Kim [a,*]

[a] Department of Bio and Brain Engineering, KAIST, Daejeon, 305-701, Republic of Korea
[b] Department of Neurosurgery, Severance Hospital, Yonsei University College of Medicine, Seoul, 120-752, Republic of Korea

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ABSTRACT

We report a formation of macroporous scaffold which is based on polyethylene glycol (PEG)-based alginate (ALG) interpenetrating polymer network (IPN) hydrogel. Using this scaffold, we assess the tumorsphere (TS)-forming ability of glioma cancer stem cells (gCSCs), which subpopulation has been highlighted as a main cause of therapeutic resistance due to self-renewal and potential of differentiation properties. Although there have been numerous methods to study the TSs, however, there is no plausible method to evaluate the formation of single gCSC cell-derived TSs, due to fusion-induced cell aggregation. To provide reliable assessment, the PEGDA hydrogel interpenetrated with ALG was fabricated as macroporous scaffold for TS formation of patient-derived gCSCs. With UV-ionic dual crosslinking process, the pore size of PEGDA-ALG hydrogel is magnified enough to be applied as a macroporous scaffold, providing increased internal voids for TS growth and expansion. As a result, within macroporous scaffold, the multiple number of single gCSC-derived TSs was successfully formed inside the structural voids.

Introduction

A rare subpopulation of neoplastic clones, cancer stem cells (CSCs) of glioblastoma multiforme (GBM), has been recently revealed as a main cause for the failure of current GBM therapies [1], exhibiting therapeutic resistance to radiotherapy [2] and chemotherapeutic agents [3]. Unfortunately, the resistance mechanisms remain unclear, as little is known about the biology and characteristics of the glioma cancer stem cells (gCSCs). Due to stem-like properties such as clonal expansion [1], a single gCSC can develop itself into floating spherical aggregates, called tumorspheres (TSs). It is well-accepted that each TS is derived from a single cell, thus TS-forming assays are considered as an evaluating method of the characteristics of gCSCs [4].

Many approaches have been suggested as TS-forming assays to develop a single cell into three-dimensional (3D) cellular clusters [5–8]. TS-forming studies include two-dimensional (2D) non-adherent culture (*i.e.* floating), hanging drop culture, and microwell-assay [9]. When cultured using such methods, CSCs are cultivated at low density in a special serum-free medium [4], called stem cell medium that is supplemented with several factors required for stem cell growth including basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). However, recentstudies reported several limitations to those culture methods, including fusion-induced growth and unintended aggregation [4,10]. This occurred because cells freely suspended in medium collide with one another, even under conditions of low cell density. Although the fusion-induced multicellular aggregates can be developed as a spherical shape, it may be different from the definition of TSs formed from single gCSC through clonal expansion.

To take into account 3D architecture of TSs and simultaneously inhibit the fusion-induced aggregation, we suggest the use of porous scaffold, which can provide a 3D physical support and confinement for each gCSC. Since TSs should be predominantly generated by the growth of a single cell, a 3D support would facilitate the confinement of the chemical gradient [11,12], while providing a compartment of single cells. To fabricate such scaffold that enables TS formation, hydrogels are required to possess non-biofouling property to lead TS formation and expansion without cell-matrix interaction. In addition, to provide internal void space

* Corresponding author.
E-mail address: pkim@kaist.ac.kr (P. Kim).
[1] These authors contributed equally.

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for TS expansion, enough porosity should be formed within hydrogels to transport nutrients and oxygen for encapsulated cells.

Polyethylene glycol (PEG)-based hydrogels have been highlighted as attractive scaffolding materials due to their bio-inert nature of PEG chains, as well as biocompatibility and facile modification [13]. PEG is easy to synthesize and modify through anionic ring-opening polymerization of ethylene oxide. A variety of end groups of PEG, including alcohol, amine, N-hydroxysuccinimidyl (NHS), or methyl ether are available for wide modification [14]. These characteristics of PEG-based hydrogels provide advantages to design desirable scaffolds such as adjustable mechanical properties and functionalized chemical modification.

A caveat to PEG-based hydrogels, however, is that the polymeric networks are too dense for the level of nutrient and oxygen transport required for TS growth. For example, the mesh size of the PEGDA hydrogel (M_r 575–20,000) is reported as 0.1–10 nm [15,16], which restricts cellular processes including proliferation [14,17]. To overcome this obstacle, several studies reported the methods to increase the porous architecture of PEG-based hydrogels by varying the precursor concentration [18], or using salt leaching technique [13], solvent-casting [19], or gas-foaming techniques [20]. However, these techniques have required complex fabrication processes that are cytotoxic because of the use of solvents.

To address these challenges, PEG-based interpenetrating polymer networks (IPNs) have been developed to modulate the mechanical or structural features of PEG hydrogel, by comprising two or more polymer networks, such as chitosan [21], poly(acrylic acid) [22], or alginate [23,24]. Among them, a highly porous alginate (ALG) hydrogel with biocompatibility and bio-inert nature [25,26], for example, was widely used as an incorporating material of a PEG-based IPN hydrogel. This PEG-based ALG IPN have been applied in cell encapsulation [24], which could not only improve the weak mechanical stability, durability, and permeability of ALG hydrogel, but also allow effective diffusion of nutrients through the IPN gel. Aforementioned studies suggested a biocompatible material with adequate mechanical strength and durability to encapsulate the cells [27], which had not been accomplished within each PEG or ALG hydrogel.

Herein, we thus focused on the properties of a PEG-based IPN gel itself, which is composed of PEG-diacrylate (PEGDA) and ALG. Through dual crosslinking, which are based on ionotropic gelation and photoactivated free radical polymerization, we could fabricate a PEGDA-ALG IPN gel with a macroporous feature. Thus, we utilized a PEGDA-ALG IPN gel as a 3D macroporous scaffold for each gCSC, which can provide the structural voids for cell cultivation with chemical gradients for TS growth and further spatial compartments to inhibit fusion-induced cell aggregation.

Materials and methods

Preparation of PEGDA-ALG hybrid hydrogel

PEGDA (MW = 6 kDa) (Sigma, St. Louis, USA) 10% (w/v) solutions were prepared in PBS. For photopolymerization, a 0.05% (w/v) photoinitiator, Irgacure 2959 (Sigma, St. Louis, USA), was used as 10% (w/v) solution in 70% ethanol. For alginate incorporated PEGDA hydrogel (PEGDA-ALG), low molecular weight sodium-alginate (Sigma, St. Louis, USA) 2% (w/v) solutions were prepared in PBS and mixed with PEGDA solutions by 1:1 volume ratio. Resulting polymer mixture pipetted into PDMS cylindrical wells (diameter and depth of wells: 6 mm and 1 mm) then covered with PET porous membrane (pore size: 8 μm). Then, simultaneous dual crosslinking occurred by pipetted 100 mM calcium chloride solution (Sigma, St. Louis, USA) on top of membrane and exposed to 365 nm UV radiation for 15 min. Resultant hydrogels were washed with PBS to remove excess ions and polymers.

Cell culture and encapsulation

The patient-derived GBM stem cell line, GSC11 was kindly provided by Dr. F. Lang (Department of Neurosurgery, The University of Texas, MD Anderson Cancer Center, TX, USA). The GSC11, were first stably transfected with green fluorescent protein (GFP). GFP-GSC11 were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air as a suspending cells in medium consisting of DMEM/F-12 (Gibco, Gaithersburg, MD, USA) with 2% 1 × B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/ml bFGF (Sigma, St. Louis, USA), 20 ng/ml of EGF (R&D Systems, Minneapolis, USA), and 1% penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). For cell loading, resultant hydrogels allow to swell for 1 d in deionized water to reach to maximum swelling ratio, then freeze in −80 °C for 12 h. Then, lyophilized sponge-type 3D scaffold is obtained by 12 h freeze-dying. The scaffolds were placed in a low-attachment 24-well plate and the dense cell suspensions (1–10 × 10^5 cells in 20–30 μl) were overlaid on top of dehydrated hydrogel to occur rapid rehydration to induce effective and homogeneous cell seeding into scaffold. The constructs were then incubated at 37 °C, 5% of CO_2 for 1 h to allow more cells to infiltrate the matrices. Finally, constructs were immerse in the culture medium and maintained in the incubator. The medium was changed every 4 day.

Analysis of tumorsphere formation and growth

The images of GFP transfected GSC11s were taken using an inverted confocal laser scanning microscope (Nikon, Tokyo, Japan). The average area and diameter of each tumorspheres were fitted to an ellipse and analyzed by adjusting the images using the particle analyzing method of the image analysis software ImageJ (NIH, Bethesda, Maryland, USA).

Release kinetics of hydrogel

The release of incorporated substance from hydrogels was investigated by examining the diffusion of fluorescein isothiocyanate-labeled dextran (FITC-dextran, M_w = 70 kDa) (Sigma, St. Louis, USA). For hydrogel preparation, 2 mg/ml FITC-dextran was dissolved in the PEGDA solution then polymerized. After washing and equilibrating with PBS containing 2 mg/ml FITC-dextran, the hydrogels were quickly rinsed with PBS before immersed into fresh PBS for measuring the release of FITC-dextran from hydrogels. After defined time points, samples of PBS containing released FITC-dextran from hydrogel were collected and analyzed in a microplate spectrofluorometer (Molecular Devices, CA, USA) at an excitation wavelength of 488 nm. The cumulative release (%) was calculated based on the concentration of released FITC-dextran by initial loading concentration using standard curve of FITC-dextran (70 kDa).

Swelling ratio of hydrogel

Swelling degrees of hydrogels were investigated by weighing water-containing hydrogel in distilled water. After 24 h from immerse samples in distilled water, the swollen samples were taken out from water, wiped superficially with a filter paper, and swollen weigh (W_s) was measured. Then, lyophilized samples were weighed to achieve dry weight (W_d). Swelling ratio is defined as the fractional increase in the weight of the hydrogel due to water absorption.

Pore size and porosity of hydrogel

Porosity and pore size distribution of lyophilized hydrogels were measured by mercury intrusion porosimetry (MIP). The measurement was performed with Autopore IV 9500 (Micromeritics, GA,