Acta Biomaterialia 21 (2015) 55–62

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Optimization of adhesive conditions for neural differentiation of murine embryonic stem cells using hydrogels functionalized with continuous Ile-Lys-Val-Ala-Val concentration gradients

Yueh-Hsun Yang [a,b], Zara Khan [a,b], Cheng Ma [a,b], Hyun Ju Lim [a,b], Laura A. Smith Callahan [a,b,c,d,*]

[a] The Vivian L Smith Department of Neurosurgery, University of Texas Medical School at Houston, United States
[b] Center for Stem Cell and Regenerative Medicine, University of Texas Brown Foundation Institute of Molecular Medicine, United States
[c] The Department of Nanomedicine and Biomedical Engineering, University of Texas Health Science Center at Houston, United States
[d] The Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston, United States

ARTICLE INFO

Article history:
Received 21 January 2015
Received in revised form 31 March 2015
Accepted 21 April 2015
Available online 27 April 2015

Keywords:
Neural differentiation
Embryonic stem cell
Tissue engineering
Gradient hydrogel
Combinatorial methods

ABSTRACT

Stem cell therapies, which aim to restore neurological function after central nervous system injury, have shown increased efficacy when a tissue engineering matrix is implanted with cells compared to implantation of the cells alone. However, much work still needs to be done to characterize materials that can be used to facilitate and direct the differentiation of implanted cells. In the current study, polyethylene glycol hydrogels functionalized with continuous Ile-Lys-Val-Ala-Val (IKVAV) concentration gradients were fabricated and utilized to systematically study and optimize the adhesive conditions for neural differentiation of mouse embryonic stem cells in two- and three-dimensional environments. The results suggest that 570 μM and 60 μM are the optimal IKVAV concentrations for 2D and 3D neural differentiation, respectively, to maximize mRNA expression of neuron-specific markers and neurite extension while minimizing apoptotic activities in cultured cells compared to those exposed to higher IKVAV concentrations. The combinatorial approach presented in this work demonstrates that hydrogels functionalized with bioactive peptides provide a defined and tunable platform that can be employed to characterize and improve culture conditions for superior survival, maturation and integration of implanted cells, leading to enhanced restoration of neurological function for those receiving stem cell therapies after traumatic brain and spinal cord injuries.

1. Introduction

Traumatic brain and spinal cord injuries have a high prevalence, enormous financial costs and lack clinical treatments that restore neurological function [1,2]. Experimental stem cell therapy treatments that aim to repair damaged tissues and organs are being developed, but often ignore the contributions of the extracellular matrix (ECM) in tissue development [3–5]. Experimental stem cell therapy treatments that have included hydrogels to provide artificial ECM support for implanted cells have shown greater improvement in neurological function than the implantation of the cells alone [6–8]. Optimization of bioactive signaling within these hydrogels could potentially lead to further improvements in the restoration of neurological function from stem cell therapy.

Laminin, a major constituent of the basement membrane that is found throughout the ECM of the central nervous system, plays a role in tissue formation and cellular function in the central nervous system [9]. Ile-Lys-Val-Ala-Val (IKVAV), one of the most studied peptide fragments from laminin, is thought to have similar biological effects to laminin [10]. IKVAV has been shown to promote neuron survival, adhesion, migration and axon extension *in vitro* [10,11], while reducing glial scar formation *in vivo* [12]. A wide range of beneficial IKVAV concentrations from 10 μM to 2.6 mM has been documented in the literature [10,13–18]. However, it has also been shown that variations in IKVAV concentration influence cell attachment and neural differentiation [13,15], implying that optimization of IKVAV concentration could further improve the beneficial effects of the peptide on neural cells and tissues. Several studies have utilized random discrete samples to optimize IKVAV concentration for both two-dimensional (2D) and three-dimensional (3D) neural differentiation and

* Corresponding author at: Vivian L. Smith Department of Neurosurgery, Center for Stem Cells & Regenerative Medicine, University of Texas Health Science Center at Houston, 1825 Pressler St. Suite 630F, Houston, TX 77030, United States. Tel.: +1 713 500 3431; fax: +1 713 500 2424.
E-mail address: laura.a.smithcallahan@uth.tmc.edu (L.A. Smith Callahan).

http://dx.doi.org/10.1016/j.actbio.2015.04.031
1742-7061/

development [14,17,19,20]. However, this approach may fail to identify the optimal peptide concentration as only a few concentrations are examined.

A systematic approach must be employed to expedite identification of the optimal concentrations for various bioactive peptides. Although many combinatory strategies have been developed [21–24], few of them are capable of both 2D and 3D cell culture [25–28]. A continuous gradient strategy is capable of maximizing the number of material parameters tested in culture and therefore is utilized in our studies of bioactive peptides. Herein, we describe a facile method to fabricate polyethylene glycol dimethacrylate (PEGDM) hydrogels embedded with a continuous concentration profile of a synthetic bioactive peptide that is defined spatially. IKVAV was chosen as the model adhesion peptide and we assessed the fidelity of our fabrication method by characterizing the IKVAV content. The well-defined samples were then used to evaluate the effects of IKVAV concentration on neural differentiation and neurite extension of murine embryonic stem cells (mESCs) both in 2D and 3D.

2. Materials and methods

2.1. Materials

PEGDM (~10,000 g mol^{-1}) was obtained from Monomer-Polymer & Dajac Labs (Trevose, PA) and dialyzed in ultrapure water for 2 days prior to experiments. Acryloyl-Gly-Ile-Lys-Val-Ala-Val (IKVAV, 640.78 g mol^{-1}) peptides were synthesized by American Peptide Company (Sunnyvale, CA) and used without further purification. Unless specified otherwise, supplies and reagents were purchased from Thermo Fisher Scientific (Waltham, MA), VWR International (Radnor, PA), Sigma–Aldrich (St. Louis, MO) or Life Technologies (Carlsbad, CA).

2.2. Hydrogel fabrication for two-dimensional and three-dimensional cell culture

Hydrogel solutions (12% w/v) were prepared by dissolving PEGDM in a mixture of 80% F-12/20% Neurobasal medium containing 0.1% Irgacure 2959 photoinitiators (Ciba Specialty Chemicals, Basel, Switzerland). Hydrogels (50 mm × 10 mm × 1 mm) embedded with a peptide concentration gradient were assembled by dispensing PEGDM solutions with and without 1.87 mM IKVAV through two syringe pumps running in inverse linear ramping profiles ranging from 0 mL h^{-1} to 52 mL h^{-1}, respectively, over 75 s into a custom mold, followed by photopolymerization with 2.3 mJ cm^{-2} UVA lights for 6 min (Fig. 1A and C). Fig. 1B demonstrates a snapshot of the gradient maker system and a representative PEGDM hydrogel with color gradients. After 3-h swelling in neural differentiation media containing 80% F-12, 20% Neurobasal medium, 1% penicillin/streptomycin, 0.8× N2, 0.2× B27, 10 mM sodium pyruvate and 2 μM retinoic acid, six disks were punched out down the length of each hydrogel using a sterile 3/8″ punch and individually placed in the wells of 48-well plates. D3 mESCs (passage 69) were then added to each well at a density of 6 × 10^5 cells per well and incubated inside a humidified incubator (37 °C, 5% CO_2) for 4 h. The cell-seeded constructs were transferred to new wells and cultivated with 500 μL of fresh neural differentiation media. Culture media were completely renewed every other day and cells were harvested with trypsin at days 3 and 6. Before mESCs were applied to each experiment, a small aliquot of cells was collected and used as the control group (ES control).

In 3D experiments, the loaded IKVAV concentration was reduced to 1.17 mM and a third syringe pump was connected to the aforementioned system, which injected mESC suspension solutions into the mold at a constant rate of 10 mL h^{-1} (Fig. 1A and D), yielding a final cell encapsulation density of 5 × 10^6 cells per milliliter. After photopolymerization and swelling processes, six cell-laden disks were punched out from each hydrogel gradient as previously described and same culture conditions were applied. Constructs were harvested at days 6 and 14.

2.3. Hydrogel characterization

Hydrogels in the absence of cells were evaluated for swelling ratio, water content, mesh size, mechanical properties and IKVAV concentration. Briefly, samples were weighed immediately after photopolymerization and weighed again after incubation with neural differentiation media at 37 °C, 5% CO_2 overnight (wet mass). The samples were then completely dried in a freeze dryer (Labconco, Kansas City, MO) and the dry mass was obtained. Swelling ratio was calculated by normalizing the wet mass to the dry mass while water content was the ratio of the water mass to the wet mass. Mesh size (ξ) was determined using the equation $\xi = V_{2,s}^{-1/3} l C_n^{1/2} n^{1/2}$ with the alternation proposed by Lu and Anseth [29] and Cruise et al. [30] where $V_{2,s}$ is the equilibrium polymer volume fraction in the gel, l is the bond length (1.50 Å) [30], C_n is the characteristic ratio of PEG (4) [31] and n is the number of bonds between crosslinks.

Young's and shear moduli of the 3/8″ hydrogel disks were quantified using a TA.XTplus texture analyzer (Stable Micro Systems, Surrey, UK) with a 1/4″ spherical probe which compressed the samples at a rate of 0.01 mm s^{-1} until 10% strain was reached. Force (F), depth of indentions (δ) and strain ε data were recorded to calculate the contact radius (a), indentation stress (σ), Young's modulus (E) and shear modulus G using the following equations [32,33]:

$$a = R^{1/2}\delta^{1/2}$$
$$\sigma = \frac{F}{\pi a^2}$$
$$E = \frac{3\pi(1-\nu^2)\sigma}{20\varepsilon}$$
$$G = \frac{3F}{16af_p\left(\frac{a}{h}\right)}, \quad f_p\left(\frac{a}{h}\right) = \frac{2.36\left(\frac{a}{h}\right)^2 + 0.82\left(\frac{a}{h}\right) + 0.46}{\frac{a}{h} + 0.46}$$

where R is the radius of the 1/4″ spherical probe, ν is Poisson's ratio (0.5) and h is the height of each sample.

IKVAV concentration in the hydrogel gradients was detected using a Lowry assay [25] after the gels were washed in ultrapure water for 3 days under shaking. Gradients were first treated with DC™ Protein Assay Reagent B (Bio-Rad, Hercules, CA) for 30 min followed by another 30-min incubation with Reagent A and finally were rinsed in ultrapure water. Disks were punched out from the treated samples using a ¼ in punch, placed in the wells of a 96-well plate and read at 750 nm in a microplate reader (Tecan Infinite M1000, Maennedorf, Switzerland). The absorbance readings were correlated to those obtained from a standard curve created using hydrogel samples with known IKVAV concentrations to quantify peptide concentrations in the gradients.

2.4. Semi-quantitative polymerase chain reaction (PCR)

RNA was extracted from trypsinized cells (2D culture) or homogenized lysate (3D culture) through a series of rinse, elution, and centrifugation using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (50 ng) isolated from each sample was then reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase reagents according to the manufacturer's protocol. PCR was executed in a thermocycler (Mastercycler Nexus Gradient, Eppendorf, Hamburg, Germany) by mixing 2 μL of synthesized cDNA with Platinum® Taq DNA Polymerase reagents and