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FGL-functionalized self-assembling nanofiber hydrogel as a scaffold for spinal cord-derived neural stem cells

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ABSTRACT

A class of designed self-assembling peptide nanofiber scaffolds has been shown to be a good biomimetic material in tissue engineering. Here, we specifically made a new peptide hydrogel scaffold FGLmx by mixing the pure $RADA_{16}$ and designer functional peptide $RADA_{16}$-FGL solution, and we analyzed the physiochemical properties of each peptide with atomic force microscopy (AFM) and circular dichroism (CD). In addition, we examined the biocompatibility and bioactivity of FGLmx as well as $RADA_{16}$ scaffold on spinal cord-derived neural stem cells (SC-NSCs) isolated from neonatal rats. Our results showed that $RADA_{16}$-FGL displayed a weaker β-sheet structure and FGLmx could self-assemble into nanofibrous morphology. Moreover, we found that FGLmx was not only noncytotoxic to SC-NSCs but also promoted SC-NSC proliferation and migration into the three-dimensional (3-D) scaffold, meanwhile, the adhesion and lineage differentiation of SC-NSCs on FGLmx were similar to that on $RADA_{16}$. Our results indicated that the FGL-functionalized peptide scaffold might be very beneficial for tissue engineering and suggested its further application for spinal cord injury (SCI) repair.

1. Introduction

Spinal cord injury (SCI) can result in devastating and permanent loss of neurological function due to the failure of axonal regeneration after injury, and all levels below the site of trauma are affected. Various attempts have been made to treat SCI with conspicuous, but, unfortunately, limited success [1]. Tissue engineering holds great promise for the axon regeneration of SCI, which usually combines cells, bioactive molecules and biomaterials. The biomaterial, a three-dimensional scaffold, plays an important role in any tissue engineering approach and should be carefully chosen. Ideal tissue engineering scaffolds should possess several properties defined as essentials in tissue engineering. Namely, scaffolds should: (i) be biocompatible; (ii) suitable microstructure; (iii) be biodegradable; and (iv) have desirable surface properties that are able to interact with surrounding cells and tissues by biomolecular recognition. Taking into account the aforementioned properties, scaffolds should be able to provide a 3-D geometry for cell attachment, migration, proliferation, differentiation, and finally temporarily act as a mechanical support for tissue regeneration [1–3].

Traditionally, different biomaterials including natural and synthetic origin have been used in tissue engineering, but both these materials have certain limitations [4]. Self-assembling peptides (SAPs) have been developed for tissue engineering in the past decades [5]. SAPs have shown a remarkable potential and they can form various nanostructures such as nanofibers [6–8], nanotubes [4,9], and nanospheres [4,10] through supramolecular assembly under solvent conditions driven by the formation of various non-covalent interactions, including hydrogen bonding, electrostatic, hydrophobic and π–π interactions. The use of peptide-based scaffolds is a novel approach in providing a nanofibrous network structure, which is similar to the native extracellular matrix. In particular, the peptide $RADA_{16}$ developed by Zhang, S. [11] has alternating positive and negative charged amino acid residues and could self-assemble spontaneously in physiological conditions into a network of interweaving nanofibers with 10–20 nm in diameter, forming hydrogel scaffolds with pores 5–200 nm in diameter and over 99% water content. Importantly, $RADA_{16}$ shows a good biocompatibility both in vitro, when used as substrate for cell culture [12–15] and in vivo, when injected in injured spinal cord [16–18] or brain tissue [19].

Abbreviations: $RADA_{16}$, AcN-RADARADARADARADA-CONH2; FGL, AcN-EVYVVAENQQGKSKA-CONH2; $RADA_{16}$-FGL, AcN-RADARADARADARADAGGEVYVVAENQQGKSKA-CONH2; FGLmx, the mixed solution of $RADA_{16}$ and $RADA_{16}$-FGL solution; AFM, atomic force microscopy; CD, circular dichroism; SC-NSCs, spinal cord-derived neural stem cells; 3-D, three-dimensional; SCI, spinal cord injury; SAPs, self-assembling peptides; NCAM, neural cell adhesion molecule; HPLC, liquid chromatography; MS, mass spectrometry; bFGF, basic Fibroblast Growth Factor; EGF, Epidermal Growth Factor; FBS, fetal bovine serum; EthD-1, Ethidium homodimer I; RA, retinoic acid; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromodeoxyuridine; MAP-2, microtubule associated protein 2; GFAP, glial fibrillary acidic protein; DAPI, 4′,6-diamidino-2-phenylindole.

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Moreover, $RADA_{16}$ could be tailor-made with functional motifs through solid-phase synthesis extension at the C-termini to guide cell behavior such as adhesion, proliferation, migration and differentiation. To date, many functional motifs have been reported, including PDSGR, IKVAV (laminin), DGRGDSVAYG (Osteopontin), RGDS (fibronectin), and PRGDSGYRGDS (collagen VI) and two bone marrow homing peptides SKPPGTSS (BMHP1) and PFSSTKT (BMHP2) [20]. Here, we specifically enriched $RADA_{16}$ with FGL motif to make a function peptide $RADA_{16}$-FGL and made a new peptide hydrogel scaffold FGLmx by mixing the pure $RADA_{16}$ and designer functional peptide solution according to our previous work [21]. FGL, the motif from neural cell adhesion molecule (NCAM), could stimulate neurite outgrowth, promote synaptogenesis, enhance presynaptic function and facilitate memory consolidation [22,23]. In addition, we found that FGL peptide functioned scaffold could promote adhesion and neurite sprouting of dorsal root ganglion neurons in comparison to non-modified peptide scaffold $RADA_{16}$ [21]. In this study, we evaluated the biocompatibility and bioactivity of the FGLmx as well as $RADA_{16}$ scaffold with SC-NSCs, which can proliferate as well as differentiate into three neural lineages of neurons, astrocytes, and oligodendrocytes and also play an important role in regenerative therapies for SCI [24]. Our results suggested that the designer peptide scaffold containing the FGL motif may be useful for SCI regeneration and tissue engineering.

2. Materials and methods

2.1. Synthesis of peptides and fabrication of scaffolds

$RADA_{16}$ (AcN-RADARADARADARADA-CONH2) and $RADA_{16}$-FGL (AcN-RADARADARADARADAGGEVYVVAENQQGKSKA-CONH2) (purity > 95%) were synthesized and purified by GL Biochem (Shanghai, China), Purity and identities of the peptides were confirmed by analytical high performance liquid chromatography (HPLC) and mass spectrometry (MS), respectively. The peptide powders were dissolved in sterile distilled H_2O (Invitrogen, Carlsbad, CA) to the desired concentration of 1% and sonicated (Aquasonic, model 50T, VWR, NJ) for 30 min for subsequent use.

The functionalized peptide solution was mixed with pure $RADA_{16}$ solution at a volume ratio of 1:1 to obtain a novel peptide scaffold named FGLmx. Each of the peptide solution (40 μl/well) was directly loaded in 96 multi-well plates (BD Biosciences) for SC-NSC adhesion, proliferation, differentiation and Live/Dead assay, followed by slow addition of 150 μl/well of basal medium. Allowed to self-assemble at 37 °C for 30 min and rinsed twice with control medium to equilibrate the environment to physiological pH.

2.2. Physiochemical property analyses

The morphology of peptide scaffolds was analyzed using AFM as described by Genove et al. [25]. In brief, peptide from stock solutions (1%) was diluted to a working concentration of 0.01% (w/v), after a 20 min sonication, a total of 5 μl of sample was deposited on a freshly cleaved mica surface for 15 s, then washed with deionized water. After air-dried, the images were obtained with a Multimode AFM microscope (Nanoscope IIIa, Digital Instruments, CA) operating in Tapping Mode. Typical scanning parameters were as follows: resonance frequency 70 kHz, spring constant 2.8 N/m, tip curvature radius 10 nm and 225 μm length, tapping frequency 75 kHz, RMS amplitude before engage 1–1.2 V, set point 0.7–0.9 V, integral and proportional gains of 0.2–0.6 and 0.4–1.0 respectively, and scan speed 1.5 Hz.

CD measurements were carried out with J-810 Spectropolarimeter (Jasco Co., Japan) to study the secondary structure of the peptide solutions with a 1 mm path length at room temperature. The spectra were collected from 260 nm to 195 nm.

2.3. Cell culture and identification

All animal experiments were conducted in accordance with China laws, previously approved by the Ethical Committee for Animal Experiments of Tongji Medical College, Huazhong University of Science and Technology.

SC-NSCs were cultured by neurospheres forming assay according to the procedures described previously by Reynolds and Weiss [26]. Thoracic spinal cord tissues were harvested from neonatal one-day Sprague–Dawley rats (Laboratory Animal Center of Tongji Medical College, China). Then the tissues were dissected and digested in 0.05% trypsin for 7 min in 37 °C water bath followed by adding an equal volume of trypsin inhibitor to stop digestion, the cell pellet was obtained by centrifugation of the suspension filtered through a 40-μm pore size sieve and was resuspended in neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 1% penicillin/streptomycin (P/S, Invitrogen), 5 μg/ml heparin (Sigma-Aldrich, St. Louis, MO), 20 ng/ml basic Fibroblast Growth Factor (bFGF, Peprotech, Rocky Hill, NJ) and 20 ng/ml Epidermal Growth Factor (EGF, Peprotech), which served as SC-NSC culture media. The cells were cultured in T25 flask and then incubated at 37 °C, 5% CO_2 for 5–7 days by which time neurospheres should have formed, then primary neurospheres were mechanically dissociated into single cells and the cells grew into neurospheres again. Secondary or tertiary neurospheres were used for subsequent experiments and all experimental procedures were carried out using monolayers of SC-NSCs except for SC-NSC identification.

For identification of SC-NSCs, neurospheres were placed on coverslips coated with 0.1% polylysine (Sigma) and incubated for 1 day in SC-NSC culture media. As for in vitro differentiation, growth factors were removed from the growth medium and 1% fetal bovine serum (FBS, Gibco) was added. The neurospheres were allowed to differentiate for 3 days. Uninduced and induced neurospheres were processed for immunofluorescence staining.

2.4. Cell viability assay

1×10^3 cells/well was seeded on peptide scaffold and cultured in culture media described above for three days, Live/Dead assay (Molecular Probes) was carried out by treating cells with 2 μM Calcein AM and 4 μM Ethidium homodimer I (EthD-1) for 30 min at room temperature, and the stain solution was then removed and washed with 100 μl phosphate buffer (PBS). Images were taken at four random points per well with a confocal microscope at 100× magnification. All samples were studied in triplicate. Quantitative analyses of live (stained green by Calcein AM metabolization) and dead (stained red by EthD-1 internalization) cells were performed by counting 100–300 cells manually with Photoshop CS6 Extended software for each of the 6 images, and live cell percentage was calculated for each sample.

2.5. Cell adhesion assay

Cell adhesion assay was performed according to previous method established by Ananthanarayanan et al. [27]. Briefly, SC-NSCs were seeded on peptide scaffold surfaces at a density of 1×10^3 cells/cm^2 and incubated for 2 h at 37 °C, 5% CO_2, then cell adhesion was assessed by inverting the culture plate to remove non-adhered cells. The adhered cell morphology images were taken randomly at several spots on each surface at 200× magnification, and the adhered cells of each image were counted manually with Photoshop CS6 Extended software.

2.6. Cell proliferation and differentiation assays

To assess the ability of the SC-NSCs to proliferate or differentiate into mature neural cells, SC-NSCs were seeded on each scaffold at a cellular