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Dual peptide-presenting hydrogels for controlling the phenotype of PC12 cells

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ABSTRACT

Controlling the cell-matrix interaction is a critical factor in the design and fabrication of tissue engineering scaffolds. A particular peptide sequence, Arg-Gly-Asp (RGD peptide), is often used as an adhesion ligand in the engineering of different types of tissues. While in some cases this has been adequate, the use of multiple ligands may be required for the successful engineering of some tissue types. We hypothesized that hydrogels presenting both the RGD peptide and the YIGSR peptide (Tyr-Ile-Gly-Ser-Arg) could successfully regulate the phenotype of PC12 cells, thereby providing a new platform for effective tissue engineering applications. We prepared alginate hydrogels modified with both RGD and YIGSR peptides at several different bulk ligand densities and determined the ways in which PC12 cells can respond to them *in vitro*. We demonstrate that alginate hydrogels presenting both RGD and YIGSR peptides successfully regulate the proliferation, morphological change, and neuronal differentiation of PC12 cells *in vitro*. Successful adhesion and proliferation of PC12 cells were dependent on the bulk density of RGD peptides, while neuronal differentiation was significantly enhanced by increasing the YIGSR density. These results suggest that hydrogels presenting multiple adhesion ligands offer many useful applications in tissue engineering approaches.

1. Introduction

Currently, the clinical treatment of nerve injury/disease typically involves the use of an autograft, which may require multiple surgical procedures to obtain a donor nerve and lead to loss of function and neuroma formation at the donor site [1]. Because of these issues, the development of engineered nerve tissues is considered a promising alternative to an autograft. In particular, nerve tissue engineering has relied on cell-laden polymer scaffolds with a tubular shape [2–4]. These scaffolds are designed to guide sprouting axons, retain neurotrophic factors, and prevent the infiltration of fibrous tissue [5]. Many types of scaffolds have been developed and reported to provide an effective synthetic extracellular matrix (ECM) in tissue engineering [6,7], including for nerve tissue. Generally, the one critical role of tissue engineering scaffolds is to control the interactions between cell surface receptors and specific adhesion ligands on the scaffold surface. These cell-matrix interactions can promote the activation of signaling pathways that regulate many critical aspects of cell adhesion, proliferation, and differentiation [8].

The most widely used adhesion ligand is an Arg-Gly-Asp (RGD) peptide, derived from ECM proteins such as collagen, fibronectin, and laminin. The RGD peptide specifically binds to integrin receptors, including the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ receptors, that otherwise function to recognize the adhesion molecules on ECM surfaces [9]. The RGD peptide is the most widely investigated type of cellular adhesion ligand [10–14], and its use in synthetic ECMs has proven effective at controlling the phenotype of many different cell types, such as osteoblasts [15], chondrocytes [16], myoblasts [17], fibroblasts [18], adipocytes [19], and stem cells [20,21]. It is not, however, the only effective peptide that has been identified. A different peptide, with the sequence Tyr-Ile-Gly-Ser-Arg (YIGSR), is also found on the β-chain of laminin. The YIGSR peptide interacts with the 67 kDa laminin receptor, which is highly expressed in neuron cells [22]. It has been shown to be involved in regulating neuronal differentiation, including neurite outgrowth [23,24]. Prior work has utilized the YIGSR peptide in synthetic scaffolds, and it has already shown to have potential for the engineering of nerve tissue [25] and vascular structure [26]. A peptide with the sequence of Ile-Lys-Val-Ala-Val (IKVAV) is also cell adhesion ligand derived from the laminin chain [27], which is also known to be involved in dif-

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ferentiation of bone marrow stromal cells into neurocytes [28]. Adhesive ligands, including RGD, YIGSR or IKVAV peptides have been reported to be associated with neuronal differentiation and neurite extension of PC12 cells [29]. Although combination of RGD and YIGSR peptides showed a synergistic effect on the adhesion and spreading of endothelial cells [30,31], there are very few studies that have investigated the function of scaffolds that combine both RGD and YIGSR peptides to successfully regulate the cellular response for application in nerve tissue engineering [5].

In this study, we hypothesized that hydrogels modified with RGD and YIGSR peptides could show a synergistic regulatory effect on the phenotype of PC12 cells compared with single-peptide-modified hydrogels. PC12 is a cell line derived from rat pheochromocytoma, which has been widely used for the studies of neurogenesis and neurite formation. PC12 cells provide a useful *in vitro* model for neuronal differentiation [32]. We selected alginate to use as a base biomaterial because it has previously been shown to provide excellent biocompatibility, low toxicity, and mild gelation conditions [33]. Alginate is a natural polysaccharide composed of β-D-mannuronate and α-L-guluronate residues and has been widely exploited for tissue engineering applications [33,34]. Alginate solutions can form hydrogels under physiological conditions in the presence of divalent cations such as calcium ions. Alginate is also inert in terms of cell and protein interactions. As such, cell-matrix interactions can be established by chemically modifying alginate gels with adhesion ligands such as the RGD peptide via simple carbodiimide chemistry [11,33,35]. We thus prepared alginate hydrogels that were modified with both RGD and YIGSR peptides at several different bulk densities, and we investigated how a control of cell-matrix interactions could influence the response of PC12 cells including neurite outgrowth during neuronal differentiation in two-dimensional environments.

2. Materials and methods

2.1. Materials

Sodium alginate (molecular weight = 200,000–300,000; guluronate unit content = 60%) was purchased from FMC biopolymers (Philadelphia, PA, USA). 2-(*N*-morpholino) ethanesulfonic acid (MES), 1-ethyl-3-(dimethylaminopropyl) carbodiimde (EDC), sodium chloride, calcium sulfate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and diethylpyrocarbonate (DEPC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Hydroxysulfosuccinimide (sulfo-NHS) was purchased from Thermo Scientific (Waltham, MA, USA). $(Glycine)_4$-arginine-glycine-aspartic acid-serine-proline (G_4RGDSP) and $(glycine)_4$-tyrosine-isoleucine-glycine-serine-arginine-serin-proline (G_4YIGSRSP) peptides were purchased from Anygen (Gwangju, Korea).

2.2. Peptide modification

Sodium alginate was dissolved in a MES buffer solution at room temperature (pH 6.5, 0.3 M NaCl). G_4RGDSP and/or G_4YIGSRSP peptides were added to the alginate solution in the presence of sulfo-NHS and EDC at a molar ratio of 1:2. The conjugation reaction was carried out overnight at room temperature. The peptide-modified alginate solution was purified by extensive dialysis against deionized water for 4 days (molecular weight cut-off = 3500), followed by activated charcoal treatment and sterilization through a 0.22-μm filter. The purified solution was then frozen and lyophilized. A ^{1}H NMR spectrometer (VNMRS 600; Varian; Palo Alto, CA, USA) was used to confirm successful peptide-alginate conjugation (D_2O, 10 mg/mL). Conjugation efficiency was determined to be roughly 60% by amino acid analysis (Hitachi L-8900; Hitachi, Tokyo, Japan).

2.3. Hydrogel preparation

RGD- and YIGSR-modified alginate solutions were mixed together at various molar ratios, and calcium sulfate was subsequently added to induce gel formation. Briefly, an alginate solution (2 wt%) was mixed with calcium sulfate (60 mM) using two syringes and a female connector to prepare the hydrogels [36]. The mixture was then placed between two glass plates with spacers (1 mm thick) for 15 min and cut into disks using a punch (10 mm diameter). Before use, gel disks were pre-swollen in Rosewell Park Memorial Institute media (RPMI-1640; Gibco; Grand Island, NY, USA). The viscoelastic properties of the peptide-modified alginate hydrogels were measured using a rotational rheometer (Gemini 150; Malvern, Worcestershire, UK) with a plate-and-plate fixture (20 mm diameter). The gap opening was set at 900 μm from the bottom plate, and temperature was maintained at 37 ± 0.1 °C (5 Pa, 1 Hz).

2.4. Cell culture

PC12 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). PC12 cells were seeded on the surface of gel disks at a concentration of 2.0×10^4 cells/disk and were then incubated with RPMI-1640 media containing 10% horse serum, 5% FBS, 1% penicillin/streptomycin, and 1% HEPES under a 5% CO_2 atmosphere at 37 °C. Media were changed every 2 or 3 days. After incubation, all samples were rinsed with PBS to remove unattached cells from the gel disks, treated with trypsin solution containing 0.05% EDTA (1 mL), and incubated at 37 °C for 5 min. Fresh media (6 mL) were added and incubated for 20 min to dissolve the gel disks. Cells were then collected by centrifugation, and the number of cells was counted using a hemocytometer. Cell numbers were measured within five days of culture and were used to calculate the growth rate.

To study the differentiation process, PC12 cells were seeded on the surface of alginate gel disks at a concentration of 2.0×10^5 cells/disk and were incubated with RPMI-1640 media containing 10% horse serum, 5% FBS, 1% penicillin/streptomycin, and 1% HEPES under a 5% CO_2 atmosphere at 37 °C. Nerve growth factor (Sigma-Aldrich) was added to the media (50 ng/mL), and the media were changed every other day.

2.5. Image analysis

The number of PC12 cells with neurite outgrowth was counted using an optical microscope (Olympus, Japan). All images of PC12 cells adhered to gel disks were analyzed with ImageJ software (NIH; Bethesda, MD, USA) to determine the number of cells with neurite outgrowth, which was defined as those with at least one neurite longer than two times the cell body diameter [5].

2.6. Immunostaining of β-tubulin

PC12 cells cultured on gel disks were fixed with 5% formalin solution containing sucrose, then incubated in cytoskeleton buffer for 20 min before being permeabilized with TBS buffer containing 0.5% Triton X-100 for 10 min. Cells were then blocked with TBS buffer containing 0.1% Triton X-100 and 2% BSA for 10 min and were treated with mouse anti-human β-tubulin III monoclonal antibody (1:200 dilution, Chemicon International; Billerica, MA, USA) for 5 min. Next, cells were treated with rhodamine-conjugated affinity donkey anti-mouse IgG (1:200 dilution, Jackson ImmunoResearch Lab; West Grove, PA, USA) for 5 min. The gel disk was rinsed five times with TBS buffer containing 0.1% Triton X-100 and mounted