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Guidance of neural regeneration on the biomimetic nanostructured matrix☆

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ABSTRACT

Biomimetic materials are used for creating microsystems to control cell growth spatially and elicit specific cellular responses by combining complex biomolecules with nanostructured surfaces. Intercellular cell-to-cell and cell-to-extracellular matrix (ECM) interactions in biomimetic materials have demonstrated potential in the development of drug discovery platforms and regeneration medicine. In this study, we developed a biomimetic nanostructured matrix by using various ECM molecular layers to create a biomimetic and biocompatible environment for realizing neuronal guidance in neural regeneration medicine. Silicon-based substrates possessing nanostructures were modified using different ECM proteins and peptides to develop a biomimetic and biocompatible environment for studying neural behaviors in adhesion, proliferation, and differentiation. The substrates were flat glass, flat silicon wafers (FWs), and nanorod-structured wafers prepared using wet etching. The three substrates were then functionalized using laminin-1 peptide, PA22-2-contained active isoleucine-lysine-valine-alanine-valine (IKVAV) peptide, and poly-D-lysine (PDL), separately. When PC12 cells were cultured and differentiated on the modified substrates, the cells were able to elongate the neurites on the glass and FW, which was coated with three types of peptide. More differentiated neurons were observed on the nanorod-structured wafers coated with laminin than on those coated with IKVAV or PDL. For achieving directional guidance of neurite outgrowth, substrates exhibiting a grating pattern of nanorods were partially collapsed by the pulling force of water, leaving few nanorods, which support the net form of laminin on the surface. Furthermore, we fabricated the topological nanostructure-patterned wafer coated with laminin and successfully manipulated the extension and direction of neurites by using more than 80 μm of a single soma. This approach demonstrates potential as a facile and efficient method for guiding the direction of single axons and for enhancing neurite outgrowth in studies on nerve regenerative medicine.

1. Introduction

The interaction between cells and extracellular matrix (ECM) is a crucial niche in determining cell fate and function (Hynes, 2009). The biomaterial plays an important role in most tissue-engineering strategies (Dvir et al., 2010; Hubbell, 1995). A major approach to bi native ECM, laminin, IKVAV, biomolecular recognition of materials by cells involves incorporation of cell-binding peptides into substrates through chemical or physical modification. In the chemical cues, the cell-binding peptides include a native long chain of ECM proteins as well as short peptide sequences derived from intact ECM, both of which incur specific interactions with cell receptors (Bergmann and Peppas, 2008). These bioactive molecules in modification of biomimetic material can be used as tissue-engineering scaffolds that provide suitable biological cues

☆ *Chemical compounds studied in this article*: Laminin (PubChem CID: 44342165); Cys-laminin A chain 2091–2108 (PubChem CID: 71308798); Isoleucyl-lysyl-valyl-alanyl-valine (PubChem CID: 131343); D-lysine (PubChem CID: 57449); Phosphate buffered saline (PubChem CID: 24978514); Ethanol (PubChem CID: 702); Glutaraldehyde (PubChem CID: 3485); Sucrose (PubChem CID: 5988); Osmium tetroxide (PubChem CID: 30318); Formaldehyde (PubChem CID: 712); Triton X-100 (PubChem CID: 5590); DAPI (PubChem CID: 2954).

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to guide new tissue formation (Matsumoto et al., 2007; Meinel et al., 2009). The crucial cue for cellular attachment and migration to the substrate is related to integrin–ligand interactions, which are presented from the cell membrane and ECM, including ligand density, integrin density, and integrin–ligand binding affinities (Chen et al., 2012; Friedl and Wolf, 2010). High ligand density may show trends to enhance cell migration (Palecek et al., 1997). Integrin and the targeting ligand are specific recognizing and signal transduction system for not only studying cellular functions but also for designing drug delivery scaffolds (Fievez et al., 2010; Garg et al., 2009; Nikanjam et al., 2007). The neural cells are well known to be an anchorage-dependent cell type to adhere to and maintain them on the ECM through ligand–receptor interaction, and require surface coating for routine culture (Kong and Mooney, 2007). Cell surface molecules bind to ECM ligands and are able to guide the direction of neurites in a manner that a growth cone of neurites adheres from one ECM molecule to others, exerting force to pull the growth cone forward and hence sprouting axons (Gomez, 2011; Myers et al., 2011). The external biochemical force of surrounding environment can act through the internal cytoskeleton of cells to affect local mechanical properties and cellular behaviors (Fletcher and Mullins, 2010). A series of ECM molecules was previously investigated to promote neuron adhesion and differentiation, and the adhesion of PC12 cells was significantly enhanced on collagen IV, poly-D-lysine (PDL), and laminin (Lee et al., 2001; Li et al., 2012; Mammadov et al., 2012; Tomaselli et al., 1987). In the ECM, the 600–800 kilodalton (kDa) laminin peptide contains different cell binding domains including IKVAV (peptide sequence, isoleucine-lysine-valine-alanine-valine) in the A chain (Dellatore et al., 2008; Hosseinkhani et al., 2013). The PDL modulates cell adhesion through a non-receptor-mediated cell-binding mechanism to promote neural adhesion through electrostatic bonds (Rao et al., 2011). In this work, we designed systematic studies for preparing biomimetic substrates by using the covalent binding of native ECM, laminin, IKVAV, and PDL separately on the nanostructured patterned surface to demonstrate the effects of neuron outgrowth on each biocompatible and topological mimetic surface.

Substrate topography engineering has become an important tool in physical modification cues to induce desired cell phenotype and genotype, and to study complex cell functions such as adhesion, migration, cytoskeleton reorganization, and cell polarization (Bettinger et al., 2009; Hoffman-Kim et al., 2010; Nikkhah et al., 2012). Previous research details the number of basic nano-topographic geometries that have been fabricated. Nano-topographic substrates have a great advantage for studying contact guidance and migration in vitro, which can be further used in tissue-engineering applications (Dvir et al., 2010; Sia, 2012). Nano-grating substrates generally appear to enhance adhesion in various cell-biomaterial geometry combinations, whereas nano-posts and nano-pits generally reduce initial cell attachment (Anselme et al., 2010; Kang et al., 2011). Previous studies indicate that mammalian cells elongated and aligned along patterns of grooves and ridges (Rebollar et al., 2008). For neurological research, misdirected axonal growth makes inappropriately connections between motor and sensory axons and their respective target tissue (Orive et al., 2009; Webber and Zochodne, 2010). The distribution of adhesive molecular cues has a clear impact on neurogenesis (Roach et al., 2010). Therefore, this study proposes biomimetic substrates that triggers PC12 cell neurite differentiation in the guidance direction by using a nanorod and nano-grating composite, which have been functionalized with adhesion molecule to create a three dimension (3D) scaffold. The biomimetic surface was able to control and restrict the direction of neurites and offer new material for further research in the field of neural engineering such as neural recording and stimulation research.

2. Materials and methods

2.1. Chemicals and materials

Ethanol, Laminin-1 in its native form from Engelbreth-Holm-Swarm murine sarcoma, water-soluble sequences CSRARKQAASIK-VAVSADR containing the active IKVAV peptide (PA22-2), poly-D-lysine, phosphate buffered saline (PBS), glutaraldehyde, osmium tetroxide, formaldehyde, Triton X-100, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (USA). Rat pheochromocytoma (PC12) cells (BCRC-60048) were obtained from the Food Industry Research and Development Institute (Taiwan). Dulbecco's Modified Eagle Medium (DMEM), horse serum, fetal bovine serum, sodium bicarbonate, and antibiotic/antimycotic solution were purchased from Gibco (USA). Nerve growth factor and goat anti-rabbit IgG secondary antibody conjugated with Cy3 were purchased from Millipore (USA). Rabbit anti-Synapsin I polyclonal antibody was purchased from Novus (USA). AlexaFluor 488 phalloidin, 4′,6-diamidino-2-phenylindole (DAPI), and Prolong Gold antifade reagent were purchased from Invitrogen (USA).

2.2. Surface process of substrates

During the surface process of substrates, all glass coverslips, pristine flat silicon wafer (FW), nanostructured wafer (NW), and patterned substrates demonstrating a periodic arrangement of nanorods were processed using O_2 plasma and washed with 75% ethanol, followed by washing with distilled water. The O_2 plasma treatment produces functional silanol groups for a coupling reaction, and has been used to graft cell-binding peptides into substrates. Covalent binding of adhesion molecules to biomaterials can effectuate strong binds between adhesion molecules and substrates. After sterilization, glass, FW, and NW substrates were separately coated with 20 μg/mL laminin-1 in its native form from Engelbreth-Holm-Swarm murine sarcoma (Sigma–Aldrich, USA) at 37 °C for 2 h, and then with either active IKVAV peptide at room temperature for 2 h, or 1 mL per 25 cm^2 of poly-D-lysine at room temperature for 5 min. The sterilized patterned substrate was coated with laminin-1 in the same condition.

2.3. Culture and differentiation of PC12 cells

Substrates were placed in the 24-well-plate, seeded PC12 cells that were adjusted to 4×10^4 cells/mL, and cultured in complete DMEM culture medium containing 10% horse serum, 5% FBS, 1.5 g/L sodium bicarbonate, and 1% antibiotic/antimycotic solution. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator for 1 day. The medium was then replaced with a differentiation medium consisting of DMEM supplemented with 1% horse serum, antibiotic/antimycotic solution, and 50 ng/mL nerve growth factor. After culturing for 1 week and once the PC12 cells were well-attached and differentiated, behaviors and morphology of neural cells were studied using scanning electron microscopy (SEM) and indirect fluorescent staining (IFA).

2.4. Scanning electron microscopy

The cell fixation processes for the observation of SEM began with cell samples first rinsed with PBS and then fixed with 4% glutaraldehyde in 0.2 M PBS (pH 7.2) containing 5% sucrose at 4 °C for 30 min. Samples were then washed with 0.2 M PBS containing 5% sucrose and then post-fixed with 1% Osmium tetroxide (Sigma–Aldrich, USA) in PBS for 1 h, then rinsed lightly in PBS and dehydrated through ascending serial concentration (50–100%, v/v) of ethanol. Finally, cells were immersed in 100% ethanol and dried in a critical point dryer (HCP-2 Hitachi, Japan) using CO_2. After drying, samples