Polydopamine-mediated surface modification of scaffold materials for human neural stem cell engineering

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A R T I C L E   I N F O

Article history:
Received 20 May 2012
Accepted 25 June 2012
Available online 17 July 2012

Keywords:
Polydopamine
Surface immobilization
Neural stem cell
Neurotrophic growth factor
Adhesion peptide

A B S T R A C T

Surface modification of tissue engineering scaffolds and substrates is required for improving the efficacy of stem cell therapy by generating physicochemical stimulation promoting proliferation and differentiation of stem cells. However, typical surface modification methods including chemical conjugation or physical absorption have several limitations such as multistep, complicated procedures, surface denaturation, batch-to-batch inconsistencies, and low surface conjugation efficiency. In this study, we report a mussel-inspired, biomimetic approach to surface modification for efficient and reliable manipulation of human neural stem cell (NSC) differentiation and proliferation. Our study demonstrates that polydopamine coating facilitates highly efficient, simple immobilization of neurotrophic growth factors and adhesion peptides onto polymer substrates. The growth factor or peptide-immobilized substrates greatly enhance differentiation and proliferation of human NSCs (human fetal brain-derived NSCs and human induced pluripotent stem cell-derived NSCs) at a level comparable or greater than currently available animal-derived coating materials (Matrigel) with safety issues. Therefore, polydopamine-mediated surface modification can provide a versatile platform technology for developing chemically defined, safe, functional substrates and scaffolds for therapeutic applications of human NSCs.

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1. Introduction

Neural stem cells (NSCs) are an attractive cell source for cell therapies and tissue engineering applications that aim to regenerate central and peripheral nerves. The ability of NSCs to self-renew and differentiate into neurons and glial cells (astrocytes and oligodendrocytes) makes them ideal candidates for the development of treatments for neurodegenerative diseases, stroke, spinal cord injury, and traumatic brain injury [1–4]. Transplanted NSCs can differentiate into neurons and glial cells, integrate into host tissue, and repair damaged tissue by enhancing neurogenesis and host axonal growth [5–8]. However, transplanted NSCs suspended in medium or buffer solution lack the physicochemical stimulation that initiates signaling cascades, and promotes proliferation and differentiation. These conditions do not support NSC survival or direct NSC differentiation in vivo [9,10]. Consequently, transplanted NSCs often undergo apoptosis and/or anoikis in the injured tissue, which can coincide with unfavorable cellular processes such as ischemia and inflammation, and reduces the therapeutic efficacy of NSC transplantation [9].

Functional biomaterial substrates and scaffolds can generate physicochemical cues that enhance proliferation and differentiation of NSCs. This is important for efficiently regenerating and recovering the functionality of injured nerve tissue through NSC transplantation. Previous studies have shown that the use of functional scaffolds in NSC transplantation improves in vivo survival of NSCs isolated from brain tissue or derived from pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [10–13]. These biomaterial substrates and scaffolds should be engineered to mimic specialized microenvironments where NSCs reside in vivo and to facilitate efficient spatial control of proliferation and differentiation by providing...
physical support and cell–matrix interactions [14,15]. Immobilization of bioactive molecules onto these substrates and scaffolds could be an efficient strategy for generating physical and biochemical cues that manipulate NSC behavior. Bioactive molecules of interest for immobilization include adhesion peptides derived from essential recognition sequences of the extracellular matrix (ECM) and neurotrophic factors that control NSC proliferation and differentiation [14,16–18].

Diverse approaches including covalent chemical conjugation and physical adsorption have been applied to immobilize peptides and growth factors onto the surfaces of substrates and scaffolds [14,19–21]. However, typical chemical conjugation methods require multistep, complicated procedures such as surface activation or functionalization steps requiring plasma or chemical treatments [19–21]. One example of this is the pretreatment of poly(lactic-co-glycolic acid) (PLGA) scaffolds using oxygen plasma to immobilize bone morphogenetic protein-2 [19]. In another study, carboxylic acid was pre-deposited and ethyl(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (NHS) chemistry was used to immobilize peptides onto polystyrene (PS) tissue culture substrates for human ESC maintenance [20]. Most studies that immobilize growth factors onto substrates and scaffolds for NSC culture employ these kinds of complicated chemical activations [14,21]. However, these treatments can significantly alter bulk material properties, can induce surface denaturation, and are characterized by batch-to-batch inconsistencies [22]. In addition, chemicals used for surface functional group activation and immobilization (e.g., NHS and maleimide) are susceptible to hydrolysis during the reaction, which leads to low surface conjugation efficiency [22]. Alternatively, physical adsorption, a common method for immobilization of ECM proteins and peptides, can be non-specific and is inefficient [22]. Therefore, simple and effective immobilization strategies are vital for the development of biologically functional culture substrates and biomimetic tissue engineered scaffolds for NSC culture.

Here, we demonstrate an immobilization strategy inspired by a mussel adhesive protein that promotes attachment to virtually any type of organic or inorganic material [23,24]. This strong attachment is attributed to an extensive repeat of 3,4-dihydroxy-L-phenylalanine (dopamine) and lysine residues in the Mytilus edulis foot protein-5 of mussel adhesive pads [23]. Dopamine containing both catechol and amine groups polymerizes at an alkaline pH, which is typical of marine environments, to form a thin polydopamine (PD) layer [23]. Dopamine polymerization is involved in oxidation of catechol to quinone [23]. Because amine and thiol groups can be covalently conjugated to a PD layer via the quinone group, PD coating exhibits latent reactivity to various nucleophiles with those functional groups [22]. Previous studies show that PD-coated substrates can be efficiently grafted with thiolated polysaccharides (hyaluronic acid) or proteins (trypsin) [22,23]. The catechol moiety strongly binds to metal ions and inorganic compounds. For these reasons, it has been suggested that a PD-mediated surface coating method could modify a wide variety of surfaces made of natural or synthetic organic/inorganic materials for a diverse array of functional applications including protein immobilization, metallization, and biomineralization [22,23].

Despite the known capacity of PD to efficiently immobilize nucleophiles, PD-mediated functionalization of polymer surfaces in order to manipulate stem cell behavior remains unexplored. In this

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**Fig. 1.** Schematic illustration of the polydopamine (PD)-mediated immobilization of bioactive molecules (ECM-derived adhesion peptides and neurotrophic growth factors). Polystyrene (PS) and poly(lactic-co-glycolic acid) (PLGA) substrates with immobilized bioactive molecules were used for NSC culture.
study, we demonstrate PD-based, facile biomimetic approach to surface modification for efficient and reliable manipulation of NSC differentiation and proliferation. This approach involves dip-coating a biomimetic polymer (PD) thin film onto the polymer surface followed by conjugation of adhesion peptides and neurotrophic growth factors to the biomimetic polymer film.

2. Materials and methods

2.1. Polydopamine (PD) coating

For PD coating, diverse substrates (PS, PLGA, polydimethylsiloxane (PDMS), silica (Si), and titanium (Ti)) were immersed into a dopamine solution (2 mg/mL in 10 mM Tris–HCl, pH 8.5, Sigma, St. Louis, MO, USA) with shaking for 18 h at room temperature to achieve a uniform coating. After rinsing with ethanol, the substrates were dried in a stream of nitrogen.

![Figure 2](image-url)  
Fig. 2. XPS analysis of the polymer substrates. (A) XPS peaks of the unmodified polymer substrates (PS and PLGA), PD-coated polymer substrates (PS-PD and PLGA-PD), and PD-coated polymer substrates with immobilized GDNF and the CGGRGD peptide (PS-PD-GDNF and PLGA-PD-CGGRGD). (B) Quantification of atomic compositions on the polymer surfaces.
temperature. The PD-coated substrates were then rinsed with distilled water to remove the unattached dopamine molecules.

2.2. Immobilization of peptides and growth factors

The PD-coated substrates (PS and PLGA) were coated with adhesion peptides (fibronectin sequences: CGGRGD, KGGRGD and laminin sequences: CCGYIGSR, KGGYIGSR, Peptron, Daejeon, Korea) and neurotrophic growth factors (nerve growth factor: NGF (PeproTech, Rocky Hill, NJ, USA) and glial cell line-derived neurotrophic factor: GDNF (PeproTech)). The PD-coated substrates were immersed into a solution of peptide (1 mg/ml in 10 mM Tris buffer, pH 8.5) and growth factor (1 mg/ml in 10 mM Tris buffer, pH 8.5) for 12 h at room temperature. The substrates were then washed with distilled water to remove the unattached peptides and growth factors. To quantify the coating efficiency of peptides and growth factors, the buffer solutions containing the unattached peptides and growth factors were retrieved immediately after finishing coating process. The concentrations of peptides and growth factors in the retrieved solutions were determined by using fluorescamine assay (Sigma) and enzyme-linked immunosorbent assay (ELISA) (R&D System, Minneapolis, MN, USA).

Fig. 3. Efficiency of PD-mediated immobilization of growth factors and peptides. (A) ELISA analysis for determining the cumulative detachment of immobilized growth factors (GDNF). (B) Fluorescamine assay for determining the cumulative detachment of immobilized peptides (CGGRGD) from the PS-PD surfaces.

Fig. 4. Fluorescent images of the synthetic polymer (PS and PLGA) substrates with immobilized, fluorescently labeled peptides (FITC-CGGRGD) and proteins (Texas-Red-BSA) at different concentrations (1 mM, 2 mM, and 4 mM). Fluorescent images of synthetic polymer (PDMS), ceramic (Si), and metal (Ti) substrates with immobilization of fluorescently labeled peptide (FITC-CGGRGD) and protein (Texas-Red-BSA) with or without PD coating.
respectively. These assays were performed according to the manufacturer’s instruction. Immobilization of peptides and proteins was also visualized by using fluorescently labeled peptides (FITC-CGGRGD) and proteins (Texas-Red-bovine serum albumin (BSA), Invitrogen, Carlsbad, CA, USA). The fluorescence signals from the immobilized FITC-CGGRGD and Texas-Red-BSA on the substrates were detected by an image analyzer (Image station 4000 MM, Kodak, New Haven, CT, USA).

2.3. Characterization of engineered polymer substrates

The atomic chemical composition of the polymer substrate surfaces was analyzed with X-ray photoelectron spectroscopy (XPS, ESCALAB 220i-XL, VG Scientific Instrument, UK). The surface morphology and roughness of the polymer substrates were analyzed by atomic force microscopy (AFM, MULTIMODE, VEECO Digital Instruments, Santa Barbara, CA, USA) and scanning electron microscopy (SEM, JSM-6700F, JEOL, Japan). The water contact angle was measured by using a Pheonix 300 (SED Corporation, Gunpo, Korea).

2.4. Culture of human neural stem cells (hNSCs)

Human fetal NSCs were derived from the telencephalon (HFT13) as previously described [25]. The cells were plated in the dishes at a cell density of $6.0 \times 10^5$/ml. Human fetal NSCs were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml; Sigma), epidermal growth factor (EGF, 20 ng/ml; Sigma), leukemia inhibitory factor (LIF, 10 ng/ml; Sigma), and N-2 supplement (Gibco) in humidified air with 5% CO$_2$ at 37 °C.

2.5. Culture of mouse neural stem cells (mNSCs)

All procedures for primary isolation of NSCs from animals were provided according to the Guide for the Care and Use of Laboratory Animals of Yonsei University. Mouse fetal NSCs were isolated as previously described [26]. In brief, brains were explanted from imprinting control region (ICR) mouse (Samtako, Osan, Korea) E13 embryos. The cortices were freed of meninges and then mechanically dissociated in culture medium using a flame polished pasteur pipette. The isolated cells were plated in the dishes at a cell density of $2.0 \times 10^5$/ml. Mouse fetal NSCs were cultured in DMEM/F12 medium supplemented with bFGF (20 ng/ml), EGF (20 ng/ml), and N-2 supplement.

2.6. Culture of human induced pluripotent stem cell (iPSC)-derived neural stem cells (NSCs)

Human NSCs were differentiated from human iPSCs established by transduction of one transcription factor (Oct4) as previously described [27]. Human iPSC-NSCs were plated in the dishes at a cell density of $5.0 \times 10^4$/ml and cultured in DMEM/
2.7. NSC culture on the engineered polymer substrates

NSCs were plated on the engineered substrates at a seeding density of \(2.0 \times 10^4\) ml for human and mouse fetal NSCs and \(2.0 \times 10^4\) ml for human iPSC-NSCs. For induction of spontaneous differentiation, NSCs were maintained in DMEM/F12 medium without supplementation of bFGF, EGF, and LIF. Uncoated polymer substrates were used as a negative control. Matrigel (BD Biosciences, San Diego, CA, USA) served as a positive control coating material. NSC differentiation was analyzed by immunocytochemical staining and quantitative real-time polymerase chain reaction assay. The cell proliferation was examined by Alamar Blue assay (Invitrogen).

2.8. Immunocytochemistry

The NSCs cultured on the polymer substrates were fixed by adding 4% (w/v) paraformaldehyde (Sigma) for 15 min and then permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 5 min. After blocking with 2% (v/v) goat serum solution for 45 min, the cells were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used for the staining: mouse monoclonal anti-neuronal class III β-tubulin (Tuj1) (1:100; Millipore, Temecula, CA, USA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:200; Millipore), and mouse monoclonal anti-O4 (1:100; Millipore). After washing with phosphate buffered saline (PBS), secondary antibodies (Alexa Fluor-488 goat anti-mouse IgG, 1:500; Invitrogen) were added and then incubated for 45 min. The cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (Sigma). The stained signals in the cells were observed by a fluorescent microscopy (Carl Zeiss, Oberkochen, Germany).

2.9. Quantitative Real-Time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Chatsworth, CA, USA) from each sample (\(n = 3\) per group) according to the manufacturer’s instructions. The RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer. A reverse transcription reaction was performed with 500 ng pure total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Universe Fast PCR Master Mix (Applied Biosystems) was used for the reaction. The profiles of gene expression in NSCs were quantified with TaqMan Gene Expression Assays (Applied Biosystems) for each target (human Tuj1: Hs00801390_s1, human GFAP: Hs00909238_g1, human glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Hs02758991_g1, mouse Tuj1: Mm00727586_s1, mouse GFAP: Mm01253034_m1, mouse oligodendrocyte transcription factor 2 (Olig2): Mm01210556_m1, and mouse GAPDH: Mm99999915_g1). The expression level of target genes was determined by the comparative Ct method, whereby the target is normalized to the endogenous reference (GAPDH) [28]. The relative expression of each marker in NSCs cultured on the engineered surfaces was normalized to that in NSCs cultured on the unmodified surface.

2.10. Statistical analysis

Quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed by the analysis of variance (ANOVA) using a Bonferroni test. A value of \(p < 0.05\) was considered statistically significant.

3. Results and discussion

In this study, we immobilized growth factors and adhesion peptides containing amine and thiol groups onto PD-coated polymer substrates in order to modulate NSC differentiation (Fig. 1). ECM protein-derived adhesion peptides, fibronectin [Arg-Gly-Asp (RGD)] and laminin [Try-Ile-Gly-Ser-Arg (YIGSR)], and neurotrophic factors, NGF and GDNF, were immobilized. These adhesion
peptides and growth factors enhance adhesion, proliferation, and differentiation in NSCs, in addition to enhancing neuronal growth and neurite extension [16,29–32]. The terminal residues of peptides were modified with cysteine (C) and lysine (K) to introduce thiol and amine groups, respectively. Two glycine residues (GG) were additionally introduced as flexible linkers between the terminal residues and ECM sequences, resulting in generation of C(K)GGRGD and C(K)GGYIGSR.

Synthetic polymer substrates, PS and PLGA, were coated with a PD layer by simple immersion in an alkaline dopamine solution (2 mg of dopamine · HCl dissolved in 1 mL of 10 mM Tris buffer, pH 8.5) for 12–18 h [22,23]. The PD-coated polymer substrates were then immersed in solutions containing peptides (1 mg/ml in 10 mM Tris buffer, pH 8.5) or growth factors (1 μg/ml in 10 mM Tris buffer, pH 8.5) for 12–18 h. The engineered polymer substrate surfaces were analyzed by XPS to confirm PD coating and immobilization of peptides (CGGRGD) and growth factors (GDNF; Fig. 2). The XPS spectra revealed that the chemical composition of the polymer surfaces was altered by the PD coating and peptide/growth factor immobilization. The unmodified polymer surfaces displayed typical photoelectron peaks, C 1s (PS), and C 1s and O 1s (PLGA) (Fig. 2A). The N 1s peaks originated from nitrogen components of the PD layer and were newly detected in the XPS spectrum from PD-coated surfaces (PS-PD and PLGA-PD) (Fig. 2A). The S 2p peaks appeared in the XPS spectrum of the PS/PLGA-PD-GDNF and PS/PLGA-PD-CGGRGD groups, indicating immobilization of GDNF and CGGRGD onto the polymer surfaces (Fig. 2A).

Quantification of atomic composition by XPS indicated that unmodified PS and PLGA surfaces did not contain a nitrogen component (N), whereas PS-PD and PLGA-PD contained approximately 6–9% nitrogen content on their surfaces (Fig. 2B). The nitrogen-to-carbon ratios (N/C) of PS-PD and PLGA-PD surfaces were 0.1 and 0.11, respectively (Fig. 2B). These values are close to the theoretical value of dopamine (N/C = 0.125) [22], indicating that PS and PLGA surfaces were modified by the dopamine polymerization. The nitrogen content (N) was further increased on PS/PLGA-PD-GDNF and PS/PLGA-PD-CGGRGD surfaces compared to PS/PLGA-PD surfaces due to the presence of amine groups and peptide bonds from GDNF and CGGRGD (Fig. 2B). XPS quantification of sulfur content (S) supported immobilizing GDNF and CGGRGD containing thiol groups onto the surfaces of PS/PLGA-PD-GDNF and PS/PLGA-PD-CGGRGD (Fig. 2B).

ELISA revealed that more than 90% of loaded GDNF was immobilized onto the PS-PD surface and the immobilized GDNF did not

Fig. 7. Human fetal NSC differentiation on the polymer substrates with and without immobilized neurotrophic growth factors and adhesion peptides. (A, C, E, G) Immunofluorescent staining and (B, D, F, H) qRT-PCR analyses examine the expression of neuronal (Tuj1) and astrocyte (GFAP) markers in human fetal NSCs. Human fetal NSCs were cultured on the unmodified polymer substrates (PS and PLGA), PD-coated polymer substrates (PS-PD and PLGA-PD), and PD-coated polymer substrates with immobilized neurotrophic growth factors (NGF and GDNF) and adhesion peptides (C(K)GGYIGSR and C(K)GGRGD) for 4 days. The relative expressions of Tuj1 and GFAP in each group were normalized to those of the unmodified polymer group (n = 3, *: p < 0.05 and **: p < 0.01, compared to the unmodified polymer group (no coating) and #: p < 0.05, compared to Matrigel group). Matrigel coatings were used as a positive control. The scale bars indicate 100 μm.
detach from the surface for up to 4 days after incubation in PBS solution at 37 °C (Fig. 3A). This indicates that PD-mediated immobilization of growth factors and cytokines may provide spatial control of stem cell proliferation and differentiation through a sustained dose of immobilized proteins, which is not possible through addition of soluble factors [14]. A fluorescamine assay indicated that the coating efficiency of the CGGRGD peptide onto the PS-PD surface was about 80% and that the immobilized peptides did not detach from the PS-PD surface for up to 4 days (Fig. 3B).

The PD-mediated coatings of peptides and proteins on the substrates were visualized using fluorescently labeled peptides (FITC-CGGRGD) and proteins (Texas-Red-BSA). The FITC-CGGRGD peptide and Texas-Red-BSA protein could not be efficiently immobilized onto the PS and PLGA surfaces without a PD coating (Fig. 4); however, they were efficiently grafted onto the PD-coated PS and PLGA surfaces in a dose-dependent manner (Fig. 4). The PD coating was also found to facilitate efficient immobilization of these bioactive molecules onto ceramic (Si), metal (Ti), and hydrophobic synthetic polymer (PDMS) substrates (Fig. 4). These results indicate that PD coating compared to physical adsorption can more efficiently mediate the grafting of proteins and peptides onto most types of material surfaces.

PD-coated polymer substrates with immobilized peptides and growth factors were analyzed by AFM and SEM to investigate surface morphology and roughness. The unmodified PS and PLGA substrates exhibited smooth surface morphology, compared with the modified substrates. AFM (Fig. 5A) and SEM (Fig. 5B) analyses confirmed the PD layer on the PS and PLGA surfaces. Self-polymerized PD particulates were also observed on the PS-PD and PLGA-PD surfaces (Fig. 5A and B). The addition of GDNF and the CGGRGD peptide increased the surface roughness of the PD-coated substrates and the size of PD particulates (Fig. 5A and B). An increase in the coating thickness measured by AFM supported that PD, growth factor, and peptide layers were coated onto the PS and PLGA surfaces.

The water contact angle of the surfaces was measured in order to analyze the change in wettability of the polymer surface. PS and PLGA surfaces typically exhibited water contact angles from 60° to 70° (Fig. 6). The PD coating rendered these polymer surfaces more hydrophilic, which was indicated by a decrease in contact angle. PS decreased from a starting angle of 65.8° ± 2.7° to 25.5° ± 4.0° and PLGA decreased from a starting angle of 68.6° ± 3.8° to 27.3° ± 6.8° (Fig. 6B and D). In a previous study, the contact angle of anodic aluminum oxide, a superhydrophobic surface, dramatically decreased after PD functionalization from a starting angle of 158.5° ± 2.8° to 37.3° ± 2.6°, indicating that even a superhydrophobic surface can become hydrophilic following PD coating [24]. This suggests that PD coatings can change the surface properties of diverse types of materials. Alternatively, the immobilization of growth factors and peptides did not significantly alter the contact angles of PS-PD and PLGA-PD surfaces (Fig. 6).

The polymer substrates with PD-mediated immobilized peptides and growth factors were tested on cultured neuronal...
lineage cells [murine neuroblastoma cell line (Neuro-2a)]. An Alamar Blue assay revealed that PD coatings on the polymer surfaces (PS and PLGA) did not cause cytotoxicity (Fig. S1). PD coatings enhanced the proliferation of Neuro-2a on the PLGA substrates (Fig. S1B). These results indicate that coating with PD provides a biocompatible method of surface modification. Furthermore, immobilization of neurotrophic growth factors (NGF and GDNF) and adhesion peptides (YIGSR and RGD) significantly promoted the proliferation of Neuro-2a 3 days after seeding (Fig. S1).

The PD polymer substrates functionalized with bioactive molecules were used to assess manipulation of NSC differentiation. Spontaneous differentiation of human fetal brain-derived NSCs was induced after 4 days in culture on the PD polymer substrates containing immobilized growth factors and peptides in the absence of mitogenic factors (bFGF and EGF). Immobilization of adhesion peptides, YIGSR and RGD, facilitated adhesion of human NSCs (Fig. S2). Immunofluorescent staining and qRT-PCR of a neuronal marker (Tuj1) demonstrated that neuronal differentiation of human fetal NSCs was significantly enhanced in the PS-PD substrates that contained immobilized GDNF, CGGYIGSR, and CGGRGD (Fig. 7A and B). Immobilization of GDNF, C(K)GGYIGSR, and KGGRGD greatly facilitated glial differentiation (astrocyte lineage) of human NSCs on the PS-PD substrates, as confirmed by immunofluorescent staining and qRT-PCR of astrocyte marker (GFAP) (Fig. 7C and D). The PD coating alone on PLGA substrates may also enhance neuronal differentiation of human NSCs (Fig. 7E and F). Tuj1 expression in differentiated human NSCs increased on PLGA substrates with immobilized NGF and C(K)GGYIGSR (Fig. 7F). Human NSCs cultured on PLGA substrates with KGGYIGSR

Fig. 8. Human fetal NSC differentiation on polymer substrates with scrambled peptides. Immunofluorescent staining of (A) neuronal (Tuj1) and (C) astrocyte (GFAP) markers in human fetal NSCs. qRT-PCR analyses examine the expression of (B) Tuj1 and (D) GFAP in human fetal NSCs. Human fetal NSCs were cultured on the unmodified polystyrene substrates (PS), PD-coated polystyrene substrates (PS-PD), and PD-coated polystyrene substrates with immobilized adhesion peptides (KGGYIGSR) and scrambled peptides (KGGYSRIG) 4 days after seeding. The relative expressions of Tuj1 and GFAP in each group were normalized to those of the unmodified polymer group (no coating) (n = 3, *: p < 0.05 and **: p < 0.01, compared to unmodified polymer group). The scale bars indicate 50 μm.
demonstrated enhanced astrocyte differentiation (Fig. 7G and H). Interestingly, immobilized scrambled peptides (KGGYSRIG) on the PD-coated polymer (PS) substrates failed to enhance neuronal or astrocyte differentiation in human NSCs (Fig. 8). This indicates that the engineered substrates exert sequence-specific control on NSC differentiation.

The results obtained from differentiation experiments with mouse fetal brain-derived NSCs are consistent with those from human fetal NSCs (Fig. S3). qRT-PCR analysis of Tuj1 showed that PD-mediated immobilization of the same neurotrophic growth factors (NGF and GDNF) and adhesion peptides (YIGSR and RGD) promoted neuronal differentiation of mouse NSCs on the PS and PLGA substrates 4 days after seeding (Fig. S3B and D). Coating with these bioactive molecules also enhanced glial differentiation (oligodendrocyte lineage) of mouse NSCs on PS and PLGA substrates, as indicated by increased expression of oligodendrocyte markers (O4 and Olig2; Fig. S3A, C and E).

Another type of human NSC, human iPSC-derived NSCs, were also tested in this study. Human iPSCs are of interest in regenerative medicine. Human iPSCs are generated from autologous somatic cells by reprogramming through transduction of four defined pluripotent stem cell transcription factors (Oct4, Sox2, Klf4, and c-Myc) [33]. The pluripotency of human iPSCs is comparable to that of human ESCs [33]. Therefore, human iPSCs should provide an autologous cell source, without the risk of immunogenicity, for cell therapies and tissue engineering applications. However, transduction of oncogenes KIf4 and c-Myc to produce iPSCs is a safety concern for tumor formation in human iPSC transplantation [27]. For this reason, Kim et al. recently established human iPSCs by transduction of only one transcription factor (Oct4) [27]. In this study, NSCs were derived from human iPSCs induced from only one factor (Oct4). They were cultured on PD-coated polymer substrates with immobilized bioactive molecules in the absence of mitogenic factors (bFGF and EGF) to induce spontaneous differentiation.

The immobilization of GDNF and adhesion peptides (KGGYIGSR and CGGRGD) that were effective for neuronal differentiation of human and mouse fetal NSCs significantly enhanced the expression of a neuronal marker (Tuj1) in human iPSC-NSCs cultured on PS-PD substrates (Fig. 9B). Immunofluorescent staining showed Tuj1-positive cells with extended neurites on PS-PD substrates that contained immobilized GDNF, KGGYIGSR, and CGGRGD (Fig. 9A). Immobilization of NGF, KGGYIGSR, and CGGRGD also promoted neuronal differentiation of human iPSC-NSCs on PLGA-PD substrates (Fig. 9C and D). PD coatings alone also enhanced the

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**Fig. 9.** Human iPSC-NSC differentiation on polymer substrates with and without immobilized neurotrophic growth factors and adhesion peptides. (A, C) Immunofluorescent staining and (B, D) qRT-PCR analyses examine the expression of the neuronal (Tuj1) marker in human iPSC-NSCs. Human iPSC-NSCs were cultured on the unmodified polymer substrates (PS and PLGA), PD-coated polymer substrates (PS-PD and PLGA-PD), and PD-coated polymer substrates with immobilized neurotrophic growth factors (NGF and GDNF) and adhesion peptides (KGGYIGSR and CGGRGD) 7 days after seeding. The relative expression of Tuj1 in each group was normalized to that of the unmodified polymer group ($n = 3$, *: $p < 0.05$ and **: $p < 0.01$, compared to the unmodified polymer group (no coating) and #: $p < 0.05$, compared to Matrigel group). Matrigel coatings were used as a positive control. The scale bars indicate 100 μm.
Fig. 10. The proliferation of various stem cells and primary cells on the polymer substrates. (A, B) The proliferation of human iPSC-NSCs cultured on unmodified polymer substrates (PS and PLGA), PD-coated polymer substrates (PS-PD and PLGA-PD), and PD-coated polymer substrates with immobilization of neurotrophic growth factors (NGF and GDNF) and adhesion peptides (C(K)GGYIGSR and CGGRGD). Human iPSC-NSCs were cultured in medium condition supplemented with mitogenic factors (bFGF and EGF). Matrigel coatings were used as a positive control. The proliferation of (C, D) human ADSCs and (E, F) HUVECs cultured on unmodified polymer substrates (PS and PLGA), PD-coated polymer substrates (PS-PD and PLGA-PD), and PD-coated polymer substrates with immobilization of growth factors (bFGF and VEGF) and adhesion peptide (CGGRGD). The cell proliferation was measured by Alamar Blue assay. The viability in each group was normalized to that in unmodified group (no coating) (n = 3; *, p < 0.05 and **; p < 0.01, compared to unmodified polymer groups).
expression of Tuj1 in human iPSC-NSCs on PLGA substrates (Fig. 9D). Interestingly, the engineered polymer substrates containing growth factors and peptides significantly enhanced the proliferation of human iPSC-NSCs when they were maintained in culture supplemented with mitogenic factors, bFGF and EGF (Fig. 10A and B). This may indicate that the functionality of the PD-coated substrates can be modulated according to stem cell culture conditions.

PD-engineered polymer substrates can be applied to other types of stem cells and primary cells. Immobilization of growth factors [bFGF and vascular endothelial growth factor (VEGF)] and RGD peptides enhanced the proliferation of human adipose-derived stem cells (ADSCs) cultured on the PS and PLGA substrates (Fig. 10C and D). The effect of immobilized peptides (CGGRGD) on ADSC proliferation was more dramatic in the PLGA substrates compared to the PS substrates (Fig. 10C and D). Interestingly, PD coatings alone on the PLGA substrates enhanced ADSC proliferation, whereas PD coatings alone did not affect ADSC proliferation on the PS substrates (Fig. 10C and D). This was also observed in our NSC experiments where we showed that PD coatings significantly enhanced neuronal differentiation of NSCs only on the PLGA substrates (Figs. 7F and 9D). When human umbilical vein endothelial cells (HUVECs) were cultured on the PS and PLGA substrates grafted with bFGF, VEGF, and the RGD peptide, they also exhibited enhanced proliferation after 3 days of culture (Fig. 10E and F).

PD-mediated immobilization could provide an alternative strategy to overcome the disadvantages of current surface coating methods for NSC culture. The coating of cationic polymers such as poly(lysine) and poly(ornithine) has been used to facilitate NS attachment and promote differentiation [3,34,35], however, these methods typically result in biologically inactive and cytotoxic substrates [36,37]. Biological materials including Matrigel, which was served as a positive control in the present study, have been widely used as coating materials for NSC culture [38]. Because Matrigel contains various ECM proteins and growth factors, it can support proliferation, differentiation, and survival of NSCs [38]. However, Matrigel's raw material is extracted from an animal source (e.g., Engelbreth-Holm-Swarm mouse sarcoma) and it is not a chemically-defined substrate [39]. Thus, there are safety concerns that include immunogenic problems and animal pathogen transmission, and these problems may impede the use of Matrigel for clinical applications [39]. This study demonstrates that PD-functionalized substrates containing immobilized growth factors and adhesion peptides do not exhibit cytotoxicity (Fig. 51 and Fig. 10), and more importantly, they enhance differentiation and proliferation of NSCs at a level comparable or greater than Matrigel (Figs. 7, 9 and 10, and S3). Therefore, PD-coated polymers with immobilized bioactive molecules may provide chemically defined, safe, functional substrates and scaffolds for NSC culture.

Further studies are required for optimizing the PD-mediated biocojunction of bioactive molecules. It has been previously reported that the reaction selectivity of PD to specific functional groups (amine, thiol, or imidazole) could be modulated by controlling the pH, which allows for the discrimination of nucleophiles on the basis of pKa value [22]. For example, under acidic pH conditions, the imidazole side chain of histidine (pKa ~6) preferentially reacts with PD, whereas the reaction to amine side chains of lysine (pKa ~10) is favored under alkaline pH conditions [22]. The selective binding of specific amino acid residues in peptides or proteins to PD via pH control may alter orientation and folding of immobilized peptides and proteins on the PD-coated surface [22]. Therefore, pH-based control of protein and peptide immobilization needs to be further optimized to induce effective peptide orientation and proper protein folding, and to ultimately maximize the activity and stability of peptides and proteins.

4. Conclusions

In summary, this study establishes bio-inspired, facile surface modification with bioactive molecules for NSC engineering. PD coatings allow for efficient surface immobilization of proteins and peptides to a diverse range of materials, including polymer scaffolds, ceramic substrates, and metal devices, for stem cell culture and transplantation. This method would also be useful for grafting other types of bioactive molecules such as nucleotides [40]. DNA or RNA-modified substrates could also be applied to genetically engineer stem cells. Therefore, PD-based immobilization methods may provide a versatile platform technology for efficient development of biomimetic substrates and scaffolds that induce desirable stem cell behavior and enhance stem cell function.

Acknowledgments

This work was supported by grants (2010-0020409, 2010-0022037, 2010-0025982, and 2010-0020277) funded by the National Research Foundation of Korea, the Ministry of Education, Science and Technology, Republic of Korea.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2012.06.067.

References


