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Bio-inspired oligovitronectin-grafted surface for enhanced self-renewal and long-term maintenance of human pluripotent stem cells under feeder-free conditions

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ABSTRACT

Current protocols for human pluripotent stem cell (hPSC) expansion require feeder cells or matrices from animal sources that have been the major obstacle to obtain clinical grade hPSCs due to safety issues, difficulty in quality control, and high expense. Thus, feeder-free, chemically defined synthetic platforms have been developed, but are mostly confined to typical polystyrene culture plates. Here, we report a chemically defined, material-independent, bio-inspired surface coating allowing for feeder-free expansion and maintenance of self-renewal and pluripotency of hPSCs on various polymer substrates and devices. Polydopamine (pDA)-mediated immobilization of vitronectin (VN) peptides results in surface functionalization of VN-dimer/pDA conjugates. The engineered surfaces facilitate adhesion, proliferation, and colony formation of hPSCs via enhanced focal adhesion, cell–cell interaction, and biophysical signals, providing a chemically defined, xeno-free culture system for clonal expansion and long-term maintenance of hPSCs. This surface engineering enables the application of clinically-relevant hPSCs to a variety of biomedical systems such as tissue-engineering scaffolds and medical devices.

1. Introduction

Human pluripotent stem cells (hPSCs) including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) show immense potential for regenerative medicine [1–6]. Current methods for culture and clonal expansion of hPSCs require animal-derived feeder cell layers (e.g., mouse embryonic fibroblasts) or animal tissue-derived matrices (e.g., Matrigel) [7–9]. However, animal-derived cells or matrices present serious safety concerns due to immunogenicity and animal pathogen transmission [8,9]. In addition, variability in animal sources often leads to batch-to-batch inconsistencies in the character and quality of hPSCs [9]. The cost of hPSC culture is significantly affected by use of these animal sources, which is a significant limitation of scaling-up hPSC culture to acquire sufficient cell numbers for clinical applications [9,10].

Therefore, establishing chemically defined, xeno-free conditions for hPSC culture is essential for production of clinically-relevant hPSCs. One method used to establish these conditions is to engineer substrates with polymers, peptides, and proteins that provide artificial extracellular matrix (ECM)-like microenvironments for feeder-free hPSC growth by enhancing cell-matrix interactions [8,9,11,12]. Several studies demonstrate that substrates modified with synthetic polymers or ECM peptides/proteins allow for maintenance of the undifferentiated state in hPSCs and support long-term self-renewal of hPSCs without animal feeder cells [13–19]. However, these approaches have been limited to specific types of material surface, mostly to polystyrene (PS) and glass. Given that there are a wide variety of materials useful for biomedical engineering, a generalized chemical approach commonly applicable for different materials allows for direct culture of hPSCs on various biomedical systems such as tissue-

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engineering scaffolds, patterned substrates, and biomedical devices. Thus, simple, effective, and material-independent surface chemistry is critical for the development of biologically functional substrates for feeder-free hPSC culture in various biomedical systems.

Peptide immobilization strategies inspired by mussel adhesion can facilitate efficient production of biomimetic polymer surfaces for hPSC expansion and maintenance in a material-independent manner. The strong attachment of mussels to various types of organic or inorganic materials is attributed to an extensive repeat of 3,4-dihydroxy-L-phenylalanine (L-Dopa) and lysine residues in the *Mytilus edulis* foot protein-5 of mussel adhesive pads [20]. Co-existence of catechol (L-Dopa) and amines (lysine) was found to be crucial for adhesion to a wide spectrum of material surfaces [20]. Therefore, dopamine, a naturally occurring catecholamine containing both catechol and amine functional groups, has been used as a bioadhesive building block for multifunctional surface coatings [20]. Dopamine can be polymerized in alkaline pH conditions to form thin polydopamine (pDA) that exhibits latent reactivity to various nucleophiles such as amines, thiols, and imidazole groups [20–23]. Thus, proteins or peptides with these functional groups can be efficiently immobilized onto the surfaces made of virtually all types of materials via pDA coating. Stem cell and tissue-engineering studies have taken advantage of these interactions by immobilizing functional peptides or proteins onto desired surfaces containing pDA [21,22]. Despite the immense potential of pDA chemistry for biomedical engineering, the mechanism of interaction between pDA and proteins/peptides has not been extensively explored.

In the current study, we applied a chemically defined, material-independent pDA-mediated immobilization of ECM peptides (vitronectin; VN) to create biologically functional culture substrates that support self-renewal and pluripotency of hPSCs. We elucidated the chemical configuration of immobilized VN peptides on the pDA coating. The engineered substrates allowed for feeder-free culture of hPSCs compatible with a variety of passaging methods, commercially available media, and hPSC lines. We found that hPSCs could be subcultured for more than 3 months on the substrates, indicating long-term maintenance of hPSC self-renewal. The VN-pDA substrates were found to enhance focal adhesion and cell–cell interaction, and to activate a series of mechanosensitive intracellular signaling pathways that are critical for hPSC self-renewal and pluripotency. Most interestingly, other polymer surfaces of nanopatterned substrates and microfluidic devices could be functionalized in this manner and then support self-renewal and feeder-free maintenance of hPSCs, indicating the versatility of this approach for hPSC culture. The pDA-mediated VN peptide coating described here can be used for chemically defined, xeno-free, and material-independent culture of hPSCs.

2. Materials and methods

2.1. pDA coating

PS culture plates were coated with a pDA layer for subsequent immobilization of VN-derived peptides. The wells of the PS culture plates were incubated with a dopamine hydrochloride solution at pH 8.5 (2 mg/ml in 10 mM Tris–HCl) (Sigma–Aldrich, St. Louis, MO, USA) for 4 h at room temperature. The pDA-coated PS culture plates were then rinsed with distilled water to remove unattached dopamine molecules. pDA coating on the poly(lactic-co-glycolic acid) (PLGA) and polydimethylsiloxane (PDMS) substrates was performed using the same protocol as that described for the PS substrate.

2.2. Immobilization of VN peptides

Immobilization of VN peptides onto the pDA-coated PS culture plates was performed as previously described [21]. Two different VN sequences were used for the immobilization (VN1: CGGPQVTRGDVFTMP [15] and VN2: CGGKKQRFRHRNRKG [16]). pDA-coated PS surfaces were incubated with peptide solutions at pH 8.5 (2 mg/ml in 10 mM Tris–HCl, Peptron, Daejeon, Korea) for 6 h at room temperature. The VN-pDA-PS surfaces were then washed with distilled water to remove unattached peptides. The immobilization efficiency of VN peptides and detachment of immobilized VN peptides under physiological conditions (in phosphate buffered saline (PBS) at 37°C for 7 days) were determined by fluorescamine assay (Sigma-Aldrich) [21]. VN peptide immobilization on the pDA-PLGA and pDA-PDMS substrates was performed using the same protocol as that described for the pDA-PS substrate. Immobilization of peptides on various material surfaces was visualized by using fluorescently labeled peptides (FITC-CGGRGD). The fluorescence signals of the immobilized FITC-CGGRGD on each substrate were detected by an imaging analyzer (OV100, Olympus Corp., Tokyo, Japan).

2.3. Surface characterization of VN-pDA-PS substrates

The atomic chemical composition of the engineered surfaces was analyzed by X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo VG Scientific, East Grinstead, UK). Surface morphology of the engineered surfaces was observed by atomic force microscopy (AFM, XE-100, Park Systems, Suwon, Korea) and scanning electron microscopy (SEM, JSM 7001F, JEOL, Tokyo, Japan).

2.4. Determination of chemical configuration of immobilized VN peptides

Dopamine hydrochloride (Sigma–Aldrich) was dissolved in PBS solution (2 mg/ml, pH 8.5) followed by incubation at room temperature for 2 h to allow for polymerization. Partially polymerized dopamine solution and VN2 peptide solution were mixed for 3 h. The prepared VN2-pDA mixture solution was filtered through a 0.22 μm microfilter before high-performance liquid chromatography (HPLC) analyses. Reverse-phase high-performance liquid chromatography/tandem mass spectrometry (RP-HPLC/MS) analyses were performed with an Agilent 1200 Infinity Series LC system equipped with a Discovery BIO wide pore hydrophobic aliphatic C18 column (15 cm × 4.6 mm, 3 μm) (Sigma–Aldrich) and a ultraviolet–Visible (UV–Vis) detector. In this system, molecules with hydrophobic nature tend to be eluted later in retention time [24]. Hydrophobicity of proteins and peptides is usually proportional to their molecular weights and therefore proteins or peptides with higher molecular weight are eluted later in retention time in the RP-HPLC with C18 column. Distilled water with 0.1% tetrafluoroacetic acid (TFA) and acetonitrile with 0.1% TFA were used as running buffers and the system flow rate was set at 0.5 ml/min. The UV absorbance of the eluate was measured at 220 nm.

2.5. Measurement of the surface elastic modulus of the substrates

The surface elastic moduli of non-coated, pDA-coated, and VN1/VN2-pDA-coated PS substrates were measured by nanoindentation using AFM. Mechanical properties of each substrate were characterized using a Molecular Force Probe 3D AFM (MFP-3D, Asylum Research, Santa Barbara, USA). A diamond-coated silicon probe (DT-NCHR, NanoWorld, Neuchâtel, Switzerland) with a tip radius of 83 ± 17 nm (manufacturer provided) was used in all measurements. The spring constant was determined to be 102.62 (N/m) using the thermal noise method. All substrates were immersed in distilled water during AFM measurements. The scan speed was set at 2 μm/s. For each sample, the measurement was performed at 5–10 different locations, and at least 5 measurements were conducted at each location. The hardness (H) was calculated using the following equation:

$$\mathrm{H} = \frac{F_{max}}{A_{projection}}$$

where F_{max} is the maximum load and $A_{projection}$ is the contact projection area, which can be estimated from following equation [25]:

$$\mathrm{A} = \frac{\pi}{2}\left(R^2 - (R-d)\right)^2$$

where R is a tip radius and d is the penetration depth. The penetration depth (d) was measured by calculating the distance along with x-axis between two specific points (A and B) in the force-distance curve obtained from AFM analysis. The point A indicates when the tip starts to touch the top layer of the pDA coating and the point B indicates when the tip pushes all the way down to the underlying solid substrates. To estimate elastic modulus (E), each loading curve was fitted using the following equation [26] ($r^2 > 0.98$ for all), which is an indentation function for a spherical tip with radius R.

$$\mathrm{F} = \left(\frac{3}{4}E_{eff}\sqrt{R}\right)\mathrm{h}^{3/2}$$

From the slope of the fitted curve, the effective modulus (E_{eff}) can be calculated, which makes it possible to calculate E from following correlation of E_{eff} with E [26]:

$$\frac{1}{E_{eff}} = \frac{1-\nu^2}{E} + \frac{1-\nu_i^2}{E_i}$$

where ν is the Poisson ratio and subindex i corresponds to the AFM probe (For SiO_2 probe, $E_i = 76$, GPa and $\nu_i = 0.17$). The Poisson ratios of each sample were assumed to