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Long-term self-renewal of human pluripotent stem cells on peptide-decorated poly(OEGMA-*co*-HEMA) brushes under fully defined conditions

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ABSTRACT

Realization of the full potential of human induced pluripotent stem cells (hiPSC) in clinical applications requires the development of well-defined culture conditions for their long-term growth and directed differentiation. This paper describes a novel fully defined synthetic peptide-decorated substrate that supports self-renewal of hiPSC in commercially available xeno-free, chemically defined medium. The Au surface was deposited by a poly(OEGMA-*co*-HEMA) film, using the surface-initiated polymerization method (SIP) with the further step of carboxylation. The hiPSC generated from umbilical cord mesenchymal cells were successfully cultured for 10 passages on the peptide-tethered poly(OEGMA-*co*-HEMA) brushes for the first time. Cells maintained their characteristic morphology, proliferation and expressed high levels of markers of pluripotency, similar to the cells cultured on Matrigel™. Moreover, the cell adhesion could be tuned by the pattern and peptide concentration on the substrate. This well-defined, xeno-free and safe substrate, which supports long-term proliferation and self-renewal of hiPSC, will not only help to accelerate the translational perspectives of hiPSC, but also provide a platform to elucidate the underlying molecular mechanisms that regulate stem cell proliferation and differentiation via SIP technology.

1. Introduction

Because human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) have the potential to grow indefinitely and differentiate into all major lineages of somatic cells in the human body [1,2], they are being studied as important resources for regenerative medicine [3,4] and for the treatment of many diseases [5,6]. The successful integration of hiPSC into clinic applications will require large-scale cell expansion without differentiation (i.e. self-renewal) and well-defined xeno-free conditions for derivation and long-term expansion of hiPSC [7,8]. However, it is difficult to precisely control the behavior of hiPSC in vitro, since environmental conditions for self-renewal and differentiation are neither completely understood nor defined.

Originally, hiPSC were grown in monolayer culture with a feeder layer of mouse cells or with conditioned media derived from these feeder cells. Rapid advancement in cell culture techniques has led to the development of chemically defined media and feeder-free hiPSC culture systems that employ animal or human-derived extracellular matrix (ECM) compounds to coat the culture substrate [7,9–12]. Matrigel™ is the most prevalent ECM analogue, which is an extraction from Engelbreth–Holm–Swarm (EHS) mouse sarcomas [13]. It contains not only basement membrane components (collagen IV, laminin, entactin and heparin sulfate proteoglycans), but also matrix degrading enzymes, numerous growth factors, inhibitors and a broad variety of other unknown proteins [14]. As such, Matrigel is a poorly chemically defined substrate. Therefore, more recent research has focused on the isolated or recombinant proteins from ECM (such as laminin and vitronectin (VN)) [7,15] and synthetic polymers (such as PMEDSAH, PAPMAAm and PMVE-alt-MA) [16–18] to replace Matrigel. These approaches achieved some promising results. Nevertheless, these ECM protein culture systems are expensive and difficult to isolate [16]. In addition, the significant variance in quality from lot to lot of protein can confound basic research attempting to dissect the molecular mechanisms underlying gene and protein expression patterns. More importantly, the presence of animal or human proteins can cause problems related to immunogenicity, microbial and

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viral contamination [19,20], and eventually limit applications in clinic. Synthetic polymer substrate represents an alternative for prolonged hiPSC culture, and it is made of defined materials. However, pure synthetic polymer is not the intrinsic constituent of microenvironments in vivo (stem cell niches) [21], and it is hard to mimic the niches. Moreover, the lack of identification of a unique set of physicochemical properties that control cell–material interactions and the remarkable distinct self-renewal mechanisms of hiPSC between synthetic polymer in vitro and ECM-rich stem cell niches in vivo also make potential transplantation applications problematic [9]. Therefore, the establishment of a chemically defined, xeno-free and safe environment that supports the self-renewal of hiPSC and promotes hiPSC in clinic applications has been a major challenge in the field ever since hiPSC were first derived and cultured on mouse embryonic fibroblasts.

Synthetic peptide has been widely used for the growth and proliferation of a variety of cell types. Specifically, RGD peptide, the well-known integrin recognition site promoting binding of cells, has been modified in various materials for the proliferation of cells [22–24], such as osteoblast, mesenchymal stem cells and neural stem cells. Synthetic peptides derived from ECM proteins to culture hiPSC offer significant advantages, because they are generated from defined chemical structures and processes, and there is no variation between batches. More importantly, eliminating the need for animal byproducts eradicates concerns regarding immunogenicity and shows greater potential for clinic [25]. Recently, synthetic peptide interfaces have achieved promising results for hESC culture. Although these studies demonstrated cell adhesion to peptide-coated surfaces, interpretation of the specificity of the peptide–cell interaction and clinical relevance is limited, owing to the use of mouse embryonic fibroblast-conditioned medium or coating of the peptide surface with bovine serum [26,27]. Furthermore, these studies did not achieve long-term self-renewal of hESC, owing to low peptide density conjugating on the surfaces [26,27].

The present study developed a technology that notably provides cell expansion and supports the long-term self-renewal of hiPSC similar to the gold standard of Matrigel, via surface-initiated polymerization (SIP) for the first time. Transparent Au-coated glasses were modified with poly(oligo(ethylene glycol) methacrylate polymer brushes, and the side ethylene glycol groups were further converted to carboxyl for a large amount of peptide tethering [28]. The peptide derived from an active domain of VN was tethered to polymer brushes using standard NHS/EDC chemistry. VN is one composition of ECM in the human body, and is reported to support the adhesion and successful propagation of hESC for >10 passages [29,30]. Many biological properties of hiPSC and hESC are similar, such as the morphology, capacity to self-renew and developmental potential [1]. The present authors believe that VN peptide could also facilitate the adhesion and self-renewal of hiPSC. In the long-term test (i.e. >10 passages), hiPSC that were cultured under the chemically defined system have adhered to the surface, remained viable, maintained the morphology and expressed the markers of undifferentiated hiPSC. A further comprehensive in vivo evaluation of hiPSC generated from the peptide-decorated substrate is currently under way in the laboratory. The completely synthetic peptide substrate developed for hiPSC culture has achieved consistent long-term self-renewal of hiPSC in chemically defined media, and will greatly accelerate the introduction of hiPSC into clinical applications.

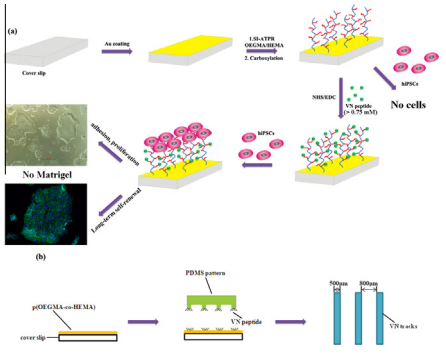
2. Materials and methods

2.1. Materials

The thiolate initiator (ω-mercaptoundecyl bromoisobutyrate) was purchased from HRBio (Suzhou, China). 1-undecanethiol, oligo-(ethylene glycol) methacrylate (OEGMA; M_n = 526), 2-hydroxyethyl methacrylate (HEMA), anhydrous *N*,*N*′-dimethylformamide (DMF), succinic anhydride and 4-(dimethylamino) pyridine (DMAP) were purchased from Sigma–Aldrich (St. Louis, USA). *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-(*N*-Morpho) ethanesulfonic acid (MES) and phosphate buffer (PBS) were obtained from Aladdin (Shanghai, China). To facilitate chemical conjugation onto the surface, the peptide was modified at its *N*-terminal with a lysine-containing spacer. The peptide (Ac-KGGPQVTRGDVFTMP sequence), provided by Chinapeptides Co. Ltd. (Shanghai, China), was synthesized by a batchwise fmoc-poly-amide method at >98% purity, according to the high-pressure liquid chromatography profile. All other chemicals were of analytical reagent grade and were used as received unless otherwise noted. All aqueous solutions were prepared with de-ionized water (DI water).

2.2. Preparation of the poly(OEGMA-co-HEMA) polymer brushes

Gold metal (50 nm) was deposited onto a cover slip using a magnetron sputtering system (KYKY Technology Development LTD, China). Gold-coated samples were cleaned with ethanol and DI water before use. Surface-initiated atom transfer radical polymerization (SI-ATRP) was carried out to prepare poly(OEGMA-*co*-HEMA) brushes, as previously described [31] (Scheme. 1a). Briefly, mixed self-assembled monolayers (SAM) of ω-mercaptoundecyl bromoisobutyrate (initiator) and 1-undecanethiol (spacer) were prepared by immersing the gold-coated samples into a 1 mM mixed solution (total concentration) of two thiols with a ratio of 1:2 for 24 h at room temperature. The surfaces modified with mixed SAM were thoroughly rinsed with ethanol to remove the physisorbed molecules and then dried in a stream of nitrogen. The poly(OEGMA-*co*-HEMA) reaction solution was prepared by mixing well with Milli-Q water (3 ml), methanol (12 ml), 2,2′-bipyridine (Bipy) (12.5 mg), monomers OEGMA (2.65 g, 5 mM) and HEMA (0.65 g, 5 mM). Another 1 ml of $CuCl_2$ solution (0.04 mM) was added to the mixed solution. After the mixed solution had been deoxygenated for 15 min, 1 ml of predeoxygenated-ascorbic acid solution (AscA, 0.04 mM) was injected with a syringe. The mixture was further deoxygenated for another 15 min, and the resulting mixture was transparent red in color as a result of reduction of the deactivator Cu(II)/Bipy complex to activator Cu(I)/Bipy complex. This mixture was then transferred to the reaction setup, and the cover slips were immersed in an inert gas glove box at room temperature. The polymerization was stopped after 6 h, and the samples were thoroughly rinsed with ethanol and Milli-Q water and dried in a stream of nitrogen. Then, the surfaces after SIP were incubated in a DMF solution containing succinic



Scheme 1. (a) Schematic of Au surface modification to present VN peptide ligands within polymer brushes supporting proliferation and long-term self-renewal of hiPSC under fully defined conditions. (b) Peptide patterns on Au-coated glass with poly(OEGMA-*co*-HEMA) brushes: microcontact printing of VN peptide.

anhydride (10 mg ml^{-1}) and DMAP (15 mg ml^{-1}) overnight to generate terminal carboxyl groups. The samples were thoroughly rinsed with methanol and Milli-Q water and dried under flowing nitrogen before further treatment.

2.3. Peptide-decorated surface preparation

VN peptide was tethered to polymer brushes using standard NHS/EDC chemistry. After being thoroughly washed, samples were incubated in 2.0 mM EDC and 5.0 mM NHS in 0.1 M MES solution (pH = 5.6) for 40 min on a super-clean bench. Then, a dilution series of VN peptide solutions (0 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1 mM and 1.25 mM in sterile PBS) were incubated on the activated surfaces in a 5 °C refrigerator for 24 h. For immediate use, the surface should be deactivated in ethanol amine (1 M, pH = 8.5) for 10 min. All samples were 75% ethanol sanitized for at least 30 min before cell culture.

2.4. Surface characterization

Polymer brushes before and after peptide conjugation were characterized by contact angle goniometry, X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). Static water contact angles were measured with a Dataphysics OCA20 contact angle system (Filderstadt, Germany) at room temperature. Six samples in each stage were used to provide an average and standard deviation. XPS (AXIS Ultra, Kratos Analytical Ltd.) was employed at 15 kV and 10 mA for both survey and high-resolution spectra. The binding energies were calibrated by the C 1s hydrocarbon peak at ~285 eV. The quantitative analysis and the curve fitting were conducted using the CasaXPS software package. AFM (PI3800/SPA400, Seiko Instruments) was used in contact mode (15-μm scanner) in dry conditions at room temperature to investigate the changes in surface morphology treated by different peptide concentrations. For the contact mode measurement, a Si_3N_4 cantilever with a spring constant of 0.12 N m^{-1} (Seiko Instruments) was used for resolution imaging. Before AFM measurement, the different Au surfaces were rinsed with ethanol and Milli-Q water and allowed to air dry.

2.5. Peptide content assessment by fluorescent peptide labeling

The gradient peptide density 24-well plates were fabricated as described above for the peptide-decorated surface. To track the change in conjugated peptide surface content, FITC-labeled VN peptide solution was used. A dilution series of FITC-labeled peptides was implemented to vary conjugated peptide surface content. The qualitative examination of fluorescence intensity was detected by inverted fluorescent microscopy (Nikon Eclipse TE 300, Japan). Furthermore, the quantitative average fluorescence intensity of the surfaces was acquired using Multilabel Reader (2300, Perkin Eimer Singapore Pte. Ltd., Singapore) with the absorbed wavelength of 488 nm and the emitted wavelength of 525 nm. Six independent measurements were performed for each surface.

2.6. hiPSC culture

UMC-C1 hiPSC generated from umbilical cord mesenchymal cells by introducing four classical factors, Oct-4, Sox-2, c-Myc and Klf-4 (provided from Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, China) were used in this work. Each peptide-decorated sample was cut into a square shape with dimensions of ~1.2 × 1.2 cm^2 and placed into 12-well plates. Cells were cultured on VN peptide-decorated cover slips and Matrigel-coated plates using chemically defined mTeSR™1 media (StemCell Technologies, Vancouver, Canada) at 37 °C in a humidified 5% (v/v) CO_2 incubator (MCO-18AIC, Japan). The culture on Matrigel™ (BD Biosciences, New Jersey, USA) served as the positive control. Matrigel was diluted with Dulbecco's Modified Eagle Medium/F12 (Gibco, USA) at a ratio of 1:80 at 4 °C. Then, 600 μl diluted Matrigel was pipetted to each well of the 12-well plates and quickly incubated at 37 °C for >30 min. Then hiPSC were seeded onto the Matrigel-coated surface. However, hiPSC were directly seeded onto the peptide-decorated substrate without additional treatments. Cells were fed daily and passaged at 1:3 splitting ratio every 3–4 days by exposure to Dispase™ (Gibco Invitrogen; Carlsbad, Canada) at 0.5 mg ml^{-1} for 5.5 min. Cells were then washed on the dish with F12 media, followed by mTeSR™1 media. Then cells were gently scraped and pipetted into smaller colonies, and passaged into new 12-well plates containing the peptide-decorated surface or Matrigel-coated surface in mTeSR™1 media. The cells were passaged to the 10th generation.

2.7. Assessment of cell adhesion and morphology

To test the cell adhesion, micropatterning experiments using polydimethylsiloxane (PDMS) stamp as a soft lithography model were applied. The present authors conceived a facile matrix method to affirm the cell-adhesion property only on the VN peptide modified surface. PDMS stamps with parallel lanes (500 μm in width and 800 μm in line spacing; Scheme 1b) were inked with VN peptide solution (0.75 mM), and EDC/NHS-activated substrates were then stamped in a certain direction for 1 h forming a "||" on the polymer-grafted surfaces, and exposed to dry air for 1 h for thorough fixation. The treated samples were finally washed three times with PBS and stored in PBS overnight to facilitate peptide rearrangement on the polymer matrices for better performance. Cells were seeded on the untreated and patterned substrates for comparison. The surfaces after 3 days of incubation were washed three times with PBS to remove the loosely adhered cells. An inverted phase contrast microscope (Nikon Eclipse TS100, Japan) was used to image the cell adhesion on the patterned surfaces.

Moreover, adhesion of hiPSC was assessed using the cell counting kit-8 assay (CCK-8, Dojindo, Japan). After cell counting, cells were seeded on the substrates conjugated with a variety of peptide concentrations at a density of 1×10^5 cells/well. After incubating for 3 days, 50 μl of CCK-8 was added into each well for 2 h incubation. Then 100 μl of supernatant from each well was transferred to new 96-well cell culture plates. The absorbance value of the supernatant optical density (OD value) was measured at 450 nm with a microplate reader (Model 680, Bio-Rad, Hercules, CA).

2.8. Assessment of cell proliferation

The hiPSC proliferation on 0.75 mM VN peptide treated surfaces was also assessed at 0.5, 1, 3, 5 and 7 days by CCK-8, as described above. The value was normalized to Matrigel at 12 h.

2.9. Immunocytochemistry

The hiPSC at passage 1 (p1), passage 5 (p5) and passage 10 (p10) were subjected to immunocytochemical analysis. The cells were fixed using 4% (v/v) paraformaldehyde in PBS buffer at room temperature for 30 min. The samples were permeabilized with 0.2% (v/v) Triton X-100 (Sigma) for 30 min before being incubated with 3% bovine serum albumin/PBS buffer at 37 °C for 2 h to block non-specific binding. Cells were incubated with a 1:50 dilution of primary antibody (Mouse Anti-Nanog IgG to Human (R&D systems, California, USA); Mouse Anti-Oct-4 IgG to Human (R&D systems); Mouse Anti-SSEA-3 IgG to Human (R&D systems); Mouse Anti-SSEA-4 IgG to Human (R&D systems)) overnight at 4 °C. The next day, cells were incubated with Invitrogen (California, USA) secondary

antibody diluent 1:150 for 1 h in dark at room temperature (Goat Anti-Mouse Invitrogen 488 IgG (Molecular Probes), Goat Anti-Rabbit Invitrogen 546 IgG (Molecular Probes)). Finally, cell nuclei were stained with 50 μg ml^{-1} 4′,6-diamino-2-diamidino-2-phenylindole for 5 min at room temperature. All staining steps were followed by three washes in PBS buffer. Cells were visualized immediately by laser confocal microscopy (Carl Zeiss, Oberkochen, Germany).

2.10. RNA isolation and real-time polymerase chain reaction

RNA was isolated from cells using TRIzol (Invitrogen, USA). Reverse transcription was performed by means of RevertAid™ First Stand cDNA Synthesis Kit (Fermentas, Canada). Quantitative polymerase chain reaction (PCR) analysis was carried out using SYBR Green (Takara, Japan) and an ABI 7500 RT-PCR machine (Applied Biosystems, USA). Items were run in triplicate, and values were normalized on the basic of GAPDH value. Primers used in this study were (5′–3′) as follows: Oct-4, forward-GCTCGAGAAGGATGTGGTCC and reverse-CGTTGTGCATAGTCGCTGCT; Nanog, forward-GC AGAAGGCCTCAGCACCTA and reverse-AGGTTCCCAGTCGGGTTCA; Sox-2, forward-CACTGCCCCTCTCACACATG and reverse-TCCCAT- TTC CCTCGTTTTTCT.

2.11. Statistical analysis

All data are presented as the mean ± standard deviation (if not mentioned otherwise). Statistical analysis was performed with Origin software. Student's *t*-test was used to determine the significant differences among the groups, and p values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Surface modification and characterization

In this study, a synthetic peptide-decorated interface was developed for the long-term self-renewal of hiPSC via SIP technology. The thiolate initiator self-assembles onto the Au surface via the Au–S bond. Compared with other SAM, such as silanes and phosphonic acid, thiolate-based SAM is easier to control the quality and density of Au-thiolate-based SAM [31]. In addition, our previous work showed that a sparse polymer coating from a low-density initiator SAM via SIP was better at immobilizing large peptide and protein [28], such as collagen, fibronectin (FN) and bone morphogenetic protein (BMP), which is an excellent choice for investigating cell–matrix interactions. Ren et al. successfully decorated poly(OEGMA-*co*-HEMA) brushes with FN and recombinant human bone morphogenetic protein-2 (rhBMP-2), and conferred the titanium surface dual-function with cell adhesion-enhancing and osseointegration-promoting properties [32]. Poly(OEGMA-*co*-HEMA) brushes were prepared via 6 h of SIP, which led to a thickness of ~20 nm that was specifically chosen for a well-balanced antifouling property and protein immobilization capacity [28], and the latter can be easily realized by altering the ratio of initiator and undecanethiol. The polymer-coated and peptide decorated surfaces were characterized by contact angle goniometry and XPS. The pristine Au substrates were hydrophobic, with a contact angle of ~82°, which decreased to an average value of 55° after polymerization (Fig. 1a). This could be due to the hydrophilicity of the grafted polymer brush with an OEG side chain. Then, after grafting peptide onto the polymer brushes, the contact angle sharply decreased to ~13°, attributed to the super-hydrophilicity of the VN peptide. These conclusions were further verified by an XPS survey scan (Fig. 1b, Fig. S1 and Table S1). The pristine Au showed strong Au 4f, Au 4d, Au 4d− and O 1s peaks as well as C 1s resulting from a contaminated hydrocarbon. After SIP from the Au surface, the Au 4d signal decreased from 21.51% to 4.68%, and a substantial increase in the C 1s and O 1s signal was detected, which indicated the existing of the poly(OEGMA-*co*-HEMA) layer. Meanwhile, the slight intensity of the Cu 2p peak was shown up owing to the residual $CuCl_2$ in the SIP process. Successful immobilization of peptide on polymer brushes was demonstrated by the appearance of N peak (4.72%) and Na peak (1.11%), because the peptide was dissolved in PBS buffer. Furthermore, XPS allowed different element species to be distinguished in different chemical environments in core-level separating spectra. The core spectra of C 1s (Fig. 1b3) clarify the three typical separating peak components corresponding to C–H/C–C (284.8 eV), C–O (286.4 eV) and C═O groups (288.8 eV) in Au-p(OEGMA-*co*-HEMA). However, the O 1s peak of polymer-grafted Au was ~1.7 times more than pristine Au. In addition, the Au 4f and O 1s core spectra (Fig. S1) show a distinctive chemical shift of the peaks (~532.5 eV for O 1s, and ~83.5 eV and 87.5 eV for Au 4f) attributed to the complicated polymer from the pristine Au (~532 eV for O 1s, and ~84 eV and 88 eV for Au 4f). Additionally, after polymer grafting, the intensity of the carbon skeleton (–C–C–/–C–H–) decreased dramatically, and the peaks of the hydroxyl and carbonyl groups increased as shown in Fig. 1b2–b3. They should be attributed to the ester and carboxyl groups of the poly(OEGMA-*co*-HEMA) brushes. It was noted that the –C–N– peak at 285.5 eV was recorded on the peptide-decorated Au, indicating the presence of VN peptide on the surface of polymer brushes, which was also proved by an ~130 Hz decrease in frequency from quartz crystal microbalance results (Fig. S2). From the data above, the successful coating of poly(OEGMA-*co*-HEMA) and successful tethering peptide on the Au surface is concluded.

3.2. hiPSC attachment to peptide decorated-poly(OEGMA-co-HEMA) surface

To immobilize peptide on the Au surface presenting polymer brushes, the hydroxyl end groups of poly(OEGMA-*co*-HEMA) were further converted to carboxyl via succinic anhydride coupling, which is reactive to the amine groups of peptides (Scheme 1a). The –COOH groups on the resulting Au–p(OEGMA-*co*-HEMA)–COOH surfaces were preactivated with EDC/NHS chemistry to facilitate the peptide tethering of terminal carboxyl groups to NHS groups, producing peptide tethering surfaces. To demonstrate hiPSC adhesion only on the VN peptide-conjugated surface, a PDMS stamp with micrometer-scale lanes was inked with VN peptide. Then the stamp was microcontact printed (as shown in Scheme 1b) onto the Au-coated surfaces presenting activated polymer matrices, with "bare" polymer-grated Au surfaces (no peptide treatment) as controls. After printing, one could clearly observe VN peptide strips on the surface (Fig. 2a). Three days after seeding, the "bare" Au–p(OEGMA-*co*-HEMA)–COOH surface showed no cell adhesions, indicating that no hiPSC adhered to the surface without VN peptide. It is known that the antifouling surfaces of poly(OEGMA-*co*-HEMA) resist mammalian cell adhesion and activation. However, hiPSC retentively adhered and grew along the VN peptide-tethered tracks to form a rectangular shape (Fig. 2c), and no hiPSC appeared on the areas between two VN tracks (Fig. 2d), which further confirmed that VN peptide played a significant role in hiPSC adhesion and proliferation. Through the technology, one could tune the hiPSC adhesion on specific or wanted tracks, as needed.

3.3. VN peptide concentration influences hiPSC proliferation

Earlier studies in the field suggested that functional peptide density on the plate surface and uniform peptide distribution were important attributes for optimal cell response [19,26].

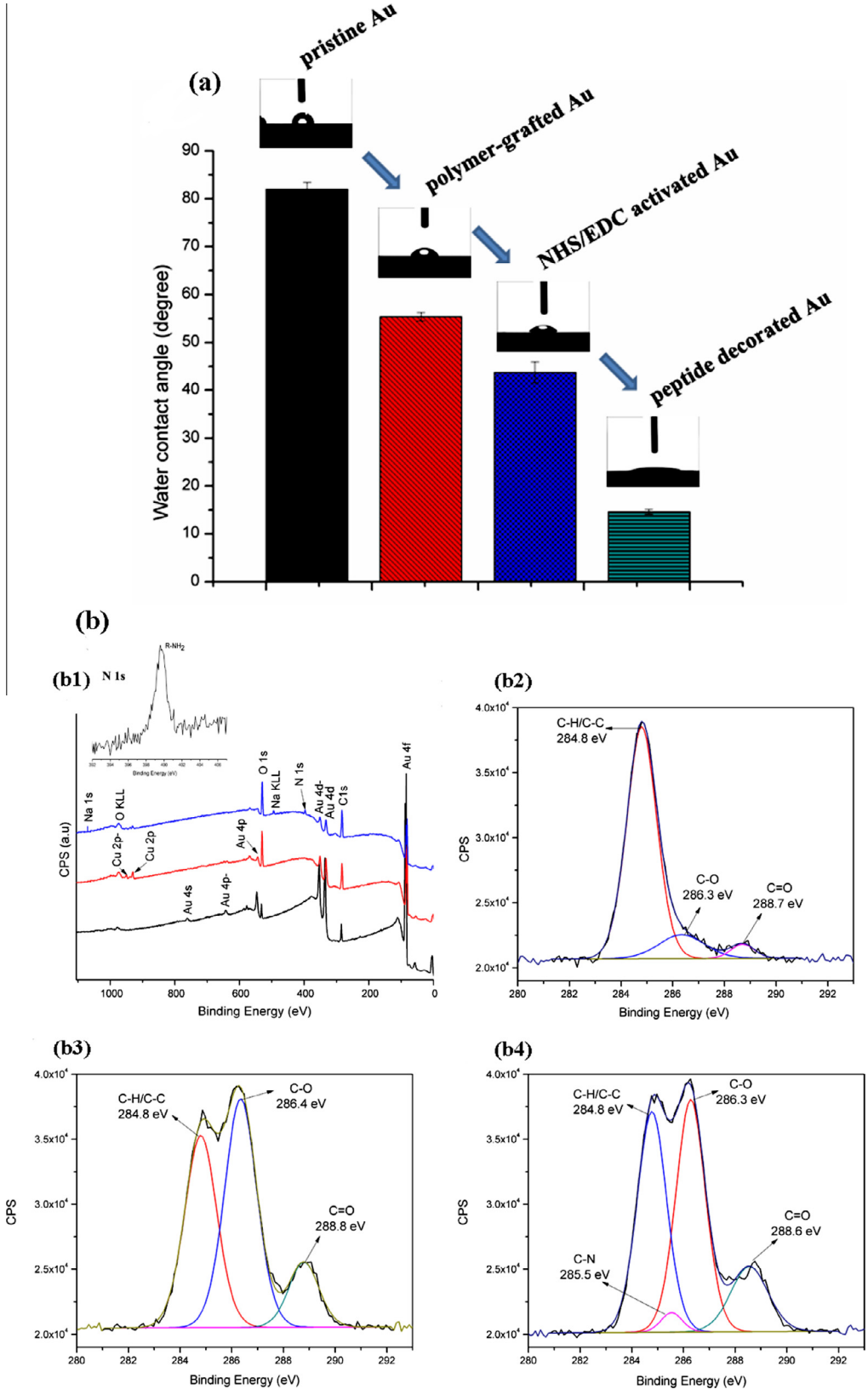


Fig. 1. (a) Water contact angles of Au surfaces in the different phases of VN peptide immobilization. (b) XPS survey scan spectra: (b1) XPS wide spectra of the pristine Au, polymer-grafted Au and peptide-decorated Au; (b2) high-resolution spectrum of carbon peaks (C 1s) for the pristine Au; (b3) high-resolution spectrum of carbon peaks for polymer-grafted Au; and (b4) high-resolution spectrum of carbon peaks for peptide-decorated Au. The insert in (b1) shows the high-resolution spectrum of nitrogen peaks (N 1s) for the peptide-decorated Au.

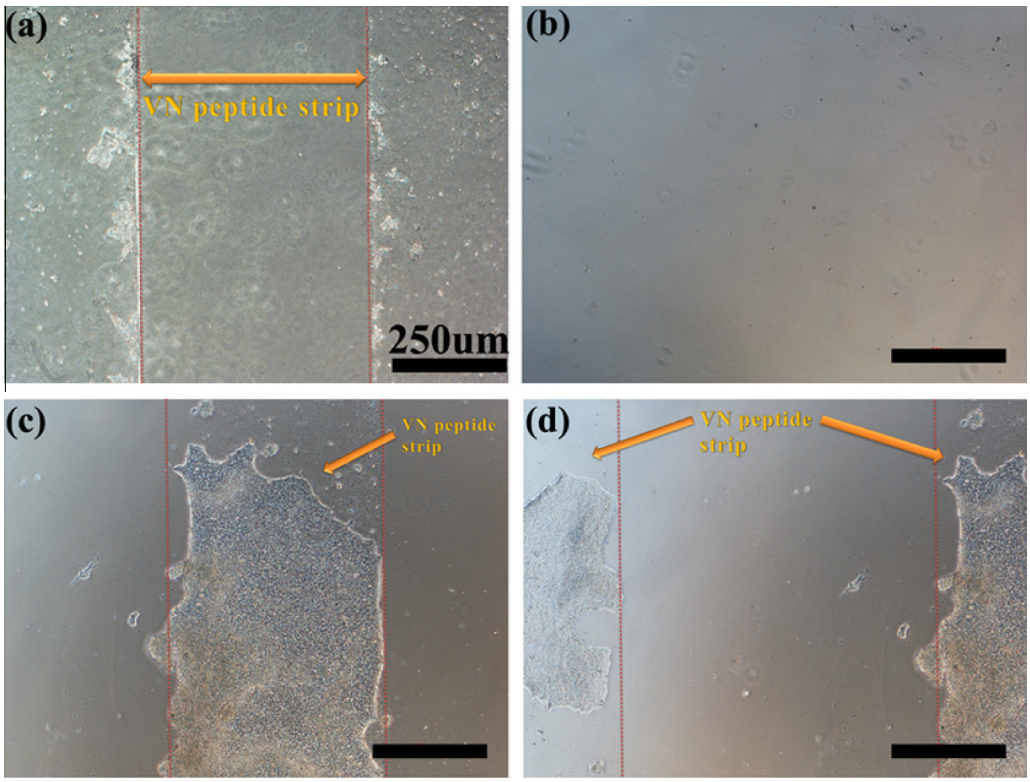


Fig. 2. (a) Microscopic images of VN peptide strips before hiPSC seeding; (b) no hiPSC on the "bare" Au-p(OEGMA-*co*-HEMA)-COOH surface after 3 days of hiPSC seeding; (c and d) there are hiPSC on the VN-patterned surface (vertical) after 3 days of hiPSC seeding.

To investigate these surface characteristics in the present system, a dilution series of VN peptide solution was used to generate different densities of VN peptide on the polymer-grated surfaces. The surface roughness of the peptide-decorated surfaces with different peptide concentrations is shown in Fig. 3a. Compared with the pristine Au (Fig. S3), the polymer-grated Au presented a slightly increased roughness, consistent with previous reports [33]. However, after decorating the peptide, the roughness changed greatly, and showed a substantial increase with the increasing peptide concentrations, which suggested that the peptide has grafted to the polymer brushes successfully. Note that the AFM images suggested that the Au surface began to be covered by peptide when the peptide concentration approached 0.75–1 mM. Excess peptide aggregations were observed on the surface at higher peptide concentrations. The poly(OEGMA-*co*-HEMA) brushes with large carboxyl groups in one polymer brush are better to immobilize more peptide and protein than other methods of conjugating peptide [28], such as aminosilane chemistry and low-temperature plasma. In order to obtain the qualitative characterization of the grafted peptide content on the polymer surface, the dilution series of FITC-labeled VN peptide solution was also applied to the surface. No green light was detected on only PBS solution (0 mM) treated polymer-grafted Au surface, and the color changed from dark to bright green on the different peptide content treated samples. It was obvious that the fluorescence intensity was enhanced with increasing peptide concentrations, which further verified the AFM results. Further, the quantitative fluorescence intensity of the surfaces was also acquired using Multilabel Reader. This demonstrated that the fluorescence intensity of the peptide-decorated substrate was concentration-dependent (Fig. 3b). The fluorescence intensity went up fast in the range of peptide concentration from 0.5 mM to 1.25 mM, suggesting that a large amount of peptide was grafted on the poly(OEGMA-*co*-HEMA) brushes.

To study the influences of the peptide concentration on the cell number and the cell morphology at the third day, the hiPSC were cultured on the surfaces treated with various ligand concentrations. As shown in Fig. 4, it is obvious that the cell numbers significantly increased with the increase in peptide concentrations. At both surfaces of "bare" plastic tissue culture (negative control) and 0 mM peptide concentration, no hiPSC adhesion was observed. At low peptide concentration (0.25–0.5 mM), although a small number of hiPSC colonies attached to the surface, they appeared as poorly spread clusters of cells surrounded by multiple areas of differentiated cells with rough colony border. Their morphologies were highly variable, and some colonies exhibited loose borders between cells, while others did not (Fig. 4a). At >0.75 mM, hiPSC almost cover the peptide-decorated surface. Qualitatively, hiPSC cultured on the surface of high peptide concentrations (0.75 mM, 1 mM and 1.25 mM) exhibited morphologies very similar to undifferentiated hiPSC on the Matrigel-coated surface, with compact colonies and a smooth colony border. In addition, uniform distribution of hiPSC colonies was obtained, implying uniformity of the peptide on the decorated Au surface. The quantitative cell numbers were measured by CCK-8. The viable cell number is directly proportional to the amount of CCK-8 assay product, formazan, which shows a linear response at 450 nm absorbance values [34]. Good correlation was observed between conjugated peptide density and hiPSC numbers after 3 days in culture on the peptide-decorated substrate in defined medium, as shown in Fig. 4b. The cell numbers were greatly enhanced with increasing VN peptide concentrations on the poly(OEGMA-*co*-HEMA) brushes from 0 mM to 0.75 mM. Subsequently, for a longer period of culture time (>1 week), it was hard for the cells to maintain adhesion and proliferation on the surfaces with lower peptide density (<0.75 mM). However, there was little difference in cell numbers on surfaces treated with higher peptide concentrations (0.75 mM, 1 mM and

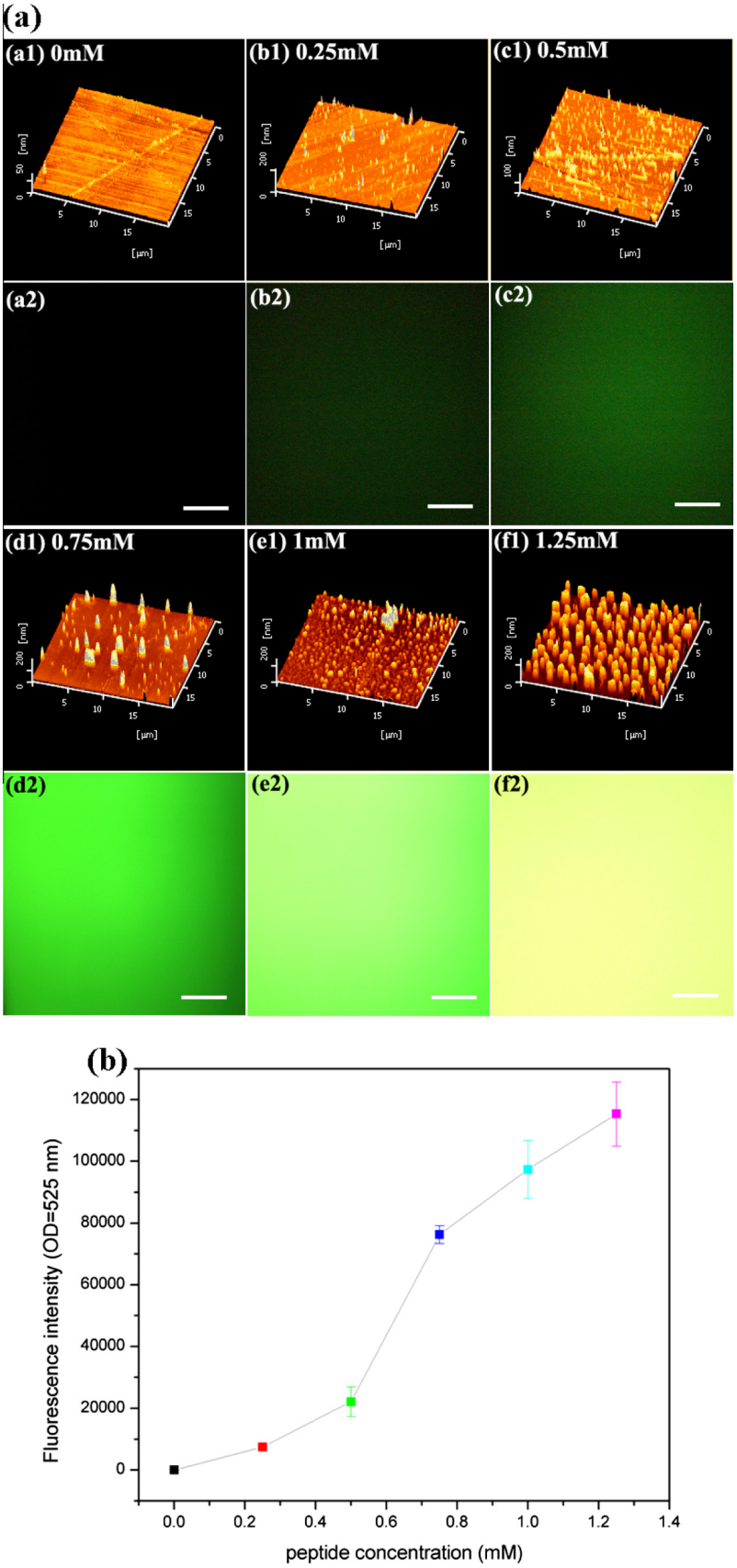


Fig. 3. (a) AFM images and corresponding qualitative fluorescence intensities of peptide-decorated substrate with various peptide ligand concentrations by inverted fluorescent microscopy. Scale bar represents 500 μm. (b) Quantitative fluorescence intensity of the peptide-decorated substrate with various peptide ligand concentrations acquired using Multilabel Reader with an absorbed wavelength of 488 nm and emitted wavelength of 525 nm. Six independent measurements were performed for each surface.

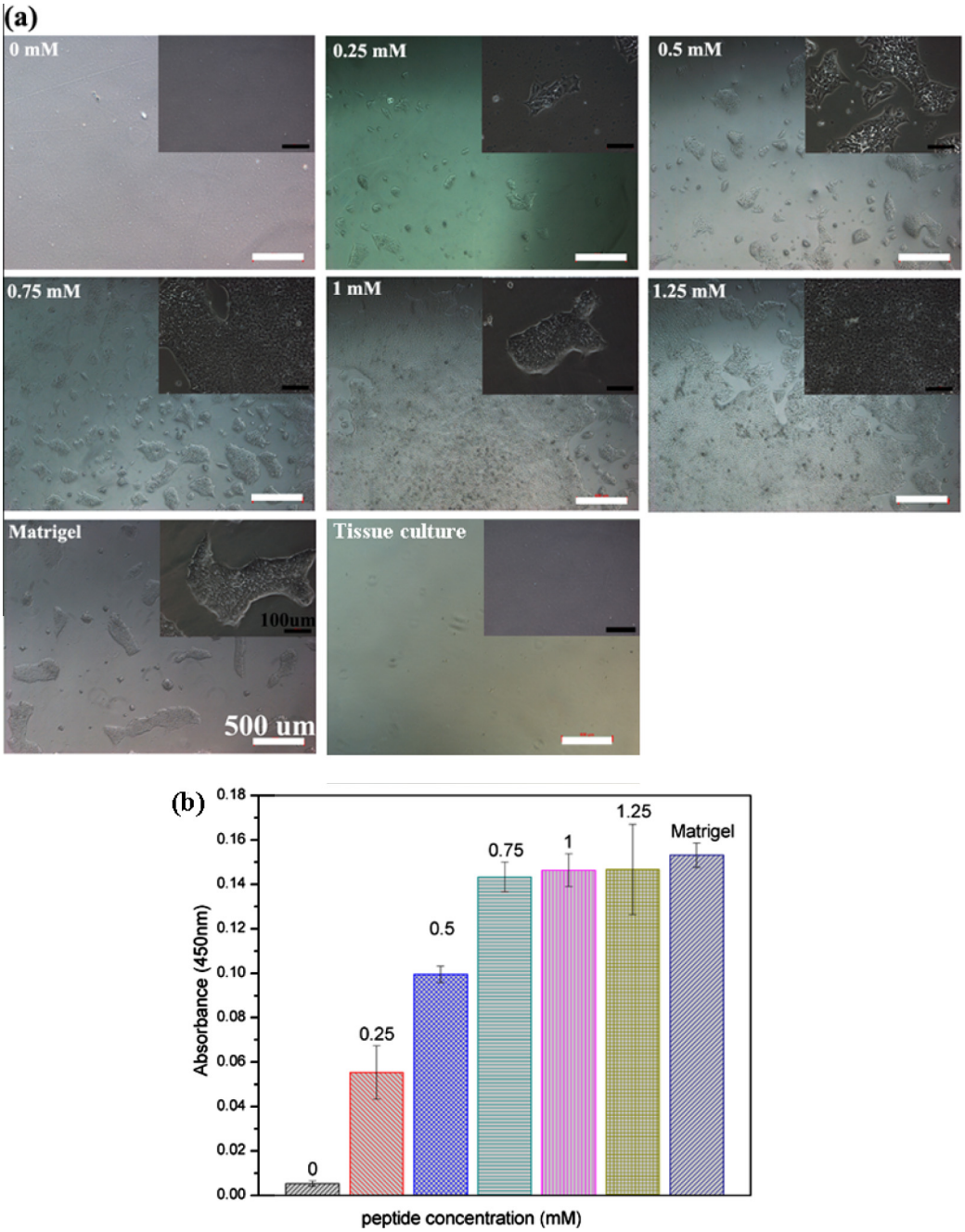


Fig. 4. (a) Morphology of hiPSC and its colonies on the peptide-decorated substrate with different peptide concentrations. The inset is the enlargement of the colony. (b) Correlation between conjugated peptide densities and hiPSC numbers after 3 days assessed by CCK-8. White scale bar represents 500 μm; black scale bar represents 100 μm. Six independent measurements were performed for each surface.

1.25 mM). These suggested that 0.75 mM peptide concentration was sufficient for the proliferation of hiPSC, and higher peptide concentration was unnecessary, which might be attributed to the peptide fully covering up the surface over the concentration of 1 mM, from AFM analysis. Other groups reported that hESC are able to adhere and self-renew in the presence of a high level of peptide concentration (>1 mM) [35]. However, the results confirm that SIP could provide large peptide conjugated sides, and a threshold surface density (0.75 mM) of the supportive peptide on the peptide-decorated surface is required to achieve hiPSC expansion.

Studies on the growth curve of hiPSC on the peptide-decorated surface at the peptide concentration of 0.75 mM and Matrigel-coated surface were conducted for 1 week via CCK-8 assay. As shown in Fig. 5, the OD value increased as time went on, indicating that the peptide-decorated substrate supported hiPSC proliferation. The viability of cells on the peptide-decorated surface displayed little statistical difference from the Matrigel group for 2 days and 3 days, showing similar proliferation in short-term days. As for 12 h, it also showed cell adherence rate (~60%) similar to that of Matrigel (Fig. 5 insert). The hiPSC attachment on the peptide-decorated substrate was ~90% of that on Matrigel. This is because Matrigel is a complex mixture of ECM proteins, growth factors and a broad variety of other unknown proteins, which could also support the adhesion of hiPSC [15]. Although only ECM protein-derived peptide existed on the peptide-decorated surface, the proliferation rate of hiPSC was faster than Matrigel at 1 day

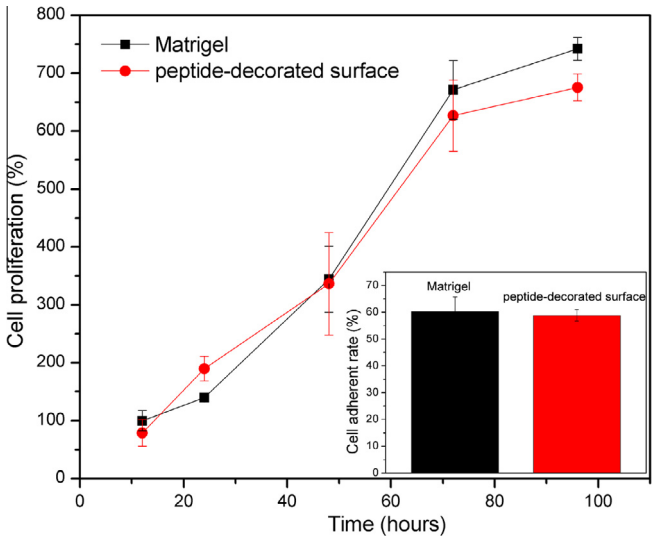


Fig. 5. Growth curves of hiPSC cultured on the peptide-decorated surface and Matrigel surface (normalized to Matrigel at 12 h). The inset is the cell adherent rate on the two surfaces after 12 h. Six independent measurements were performed for each surface.

and 2 days. In future work, the present authors will try to raise the production of hiPSC in their synthetic peptide-decorated substrate through grafting some other chemically defined cell-proliferation-promoting material on the surface.

3.4. Long-term culture of hiPSC on peptide-decorated surfaces

The primary goal of this study was to develop a synthetic substrate for sustained self-renewal of undifferentiated hiPSC in a medium that is free from non-human, animal-origin raw materials (xeno-free) and that is chemically defined. To this end, UMC-C1 hiPSC were cultured on the peptide-decorated substrate for >10 serial passages in a commercial defined medium (mTeSR™1). To assess the ability of the peptide-decorated surface to support the self-renewal of hiPSC, several characteristics were studied: colony attachment, colony morphology, cell viability and the presence of hiPSC markers. Morphological changes were one of the early indicators of differentiation. Undifferentiated hiPSC colonies that were cultured on Matrigel (positive control) are shown in Fig. 6b. Undifferentiated hiPSC exhibited a high nucleus-to-cytoplasm ratio, formed tightly packed colonies with defined colony borders, and expressed pluripotency markers such as the transcription factor Oct-4, and surface carbohydrate moieties SSEA-3 and SSEA-4 [26,36]. The newly planted colonies were able to attach robustly one day after seeding and showed significant spreading on the peptide-decorated substrate. The cells continuously maintained typical undifferentiated morphology on the peptide-decorated substrate, as characterized by compact colonies with defined edges and large nuclei-to-cytoplasm ratios, as shown in Fig. 6a. The hiPSC colonies were able to grow continuously into larger sizes and became ready for passaging in 3–4 days. Spontaneous differentiation on the edges or in the center of the colonies was rarely observed in hiPSC on the peptide-decorated substrate. It was obvious that, throughout p1, p5 and p10, hiPSC cultured on the peptide-decorated substrate maintained typical stem cell morphology and grew in colonies similar to that of the Matrigel control.

Maintenance of pluripotency is a critical parameter when evaluating new surfaces for hiPSC culture. Immunofluorescence staining was conducted to assess whether cells retained markers of undifferentiated hiPSC. The POU family transcription factor Oct-4 is a highly specific and necessary marker for undifferentiated hiPSC, and SSEA-4 is a glycolipid cell surface antigen strongly expressed in undifferentiated hiPSC [37]. Results showed the strong positive staining of Nanog, Oct-4, SSEA-3 and SSEA-4 in cultures of the peptide-decorated surface throughout p1, p5 and p10 (Fig. 7), indicating that hiPSC could maintain their undifferentiated characteristics very well on the peptide-decorated substrate for long-term passages. To quantify the pluripotency expression of the cells, three pluripotent markers (Nanog, Oct-4 and Sox-2) were selected for use in real-time PCR analysis (RT-PCR). The results showed the hiPSC cultured on the peptide-decorated substrate expressed similar levels of the three pluripotent markers, and ~98.2 ± 2.4%, 91.7 ± 16.1%, and 96.2 ± 4.3% of hiPSC coincidently expressed pluripotency markers after maintenance for 10 passages on the peptide-decorated substrate compared with the Matrigel

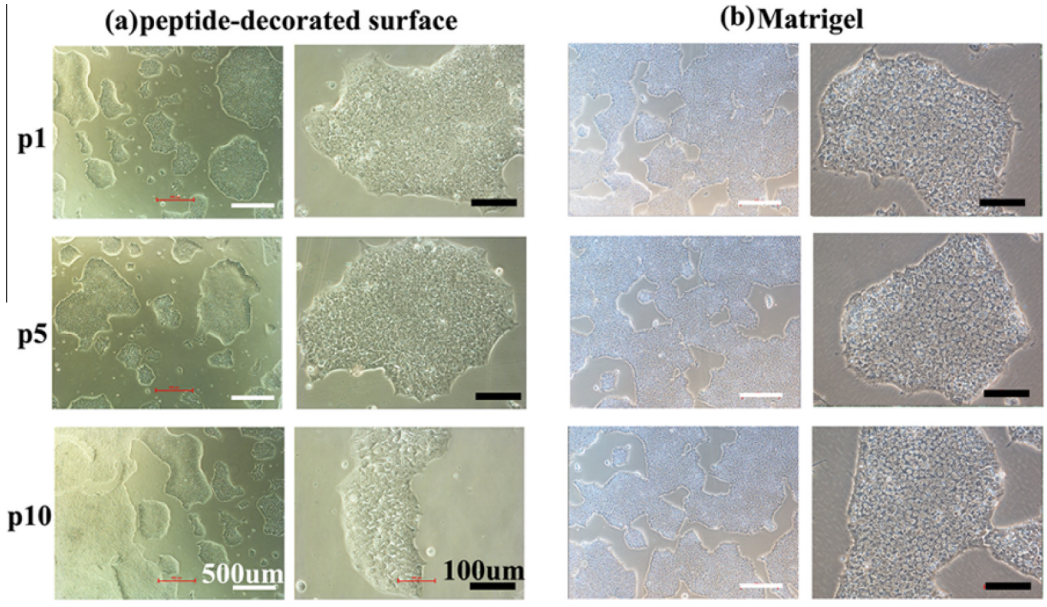


Fig. 6. The microscopic images of morphology of hiPSC colonies (4× and 20×) continuously cultured at passage 1, passage 5 and passage 10: (a) peptide-decorated substrate; (b) Matrigel. White scale bar represents 500 μm; black scale bar represents 100 μm.

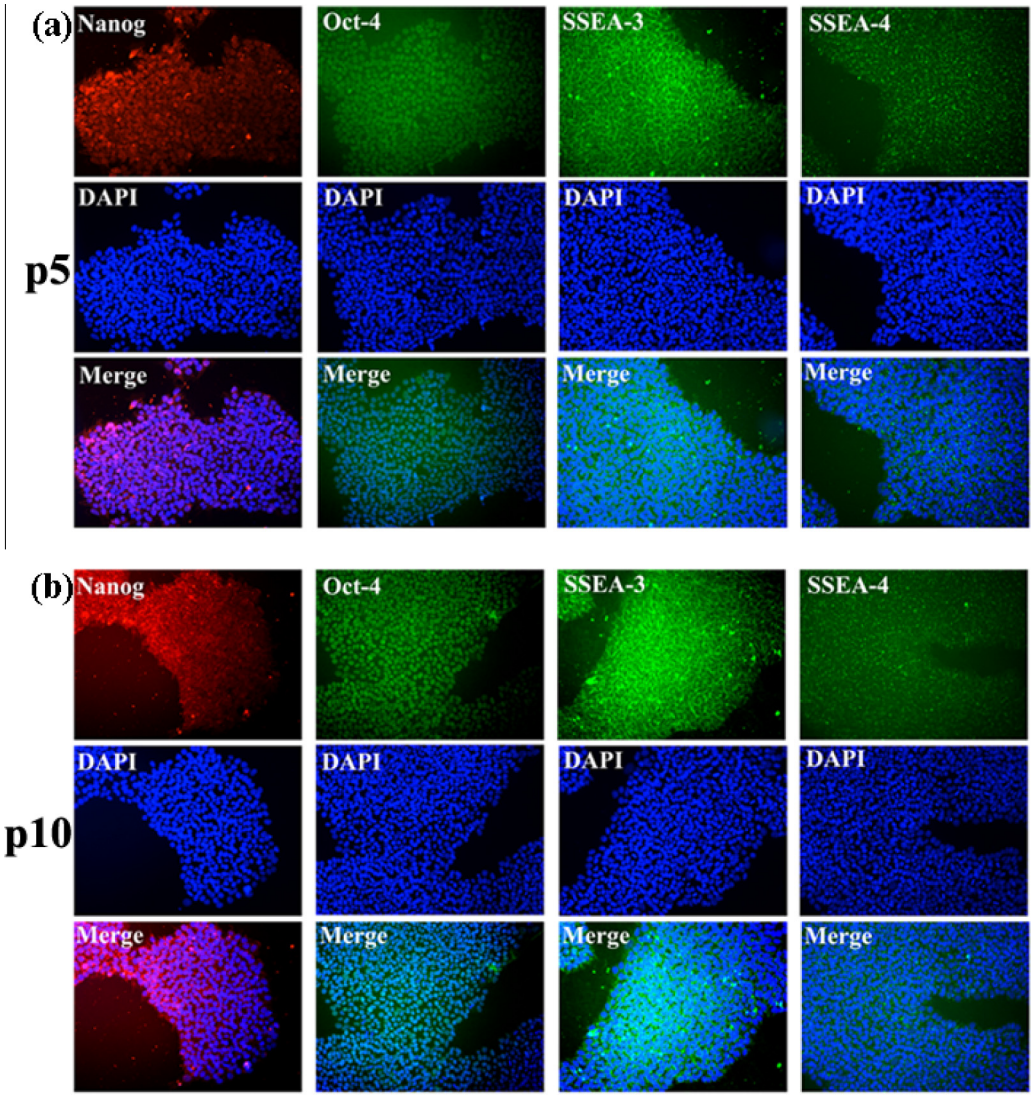


Fig. 7. Fluorescence micrographs of colonies of hiPSC continuously cultured on the peptide-decorated substrate for (a) passage 5 and (b) passage 10 in mTeSR™1 media visualized by laser confocal microscopy, showing expression of hiPSC markers: Nanog, Oct-4, SSEA-3 and SSEA-4.

surface (Fig. 8). It verified that the hiPSC robustly maintained their pluripotency after culture on the peptide-decorated substrates. Overall, the results demonstrate that a short peptide sequence of VN protein conjugated on the poly(OEGMA-*co*-HEMA) brushes is sufficient to support long-term self-renewal of hiPSC under a fully defined medium. The fully synthetic surface greatly reduces the risk of cellular contamination with animal-derived pathogens and provides a scalable, robust platform for the culture of hiPSC, as well as providing primary insights into exploring the hiPSC responses or hiPSC–matrix interactions through SIP technology. A comprehensive exploration of the hiPSC–matrix interactions (such as migration and differentiation) is currently under way using the peptide-decorated surface developed.

4. Conclusion

In summary, a completely synthetic, xeno-free, chemically defined peptide-decorated poly(OEGMA-*co*-HEMA) substrate was developed, which allows for long-term hiPSC growth and self-renewal under a fully defined medium. A threshold concentration at 0.75 mM of the VN peptide on the surface was found to achieve hiPSC expansion. The peptide-tethered surface could support hiPSC adhesion, proliferation and self-renewal, maintaining characteristic morphology, adherence rate, proliferation, and expressed high levels of markers of pluripotency, similar to Matrigel. Given that in vivo maintenance of pluripotency is paramount to myriad applications using hiPSC, the next step is to evaluate the trilaminar differentiation potential of hiPSC generated from the peptide-decorated substrate in vivo. This system has the advantage of eliminating risks of microbial and viral contamination picked up from mouse feeder cells and animal or human-derived proteins. The present authors believe that the synthetic defined environment incorporation lithography for culturing hiPSC may be helpful in investigating the molecular mechanisms of self-renewal and in controlling differentiation into specific cell types for applications in regenerative medicine.

Acknowledgments

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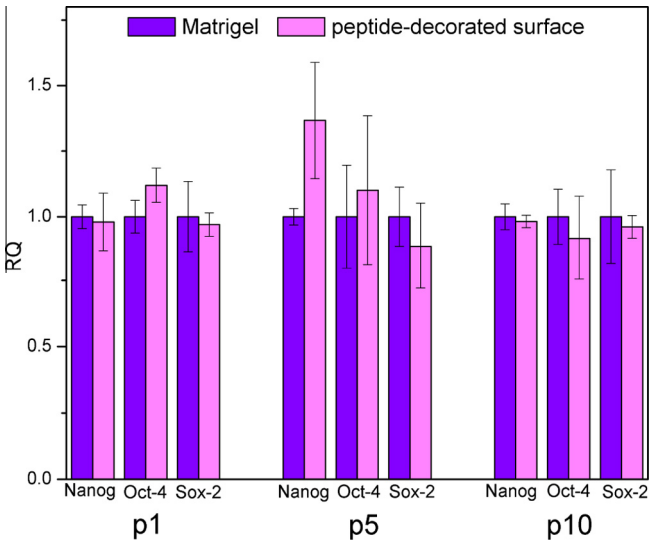

Fig. 8. Relative three transcript levels of Nanog, Oct-4 and Sox-2 from hiPSC continuously cultured on peptide-decorated and Matrigel surface at passage 1, passage 5 and passage 10 by RT-PCR. Items were run in triplicate. Results indicate that hiPSC maintained their undifferentiated characteristics very well on the peptide-decorated substrate for long-term passages.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.07.017.

Appendix B. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–8, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: http://dx.doi.org/10.1016/j.actbio.2013.07.017

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