

PROTEIN ADSORPTION AND CELL ADHESION ON RGD-FUNCTIONALIZED SILICON SUBSTRATE SURFACES*

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Abstract A method was developed to modify silicon surfaces with good protein resistance and specific cell attachment. A silicon surface was initially deposited using a block copolymer of *N*-vinylpyrrolidone (NVP) and 2-hydroxyethyl methacrylate (HEMA) (PVP-*b*-PHEMA) film through surface-initiated atom transfer radical polymerization and then further immobilized using a short arginine-glycine-aspartate (RGD) peptide. Our results demonstrate that the RGD-modified surfaces (Si-RGD) can suppress non-specific adsorption of proteins and induce the adhesion of L929 cells. The Si-RGD surface exhibited higher cell proliferation rates than the unmodified silicon surface. This research established a simple method for the fabrication of dual-functional silicon surface that combines antifouling and cell attachment promotion.

Keywords: Cell adhesion; Protein adsorption; RGD; Poly(*N*-vinylpyrrolidone); ATRP.

INTRODUCTION

In recent years, silicon substrates have provided a versatile platform for applications in the development of bioelectrical sensors and devices^[1, 2], biomicrofluidics^[3], cell-based disease diagnosis probes^[4], DNA transformation nanocatalyst^[5], nanowire-based biosensors^[6], protein biochips^[7] and silicon-neuron interfaces^[8]. However, silicon surfaces exposed to air or water are prone to developing a native oxide layer with surface silanol groups. These silanol groups are ionizable in water. Consequently, they can create a streaming potential in the fluid flow, promote biofouling and thereby limit the long-term functioning of these microdevices^[9].

Many efforts have focused on silicon surface modification to enhance the performance of a given material in a certain application. Such goals could be partially reached by immobilizing biological molecules, such as antibodies, enzymes, plasma proteins, extracellular matrix proteins, growth factors and peptide fragments on the surface^[10–12], which render the material biologically functional. In addition, promoting the specific binding of biological targets can prevent biofouling, which is a major obstacle in many potential biological applications^[13]. The problem associated with biofouling can be solved by functionalizing the surfaces with poly(ethylene glycol) (PEG), oligo(ethylene glycol), or PEG alternatives to impart adhesion resistance^[14].

Ideal platforms on silicon substrates potentially allow the specific binding of biological targets. This requires novel surface functionalization strategies. Surface-initiated atom transfer radical polymerization (SI-ATRP) has been proven to be an effective method for the production of biomedically and biotechnologically

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interesting polymer brushes^[11]. The modified silicon surfaces based on thermosensitive poly(*N*-isopropylacrylamide)^[15–17] and pH-responsive poly(methacrylic acid) polymer brushes^[18] have been prepared through SI-ATRP to regulate cell adhesion and protein adsorption. We developed poly(*N*-vinylpyrrolidone) (PVP)-modified surfaces that are highly protein resistant through SI-ATRP to block non-specific binding^[19–21]. Selective attachment of bioactive molecules on a biologically inert polymer brush is beneficial in performing biospecific interactions^[22].

Arginine-glycine-aspartate (RGD) is a conserved motif found in several matrix proteins such as fibronectin, fibrinogen, vitronectin and many other glycoproteins that exhibit high binding affinity to integrin receptors present on the cell surface^[23]. RGD-containing molecules are widely used as cell adhesion promoters in biomaterials sciences^[24]. In the current study, we demonstrated the use of RGD-functionalized PVP-*b*-PHEMA brushes prepared *via* consecutive SI-ATRP to establish silicon surfaces with good protein resistance and specific cell attachment. By this design, the PVP segment is an ideal inert polymer to resist nonspecific protein adsorption, and the poly(2-hydroxyethyl methacrylate) (PHEMA) segment is a good and suitable candidate to perform functions by immobilization of RGD peptide, which is known to strongly accelerate cell adhesion. The antifouling, cell adhesion and proliferation properties of the RGD-functionalized surfaces were evaluated.

EXPERIMENTAL

Materials

NVP (Acros, 98%) and HEMA (Aldrich, 99%) were purified before use through distillation under reduced pressure to remove any present inhibitors. CuCl (Aldrich, 98%) was purified by stirring in acetic acid, washed with methanol and then dried in vacuum. 3-Aminopropyl triethoxysilane (APTES) and 4-nitrophenyl chloroformate (NPC) were purchased from Aldrich and used as received. The cyclic ligand 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradecane (Me₆TATD) was prepared according to a previously described method^[25]. RGD was purchased from GL Biochem. Co., Ltd (Shanghai, China). Silicon wafers (0.56 mm thick, Guangzhou, China) were cut into square chips of approximately 0.5 cm × 0.5 cm using a diamond-tipped glass cutter. 2,2'-Bipyridine (Bipy), ascorbic acid, triethylamine (TEA) and all other solvents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and purified according to standard methods. Na¹²⁵I was purchased from Chengdu Gaotong Isotope Corporation (China Nuclear Group). The China Center of Type Culture Collection supplied the L929 and L02 cells.

Immobilization of RGD

*Grafting of PVP-*b*-PHEMA block copolymers on silicon surface (Si-PVP-HEMA)*

The pretreatment and synthesis of PVP-grafted silicon surfaces (Si-PVP) were performed as previously reported^[19]. PHEMA blocks were prepared from the PVP-grafted silicon surfaces by separately adding HEMA (2.6 g, 20.0 mmol), CuCl₂ (13.4 mg, 0.1 mmol), Bipy (31.2 mg, 0.2 mmol), ascorbic acid (17.6 mg, 0.1 mmol) and 5.0 mL of the mixture of methanol (50 vol%) and water (50 vol%) in a reaction tube. The tube was sonicated for approximately 2 min and then degassed by three freeze-pump-thaw cycles. The PVP-grafted silicon surfaces and the tube were transferred to a nitrogen-purged glove box (Vigor Gas Purification Technologies). The reaction solution in the tube was added to a glass vessel in which the PVP-grafted silicon surfaces were placed. Polymerization was carried out at 25°C for 3 h. The silicon surfaces grafted with PVP-*b*-PHEMA diblock copolymers were removed from the solution, rinsed thoroughly with distilled water and methanol and dried under nitrogen flow.

*NPC activation of PVP-*b*-PHEMA grafted silicon surfaces (Si-NPC)*

PVP-*b*-PHEMA grafted silicon surfaces were activated by exposing to a mixture of NPC (0.51 g, 2.5 mmol) and TEA (0.25 g, 2.5 mmol) in anhydrous acetonitrile for 12 h at room temperature with stirring. Excess NPC was removed by thoroughly rinsing with acetonitrile and acetone. Finally, the obtained NPC-activated silicon surfaces were dried under nitrogen flow.

Conjugation of RGD to NPC-activated silicon surfaces (Si-RGD)

NPC-activated silicon surfaces were treated with a phosphate buffered saline (PBS, pH 7.4) solution containing 0.1 mg/mL RGD for 12 h at room temperature. The obtained surfaces were removed from the reaction solution, rinsed thoroughly with PBS buffer, and dried in nitrogen stream.

The RGD graft densities were assayed by measuring the liberation of *p*-nitrophenol (equivalent to the RGD on the surface) from the NPC-activated silicon surface substrate. A molar extinction coefficient of $\epsilon = 17700 \text{ L}/(\text{mol}\cdot\text{cm})$ at 402 nm was used for *p*-nitrophenol^[26].

Surface Characterization

The chemical composition of the silicon surfaces was determined through X-ray photoelectron spectroscopy (XPS, ESCALAB MK II X-ray photoelectron spectrometer, VG Scientific). Static water contact angles were measured on an SL200C goniometer (USA KINO Industry Co.) using the sessile drop method at room temperature.

Protein Adsorption

Fibrinogen and human serum albumin (HSA) were radiolabeled as previously described^[21]. The labeled proteins were mixed with unlabeled proteins (1:9, labeled:unlabeled) at a total concentration of 1 mg/mL. The unmodified and modified silicon surfaces were equilibrated in PBS (pH 7.4) for 12 h prior to fibrinogen exposure and then incubated for 3 h in the radiolabeled protein solutions at room temperature. After incubation, the surfaces were rinsed thrice with PBS for 10 min each, wicked onto filter paper, and transferred to clean tubes for radioactivity determination (Wizard 3" 1480 Automatic Gamma Counter, Perkin-Elmer Life Sciences). The quantities of the adsorbed protein are expressed as mass per unit area ($\mu\text{g}/\text{cm}^2$).

Assessment of Cell Adhesion and Morphology

L929 cells were cultured in RPMI medium 1640 (Gibco) containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin at 37°C in 5% CO₂ atmosphere. The cells were seeded on silicon substrates in a cell culture plate at a density of $1.0 \times 10^4 \text{ cells}/\text{cm}^2$. After 24 h incubation, the cultured cells were fixed in PBS containing 2.5% glutaraldehyde at room temperature for 20 min and washed twice with PBS buffer. The L929 cells were treated with 4',6-diamidino-2-phenylindole solution at room temperature for 10 min and then washed thrice with PBS buffer for 15 min each. A fluorescence microscope (Olympus, IX71, Germany) was used to record the fluorescence images of the stained cells.

Assessment of Cell Proliferation

Evaluation of cell proliferation on silicon substrates on days 1, 2, and 3 was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method described by Mosmann^[27]. MTT solution (5 mg/mL) was prepared by dissolving 50 mg MTT into 10 mL PBS (pH 7.4). L02 cells were seeded on the sterilized substrates at $1.0 \times 10^4 \text{ cells}/\text{cm}^2$. Absorbance was determined at the end of each time point using the MTT assay. Briefly, 20 μL of the MTT reagent was added to 400 μL of culture medium in each well after incubating for 4 h at 37°C. The MTT solution was then carefully removed from each well, and 120 μL of dimethyl sulfoxide was added to dissolve MTT formazan crystals. Absorbance was measured at 570 nm using a spectrophotometer (Varioskan Flash unit, Thermo Scientific, USA). Six samples were measured for each group at different time points.

RESULTS AND DISCUSSION

Biofunction of Silicon Surfaces

The fabrication of the RGD-functionalized brushes is outlined in Fig. 1. The process starts with the modification of a silicon substrate with ATRP initiator containing an α -chloroacetyl group. The functionalization of the surface with the α -chloroacetyl initiator can be monitored through XPS, as evidently observed by the appearance of the Cl2p signal at 200 eV (Fig. 2). The initiator-functionalized surfaces were used to initiate the ATRP of NVP monomer. The grafting process was stopped after 30 min to ensure that the graft chain ends remained

active. The PVP-grafted silicon surfaces exhibited more intense signals of carbon and nitrogen compared with the initiator-functionalized surface. This result can be attributed to the PVP brush.

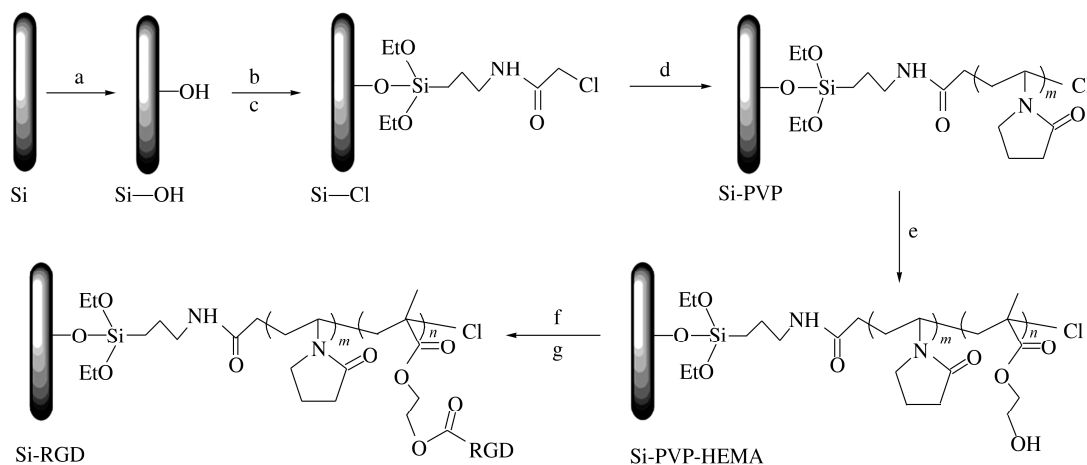


Fig. 1 Preparation of Si-RGD: (a) “Piranha” solution, (b) 2% (V/V) APTES, (c) TEA, chloroacetyl chloride, (d) [NVP]/[CuCl]/[Me₆TATD] (100:1:1), (e) [HEMA]/[CuCl₂]/[Bipy]/[ascosic acid] (200:1:2:1), (f) NPC and (g) RGD

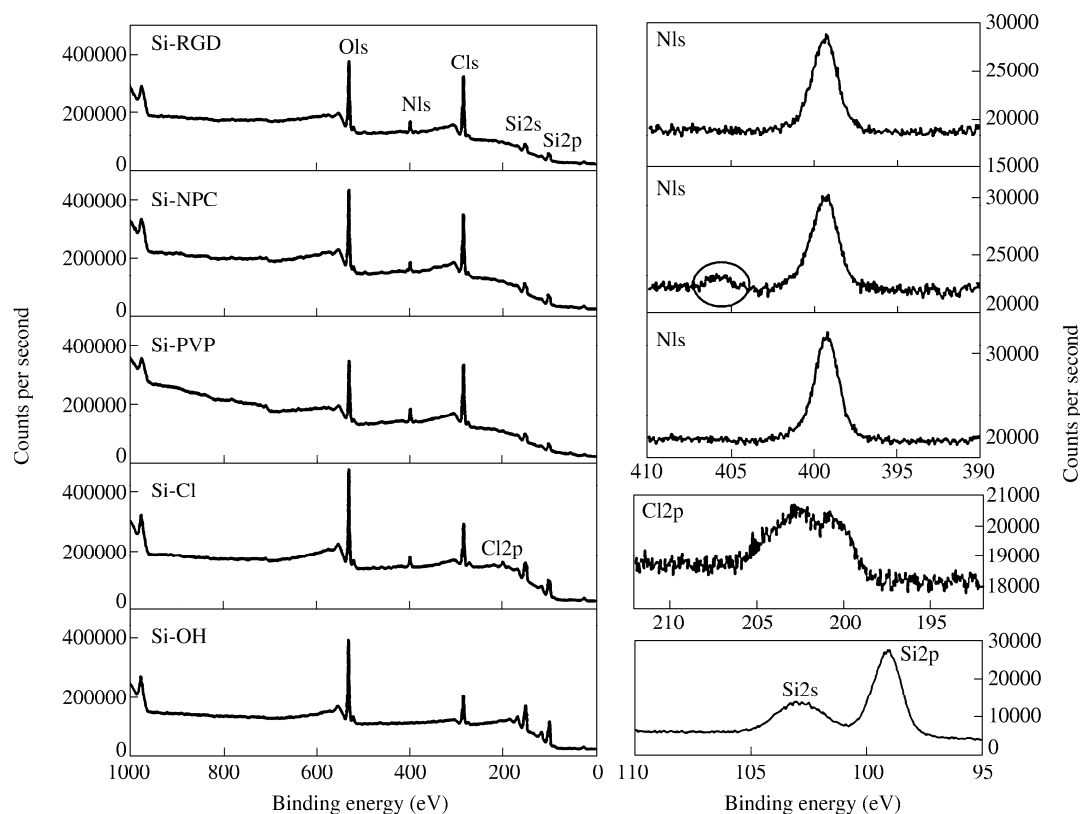


Fig. 2 XPS survey spectra (left) and selected elemental scans (right) of Si-RGD, Si-NPC, Si-PVP, Si-Cl and Si-OH (top to bottom)

The functionalization of the PVP-*b*-PHEMA grafted silicon surfaces started with the activation of the hydroxyl groups with NPC in anhydrous acetonitrile in the presence of TEA^[28]. The introduction of the

p-nitrophenyl groups resulted in the appearance of an additional N1s signal at 406 eV in the XPS spectrum (Fig. 2), which provides support for the introduction of the activating groups^[29]. The NPC-activated surfaces were then treated with RGD for 12 h at room temperature to produce Si-RGD surfaces. The N1s signal disappeared at 406 eV in the XPS spectrum, which can be attributed to the nitro group of the NPC moiety. This result suggests that the immobilization process was successfully completed. The surface RGD density is an important property that determines cell adhesion and proliferation^[24]. The surface RGD density in the current work was determined to be 0.6 nmol/cm² by directly measuring the liberation of *p*-nitrophenol from the NPC-activated surfaces upon RGD treatment. The present method may give a higher RGD density because the liberated *p*-nitrophenol includes two parts: the conjugation of RGD and the hydrolysis of NPC.

The measurement of advancing water contact angles is a simple, quick and effective way to examine the hydrophilic/hydrophobic properties of the prepared surfaces. Figure 3 summarizes the water contact angle results obtained for the silicon surfaces. The contact angle of the PVP-grafted silicon surface was 24°, which is approximately equal to the reported values obtained for silicon wafers^[19]. In this case, the hydrophilic PVP dominates the surface property. After RGD was immobilized onto the PVP-*b*-PHEMA layer, the contact angle increased to 47°. This result was probably caused by the presence of ionic peptide molecules on the surface^[30].

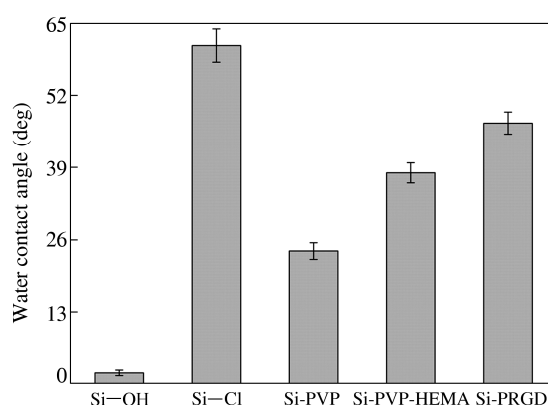


Fig. 3 Sessile drop water contact angles (advancing) of unmodified and modified glass surfaces. Water contact angle was measured at room temperature. Data are mean $\pm$ standard error ($n = 3$).

Assessment of Protein Adsorption

Fibrinogen and HSA were chosen as model proteins in these experiments to study the protein adsorption of unmodified and modified silicon surfaces. Fibrinogen is a prototypical, large protein (340 kDa, 45 nm $\times$ 9 nm $\times$ 9 nm) with a strong tendency to adsorb^[31]. HSA is the most abundant protein in human blood plasma. Figure 4 shows the amount of fibrinogen and HSA adsorption on the unmodified and modified silicon surfaces. The results clearly indicate that the unspecific adsorption of fibrinogen and HSA is effectively suppressed on the Si-RGD surfaces. This result is in good agreement with our previous studies and the general notion that PVP-based materials can prevent non-specific adsorption of proteins^[19, 32].

Cell Adhesion

Cell adhesion experiments were carried out to evaluate the ability of the RGD functionalized surfaces to induce the specific adhesion of cells. The Si-RGD surfaces were compared with two reference samples to exclude cell adhesion through non-specific interactions: (i) unmodified silicon surface and (ii) PVP-modified silicon surface. One day after seeding (Fig. 5), very few cells adhered to the Si-PVP surface (*ca.* $0.33 \times 10^4/\text{cm}^2$) compared with the significantly adhered cells on the unmodified silicon surface (*ca.* $3.11 \times 10^4/\text{cm}^2$). This result is in good agreement with our previous works^[20]. Cell adhesion is highly dependent on the adsorption of adhesive proteins^[33]. Thus, the low cell adhesion observed on the Si-PVP surface is likely related to its resistance to protein adsorption in the cell culture medium. In contrast to the Si-PVP antifouling surface, the cells adhered to

and spread well on the Si-RGD surface ($ca. 3.08 \times 10^4/cm^2$). This result can be explained by the fact that cell adhesion generally occurs through the interactions of cell surface receptors with characteristic peptide sequences (ligands) such as RGD^[24]. These results demonstrate that cell adhesion on the RGD-functionalized silicon surface is mediated by specific interactions and does not involve non-specific interactions.

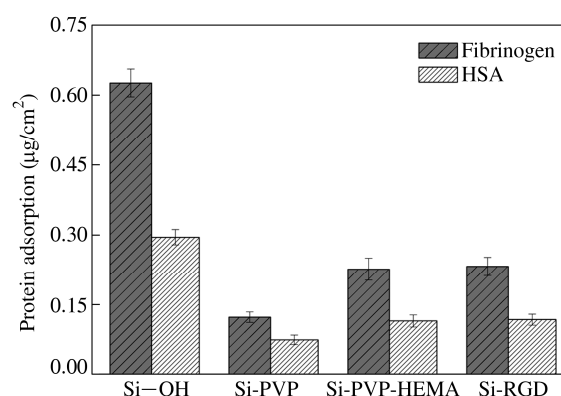


Fig. 4 Fibrinogen and HSA adsorption on silicon surfaces
Data are mean $\pm$ standard error ($n = 3$).

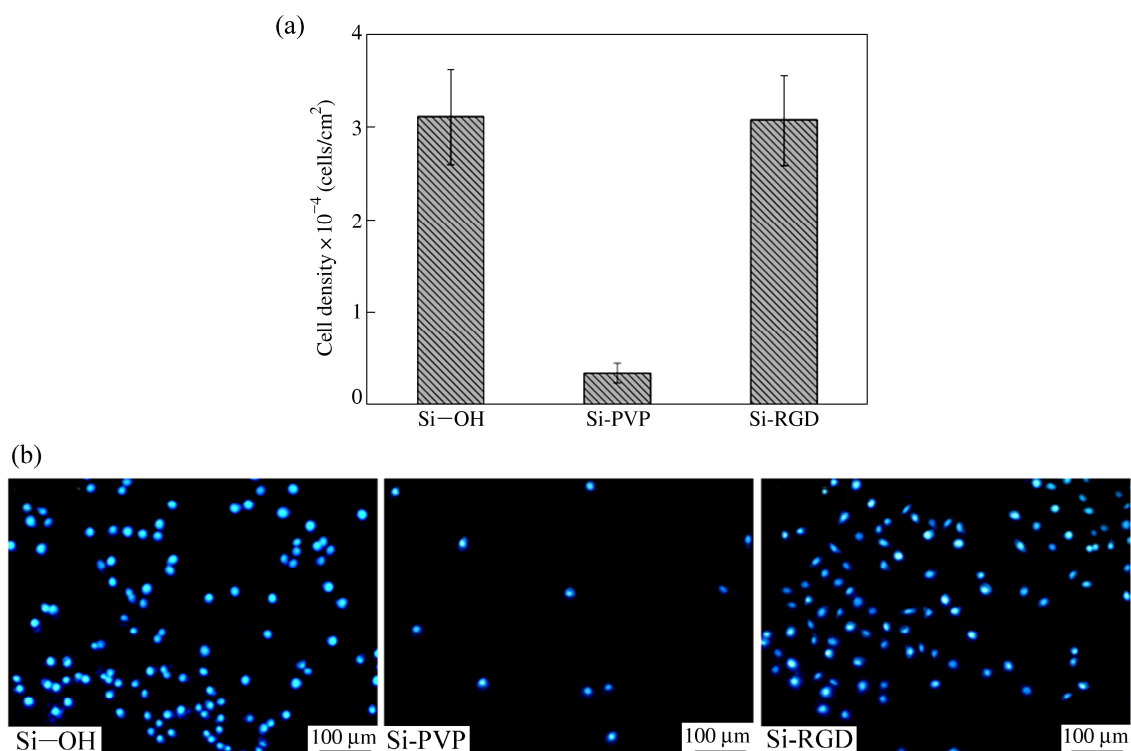


Fig. 5 (a) Cell density on Si-OH, Si-PVP, and Si-RGD surfaces (Data are mean $\pm$ standard error ($n = 3$)); (b) Fluorescence microscopy images of L929 cells (the scale bar is 100 µm) on Si-OH, Si-PVP and Si-RGD surfaces, respectively

Cell Proliferation

The proliferation of L02 cells on unmodified and RGD-functionalized silicon surfaces was performed using the MTT assay (Fig. 6) to investigate further the long-term cell performance. One day after seeding, the Si-RGD surface supported lower levels of cell adhesion compared with the unmodified silicon surface. This result can be attributed to the resulting compromise of the cell-resistant form of the PVP-based substrate and the tethering of RGD for cell adhesion promotion. The proliferation rates of the cells increased significantly on the Si-RGD surface with the passage of culture time. However, the cells showed a low proliferation rate on the Si—OH surface. This result may be attributed to the high binding affinity of the RGD peptide to integrin receptors on the cell surface^[23]. The above results clearly indicate that the dual-functional silicon surface with antifouling and cell adhesion properties may be achieved conveniently using the RGD-functionalized method.

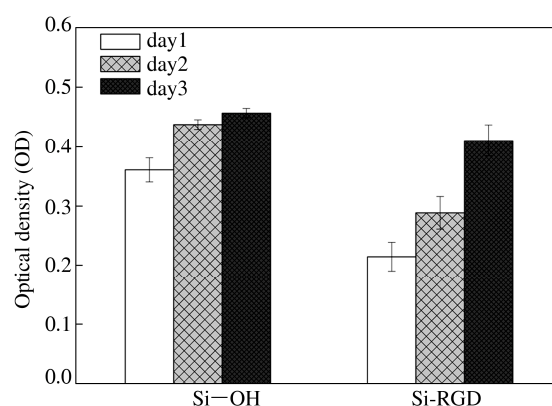


Fig. 6 Proliferation of L02 cells on Si—OH and Si-RGD surfaces
The number of cells per well was measured using the MTT assay and expressed as optical density (OD).

CONCLUSIONS

We developed a protocol using polymer brushes prepared through SI-ATRP as platforms for the covalent immobilization of peptides. The RGD-functionalized silicon surfaces are effective in resisting non-specific protein adsorption and promoting cell adhesion and proliferation. Thus, the simple method presented in this study offers a promising strategy for the fabrication of Si-based biomedical devices.

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