Preferential adsorption of cell adhesive proteins from complex media on self-assembled monolayers and its effect on subsequent cell adhesion

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Abstract

We examined the effect of surface chemistry on adsorption of fibronectin (Fn) and vitronectin (Vn) and subsequent cell adhesion, employing self-assembled monolayers (SAMs) of alkanethiols carrying terminal methyl (CH_3), hydroxyl groups (OH), carboxylic acid (COOH), and amine (NH_2). More Fn and Vn adsorbed to COOH- and NH_2-SAMs than to CH_3- and OH-SAMs from a mixture with bovine serum albumin (BSA) and from 2% fetal bovine serum. Adhesion of human umbilical vein endothelial cells (HUVECs) on CH_3- and OH-SAMs preadsorbed with Fn and BSA decreased with decreasing adsorbed Fn; however, HUVECs adhered to COOH- and NH_2-SAMs even in the presence of BSA at 1000-fold more than Fn in a mixture because of the preferential adsorption of Fn and/or displacement of preadsorbed BSA with Fn and Vn in a serum-containing medium. SAMs coated with a mixture of Vn and BSA exhibited adhesion of HUVECs regardless of surface functional groups. A well-organized focal adhesion complex and actin stress fibers were observed only for COOH- and NH_2-SAMs when SAMs were preadsorbed with Vn and BSA. These results suggest that COOH- and NH_2-SAMs allow for both cell adhesion and cell spreading because of the high density of cell-binding domains derived from adsorbed Vn.

Key Words: Self-assembled monolayers; Cell adhesion; Preferential protein adsorption; Fibronectin; Vitronectin

1. Introduction

Much effort has been devoted to understanding cell interactions with artificial materials to facilitate the development of medical devices and scaffolds for tissue engineering. However in most cases, cell-material interactions are not clearly understood, and many studies are required to thoroughly characterize many factors relevant to the cell-material interactions for a particular system. These factors include material surface energy, surface electrostatic properties, macro- and micro-surface morphology, surface heterogeneity, functional groups, and the mobility of functional groups on surfaces. Systematic studies of biological responses to artificial materials require surfaces with well-controlled properties. Self-assembled monolayers (SAMs) of alkanethiols, $HS(CH_2)_nX$, where X denotes various functional groups [1,2], are suitable for studying correlations between biological responses and surface properties. It was reported that cell adhesion behavior is highly dependent on the outermost functional groups of SAMs [3-10].

The process of cell adhesion onto a material's surface includes the interaction between integrins in the cell membrane and adhesive proteins, such as fibronectin (Fn) and vitronectin (Vn), adsorbed on the surface. Role of Fn and Vn in cell adhesion on material surfaces has been extensively studied using polymer substrates [11-16] and SAMs [17-22]. Pretreatment of artificial materials with a solution containing either Fn only or Vn only improves cell adhesion to the surfaces. Realistically, however, cell adhesion onto surfaces occurs in tissue fluids, plasma, or culture media supplemented with serum, all of which contain various kinds of proteins. The adsorption behaviors of Fn-only and Vn-only solutions differ from that of complex media, because concentrations of cell adhesive proteins in serum (Fn: 30 μg/mL [23]; Vn: 200 μg/mL [24]) are several orders of magnitude lower than some other proteins, such as bovine serum albumin (BSA, 35–55 mg/mL) and immunoglobulin G (IgG, 0.8–1.8 mg/mL), which lack the capacity to interact

with cells. This would imply that a protein-adsorbed layer mainly consists of serum proteins that do not mediate cell adhesion.

We aimed to understand how minute amounts of Fn and Vn in complex media can mediate cell adhesion to SAMs presenting different surface properties. In this study, we examined adsorption of Fn and Vn to SAMs in the case of mixed solutions with BSA or fetal bovine serum (FBS), as well as displacement of initially adsorbed proteins by Fn and Vn. We also tested adhesion of human umbilical vein endothelial cells (HUVECs) and formation of focal adhesion complexes on these SAMs to elucidate the relationship between adsorption of Fn and Vn and cellular behavior.

2. Materials and methods

2.1 Materials

1-Dodecanethiol (CH_3: Wako Pure Chemical Industries, Ltd., Osaka, Japan), 11-mercapto-1-undecanol (OH: Sigma-Aldrich, St. Louis, MO, USA), 11-mercaptoundecanoic acid (COOH: Sigma-Aldrich), and 11-amino-1-undecanethiol hydrochloride (NH_2: Dojindo Laboratories, Kumamoto, Japan) were used as received. Dulbecco's (D) phosphate-buffered saline (PBS) (DPBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Human plasma Fn (F2006), bovine plasma Vn (V9881), and BSA (A4503) were purchased from Sigma-Aldrich and used as received. Antibodies against integrin α_5 (AB1928, Millipore, Billerica, MA, USA) and integrin α_v (ab16821, Abcam, Cambridge, UK) were used as received. Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 488 anti-mouse IgG, and Alexa Fluor 594 phalloidin were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.2. Preparation of SAMs

Cover glasses (diameter; 15 mm, Matsunami Glass Ind., Ltd., Osaka, Japan) were cleaned by oxygen plasma treatment using a plasma reactor (PA300AT, O-kuma Engineering Co. Ltd., Fukuoka, Japan) for 1 min and rinsed with highly purified water (18.2 MΩ) and with ethanol three times. Cleaned cover glasses were coated with a chromium underlayer of 1 nm and then a gold layer of 19 nm in thickness by a thermal evaporation apparatus (V-KS200, Osaka Vacuum Instruments, Osaka, Japan). The gold-coated cover glasses were immediately immersed in a 1 mM solution of alkanethiols overnight to form SAMs. The cover glasses were then rinsed with ethanol and water twice and finally with ethanol, and then dried under a stream of nitrogen gas.

2.3. Surface characterization

Elemental compositions of the SAM surfaces were determined by X-ray photoelectron spectroscopy (XPS) using an ESCA 850V (Shimadzu Co., Kyoto, Japan) equipped with a Mg Kα source. The take-off angle was 90°, and the operating pressure was lower than 1×10^{-5} Pa. All spectra were shown referring to Au ($4f_{7/2}$) at 83.8 eV.

Static water contact angles were determined by the sessile drop method using a contact angle meter (CA-X; Kyowa Interface Science Co. Ltd., Saitama, Japan) at room temperature. A droplet (10 μL) of water was placed on a SAM surface, and 10 sec later, the contact angle was determined three times. This procedure was repeated five times at different sites on the same surface, and the contact angle of a sample was expressed as the mean value of five contact angle measurements with SEM.

2.4. Adsorption of Fn and Vn

Fn and Vn were radiolabeled using the chloramine-T method [25]. Five microliters of $Na^{125}I$ (Iodine-125, PerkinElmer Inc., Boston, MA, USA) and 100 μL of chloramine-T (Nacalai Tesque, Kyoto, Japan; 0.2 mg/mL in 0.5 M phosphate buffer containing 0.15 M NaCl, pH 7.4) was added to 200 μL of Fn or Vn (500 μg/mL in 0.5 M phosphate buffer containing 0.15 M NaCl). For Vn, 50 μg/mL of stock solution was concentrated by ultrafiltration (Amicon Ultra-0.5, nominal molecular weight limit: 10000; Millipore) before radiolabeling. The reaction mixture was incubated for 2 min at room temperature. Then 100 μL of sodium metabisulfite (Nacalai Tesque; 4 mg/mL in water) was added to stop the reaction. Labeled proteins were separated by size-exclusion chromatography using a Sephadex G-25 column (PD-10; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Fractions were collected and examined for radioactivity using a gamma counter (ARC-380CL, Aloka Co., Ltd., Tokyo, Japan). Protein concentration was also determined by the Micro BCA™ protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Labeled proteins were mixed with unlabeled proteins, and specific activity (cpm/ng-protein) was determined. The sample was stored at -20°C until use. A control protein adsorption experiment was performed using ^{125}I-labeled proteins with a different ratio to non-labeled proteins. The adsorbed amount of proteins was constant regardless of the ratio (data not shown), suggesting that the iodination of Fn and Vn does not change their adsorption behavior.

A 100 μL droplet of protein solution, dissolved in DPBS containing 10 mM NaI (PBSI) [26], was placed on a 35-mm non-treated polystyrene dish (Asahi Techno Glass Corp., Tokyo, Japan), and substrate-carrying SAM was immediately floated on the droplet so that the SAM surface faced the protein solution. The substrate was placed on the droplet immediately in order to avoid formation of protein layer at air-water interface of the droplet. The concentration of Fn or Vn was kept constant at 0.01 mg/mL while BSA concentration was varied at 0.01–10 mg/mL in PBSI.

After 30 min incubation at 37°C, the substrates were rinsed with PBSI and placed in polystyrene tubes. The radioactivities (cpm) were measured by the gamma counter and converted to the amount of adsorbed Fn or Vn (ng/cm^2). In some experiments, to observe displacement of adsorbed proteins, SAMs were first incubated with 10 mg/mL BSA for 30 min and then rinsed with PBSI followed by incubation with either ^{125}I-labeled Fn or Vn for 30 min.

Protein adsorption to SAMs from FBS was also tested. Fn contained in native FBS was removed using gelatin Sepharose [27]. FBS was mixed with gelatin Sepharose 4B (GE Healthcare) and incubated for 2 h at room temperature. Fn-depleted FBS was collected by centrifugation at 1000 ×g for 2 min using a filter unit (ULTRAFREE-MC; Millipore). Removal of Fn was confirmed by ELISA. ^{125}I-labeled Fn was then added to a concentration of 30 μg/mL. For Vn, ^{125}I-labeled Vn was added to native FBS at a concentration of 20 μg/mL, which is one tenth of the Vn concentration in native FBS [24]. A protein adsorption experiment was performed as described above. The amount of adsorbed Fn was calculated from radioactivity while that of adsorbed Vn was calculated from radioactivity and then multiplied by 11 because the FBS included ^{125}I-labeled Vn and native Vn with a presumed ratio of 1:10.

2.5. Cell culture

HUVECs (Lonza, Walkersville, MD, USA) were maintained on tissue culture dishes at 37 °C in 5% CO_2. The culture medium, prepared by following the instructions from the HUVECs supplier, was Endothelial Basal Medium (EBM-2, Lonza) supplemented with 2% FBS (Lonza) and with the growth factors hFGF-B, VEGF, R3-IGF-1, and hEGF, and also containing hydrocortisone, ascorbic acid, heparin, gentamicin, and amphotericin-B (Lonza). Cells were collected by the addition of a 0.025% trypsin/0.01% EDTA solution (Lonza). Cells in passages from 4 to 5 were

collected and used for the cell adhesion assay. The culture medium containing EBM-2, 2% FBS, gentamicin, and amphotericin-B was used in the cell adhesion tests and is referred to here as the assay medium.

2.6. Cell adhesion to SAMs preadsorbed with proteins

SAMs, formed on the gold-coated cover glasses as described above, were immersed in 70% ethanol and placed in a 24-well non-treated polystyrene plate (Asahi Techno Glass Corp.). The SAMs were incubated with 10 μg/mL Fn or Vn solutions in DPBS or 0.01-10 mg/mL BSA at 37 °C for 30 min. The surface was then washed with DPBS three times and once with the assay medium. Collection of HUVECs by the addition of trypsin/EDTA was carefully monitored and terminated at the point of rounding up of the cells in order to minimize damage to cell surface components. Cells were washed once and then suspended in either the assay medium, or the serum-free medium which contains only 1 mg/mL BSA (same as BSA concentration in 2% FBS). A suspension of HUVECs was added to each well at a density of 1×10^4 cells/cm^2 and incubated at 37 °C in 5% CO_2. After 1 h of culture, phase-contrast images of the cells were taken using an inverted microscope (IX71, Olympus, Tokyo, Japan) with a 10× objective. Cells were washed with warm DPBS three times to remove non-adherent cells and then fixed with 4% paraformaldehyde (PFA; Nacalai Tesque) in DPBS for 20 min. After the fixed cells were washed with DPBS three times, cell nuclei were stained with 2 μg/mL of Hoechst 33258 (Dojindo Laboratories) in 20 mM Tris-HCl buffered solution (pH 8.0) for 20 min. The number of adherent cells on each surface was determined by counting the number of stained nuclei using a fluorescence microscope (10× objective lens) in 10 different fields.

2.7. Immunostaining

HUVECs were cultured on SAMs preadsorbed with Fn or Vn as described above. After 1 h of incubation, cells were rinsed with warm DPBS three times and fixed with PBS solution containing 4% PFA for 20 min. Cells were permeabilized by treatment with 0.5% TritonX-100 solution at room temperature for 5 min and then treated with 2% skim milk solution for 1 h to block non-specific adsorption of antibodies, followed by incubation with the primary antibody against integrin α_5 (1:200 in 2% skim milk) for Fn-coated SAMs or integrin α_v (1:200 in 2% skim milk) for Vn-coated SAMs for 1 h at 4 °C. After washing with PBS containing 0.05% Tween-20, cells were treated with Alexa Fluor 488 anti-rabbit IgG (integrin α_5) or Alexa Fluor 488 anti-mouse IgG (integrin α_v) (1:500 in 2% skim milk) for 1 h at 4 °C and washed with PBS containing 0.05% Tween-20. Actin filaments were stained with Alexa Fluor 594-conjugated phalloidin (1:40 in DPBS) for 20 min at room temperature and rinsed with DPBS. Samples were mounted on cover glasses with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA) and observed with a confocal fluorescence microscope (FV10i, Olympus).

2.8. Statistical analysis

Significant differences between two groups were examined using the Tukey's HSD test. A $p < 0.05$ was considered as statistically significant. All statistical calculations were performed using JMP, version 7.0.1 (SAS Institute, Cary, NC).

3. Results

3.1. Surface characterization

SAMs carrying four different functionalities (CH_3-, OH-, COOH-, NH_2-) were chosen since they are typical functional groups present in polymer substrates and hence commonly examined for protein adsorption and cell adhesion studies [3-10,17-22]. Surface properties of the SAMs were characterized by XPS and water contact angle measurements (Table 1). Most of the atomic compositions obtained were in good agreement with those expected from the molecular structures of alkanethiols. The water contact angles were in agreement with those reported in the literature [19]. These analyses confirmed that the expected SAM surfaces were formed on the gold surface.

3.2. Adsorption of Fn and Vn

3.2.1. Adsorption from single and mixed solution with BSA

We measured the amounts of Fn and Vn adsorbed to SAMs in either single or mixed protein solution using ^{125}I-labeled proteins. Concentrations of Fn or Vn were kept constant at 10 μg/mL while BSA concentration was varied from 0 to 10 mg/mL. Total amounts of adsorbed proteins were also determined by a surface plasmon resonance (SPR) apparatus. Total amounts of adsorbed Fn and BSA showed no dependence on Fn/BSA ratios, while those of Vn and BSA to COOH- and NH_2-SAMs showed maximum at Vn/BSA of 0.001 (Supplementary Figure 1). When SAM surfaces were exposed to single Fn solution, a larger amount of Fn was adsorbed to CH_3-, COOH-, and NH_2-SAMs than to OH-SAMs (Fig. 1a), in consistent with other reports [19]. The adsorbed amounts of Fn decreased with a decrease in the Fn/BSA ratio. However, the decrement differed with the surface functional group of the SAMs: The adsorbed amount of Fn was greatly reduced for CH_3- and OH-SAMs but remained large for COOH- and NH_2-SAMs. Even for Fn/BSA = 0.001, which is similar to the ratio of Fn and BSA in FBS (Fn: 30 μg/mL; BSA: 35–55 mg/mL), Fn

adsorbed to COOH- and NH_2-SAMs at 40% and 15% of that from the single Fn solution, respectively.

Adsorption of Vn exhibited a similar tendency to that of Fn. Larger amounts of Vn were adsorbed to CH_3- and NH_2-SAMs than to OH- and COOH-SAMs from single Vn solution (Fig. 1b). Although adsorption of Vn to CH_3- and OH-SAMs was markedly decreased in the presence of BSA, it was still observed for COOH- and NH_2-SAMs. For Vn/BSA = 0.01, which is similar to FBS (Vn: 200 μg/mL, BSA: 35–55 mg/mL), the amount of adsorbed Vn decreased to only 60% for both COOH- and NH_2-SAMs while it decreased to ~ 10% for CH_3- and OH-SAMs.

3.2.2. Adsorption of Fn and Vn from FBS

We also examined adsorption of Fn and Vn to SAMs from FBS using ^{125}I-labeled Fn or Vn. To ensure that radioactivity was in the measurable range, ^{125}I-labeled Fn (final concentration: 30 μg/mL) was added to Fn-depleted FBS while ^{125}I-labeled Vn (final concentration: 20 μg/mL, one tenth of that in native FBS) was added to native FBS. Adsorption of Fn and Vn from 2% FBS, which corresponds to the culture condition of HUVECs, was greatly influenced by the surface functional group of SAMs as follows (Fig. 2): COOH- > NH_2- > CH_3- ≈ OH-SAM for Fn and NH_2- > COOH- > OH- ≈ CH_3-SAM for Vn, respectively. Preferential adsorption of Fn and Vn were also observed from FBS, and larger amounts of Vn adsorbed to SAMs than Fn. Additionally, the tendency of adsorption was similar to protein adsorption from the mixed solution with BSA where ratios of cell adhesive proteins to BSA were similar to those in FBS (Fn/BSA = 0.001 for Fn and Vn/BSA = 0.01 for Vn, respectively). These results suggest that adsorption of cell adhesive proteins from FBS mainly results from competitive adsorption with BSA.

3.2.3. Adsorption of Fn and Vn to SAMs preadsorbed with BSA

Considering that the concentration of BSA in serum is much higher than that of Fn or Vn, it is to be expected that a protein-adsorbed layer from FBS mainly would consist of BSA at the initial phase of adsorption. In such a case, adsorbed BSA would need to be displaced with Fn and Vn. To examine displacement of BSA with cell adhesive proteins, SAMs were incubated with 1 mg/mL BSA for 30 min followed by incubation with either ^{125}I-labeled Fn or Vn. The adsorbed amounts of both Fn and Vn on COOH- and NH_2-SAMs were higher than those on CH_3- and OH-SAMs (Fig. 3), suggesting that COOH- and NH_2-SAMs allow for displacement of preadsorbed BSA with Fn and Vn.

3.3. Cell adhesion to SAMs preadsorbed with Fn and Vn

HUVECs were used to examine cell adhesion behavior to SAMs preadsorbed with Fn or Vn mixed with BSA. Figure 4 shows the number of adhered HUVECs on SAMs preadsorbed with different ratios of Fn/BSA or Vn/BSA mixtures. Most of adherent cells (> 95%) were viable for all conditions as tested by staining with calcein-AM (live cells) and propidium iodide (dead cells) (data not shown). When BSA/Fn ratio was relatively high (Fn only and Fn/BSA $\geq$ 0.1), the numbers of adhered cells were similar among the SAMs (Fig. 4a). The numbers of adherent cells drastically decreased for CH_3-SAMs and for OH-SAMs with decreasing Fn/BSA ratio. In contrast, large amounts of cells adhered on COOH- and NH_2-SAMs preadsorbed with Fn/BSA of 0.001. To study the role of FBS in culture medium on cell adhesion, HUVECs were incubated on SAMs preadsorbed with Fn/BSA in serum-free medium containing only 1 mg/mL BSA (Fig. 4b). HUVECs adhered to COOH-SAM preadsorbed with Fn/BSA of 0.001 even in the serum-free medium and hardly adhered to that preadsorbed with only BSA, coinciding with preferential

adsorption of Fn on COOH-SAM. In contrast, adhesion of HUVECs observed for NH_2-SAM preadsorbed with only BSA. This suggest that some non-biospecific interaction or electrostatic interaction between cell surface and NH_2-SAM covered with BSA contributed to initial cell adhesion.

In addition, cells adhered to COOH-SAM preadsorbed with only BSA (~50% of those for Fn-adsorbed SAMs), in agreement with our previous studies [9,10]. Considering the experimental conditions in which cells are incubated in a medium supplemented with 2% FBS, preadsorbed BSA is thought to be displaced with serum proteins included in FBS. Cell adhesion was greatly suppressed for COOH-SAM preadsorbed with single BSA when cells were incubated in the serum-free medium. These results suggest that displacement of BSA with Fn or Vn, both of which are included in a serum-containing medium, is also important to mediate cell adhesion to surfaces.

Preadsorption of single Vn solution enabled HUVECs to adhere to SAMs independently of the SAM surface functional group (Fig. 4c). Of note, no significant difference in the numbers of adhered cells was observed among SAMs preadsorbed with a Vn/BSA of 0.001. This was also observed when cells were incubated in the serum-free medium (Fig. 4d). Preadsorption of SAMs with only BSA did not change the number of adherent cells for NH_2-SAMs and slightly decreased for CH_3- and OH-SAMs, suggesting the contribution of non-biospecific or electrostatic interaction to cell adhesion. In contrast, for COOH-SAM, presence of Vn greatly increased the number of adherent cells in both cases. These results clearly suggest that preferential adsorption of Vn mediate adhesion of HUVECs to COOH-SAM.

3.4. Formation of focal adhesion and actin stress fibers

Spreading of HUVECs on SAMs preadsorbed with either Fn or Vn was evaluated in terms of formation of focal adhesion complexes and actin stress fibers. Cells were immunostained with antibodies against integrin α_5 for Fn-adsorbed surfaces and integrin α_v for Vn-adsorbed surfaces, which are major integrins interacting with Fn ($\alpha_5\beta_1$) and Vn ($\alpha_v\beta_3$), respectively. F-actin was also stained with fluorescently labeled phalloidin to observe stress fiber formation.

HUVECs adhered on SAMs preadsorbed with single Fn exhibited clear clusters of integrins α_5, which are co-localized with actin stress fibers (Fig. 5a), indicating formation of well-organized focal adhesion. When HUVECs were cultured on SAMs preadsorbed with Fn/BSA of 0.001, less-developed F-actin fibers and no obvious cluster of integrins were observed for COOH-SAMs, indicating reduced formation of focal adhesion (Fig. 5b). For NH_2-SAMs, diffuse F-actin and no cluster of integrins were observed. Fewer HUVECs adhered to CH_3- and OH-SAMs preadsorbed with Fn/BSA of 0.001 as shown in Figure 4a, and adherent HUVECs exhibited no clustering either of integrin or F-actin.

In the case of SAMs preadsorbed with single Vn, robust clusters of integrin α_v and actin stress fibers were observed for all SAMs (Fig. 6a), indicating formation of well-organized focal adhesions on Vn-coated SAM surfaces. For SAMs preadsorbed with a Vn/BSA of 0.001, however, no clustering of integrin or F-actin was observed for CH_3- and OH-SAMs whereas HUVECs on COOH- and NH_2-SAMs exhibited clusters of integrin and actin stress fibers (Fig. 6b). These results suggest that Vn adsorbed on COOH- and NH_2-SAMs efficiently mediates formation of the focal adhesion complex while that adsorbed on CH_3- and OH-SAMs supports attachment of HUVECs but not formation of focal adhesion complexes.

4. Discussion

We have employed SAMs carrying four different functionalities (CH_3-, OH-, COOH-, NH_2-) and their mixed SAMs for protein adsorption and cell adhesion studies since they are typical functional groups present in polymer substrates and hence commonly examined [3-10,17-22]. XPS analysis of the SAMs indicated that most of the atomic compositions obtained were in agreement with those expected from the molecular structures of alkanethiols (Table 1). Sulfur percentages were smaller than expected values estimated from molecular formula in all SAMs. This might be due to the attenuation of the photoelectrons emitted from the S-Au interface which must reach the detector through the SAM layer. In addition, the presence of oxygen in NH_2-SAM surfaces might have been the result of oxidized sulfur species, as Wang et al [28] suggested. The water contact angles were in agreement with those reported in the literature [19]. These analyses confirmed that the expected SAM surfaces were formed on the gold surface.

We have shown that HUVECs adhere well to COOH- and NH_2-SAMs but poorly to OH- SAMs and hardly at all to CH_3-SAMs [9]. In our study which examined the effect of surface wettability on protein adsorption and subsequent cell adhesion using mixed SAMs of two different alkanethiols [10], we found that the number of adherent cells was at its maximum on SAMs with moderate wettability (40–60°), although wettability varied slightly by the combination of surface functional groups and cell type. Our previous investigation of real-time monitoring of protein adsorption and cell adhesion using an SPR apparatus and a total internal reflection fluorescence microscope also demonstrated that serum proteins rapidly adsorbed to SAM surfaces within 1 min while cells adhered during the first 1 h [9], demonstrating clearly that adsorption of serum proteins contributes to a SAM's suitability for cell adhesion. Moreover, on COOH- and NH_2-SAMs, HUVECs adhesion to BSA-preadsorbed surfaces in medium containing 2% FBS was delayed by several minutes compared to HUVEC adhesion to bare surfaces, suggesting that displacement of

preadsorbed BSA with cell adhesive proteins, such as Fn or Vn, supports cell adhesion to these surfaces. These previous results led us to further examine the competitive adsorption of cell adhesive proteins onto SAMs carrying different functional groups.

This study focused on adsorption of Fn and Vn to SAMs from complex media including mixed solution with BSA and 2% FBS, and its role on cell adhesion. HUVECs adhered well to all SAMs preadsorbed with single solution of either Fn or Vn, indicating that Fn or Vn with high coverage effectively mediates cell adhesion. In contrast, the surface functional group of SAMs greatly affected adsorption of Fn and Vn in the presence of BSA (Fig. 1); larger amounts of Fn and Vn adsorbed on COOH- and NH_2-SAMs than on CH_3- and OH-SAMs. A similar tendency was observed for adsorption of Fn and Vn from 2% FBS (Fig. 2). The difference in the adsorbed amount between Fn and Vn arises from the difference in their concentrations in FBS. Greater adsorption of Fn and Vn to surfaces having carboxylic acid and amine are consistent with other studies using tissue culture polystyrene (TCPS) and Primaria^{TM} [15] and self-assembled monolayers of alkyl silanes [8]. We previously measured the total amounts of adsorbed serum proteins on the SAMs from culture medium supplemented with 2% FBS using SPR [9], identifying 198 ng/cm^2 for CH_3-, 180 ng/cm^2 for OH-, 226 ng/cm^2 for COOH-, and 256 ng/cm^2 for NH_2-SAMs, respectively. The amounts of adsorbed Fn determined in this study were calculated to be 0.11% for CH_3-, 0.06% for OH-, 1.32% for COOH-, and 0.29% for NH_2-SAMs, of total amount of adsorbed serum proteins. The amounts of adsorbed Vn were also calculated to be 0.67% for CH_3-, 1.09% for OH-, 3.57% for COOH-, and 6.84% for NH_2-SAMs, respectively. Percentages of Fn and Vn included in the protein-adsorbed layers on COOH- and NH_2-SAMs were much larger than those in FBS (Fn: 0.04%, Vn: 0.29%), which are estimated by assuming total protein

concentration of FBS to be 70 mg/mL. Our results clearly demonstrate that Fn and Vn are effectively accumulated on COOH- and NH_2-SAMs from complex media.

Vroman showed that fibrinogen from plasma at intermediate dilution adsorbs only transiently over a short interval [29]. This phenomenon, referred to as the Vroman effect, has been shown to result from competitive adsorption and displacement of adsorbed proteins with other proteins in the fluid phase [30]. Because of the low content of Fn and Vn in FBS, a protein layer at the initial phase of protein adsorption mostly consists of major serum proteins lacking cell-binding activity such as BSA and IgG. Displacement of initially adsorbed proteins with Fn and Vn is considered to be involved in preferential adsorption of Fn and Vn according to the Vroman effect, in which faster diffusing molecules are considered to be displaced by proteins with a higher affinity for surface [30,31]. Our result indicated that COOH- and NH_2-SAMs exhibited better adsorption of Fn and Vn after preadsorption of BSA for 30 min (Fig. 3), suggesting displacement of preadsorbed BSA with Fn and Vn. Displacement of preadsorbed BSA might be related to its conformational change [32,33]. Circular dichroism (CD) spectroscopic study by Sivaraman et al demonstrated that adsorption of albumin on hydrophobic CH_3-SAM induced greater conformational change than on hydrophilic surfaces (OH-, COOH-, NH_2-SAMs) [34]. Conformational change of adsorbed proteins on hydrophobic surfaces is also shown by molecular simulation [35] and Fourier transform infrared spectroscopy [36]. While strongly (or irreversibly) adsorbing albumin to CH_3-SAM would inhibit displacement with other proteins in liquid phase, weakly (or reversibly) adsorbing albumin to hydrophilic SAMs would allow for the displacement [32,37]. Affinity of Fn with OH-SAM, however, was not so strong as shown by poor adsorption of Fn to OH-SAM (Fig. 1a). Therefore, preferential adsorption of Fn did not occur for OH-SAM. Preferential adsorption of Fn and Vn for COOH- and NH_2-SAMs is considered to result from displacement of initially

adsorbed BSA with Fn and Vn due to relatively weak interaction of BSA with COOH- and NH_2-SAMs.

Despite the similarity of adsorption behavior to SAMs between Fn and Vn, cell adhesion behavior was greatly different. HUVECs adhered to SAMs preadsorbed with mixtures of Fn and BSA (Fig. 4a), correlating to the number of adsorbed Fn (Fig. 1a). However, HUVECs adhered to SAMs preadsorbed with Vn and BSA regardless of surface functional groups (Fig. 4c), although a much smaller amount of Vn adsorbed on CH_3- and OH-SAMs than on COOH- and NH_2-SAMs (Fig. 1b). We consider that this finding can be explained in terms of the number of adhesion sites on SAMs. The tripeptide sequence Arg-Gly-Asp (RGD) is one of the important adhesion sites included in both Fn and Vn and interacts with both integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [38]. We calculated RGD density on SAMs carrying adsorbed Fn or Vn from Figure 1 according to their molecular weight (Fn: 440 kDa; Vn: 69 kDa) and number of RGDs (Fn: two; Vn: one per molecule). The numbers of adherent cells (Fig. 4a, 4c) were then plotted against the RGD density (Fig. 7). The RGD densities on CH_3-SAM and OH-SAMs preadsorbed with Vn/BSA of 0.001 (Fig. 7b) were much higher than those preadsorbed with Fn/BSA of 0.001 (Fig. 7a). This result suggests that the higher RGD density for adsorbed Vn than that for adsorbed Fn enables cells to adhere to CH_3-SAM and OH-SAMs preadsorbed with Vn/BSA of 0.001. Results in Figure 7 also shows that most of prepared surfaces are located at higher RGD density for COOH- and NH_2-SAMs regardless of Fn/BSA or Vn/BSA, clearly demonstrated that preferential adsorption of Fn and Vn onto COOH- and NH_2-SAMs supports cell adhesion on these SAM surfaces.

While good adherence of HUVECs on COOH- and NH_2-SAMs preadsorbed with Fn agrees with other study [19], good adherence on COOH- and NH_2-SAMs preadsorbed with Vn has not been reported. Our result is consistent with other study using polymer substrates. Steele et al. examined

attachment of HUVECs to TCPS (negatively charged) and Primaria^{TM} (positively charged) adsorbed with either Fn or Vn [15]. The density of Fn for half-maximal attachment was calculated to be 54 fmol/cm^2 for TCPS and 27 fmol/cm^2 for Primaria^{TM}, while the density of Vn was 246 fmol/cm^2 for TCPS and 87 fmol/cm^2 Primaria^{TM}. Keselowsky et al. examined attachment of osteoblast-like cell line (MC3T3-E1) cells to SAMs preadsorbed with Fn [19]. The density of Fn for half-maximal attachment of cells was calculated to be 1090 fmol/cm^2 for CH_3-, 86 fmol/cm^2 for OH-, 250 fmol/cm^2 for COOH-, and 370 fmol/cm^2 for NH_2-SAMs, respectively. Our study showed adhesion of HUVECs at a lower RGD density than previous reports. This reduction is probably attributable to cell adhesion experiments performed in a medium supplemented with 2% FBS, which allows for displacement of preadsorbed proteins with serum proteins in the medium. In fact, around 50% of seeded cells could adhere to COOH-SAMs preadsorbed with only BSA (Fig. 4a) whereas HUVECs did not adhere in a serum-free medium (Fig. 4b). Although role of 2% FBS in the medium on cell adhesion for NH_2-SAM is not clear due to the presence of some non-specific interaction between cells and BSA-adsorbed NH_2-SAM (Fig. 4b), these findings suggest that HUVECs adhere well to COOH-SAMs because of preferential adsorption of both Fn and Vn and displacement of adsorbed BSA with Fn and Vn in a medium.

Although HUVECs adhered to SAMs preadsorbed with Vn and BSA (Vn/BSA: 0.001) regardless of surface functional groups (Fig. 4c), focal adhesion and actin stress fibers were well organized for HUVECs on only COOH- and NH_2-SAMs (Fig. 6b). The RGD densities on Vn-adsorbed COOH- (535 fmol/cm^2) and NH_2-SAMs (398 fmol/cm^2) were an order of magnitude higher than those on CH_3-SAM (60 fmol/cm^2) and OH-SAMs (40 fmol/cm^2). This result is consistent with the study reported by Massia et al. [39], which demonstrated that cell spreading and formation of focal contact and stress fiber required more RGDs than cell attachment. In

addition, the RGD densities on Vn-adsorbed COOH- and NH_2-SAMs were higher than those on Fn-adsorbed COOH- (225 fmol/cm^2) and NH_2-SAMs (83 fmol/cm^2) in the presence of 1000-fold BSA. Greater adsorption of Vn than Fn was also observed in protein adsorption from 2% FBS, consistent with earlier studies using a variety of polymer substrates [15,27,40,41]. Taken together, these results suggest the importance of preferential adsorption of Vn in mediating cell adhesion on COOH- and NH_2-SAMs as reported by the reduction of cell adhesion in a medium supplemented with Vn-depleted serum but not Fn-depleted serum [42].

Present study demonstrated that preferential adsorption of Fn and Vn from complex media is important for subsequent cell adhesion. It is known that conformation of adsorbed protein also plays an important role in its biological function. This is implied by our results that the RGD density necessary for cell adhesion is much higher than that obtained in studies using substrates presenting RGD peptides [39,43]. Our results suggest that some Fn and Vn adsorb to SAMs so that their cell-binding domains are inaccessible to integrins because of changes in orientation and conformation upon adsorption. For Fn adsorption, the CH_3-SAM required the highest RGD density while the OH-SAM required the least RGD density (Fig. 7a), suggesting surface-dependent conformational change of adsorbed Fn. Conformational change of adsorbed Fn has been widely studied using SAMs [19,20,22,44,45] and polymer substrates [11,14,16]. Keselowsky et al. reported that surface functional groups of SAMs modulate conformation of adsorbed Fn qualitatively determined by binding of monoclonal antibody against their cell binding: OH- > COOH- = NH_2- > CH_3-SAMs [19]. These works studied the conformational change of adsorbed Fn from a single solution. In complex media, coadsorbing proteins affect conformation of adsorbed Fn [46]. To identify the true effect of protein adsorption on cell adhesion in complex media, the density of effective (active) adhesion sites accessible to cells needs to be further quantified by

characterizing the conformational change of adsorbed Fn and Vn as well as the adsorbed amounts in complex media. Additionally, proteomics and bioinformatics analyses revealed the contribution of other adsorbed proteins to cellular behavior [47]. Combination of these techniques is required to further understand the role of adsorbed protein layer on cellular responses.

This study employed HUVECs to examine cell adhesion behavior to SAMs. Results will provide insights into endothelialization of artificial vascular grafts in vitro for preparing thromboresistant surface. The aim of this study is to understand the role of protein adsorption on cell adhesion at initial phase of cell adhesion process (~1 h). For effective coverage of materials surface with endothelial cells, cellular behaviors at later stages including growth or thromboresistant ability [48] need to be studied. In addition, relationship between protein adsorption and cell adhesion needs to be examined for a wide variety of cells to further understand cellular behavior on artificial substrates and to design substrates for specific cell types [49-52].

5. Conclusion

Adsorption of Fn and Vn was preferential for COOH- and NH_2-SAMs compared to CH_3- and OH-SAMs in both mixtures with BSA and a medium supplemented with 2% FBS. HUVECs adhered to SAMs preadsorbed with Fn and BSA depending on the adsorbed amount of Fn while they adhered all SAMs preadsorbed with Vn and BSA. This result was well related to the higher density of RGDs derived from adsorbed Vn than that derived from adsorbed Fn. HUVECs adhered to COOH- and NH_2-SAMs preadsorbed with Vn and BSA exhibited clustering of integrins and well-organized actin stress fibers. These results demonstrate that Vn preferentially adsorbs to COOH- and NH_2-SAMs sufficiently to support both cell adhesion and cell spreading.

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Nanomedicine Molecular Science" (No. 2306) and a Grant-in-Aid for Young Scientists (B) (No. 23700559) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Figure legend

Figure 1. The adsorbed amount of Fn (a) and Vn (b) on SAMs after 30 min of incubation as a function of the weight ratio of Fn or Vn to BSA. Fn and Vn in X-axes indicate single Fn or Vn solution used for protein adsorption experiments. Concentrations of Fn and Vn were kept constant (10 μg/mL) for a series of experiments. Data shown are means ± SD (n = 5 for Fn; n = 4 for Vn).

Figure 2. The adsorbed amounts of Fn and Vn on SAMs from 2% FBS after 30 min of incubation. Data shown are means ± SD (n = 5). Asterisks indicate significant differences ($p < 0.05$).

Figure 3. The adsorbed amounts of Fn and Vn on SAMs preadsorbed with BSA. SAMs were preadsorbed with 10 mg/mL BSA followed by incubation in either Fn or Vn solution (10 μg/mL). Data shown are means ± SD (n = 5 for Fn; n = 4 for Vn). Asterisks indicate significant differences ($p < 0.05$).

Figure 4. The numbers of adherent HUVECs on SAMs preadsorbed with a mixture of Fn (a, b) or Vn (c, d) with BSA. Cells were incubated in a medium supplemented with 2% FBS (a, c) or a serum-free medium containing only 1 mg/mL BSA (b, d) for 1 h. Data shown are means ± SD (n = 4).

Figure 5. Confocal fluorescence images of HUVECs adhered to SAMs preadsorbed with single Fn (a) and Fn/BSA of 0.001 (b). Cells were incubated for 1 h, fixed, permeabilized, and stained for integrin α_5 (green), F-actin (red), and nucleus (blue). Scale bar: 20 μm.

Figure 6. Confocal fluorescence images of HUVECs adhered to SAMs preadsorbed with single Vn (a) and Vn/BSA of 0.001 (b). Cells were incubated for 1 h, fixed, permeabilized, and stained for integrin α_v (green), F-actin (red), and nucleus (blue). Scale bar: 20 μm.

Figure 7. Relationship between RGD densities and adhesion of HUVECs for SAMs preadsorbed with Fn/BSA (a) and Vn/BSA (b). The RGD density is calculated from Fig. 1, and the number of adherent cells is the same as in Fig. 4a and c.

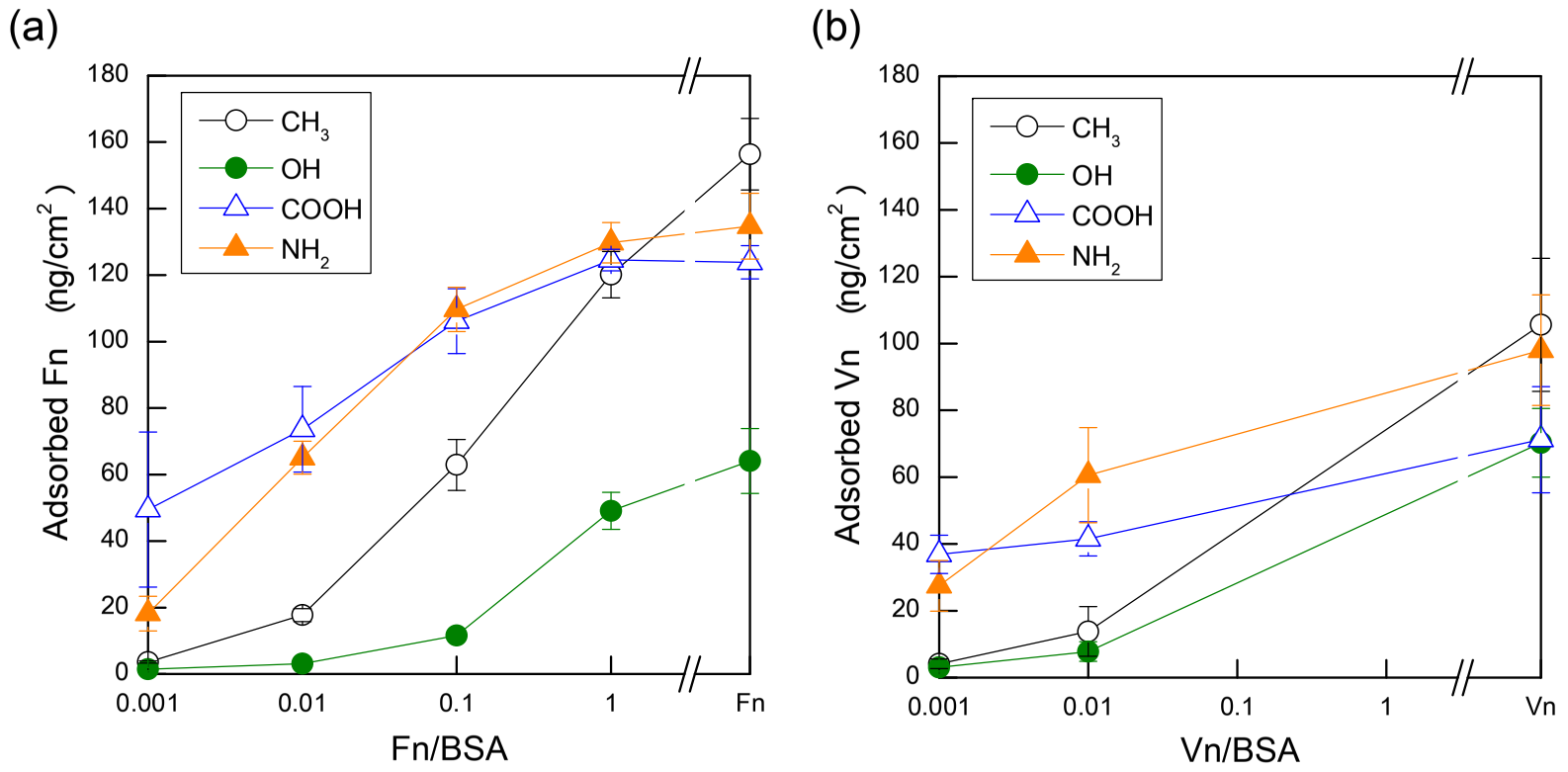
(a)
(b)
Adsorbed Fn (ng/cm^2)
Adsorbed Vn (ng/cm^2)
CH$_3$
OH
COOH
NH$_2$
0
20
40
60
80
100
120
140
160
180
0.001
0.01
0.1
1
Fn
Vn
Fn/BSA
Vn/BSA

Figure 1

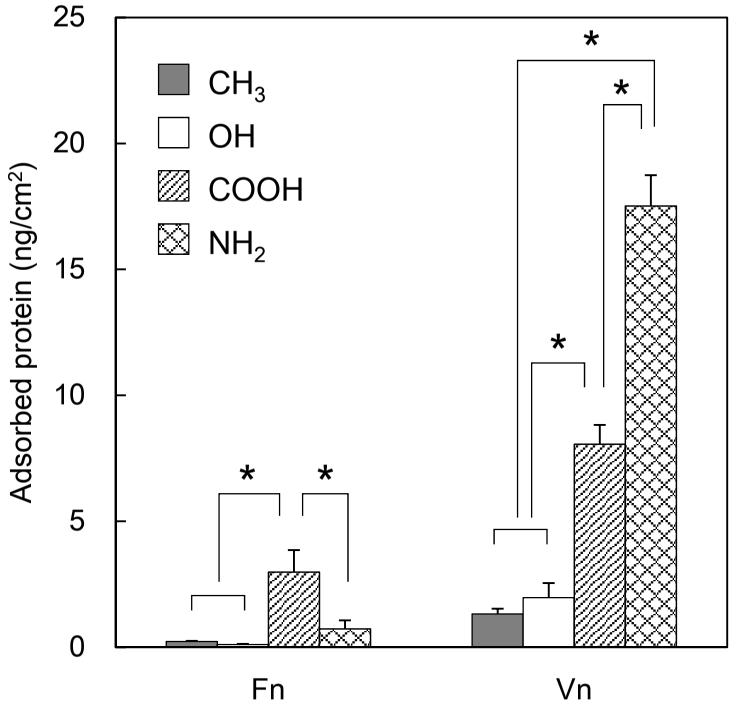

Figure 2

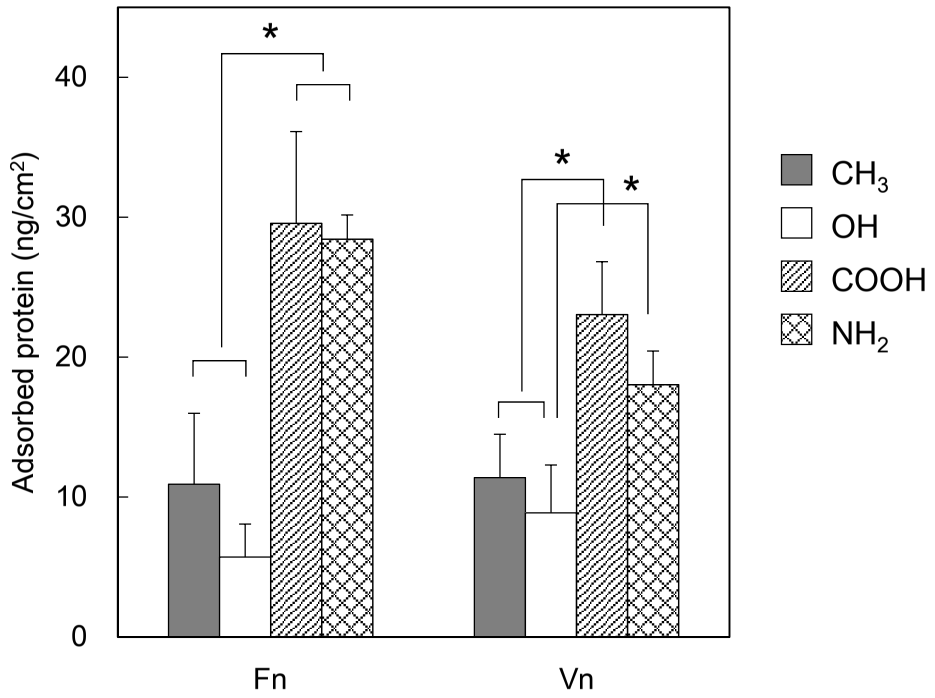

Figure 3

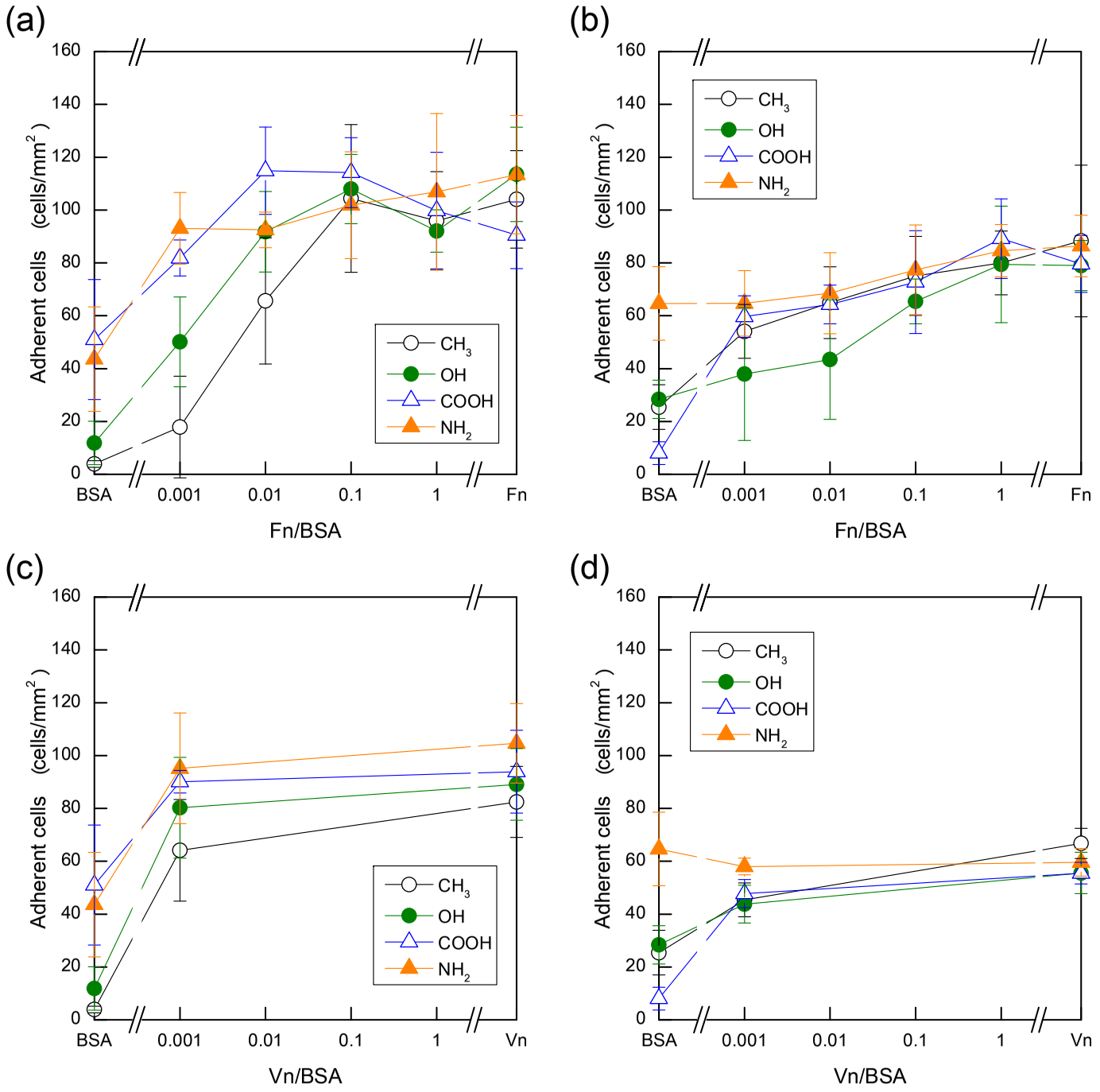
(a)
(b)
(c)
(d)
Adherent cells (cells/mm^2)
0
20
40
60
80
100
120
140
160
BSA
0.001
0.01
0.1
1
Fn
Vn
Fn/BSA
Vn/BSA
CH$_3$
OH
COOH
NH$_2$

Figure 4

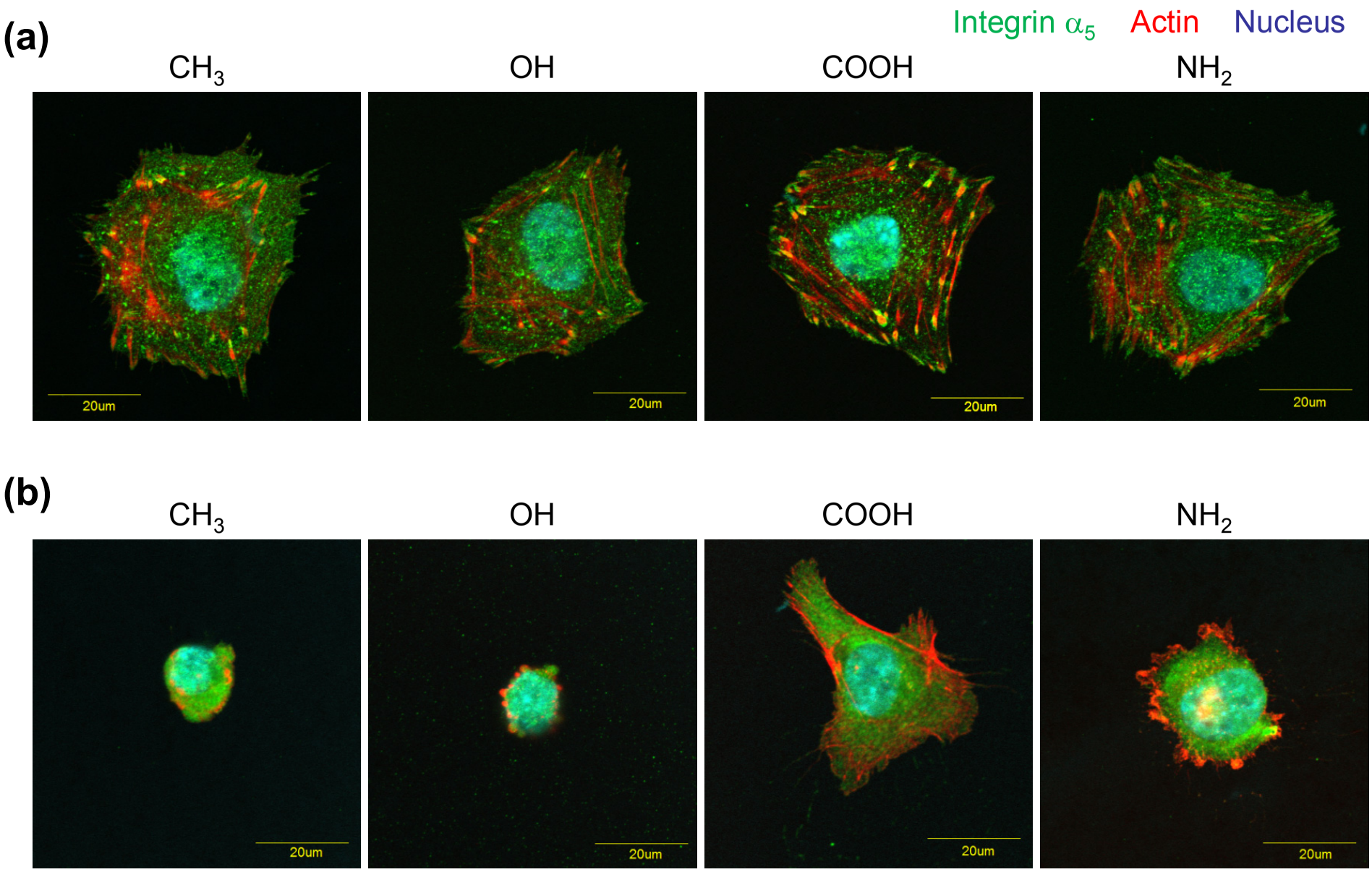
(a)
Integrin α_5
Actin
Nucleus
CH_3
OH
COOH
NH_2
20um
20um
20um
20um
(b)
CH_3
OH
COOH
NH_2
20um
20um
20um
20um

Figure 5

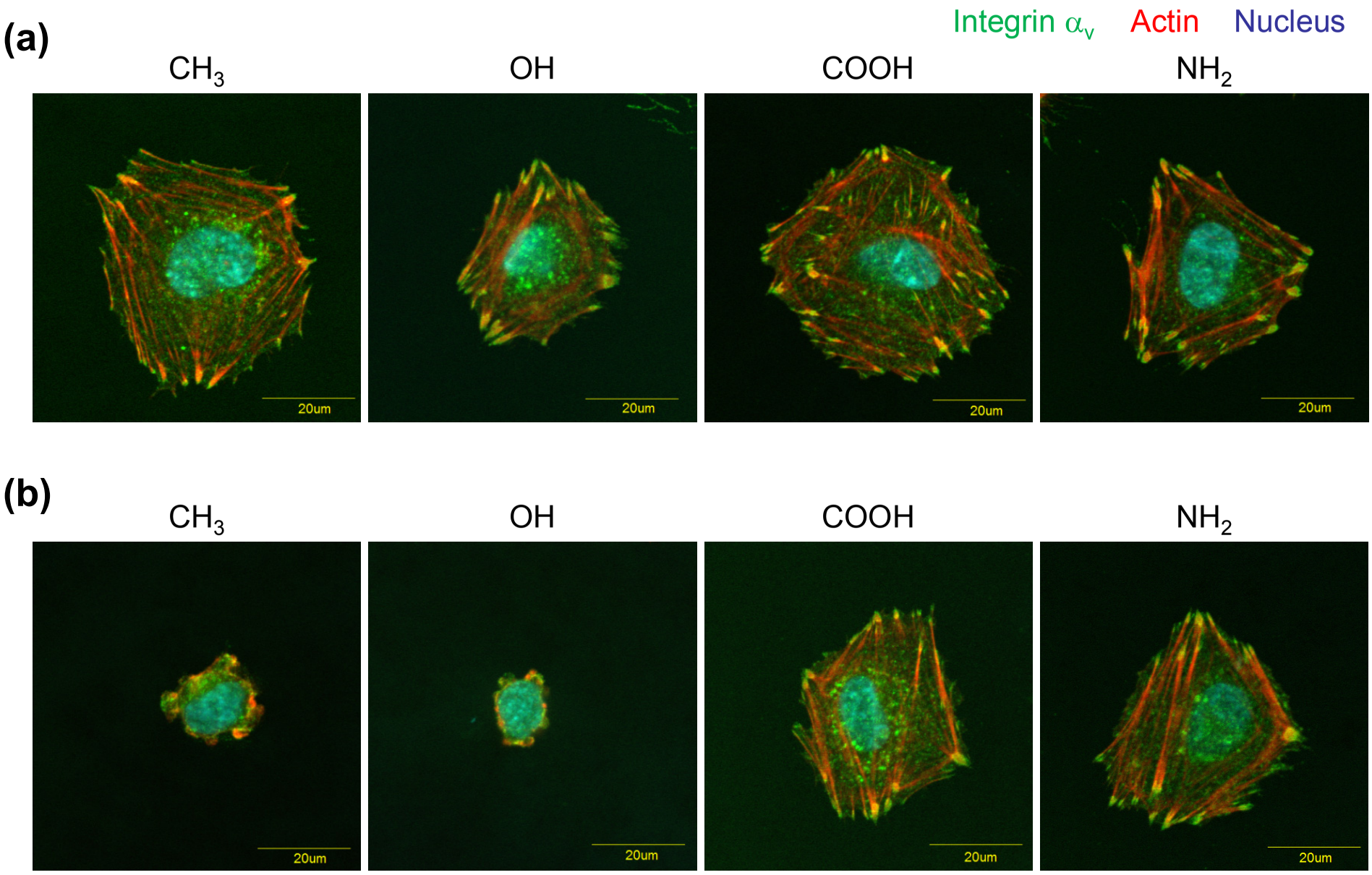
(a)
Integrin α_v
Actin
Nucleus
CH_3
OH
COOH
NH_2
20um
20um
20um
20um
(b)
CH_3
OH
COOH
NH_2
20um
20um
20um
20um

Figure 6

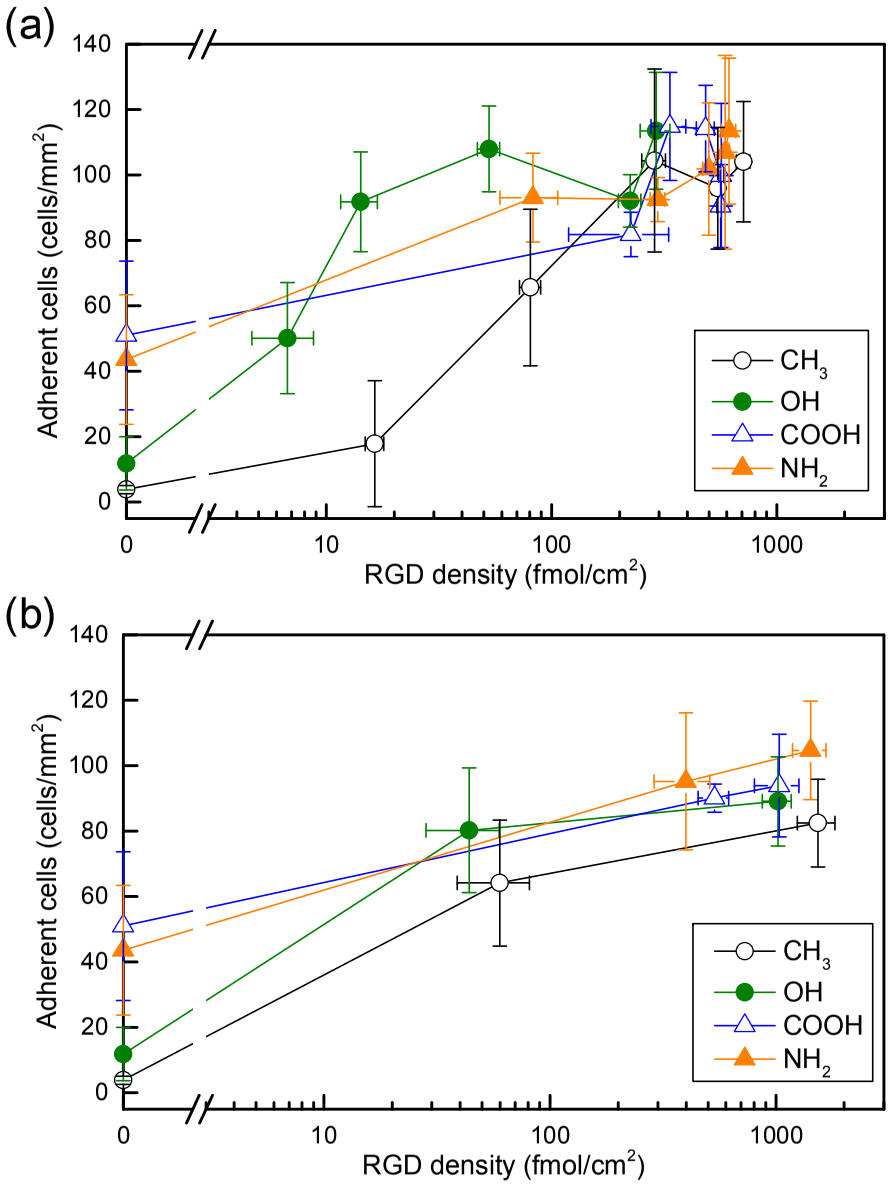
(a)
Adherent cells (cells/mm^2)
RGD density (fmol/cm^2)
CH$_3$
OH
COOH
NH$_2$
(b)
Adherent cells (cells/mm^2)
RGD density (fmol/cm^2)
CH$_3$
OH
COOH
NH$_2$

Figure 7

Supplementary Figure

Preferential adsorption of cell adhesive proteins from complex media on self-assembled monolayers and its effect on subsequent cell adhesion

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Total amount of adsorbed proteins onto SAMs

Experimental

BK-7 glasses (10 x 25 mm, thickness: 1 mm) were cleaned by oxygen plasma treatment using a plasma cleaner (FEMTO; Diener Electronic GmbH + Co., Germany) for 1 min, and rinsed with highly purified water (18.2 MΩ) and with 2-propanol three times. The cleaned glasses were coated with a chromium underlayer of 1 nm and then a gold layer of 49 nm in thickness by a thermal evaporation apparatus (V-KS200, Osaka Vacuum Instruments, Osaka, Japan). The gold-coated glasses were immediately immersed in 1 mM alkanethiol solutions overnight at room temperature to form mixed SAMs. The glasses were then rinsed with ethanol and water twice and finally with ethanol, and then

dried under a stream of nitrogen gas.

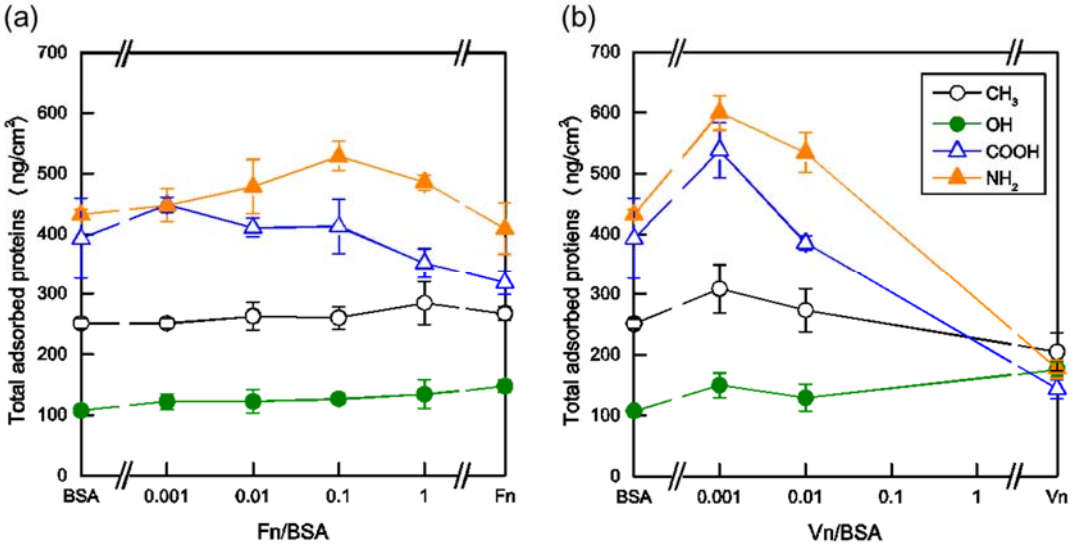
A surface plasmon resonance (SPR) instrument was assembled by utilizing the Kretschmann configuration as previously reported [1]. The SAM-coated glass plate was coupled to a hemicylindrical prism with an immersion oil (n = 1.515, Cargille Laboratories, Ceder Grove, NJ) and the SPR flow cell was attached on the plate. A peristaltic pump (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) delivered the liquid sample to the flow cell at 1 mL/min. For background correction, a blank buffer solution of DPBS was allowed to flow across the SAM surface. All of experiments were performed at 37 °C.

The SAM surface was first exposed to DPBS, and a protein solution (mixture of Fn/BSA or Vn/BSA) was injected in the flow cell, and then left for 30 min. DPBS was then circulated for 5 min to wash out the protein solution and weakly adsorbed proteins. The total amount of adsorbed proteins was determined by SPR angle shifts, using the following relationship [1], supposing refractive indices and densities of proteins to be 1.45 and 1 g/cm^3

The amount of adsorbed protein (ng/cm^2)

= 500 × increase of the resonance angle (degree)

Result



Supplementary Figure. Total amount of adsorbed proteins onto SAMs carrying different functionalities as a function of the weight ratio of Fn to BSA (a) and Vn to BSA. Concentrations of Fn and Vn were kept constant (10 μg/mL) for a series of experiments. Fn, Vn, and BSA in X-axes indicate single solution used for protein adsorption experiments (Fn, Vn: 10 μg/mL, BSA: 10 mg/mL). Data shown are means ± SD (n = 3).

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