Colloids and Surfaces B: Biointerfaces 84 (2011) 280–284

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Short communication

Bioinert surface to protein adsorption with higher generation of dendrimer SAMs

Erino Matsumoto[a], Tomohiro Fukuda[a,b], Yoshiko Miura[a,b,*]

[a] School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan
[b] Department of Chemical Engineering, Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

ARTICLE INFO

Article history:
Received 31 October 2010
Received in revised form 1 January 2011
Accepted 1 January 2011
Available online 7 January 2011

Keywords:
Dendrimer
Self-assembled monolayer
Protein adsorption
Polymer brush

ABSTRACT

Interactions between proteins and biomaterial surfaces correlate with many important phenomena in biological systems. Such interactions have been used to develop various artificial biomaterials and applications, in which regulation of non-specific protein adsorption has been achieved with bioinert properties. In this research, we investigated the protein adsorption behavior of polymer brushes of dendrimer self-assembled monolayers (SAMs) with other generations. The surface adsorption properties of proteins with different p*I* values were examined on gold substrates modified with poly(amidoamine) dendrimer SAMs. The amount of fibrinogen adsorption was greater than that of lysozyme, potentially because of the surface electric charge. However, as the generations increased, protein adsorption decreased regardless of the surface charge, suggesting that protein adsorption was also affected by density of terminal group.

1. Introduction

Interactions between proteins and biomaterial surfaces correlate with many important phenomena in biological systems, and such interactions have been used to develop various artificial biomaterials and applications. While biomimetic techniques such as enzyme immobilization are required for the fabrication of biochips and biosensors [1], the suppression technique of non-specific adsorption is required for most biomaterials, such as artificial blood vessels and stents, in addition to the biosensing devices. Protein adsorption is often unfavorable for some biomaterials due to induced side effects, such as thrombosis and immunological responses [2]. Additionally, suppression of non-specific protein adsorption at the interface is necessary for biosensing devices due to the improvement of the sensitivity and signal/noise ratio.

Regulation of non-specific protein adsorption has been achieved by the fabrication of surfaces with bioinert properties. Such bioinert surfaces have been investigated by many groups and have been reported to reduce protein adsorption by surface coating with hydrophilic polymer brushes. For example, modification with hydrophilic polymers such as poly(ethyleneglycol) (PEG) [3], dextran [4] and poly(phosphorylcholine methacrylate) (PMPC) [5] have been reported to inhibit non-specific adsorption of proteins due to their highly hydrated properties [6]. Protein adsorption has also been contributed to complicated factors involving protein conformation [7], protein charge distribution [8], degree of intermolecular coupling [9], surface charge [10], surface roughness [11] and functional groups [12] on the surface. However, an optimal design for bioinert surfaces remains to be developed.

The densities of polymer brushes have also been reported to be strongly related to protein adsorption [13,14]. Well-defined linear polymer brushes at the interface provide a precisely controlled surface, and the interfacial force for protein adsorption has been formulated using parameters such as chain density and layer thickness [15,16]. Well-defined polymer brushes have been achieved by living radical polymerization [17] and Langmuir–Blodgett membranes [18]. In these reports, the layer thickness of the polymer brush and the densities of the polymer were reported to play important roles in protein adsorption and the bioinert properties in terms of the interfacial force.

In this research, we investigated the protein adsorption behavior of polymer brushes of dendrimer self-assembled monolayers (SAMs) with the other generations (Fig. 1). A dendritic polymer brush constitutes well-defined and multi-branched structures of nanoscale size on a substrate. For example, interfaces with poly(amidoamine) (PAMAM) dendrimer SAM have been prepared with various generations [19,20]. However, there have been few reports regarding protein adsorption on the dendritic polymer brush, except for some reports representing excellent bioinertness for protein adsorption on high generation dendrimer coated surfaces [21,22]. The dendritic polymer brush has a high density of terminal group, which has the advantage of regulating protein

* Corresponding author at: Department of Chemical Engineering, Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan. Tel.: +81 92 802 2749; fax: +81 92 802 2769.
E-mail address: miuray@chem-eng.kyushu-u.ac.jp (Y. Miura).

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doi:10.1016/j.colsurfb.2011.01.003

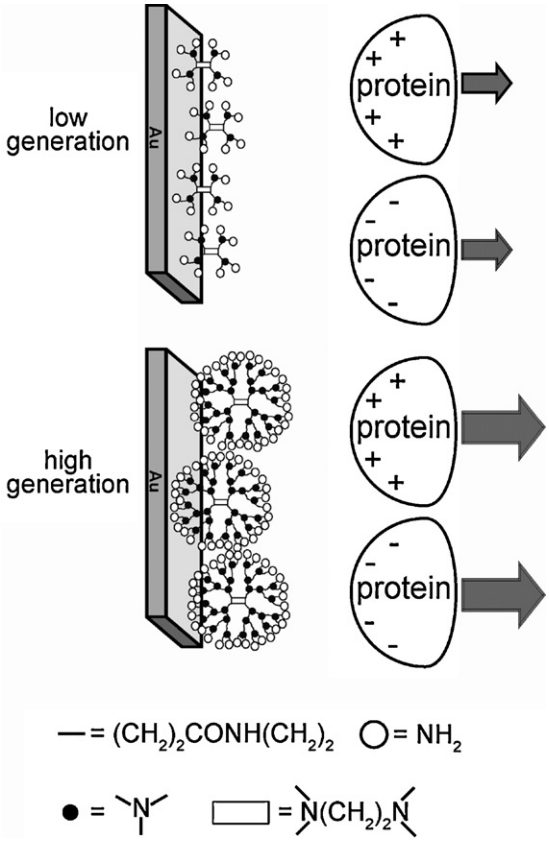

Fig. 1. Schematic illustration of protein adsorption on dendrimer SAMs.

adsorption, compared with the linear polymer brush [23,24]. A higher generation PAMAM–COOH-coated surfaces had low non-specific protein adsorption as compared to its lower analogues or succinic acid surfaces [25]. In our research, the adsorption properties of proteins with different p*I* values were examined on gold substrates modified with PAMAM dendrimer SAMs. The thickness and the densities of the polymer brushes were controlled by the generation of dendrimers. Consequently, the relationship between proteins and the dendritic polymer brush interface was investigated.

2. Materials and methods

2.1. Materials

The following reagents were used as received: PAMAM dendrimers, bovine serum albumin (BSA), lysozyme and fibrinogen (Sigma–Aldrich, MO, USA), and ethanol (EtOH) (Kanto Chemical, Tokyo, Japan). The gold substrates used were obtained from GE Healthcare Bioscience (Chalfont, St. Giles, UK) for surface plasmon resonance (SPR) measurements and from Moritex (Tokyo, Japan) for surface characterizations.

Modifications of the gold substrates were measured with X-ray photoelectron spectroscopy (XPS) by Axis-Ultra DLD (Shimadzu, Kyoto, Japan) and Fourier transform infrared-reflection adsorption spectroscopy (FTIR-RAS) using a Spectrum100 (PerkinElmer, Inc., MA, USA) with an accumulation of 1000 interferograms. FTIR-RAS measurements were conducted using a Reflector 2 (Harrick Scientific, NY, USA) and a liquid-N_2-cooled MCT detector. The static contact angles of water in air and air in water on the substrate were measured at room temperature by a Drop Master 300 (Kyowa Interface Science, Saitama, Japan) and a DSA10-Mk2 (Krűss GmbH, Hamburg, Germany). Protein adsorption was measured using a BIAcore 3000 (GE Healthcare Bioscience). The atomic force microscope (AFM) images were measured by a tapping mode with a cantilever of the NCHV-10V using a D-3000, which is equipped with NanoScope IIIa (Veeco Instruments Inc., NY, USA).

Table 1
Contact angles of bare Au surface and dendrimer surface.

	Water (in air) (°)	Air (in water) (°)
Bare Au	60.6 ± 1.2	109.4 ± 4.6
G1	30.6 ± 0.7	131.3 ± 1.3
G2	31.3 ± 1.8	117.1 ± 8.3
G3	29.1 ± 0.8	128.3 ± 6.1
G4	28.8 ± 0.6	132.7 ± 2.4

2.2. Preparation of dendrimer SAMs

The gold substrates were cleaned by immersion into a piranha solution (concentrated H_2SO_4:H_2O_2 = 3:1), exposure to UV/ozone (UV. TC. NA. 003, Bioforce Nanoscience Inc., Ames, USA) for 30 min and subsequently rinsed with ultra-pure water and EtOH. The clean gold substrates were immersed into an aqueous solution of dendrimers (0.10 mM) for 12 h. The substrates were rinsed with ultra-pure water, and the solvent was removed under N_2.

2.3. Protein binding assay by SPR

Protein binding assays were performed by SPR using the BIAcore 3000 biosensor system. The analyses of proteins were performed using a HBS-N buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, GE Healthcare Bioscience). Analyses were performed on the chip surface by an injection of 0.1 mg/ml protein solution into flow cells at 25 °C at a flow rate of 20 μl/min for 1 min.

3. Results and discussion

The SAMs of dendrimers were easily prepared by immersing gold substrates in aqueous solutions of the dendrimer compounds [19,20]. The SAM formation was monitored by contact angle goniometry, FTIR-RAS and XPS. The water contact angle of the SAMs decreased from 61° to approximately 30° on each dendrimer substrate (Table 1). On the other hand, the air contact angle of the SAMs increased from 109° to approximately 125°, and the results indicated that the dendritic surface had moderate hydrophilicity. FTIR-RAS spectra showed the peaks from the dendrimers; the bands of the N–H vibration were observed around 3317 cm^{-1}, and the amide I and amide II emerged at 1680 and 1560 cm^{-1}, respectively (Fig. 2 (top)). These amide peaks increased as the generation number increased (Fig. 2 (bottom)). The XPS spectra of the dendrimers also indicated the SAM formation, which showed the shoulder peaks of C=O, C–N and C–O bands. The band intensities increased as the generation increased. XPS spectra and a detailed analysis are described in supplementary data. The results of the contact angles and FTIR-RAS were consistent with a previous report [19]. Particularly, the absorbance of the IR spectra was in accordance with previous reports demonstrating that dendrimers have densely packed monolayers on an Au surface. Spectroscopic analyses of XPS and FTIR-RAS showed that almost the same amount of molecules was detected on the surface of each generation of dendrimer. The surface geometry of the dendrimer SAM was measured by a tapping mode AFM and monitored as topographic and phase images (see supplementary data). The surface roughness of the dendrimer SAM increased as the generation increased in the topographic image. As seen from the topographic image, the spherical objects were densely packed on the substrate in the case of G3- and G4-

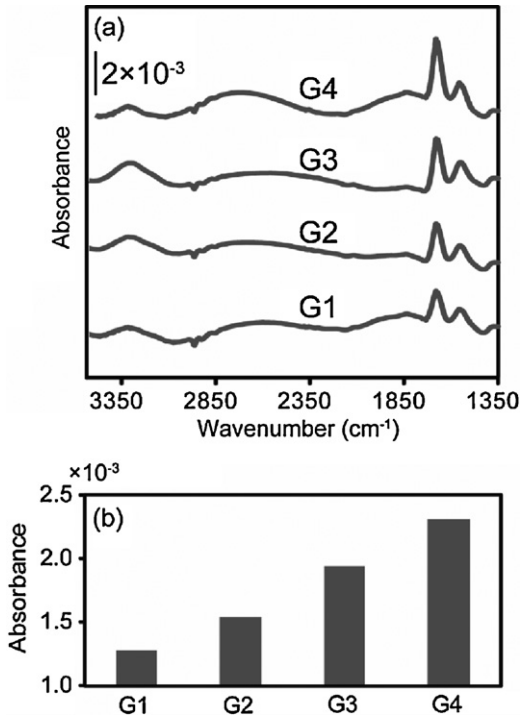

Fig. 2. (a) FTIR-RAS spectrum of dendrimer surface and (b) absorbance comparing at 1680 cm^{-1} in the amide I region.

dendrimers, although apparent images of G1- and G2-SAM were not observed due to both the difficulty of the measurement and the small size of the dendrimers [26]. On the G4-SAM, the molecules indicated a circular pattern with a diameter ca. 7.5 nm, which was much larger than the theoretical diameter of 4.4 nm [27]. These data indicate that dendrimer SAMs were successfully formed as an ordered polymer brush.

For the biological evaluation, the PAMAM dendrimer SAMs were evaluated by adsorption of three proteins (lysozyme, BSA and fibrinogen) using SPR (Table 2, Fig. 3 and supplementary data). The SPR curve with BSA and fibrinogen showed a gradual increase after injection, but that with lysozyme only showed a bulk effect with a small amount of adsorption [28]. The amount of protein bound (RU change) was observed to be in the order of fibrinogen > BSA > lysozyme in each generation of dendrimer SAM. For example, the amount of fibrinogen bound on the G4-SAM was over 50-fold larger than that of lysozyme. This adsorption behavior may be explained as follows: the PAMAM dendrimer on the surface had amine termini and most likely contributed to the brush formation with a cationic charge at the interface. Fibrinogen and BSA obtain a negative net charge at pH 7.4, which had a possibility of binding to the surface via an electrostatic interaction, while lysozyme with the positive charge did not bind [10,29]. Therefore, the amount of protein bound to the surface was highly affected by the electrostatic interaction.

Subsequently, the amount of protein bound was compared between the generations. Remarkably, the amount of all protein

Table 2
The amount of protein adsorbed on dendritic SAMs by SPR (ΔRU = change in refractive units).

	Au	G1	G2	G3	G4
Lysozyme (14.3, 10.9)[a]	136	129	58	26	11
BSA (69, 4.8)[a]	466	208	141	133	53
Fibrinogen (340, 6.0)[a]	2617	1576	1067	780	725

[a] MW (kDa) and p*I*.

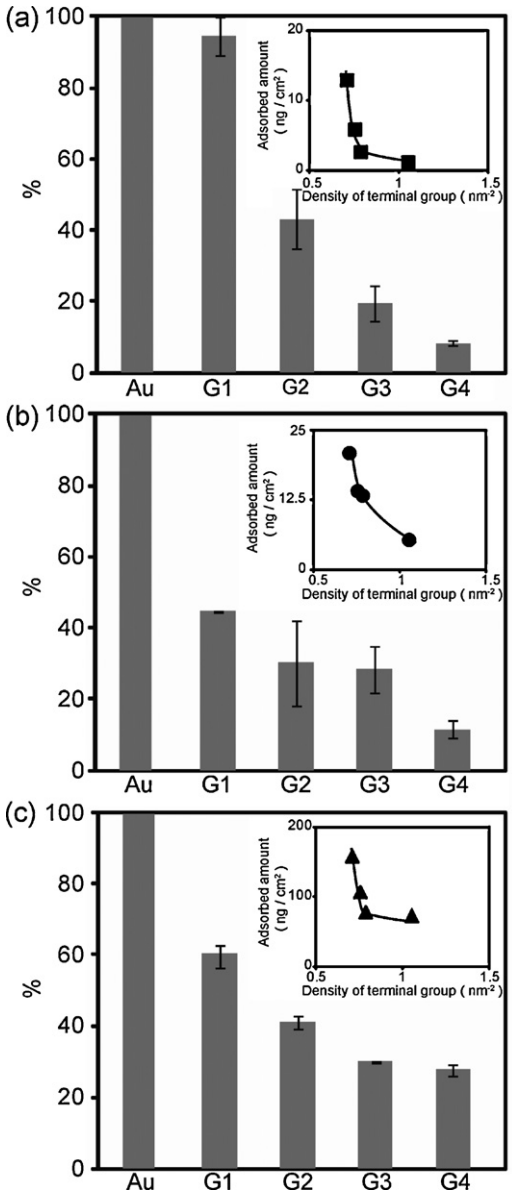

Fig. 3. The amounts of protein adsorption (RU) on dendrimer SAMs: (a) lysozyme, (b) BSA, and (c) fibrinogen. The amount of each protein bound on Au was set as 100%. Inset: chain density of terminal functional group *vs.* protein adsorbed amount.

bound decreased as the generation increased, regardless of the isoelectric point of the proteins; for example, there was more than a 12-fold difference in the amount of bound lysozyme between the G1- and G4-SAMs. This result indicates that the dendrimer brush surface with a high generation had higher bioinertness. The terminal group of the PAMAM dendrimer was amine, and the density of both the terminal group and the positive charge increased as the generation increased. Taking into account less adsorption of the proteins, there was a possibility that protein adsorption was more affected by the density of the terminal group than the electrostatic interaction between the protein and the dendrimer surface. The relationship between the amount of protein adsorption and dendrimer density of terminal group is illustrated in Fig. 3 (inset). A series of decay curves show a dependency on the increase of the density of terminal group in all proteins. These decay curves are similar to the curve on the hydroxyl group terminated PEG surface reported by Unsworth et al. [14], in which a dense polymer brush induced a repulsion force to the adsorbed proteins. This

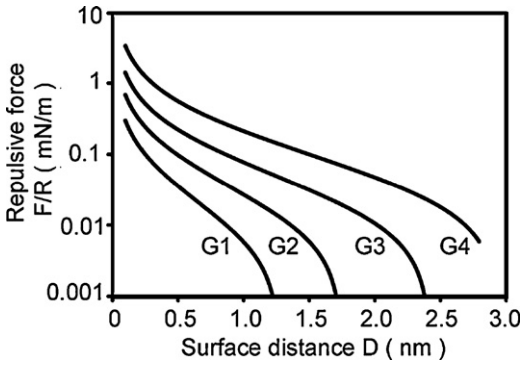

Fig. 4. Steric repulsion free energy on dendrimer surface calculated using Eq. (1) with parameters, (G1) L_0 = 1.9 nm, N = 4, s = 1.18 nm, and (G2) L_0 = 2.6 nm, N = 6, s = 1.15 nm, and (G3) L_0 = 3.6 nm, N = 8, s = 1.12 nm, and (G4) L_0 = 4.4 nm, N = 10, s = 0.975 nm.

correlation indicates that the PAMAM dendrimer surface had a higher density of terminal group and was highly hydrated. Therefore, the surface with the dendrimer exhibited bioinertness with respect to the various kinds of proteins evaluated, similar to the surface with the dense polymer brush, regardless of the isoelectric point. Other group had reported the less adsorption of protein such as cytochrome-*c* and fibronectin has been found at the highest grafting densities on PEG surface [30]. Our tendency of the correlation between density of terminal group and protein adsorption was corresponded to the previous report, and therefore our system was expected to represent same trends in other protein adsorption behavior.

Interactions between proteins and surfaces include hydrogen bonding, hydrophobic interaction, electrostatic interaction and steric repulsion, which closely relates to chain density [14]. The surface with higher steric repulsion was obtained by a surface modification with a dense polymer brush. The dendrimers have high density of terminal group and thus act as a building block of a precise dense polymer brush with a molecule. To evaluate the dendrimer surface as a dense polymer brush, the repulsive energy on the dendrimer surface was calculated using Eq. (1) (Fig. 4). Bioinertness, based on the effect of polymer density, was predicted by de Gennes et al. [15], which was estimated by a presumptive calculation of intermolecular repulsion between the surface with the polymer brush and particles using Eq. (1) [16,31]. Dendrimer SAMs act like polymer brush, and the densities of the polymer brushes are controlled by the generation of dendrimers [21,22]. We approximately regarded dendrimer SAMs as one of the polymer brush because Eq. (1) is composed of common parameters and similar parameters, such as the equilibrium layer thickness and the distance between the terminal groups, though Eq. (1) was originally applied to the interface with linear polymer brush. Therefore, we calculated this model equation to study the effect of polymer density with the structural parameter of the dendritic brush such as the parabolic segment density (Fig. 4). F/R (mN/m) indicates repulsive energy on the dendrimer surface when object close to surface, which is expressed as surface distance (D).

$$\frac{F(D)}{R} = 2\pi E(D) = -2\pi \int p(D)\ \mathrm{d}D = 4\pi P_0 \left[\frac{L_0}{D} + \left(\frac{D}{L_0}\right)^2 - \left(\frac{D}{L_0}\right)^5 - \frac{9}{5}\right] \quad (1)$$

$$P_0 = \frac{kTN}{2}\left(\frac{\pi^2}{12}\right)^{1/3}\frac{a^{4/3}}{s^{10/3}}$$

Each parameter is indicated as follows: F is the surface force, which is dependent on surface distance (D); R is the radius of the particle surface; L_0 is the dendrimer SAM thickness; P_0 is the pressure between the surfaces; k is the Boltzmann constant; T is the temperature (298 K); N is the number of branches; a is the length of branch (0.7 nm); and s is the distance of the terminal group. In Fig. 4, the repulsive energy F/R (mN/m) indicates that the energy increased as the generation of the dendrimer increased. The calculation corresponded to our experimental result, and these curves were similar to the curve of the dense polymer brush.

4. Conclusions

PAMAM dendrimers provided the SAMs with high coverage, which can be regarded as well-defined polymer brushes. The proteins bound to the dendrimer SAMs depended on the generation of dendrimers and the type of proteins. The amount of protein bound decreased with the generation of dendrimers, indicating the bioinertness of the dendrimer SAMs with a higher generation. The bioinert surface properties of the dendrimers were based on the steric repulsion of the dendrimers due to the dense brush structures. We believe that the dendrimer interface contributes to the bioinert properties of an interface, and thus, the bioinert dendrimer interface of phosphorylcholine groups with stronger bioinertness is under investigation for future studies.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (20106003). We thank Prof. Shinichi Ikushiro (Toyama Pref. Univ.) for the SPR measurement and Dr. Tatsuya Murakami (JAIST) for the XPS measurement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.colsurfb.2011.01.003.

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