Protein−Ligand Interactions at Poly(amidoamine) Dendrimer Monolayers on Gold

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Avidin−biotin interactions as a typical protein−ligand model were investigated on the monolayers of a fourth-generation poly(amidoamine) dendrimer that were constructed on the self-assembled monolayers (SAMs) of 11-mercaptoundecanoic acid (MUA) on gold. Surface plasmon resonance (SPR) spectroscopic analysis revealed a resonance angle shift of 0.34° ± 0.03° for the formation of dendrimer monolayers on reactive SAMs, which indicates that about 89% of the gold surface is covered with dendrimer molecules. The dendrimer monolayers were functionalized with biotin, and the efficacy of dendrimer monolayers as a biomolecular interface was evaluated in terms of the surface density of biotin ligands and the avidin binding level. For comparisons, the mixed SAMs and polymeric layers of poly-L-lysine (PLL) on MUA SAMs were prepared and examined by a similar procedure. The specific binding of avidin to the biotinylated dendrimer monolayers approached a surface density of 5.0 ± 0.2 ng·mm^{-2}, which corresponds to about 88% surface coverage by avidin, showing a much higher level than those from mixed SAMs (2.3 ± 0.1 ng·mm^{-2}) and PLL layers (3.2 ± 0.2 ng·mm^{-2}). Interestingly, the fully biotinylated dendrimer monolayers gave rise to efficient avidin−biotin interactions, resulting in about 80% of the maximum avidin binding level, even under the condition that a serious steric hindrance would occur due to densely packed biotin ligands. These results strongly imply that efficient avidin−biotin interaction originates from a structural feature of dendrimer monolayers such as a surface exposure of derivatized biotin ligands and a corrugated surface.

Introduction

Along with gene chip technology,[1] significant efforts have been devoted to identifying the function of gene products (proteins) by analyzing the complementary recognition processes such as protein−protein,[2] protein−DNA,[3] protein−ligand,[4] and protein−RNA interactions.[5] Recently, a variety of attempts related to protein chip based approaches have been reported,[6] but several issues still remain to be addressed in the development of viable chips: the immobilization/functionalization of biomolecules with a high density on a solid surface is required. In addition, steric hindrance and nonspecific protein binding should be minimized for a sensitive detection of biospecific interactions. With similar viewpoints, the development of biosensors and biochips has been intensively pursued to date by employing well-defined composite films such as alkanethiolate-based self-assembled monolayers (SAMs)[7] and silane-based SAMs,[8] Langmuir−Blodgett,[9] and polymers[10] as the biomolecular interface. In these approaches, introduction of the versatile chip interfaces and the strategy for controlled immobilization of biomolecules onto the interfaces, forming molecularly organized layers with preserved biological activity and minimized nonspecific protein adsorption, have been of great interest.

Keeping pace with the current trends, highly branched dendritic macromolecules (dendrimers) have been recognized as promising building units of molecularly organized nanostructures. Crooks and colleagues reported the construction and characterization of the dendrimer monolayers on metal surfaces using various techniques and proposed their versatile potential for technological applications.[11,12] Benters et al. presented the use of poly(amidoamine) dendrimers as a linker system on a glass surface and fabrication of the dendrimer-based DNA microarray for the analysis of single nucleotide polymorphisms.[13] We have recently reported the construction of

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molecularly organized biocomposites by taking advantage of unique properties of dendrimers. The molecularly organized layers of fourth-generation poly(amidoamine) (PAMAM) dendrimers on reactive SAMs, which was devised by the Crooks group, were successfully employed as building units[14] and affinity biosensing interfaces.[15]

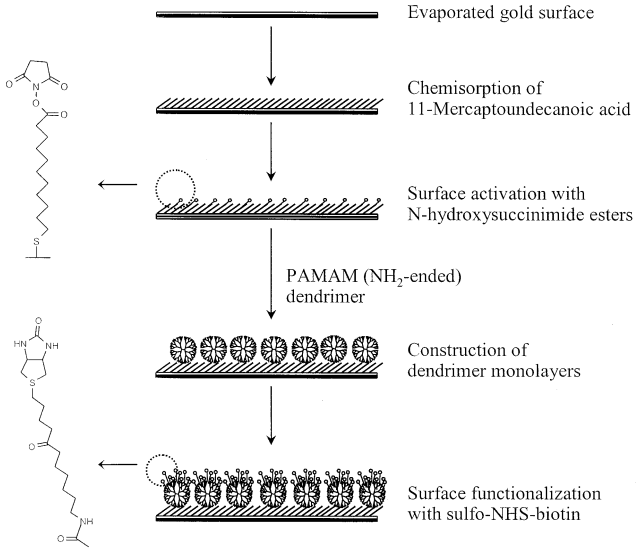
In this paper, the efficacy of the dendrimer monolayers as a biomolecular interface was evaluated for avidin−biotin interactions as a typical protein−ligand model. The dendrimer monolayers were functionalized with different concentrations of biotin, and the avidin association level as a function of the surface density of biotin was investigated by using optical methods of UV/vis spectroscopy and surface plasmon resonance spectroscopy (SPR). Other biomolecular surfaces including various SAMs and poly-L-lysine layers were also tested using a similar procedure and compared with the dendrimer monolayers. Details are reported herein.

Materials and Methods

Chemicals and Reagents. The fourth-generation PAMAM dendrimer manufactured by Dendritech, Inc. (Midland, MI) was purchased from Aldrich. Poly-L-lysine (PLL) (MW 15−30 kDa), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), and biotinyl-ϵ-amidocaproic acid *N*-hydroxysulfosuccinimide ester (sulfo-NHS-biotin) were purchased from Sigma. Cystamine dihydrochloride, 11-mercaptoundecanoic acid (MUA), 16-mercaptohexadecanoic acid, 11-mercaptoundecanol, 2,2′-(ethylenedioxy)bis(ethylamine), pentafluorophenol, and 4-nitrobenzaldehyde were used as received from Aldrich. *N*-Hydroxysulfosuccinimide (sulfo-NHS), biotinyl-3,6,9-trioxaundecanediamine (biotin-PEO-LC-amine), and immunopure avidin were obtained from Pierce. All other reagents used were of the highest quality available and purchased from the regular source. Doubly distilled and deionized water with a specific resistance over 18 MΩ·cm was used throughout the work. For the buffer solutions, a phosphate buffered saline solution containing 10 mM phosphate, 17 mM KCl, 138 mM NaCl, and 0.05% (v/v) Tween 20 (PBST, pH 7.4) and a triethanolamine (TEA) buffer (50 mM, pH 8.0) with 0.25 M NaCl were used.

SPR Analysis of Dendrimer Layer Formation and Avidin−Biotin Interaction. All the SPR measurements were performed with Biacore-X instrument (BIAcore) and a commercial sensor chip (Pioneer J1, BIAcore) with an evaporated gold surface. The chip surface was first cleaned with 0.1 N NaOH containing 1% Triton-X for 5 min. According to the procedure depicted in Scheme 1, PAMAM dendrimers and PLLs were covalently attached to the activated MUA SAMs on the gold surface by batch reaction, which is basically identical to the methods reported by the Crooks and Corn groups.[12,16] Briefly, the SAMs were prepared from the chemisorption of 11-mercaptoundecanoic acids (MUA, 2 mM) representing reactive carboxylate groups in ethanol for 2 h on a gold surface. After SAM formation and ethanol rinsing, terminated carboxylate groups of chemisorbed MUA SAMs on gold were activated with the aqueous solution containing EDAC (0.4 M) and sulfo-NHS (0.1 M) to give the amine-reactive ester groups for 1 h. After rinsing with deionized water, the gold substrate was transferred to a methanolic solution of dendrimer (22 μM, based on the primary amine concentration) or aqueous solution of PLL (2 mg/mL, in TEA buffer). After reaction for 2 h, the gold substrate was incubated with bicarbonate buffer (0.1 M, pH 9.5) for 30 min to hydrolyze the remaining reactive esters and dissolve out adsorbed molecules as described elsewhere.[17] The dendrimer coupling to the MUA-modified chip surface was conducted by batch reaction after undocking the chip, because a microfluidic part of the Biacore instrument is not compatible with an organic solvent. After the coupling reaction, the dendrimer-modified chip was redocked and a shift in the SPR angle before and after dendrimer layer formation was measured. To confirm that docking/undocking of the sensor chip has no significant effect on the reproducibility of SPR data, we also conducted the SPR measurements for all the processes including dendrimer coupling and avidin interactions in situ by using Spreeta (Texas Instruments Co., Dallas), a commercial SPR-sensing system. In this case, a rubber flow cell system was used, which enabled in situ SPR measurements even for the dendrimer coupling reaction in methanolic solution. Biotinylation was carried out by passing the aliquots of sulfo-NHS-biotin in bicarbonate buffer (0.1 M, pH 9.5) over the sensing surface. Under this reaction condition, the coupling reaction of NHS groups in the biotin reagent with amine-terminated dendrimers proceeds very fast and is completed in the range of minutes as reported elsewhere.[18] Accordingly, the effect of hydrolysis of sulfo-NHS-biotin itself on the coupling reaction seems to be negligible. The biotin reagent with a specified concentration was prepared by a proper dilution with deionized water before treatment, thoroughly mixed with the same volume of bicarbonate buffer, and immediately injected into a gold surface. After biotinylation, the running solution was sequentially changed into deionized water.

Scheme 1. Schematic Depiction of Monolayer Formation of Fourth-Generation PAMAM Dendrimer and Surface Functionalization with Biotin Ligands



For the mixed SAMs, the gold surface was chemisorbed with the ethanolic solution (final concentration, 2 mM) containing 11-mercaptoundecanol and 16-mercaptohexadecanoic acid in a 12:1 ratio that was adopted from the experimental data by the Campbell group.[19] The resulting surface was activated to give the reactive ester groups and derivatized with aliquots of biotin-PEO-LC-amine according to procedures similar to Scheme 1. The cystamine SAMs were constructed by chemisorption of cystamine dihydrochloride onto a gold surface and modified with sulfo-NHS-biotin. As for the extended layers of SAMs, 2,2-(ethylenedioxy)bis(ethylamine) was covalently attached to the activated MUA SAMs on gold and biotinylated by using the same procedure.

To analyze the avidin binding, the biotinylated surfaces were prerinsed with PBST for 10 min, and the avidin solution (50 μg/mL, in PBST) was injected with a flow rate of 1 μL·min^{-1} for 1.5 h. After washing with PBST, a stream of deionized water was followed, and a relative SPR angle shift for avidin association was estimated. To evaluate nonspecific adsorption of avidin, two approaches were attempted. First, a solution of avidin was injected over the polymeric layers that had not been biotinylated,

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and second, prebound avidin with biotin was loaded onto the biotinylated polymeric layers. Avidin binding reaction continued for 30 min, in which time biospecific association of avidin reached more than 95% of the maximum level. Then the surfaces were washed with PBST for 20 min to eliminate reversibly bound avidin, followed by a stream of deionized water, and the angle shifts by nonspecific avidin binding were measured.

Determination of the Surface Density of Functionalized Biotin Ligands. To estimate the surface densities of amine groups and biotin ligands, a freshly evaporated gold surface was prepared by the resistive evaporation of Au (200 nm) onto titanium-primed (20 nm Ti) Si[100] wafers. Prior to the layer-forming process, the gold-coated substrate (25 mm × 25 mm) was cleaned for 5 min by piranha solution (1:4 = 30% H_2O_2: concentrated H_2SO_4 (v/v)).

CAUTION: *Piranha solution reacts violently with most organic materials and must be handled with extreme care.*

As depicted in Scheme 1 and described above, the PAMAM dendrimers and PLLs were covalently attached to the activated MUA SAMs on a gold surface. Each layer was biotinylated by adding an aqueous solution of sulfo-NHS-biotin with a specified concentration to the gold substrate immersed in bicarbonate buffer solution (0.1 M, pH 9.5). After 30 min, the biotinylated gold substrate was rinsed with deionized water, followed by thorough washing with anhydrous ethanol, and dried with argon gas.

Titration of free amines at the chemically modified gold surface was performed according to the spectroscopic method of Moon et al.[20] Briefly, 4-nitrobenzaldehyde (4-NB) is used to completely react with free amines through imine formation, and the amount of amine groups can be determined by analyzing the released 4-NB after acid hydrolysis. In this work, biotinylation was performed after the corresponding layers on a gold surface were formed, and the surface density of biotin ligands could be estimated by assaying the amount of amine groups before and after biotinylation of the layers. The differently treated substrates were immersed into anhydrous ethanol containing 4-NB (1 mg/mL) and acetic acid (0.02 mL) at 50 °C for 6 h under argon atmosphere. After the condensation, the substrates were washed with and sonicated in absolute ethanol for 1 min, and finally dried with argon gas. The imine-coupled 4-NB was hydrolyzed by incubating the substrates with water (1 mL) containing acetic acid (0.02 mL) and heating the aqueous solution at 30 °C for 1 h. The hydrolyzed solution was sampled, and the absorbance of 4-NB was measured at 265 nm. The concentration of 4-NB was determined by using the extinction coefficient ($\epsilon_{max} = 1.45 \times 10^4$ $M^{-1}\cdot cm^{-1}$ in water or 0.2% acetic acid).

Results and Discussion

Characterization of Dendrimer Layers on Reactive SAMs. According to the methods devised by the Crooks group[12] and also applied in our previous works,[14,15] the layers of G4 PAMAM dendrimers were made by covalent coupling of the amine groups of dendrimers with the activated MUA SAMs presenting the amine-reactive ester groups on the chip surface. In these approaches, formation of closely packed dendrimer monolayers on the gold has been successfully characterized by analyzing the thickness and coverage of the ferrocenyl-modified dendrimer monolayers. In the present work, formation of dendrimer monolayers was alternatively confirmed by using SPR spectroscopy. We have noticed that when G4 PAMAM dendrimer was deposited on a flat surface, it has a thickness of 26 ± 2 Å with a substantial distortion,[14a] which is well coincident with the data by Tokuhisa et al.[11] Geometric calculations revealed that a monodisperse and spherical G4 PAMAM dendrimer in a monolayer format has a packing density similar to that of a globular protein. With these viewpoints, we reasoned that the surface coverage of immobilized dendrimers on a MUA-modified sensor chip can be calculated from a SPR angle shift. This estimation is based on the generally accepted and used assumption that 0.1° of angle shift in SPR sensorgram corresponds to 1 $ng\cdot mm^{-2}$ mass change at the sensing surface.[21] The maximum amount of molecules that can be immobilized in a defined area ($ng\cdot mm^{-2}$), M, can be theoretically estimated based on the geometric property and mass of the immobilized molecules on the surface as follows.[7c]

$$M = \frac{10^{14}/\pi r^2}{10^{-9}(N_A/\mathrm{MW})}$$

where r, MW, and N_A represent the radius (Å) and molecular weight of a molecule and Avogadro's number, respectively. Consequently, the surface fractional coverage by dendrimers is calculated by dividing the mass change caused by immobilized dendrimers on the chip surface ($ng\cdot mm^{-2}$) by M.

In a series of measurements using two SPR-sensing systems (BIAcore and Spreeta), it was found that the angle shifts by two systems are approximately equivalent. From the measurements using Biacore-X, the SPR angle shift for formation of dendrimer layers was observed to be 0.34 ± 0.03°, and this value roughly corresponds to a mass change of 3.4 $ng\cdot mm^{-2}$ and coverage of 2.39×10^{-11} $mol\cdot cm^{-2}$. This estimation is in good agreement with dendrimer coverage of 2.25×10^{-11} $mol\cdot cm^{-2}$, which had been calculated from the ferrocene density integrated via cyclic voltammetry for the ferrocenyl-modified dendrimer monolayers.[14b] From the above equation, the surface coverage by dendrimers was estimated to be 89%, which coincides well with those reported in our previous work[14b] and elsewhere.[11] This result also strongly implies that dendrimers yield densely packed monolayers on reactive SAMs and the surface fractional coverage by immobilized dendrimers can be analyzed by SPR spectroscopy. In addition, it is known that dendritic polymers can cover some surface defects on SAMs, and the use of dendrimers might offer an advantage for construction of monolayers on reactive SAMs due to the unique chemical and structural properties.

Quantitative Analysis of Biospecific Interaction on the Dendrimer Monolayers. Based on the observations that dendrimers form compact monolayers on reactive SAMs, particularly yielding coverage of around 89% in the SPR study, we reasoned that dendrimer monolayers can be used as a platform for further functionalization and biospecific interactions. To test the dendrimer monolayers in the aspects of functionalization level of ligands and surface coverage of biospecifically associated proteins, an avidin−biotin couple was employed as a typical example of protein−ligand interactions. For this, the dendrimer monolayers on reactive SAMs were biotinylated as described under Materials and Methods, reacted with avidin, and analyzed with SPR spectroscopy.

First, we confirmed the occurrence of biospecific interaction between avidin molecules and biotin ligands on the dendrimer monolayers. In a series of experiments, interaction of avidin with biotinylated dendrimer monolayers resulted in an angle shift of 0.5° in the SPR analysis as seen in a typical sensorgram (Figure 1). To verify that the avidin molecules were specifically associated with biotin ligands, two control experiments were conducted. The dendrimer monolayers modified with 0.1 mg/mL biotin reagent were reacted with a solution of avidin that had

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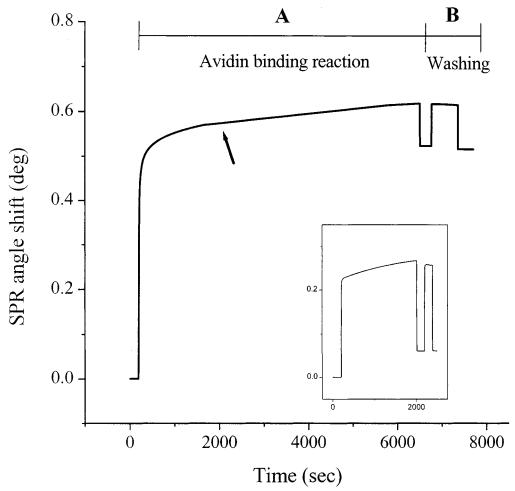

Figure 1. SPR sensorgram for specific binding of avidin and nonspecific adsorption of avidin (inset) to the biotinylated dendrimer monolayers. Dendrimer monolayers onto reactive SAMs that had been biotinylated with sulfo-NHS-biotin (1 mg/mL) were subjected to avidin binding reaction (50 μg/mL, in PBST) with flow rate of 1 μL/min (A). The extent of nonspecifically adsorbed avidin was measured after prebound avidin with biotin was added to the biotinylated surface for 30 min (arrow), in which time specific binding of avidin reached a level higher than 95% of a maximum change. After the avidin reaction, the avidin-bound surface was washed with PBST to eliminate the reversibly bound avidin (B), followed by a stream of deionized water, and SPR response was recorded.

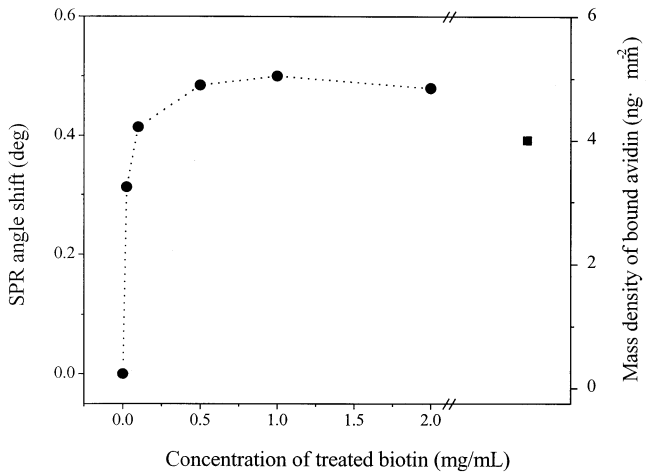

Figure 2. Specific binding level of avidin on the dendrimer monolayers that had been biotinylated by using different concentrations of sulfo-NHS-biotin in the reaction mixture. The avidin binding level was obtained from SPR angle shift. The symbol (■) represents the SPR angle shift when the fully biotinylated dendrimer monolayers were used as described under Results and Discussion.

been presaturated with biotin, and the relative change in the SPR angle due to nonspecific adsorption of avidin was estimated for 30 min compared to the specific binding of avidin (arrow in Figure 1). As can be seen in the inset of Figure 1, the nonspecific adsorption level by avidin was about 12% relative to the specific avidin binding. When a solution of avidin was added to the dendrimer monolayers that had not been biotinylated, a similar angle shift was also observed. These observations indicate that the registered angle shift came mainly from the biospecific avidin−biotin interaction. The repulsive interactions between positive surface charges of the dendrimer layers (pK_a value of ∼9.5) and avidin molecules (pI value of ∼10) under the reaction condition (pH 7.4) might decrease the probability of nonspecific avidin adsorption.

To evaluate the binding event on the biotinylated dendrimer monolayers in more detail, the association level of avidin was quantitatively analyzed with respect to the amount of biotin ligands on the layers. The preformed dendrimer monolayers were biotinylated with different concentrations of the sulfo-NHS-biotin reagent, and reacted with avidin (50 μg/mL, in PBST). As shown in Figure 2, the association level of avidin radically increased with respect to the concentration of treated biotin when the surface was reacted with a low concentration of sulfo-NHS-biotin (<0.5 mg/mL), and reached a maximum coverage slowly due to a steric limitation. Then the avidin coverage slightly declined with an increase in the concentration of sulfo-NHS-biotin. Maximum mass density of avidin (∼5 ng·mm^{-2}) was observed when the dendrimer monolayers were functionalized with a biotin concentration of 1 mg/mL. When considering the dimension (40 × 55 × 55 Å) and molecular weight (67 kDa) of the avidin molecule, mass density of 5 ng·mm^{-2} corresponds to about 88% surface coverage by avidin from the calculation using the aforementioned equation. This indicates that the biotinylated dendrimer monolayers are almost completely covered with avidin molecules through specific avidin−biotin interaction.

Table 1. SPR Angle Shift by Specific Binding of Avidin to Various Layers

layer	angle shift[a] (deg)	surf. coverage[b] (%)
PAMAM dendrimer (G4)	0.50 ± 0.02	88 ± 4
poly-L-lysine (MW 15−30 kDa)	0.32 ± 0.02	56 ± 4
mixed SAMs	0.23 ± 0.01	41 ± 2
cystamine SAMs	0.18 ± 0.01	32 ± 2
2,2-(ethylenedioxy)bis(ethylamine)	0.18 ± 0.01	32 ± 2

[a] The binding of avidin was measured after biotinylation under the condition yielding a maximum avidin binding for the corresponding layers. Data represent the mean and deviation of duplicate experiments. [b] The surface coverage by avidin was calculated based on the equation described under Results and Discussion.

For comparisons, the PLL layers[22] and mixed SAMs,[19,23−24] which have been extensively studied by a number of approaches, were chosen and investigated in the same aspects. In addition, cystamine SAMs and extended layers of 2,2-(ethylenedioxy)bis(ethylamine) modified to MUA SAMs, which were also expected to form the molecularly organized layers presenting the surface amine groups, were tested. First, PLL layers were formed by a covalent attachment onto reactive SAMs according to the method proved by Corn and colleagues.[16] Then, the resulting layers were biotinylated by using the aqueous solution of sulfo-NHS-biotin with different concentrations, and reacted with a solution of avidin as described above. Maximum binding of avidin was observed when the PLL layers were modified with the biotin reagent of 1 mg/mL in the reaction mixture. The trend in the avidin association level with respect to the biotin concentration was similar to the dendrimer monolayers. However, the maximum mass density of avidin was reduced to about 64% level (3.2 ng·mm^{-2}) of the dendrimer monolayers (Table 1). As the concentration of treated biotin gradually increased to >1 mg/mL, the avidin binding level slightly declined to ∼3 ng·mm^{-2} and maintained. By similar procedures,

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Table 2. Surface Densities of Amine Groups and Biotin Ligands

structural element of interfaces	biotin treatment[a]	SPR angle shift for avidin binding (deg)	absorbance[b] (cm^{-2})	density of amines[c] (100 $Å^{-2}$)	density of derivatized biotin[d] (100 $Å^{-2}$)
PAMAM dendrimer (G4)	nt		0.0074 ± 0.0002	3.08 ± 0.08	
	A	0.50 ± 0.02	0.0041 ± 0.0001	1.70 ± 0.04	1.38 ± 0.04
	B	0.40 ± 0.02	0.0010 ± 0.0001	0.04 ± 0.04	3.04 ± 0.04
poly-L-**lysine**	nt		0.0108 ± 0.0017	4.49 ± 0.71	
	A	0.32 ± 0.02	0.0059 ± 0.0004	2.46 ± 0.17	2.03 ± 0.17

[a] nt, not treated; A, sulfo-NHS-biotin concentration in the reaction mixture was 1 mg/mL; B, biotinylation was repeated three times using a 2 mg/mL sulfo-NHS-biotin solution. [b] The polymeric layers were formed on a gold surface (25 mm × 25 mm) as described under Materials and Methods, and then reacted with 4-NB. After acid hydrolysis, the absorbance was measured at around 265 nm and divided by the surface area of the respective layers. Data represent the mean and deviation of duplicate tests. [c] Calculated from the absorbance of hydrolyzed 4-NB using the extinction coefficient ($\epsilon_{max} = 1.45 \times 10^4$ $M^{-1}\cdot cm^{-1}$ in water or 0.2% acetic acid) for 4-NB. [d] Estimated by assaying the amount of amine groups before and after biotinylation of the corresponding layers.

avidin binding was analyzed for the mixed SAMs consisting of 11-mercaptoundecanol and 16-mercaptohexadecanoic acids as described under Materials and Methods. The avidin association level at the mixed SAMs was about 2.3 ng·mm^{-2}, which corresponds to about 46% of that from the dendrimer monolayers. This level is comparable to that of optimum streptavidin binding in the mixed SAMs reported by Campbell and colleagues.[19] On the other hand, when cystamine SAMs and extended layers of 2,2-(ethylenedioxy)bis(ethylamine) were employed, SPR angle shifts for avidin association were relatively low compared to other systems, showing about 36% of that from the dendrimer monolayers under the experimental conditions yielding a maximum avidin binding (Table 1). From these results, it is evident that the biotinylated dendrimer monolayers led to a higher avidin-binding level, reaching a full coverage, compared to other tested layers. Known by the approaches having dealt with SAMs and polymeric layers[19,22−25] was that the association level of strept(avidin) is significantly affected by the surface density of biotin ligands on the layers. In general, the strept(avidin) binding level is known to be proportional to the surface density of biotin ligands when biotin density is very low, and to drop beyond a certain level of biotin density mainly due to steric hindrance by preoccupied strept(avidin) molecules. Recently, Ruiz-Taylor et al. noted that the surface coverage by streptavidin linearly increases within a low biotin concentration up to 70 pmol·cm^{-2} when biotin-derivatized and PLL-*graft*-poly(ethylene glycol) copolymers were adopted.[25]

To understand and get some insights into a high coverage by avidin and the steric effect in the avidin binding on the biotinylated dendrimer monolayers, surface densities of reactive amine groups and functionalized biotin ligands were estimated from the dendrimer and PLL layers by using a spectroscopic analysis method.[20] Due to a low molecular weight of biotin, estimation of the amount of derivatized biotin by using SPR analysis is impossible, and the surface density of biotin ligands was spectroscopically determined. In this procedure, 4-NB is used to completely react with free amines through imine formation, and the amount of amine groups can be determined by analyzing the concentration of released 4-NB after acid hydrolysis. In this work, biotinylation was performed after the corresponding layers were formed on a gold surface, and surface density of biotin ligands was estimated by assaying the amount of amine groups before and after biotinylation of the layers. As shown in Table 2, the dye-labeling assay, which has been used for the determination of surface amine density on a variety of modified surfaces,[26] was sensitive and reliable. The MUA SAM modified surfaces without amine groups as a blank test showed no peak at the specified wavelength (data not shown). As mentioned above, a maximal binding occurred when sulfo-NHS-biotin concentration of ∼1 mg/mL was used for biotinylation. Thus, surface density of biotin ligands was determined for the layers that had been biotinylated under this condition. To assess the maximum density of biotin ligands, the corresponding layers were modified by using a high concentration of sulfo-NHS-biotin (more than 2 mg/mL) and successive biotinylation reactions (three times). By consecutive treatments, dendrimer monolayers were found to be fully biotinylated under the above-mentioned reaction conditions (vide infra). From the spectroscopic analysis of released 4-NBs after hydrolysis, the densities of free amine groups and biotin ligands were reliably estimated from the dendrimer and PLL layers. The association level of avidin was additionally measured by using SPR spectroscopy.

As shown in Table 2, the densities of freely accessible amine groups were estimated to be 3.04 and 4.49 per 100 $Å^2$ for the dendrimer and PLL layers, respectively, by dividing the total number of amine groups by the area of gold surface. The number of reactive amine groups per dendrimer molecule was determined to be about 21 by taking into consideration the surface density of the dendrimer monolayers. The calculated value indicates that about 33% of 64 amine groups per dendrimer molecule is available for further functionalization. When considering that dendrimers were assembled in a molecularly organized and closely packed manner as described above, this estimate is likely to be reasonable. From the surface amine density, the surface area occupied by an amine group in the PLL layers was calculated to be 22 $Å^2$, which is in good accordance with that estimated by Frey et al.[22] Next, under the optimized biotinylation condition resulting in a maximal binding of avidin, the surface density of biotin ligands for the dendrimer monolayers was about 1.38 per 100 $Å^2$, showing a lower biotin density than for the PLL layers (2.03/100 $Å^2$). Nonetheless, the dendrimer monolayers displayed a much higher avidin binding level (5 ng·mm^{-2}) compared to the PLL layers (3.2 ng·mm^{-2}). This phenomenon might be explained by the structural features of adopted layers. The G4 PAMAM dendrimer has a spherical shape and most biotin ligands might be surface exposed, thus becoming easily accessible by avidin molecules. On the other hand, PLLs of a linear polymer typically form entangled layers, and biotin ligands located inside the polymeric networks are not easily accessible by avidin molecules; consequently the surface-exposed por-

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tion of biotin ligands might be relatively low. From these observations, it is plausible that the density of surface-exposed biotin ligands is one of the crucial factors leading to efficient biospecific interaction on a solid surface. In this sense, the dendrimer monolayers confer a merit as platforms for protein−ligand interactions. Recently, Benters et al. reported an interesting observation regarding the property of dendrimer layers.[13] The layers of PAMAM dendrimers (G4) resulted in a very high immobilization efficiency for amino-tethered DNA oligomers and consequently nearly a 10-fold higher fluorescence signal for detecting the hybridization than the PLL-coated surfaces.

In general, the association level of strept(avidin) significantly decreases due to steric hindrance on the layers presenting a high surface density of biotin ligands.[23−25] As seen in Figure 2, however, the dendrimer monolayers exhibited a tendency that the binding level of avidin is maintained even though the concentration of treated sulfo-NHS-biotin increased. In an effort to evaluate the steric effect in the avidin binding, we fully derivatized the dendrimer monolayers with biotin ligands as described above and investigated the surface coverage by avidin. Interestingly, although the surface density of biotin ligands reached a saturated level of 3.04 per 100 Å^2 as shown in Table 2, the biotinylated dendrimer monolayers still displayed a high avidin-binding level of about 4 ng·mm^{-2} that corresponds to 80% of a maximum binding level (5 ng·mm^{-2}). It was reported by Ruiz-Taylor et al. that streptavidin binding reaches a maximum level of 6.3 pmol·cm^{-2} (corresponding to 0.42° angle shift by avidin binding) on the surface presenting a low range of biotin density (∼0.42/100 Å^2), causing no steric hindrance in the streptavidin binding.[25] In this regard, an angle shift of 0.4° by avidin obtained even from the fully biotinylated dendrimer monolayers strongly implies a benefit from the unique structural feature of dendrimers.

From the above results, it is evident that dendrimer-assisted surfaces exhibit different features in avidin−biotin interactions compared to the commonly used systems. Comparison of avidin−biotin interactions at the dendrimer monolayers with the well-characterized mixed SAMs[19,24] is expected to provide a clue to this presumption. Pérez-Luna et al. constructed the mixed SAMs by using the solution of thiolates and biotinylated thiolates on the gold surface, and investigated the binding level of streptavidin as a function of the mole fraction of a biotinylated thiolate; the coverage by streptavidin reached a maximum when the mole fraction of biotinylated thiolates was 0.15, and then declined seriously due to steric limitation. An average density of biotin occupied by a streptavidin molecule was estimated to be 18 biotin groups, when maximum streptavidin binding occurred, by taking into consideration the predicted projection area of streptavidin (∼2500 Å^2).[24] In the case of dendrimer monolayers, an average density of biotin per avidin molecule was found to be about 35 when a maximal binding of avidin was achieved. On the mixed SAMs, the surface density of 35 biotin ligands gave rise to a slightly reduced avidin binding from the maximum binding level. However, the dendrimer monolayers led to an avidin association level twice as much as the mixed SAMs, even though the surface density of biotin ligands on the dendrimer layers was 2-fold higher than on the mixed SAMs. Furthermore, the fully biotinylated dendrimer monolayers having an average density of 76 biotin groups per 100 Å^2 exhibited about 80% of maximum avidin-binding level, and this level was also much greater than a maximum level observed from the mixed SAMs.

The above observations and comparisons strongly suggest that specific binding of avidin to the biotinylated layers is affected not only by the surface density of freely accessible biotin ligands but also by the structural feature of the layer surface. As mentioned earlier, the G4 PAMAM dendrimer has a well-defined globular structure, and the resulting dendrimer monolayers on reactive SAMs might exert a corrugated architecture, displaying a distinctively different surface structure from the SAM-based surfaces as proposed and observed by other groups.[12,13] Thus, a corrugated structure of the dendrimer monolayers seems to enable an efficient avidin−biotin association, resulting in a high avidin-binding level, even under the condition that serious steric hindrance would occur due to densely packed biotin ligands. In these regards, the need to carefully control the surface density of ligands at the dendrimer monolayers to maintain high binding levels might be eliminated. The dendrimer monolayers showing the above-mentioned features are expected to be applied as the interfacing layer to various solid substrates such as metals, silicon, and glass.

Conclusions

We have demonstrated that the monolayers of PAMAM dendrimer (G4) on reactive SAMs offer several advantages as the biomolecular interface for achieving avidin−biotin interactions on a solid surface over commonly used SAMs and polymeric layers. From the SPR study, it was revealed that the dendrimer monolayers on reactive SAMs are effective as platforms for biotin functionalization and consequent avidin−biotin interactions. The biotinylated dendrimer monolayers led to higher association level of avidin than the PLL layers and various SAMs, resulting in about 88% surface coverage by avidin. From the analysis of surface density of biotin ligands, it was obvious that steric hindrance does not occur in this system even with a large surface density of biotin ligands. These advantages are likely to originate from the structural feature of the dendrimer monolayers such as the surface exposure of derivatized biotin ligands and a corrugated surface.

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