# Efficient Biosensor Interfaces Based on Space-Controlled Self-Assembled Monolayers

Hideo Tokuhisa,*,† Jun'an Liu,† Kazuhiro Omori,‡ Masatoshi Kanesato,† Kazuhisa Hiratani,§ and Lane A. Baker*,||

*Photonics Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan, Department of Material Technology, Industrial Technology Center of Tochigi Prefecture, 367-1 Karinuma, Utsunomiya, Tochigi 321-3224, Japan, Department of Applied Chemistry, Utsunomiya University, 7-1-2 Youtou, Utunomiya 321-8585, Japan, and Department of Chemistry, Indiana University, Bloomington, Indiana 47405-7102*

*Received October 8, 2008. Revised Manuscript Received December 2, 2008*

In this paper we demonstrate control over the spacing of surface-modifying probe molecules through the use of labile dendron spacers. During this process, anchor molecules are first adsorbed to a surface, with dendron modifiers attached. Steric interactions of the bulky dendrons control the density of anchor molecules bound to the surface. The dendron branches are subsequently detached from the anchor molecules, and the anchors are chemically modified with probe molecules, resulting in a surface with controlled spacing between probe molecules. Control over this spacing is important when the probe size is small in comparison with the target molecule. This importance is demonstrated for the binding of protein (streptavidin) targets to the probe (biotin) surface. The effect of probe space control on the efficiency of target capture is evaluated by examining the binding of streptavidin to thiolated biotin for a series of mixed monolayers. Surface modification is monitored by Fourier transform infrared reflection absorption spectroscopy (FTIR-RAS). The relative concentration of probe molecules at the surface is measured using X-ray photoelectron spectroscopy (XPS) measurements. Thiolated-biotin surfaces with optimized spacing show an increased capture efficiency for streptavidin relative to surfaces with nonoptimal or no control over probe spacing, as measured by surface plasmon resonance (SPR) spectroscopy. These results are of potential significance for the optimization and fabrication of micro- and nanoarrays used in chemical and biochemical measurements.

## Introduction

Microarrays are an attractive platform for a number of bioanalytical applications, including drug development,[1] enzyme−substrate profiling,[2] and DNA sequencing.[3] The advantage of microarrays lies in the combinatorial approach to screening samples using a high density of heterogeneous chemical or biochemical probe molecules attached to a solid support. A critical aspect of microarray performance is optimizing probe−target interaction. Self-assembly is an appealing choice for immobilization of probe molecules at surfaces, as the method is simple and allows for a number of useful attachment chemistries to be realized.[4,5] Probe-target binding at highly packed probe arrays, however, can differ significantly from binding of the same molecules when in solution, or in a biological setting.[6] These differences can be caused by a number of probe−probe interactions (e.g., steric or electrostatic interactions between probe molecules) that prevent target binding.

Probe−probe interactions may be reduced by lowering the overall density of probe molecules at a surface. Lowering the density of probe molecules, however, may preclude detection of target molecules in the case of low signal-to-noise measurements. This may be especially troublesome in specific cases, such as when detecting low copy number proteins in a complex cell lysate. It is therefore important to optimize the density of probe molecules at a surface, providing an opportunity for maximal target binding and detection. Additionally, it is well known that mixed monolayers often form island-like features with different components segregated within the monolayer.[7,8] Thus for self-assembled probe molecules, simply lowering the density of probe molecules does not explicitly generate an optimal surface for target binding. An optimal method for generating probe surfaces allows control over the space around each probe individually. This serves to both reduce probe−probe interaction and to maximize the density of probe molecules (and thus target binding capacity), attributes that are desirable for applications that demand highly sensitive sensing or arrayed interfaces.

To date, several methods to mitigate steric hindrance between neighboring probe molecules have been reported.[9−12] For example, in oligonucleotide arrays, surface density or probe oligos

* To whom correspondence should be addressed. Tel: +81-29-861-2442 (H.T.); +1-812-856-1873 (L.A.B.). Fax: +81-29-861-3029 (H.T.); +1-812-855-3500 (L.A.B.). E-mail: h-tokuhisa@aist.go.jp (H.T.); lanbaker@indiana.edu (L.A.B.).

† National Institute of Advanced Industrial Science and Technology.

‡ Industrial Technology Center of Tochigi Prefecture.

§ Utsunomiya University.

|| Indiana University.

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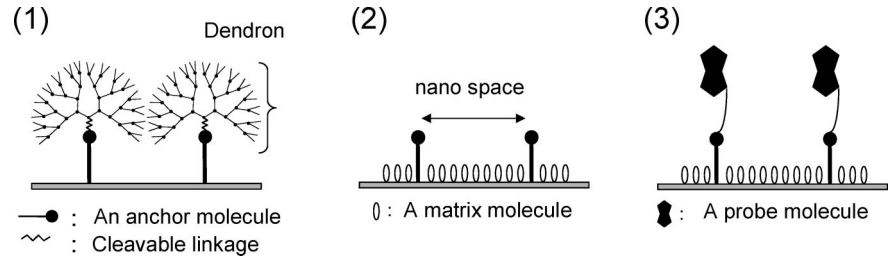
10.1021/la8033148 CCC: $40.75 
Published on Web 12/31/2008

**Scheme 1. An Immobilization Method That Controls the Space around Individual Probe Molecules[a]**




[a] (1) Fabrication of a dendrimer monolayer. (2) Removal of dendron spacers and introduction of a matrix molecule. (3) Modification of a probe molecule.

are a key factor for binding between probe−target oligonucleotides. Oligonucleotide probe density is commonly controlled by varying immobilization conditions, including the ionic strength of solution, interfacial electrostatic potential, and in cases where duplex or single-stranded oligonucleotides are used.[11] For oligonucleotides, it is much simpler to optimize the spacing between probe molecules because of the ease of denaturing the probe-target pairs. For example, a probe can be bound to the surface prehybridized with the target oligo. The target oligo can then be removed by thermally denaturing the hybridized probe-target, leaving a perfectly spaced probe array. Such strategies are commonly employed to optimize sensor response, and can result in marked improvement in both the amount of target captured and the kinetics of target capture. For probes designed to detect proteins, harsh conditions (thermal, pH, etc.) must often be applied to denature probe−target interactions. These conditions can desorb or damage the surface-bound probe molecules, limiting the applicability of this approach for protein targets. Further, it may be difficult to obtain suitable target proteins to generate an optimally spaced probe surface.

Park and co-workers have reported dendron-controlled spacing of probes for both oliogonucleotides and proteins.[9,10] In these studies, a conical-shaped dendron was attached to each probe molecule, and the dendron was subsequently adsorbed to the surface of interest, with the dendron providing spacing between probe molecules. When modified with biotin, the dendron−probe surface showed binding efficiency toward streptavidin, comparable to a mixed monolayer with a lower concentration of immobilized biotin.

In this paper we describe an immobilization method that controls the space around individual probe molecules using dendron spacers[13] (Scheme 1). First, an anchor molecule is focally substituted within a dendron structure through bonds that are labile to external stimuli (in this case, alkali solution). The anchor molecule is bifunctional in that it contains one functionality that is later chemically modified with a probe molecule, and a second functionality that serves to chemically adsorb the molecule to a substrate. Second, a self-assembled monolayer of dendron-spaced anchor molecules is formed. The packing of the monolayer is dictated by the sterics of the dendron spacers, and chemisorption to the substrate is controlled by the bifunctional anchor. Third, the dendron spacers are removed by an external trigger. The dissociated dendrimer is removed from the surface, leaving behind individual anchor molecules chemisorbed to the substrate. Each anchor molecule is spaced by a minimum distance determined by the steric bulk of the dissociated dendron spacer.

While conceptually similar to the method described by Park and co-workers,[10] our method affords a near ideal comparison by producing surfaces that differ only in probe spacing, as regulated by the dendron spacers. We believe our approach offers more insight into the important role that probe spacing plays in overall sensitivity. Specifically, in this study, we fabricate a biotinylated surface in which the biotin-probe density is controlled through our previously reported dendron-spacer method,[13] as described above. We compare the space-controlled biotin surface to monolayers of mixed composition. These mixed self-assembled monolayers are formed by immersing a Au substrate in a solution containing two thiol molecules. One thiol (e.g., thioctic acid) serves as an anchor for subsequent attachment of biotin probes. A second thiol (e.g., mercaptohexanol) serves to prevent nonspecific adsorption to the Au surface in regions absent of the biotin probe. On the basis of the relative biotin concentration in the mixed monolayers and in the dendron-spacer controlled monolayers, as determined by X-ray photoelectron spectroscopy (XPS) measurements, we demonstrate the advantageous effect of free space around the biotin molecules on the affinity for streptavidin obtained by surface plasmon resonance (SPR) meaurements.

## Experiment

**Materials.** Unless stated otherwise, all reagents and chemicals for this research were purchased from commercial sources and used without further purification. 1,2-Bis(2-aminoethoxy)ethane, *N*-hydroxysuccinimide (NHS), DL-α-lipoic acid, and 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC) were obtained from Tokyo Chemical Industry Co., Ltd. (TCI). d-Biotin, and 6-mercapto-1-hexanol 97% were purchased from Sigma-Aldrich Corporation. Streptavidin was obtained from Wako Pure Chemical Industries, Ltd.

**Synthesis.** Dendrimers (**Dn1**, **Dn2**) were synthesized according to a previous report.[13] Briefly, the dendritic benzyl alcohols were synthesized by repetitive protecting and deprotecting procedures including bromination and etherification.[14] The mixture of thioctic acid (2.0 equiv), the corresponding dendritic benzyl alcohol (1.0 equiv), EDC-HCl (2.0 equiv), 4-(dimethylamino)-pyridine (0.2 equiv), and dimethylformamide (DMF) were stirred at room temperature overnight. After removal of the solvent under reduced pressure, the residue was purified by preparative gel permeation chromatography to afford the dendrimers **Dn1** and **Dn2**.

**The Activated Pentafluorophenolic Ester of Biotin.** A mixture of biotin (500 mg, 2.05 mmol), 2,3,4,5,6-pentaflurophenol (472 mg, 2.56 mmol), and EDC-HCl (491 mg, 2.56 mg) was stirred overnight at room temperature in 20 mL of DMF. The DMF was evaporated, and the crude product was subjected to silica gel chromatography, eluting with 10% methanol in chloroform to yield 749 mg of the activated pentafluorophenolic ester of biotin (yield: 89%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ (ppm) = 5.13 (br, 1H), 4.81 (br, 1H), 4.53−4.55 (m, 1H), 4.33−4.36 (m, 1H), 3.17−3.21 (m, 1H), 2.95 (dd, 1H, $J$ = 5.1 Hz, $J$ = 12.8 Hz), 2.75 (d, 1H, $J$ = 12.8 Hz), 2.71 (t, 2H, $J$ = 7.4 Hz), 1.68−1.87 (m, 4H), 1.48−1.63 (m, 2H). ESI−MS: (410.36) 433.3(M+Na).

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**3,6-Dioxa-8-amino-octane-1-*N*-biotinamide ($NH_2$EG-Biotin).** A mixture of biotin pentafluorophenyl ester (749 mg, 1.83 mmol), 1,2-bis(2-aminoethoxy)-ethane (2.67 g, 18.3 mmol), and triethylamine (554 mg, 5.49 mmol) was stirred overnight at room temperature in DMF. The DMF was removed under vacuum, and the residue was purified by amine-silica gel chromatography with chloroform/methanol (8:2) to afford $NH_2$EG-biotin as a white powder with a satisfactory yield (71%).

$^1$H NMR (500 MHz, $CDCl_3$): $\delta$ (ppm) = 6.78 (br, 1H), 6.16 (br, 1H), 5.08 (br, 1H), 4.49−4.52 (m, 1H), 4.31−4.34 (m, 1H), 3.51−3.64 (m, 8H), 3.45 (dd, 2H, $J$ = 5.4 Hz, $J$ = 10.1 Hz), 3.15 (dt, 1H, $J$ = 4.6 Hz, $J$ = 7.4 Hz), 2.87−2.94 (m, 3H), 2.73 (d, 1H, $J$ = 12.8 Hz), 2.18−2.28 (m, 2H), 1.63−1.77 (m, 4H), 1.42−1.48 (m, 2H). ESI−MS: (374.5) 375.4(M+H).

**Gold-Coated Glass Substrates.** Chromium (5 nm) and then gold (50 nm) were evaporated onto glass substrates for SPR measurements. The gold films for Fourier transform infrared (FTIR) and XPS measurements were deposited by evaporation of 10 nm Ti and 100 nm Au on a glass. Prior to immobilization of thiol molecules, gold substrates were cleaned by immersion in a freshly prepared piranha solution (3:1 v/v of 98% $H_2SO_4$ and 30% $H_2O_2$) for 10 min, followed by washing with Milli-Q water, and then dried under a stream of $N_2$ (*Caution: piranha solution reacts violently with organic compounds and should be handled with great care*).

**Preparation of Self-Assembled Monolayer on Gold.** To prepare a dendrimer monolayer, piranha-cleaned substrates were immersed in a 1,2-dichloroethane solution of the dendrimer (0.1 mg/mL) for 24 h at room temperature, followed by copious washing with 1,2-dichloroethane, and dried under a stream of $N_2$. The dendron protecting groups were then removed from the thioctic acid cores by cleaving the ester in ethanolic 1 M KOH containing 1 mM 6-hydroxy-1-hexanethiol as a matrix molecule for 24 h, followed by washing with ethanol, 1,2-dichloroethane, water, and then dried under a stream of $N_2$. The remaining acid groups were activated with NHS by exposing the surface to 10 mL of aqueous solution containing 11.5 mg of NHS and 40 mg of EDC for 2 h, followed by copious washing with water, and then dried under a stream of $N_2$. Finally, the activated acid groups were exposed to 0.1 mM aqueous solution containing $NH_2$EG-biotin and triethyl amine at room temperature overnight, followed by copious washing with water, and then dried under a stream of $N_2$.

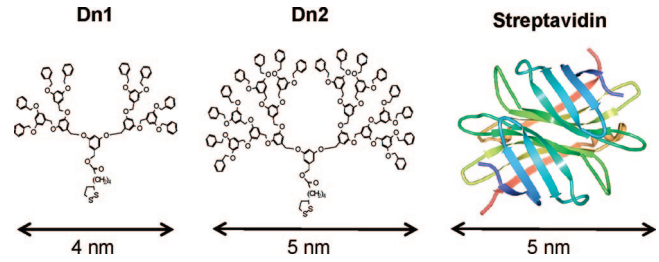
To prepare a mixed monolayer, Au substrates were immersed in two-component thiol solutions made by dissolving the desired ratio of thioctic acid and 6-hydroxy-1-hexanethiol in ethanol. The total thiol concentration was kept at 1 mM. After immobilization at room temperature for 24 h, the substrates were thoroughly rinsed with ethanol and then dried under a stream of $N_2$. The biotin modification of the acid surfaces was performed in the same way as described above.

**Instrumentation.** FTIR-reflection absorption spectroscopy (RAS) measurements were performed on a Digilab FTS 7000 FT-IR spectrometer equipped with a Spectra-Tech FT-80 grazing angle reflectance accessory and a liquid-$N_2$-cooled MCT detector. XPS spectra were acquired using a Thermo VG Scientific Theta Probe system. XPS data acquisition was employed with a pass energy of 100 eV, a step increment of 0.05 eV, and a Mg anode power of 400 W. SPR measurements were performed using the SPR biosensor SPR-MACS (Moritex). Measurements were performed using phosphate-buffered saline (PBS, pH 7.4) as the running buffer and changes in resonance angle ($\Delta\theta$) as a function of time were recorded before streptavidin injection and 20 min after streptavidin injection.

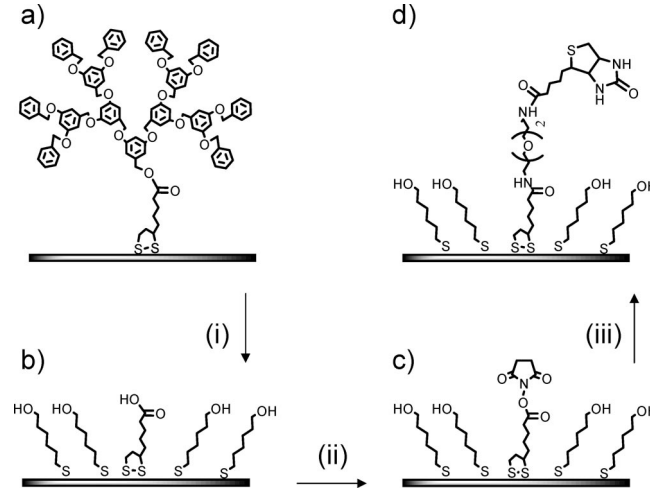
## Results and Discussion

Two different generation dendrimers, **Dn1** and **Dn2**, were synthesized by introducing Fréchet-type benzylether dendrons to thioctic acid anchor through an alkali labile ester linkage (Scheme 2). Monolayers of dendron-spaced anchors **Dn1** and **Dn2** were self-assembled on respective Au surfaces. Biotin surfaces were fabricated from these dendron-spaced anchors in three steps (Scheme 3): (i) Removal of dendrons from the anchors by alkali hydrolysis and subsequent addition of hydroxy hexane thiol; (ii) Activation of anchor carboxylic acids with NHS; and (iii) reaction of the activated carboxylic acid of the anchor with $NH_2$EG-biotin, forming a biotin-probe surface with controlled spacing. For comparison, a series of mixed monolayers were prepared in solutions containing different ratios (100:0, 50:50, 10:90, 5:95) of thioctic acid to mercaptohexanol. These mixed monolayers were then NHS-activated and modified with biotin as described above in steps ii−iii. This provided four surfaces with random organizations of biotin and with different densities of biotin.

**Scheme 2. Structures of Dendrimers Dn1 and Dn2**




**Scheme 3. Schematic Drawing of the Synthesis of a Space-Controlled Biotinylated Surface from a Self-Assembled Monolayer of Dn1[a]**




[a] (i) Dendron removal with KOH hydrolysis. (ii) Activation of carboxylic acid with NHS and EDC. (iii) Introduction of biotin moiety through amide formation.

Progression of surface modification at each step described above was confirmed by FTIR-reflection absorption spectroscopy (RAS). Figure 1 shows FTIR-RAS spectra for each step of the surface modification in the case of **Dn1**. The FTIR spectrum of the self-assembled monolayer of **Dn1** on Au shows the characteristic signals for the dendron branches: a signal at 1733 $cm^{-1}$ arising from C=O stretching mode of the ester linkage, and three strong signals at 1598 $cm^{-1}$, 1454 $cm^{-1}$, and 1163 $cm^{-1}$ due to the large benzyl ether backbone of the dendron. After exposing the dendrimer surface to an ethanolic solution of 1 N KOH for 24 h, peaks corresponding to the dendron-spacers completely disappeared. A new peak at ca. 1726 $cm^{-1}$ appears, which is attributed to the alkali-liberated carboxylic acid. To confirm this peak is indeed a carboxylic acid, the pH was altered and the peak characteristically shifted to lower wavenumber, a feature which is strongly indicative of a carboxylic acid. Formation of an NHS-ester of the carboxylic acid using a carbodiimide (EDC) was confirmed through the appearance of three characteristic signals: 1745 and 1792 $cm^{-1}$ due to the imide asymmetric

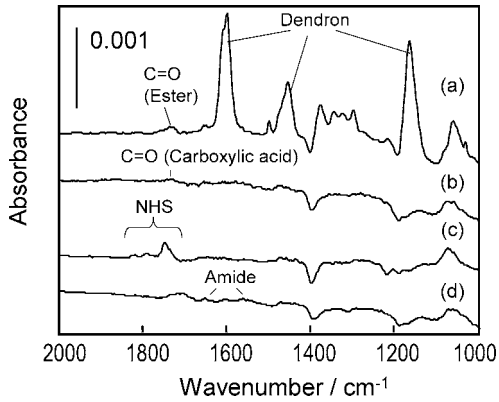



**Figure 1.** FTIR spectra of **Dn1** surface at each step for surface modification: (a) self-assembled monolayer formation of **Dn1** on a Au surface; (b) removal of dendron with KOH hydrolysis; (c) activation of carboxylic acid with NHS; and (d) introduction of biotin moiety by amide formation.

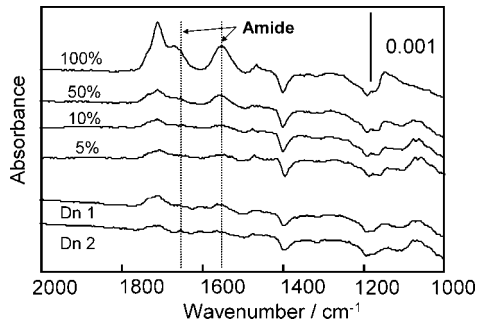



**Figure 2.** FTIR spectra of the resulting biotin surfaces prepared from 100, 50, 20, and 10% thioctic acid and **Dn1** and **Dn2** surfaces.

and symmetric stretching modes, respectively, and 1819 $cm^{-1}$ due to the ester stretching mode.[15] Finally, the addition of amine-terminated biotin to the NHS ester resulted in the appearance of signals at 1655 and 1543 $cm^{-1}$ due to amide bond formation and complete disappearance of the signal arising from the NHS-ester, indicative of nearly full modification of biotin probe molecules. It was thus confirmed that each reaction proceeded satisfactorily.

The same procedures were performed on **Dn2** dendrimer surface (Figure S1) and the mixed monolayers. The FTIR spectra of all the resulting biotin surfaces are shown in Figure 2. They show a similar response in the spectral region of 1000 to 2000 $cm^{-1}$ overall, although the peak intensities are different among the surfaces. Given that amide signals are only attributed to amine-terminated biotin, the difference in the peak intensity due to the amide bonds depends on the biotin concentration at the surface. As expected, the intensity of the amide bonds decreases with decreasing thioctic acid content of the surface, and also decreases as the size of the dendron grows from **Dn1** to **Dn2**. It is difficult, however, to quantitatively describe this difference using surface FTIR spectra since orientation effects prevent direct comparison.

XPS measurements on the different biotin-probe surfaces were performed to quantitate the amount of biotin present at each surface. Since the nitrogen N(1s) region is only attributed to amine-terminated biotin, we determined the relative concentration of biotin-probe at the surface based on the nitrogen peak area, with the 100% thioctic acid surface as a reference. The resulting XPS spectra are shown in Figure 3. As expected, the relative

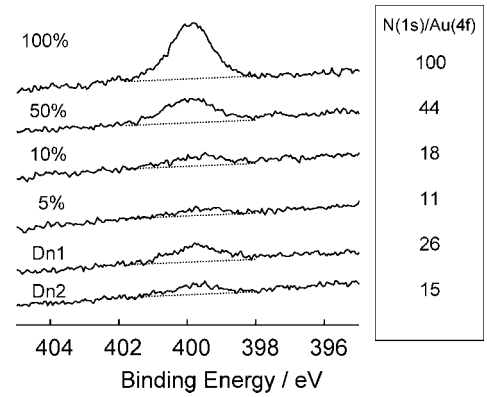



**Figure 3.** XPS spectra of the N(1s) region for all the biotin surfaces and the relative peak area ratios of the nitrogen signal normalized with a Au(4f) peak area among all the surfaces when 100% thioctic acid surface is 100.

concentration of biotin probe molecules is observed to decrease as the component ratio of thioctic acid decreases in the mixed monolayer surfaces, or as the size of the dendron spacer increases. For mixed monolayers, differences in the relative peak area of the nitrogen region are attributed to several factors, including the preferential adsorption of thioctic acid on Au over the hydroxyl thiol during the mixed monolayer formation, and the difference in the surface reaction efficiency for introduction of the biotin probes in steps ii−iii described above. On the basis of XPS measurements, the biotin surface fabricated using the dendron **Dn2** has a similar concentration of the biotin probe as the 10% thioctic acid surface. Comparison between the two surfaces in terms of affinity toward protein therefore allows the effect of the controlled space around the biotin probes on the probe-target binding to be established.

SPR measurements were performed for all biotin surfaces prepared to verify the activity of immobilized biotin-probe molecules. Streptavidin was chosen as the target protein for two reasons. First, interaction between biotin and streptavidin is well-established and often serves as a model for immunoassays.[16] Second, the size of streptavidin (ca. 55 × 45 Å)[17] matches the size of benzyl-ether dendron **Dn2** (diameter: c.a. 50 Å) used in this study (for comparison, **Dn1** diameter: c.a. 40 Å). We thus expect the largest dendron spacer to provide optimal free space around each probe molecule for binding with streptavidin, as well as an optimal density of probe molecules at the surface, leading to an enhanced response relative to the other surfaces prepared.

The SPR response ($\Delta\theta$) to the binding of streptavidin on the 10% thioctic acid surface and the **Dn2**-footprint surface is presented as a change in resonance angle as a function of time in Figure 4. For binding analysis, biotin-probe surfaces were prerinsed with PBS buffer solution for 20 min. A solution of streptavidin (100 $\mu$g/mL) was then introduced under flow to the cell chamber. Binding of streptavidin to the biotin-probe interface reached saturation in 5 min at a flow rate of 15 $\mu$L/min. Following the removal of the nonspecifically bound streptavidin by washing with PBS buffer solution for 15 min, the increase in resonance angle was determined for each biotin-probe surface. The surface prepared using the **Dn2** dendron-spacer exhibited the greatest binding of streptavidin (ca. 40% greater) compared to the biotin-probe interface fabricated from the 10% thioctic acid surface.

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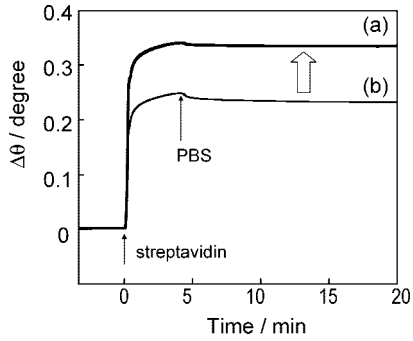


**Figure 4.** SPR sensorgrams illustrating the interaction of (a) **Dn2** and (b) 10% thioctic acid surfaces with 0.1 mg/mL of streptavidin in 0.1 mol/L PBS.

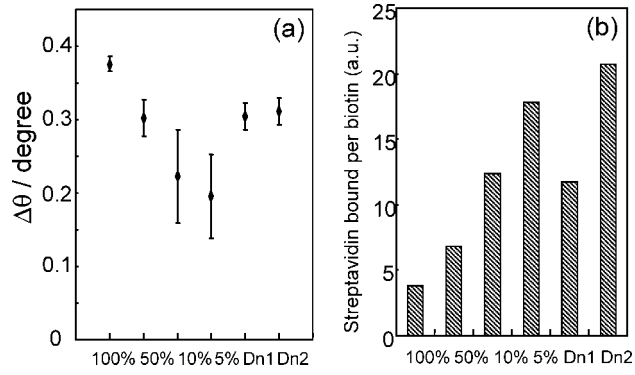


**Figure 5.** (a) SPR signals for the recognition of 0.1 mg/mL of streptavidin on all the biotinylated surfaces. Error bars indicate the absolute standard deviation of two lines on at least two samples. (b) SPR signals divided by the relative N(1s)/Au(4f) peak area ratios demonstrating the relative efficiency to bind to streptavidin for one biotin probe.

On the basis of the XPS results described above, the biotin concentrations at the **Dn2** and 10% thioctic acid surface were approximately equal, clearly indicating that space control around the immobilized probe molecules is very important to enhance the binding of bulky proteins.

Figure 5 demonstrates a summary of the SPR results for all biotin-probe surfaces prepared in this study. In the case for the series of the mixed monolayers, the lower the biotin-probe concentration at the surface, the lower the amount of bound streptavidin. This is different from the report of Campbell's group[18] demonstrating that there are optimal biotin concentrations around 10−40% to maximize the adsorption of streptavidin. This difference may be rationalized by three facts: (1) thioctic acid itself can provide some space around the biotin molecule because it has two anchoring points to the Au surface for each biotin; (2) as reported here, biotin is introduced through surface reactions, as opposed to biotin-modification of the thiol prior to surface adsorption; (3) the height of mercaptohexanol as a matrix layer relative to biotin probes is so low as to provide some free space around the biotin moiety. Taken together, the use of a dithiol with surface probe-modification is expected to result in a different overall coverage, relative to previous reports. In fact, according to a literature method, the amount of streptavidin adsorption in the case of the biotin-probe interface fabricated from 100% thioctic acid surface was estimated to be 2.0 molecules/cm$^2$,[19] which is higher than that of the 100% biotin surface described by Campbell and coworkers (∼1.6 molecules/cm$^2$) (see Supporting Information). For biotin-probe surfaces prepared using dendron spacers, the sensitivity increases slightly even though the biotin concentration decreases, indicating biotin probes with appropriate space (provided by **Dn2**) are more effective at binding streptavidin. This point becomes more evident when the SPR response ($\Delta\theta$) is divided by the relative biotin concentration obtained in the XPS measurements (Figure 5). The biotin probe surface fabricated using the dendron-spacer **Dn2** binds the most streptavidin per biotin among all surfaces examined in this study. This implies that the proper control of space around the probe molecules can augment the binding efficiency considerably.

## Conclusions

In this report, biotin-probe surfaces have been prepared using dendrons as space-controlling modifiers. The effect of free space on the amount of streptavidin retained per biotin was evaluated through a combination of SPR and XPS measurements. Dendron-spaced biotin-probe surfaces were compared with biotin-probe surfaces prepared from mixed solutions containing a thioctic acid and a hydroxyl terminated alkane thiol at four different ratios. The biotin-probe surface fabricated using a dendron spacer (**Dn2**) with a size equivalent to streptavidin exhibited an ∼40% greater total binding of streptavidin when compared to the mixed monolayer (10% thioctic acid) of similar biotin content. The **Dn2**-spaced surface also exhibited the highest streptavidin binding when considered on a per-biotin basis. From this comparison, we conclude that optimizing the free space as well as the probe density around immobilized probe molecules is an important factor to consider for enhancing the performance in capturing proteins, such as streptavidin, at surfaces. The total sensitivity using the dendron-spaced biotin-probe surface can potentially be improved by increasing the initial density of chemisorbed dendrimer. Kinetic studies designed to provide more information related to the free-space effect on the adsorption of the proteins are currently under investigation.

**Acknowledgment.** This research was supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (A), 17681017, 2004−6. Additional support was provided by a grant from The New Energy and Industrial Technology Development Organization (NEDO) in 2006−8.

**Supporting Information Available:** FTIR-RAS spectra for each step of the surface modification in the case of **Dn2** and an estimation of streptavidin coverage. This material is available free of charge via the Internet at http://pubs.acs.org.

LA8033148

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