Creation of a mixed poly(ethylene glycol) tethered-chain surface for preventing the nonspecific adsorption of proteins and peptides

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Using a heterotelechelic poly(ethylene glycol) (PEG) possessing a mercapto group at one end and an acetal group at the other end (acetal-PEG-SH), the authors constructed a reactive PEG tethered-chain surface on a surface plasmon sensor (SPR) gold chip for biosensing with high sensitivity. Nonspecific bovine serum albumin adsorption on the PEG tethered-chain surface was significantly influenced by the density of the PEG chain, and was almost completely suppressed by increasing the PEG density through the repetitive treatment of the chip surface with acetal-PEG-SH. The PEG density was increased even more by adding an underbrushed layer made of shorter-PEG-SH-chain molecules (2 kDa, hereafter 2k) to the surface made of longer-PEG-SH-chain molecules (5 kDa, hereafter 5k). SPR measurement then gave an estimate of the adsorption of a series of proteins with varying sizes and isoelectric points on the PEG chain surface having an underbrushed layer (PEG5k/2k surface). As compared to other SPR surfaces, viz., a commercial carboxymethyl dextran and conventional PEG5k tethered-chain surface without an underbrushed layer, the mixed PEG5k/2k surface showed almost complete inhibition of the nonspecific adsorption not only of high-molecular-weight proteins but also of low-molecular-weight peptides. [DOI: 10.1116/1.2800754]

I. INTRODUCTION

Modifying the substrate with poly(ethylene glycol) (PEG) as a tethered chain leads to reduced nonspecific interaction with biomolecules such as proteins and cells. This is because PEG has a low interfacial free energy in water, as well as high chain mobility, which induces excluded-volume effects.[1–8] Furthermore, a PEG chain surface possessing a reactive group at the free-chain end of the tethered PEG has recently attracted attention as a platform for installing biospecific ligands, with the expectation of achieving the highly sensitive biorecognition of target molecules without any nonspecific interaction. A detailed electron spin resonance (ESR) study conducted by our group on the mobility of tethered PEG end-functionalized with an ESR probe has revealed that the mobility of the PEG chain end nicely correlates with an increase in the molecular weight of the PEG chain.[9] This result is consistent with the specific binding ability of lectin to the sugar moiety installed at the distal ends of the tethered PEG chains with varying molecular weights. Nevertheless, an increase in the PEG molecular weight results in a decrease in the chain density of the PEG layer due to the increased excluded-volume effect, with the result that the solute penetration into the PEG layer induces nonspecific interaction with the substrate. Thus, the length and density of the tethered PEG chain are in a tradeoff relationship in terms of the sensitivity and selectivity of biomolecule recognition.[3,10,11] To construct a surface with highly selective biosensing through PEGylation, a tethered PEG layer is required to satisfy the creation of both high mobility to facilitate biorecognition and high density to ensure nonfouling. To overcome

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TABLE I. PEG tethered-chain surfaces made by different protocols of modification.

Sample	[PEG][a]	MW_{PEG} [b]	Flow time	Modification cycles[c]	Backfilling cycles[d]	Total SPR angle shift[e]
	mg/mL	kDa	min	times	times	deg ($\times 10^4$ RU)
1	0.1	5	5	1	···-	0.075±0.013
2	0.1	5	10	1	···	0.12±0.021
3	1	5	20	1	···	0.15±0.015
4	1.0[f]	5 and 2	20	1	···	0.16±0.019
5	1	5	20	1	1	0.21±0.011
6	1	5	20	1	3	0.30±0.016
7	1	5	20	4	3	0.32±0.012

[a]Concentration of the α-acetal-ω-mercapto-PEG solution.
[b]Molecular weight of acetal-PEG-SH introduced onto a gold-sensor surface.
[c]Number of PEG modification cycles using acetal-PEG-SH (5k) onto a gold-sensor surface.
[d]Number of injections of acetal-PEG-SH (2k) solution onto a PEG5k tethered-chain surface.
[e]Total SPR angle shift induced by PEG modification onto a gold-sensor chip (n=4, ±SEM).
[f]Concentration of the mixture of acetal-PEG-SH with 5k and 2k (1:1) solution.

this tradeoff, a mixed PEG tethered-chain surface with a filler layer of short PEG (2k) underlaid on the preconstructed longer-PEG-brushed layer (5k) was prepared from α-acetal-ω-mercapto-PEG.[12,13] The surface of mixed PEG tethered chains almost completely prevented nonspecific adsorption of bovine serum albumin (BSA).[14] The present paper reports the details of our study using surface plasmon resonance (SPR) (Refs. 15–29) on the relation between the density of the surface PEG layer and the adsorption of a series of proteins with varying sizes and isoelectric points.

II. EXPERIMENT

A. Materials

Two lots of α-acetal-ω-mercapto-PEG (acetal-PEG-SH) with different molecular weights were synthesized, as previously reported.[12,13] The molecular weight and polydispersity index of the synthesized PEGs, denoted as PEG5k and PEG2k, were 4990 and 1.04, and 1920 and 1.03, respectively. A gold chip (SIA KIT Au) for the SPR analysis was purchased from Biacore AB (Uppsala, Sweden). Bovine serum albumin, fibrinogen from bovine plasma, myoglobin from equine hearts, and lysozyme from chicken egg whites were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Oligo-peptides, bradykinin, and RGDS were obtained from Peptide Research (Osaka, Japan). A 0.01 M HEPES buffer (pH 7.4, containing 0.15 M sodium chloride, 3 mM EDTA, and 0.005% surfactant P20) was purchased from Biacore AB (Uppsala, Sweden). As a control, a carboxymethyl dextran-modified gold chip (CM chip) for SPR measurements was obtained from Biacore AB (Uppsala, Sweden). The SPR evaluations were carried out on a Biacore 3000 device (Biacore AB, Uppsala, Sweden).

B. Preparation of α-acetal-ω-mercapto-PEG tethered-chain surface having different chain densities

Introducing acetal-PEG-SH onto a gold chip was done using a SPR instrument (Biacore 3000). After a bare gold sensor chip was placed in the instrument, a sodium phosphate buffer (pH 7.4, 0.05 M, containing 1 M NaCl) solution of acetal-PEG-SH was injected at a constant flow rate of 20 μL/min. After the injection, the gold chip was washed with a solution of 0.05 M NaOH for 1 min. This washing was repeated twice. In this paper, we define this adsorption/rinsing procedure of acetal-PEG-SH as one PEG modification cycle. We monitored the SPR sensorgram of this PEG modification on the gold surface, and the amount of immobilized PEG chain was assessed based on the SPR angle shift. To construct various PEG chain surfaces having different chain densities on a bare gold surface, we changed several parameters, such as the concentration of the acetal-PEG-SH solution (0.1 or 1.0 mg/mL), the injection time (5, 10, or 20 min), the chain length of acetal-PEG-SH (2k or 5k), and the number of PEG modification cycles (1–7 times). In this study, seven kinds of PEG chain chips having different PEG chain densities were prepared. The preparation conditions are summarized in Table I. The BIAcore system tracks the change of the resonance angle, which is expressed as resonance unit (RU). An SPR angle shift of 1×10^{-4} deg corresponds to 1 RU.

C. Nonspecific adsorption of BSA on PEG-grafted surfaces having different chain densities

A 1.0-mg/mL BSA in 0.01 M HEPES buffer was prepared. The solution was allowed to flow onto PEG-grafted surfaces having different chain densities for 10 min at a flow rate of 20 μL/min at 25 °C, followed by the injection of HEPES buffer for 3 min at the same rate. We measured the magnitude of the SPR angle shift on the PEG chain surfaces caused by this successive injection. The SPR angle shift of adsorbed BSA on PEG-grafted surfaces was converted to the following relation: 1 RU=0.088 ng/cm^2, which was determined by a radiolabeling protein method.[30]

TABLE II. Proteins and peptides used for studies of adsorption.

Proteins and peptides	MW [-]	pI [-]
Fibrinogen	340 000	6.0
BSA	68 000	4.8
Myoglobin	17 600	6.8
Lysozyme	14 300	10.9
Bradykinin	1 060	12.5
RGDS	433	6.7

D. Nonspecific adsorption of biomolecules with different sizes and isoelectric points on PEG tethered-chain surfaces

First, six kinds solutions of 0.1-mg/mL fibrinogen, BSA, myoglobin, lysozyme, bradykinin, and RGDS in 0.01 M HEPES buffer were prepared. These proteins and peptides were listed in Table II. Each prepared solution was allowed to flow onto the mixed PEG (5k/2k) chain surface with a SPR angle shift of 0.32° for PEG modification for 10 min at a flow rate of 20 μL/min at 25 °C, followed by the injection of HEPES buffer for 3 min at the same rate. The magnitude of the SPR angle shift on the PEG chain surface caused by this successive injection was measured. A conventional PEG5k tethered-chain surface with the SPR angle shift of 0.15° was used as a control. Additionally, conventional CM chips before and after blocking treatment of the carboxylic groups were used as control surfaces. For the blocking of the carboxylic groups in the CM surface, the carboxyl groups were activated by NHS/EDC solution for 20 min and were converted into hydroxyl groups through reaction with ethanolamine for 20 min.[28] For the mixed PEG surface, 0.1 mg/mL biocytin hydrazide (MW=386.5) in 0.01 M HEPES buffer was assessed by the SPR instrument under the same conditions. The SPR angle shifts of adsorbed protein on PEG-grafted and CM surfaces were converted to the following relation: 1 RU=0.088 ng/cm^2 (Ref. 30) and 0.1 ng/cm^2,[18,29] respectively.

III. RESULTS AND DISCUSSION

As we have reported previously, the bare gold surface integrated into the SPR instrument was prepared by letting an aqueous solution of heterotelechelic PEG possessing a mercapto group at one end and an acetal group at the other chain end flow into the SPR channel.[14] We have so far revealed that the nonfouling property of the mixed layer of PEG5k and PEG2k (5k/2k) significantly reduced the nonspecific adsorption of BSA. Recently, Norde and co-workers confirmed that the densely packed PEG chain layer was constructed by using bimodal PEG brushes, and the constructed surface reduced an adsorption of BSA on the substrate surface regardless of PEG chain length.[31] To further evaluate the effect of the density of the mixed PEG layer on nonspecific protein adsorption, the PEG chain density was varied in this study using different PEG-immobilization treatment protocols. The idea of our work is how the repeated PEG treatment using different molecular weight works for both chain density and nonfouling character. The details of the results are shown in Table I. The SPR angle shift for grafting PEG was significantly influenced by PEG concentration, reaction temperature, reaction time, and salt composition in PEG solution (data not shown). First, by varying reaction time, the amount of grafting PEG was controlled (conditions 1 and 2). In the case of PEG modification by using a 0.1 mg/mL PEG5k solution, apparent saturation of the SPR angle shift profile for PEG binding was reached after about 10 min (data not shown). To increase grafting of PEG, a 1.0 mg/mL PEG5k solution was used (condition 3). Apparent saturation of the PEG-binding curve was reached after about 15 min (data not shown). Owing to further increase of grafting, we introduced shorter PEG (PEG2k). By using a mixture solution of PEG5k and 2k, the grafting was increased. The concentrations of PEG5k and 2k in the mixture solution were both 0.5 mg/mL. Thus, total concentration of PEG chain was 1.0 mg/mL (condition 4). Additionally, repeated introduction of PEG to the preconstructed PEGylated surface induced further reincrease in SPR angle shift for PEG grafting (conditions 5 and 6). The maximum SPR angle shift for grafting was provided by three repeated injections of PEG2k after four repeated injections of PEG5k. The SPR angle shift of 0.24° by PEG5k and the further shift of 0.08° by PEG2k were observed (condition 7). Therefore, various PEGylated surfaces having a SPR angle shift not more than 0.32° were constructed. A 1.0 mg/mL solution of BSA was then flowed over the PEG tethered-chain surfaces with varying densities. Nonspecific adsorption of BSA on the PEG surfaces with varying densities was assessed by SPR. As anticipated, the increase in the amount of PEG modification, viz., PEG density, on the SPR chip decreased the nonspecific adsorption of BSA, as shown in Fig. 1. In particular, BSA adsorption was reduced significantly when the SPR angle shift for PEG modification was above about 0.16°. Nevertheless, a slight, but definite, adsorption of BSA was still observed on the surface with a SPR angle shift of 0.16° for PEG modification, as shown in the inset of Fig. 1. Thus, it is preferable to construct a PEG tethered-chain surface having as high a chain density as possible. As we reported previously, the PEG density was increased by introducing an underbrushed layer made of shorter-PEG-SH-chain (2k) molecules onto a surface having longer-acetal-PEG-SH-chain (5k) molecules.[14] As seen in the inset of Fig. 1, the PEG chain density of the mixed PEG (5k/2k) surface increased and the surface was almost completely nonfouling. Remarkably, almost no BSA absorption was observed above the PEGylated sensor surface with the SPR angle shift of 0.3° for PEG modification. To our knowledge, it is difficult to increase the PEG chain density up to the SPR angle shift of about 0.32° using only PEG-SH treatment. Thus, we confirmed that the mixed PEG tethered chain is effective in rejecting BSA absorption on the sensor-chip surface.

Though the nonspecific absorption of the BSA molecule (MW=68 kDa) was confirmed as stated above, it is important to investigate the effect of the molecular size of proteins on the nonspecific adsorption of the constructed PEG chain

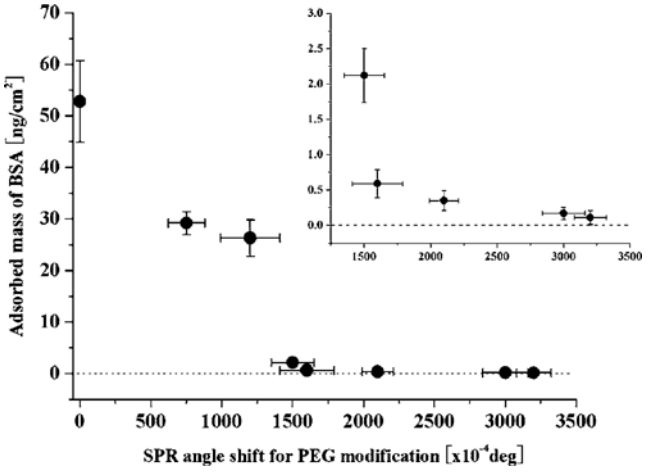

FIG. 1. Relationship between adlayer PEG density and nonspecific BSA adsorption on a gold surface. The PEG-brushed layer was constructed using α-acetal-ω-mercapto-PEG. PEG density on gold surfaces was represented as SPR angle shift. On the other hand, adsorbed mass of BSA on these PEG surfaces were calculated by using the relation that SPR signal of 1 RU corresponded to 0.088 ng/cm^2 (Ref. 30). Immobilized amount of α-acetal-ω-mercapto-PEG was modulated by changing the conditions of the PEG-modification (details of the conditions are shown in Table I). Inset in the figure shows a magnified portion of the high-PEG-density region (n =4, ±SEM).

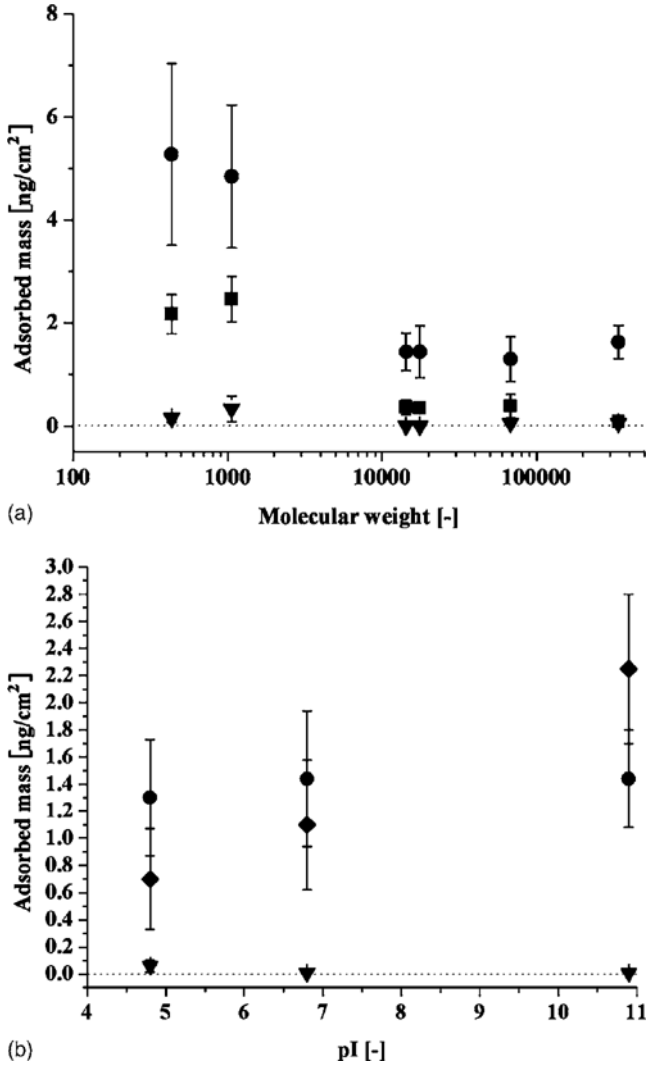

FIG. 2. (a) Molecular-weight dependency of the nonspecific adsorption of proteins and peptides on the PEG chain surface and the values given by the carboxyl-dextran sensor chip. The 0.1-mg/mL solutions of the proteins and peptides listed in Table II were analyzed using a conventional PEG (5k) chain surface (square), a mixed PEG (5k/2k) chain surface (triangle), and a commercial CM5 chip (circle) blocked by ethanol amine via the active-ester method (n=4, ±SEM). (b) Isoelectric-point dependency of the nonspecific adsorption of proteins and peptides on a mixed PEG (5k/2k) chain surface (triangle) and a commercial CM5 chip with (circle) and without (diamond) blocking treatment with ethanolamine via the active-ester method (n =4, ±SEM). Nonspecific adsorbed mass on PEG surfaces and on CM surfaces was calculated by using 1 RU as 0.088 ng/cm^2 (Ref. 30) and as 0.1 ng/cm^2 (Refs. 18 and 29), respectively.

surface. Using various types of proteins in terms of size and isoelectronic point, we conducted protein adsorption experiments on the prepared PEG chain surfaces. Figure 2(a) shows plots of the amount of adsorbed proteins on the mixed PEG (5k/2k) chain surface with the SPR angle shift of 0.32° for PEG modification. As controls, we used a commercially available carboxymethyl dextran (CM)-modified surface and a conventional PEG5k tethered-chain surface with the SPR angle shift of 0.15°. Under such a general PEG modification condition without treatment of underbrushed PEG, this SPR angle shift for PEG modification is considered to be almost maximal. A CM chip whose carboxyl groups were blocked by ethanolamine via the active ester technique was also employed (blocked CM).[28]

When the commercially available CM sensor chip was used after the blocking treatment, the nonspecific adsorption of large proteins (MW$>10^4$) was reduced to some extent. However, fairly large amounts of low-molecular-weight peptides, such as bradykinin and RGDS, were adsorbed on the blocked CM surface. The thickness of the CM layer was reported to be about 100 nm.[29] Thus, small molecules may have penetrated into the CM layer or been adsorbed on the defects of the CM layer, which caused the nonspecific signal of the small molecules. In the case of the conventional PEG5k chain chip, the nonspecific adsorption of large proteins was almost completely suppressed. Low-molecular-weight peptides, however, showed a tendency similar to that of the CM surface, although the amount of adsorbed peptides was much lower than in the case of the blocked CM chip. In contrast, the nonspecific adsorption of high-molecular-weight proteins as well as low-molecular-weight peptides was completely suppressed on the mixed PEG (5k/2k) tethered-chain surface. The smallest peptide used in this study was RGDS, a tetrapeptide (MW=433). It is further surprising that when the biocytin hydrazide molecule (MW =386.5) contacted the mixed PEG (5k/2k) chain surface, the SPR angle shift for the adsorption was about 4×10^{-4} deg (0.35 ng/cm^2, which was estimated by using a radiolabeled protein), viz., the adsorption was almost completely suppressed also. This means that the mixed PEG chain surface repelled the low-molecular-weight peptides, but also other the low-molecular-weight compounds such as biotin derivatives.

The characteristics of the nonspecific protein adsorption were also investigated in terms of the isoelectric points of the proteins. When nonblocked CM, which had free-carboxylic groups in and on the substrate layer, was used as the sensor chip, significant protein adsorption was observed, especially in the high isoelectric region due to the electrostatic interaction between the anionic dextran surface and the cationic proteins [Fig. 2(b)]. In the case of the blocked CM chip, the electrostatic attractive force decreased because of the shield of negative charge on the CM surface induced by the blocking treatment, which resulted in a reduction of the adsorption amount of positively charged proteins. The blocking surface, however, rather increased adsorption of the proteins with a low isoelectric point. As a result of the blocking of the anionic site, the repulsive force between the surface and anionic proteins decreased. This alternatively induced an increase in the nonspecific interaction, a part of which may have been caused by hydrogen bonding between amino groups of the proteins and the hydroxyl group or amide group on the blocked CM. Thus, regardless of the isoelectric point of the proteins, a consistent amount of proteins was nonspecifically adsorbed on the ethanolamine-blocked CM surface. The acetal-PEG chain surfaces constructed in this study should be nonionic. Indeed, we confirmed that the zeta potential of the acetal-PEG-modified gold surface was almost zero.[32] Regardless of the existence of a charge on the protein molecule, the acetal-PEG (5k/2k) chain surface was completely nonfouling [triangular plots in Fig. 2(b)]. Because the mixed PEG (5k/2k) chain surface was almost completely nonfouling for a wide range of proteins and peptides, we anticipated that it will be useful as a functional sensor chip with high sensitivity due to the low background adsorption.

IV. CONCLUSIONS

In conclusion, by using acetal-PEG-SH having 5k and 2k, a mixed PEG tethered-chain surface, viz., a PEGylated surface with an underbrushed layer, was constructed on a SPR gold-sensor chip. The obtained mixed PEG brushed surface showed a complete nonfouling characteristic of high-molecular-weight proteins, but also of small-molecular-weight peptides. In addition, the isoelectric point of proteins did not influence the nonspecific protein adsorptions on the mixed PEG tethered-chain surfaces.

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[30]See **EPAPS** Document No. **E-BJIOBN-2-003704** for details on using radiolabeling protein, the absolute amount of adsorbed protein on a PEG tethered chain surface was estimated. This document can be reached through a direct link in the online article's HTML reference section or via the **EPAPS homepage** (http://www.aip.org/pubservs/epaps.html).
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