Construction of mercapto-ended poly(ethylene glycol) tethered chain surface for high performance bioconjugation

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Abstract

Protein chips technology has emerged as a promising tool for screening protein-protein interactions and characterizing the levels of proteins expressed in cells. Novel surface technologies must be improved to satisfy the specific protein orientation on the surface without denaturation, retaining non-fouling character of the surface. In order to construct facile immobilization of protein on the mixed PEG tethered chain on gold surface, we designed mercapto-ended PEG tethered chain surface using telechelic PEG possessing a mercapto group at both end. Marcapto-ended PEG tethered chain surface was constructed on a gold surface by the consecutive treatments with the short MeO-PEG-SH (2k), followed by the treatment with long SH-PEG-SH (5k). By changing the density of pretreated MeO-PEG-SH, the amount of modified SH-PEG-SH could be controlled. Under the suitable modification conditions, maleimide-installed protein or antibody Fab' fragment was immobilized easily on the constructed PEG surface. The constructed surface showed fairly low non-specific protein adsorption and high specific affinity, indicating that the immobilized proteins at the distal end of PEG tethered chain remained their activity. The surface possessing the mercapto-ended PEG tethered chain thus prepared can be regarded as a promising tool for protein analyzing surface.

Introduction

Protein chips technology has emerged as a promising tool for screening protein-protein interactions and characterizing the levels of proteins expressed in cells[1,2]. Protein chips have also been used as a proteomics screening tool to characterize biochemical processes by identifying novel protein-protein and protein-DNA binding interactions. Particularly, the exploitation of surface plasmon resonance (SPR) analyzer has been widely utilized as a label-free, real-time measurement system for biomolecular interaction[3,4]. The development of surface-based proteomics tools required general and facile methods for the immobilization of protein on the surface by a specific orientation, retaining its functionality[5,6]. The present demand is to satisfy the specific protein orientation on the surface without denaturation, retaining non-fouling character of the surface[7,8]. We have been focusing on preparation of complete non-fouling surface using PEG tethered chain surface. There are two key technologies for our surface modification. One is the use heterotelechelic poly(ethylene glycol) (heteroPEG), which as prepared by our original synthetic method[9]. The other is to construct mixed PEG tethered chain surface, which means that the PEG tethered chain consists of both long (5k) and short (2k) chain length[10,11]. However, protein rejection and conjugation of ligand on the mixed heteroPEG tethered chain surface are trade-off relation. Due to the protein repellant character, it is difficult to conjugate biomolecules such as antibodies and membrane proteins on their surface.

In this study, in order to construct facile conjugation of proteins on the non-fouling PEG tethered chain surface, we constructed mercapto group for protein immobilization at the distal end of PEG tethered chain surface. The idea was newly constructed by the consecutive treatments with the short mercapto-ended semitelechelic PEG (MeO-PEG-SH) (2k), followed by the treatment with long mercapto-ended telechelic PEG (SH-PEG-SH) (5k). In this treatment, mixed PEG chain possessing the mercapto group at the distal end of the long PEG chain was constructed avoiding loop formation. Mercapto group installed the distal end of tethered PEG chain on the surface enable immobilized of protein such as Maleimide-installed protein via the Michael reaction or antibody Fab'-fragment via disulfide linkage. Thus the immobilized of protein on the surface can be convenient without changing the distal end of PEG chain. The immobilized proteins thus obtained were anticipated to retain a high activity for the specific interaction with antibody or antigen. PEG tethered chain surface was constructed on a gold surface by the consecutive treatments, which was monitored by SPR sensor. And also immobilization of proteins via a maleimide addition as well as a disulfide conjugation and the specific interaction of the immobilized protein was monitored using SPR sensor.

Experimental Section

Materials

Tetrahydrofuran(THF), triethylamine, *N-N'*-dimethylformamide(DMF), sodium hydride(NaH), and methanesulfonyl chloride were

purified by conventional methods. Hydroxyl-ended telechelic poly(ethylene glycol)(OH-PEG-OH, M_n = 4600)(Aldrich) and mercapto-ended semitelechelic poly(ethylene glycol)(MeO-PEG-SH, M_n = 2000) (NOF) were used as received. N-proplyamine (Wako), sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexa ne-1-carboxylate(Sulfo-SMCC)(Pierce), Bovine Serum Albumin, (BSA)(Sigma) and all other reagents were used as received. A gold sensor chip (SIA kit Au) for SPR measurements was purchased from Biacore AB. SPR evalutions were carried out on a Biacore 3000 device (Biacore AB).

Synthesis of mercapto-ended telechelic poly(ethylene glycol)

For the preparation of mercapto-ended telechelics, PEG having methane sulphonyl group at both ends (MS-PEG-MS) was prepared from hydroxyl-ended telechelics. The OH-PEG-OH (1.0 mmol) and THF (20 ml) were added to a 100 mL round-bottom flask containing an argon atmosphere. After the PEG was dissolved, NaH (20 mmol) and triethylamine (9.0 mmol) were added to the PEG solution. The prepared PEG solution was added to the THF solution of methanesulfonyl chloride (7.0 mmol) at the room temperature with stirring. The mixture was further stirred for over night. The obtained polymer (MS-PEG-MS) was precipitated into an excess amount of diethyl ether and separated by filtration. The precipitate was dried in vacuo and then finally freeze-drying with benzene.

For the preparation of thiol-ended PEG telechelics, the potassuim-□-ethyldithiocarbonate (8.8×10^{-4} mol), THF (50 mL) and DMF (3.6 mL) were added to the round-bottom flask under an argon atmosphere and stirring for several minutes. The mixture solution was added to the THF solution of MS-PEG-MS and stirred for several hours. After the reaction, the obtained polymer (DTC-PEG-DTC) was recovered in the same way as described above. In order to convert *O*-ethyldithiocarbonate-end to thiol group, 14 mmol of propylamine was added to THF solution of the obtained DTC-PEG-DTC (2.2×10^{-5} mol) and stirred for several hours. After the reaction the obtained polymer (SH-PEG-SH) was recovered in the same way as described above.

Preparation of mercapto-ended telechelic PEG brush surface

After the bare gold SPR sensor chip was cleaned by piranha solution (H_2SO_4:H_2O_2=3:1) for several minutes. This chip was docked into the SPR instrument. Sodium phosphate buffer (pH 7.4, 50 mM, containing 1 M NaCl) solution of methoxy-PEG-SH (MeO-PEG-SH) (M_n = 2k, 1 mg/mL) was injected at a constant flow rate of 5μL/min at 37 °C for several minutes by monitoring the amount of immobilized MeO-PEG-SH by the SPR angle shift (0.075 - 0.1°). To the preconstructed MeOPEG(2k) tethered chain with the controlled brush density, mercapto-ended PEG telechelics (5k) was modified (1 mg/mL, pH 7.4, 50 mM, containing 1 M NaCl). The mercapto-ended PEG telechelics was obtained just before use by the reaction of 57.5 μL of 1.4 M n-propylamine with 500 μL of DTC-PEG-DTC aqueous solution (1 mg/mL) for 30 min at 37 °C. The obtained SH-PEG-SH was purified by a column separation using NAP-5 column with sephadex G-25 (Amersham Biosciences), 1 mL of SH-PEG-SH fraction was eluted by PBS solution (pH 7.4, 50 mM, containing 1 M NaCl). After the immobilization of SH-PEG-SH, the solution of MeO-PEG-SH was injected again at a constant flow rate of 5 μL/min at 37 °C three times to improve the brush density maximum.

Nonspecific adsorption of protein to PEG modified surface

Adsorption of BSA on the prepared PEGylated surface was measured by SPR. A solution of 10 μM BSA was allowed to follow onto the constructed PEG surface at the a constant flow rate of 5 μL/min for 30 min at 25 °C and then the SPR response shift was measured. As a control, BSA adsorption on a bare gold chip was examined.

Immobilization of Protein to PEG modified surface

For the preparation of maleimide-BSA, 54.4 μL of 1 mM sulfo-SMCC solution was added to 500 μL of 10 μM BSA solution and stirred 30 min at 37 °C. The obtained protein solution was purified by using NAP-5 column and 1mL fraction of BSA-maleimide solution was eluted by PBS buffer (pH 7.4, 50 mM, containing 0.15 M NaCl). Immobilization of the BSA-maleimide on the SH-installed PEGylated surface was carried out using the SPR instrument, monitoring the angle shift. A solution of 10 μM BSA-maleimide was allowed to follow onto the constructed PEG surface at a constant flow rate of 1 μL/min for 180 min at 25 °C. As a control, 10 μM BSA solution was injected onto the constructed PEG surface at the a constant flow rate of 5 μL/min for 30 min at 25 °C and then the SPR response shift was measured. For the preparation of anti-FITC antibody Fab' fragment, 12 μL of 10 mM DTT solution was added to 200 μL of 10 μM anti-FITC antibody $F(ab')_2$ solution and stirred 2 hours at room temperature. The obtained protein solution was purified by using centrifugal column and the fraction of Fab' fragment solution was eluted by PBS buffer (pH 6.5, 50 mM, containing 5.5 mM EDTA). Immobilization of Fab' fragment on the SH-installed PEGylated surface was carried out using SPR instrument. A solution of 1 μM Fab' fragment was allowed to follow onto the

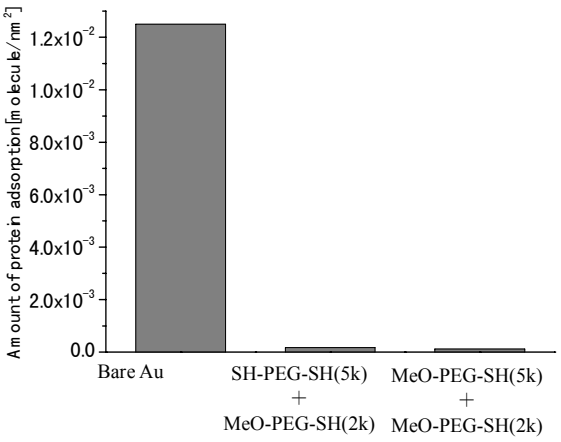

Figure.1 Amount of BSA adsorption via nonspecific adsorption versus various surface.

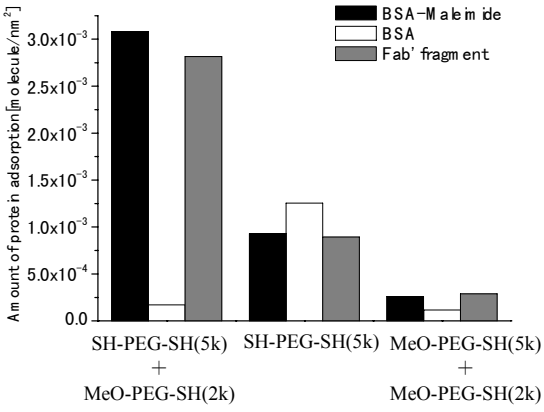

Figure.2 Amount of protein adsorption via specific or nonspecific adsorption versus various PEG surface.

constructed PEG surface at a constant flow rate of 5 μL/min for 30 min at 25 °C.

Results and Discussion

The purpose of this study was to construct a PEG tethered chain surface having mercapto group at the distal end of the PEG chain and immobilization of specific proteins at the end of the PEG chain end for high performance detection of protein-protein interaction, retaining the low non-specific interaction of environmental proteins. Our idea was to employ the consecutive treatments of bare gold surface with the short MeO-PEG-SH (MW= 2k), followed by the treatment with long SH-PEG-SH (MW= 5k). The pre-immobilized short PEG chain may prevent loop formation when the surface was modified with long SH-PEG-SH (5k). From the SPR measurement, the surface density of immobilized PEG was monitored. With increasing the amount of the preinstalled short PEG(2k) chain, the amount of immobilized SH-PEG-SH(5k) was decreased. For example, the SPR angle shifts of SH-PEG-SH were 0.14°and 0.09°, when the surfaces were pre-immobilized by the short PEG chain, which angle shifts were 0.05° and 0.1°, respectively.

Under the suitable conditions, non-specific adsorption of BSA was assessed as shown in Figure 1. The amount of protein adsorption was measured by SPR angle shift and the surface density was calculated according to the calculation for estimating from the measured SPR angle shift [12, 13]. When BSA was contacted with bare gold surface, significant amount of non-specific adsorption took place. On the contrary, the MeO-PEG-SH(2k)/SH-PEG-SH(5k) mixed PEG chain surface showed almost complete non-fouling surface, which was the same tendency as that of MeO-PEG-SH(2k)/MeO-PEG-SH(5k) mixed surface[14]. This result shows that the PEG brushed layer was effectively constructed by the consecutive treatment of MeO-PEG-SH(2k) followed by the treatment with SH-PEG-SH(5k).

From the data obtained in the Figure 1, it is anticipated that the SH-PEG-SH(5k) forms tethered chains on the surface, but not loop formation. Thus, the immobilization of specific proteins can be anticipated via the specific reaction with the SH end group.

The immobilization of specific proteins can be anticipated via the specific reaction with the SH end group. In order to confirm the specific conjugation reaction of the protein, maleimide-BSA was conjugated via the Michael type addition reaction with mercapto group on the surface. Furthermore antibody Fab' fragment could be also conjugated via disulfide linkage with mercapto group on the surface. The specific protein immobilization data were summarized in Figure 2.

On the SH-PEG-SH(5k)/MeO-PEG-SH(2k) mixed surface, very large amount of the maleimide-BSA and antibody Fab' fragment were immobilized, which were sharp contract to that with BSA. These results indicate that the immobilization of proteins via specific reaction with the SH end group. On the other hand, when the PEG surface was constructed with only SH-PEG-SH(5k), the amount of immobilized maleimide-BSA and antibody Fab' fragment were decreased and almost equal to BSA, indicating non-specific adsorption of proteins on the SH-PEG-SH(5k) surface, but not the immobilization via the specific reaction with the maleimide and mercapto groups. It can be explained by the loop formation on the gold surface by SH-PEG-SH(5k) chains. In the case of MeO-PEG-SH(5k)/ MeO-PEG-SH(2k) mixed surface, neither specific protein nor free protein showed very low adsorption. On the basis of the obtained results, in the SH-PEG-SH(5k)/MeO-PEG-SH(2k) mixed surface, the SH-PEG-SH(5k) forms tethered chain formation

avoiding loop formation. The mercapto group could be installed at the distal end of the PEG tethered chain surface and can be utilized for the immobilization of the specific biomolecules.

Conclusion

In this paper, we described the surface modification with the mercapto-ended telechelic PEG. In order to immobilize proteins on the gold surface, retraining their functionality, mercapto-ended poly(ethylene glycol) (PEG) tethered chain surface was newly constructed by the consecutive treatments with the short mercapto-ended semitelechelic PEG (MeO-PEG-SH) (2k), followed by the treatment with long mercapto-ended telechelic PEG (SH-PEG-SH) (5k). The pre-constructed short MeO-PEG-SH (2k) prevented a loop formation of the long SH-PEG-SH (5k) on the surface. By using the mercapto group at the distal end of SH-PEG-SH (5k), maleimide-installed protein and antibody Fab' fragment were immobilized easily on the constructed PEG surface. Thus the protein immobilized surface having high non-fouling character can be easily obtained and it is promising tool as a new bio-conjugation for high performance protein immobilization.

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