Improvement of the Thermal Stability of Streptavidin Immobilized on Magnetic Beads by the Construction of a Mixed-poly(ethylene glycol) Tethered-chain Layer

Running head: Improvement of the thermal stability of streptavidin

Key Words: DNA/ thermal stability/ magnetic beads/ PEG tethered-chain layer/ Poly(ethylene glycol) (PEG)/ streptavidin

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INTRODUCTION

Protein-immobilized substrates have been widely used in a number of applications for biosensing and bioseparation. Many kinds of sensor have been based on the change in colorimetric, fluorometric, and luminnometric signals derived from the immunoreactions of protein-immobilized sensor chips and particles [1 - 4]. The protein-immobilized substrates such as column and magnetic beads are also used as separation materials that can separate target biomolecules rapidly and simply in sample solution.

To develop the high-performance materials for biosensing and bioseparation, the effective blocking treatment of protein-immobilized substrate is very important to decrease nonspecific adsorption on substrate surface and increase the dispersion stability of particles. Poly(ethylene glycol) (PEG) is known as one of the excellent blocking agents. Because of the nonionic property, hydrophilicity and large steric exclusion effect of PEG[5], PEGylated surfaces and nano-/micro-scale particles show excellent non-fouling properties for various molecules[6] and high dispersion stability[7-9], respectively. Furthermore, in our recent works, it was revealed that the PEGyation improves the function of immobilized proteins on solid surfaces and particles. For example, the antigen-binding efficiency of anti C-reactive protein antibody-immobilized gold sensor surface[10] and anti ferritin antibody-immobilized latex particles were improved by the co-immobilization of densely packed PEG layers that were constructed by SH terminated PEGs and oligoamine terminated PEGs. The substrate reactivity of glucose dehydrogenase-immobilized gold nanoparticle (GNP) was improved by the co-immobilization of PEG/polyamine block copolymer[8,] and lipase/PEG-polyamine/GNP hybrids showed the almost same initial enzymatic activity after five-times repeated thermal treatments at 58 ºC for 10 min[12]. These results indicate that the co-immobilization of PEG derivatives onto the protein-immobilized substrates is a potential treatment to strongly improve the performance of biosensing and bioseparation materials.

The heat-induced inactivation of protein is one of negatively points which often restrict their field of use. It was thought that the heat-induced inactivation was occurred due to dissociation of intramolecular interaction of protein[13]. Here, we report the prevention effect of the densely packed PEG layer on the heat-induced inactivation of streptavidin-immobilized magnetic beads (SA-MB). The SA-MB co-immobilized with PEG tethered-chain layer (PEG/SA-MB) was constructed by the co-immobilization of oligoamine terminated PEGs onto SA-MB and its biotin binding efficiency was evaluated using the capture amount of biotinylated single-stranded DNA (ssDNA).

During the course of this study, it was revealed that the capture efficiency of biotinylated ssDNA by PEG/SA-MB was twice larger than that by SA-MB after repeated thermal treatments at 75 ºC for 5 min.

EXPELIMENTAL

Materials

Carboxylated magnetic beads (Magnosphere^{TM} MS300/High Carboxyl, 10% solid content in slurry, particle diameter was 3 μm), α-methoxy-poly(ethylene glycol)-pentaethylenehexamine (N6-PEG) with different two molecular weights, N6-PEG 5k (Blockmaster^{TM} CE510, M_w = 5k) and N6-PEG 2k (Blockmaster^{TM} CE210, M_w = 2k) were a kind gift from the JSR Corporation. Other chemicals were used as purchased. The sequence of biotinylated ssDNA used in this study is as follows; (5'-biotin-ATA GGA GTC GAC CGA CCA GAA-3')

Construction of PEG/SA-MB and SA-MB

Figure 1 shows a schematic illustration of PEG/SA-MB. Streptavidin, ethanolamine and N6-PEGs were adhered onto the surface of carboxylated magnetic beads via the active ester reaction method in the same manner as previous report[9,11]. Ten μL of magnetic bead suspension were poured into a 1.5-mL plastic tube, and the tube was placed on a magnetic separator to remove the suspension. After removing the suspension, the beads were dispersed into 1 mL of NaH_2PO_4 solution (10 mM, pH 4.7). Then, 100 μL of 10 mg/mL EDC solution (dissolved in NaH_2PO_4 solution) were added to the solution, followed by shaking for 30 min at room temperature and pH 4.7. After removing the suspension using the magnetic separator, the beads were dispersed into 1 mL of streptavidin solution at various concentrations (dissolved in NaH_2PO_4 solution) and pH 4.7. The reaction time for streptavidin immobilization was 15 min at room temperature. To construct a densely packed PEGylated surface, we employed our original blocking procedure[9-10,14-16] in this study viz., mixed PEG tethered chains layer was constructed on the SA-MB surface by the use of longer PEG (M_w = 5k) and shorter PEG (M_w = 2k) as descrived follows; immediately after the immobilization of streptavidin, the beads were dispersed into 1 mL of N6-PEG solution overnight. The N6-PEG solution was a mixture of 600 μL of N6-PEG (5k) (1.5 wt%, pH 7.4) and 400 μL of N6-PEG (2k) aqueous solution (1.5 wt%, pH 7.4). As a control, SA-MB was prepared by treating streptavidin-modified beads with ethanolamine (the beads were dispersed into 1 mL of ethanolamine aqueous solution (6.8×10^{-3} M, pH 7.4) overnight). The resulting beads

were washed twice with PBS (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4), and were resuspended in 1 mL of PBS.

Measurement of the amount of biotinylated ssDNA captured by PEG/SA-MB and SA-MB

In order to measure the amount of biotinylated ssDNA captured by PEG/SA-MB and SA-MB, the change in the absorbance of the ssDNA solution at 260 nm was measured before/after bead incubation on a Nano Drop 1000 (Thermo Fisher Scientific, Inc.), as follows. Forty μL of bead suspension were poured into a 0.2-mL plastic tube and washed once with binding buffer (10 mM Tris-HCl containing 0.5 mM EDTA, 1 M NaCl, 0.1 wt% Tween-20, pH 7.4). Then, the supernatant was removed, and 10 μL of biotinylated ssDNA solution (5.0 μM, dissolved in binding buffer) were added. After incubation for 1 h at room temperature, the beads were separated, and the absorbance of the supernatant at 260 nm was measured. The amount of biotinylated ssDNA captured by PEG/SA-MB and SA-MB was estimated using the obtained absorbance, and the extinction coefficient of biotinylated ssDNA was estimated to be 220,200 L/ (mole·cm).

Measurement of the ζ-potential

The ζ-potential of PEG/SA-MB and SA-MB was measured on a Zetasizer Nano (Malvern). Measurements were carried out at room temperature. All samples were dispersed into PBS and the bead concentration was 0.05 mg beads/mL.

Evaluation of the amount of biotinylated ssDNA captured by the constructed magnetic beads after heat treatment

Streptavidin is reported to have melting point more than 75 °C at neutral pH[13], which indicates that local motions in streptavidin start at around this temperature area. Thus, utility of our materials was investigated below the melting point. Before the incubation of the magnetic beads with the biotinylated ssDNA solutions, PEG/SA-MB and SA-MB were heated on an iCycler (Bio-Rad Laboratories, Inc.). One to three cycles of heat treatment at 75 ºC for 5 min were performed. The estimation of the amount of biotinylated ssDNA captured by the constructed magnetic beads was carried out using the same procedures as described above. The same evaluation was carried out in Methoxy-PEG-OH (10 wt%, M_w = 5k) containing PBS buffer.

RESULTS AND DISCUSSION

The immobilization of streptavidin on the magnetic bead (SA-MB) surface was carried out by the active ester method. The functionality of the obtained SA-MB was estimated by the amount of biotinylated ssDNA captured. The quantitative data are listed in Figure S1 in the Supplementary information section. As shown in the figure, the amount of biotinylated ssDNA captured by SA-MB increased with increasing streptavidin concentration in the immobilization reaction. These results indicate that the effective immobilization of streptavidin onto the magnetic beads was accomplished under the present experimental conditions, and the amount of immobilized streptavidin can be controlled by changing the streptavidin concentration in the solution.

The immobilization of PEG as a form of a tethered-chain layer on the SA-MB surface was carried out using N6-PEG, which possesses six amino groups at the end of a PEG chain. Although a monoamine end group is theoretically sufficient to conjugate the surface active-ester moiety, it is experimentally difficult to conjugate desirable amounts of PEG molecules due to the steric repulsion between the PEG chains. Because the SA-MB surface is negatively charged, the oligoamine moiety tends to access to the SA-MB surface. This electrostatic attractive force makes it easier for N6-PEG not only to react with the active-ester moiety but also to form a polyion complex with the liberated carboxylic acids on the surface of the magnetic beads. In this work, a mixed-PEG tethered-chain layer was constructed on the SA-MB surface in order to increase the chain density.

The amount of biotinylated ssDNA captured by SA-MB was slightly higher than that by PEG/SA-MB (Figure S2). To confirm the construction of the PEG layer on the magnetic beads, ζ-potential analysis and observation of the dispersion stability in cell lysis buffer were performed. Figure 2 shows the changes in the ζ-potential of PEG/SA-MB and SA-MB according to the immobilized amount of streptavidin. The ζ-potential of SA-MB increased with increasing amount of streptavidin immobilized on the bead surface. For example, the ζ-potential of SA-MB possessing 11.1 pmol streptavidin/mg beads was -20.9 mV, and it increased with increasing amount of streptavidin immobilized on the bead surface. Finally, it reached -11.5 mV. Because streptavidin has its isoelectric point at 6.8~7.5, it is slightly negative under these conditions. In contrast, the ζ-potential of PEG/SA-MB was about -5 mV, regardless of the amount of immobilized streptavidin, indicating that the negatively charged surface of the magnetic beads was shielded almost completely by the constructed PEG layer. Additionally, the dispersion stability of the obtained beads was also evaluated. Figure 3 shows images of the SA-MB and PEG/SA-MB solution in cell lysis buffer, contains non-ionic and zwitterionic surfactants. As shown in the figure, most of the SA-MB was

precipitated after 1 h, while no precipitation was observed in the PEG/SA-MB solution. These results indicated that a PEG layer was formed effectively on the SA-MB surface.

Prior to the investigation of PEG co-immobilization on the SA-MB surface, we checked the effect of free PEG in the buffer solution on the stabilization of streptavidin on the magnetic bead surface. It is well known that a free PEG stabilizes both protein structure and protein activity. For example, free PEG acts in the reaction medium as a stabilizer of penicillin acylase and Ca-ATPase[17, 18]. Increasing intramolecular hydrophobic interaction of the surrounded proteins by large water-excluding volume, flexibility and hydration of PEG is believed to stabilize the compact structure of proteins[19]. Figure 4 shows the changes in the capture efficiency of biotinylated ssDNA by SA-MB after the three cycles of heat treatment at 75 ºC in PBS with and without sufficient concentration (10 wt%) of methoxy-PEG-OH (*Mw* = 5k). The capture efficiency was defined as the percentage of the initial capture amount (without heat treatment). In this experiment, SA-MB possessing 26.4 pmol streptavidin/mg beads was employed. When SA-MB was subjected to three cycles of heating treatment in PBS without PEG additive, the capture efficiency of biotinylated ssDNA was drastically decreased, reaching 29 % of the initial capture amount. In the presence of 10 wt% commercial PEG, on the other hand, 41 % of the capture efficiency of the surface streptavidin remained after the three cycles of heat treatments. These results indicate that the addition of free PEG into the buffer solution improves only slightly thermal stability of the immobilized-streptavidin.

Then, we evaluated the change in the ssDNA capture ability of PEG/SA-MB after heat treatment. Figure 5 shows the change in the capture efficiency of biotinylated ssDNA according to the number of heat treatment cycles. In this experiment, PEG/SA-MB and SA-MB possessing 26.4 pmol streptavidin/mg beads was employed. In order to construct PEG layer with sufficient density on streptavidin immobilized surface, we selected 0.2 μM of streptavidin concentration at the preparation step to make space for consecutive PEG immobilization. The capture efficiencies were defined as percentages of the initial values (without heat-treatment). These efficiencies decreased with increasing number of heat treatment cycles. In the case of SA-MB, only about 29 % of the biotinylated ssDNA was captured (○) when the SA-MB was heated three times. On the contrary, in the case of PEG/SA-MB, about 62 % of the biotinylated ssDNA was captured (●) even after three cycles of heat treatment. These results clearly indicate that the co-immobilization of PEG tethered-chain layer with streptavidin on the magnetic bead surface show great improvement in the thermal stability of the immobilized-streptavidin.

CONCLUSION

This is the first report to reveal that the co-immobilized PEG layer prevented the heat-induced inactivation of biotin recognition ability of SA-MB and the co-immobilized PEG tethered-chain layer is effective treatment in improving not only enzymatic activity[12] but also molecular recognition ability of protein under the high temperature condition. Furthermore, it was also revealed that the prevention effect on heat-induced inactivation of SA-MB by the co-immobilization of PEG tethered-chain layer was more effective than that by the addition of free PEG into the solution, indicating the importance of polymer/protein hybrid surface to develop a high-performance biomaterials. Recently, SA-MB has been widely used as a bioseparation material in versatile applications for biopanning, biomacromolecule isolation, immunoassays, and genome sequencing. In these cases, SA-MB often has to be used in extreme conditions such as high temperature, low pH, and high surfactant concentration. The constructed PEG/SA-MB with high thermal stability would be expected to work effectively in these extreme conditions.

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Supplementary information is available at Polymer Journal's website

Figures and their legends

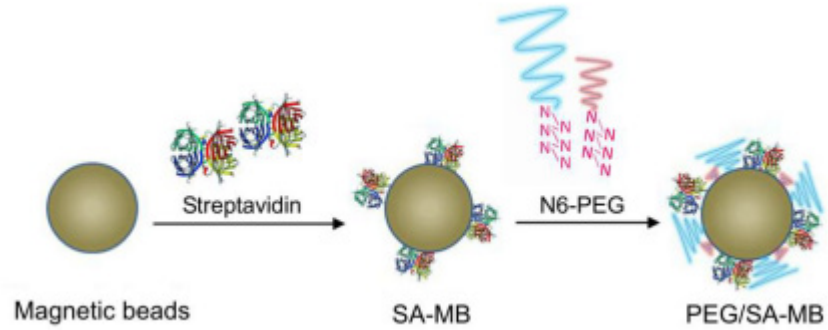

Figure 1. Schematic illustration of the preparation of Streptavidin immobilized magnetic beads (SA-MB) and PEG/streptavidin co-immobilized magnetic beads (PEG/SA-MB).

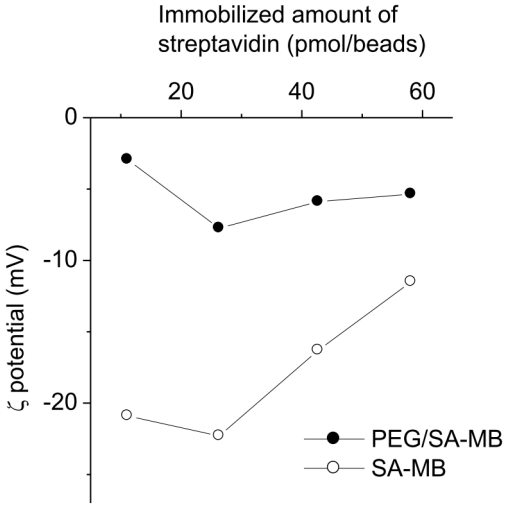

Figure 2. Changes in the ζ-potential of (●) PEG/SA-MB and (○) SA-MB according to the amount of immobilized streptavidin. All samples were dispersed into 10 mM sodium phosphate buffer containing 150 mM NaCl at pH 7.4, and measurements were carried out at room temperature. The bead concentration was 0.05 mg/mL.

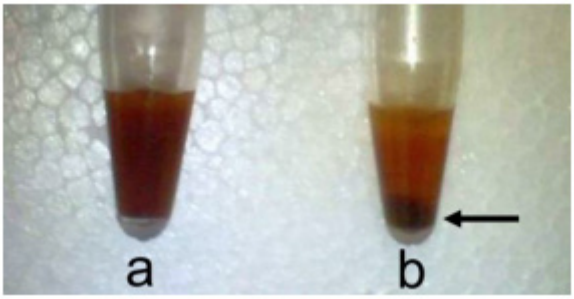

Figure 3. Images of (a) PEG/SA-MB and (b) SA-MB dispersed into 100 μL of 5x cell lysis buffer (pH 8.0). The amount of streptavidin immobilized on these beads was 26.4 pmol/mg beads. Two-mg samples of each type of bead were incubated for 1 h at room temperature. The aggregation is indicated by arrow.

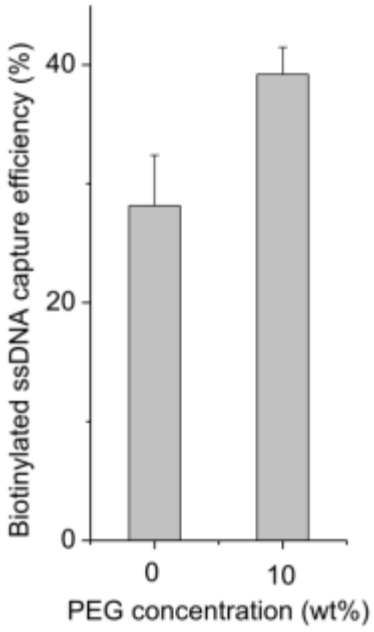

Figure 4. The capture efficiency of biotinylated ssDNA by SA-MB (the amount of immobilized streptavidin was 26.4 pmol/mg beads) after heat treatment with three cycles of heat treatment at 75 ºC for 5 min with and without 10 wt% PEG(5k) in PBS buffer. The efficiency was defined as the percentage of the initial value (without heat treatment).

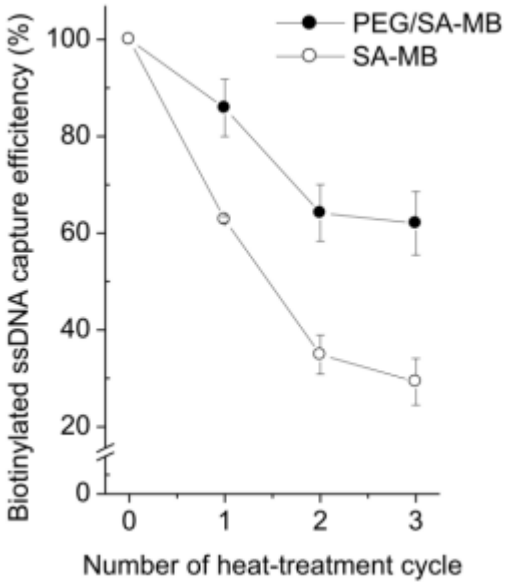

Figure 5. Changes in the capture efficiency of biotinylated ssDNA by PEG/SA-MB (●) and SA-MB (○) as a function of the number of heat-treatment cycles. The efficiencies were defined as percentages of the initial values (without heat treatment). The amount of streptavidin immobilized on PEG/SA-MB and SA-MB was 26.4 pmol/mg beads. Heat treatment at 75 ºC for 5 min was performed at each cycle.

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