Effect of Linker for Immobilization of Glutathione on BSA-Assembled Controlled Pore Glass Beads

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Controlled pore glass bead was modified with bovine serum albumin (BSA), and glutathione (GSH) was immobilized through three kinds of linkers on top of BSA. Bis(3-sulfo-*N*-hydroxysuccinimide suberate) sodium salt (BS^3), *N*-hydroxysuccinimide 3-(2-pyridyldithio)propionate (SPDP), or *N*-hydroxysuccinimide 4-maleimidobutyrate (GMBS) was introduced into the BSA-bound matrix. Subsequently, GSH was immobilized by addition of thiol side chain into the maleimido moiety, replacing a disulfide group, or formation of an amide group upon releasing 3-sulfo-*N*-hydroxysuccimide group. It was observed that conjugation methodology played a critical role for activity of the immobilized GSH. SDS-PAGE chromatogram showed that the matrix of glutathione immobilized on BSA through GMBS manifested high selectivity towards glutathione-S-transferase (GST) in cell lysate.

Key Words : Glutathione (GSH), Glutathione-S-transferase (GST), Controlled pore glass (CPG), Linker, Bovine serum albumin (BSA)

Introduction

Since Wilchek *et al.* reported for the first time purification of staphylococcal nuclease, affinity chromatography has become a more and more powerful and effective fractionation technique for the purification of proteins.[1] A general approach is to attach a relevant ligand to an insoluble matrix covalently. As a result, specific biomolecules can be isolated from a complex mixture due to the selective interaction between the target molecule and the complementary ligand. Successful affinity chromatography requires matrices[2] of high selectivity[3] and binding capacity.[4]

Natural polymers such as agarose gel and cross-linked dextran are materials of choice for affinity chromatography due to numerous advantages. Unlike the conventional supports, controlled pore glass (CPG) is rigid and mechanically and chemically stable to the condition of coupling and elution. In addition, it exhibits desirable elution properties. However, CPG surface, even when coated, is polar and retains partial negative charge.[5] It is one of factors resulting in serious nonspecific binding of proteins. Therefore, application on both affinity chromatography and solid-phase peptide synthesis has been limited. Once the obstacles are eliminated, widespread use of the materials can be expected.

Here we present modification of the CPG beads with BSA, subsequently with GSH through a linker (BS^3, SPDP, or GMBS), and characteristics of the surface materials in terms of GST binding. BSA has been known to reduce nonspecific binding of proteins on the surface and applied to form a molecular layer for a biosensor or protein microarrays.[6a-c] Glutathione was chosen as a ligand to be tethered on the BSA self-assembled matrix due to widespread use and fair understanding of glutathione S-transferase gene fusion system.[7]

Experimental Section

General Supplies and Equipment. Aminopropyl-tethered controlled pore glass beads (AMPCPG; 80-120 mesh; mean pore diameter, 50 nm) were purchased from CPG, Inc. Bis(3-sulfo-*N*-hydroxysuccinimide suberate) sodium salt (BS^3), *N*-hydroxysuccinimide 3-(2-pyridyldithio)propionate (SPDP), or *N*-hydroxysuccinimide 4-maleimidobutyrate (GMBS), reduced glutathione (GSH), bovine serum albumin (BSA), *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-ONSu), piperidine, 2-mercaptoethanol, and phosphate buffered saline tablets (PBS) were obtained from Sigma-Aldrich. All other chemicals were of analytical reagent grade and were used without further purification. Deionized water (18 MΩ·cm) was obtained by passing distilled water through a Barnstead E-pure 3-Module system. UV-vis spectra were recorded on a Hewlett-Packard diode-array 8453 spectrophotometer.

Preparation of BSA-bound AMPCPG Bead: (i) Surface activation: AMPCPG beads (0.15 g, surface capacity: 98.2 μmol/g, surface area: 42.9 m^2/g) were washed thoroughly with deionized water and subsequently with acetone by using a glass filter. In a separate vessel, suberic acid bis(3-sulfo-*N*-hydroxysuccinimide ester) sodium salt (BS^3, 10.0 mg, 17.6 μmol) was dissolved in a mixture of DMF and

sodium bicarbonate buffer (1.0 mL, 3 : 7(v/v), 50 mM, pH 8.5). Subsequently, the solution was introduced into a vial containing the beads. The beads were shaken for 2 h at room temperature. The resulting porous materials were separated from the solution by filtration and washed thoroughly with DMF, deionized water, and acetone sequentially. (ii) Conjugation of BSA: The beads were placed into a vial containing PBS buffer solution of BSA (1.0 mL, 1.0 μM) and shaken for 12 h at room temperature. Finally, the resulting beads (**3**) were separated with a glass filter, washed thoroughly with distilled water, and dried in vacuum.

Immobilization of Glutathione on the BSA-bound AMPCPG Bead: (i) Surface activation: 4-Maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS) (5.0 mg, 18 μM) in sodium bicarbonate buffer (1.0 mL, pH 8.5) was introduced into an e-tube holding sample (**3**) (0.15 g). Beads were shaken for 2 h at room temperature, washed thoroughly with DMF and subsequently with DI water. (ii) GSH-immobilization step: Reduced glutathione (5.4 mg, 18 μM) in PBS buffer (1.0 mL, pH 7.4) was added into the e-tube holding the above activated materials. After allowing 12 h at room temperature for the reaction, the beads were washed thoroughly with deionized water. (iii) Capping step: 2-Mercaptoethanol (200 μL) in PBS buffer (800 μL) was introduced into the e-tube containing the previously prepared beads, and 2 h is allowed for the capping at room temperature. Subsequently, the GMBS-treated beads (**6**) were washed and dried. The similar procedure was followed for the preparation of the other matrices except use of BS^3 (**4**) or SPDP (**5**) instead of GMBS.

Determination of Surface Amine Density: BSA-bound beads (10 mg) were taken into an e-tube. In parallel, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-ONSu) (2.5 mg) was placed into a separate glass vial, and a mixture of DMF and $NaHCO_3$ buffer (2.5 mL, pH 8.5, 3 : 7 (v/v)) was added to dissolve the reagent. One fifth of the solution was taken and transferred into the e-tube containing the beads. The tube was placed into a vial, and the vial was shaken for 30 min at room temperature. The beads were separated with a glass filter. The porous materials were washed with DMF several times and subsequently with deionized water. After being dried in vacuum, 20% piperidine in DMF (0.5 mL) was added into an e-tube holding the beads. The beads were allowed to react with piperidine for 30 min. Then the resulting solution from the tube was transferred carefully into a new e-tube, and the beads were washed with 20% piperidine in DMF (0.25 mL) twice. All of the solution were collected and combined into the previous e-tube. Then the solution was mixed with a certain volume of methanol to adjust the absorbance. The absorbance at 301 nm was measured using a UV/Vis spectrometer, and a relevant solvent was used for background correction. In total, the above measurement was repeated five times. Various reaction times from 0.5-9 h were employed to observe saturation of the density. The calibration curve was obtained according to the previously reported method.[8]

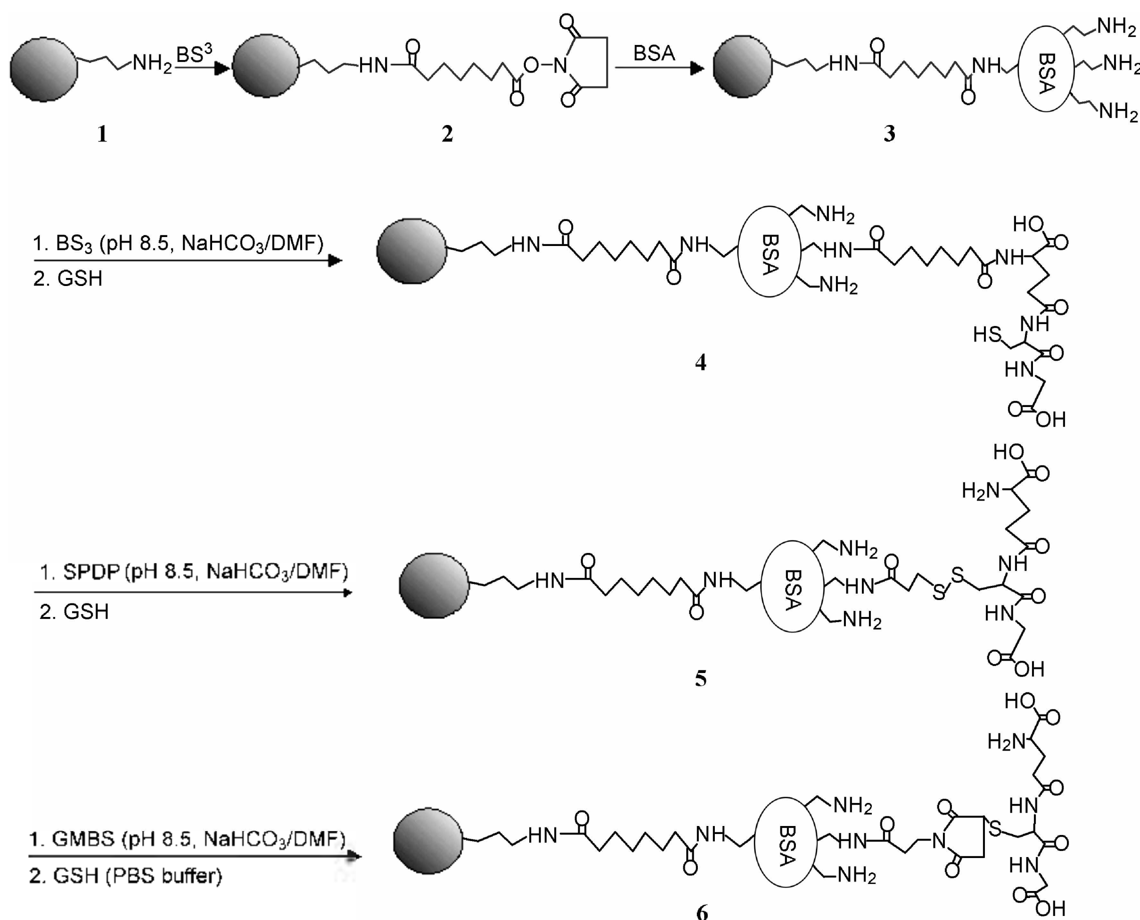
Preparation of Cell Lysate Containing Overexpressed GST: The single colony containing a recombinant pGEX plasmid was incubated in 200 mL of 2X YTA medium. After change into the log phase, gene expression was induced with IPTG for 6 h. Subsequently, cells were pelleted by centrifugation and washed with 1X PBS. Then E. coli was lysed in 10 mL hypotonic buffer (20 mM Tris, 150 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4) containing 0.50 mM PMSF by a sonicator. The lysates were obtained by the removal of insoluble materials.

Elution of GST: Immobilized beads (3.6 mg) were transferred to an 1.5 mL Eppendorf tube, and hypertonic buffer (1.0 mL, 20 mM Tris, 150 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4) was added twice to wash the beads. Subsequently, cell lysate containing overexpressed GST (40 μL) or pure GST and washing buffer (500 μL, 20 mM Tris, 150 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM EGTA, 1.0% TX-100, 0.10 mM PMSF, pH 7.4) were placed in the e-tube. The mixture was allowed to interact for 1 h at 4 °C while shaking. The beads were washed with 1.0 mL of the washing buffer three times. After all the supernatant was removed, the e-tube with sample buffer (70 μL, 0.16 M Tris/HCl, pH 6.8, 12.5% (w/v) SDS, 25% (v/v) glycerol, 12.5% (v/v) 2-mercaptoethanol, 0.05% (v/v) bromophenol blue) was heated to boil for 7 min. Then samples were run for SDS-PAGE, and the chromatogram was colored by CBB G-250 staining solution.

Results and Discussion

CPG: In affinity chromatography, dextran and agarose, the most frequently used chromatography supports, exhibit extremely low nonspecific adsorption. Nevertheless, agarose gel, typically in a bead shape, suffers several drawbacks. Moderate flow (or elution) rate due to its soft nature, severe and irreversible shrinkage of beads after being dried or frozen, and poor stability in some organic solvents are examples.[9] Unlike the traditional supports, controlled pore glass (CPG) exhibits many exceptional properties as a support: 1) it is mechanically stable, 2) it is chemically stable from pH 1 to pH 14, 3) it is inert to a broad range of nucleophilic and electrophilic reagents, 4) it is stable against heating, 5) it exhibits excellent flow (or elution) properties. All of these characteristics support potential usefulness in many fields such as permeation chromatography, solid phase synthesis, affinity purification, etc.

Pore size: Accessibility of protein to the immobilized ligand crucially depends on the pore size of the matrix. While a larger average pore radius increases diffusion rate of a protein into the matrix, the correspondingly diminished surface area reduces the binding capacity of the adsorbent. To achieve right balance between the pore size and the surface area, porosity of the support has to be optimized for each specific protein. Because GST (23 kDa), whose dimension is $53 \times 62 \times 56$ $Å^3$,[10] was employed in our investigation, CPG of 50 nm was selected. CPG with the pore size has been reported to allow inclusion of the



Scheme 1. Schematic presentation of the procedure for three samples on AMPCPG matrix.

complete range of molecular subunits normally found in proteins.[11]

Immobilization of Glutathione on the BSA-bound AMPCPG: One of key concerns for affinity matrix is degree of nonspecific binding (or NSB). It is a ubiquitous problem in affinity purification and solid-phase synthesis. CPG surfaces, even when coated, are polar and retain partial negative charge,[5] which may result in serious nonspecific binding during the application. We, first, modified the CPG with BSA, which formed a molecular layer that reduced NSB of proteins significantly.[6] Also, amine group of lysine in BSA was allowed to react with a linker, and finally GSH was tethered. Summarized modification steps were outlined in Scheme 1.

Controlled pore glass bead was modified with BSA using BS^3. Subsequently, one of selected linkers (BS^3, SPDP, and GMBS) was incorporated in the BSA-bound matrix. Although SPDP and GMBS, heterobifunctional linkers, are reactive with both thiol and amine group, reaction of the amine groups of BSA to release *N*-hydroxysuccinimide group was dominant during the conjugation process due to low concentration of sulfhydryl group of BSA.[12] At a subsequent step, the intact sulfonated *N*-hydroxysuccinimide group of BS^3 is displaced by the free amine group of GSH to form a stable amide group. Meanwhile, the sulfhydryl group of the GSH forms a covalent bond by cleaving the disulfide bond of SPDP or covalently adds into the maleimido moiety of GMBS. After the immobilization of GSH, ethanolamine or 2-mercaptoethanol was introduced to block the remained active group.

As a result of two different reaction sites, the tethering mode of sample (**4**) is different from the other two cases,

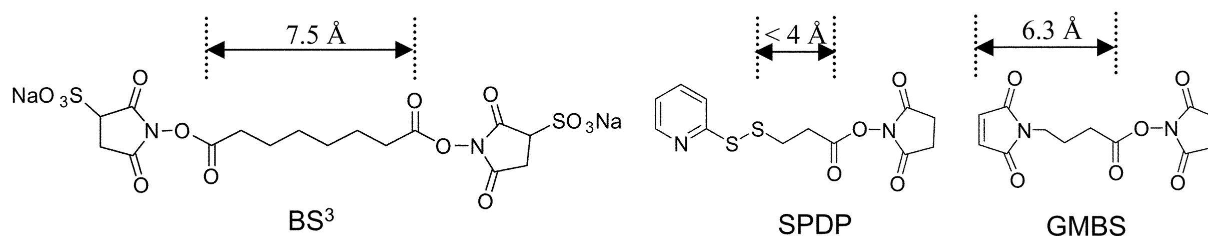


Figure 1. Molecular structure of three linkers and calculated length of the spacers.

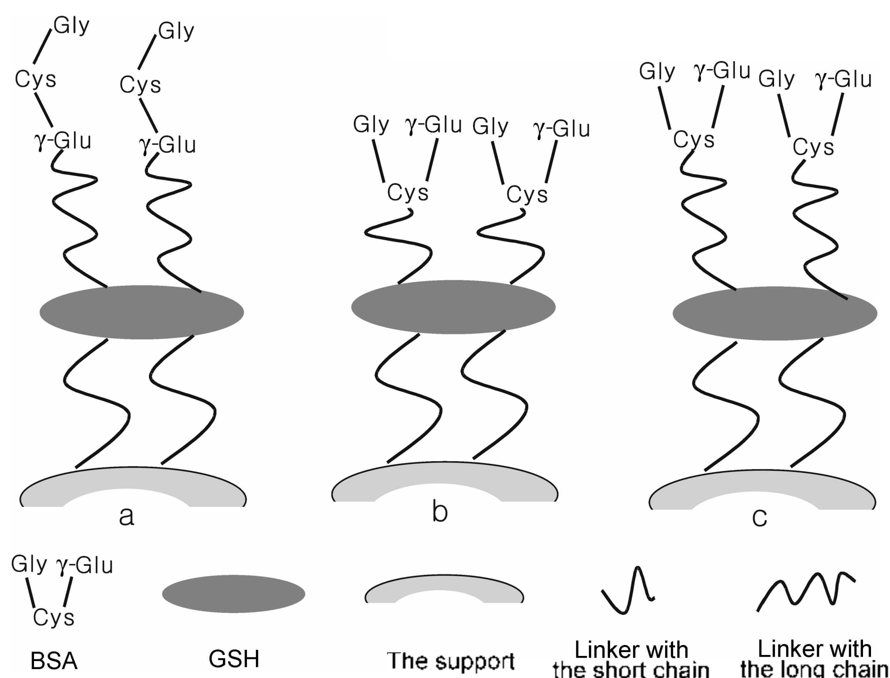


Figure 2. Schematic diagram showing difference among the immobilization modes (**a**): BS[3] linker provides linear linkage with a long spacer. (**b**): SPDP linker produces branched linkage with the shortest spacer. (**c**): GMBS linker generates branched linkage.

sample (**5**) and sample (**6**). Also, because each linker has characteristic length of the bridge, the longest spacer (7.5 Å) is expected for sample (**4**), while the shortest spacer (less than 4 Å) for sample (**5**) (Figure 1). As a result, different binding modes and spacer lengths generated three different status of GSH immobilization (Figure 2).

Ligand Density Measurement: Due to the difficulties in measuring amount (or density) of immobilized glutathione directly, an indirect method was employed in our research. Fmoc-ONSu was incorporated on BSA-bound matrix instead of a linker. Because three employed linkers have the *N*-hydroxysuccinimide functional group in common, we assume that the reaction with Fmoc-ONSu mimics the one with the linkers. After completion of the reaction, Fmoc group was deprotected, and released dibenzofulvene was measured. The 9-fluorenylmethoxycarbonyl (Fmoc) group is stable toward acids and readily cleaved by a variety of bases. In this study 20% piperidine in DMF is employed to deprotect the Fmoc functional group. Piperidine forms an adduct with the dibenzofulvene, and the adduct absorbs at 301 nm.[13] Typical ligand density obtained with this method is 11.2 μmol/g. Given the surface area of 42.9 m^2/g, the observed density corresponds to 0.16 amine/100 $Å^2$. In average each ligand occupies 625 $Å^2$, and there is a significant spacing between the ligands. Therefore, the immobilized GSHs expect spacing of about 25 Å between them.

Interaction between GST and GSH on the CPG: To compare binding property of the beads towards GST, the same amount of the beads was taken, and the purified GST was eluted off from sample (**4**), sample (**5**), and sample (**6**). Chromatographic analysis (Figure 3, lane 2 and 4) showed that both sample (**4**) and (**6**) bind GST, and binding of the latter sample is more efficient than the former case. Because spacer length is comparable in the two cases, the only difference is mode of the immobilization. It is well-known

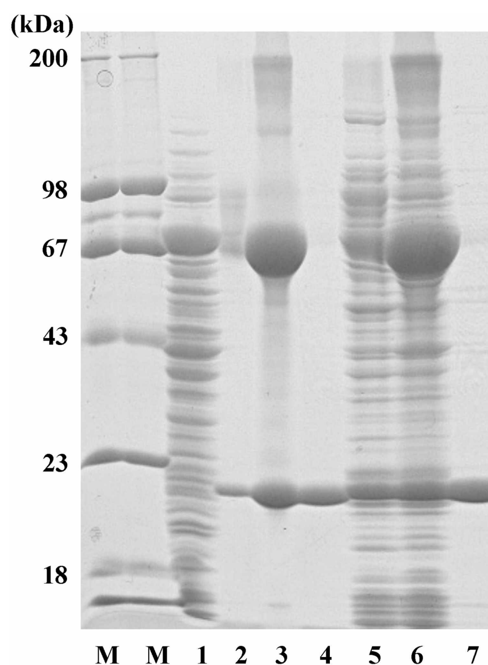


Figure 3. Purified GST and cell lysate containing overexpressed GST were employed to check the binding characteristics of sample (**4**), (**5**) and (**6**). M: Size marker. Lane 1: Cell lysate containing overexpressed GST was eluted from AMPCPG. Lane 2, 3 and 4: Purified GST (23 kDa) was eluted off from sample (**4**), (**5**) and (**6**), respectively. Lane 5, 6 and 7: Proteins in cell lysate were eluted off from sample (**4**), (**5**) and (**6**), respectively.

that an appropriate anchoring site is important for a successful affinity chromatography. The data showed that the anchoring through the cysteine side chain is the better choice. The crystal structure data of GST shows that domain I has a deep (19 Å) cavity, and this domain is considered to be the GSH binding domain.[10] For sample (**6**), the backbone of GSH faces domain I and fifteen hydrogen bonds or salt-bridge contacts are involved in the interaction with GST. However, if GSH were conjugated with BSA through the amine side chain, its resulting structure should be definitely different from the former (Figure 2). It is expected that the remaining twelve hydrogen bonds or salt-bridges become destabilized because of the profound change between active site and GSH.

Interestingly, for sample (**5**), an undesirable phenomenon occurred during the elution procedure. BSA was eluted off from the matrix together with GST (Figure 3, 67 kDa band in lane 3 and 6). It is very likely that 2-mercaptoethanol in the elution buffer cleaved the disulfide bond holding GSH, and triggered the undesirable secondary reaction. While the detailed mechanism is not clear yet, it is worthwhile to point out that combination of BSA and the use of SPDP leading to a disulfide linkage with GSH is problematic.

When cell lysate containing overexpressed GST was introduced into AMPCPG, as a control, nonspecific binding was significant (Figure 3, lane 1). Test of samples with the lysate made the comparison more clear. As evident in Figure 3, both sample (**4**) and sample (**5**) exhibited significant non-specific protein binding (lane 5, 6), while sample (**6**) captured GST selectively (lane 7). The chromatogram clearly shows that nonspecific protein binding is effectively suppressed with the use of BSA and specific binding of GST is amenable with a right choice of immobilization mode. In this particular example, GMBS resulted in the best binding selectivity and capability for GST.

In summary, for the successful use of CPG for selective binding GST, use of BSA interlayer and a right linker is critical. The beads tethering GSH with three different binding modes were tested with cell lysate containing overexpressed GST, and only GMBS turned out to be a linker that enabled the immobilized GSH to capture GST selectively.

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