A Development of Anchoring Adapter Molecule for Antibody Immobilization: Monomeric Streptavidin / Antibody-binding Domain Fusion Protein

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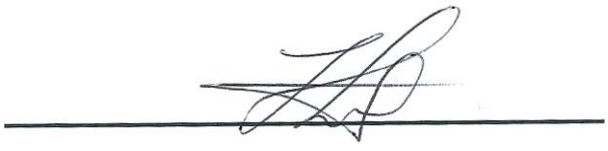
Somin Heo

12. 18. 2015

Approved by



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12. 18. 2015

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Abstract

The enzyme-linked Immunosorbent assay (ELISA) has been developed as a format of a diagnostic tool in medicine recently to detect the presence of an antigen in a liquid sample or wet sample. Especially, sandwich type ELISA has been commonly commercialized and preferred because of high specificity and sensitivity among the systems by using two antibodies to capture and detect its antigens. In addition, immobilization of antibodies is a key process to improve specificity and sensitivity in ELISA. Here we report a novel method to immobilization antibodies on the surface of the solid support with a recombinant antibody binding domain (ABD) fused with mono-streptavidin (mSA). The Z domain derived from protein A which has high binding affinity to the Fc region of various antibodies such as rabbit, rat and mouse. mSA is monomeric form of streptavidin which consists of tetramer and has very high affinity with bioitin (vitamin H). We have genetically inserted ABD with mSA (mSA-ABD) to obtain dual functions separately. mSA-ABD fusion protein is overexpressed in E.coli system and successfully purified by affinity chromatography. The binding affinity of ABD of mSA-ABD is verified with quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) analysis. The other binding affinity of mSA with biotin was confirmed with binding activity test using biotin coated plates. Finally, we treat mSA-ABD as an anchoring adapter molecule in sandwich type of ELISA taking advantage of dual binding affinity and we can obtain the typical ELISA result from the series of experiments.

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Chapter 1. Introduction

1.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technique widely used for detecting and quantifying substances such as proteins, peptides and hormones. In the Enzyme-linked immunosorbent assay, an antigen or capture antibody must be immobilized onto a solid phase support and then mixed with an antigen or antigen and detecting antibody depending on the types of Enzyme-linked immunosorbent assay you choose to perform. Finally, an antibody which is conjugated to an enzyme is treated for the detection. Detection is processed by the conjugated enzyme activity via incubation with a substrate to produce a measureable product.

The enzyme-linked immunosorbent assay has been known as one the most powerful technique to detect a variety of antigens or other substances you might be interested in due to its high sensitivity which result from the detection strategy of highly specific antibody-antigen interaction[1]. Furthermore, this technique has numerous other fallowing advantages. For example, the reaction products between enzyme conjugated antibody and substrates has stable for lone time periods and the estimation of results can either be visualized or simply detected by spectrophotometer in most laboratories[2].

There are various types of the enzyme-linked immunosorbent assay systems have been studied with modification to robust sensitivity. (Figure 1-1) The key step of discrimination between indirect ELISA and direct ELISA depends on how to detect antigen whether adhering antigens or immobilizing antibodies onto the solid surfaces respectively. The most universally and widely used type of enzyme-linked immunosorbent assay is so called sandwich ELISA included in the indirect ELISA. The sandwich technique is adapted to identify specific antigens[3]. In the sandwich ELISA, an antigen is bound between a capture antibody and detection antibody. Immobilized capture antibody can pull down multivalent antigens as much as it can and then a specific detection antibody is added. The detection antibody could be coupled with an enzyme conjugated secondary antibody which produces colorimetric signal amplification with chromogenic substrates.

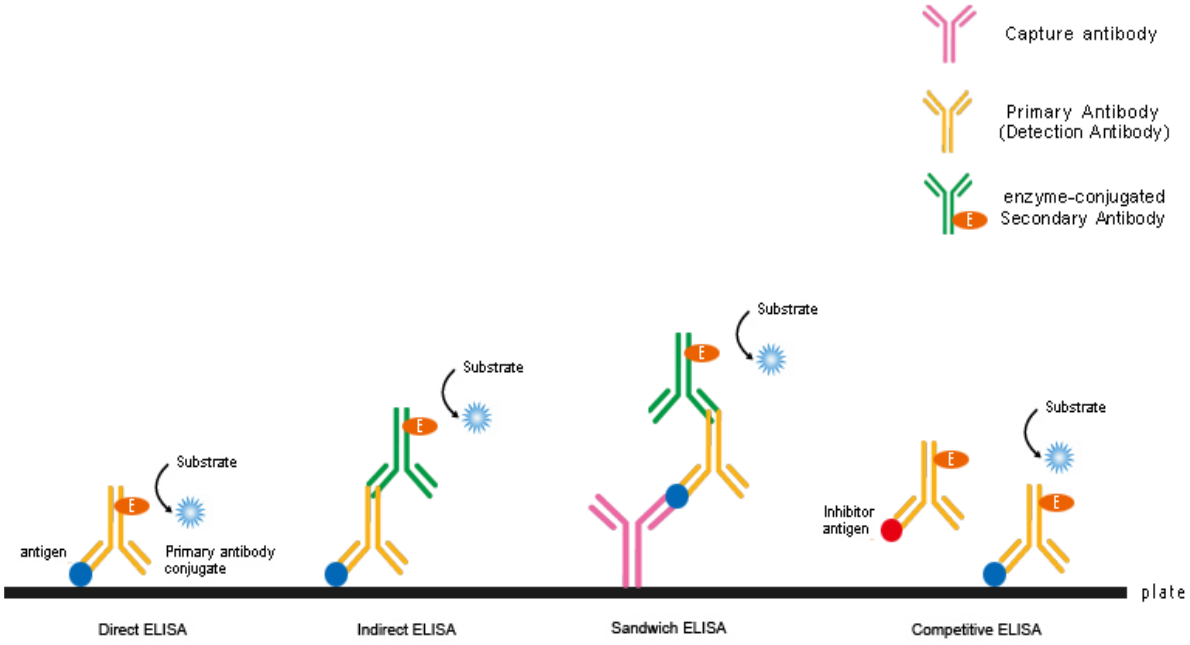


Figure 1-1. Illustration of various types of Enzyme-linked immunosorbent assay [4]

1.2 Antibody immobilization

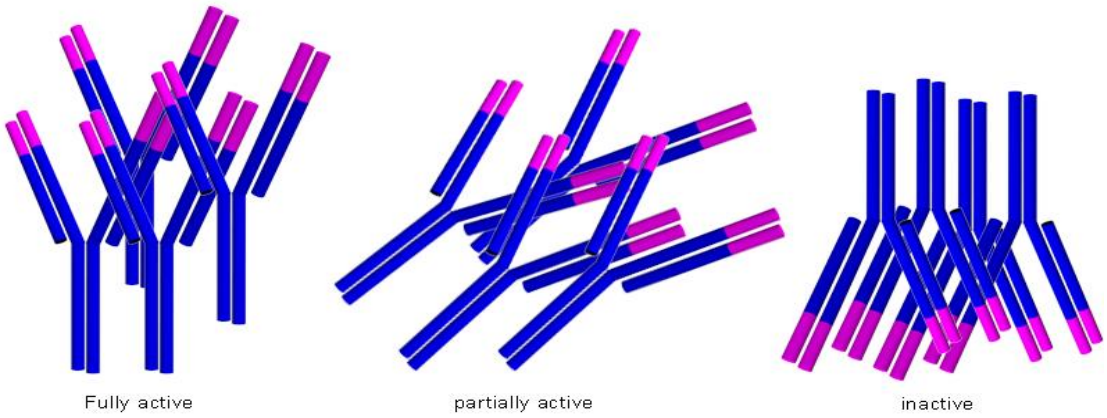
The immobilization of antibody on the surface of solid support is an indispensable process for the development of most immune-based assay systems. The selection of the method of antibody immobilization considerably affects antibody-antigen interactions. For the past several years, a variety of strategies have been studied to control antibody immobilization, mainly by directing the orientation, stability and density of bound antibodies for many fields such as purification of materials, diagnostic immunoassays and immunosensors.[5, 6] The simplest method to immobilize antibodies is accomplished by physical adsorption using van der Waals forces onto plastic or poly-lysine glass or other surfaces.[7] However, these types of approaches typically cause denaturation of majority of adsorbed proteins.[8]

As an alternative option, antibodies can be covalently attached on chemically activated surfaces through the reaction of lysine side chain. It helps antibodies easily and directly be coupled to amine-reactive surfaces.[9] Various approaches using chemical reagents such as succinimide ester and maleinimide for covalent attachment of antibodies on different surfaces have been widely reported. However, problems associated with these strategies may cause loss of biological activity in many cases. When antibodies are covalently coupled to solid supports, their specific binding capacity is generally less than those of soluble antibodies. The main reason could be the random orientation of antibodies. Multiple lysine groups are presented on an antibody molecules, it can induce multiple attachment may

arise and some inadequately oriented antibodies could have reduced sensitivity in immune-based assay.[10, 11] Three optional possibilities of antibody orientation is illustrated (Figure 1-2).

To overcome these disadvantages, several strategies for achieving well oriented antibody coupling have been developed. One of the most promising candidates for immobilization of antibodies is affinity protein such as protein A or protein G.[11] Protein A and protein G are recombinant forms of bacterial wall proteins from *Staphylococcus aureus* having Fc binding domains. When affinity proteins bind selectively to the Fc regions of antibodies, the Fab fragments are able to point away from the surfaces and exposing its fragments to bind the antigens more readily.[12] Especially, antibody binding domain, Z domain, derived from protein A has five homologous Fc-binding domains will be introduced for the immobilization of antibodies.

(A)



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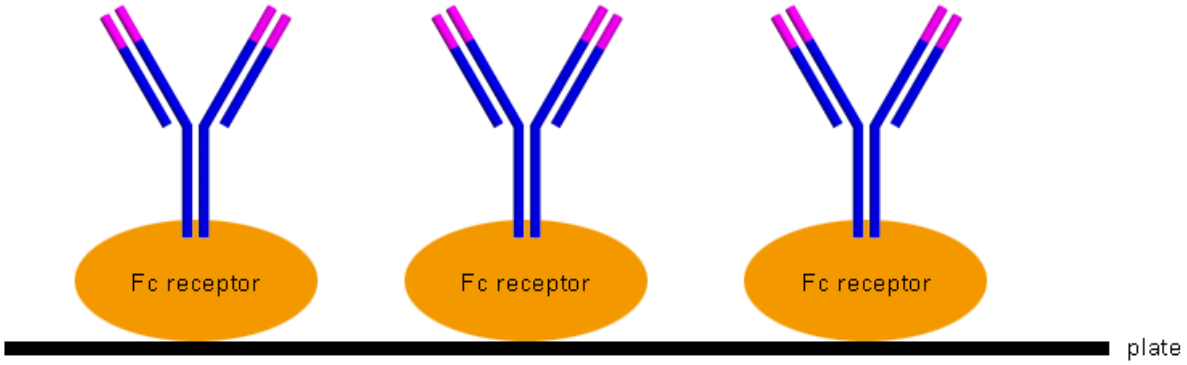


Figure 1-2. (A) Schematic illustration of the three optional possibilities of antibody orientation. (B) Schematic representation of immobilization of antibodies on the solid phase support. [11]

1.3 Research Outline

In this thesis, a recombinant fusion protein which has both high binding affinity with biotin and antibodies simultaneously that could be utilized as an anchoring adapter molecular in ELISA. We choose a monomeric type of streptavidin to immobilize the molecule on the biotin coated plates. The streptavidin is a tetrameric protein which has known as a remarkable protein for its ability to bind to four d-biotin molecules, which is unusually selective and powerful non-covalent interaction [dissociation constant $K_d = 10^{-15}$ M].[12,13] So, the streptavidin and biotin binding system has been successfully introduced in molecular biology and biotechnology, such as labelling and binding experiments. Although the streptavidin and its homology isolated from the *actinobacterium Streptomyces avidinii* have highly stable and strong affinity with biotin in spite of the binding environment of extreme temperature, pH and the presence of detergents, it has some limitations to overcome. For example, the tetramer form of streptavidin has four binding sites with biotinylated ligands at each unit and it could cause target aggregation. Since the aggregation can change biological functions of proteins, the use of wild type streptavidin is needed to be modified for certain application field especially, in live cell experiments. [14]

(A) Tetrameric streptavidin (B) Monomeric streptavidin

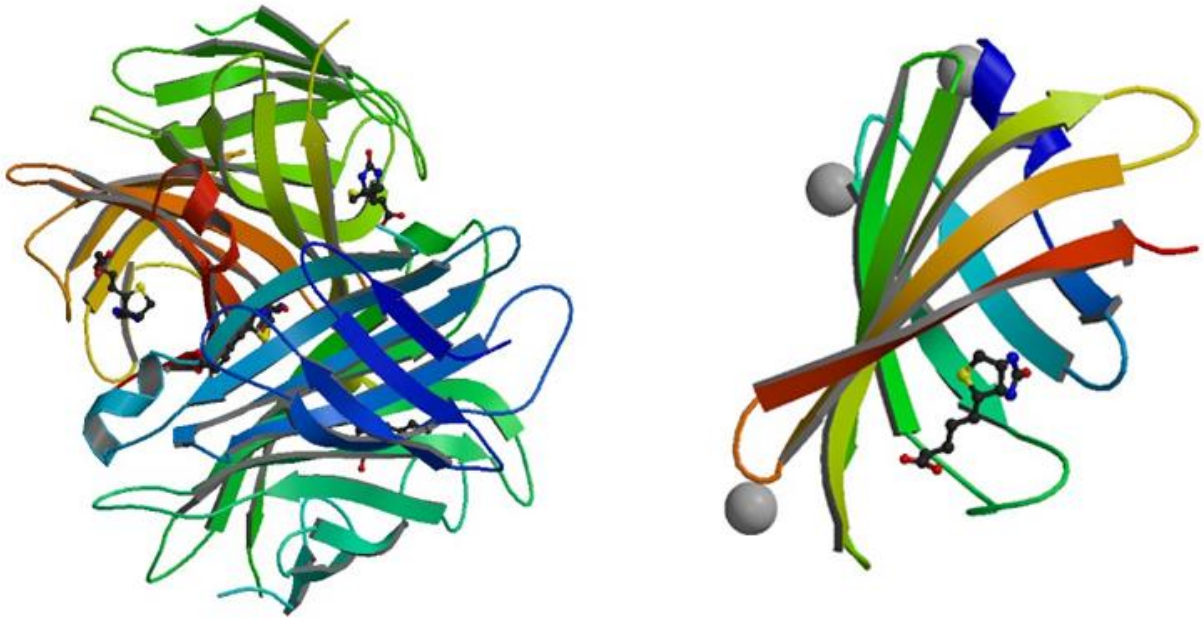


Figure 1-3. The crystal structures of (A) Tetrameric Streptavidin (PDB ID: 1N7Y) and (B) Monomeric streptavidin (PDB ID: 4JNJ).

For this reason, we chose a monomeric form of streptavidin (mSA), which is a 13 kDa protein and still has high affinity with biotin. In detail, there is big difference between monomeric and monovalent streptavidin. Monovalent streptavidin is derived from a tetramer streptavidin with three dead units not to bind with biotin (Figure 1-4). Otherwise, monomeric streptavidin is monomer form of streptavidin which is supposed to maintain to be stable and favorable with biotinylated ligands. For the past, monomeric streptavidin has known as having low affinity with biotin than monovalent streptavidin but, recently park's group has engineered monomeric streptavidin (mSA) by combining streptavidin and rhizavidin sequences, which is stable and binds to biotin with low nanomolar affinity or even less concentration. [15,16]

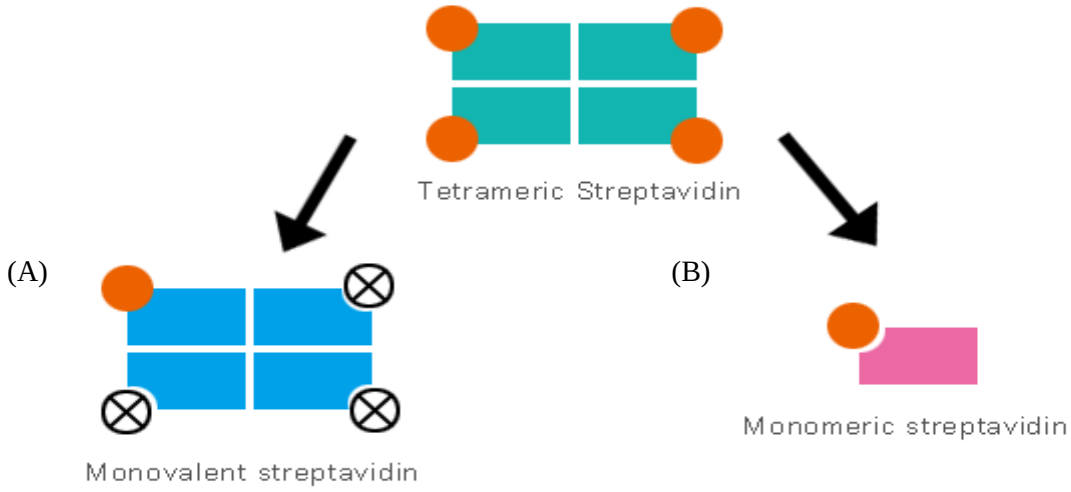


Figure 1-4. The design illustration of (A) Monovalent streptavidin and (B) Monomeric streptavidin originated from wild type of tetrameric streptavidin.[14]

To impart another binding ability, the Z domain derived from protein A was introduced as an antibody binding domain (ABD). Furthermore, the Z domain is an engineered analog of the B domain developed as affinity purification for fusion protein production.[17] ABD has high specific affinity to bind with a fragment crystallizable (Fc) region of the numerous kinds of antibodies originated from mouse, rat, and rabbit. This versatile binding affinity with various antibodies is mainly used to immobilize antibodies on the solid phase supports.

Until now, streptavidin was commonly used in labeling biotinylated antibodies to amplify signals in result immunoassay system. However, in this project, it was utilized it with a different approach. In order to impart two binding affinity to one molecule at the same time, we combined those

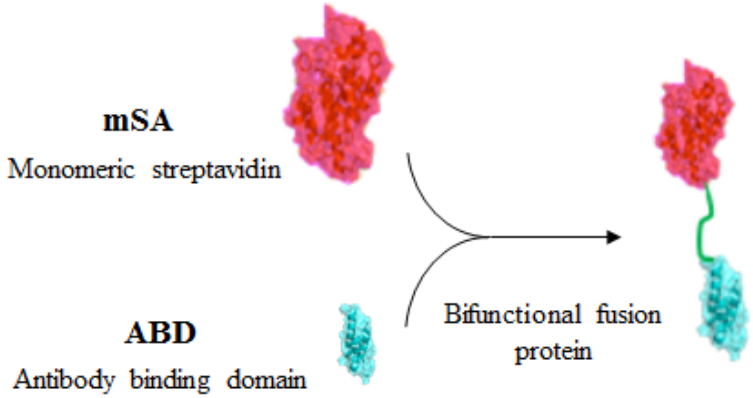
two proteins genetically and the engineered the novel fusion protein between mSA and ABD as an antibody anchoring adapter molecule which could be used in sandwich type of ELISA system.

Chapter 2. An anchoring adapter molecule for antibody immobilization

2.1 Summary

mSA is monomeric form streptavidin, which has improved binding affinity with biotin. The binding affinity has been known one of the strongest interactions in the nature. Here, we developed the mSA-ABD fusion protein which has Ig-binding affinity as well as biotin binding capability which can be an anchoring adapter for antibody immobilization for proper orientation to enzyme-linked immunosorbent assay system.

(A)



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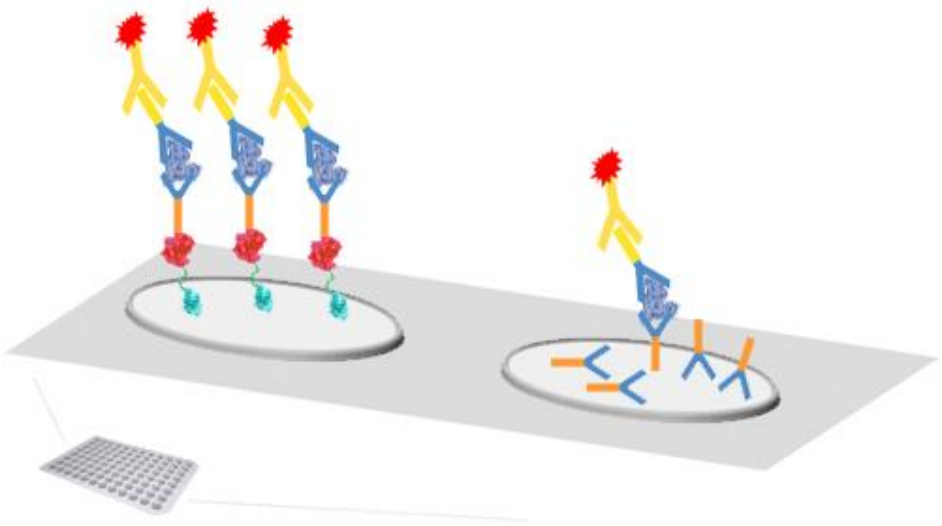


Figure 2-1. (A) Genetically engineering mSA-ABD fusion protein. (B) Schematic image of an anchoring adapter molecule for antibody immobilization using of mSA-ABD fusion protein.

2.2 Materials and Methods

2.2.1 Antibody-binding Domain insertion and Fusion Protein purification

The Z domain with 29 amino acids linker was fused into C-terminus of mSA into pETdueT bacterial high expression vector. The recombinant DNA was transformed into *Escherichia coli* strain BL21 (DE3) competent cells. One colony was selected and amplified in 5 mL in starter media (LB media, 50 mM Monopotassium phosphate, 50 mM Disodium phosphate, 25 mM Ammonium sulfate, 2 mM Magnesium Sulfate and 1% glucose) with ampicillin. Subculture was processed at 37℃ to amplify cells in the starter media overnight and it is subsequently seeded into 1L induction media (LB media, 50 mM Monopotassium phosphate, 50 mM Disodium phosphate, 25 mM Ammonium sulfate, 2 mM Magnesium Sulfate and 0.05% glucose). mSA-ABD was induced overnight by adding 50 uM isopropyl p-D-1-thiogalactopyranoside (IPTG) with appropriate antibiotics at 20℃.

The amplified cells in the induction media were harvested by high speed centrifugation and re-suspended with 25 mL of lysis buffer (50 mM Tris, 150 mM Sodium chloride, 10 % glycerol and 0.2% triton x-100, pH 7.5). To catalyze the hydrolysis reaction in bacterial cell walls, lysozyme (50 ug/mL) was added to the re-suspended solution and incubated at least 30 min at 4 ℃ on an orbital shaker. The lysate was proceeded to sonicate for 10 min in 30 sec intervals to entirely break down bacterial cell walls, followed by centrifugation at 25000 g for 1h 30 min at 4 ℃. After centrifugation, the supernatant was purified by an immobilized metal affinity chromatography (IMAC) taking advantage of the presence of hexa-histidine tag (His-tag) in the N-terminus of mSA-ABD. The extracted solution was loaded into 1 mL HisTrap HP column which is prepacked with Ni Sepharose High Performance and designed for simple, high-resolution purification of histidine-tagged proteins by IMAC (GE healthcare). The protein extract with his tag was eluted Imidazole elution buffer (50 mM Tris, 150mM Nacl and 1M imidazole pH 7.5) with a linear gradient from 5 to 100 %. The excessive imidazole residues were removed by overnight dialysis with buffer (50 mM Tris, 150 mM Nacl, pH 7.5) and the purified mSA-ABD measured by SDS-PAGE.

2.2.2 Quartz crystal microbalance (QCM) measurements

To confirm the biding ability of Z domain to various types of immunoglobulin, Q-Sense E4 and Standard gold QCM sensors (Q-Sense, Sweden) were used. The detailed method was described previous study.[18,19] At first, the system was maintained in flow mode with a pump. Temperature should be maintained at 25.0 ± 0.1 ℃. The equilibrium of the system was achieved by phosphate buffer (100 mM NaCl and 50 mM sodium phosphate, pH 6.5) and 0.5 mg/mL mSA-ABD or mSA-GST dissolved in PBS buffer was injected. After keeping the signal balanced using phosphate buffer, 0.2 mg/mL rabbit,

mouse or rat antibodies in phosphate buffer was treated. The QCM chips were washed to remove unwanted residues by phosphate buffer at each step and the final washing step was continued for a long time to verify tight bindings between Z domain and various types of IgGs.

2.2.3 Surface plasmon resonance (SPR) analysis

SPR experiments were conducted with CM-5 gold chips via Biacore 3000 device (GE Healthcare) at 25 ℃. Sterile and filtered PBS buffer was used as a running buffer. A CM-5 gold chip is coated with carboxymethylated dextran which can be coupled with a variety of immunoglobulin on the surface by standard amine-coupling chemistry.[20] For pre-concentration of the sensor chip, 0.1 mg/mL of each Ig G was injected shortly until respond units (RU) reached to certain of ranges. To activate carboxyl groups on the surface of sensor chip, 60uL of 1:1 mixture: 0.5 mg/mL of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 0.5 mg/mL of N-hydroxysuccinimide (NHS) was introduced at a flow rate of 10 μL/min. 0.1 mg/mL of rabbit, mouse or rat IgG was injected individually at flow rate for 7 min. Injected volume was determined according to desired RU value. Excessively remaining reactive carboxylated groups were quenched with 1 M ethanolamine (pH 8.0). mSA-ABD was injected and flowed for 3 min at various concentrations to optimize the binding ability of ABD. The binding kinetics of mSA-ABD to various types of IgG was measured using Biaevaluation software with 1:1 Langmuir binding model.

2.2.4 Monomeric streptavidin binding Affinity Test

To demonstrate the affinity between mSA and biotin, biotin coated plates (Clear, 8-Well Strip, Pierce, code number 15151) were introduced. The mSA-ABD was dissolved in phosphate-buffered saline (PBS; 35 mM phoshate and 150 mM NaCl, pH 7.3). 100 uL of the solution was loaded in each wells of the plate and incubated in various concentrations on a rocking plate overnight at 4 ℃. To remove not immobilized proteins, the plate was vigorously washed 5 times with 250 uL of PBST buffer (100 mM phosphate buffered saline and 0.05% tween-20). Anti-bovine serum albumin antibody (rabbit from abcam, code number ab 7637) which is conjugated with horseradish peroxidase (HRP) was treated in each well and incubated in 2 h at 37℃ and washed 3 times using 250 uL of PBST buffer. To prepare the substrate solution, we used SIGMAFAST OPD tablet kit (Sigma-Aldrich). According to the instruction, 0.4 mg/mL o-phenylendiamine (OPD) is dissolved in 0.4 mg/mL hydrogen peroxide (H_2O_2) and 12.5 ml of 0.05 M phosphate-citrate, pH 5.0. 100 μL of the solution was loaded incubated for 2 min at room temperature. After incubation, 100 μL of 2 N H_2SO_4 solutions was added into the each well to

stop the enzymatic reaction. The absorbance at 450 nm of each well was measured by a multi-scanner (Infitite 200, TECAN).

2.2.5 Sandwich type of Enzyme-Linked Immunosorbent Assay

mSA-ABD was dissolved in phosphate-buffered saline (PBS; 35 mM phoshate and 150 mM NaCl, pH 7.3) at 10 nM respectively. Each well of a biotin coated pates (Clear, 8-well strips, Pierce) were filled in 100 uL of the solutions to immobilize the proteins on the surfaces of the plates by interaction between biotin and mSA. After the immobilization for overnight at 4 ℃, each well was washed vigorously 5 times with 250 μL of PBST buffer. The primary antibodies against BSA (rabbit from Santa Cruz Biotech) and EpCAM (rabbit from Sino Biological Inc.) solutions were prepared with the PBS buffer. 100 uL of the antibody solutions were loaded on the wells of the mSA-ABD-immobilized plate. After overnight incubation, each well was washed three times with 250 μL of PBST buffer. 100 uL of BSA and EpCAM solutions were loaded on the wells of the plate and incubated for 2 h at 37°C. After incubation, each well was washed three times with 250 μL of PBST buffer. The primary antibodies against BSA (mouse from Abcam) and EpCAM (mouse from Sino Biological Inc.) solutions were dissolved in the PBS buffer and 100 uL of the primary antibodies solution were loaded on the wells of the plate. Sequentially, HRP-conjugated anti-mouse secondary antibodies (Biolegend) were diluted and 100 uL of the solutions were loaded on the wells of the plate. To prepare the substrate solution, we used SIGMAFAST OPD tablet kit (Sigma-Aldrich) and followed instructed manners provided from the company.

2.3 Results and Discussion

2.3.1 Antibody-binding Domain insertion and Fusion Protein purification

To acquire the construction of a fusion protein which has high affinity to biotin and also has binding affinity with various types of antibodies, we prepared antibody binding domain (ABD) isolated from protein A and genetically fused to the C-terminus of mSA. The 27 extra amino acids (PNSGGGLVPRGSGGGCGGGTGGGSGGG) were inserted as a linker between mSA and ABD to reduce steric hindrance between two domains which should be fully functional. The linker also provides conformational flexibility to the recombinant protein be more stable during the assembling process.[21] To obtain a fusion protein of mSA- Glutathione S-transferases (mSA-GST) as a negative control of Z domain, we eliminated Z domain using restriction endonucleases and inserted the sequence of GST to the C-terminus of mSA.

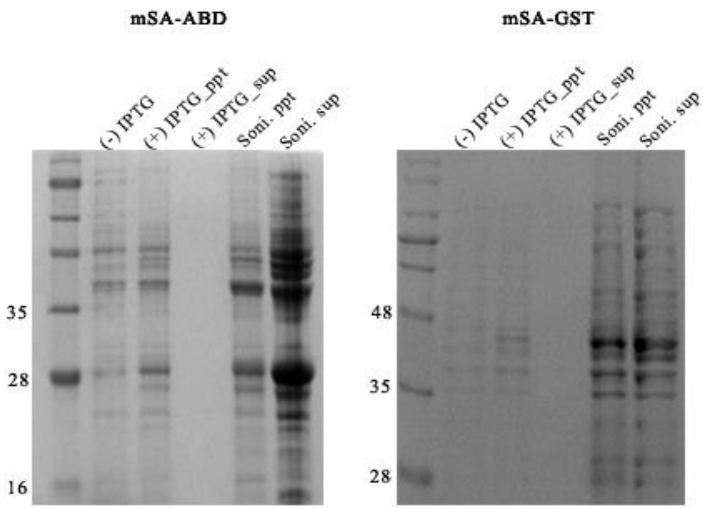
The mSA-ABD fusion protein (mSA-ABD) and the mSA-GST fusion protein (mSA-GST) were overexpressed in *E. coli* strain BL21 (DE3). We could finally set the most optimal culture conditions through a chain of experiments. Culture was preceded at 20℃, overnight culture and the concentration of IPTG was 50 uL to yield the low rate of soluble proteins synthesis.[22] In order to maximize the efficiency of protein expression, we introduced several additives to LB culture media. The addition of glucose is able to prevent leaky mSA expression, which could reduce cell viability and overall protein yield. Furthermore, adding 2 mM of MgSO4 can double the total biomass after overnight induction.[23, 24]

We could acquire a soluble fraction of the protein after sonication process to break bacterial cells membranes efficiently (Figure 2-2A). Usually, Biotin or streptavidin was purified using streptavidin coated agarose beads or biotinylated agarose beads. But, using these methods requires very low pH and high salted buffer system to elute high affinity molecules from the affinity beads, which causes harsh conditions for proteins to be stable in aqueous phase. Therefore, we were purified the lysate by immobilized-metal affinity chromatography (I-MAC), because the recombinant protein has hexa-histidine tags (His-tag) of mSA-ABD and mSA-GST. The His-tags provides binding affinity to Ni-NTA columns and the purification steps were processed with a mild condition buffer system. (Figure 2-2B)

The molecular weights of mSA-ABD and mSA-GST were identified by SDS-PAGE (Figure 2-3C). According to the calculated value 23517.2 Da and 42574.7 Da respectively suggests that the fusion of the ABD and GST to mSA protein was successful. The concentration of the protein was estimated by Bradford assay.

Figure 2-2. Characterization of mSA-ABD and mSA-GST. (A) SDS-PAGE analysis presents the soluble fraction of the mSA-ABD and mSA-GST. (B) Immobilized-metal affinity chromatography profiles of the mSA-ABD and mSA-GST with a linear gradient of 1M imidazole elution buffer from 5-100%. (C) SDS-PAGE analysis shows the estimated molecular size of the mSA-ABD and mSA-GST after dialysis.

(A)



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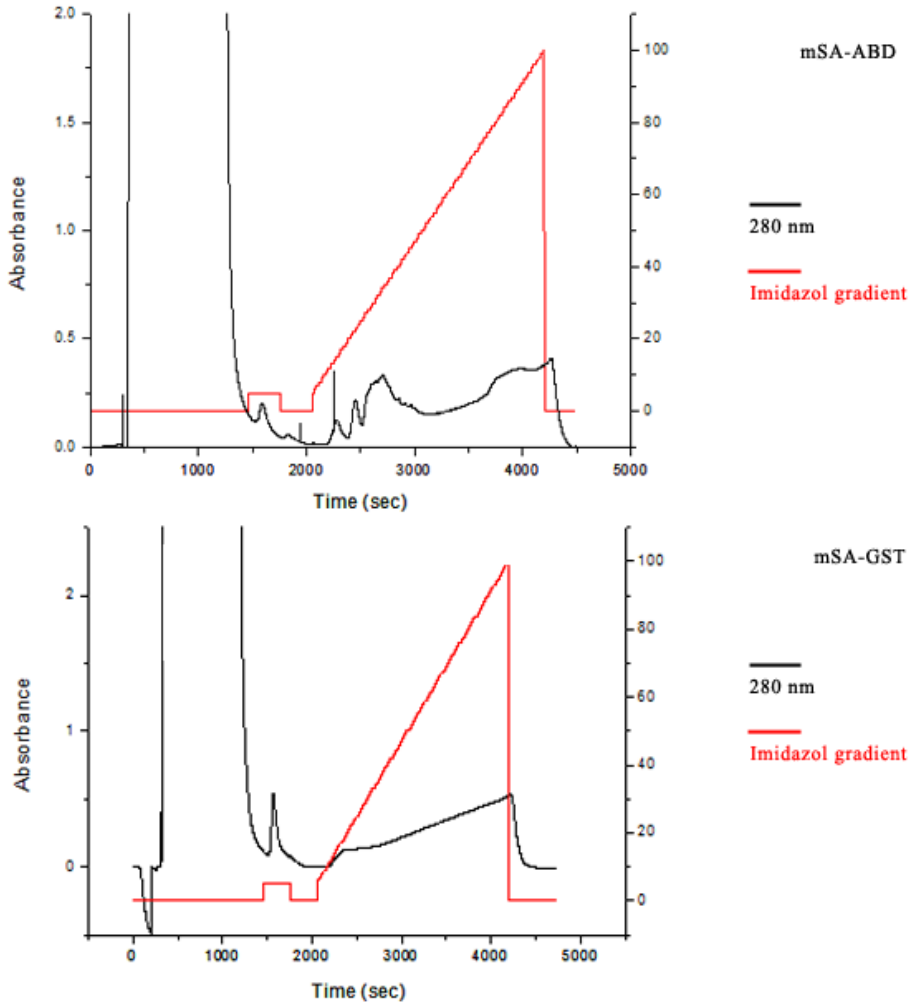

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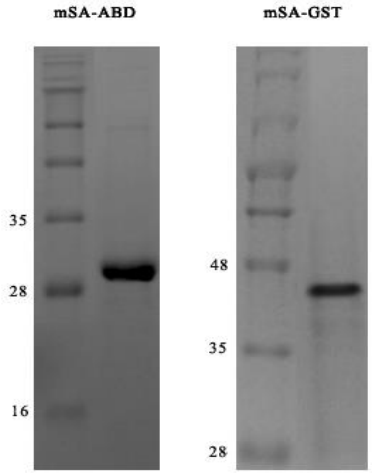


2.3.2 Antibody-binding Domain

To confirm binding affinity of the Z domain derived from protein A with various types of IgG, we introduced quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) for estimation of binding kinetics and affinity between biomolecules in real-time.

Firstly, the QCM experiment measures the change of the frequency depending on the mass variation per unit area in liquid environment when biomolecules are deposited on the surface of a sensor chip. As the mSA-ABD and mSA- glutathione S-transferase (mSA-GST) were treated onto the QCM sensor to be deposited, the resonance frequency was decreased and maintained its value until the values reached to the saturation point to completely cover the surface of the sensor chip with sample. Washing step was required to eliminate simply attached non-specific proteins. Subsequently, we treated mouse, rabbit and rat IgG solution over the sample-layered QCM sensor. The value of frequency was notably decreased otherwise, there was no change in case of mSA-GST fusion protein (Figure 2-4). After that, we performed an extensive washing step for a long time to create harsh conditions to remove loosely bounded proteins. In spite of including the harsh washing step, the frequency value of mSA-ABD was still consistent. From this results, we suggest that the mSA-ABD specifically can bind with various types of the antibodies. Also, the complex of mSA-ABD and antibody is stable and the bimolecular interaction is intact during the experiments.

Secondly, SPR analytical technique was chosen to examine the binding affinity between mSA-ABD and several types of IgGs more specifically. In the contrast with QCM experiment, first we introduced three kinds of IgGs originated from rabbit, mouse and rat to immobilize on the surface with carboxylated dextran which coupled to the gold metal surface via EDC/NHS amine coupling reaction. 1M ethanolamine is treated to avoid undesired binding among the following sample with reactive amine group residues on the surface. Then, we injected the mSA-ABD with variously different concentrations. Each experiment was carried out after regeneration of the surface by using base washing solution and subsequent equilibration with a proper binding buffer. Immediately after mSA-ABD was injected at various concentrations, a curved line was significantly and rapidly increased in respond units (RU). These data suggest that ABD of mSA-ABD is fully functional in order to tightly capture several types of IgGs successfully.

We estimated the kinetics between mSA-ABD and each type of antibodies by using global curve fitting in the SPR sensorgrams with 1:1 Langmuir binding models. The binding kinetic data of the mSA-ABD includes association (on) and dissociation (off) rates (ka and kd) and binding affinity constant (KD). The most important value to analyze in SPR is KD value which usually represents the equilibrium dissociation constant between antibodies and its antigens. KD value and binding affinity are inversely related. The KD value relates to the concentration of antibody and so the lower the KD

value indicates that the substance has higher binding affinity to antibodies. According to the table (figure), each binding affinity constant of the mSA-ABD which is bound to the mouse, rabbit and rat Ig Gs is 199 nM, 16.1 nM and 16 nM respectively. Based on facts, so called high affinity molecules have typically nanomolar (nM) range of KD value. The SRP analysis data of mSA-ABD reveals that mSA-ABD is tightly bound to the antibodies originated from rat and rabbit with strong affinity. Otherwise, the binding affinity between mSA-ABD and the mouse antibody is not enough low as rabbit or rat antibody. This result indicates mSA-ABD not efficiently and strongly captures the mouse antibody but, the KD value about the mouse antibody is acceptable. The significant difference results from almost 40 times larger dissociation (off) value (kd) of the mSA-ABD for the mouse IgGs than that of the mSA-ABD for the rabbit and rat IgGs. The fast dissociation rate of the mouse IgGs is understandable because ABD derived from protein A which has a tendency with low binding property for mouse IgGs.

From QCM and SPR experiments, these results demonstrate that mSA-ABD could be a universal and versatile IgG binding molecules, because they can bind a variety of IgGs originated from different species by simple molecular recognition. We think mSA-ABD can be used as an effective adapter molecule to immobilize antibodies with those properties.

Figure 2-3. QCM analysis of mSA-ABD (black lines) and the mSA-GST (red lines). All three types of antibodies were used; mouse (left), rabbit (center) and rat (right).

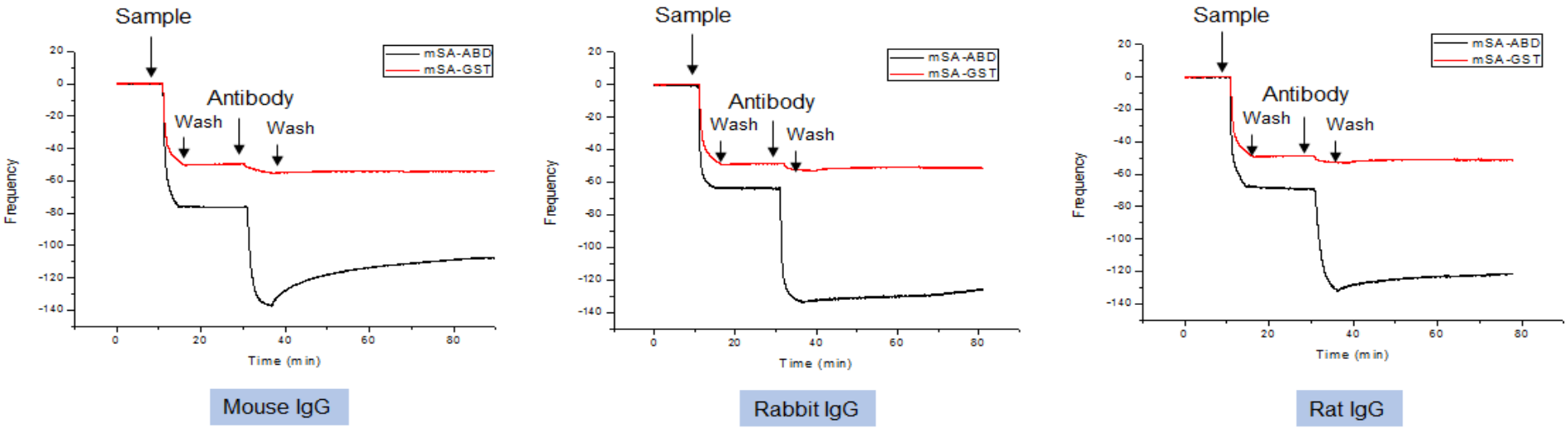
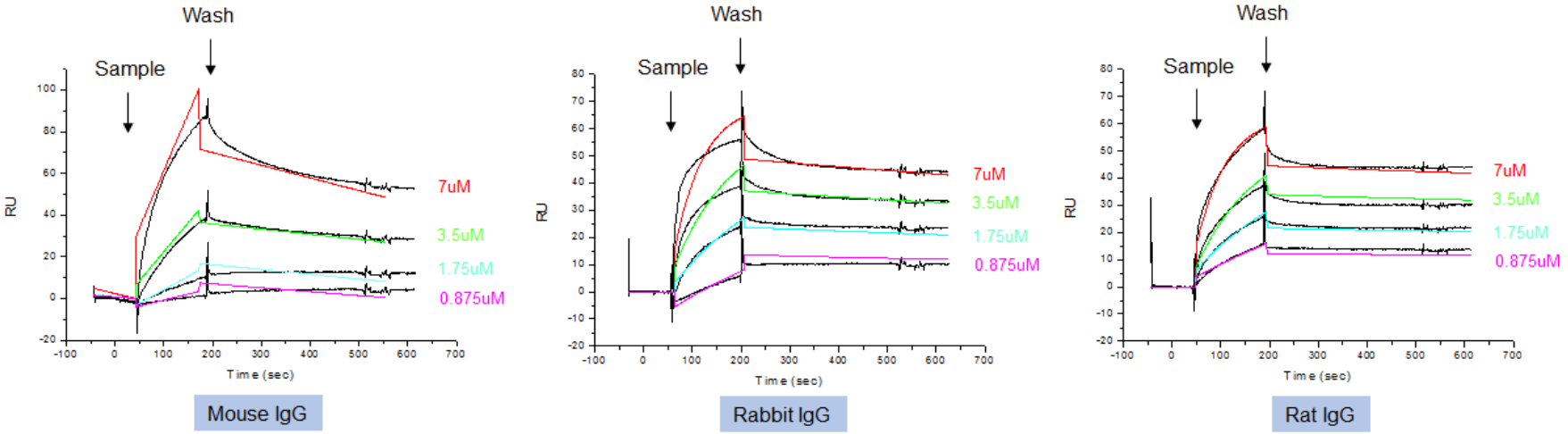


Figure 2-4. SPR analysis of the mSA-ABD using 1/2 serial dilution stating from 7 uM. All three types of antibodies were used; mouse (left), rabbit (center) and rat (right). 1:1 Langmuir binding models was to fit the curved lines to fitting lines depending on the concentration gradients (bottom, table).



Antibody	k_{ON}(1/mM·s)	k_{OFF}(1/ms)	K_A(1/nM)	K_D(nM)	Chi^2
(A) mouse	0.241	0.0048	0.0050	199	3.35
(B) rabbit	19.1	0.308	0.0622	16.1	5.85
(C) rat	9.3	0.149	0.0624	16	2.89

2.3.3 Monomeric streptavidin binding Affinity Test

To simply verify binding affinity between biotin and mSA, commercially available 96-Well Strip Plates coated with biotin onto the surface were arranged. We loaded mSA-ABD fusion protein samples with various concentrations on each well in the plates and incubated them to immobilize on the surface of the plate in time and temperature dependently both 1 hour at 37 ℃ and overnight at 4 ℃. The amounts of immobilized mSA-ABD on the surface of the plate were evaluated by treating HRP-conjugated antibodies which bind to ABD of mSA-ABD. According to the graph (Figure 2-5), HRP conjugated antibody binds to the ABD of mSA-ABD, so the signals generated from its substrate and the presence of H_2O_2 were measured by absorbance at 450 nm. The detected signals indicate that mSA successfully binds to the surface of biotin coated plates, which means even a monomeric form of streptavidin has high binding affinity with biotin because the concentration of limit of detection (LOD) is almost three digits picomolar range. In addition, the results which consist of baseline, exponentially increased line and saturation line suggest a tipping point between exponentially increased line and saturation line. The concentration of the tipping point is 10 nM in the experiment and it critical to determine the optimized concentration of mSA-ABD which is supposed to be loaded in each well of the plates in order to avoid steric hindrances between antibodies and help to expose ABD of mSA-ABD to antibodies to offer more opportunities to be effectively contacted each other. There was no significant difference when mSA-ABD was incubated by different time and temperature. Since short incubation time at moderate temperature is enough to immobilize mSA-ABD on the surface of the biotin coated plates, the binding affinity between mSA and biotin was rapidly formed and this condition can save much time to proceed with the next step which is different from other immobilization methods which usually incubate its samples for overnight.

Furthermore, GST(Glutathione-*S*-transferase)-ABD fusion protein which is a positive control sample of ABD and was introduced to confirm nonspecific bindings of other molecules on the surface of the biotin coated plates which brings false signals. The graph shows that the signals from GST-ABD was barely detected as baseline signals in the mSA-ABD binding test and this result implies there is no nonspecific binding on the surface of the biotin coated plates even though, GST-ABD also has binding affinity to antibodies. To ensure that even HRP conjugated antibodies are not directly bound to the surface of the biotin coated plates, streptavidin (Sigma) was purchased and dissolved in PBS to be loaded on each well on the plates. The solution of streptavidin was loaded with various concentrations to occupy the rest of spaces without mSA-ABD as a blocking agent and we obtained almost consistent results within Standard deviation in the presence of streptavidin with even low or high concentration. From this experiment, we are certainly confident that even HRP conjugated antibodies are not bound

onto the surface of the biotin coated plates and cannot produce any false signals. In addition, mSA is tightly bound to the surface of the biotin coated plates, which is not be interrupted by the presence of streptavidin (Figure 2-5)

. (A) (B)

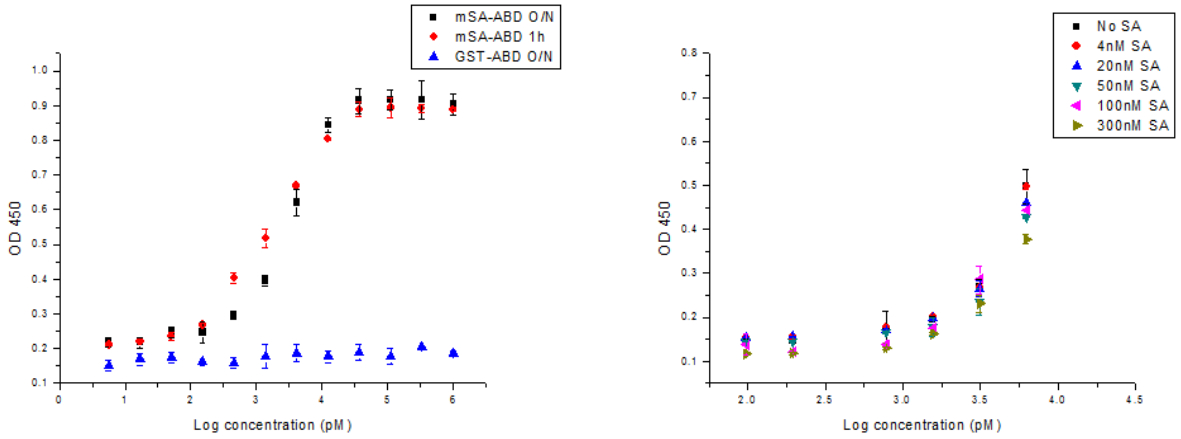


Figure 2-5. The graph of mSA binding affinity test with HRP conjugated antibodies (A) and streptavidin concentration dependent. (B)

Since mSA-ABD has two different binding affinities including mSA which is strongly bound to biotin and ABD which is species independently bound to various types of antibodies on the end of both side of the protein, we finally aimed to use it as an anchoring capture antibody molecule in sandwich ELISA systems in an oriented-controlled manner without any chemical modifications. The mSA part binds to the biotin coated surface of the plates and the ABD part is exposed to opposite side of the biotin coated surface. So, ABD readily binds to Fc region of capture antibodies and their variable region is displayed to catch target antigens effectively with full active configuration in immobilization forms of antibodies.

2.3.4 Sandwich type of Enzyme-Linked Immunosorbent Assay

Finally, to test the ability of mSA-ABD as an anchoring adapter molecule for immobilizing capture antibody in sandwich ELISA systems, we immobilized fixed amounts of mSA-ABD (10 nM) which was determined by previous experiment on the surface of the biotin coated plates. To saturate the proteins with capture antibodies, we loaded excessive amount of either anti-BSA or anti-EpCAM rabbit IgGs individually and added various concentrations of bovine serum albumin (BSA) or epithelial cell adhesion molecule (EpCAM) as a role of their antigens. To avoid exchanges between capture and detection antibodies, we used rabbit-originated IgGs for capture antibodies and mouse-originated IgGs

for detection antibodies according to the analysis of binding affinity of SPR. In order to eliminate unbound BSA or EpCAM, we performed extensive washing steps subsequently. Finally, we obtained LODs in the range of 0.1 nM both BSA and EpCAM similarly and the linear signal response values in the ranges of 2.5 ~ 100 nM corresponding to the various concentrations of BSA and EpCAM. This approach is regarded as a typical method of sandwich ELISA systems to figure out the amounts of antigens which are presented.

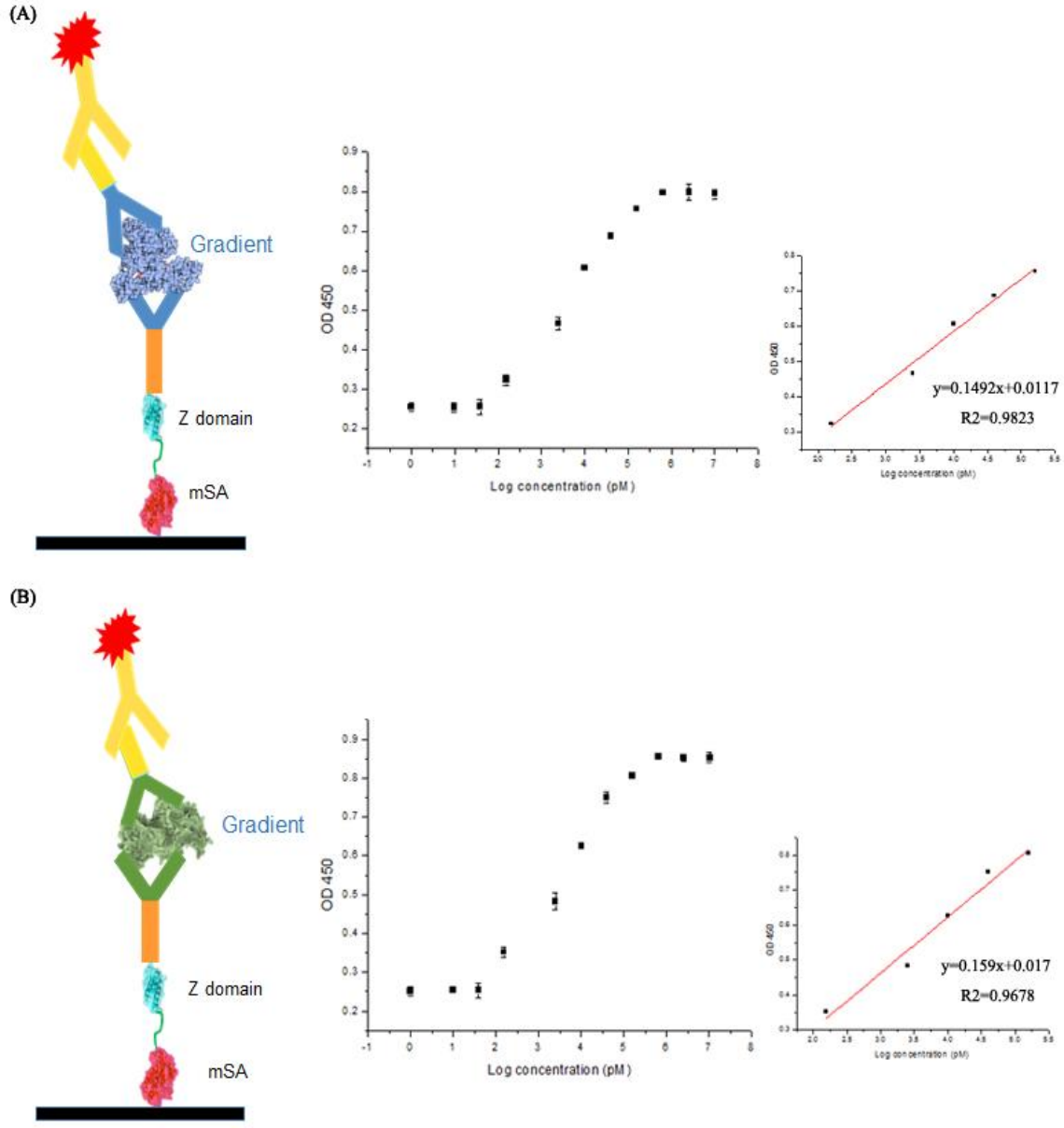


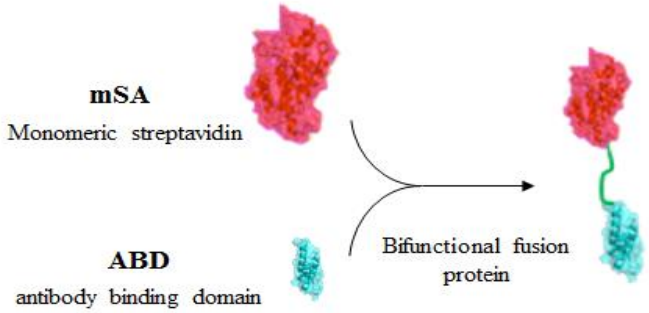
Figure 2-6. The results of sandwich type of ELISA using BSA (A) and EpCAM (B) as target antigens.

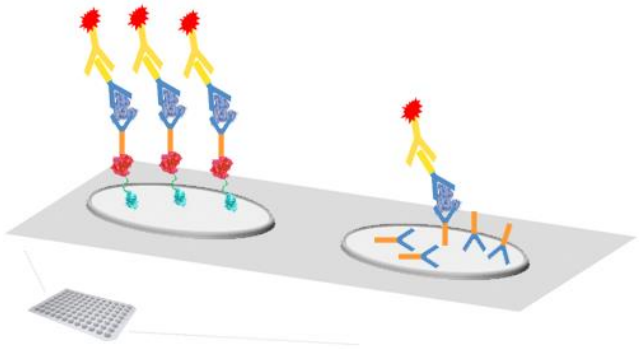
We believe that all the immobilization processes of antibodies used mSA-ABD fusion proteins were achieved simply by biomolecular recognition and interactions between proteins which have high

binding affinities. We would like to explain mSA-ABD as an anchoring adapter molecule in ELISA systems for immobilizing of antibodies originated from various species. These whole processes are simply achieved by their intrinsic molecular recognitions and interactions. Therefore, this method can maximize the interaction between capture antibodies and target antigens. It can also assure reproducibility in ELISA results by minimizing any disadvantages such as antibody inactivation and denaturation or misconfiguration by using chemical modifications.

2.4 Conclusions

In conclusion, the recombinant fusion protein, mSA-ABD which both have the high binding affinity with biotin and ability of selectively combing with Fc regions of the antibodies originated from various kinds of animals such as rat, rabbit and mouse, has been developed as an anchoring adapter molecule for capture antibody immobilization in the sandwich type of ELISA system. In contrast with other chemical cross linker reagents, mSA-ABD can be uniformly produced and overexpressed in E.coli system with reproducibility. Moreover, because of the presence of ABD of mSA-ABD, we did not need to think additional chemical modification processes which would cause severe denaturation and misconfiguration of antibodies to immobilize them on the solid phase. To found optimized culture conditions and purification methods with series of experiments, the soluble fraction of the protein was obtained by inserting Z domain which is known to improve the solubility of monomeric streptavidin proteins. Also, the binding affinity of ABD of mSA-ABD, compared with mSA-GST, was verified by QCM and SPR experiments. Furthermore, we conducted mSA binding activity test with the biotin coated plates to figure out high, stable and rapid binding affinity between them and we acquired typical immunoassay results using both BSA and EpCAM ELISA tests by introducing mSA-ABD as an anchoring proteins. Therefore, we have been developed the novel recombinant fusion protein, mSA-ABD in order to utilize it as the anchoring adapter molecule in sandwich type of ELISA system. This approach is a huge potential candidate of immobilization of antibodies on the solid surface using simple biomolecular recognitions and interactions.





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2015.12.15

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