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Orientation and capturing of antibody affinity ligands: applications to surface plasmon resonance biochips

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Abstract

A surface plasmon resonance (SPR) sensor chip with immobilized protein G was used for simultaneously capturing, purifying and orienting antibody ligands. The ligands were further stabilized by chemical cross-linking. This procedure of designing the sensor chip improved efficient use of the ligands and could prolong the analytical use.

The procedure was evaluated on standard dextran-coated sensor chips onto which commercial semi-purified antibodies toward human serum albumin and human troponin were captured and used for analysing their antigens.

The procedure demonstrates a general design approach for presenting the biorecognition element on a biosensor surface which enhances sensitivity, stability and selectivity at the same time as an impure ligand is purified.

Keywords

Biosensor; Affinity interaction; SPR; Biacore; Protein G; Sensor chip

1. Introduction

Surface sensitive biosensors, such as surface plasmon resonance (SPR) and surface acoustic wave sensors, require elaborate design of the sensing surfaces in order to accomplish favourable orientation, high purity, long-term stability, and high activity of their capturing ligands [1-4]. By applying a primary layer of ligand on the surface with more than one capturing site, *e.g.*, protein G or protein A, the orientation and purity can be enhanced and allow regeneration of a secondary capturing ligand [2, 5-9]. By carrying out the orientation and purification of the candidate ligand antibodies simultaneously the need for purification prior to immobilisation is circumvented. Also, evaluation of different ligands, such as antibodies or antibody fragments, can be done with a lesser number of sensor surfaces. By subsequently cross-linking the ligands, their stability can be further improved [2].

Both protein G and protein A are well established as ligands in analytical and preparatory affinity chromatography [10-15]. Protein G, in particular, has been shown to be useful in a variety of bioanalytical applications for efficient capturing and orientation of IgG antibodies for bacterial detection [16-19] in kinetic studies [20] and for IgG quantification in industrial applications [5]. Protein G is derived from group C and G *Streptococcus*, while protein A is from *Staphylococcus aureus* [21]. Although they have overlapping binding sites on the antibody Fc region, there are no structural similarities between protein G and protein A. Protein G binds a wider range of antibody subtypes, often with higher affinity. The main interaction between protein G and the antibody is at the Fc part of the antibody in the interface between heavy chain constant domain 2 and 3. Native protein G also exhibits an affinity for the antibody Fab region, although with an affinity of only 10% of that shown for

the Fc region. Modern genetically engineered protein G products often lack this affinity for the Fab region [22].

In this article SPR chips with protein G are used to orient and capture of ligands to antibodies towards human serum albumin (HSA) and troponin T (TnT). Human serum albumin is a common model protein and also a critical biomarker for hepatocyte differentiation and function often used *in vitro* [23, 24]. Troponin T is an important clinical biomarker for myocardial states, and is one of the endpoints for *in vitro* cardiotoxicity testing [25, 26]. However, the same surface design and assaying principle can be applied to numerous other immunoanalytical systems.

2. Materials and methods

2.1. Materials

A genetically engineered protein G from *Streptococcus sp.* expressed in *E. coli* was used (Sigma Chemical Company, St. Louis, MO, USA). The protein had retained its IgG affinity but lacked the albumin, Fab, and membrane binding regions. Dimethyl pimelimidate (DMP) was purchased from Sigma. Buffer exchange was performed on a gel filtration column (PD-10, Sephadex G25 M, GE Healthcare AB, Uppsala, Sweden) and subsequent concentration on an Amicon Ultra 4 filter (cut-off 10 kDa; Millipore Inc, Billerica, MA, USA).

The HSA antibody was a polyclonal rabbit anti-human HSA antibody (Dako Denmark A/S, Glostrup, Denmark), and the TnT antibody was a monoclonal mouse anti-human TnT antibody (clone 7G7; Abcam Cambridge, MA, USA).

HSA and bovine serum albumin (BSA) (>96%) were obtained from Sigma and TnT from HyTest Ltd (Turku, Finland).

HBS-EP buffer (10 mM HEPES, pH 7.3; 3.4 mM EDTA; 150 mM NaCl and 0.05% polysorbate 20), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS), and CM5 chips for SPR experiments were purchased from GE Healthcare. PBS buffer 0.140 M NaCl, 0.0027 M KCl, 0.010 M phosphate buffer pH 7.4 was prepared from PBS tablets (Medicago AB, Uppsala, Sweden).

2.2. Instrumentation

A Biacore 2000 (GE Healthcare) was used in all SPR measurements. A Varian Cary 100 Bio

UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA, USA) was used for colorimetric HSA determination.

2.3. Procedures

SPR experiments were carried out on CM5 chips at a flow rate of 5 μ L/min using HBS-EP as running buffer.

2.3.1. Protein G preparation

Protein G was delivered in Tris buffer. The Tris buffer, which contains amine groups that would have interfered with the amine coupling procedure, was exchanged with PBS buffer using a gel filtration column. The resulting elute was concentrated to a concentration of \sim 500 μ g/ml using a spin filter with a cut-off of 10 kD.

2.3.2. Surface preparation

Protein G was immobilized in two channels of the sensor chip, one used for the active ligand system and the other as reference. The protein G was coupled to the CM5 chip by EDC/NHS. The carboxy dextran surface of the chip was activated by treatment of 200 mM EDC and 50 mM NHS for 7 min. The concentrated protein G was diluted 1:10 in 10 mM acetate buffer, pH 3.6, and injected over the activated chip for 60 min. Subsequently, the surface was deactivated with 1 M ethanolamine for 7 min. Between measurements, chips were stored in a nitrogen atmosphere in refrigerator at 4 $^{\circ}$ C [27].

Cross-linking of sensor chips with protein G-antibody complexes was performed as follows [2]. Antibody was injected onto the protein G chip (prepared as described above) for 20 min at a concentration of 5 μ g/ml. The affinity bound antibody was cross-linked with protein G by injection of freshly prepared 30 mM DMP in 200 mM sodium borate buffer pH 9 for 25 min.

Subsequently the surface was blocked by 1M ethanolamine for 25 min.

2.3.3. Surface plasmon resonance measurements

The analytes, TnT and HSA, were assayed on the sensor chips prepared as described above by a 20 min injection of antibody at a concentration of 5 $\mu\text{g/ml}$ and a 4 min injection of the analyte. After each injection of analyte the surface was regenerated by two 1 min pulses of glycine at pH 1.5.

2.3.4. Albumin reference assay

The HSA concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) using a microassay procedure devised by the manufacturer [28]. Four parts of sample were pipetted into a test tube and one part of reagent was added. Samples were vortexed and left for 5 min before measurement in the spectrophotometer.

3. Results and discussion

3.1 *Biosensor surface design*

The design of a biosensor surface for immuno-affinity assays should favour high yield at the capture of the affinity ligands, orient the interacting sites of ligands towards the test solution with the analyte molecules, stabilize the ligands on the surface, and allow reuse or regeneration of the specific antibodies of the assay. The methodology that we described here, by which these qualities are ensured, are based on several preconditions.

The sensing surface is covered by a carboxymethylated dextran matrix extending approximately 100 nm from the surface for reducing non-specific adsorption to the surface [29] and providing sites for immobilization. The immobilization of analytical antibodies to such dextran matrices predominately uses covalent coupling methods [30, 31], and results in randomly oriented antibody ligands. In a few methods, the analyte recognizing molecules are captured by an affinity ligand, for example, using anti-IgG antibodies for capturing IgGs, avidin or streptavidin [26] for biotin conjugated biomolecules, Ni-nitriloacetic acid (NTA) for His-tagged molecules, glutathione for glutathione-S-transferase (GST), and protein A or protein G for antibodies [8, 9, 31].

The ligand capturing methods may have advantages, but there are also drawbacks. An anti-IgG antibody may have a higher affinity to the target IgG but could be species-selective as well as sensitive to harsh pH during regeneration and storage of chips. Avidin and streptavidin require chemical modifications, GST and His-tags require a tailored expression system for with the antibody of interest.

Protein G on the other hand, has the advantage of having affinity for several antibody subclasses and orients the antibody spatially (Fig. 1). In addition, regeneration of the ligand is

possible. This has also the advantage of saving the number of chips required and reducing the number of manual interventions, *e.g.* chip or buffer exchange.

The procedure applied here consisted of three steps: (1) protein G was immobilized using EDC/NHS, (2) the IgG molecules with the specificity of choice were bound to the protein G layer, and (3) the antigen (*i.e.*, the assayed analyte) was injected over the surface and the degree of binding monitored. The steps are schematically shown in Fig. 1. The figure does not depict the 3D structure and reaction mechanism on the chip in detail. However, based of previous studies of dextran polymers on modified gold surfaces it is known that immobilized protein ligands are evenly distributed along the linear dextran chains [32, 33] and that the average distance between the end-point bound chains is 2.5 nm [34].

Figure 2 shows sensorgrams for these steps with signals for binding of protein G to dextran (A), affinity binding of antibodies to the protein G derived surface (B), and binding of analytes to the antibody-protein G complex (C).

A high titre of antibody binding to protein G improves the performance of the subsequent assay, since the resulting binding capacity of the antibody is directly dependent on the surface density of protein G [30]. Thus, by maximizing the protein G binding in the first step, the sensitivity of the assay step is increased.

The species and even subgroup from which the antibody originates, may determine the affinity between the antibody and protein G [11, 21]. Moreover, the affinity towards the analyte of the chosen ligand can be used to tune the dynamic range of the assay [35]. Thus, a careful selection of an antibody ligand that addresses both affinity towards protein G and the analyte is important for the performance of the assay. Figure 2 results demonstrate this, exhibiting a mean affinity constant (K_D) of $5 \cdot 10^{-12}$ and dissociation rate constants (k_{diss}) ranging from 1 to $3 \cdot 10^{-5} \text{ s}^{-1}$ for the investigated concentrations. The estimations were

obtained with a Langmuir isotherm fit and had residuals similar to those previously described for protein G [20].

In order to enhance electrostatic attraction between the protein and dextran matrix the binding of protein G to the dextran surface was optimized by choosing a coupling pH between the isoelectrical point of protein G and the protonation pH of the carboxylic groups on the dextran matrix. For protein G this was noted as being critical for the amount of immobilized protein, as also previously reported [20]. Optimization was achieved by a pre-concentration in the dextran matrix, where a pH between 3.6 and 3.8 was found suitable for immobilization. Immobilization levels of protein G between 2500 and 5000 RU were reached with an immobilization buffer at pH 3.6.

The binding of anti-HSA to protein G resulted in 3000 RU (5 μ L/mL for 20 min) on a chip with 4700 RU of immobilized protein G (Fig. 2 A and B). When binding anti-TnT to the protein G chip, approximately 1000 RU of protein was captured on a surface with 3800 RU of immobilized protein G. The lower binding observed for anti-TnT compared to the anti-HSA antibody was assumed to be caused by a higher off-rate. In comparison to earlier work [36], approximately tenfold higher binding of anti-TnT antibody can be attained by direct binding to the dextran matrix. However, in the present study an oriented antibody with only one tenth of the antibody bound to the sensor surface still achieved half the activity. Thus, the oriented antibodies showed a higher activity per ligand.

The higher off-rate of the mouse-anti-TnT antibody from protein G made it necessary to subtract parallel injections of buffer to compensate for the continuous loss of antibodies from the surface. The better performance of the rabbit anti-HSA is surprising, as this antibody subgroup is predicted to have a less good affinity to protein G than the mouse IgG1 anti-TnT antibodies [11]. However, there has also been a report of the opposite relationship [37].

Some desorption of immobilized protein G from newly prepared surfaces occurred. In order to wash the sensor surface, repeated steps of antibody binding and regeneration were performed before starting assaying. Although the immobilized chip could be stored for over three months, sensor chips that had been stored in the refrigerator and used again by being re-docked with the SPR instrument, showed a lower binding of IgGs. This could be explained by misalignment of the sensor surface at re-docking and by denaturing of protein when dried.

Previous characterisation of a similar composite structured chip surface (Fig. 1) has been carried out using spectroscopic ellipsometry [7]. This has shown the formation of IgG-Protein G complexes on gold chip in the order of 4 nm. Also orientation of protein A using a His-tagged protein A on a Ni-NTA surface have been described [9]. That approach, however, introduces unspecific binding of metal binding proteins and a non-covalent binding of protein G.

3.2 Application of the oriented protein G sensor chip

To verify the application on the SPR sensor chip, oriented antibodies were applied for assaying two analyte proteins – troponin T and human serum albumin. A polyclonal antibody was used for HSA, and a monoclonal antibody directed towards the amino acid 60-70, as previously applied with SPR [36] was used for troponin T.

Figure 3 shows the SPR response versus analyte concentrations of HSA and TnT where the antibodies were repeatedly captured to the protein G chip before each injection of analyte. Thus, the assay cycle began with regeneration of used antibody by flowing a buffer solution of pH 1.5 over the chip, washing with running buffer, charging the bound protein G with fresh specific antibody, washing with running buffer, injecting the analyte at pH 7.4, and finally

washing with running buffer.

As is evident from Figure 3, the dynamic ranges for the two tested analytes differed. The analytes showed a dynamic range of detection from 0.01 to 7.5 $\mu\text{g/ml}$ for albumin and from 0.03 to 2 $\mu\text{g/ml}$ for TnT. The TnT antibody was saturated much faster than the HSA surface. This might partly be explained by the higher level of anti-HSA bound to the surface and partly by different affinity of each epitope of the antibodies to the analytes. The deviating molecular weights of the analytes, 67 kD for HSA and 36 kD for TnT, could also explain some of the difference of level.

The role of using a surfactant for negating formation of aggregates has previously been demonstrated by Choi and colleagues both for Protein A and Protein G complexes with IgGs [3, 16]. In particular, they have investigated the effects of polysorbate (Tween 20) using atomic force microscopy and shown how the roughness of the surface was reduced by applying the surfactant. All experiments shown in this article have used polysorbate 20 in the running buffer.

Previous applications of using orientation of ligands have been dedicated to the capture of bacteria and yeasts through their surface specific antigens. In particular, this has concerned the pathogenic species of *Yersinia enterocolitica* [17], *Vibrio cholerae* [18] and *Salmonella typhimurium* [16], *Escherichia coli* [19] and allowed quantification of the species in the range of 10^2 to 10^9 colony forming units/ml. Using orienting ligands for affinity binding of large analytes as whole cells is noteworthy. However, seems that this methodology is more apt for use with smaller analytes, such as proteins, where the sensitivity range of SPR is fully exploited [2, 5, 20, 38]. Other methods of choice where the ligand orientation would favor the sensing mechanism could be the resonant mirror technique [39], optical waveguide lightmode spectroscopy [40, 41] and dual polarization interferometry [42].

3.3 Stabilization of the sensor chip by cross-linking

The complex of protein G with its affinity bound antibody was stabilized by cross-linking by DMP [2]. This allowed an extended use of the chip. The cross-linking reaction with DMP is already well characterized and often used in affinity chromatography applications [15, 43, 44]. The HSA and TnT antibodies used in this study were readily cross-linked with DMP, although a loss in activity was noted. As a consequence, subsequent binding of HSA and TnT analytes to the cross-linked complexes was lower compared to protein G sensor surfaces with affinity-bound but not cross-linked antibodies. The obtained dynamic ranges for the cross-linked antibodies were 0.5-7.5 $\mu\text{g/ml}$ for HSA and 0.03-1 $\mu\text{g/ml}$ for TnT (Fig. 4). Thus, the stabilized sensor surfaces showed retained analytical performance that allowed quantification of both analytes, as seen in the figure.

Repeated injections of the analytes and regeneration of cross-linked antibodies slowly lowers the surface activity. Figure 6 shows repeated injections of TnT over a cross-linked anti-TnT surface at three concentrations. The activity is reduced at the higher concentrations, while the lower concentrations retain their activity to a greater extent.

To verify the specificity after cross-linking, injections of 5 $\mu\text{g/ml}$ of HSA and BSA were made over a cross-linked anti-HSA surface and the signals were compared with the signals from an unmodified protein G surface and dextran surfaces. As can be seen in Figure 5A, a significant amount of HSA binds to the cross-linked anti-HSA chip while only a small amount of bovine serum albumin binds the antibody. The noted unspecific binding to the un-coupled protein G surface is comparable to that of unmodified dextran (Fig. 5B). The K_D value for the binding was determined to $8.45 \cdot 10^{-10}$ M. The deviation from the K_D value from the non-cross-linked surface (cf. Fig. 2; $3.1 \cdot 10^{-12}$ M) was assumed to be due to a faster dissociation from the cross-linked surface (*i.e.*, a larger k_{diss}).

We also compared the SPR response for HSA binding to the protein G oriented antibody with an established spectrophotometric protein assay method (the Bradford assay) (Fig. 7). Thirty randomly distributed HSA samples were analyzed with both methods. As Figure 7 shows, the SPR response declines at high concentrations due to saturation of the chip surface. This observation is comparable with the SPR graph in Figure 5.

The cross-linking method has the advantage of both purifying commercial antibody preparations and reusing exclusive antibodies; this may favour routine assay applications. A drawback is a rather substantial loss of activity. However, this is compensated for by (1) the increased capturing efficiency of the oriented ligands, and (2) increased stability of the ligand, which favors its long-term use. The latter is of substantial benefit for applications in toxicity testing [45, 46] and in bioprocess monitoring and control [47].

4 Conclusion

In this study, the possibility of simultaneously purifying, immobilizing, and orienting antibody ligands from crude ligand extracts by protein G was exploited. This has clear advantages. Compared with, for example, a streptavidin-biotin procedure, the antibody does not have to be labelled, thereby avoiding the risk of compromising the affinity properties.

The analytical capacity of the methodology was demonstrated for SPR dextran-coated biochips with antibodies towards HSA and human TnT. However, the method is applicable to most IgG antibodies.

The capturing protein G ligand permits reuse of the surface, *e.g.*, for screening of large amounts of antibodies.

Cross-linking of the surface was applied for providing robustness of the method. The

possibility to fixate a surface after ligand capturing may be of particular value when the amount of ligand available is limited.

Thus, the use of oriented protein G-antibody complexes, cross-linked or not, in order to increase efficiency in performance of SPR and other biosensors, is an easy procedure to extend their applicability. The method can be adapted to other antibody-binding proteins such as protein A, protein A/G or protein L, to further increase the span of possible antibodies and applications.

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Figure Legends

Figure 1:

Schematic of the assay principle: 1. Protein G (green) is randomly immobilized to the dextran matrix. 2. Antibodies (blue) are captured by protein G with their antigen binding domains pointing away from protein G. 3. The antibodies are the discriminating component binding the analyte (red).

Figure 2:

Sensorgrams showing the steps in the procedure for an HSA assay: (a) time course for immobilization of protein G to the dextran chip; (b) binding of IgG to protein G (from three repetitions) followed by binding of HSA (from bottom to top 1, 5 and 10 $\mu\text{g/ml}$); (c) close-up of the HSA binding. Arrows indicate activation of surface (1), injection of protein G (2), deactivation of surface (3), injection of antibody (4), injection of analyte (HSA) (5) and regeneration (6).

Figure 3:

SPR response versus concentration for HSA (lower graph) and TnT (upper graph) The graphs show SPR responses where the antibodies were regenerated after each injection of the analytes. The SPR values shown are reduced with the non-specific binding from a protein G reference surface.

Figure 4:

SPR response versus concentration for HSA (lower graph) and TnT (upper graph) The graphs show SPR responses where the antibodies were cross-linked to protein G. The SPR values shown are reduced with the non-specific binding from a protein G reference surface.

Figure 5:

The upper (pink) graph is from a channel with anti-HSA cross-linked to protein G, the blue graph is from a channel with protein G, and the yellow and cyan graphs are from channels with unmodified dextran. a) HSA 5 μ g/ml and b) BSA 5 μ g/ml injected over the surface.

Figure 6:

Stability of cross-linked surface with repeated injections of troponin T over cross-linked antibody. The first group of bars represents injections of samples 5-11, the middle group of bars samples 9-19, and the last group of bars samples 23-28. Samples were the first, middle, and last at concentrations of 0.03, 0.5, and 3 μ g TnT/ml respectively.

Figure 7:

Comparison of analytical responses for HSA samples in the range 01-10 μ g/ml analyzed by SPR and spectrophotometric assay (Bradford). The SPR responses are represented as resonance units and spectrophotometric assay as absorbance units at a wavelength of 595 nm.

Figure 1

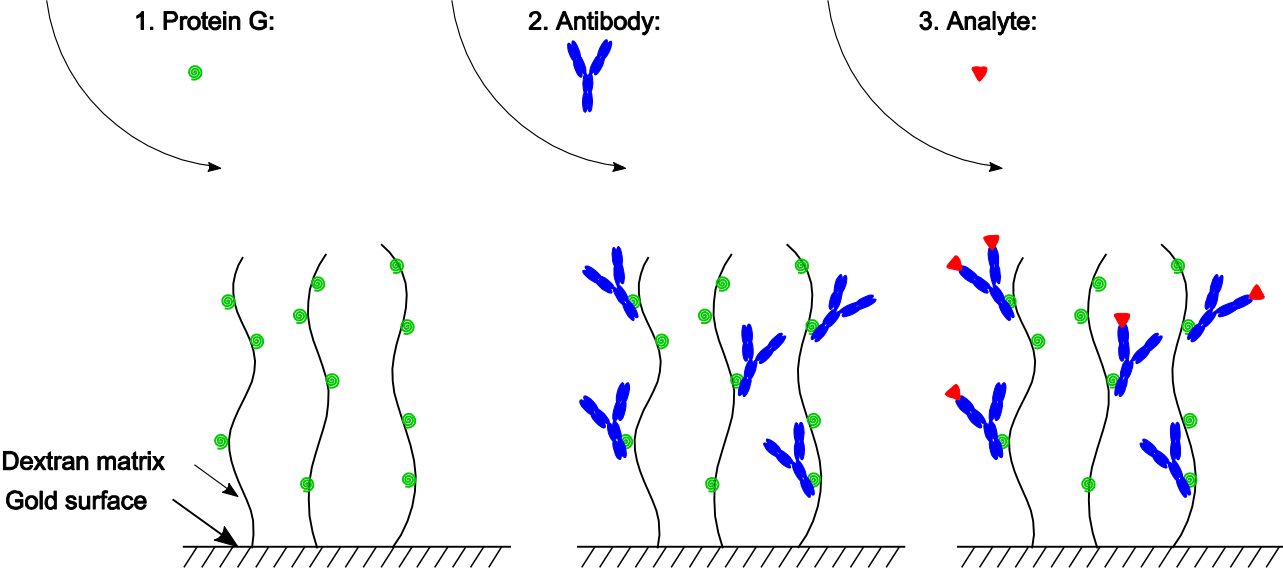
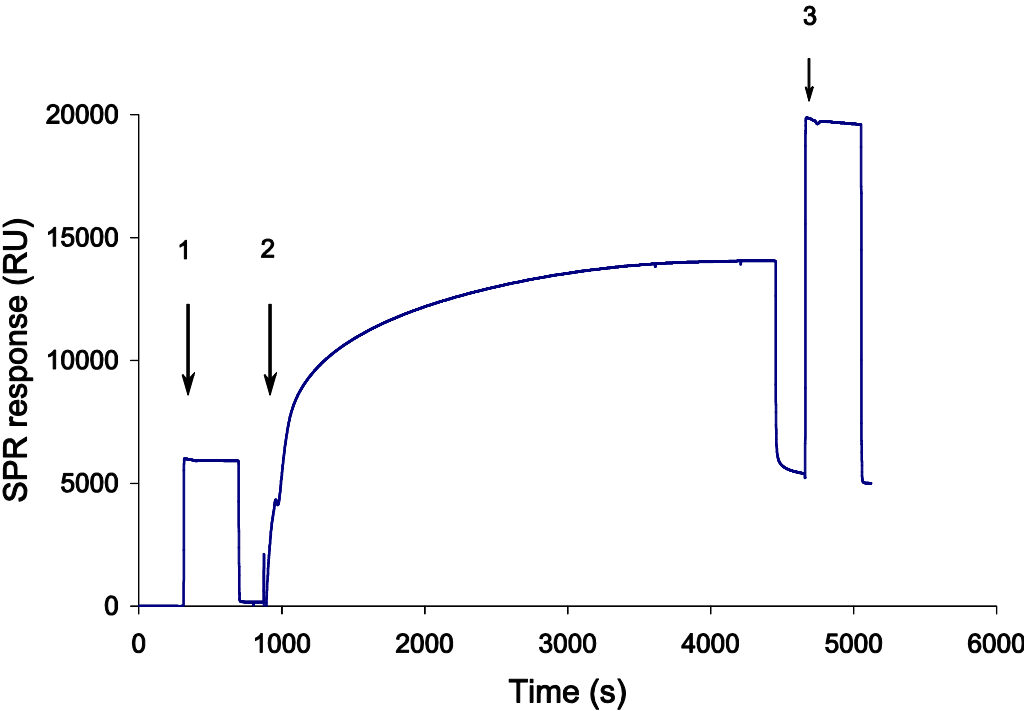
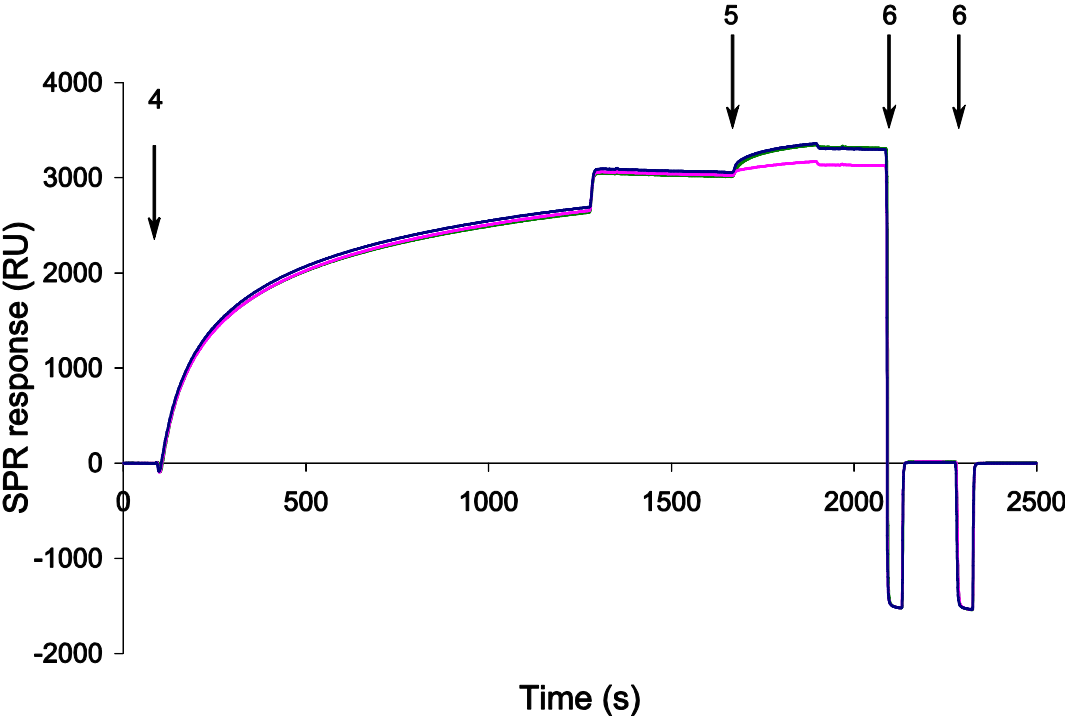


Figure 2

(A)



(B)



(C)

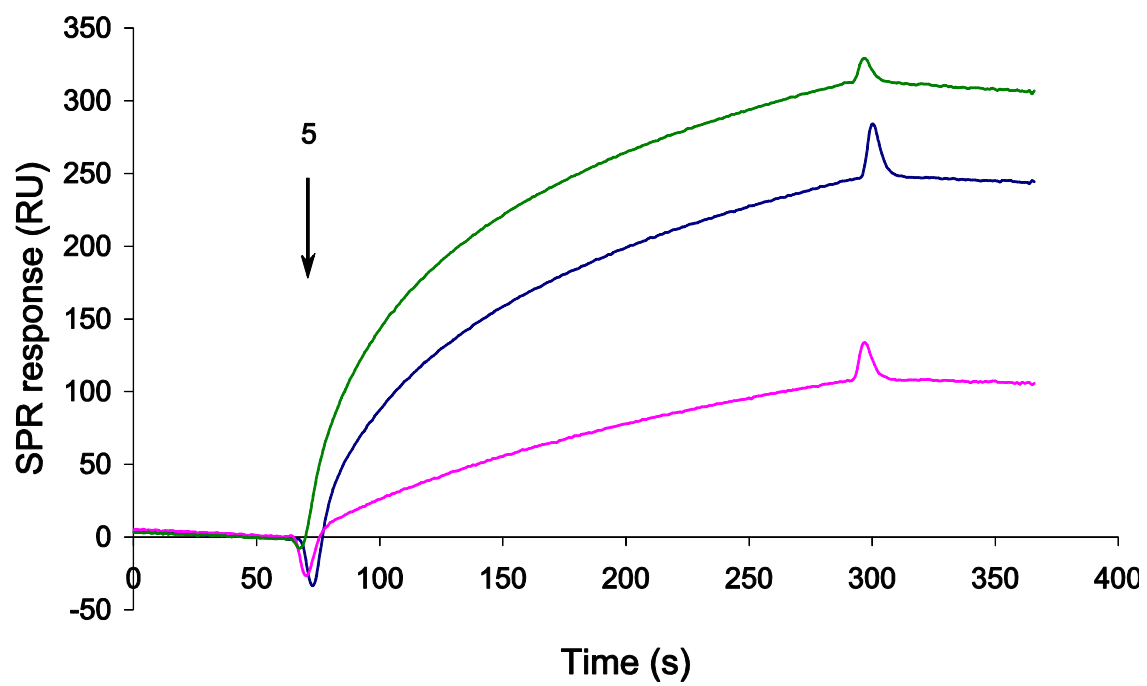


Figure 3

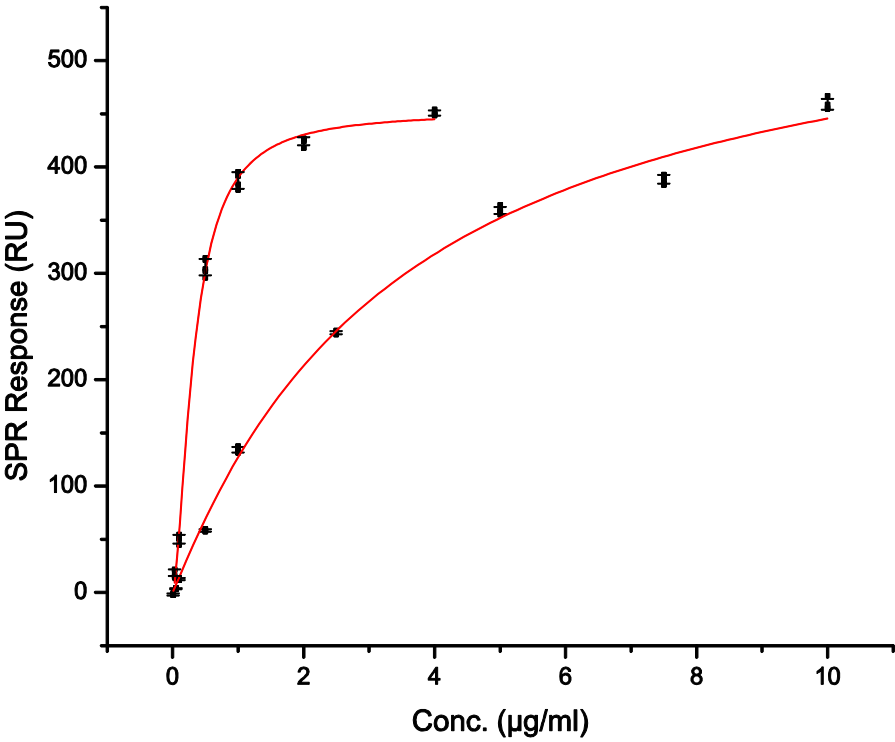


Figure 4

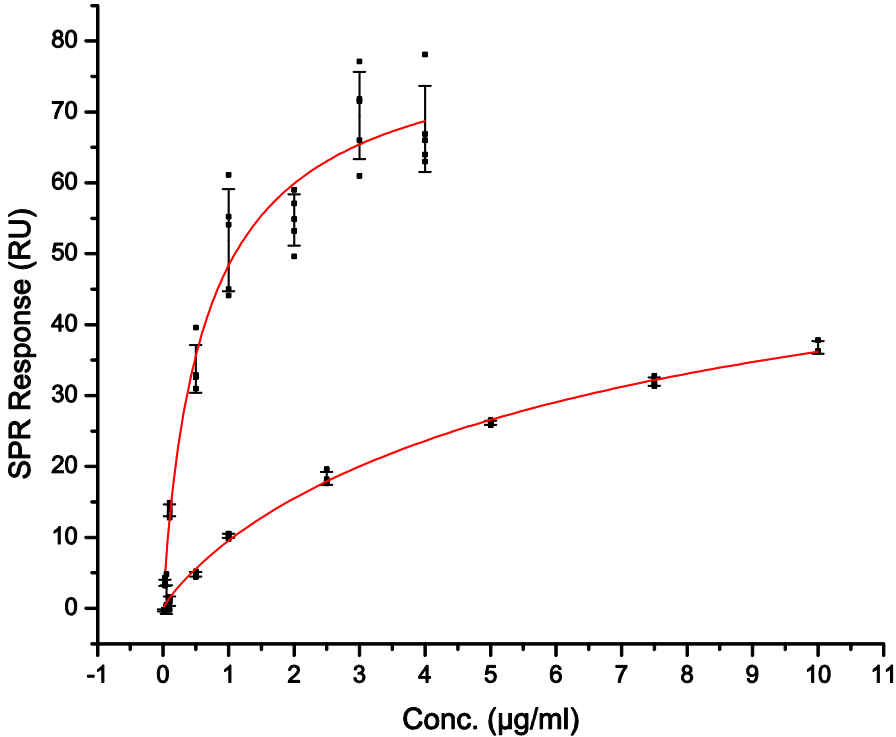
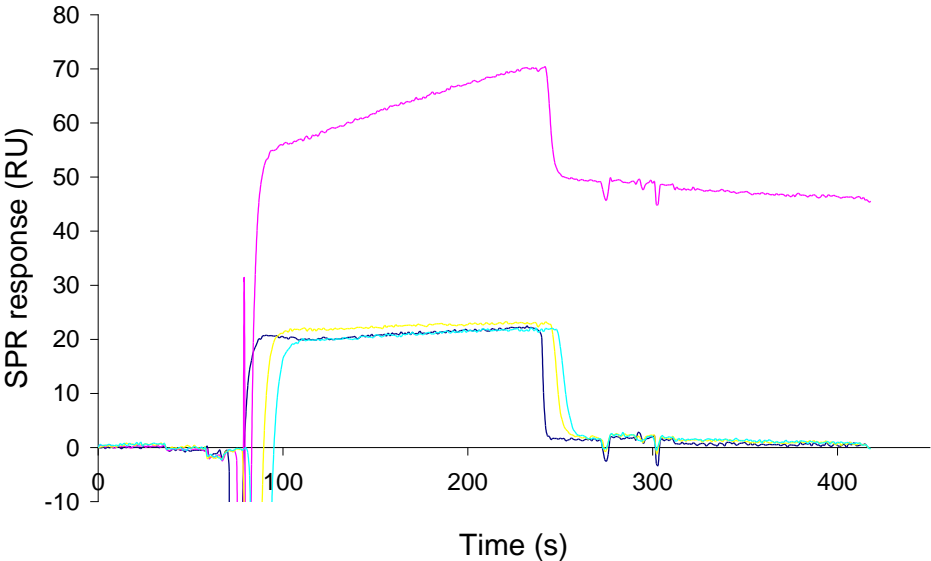


Figure 5

(A)



(B)

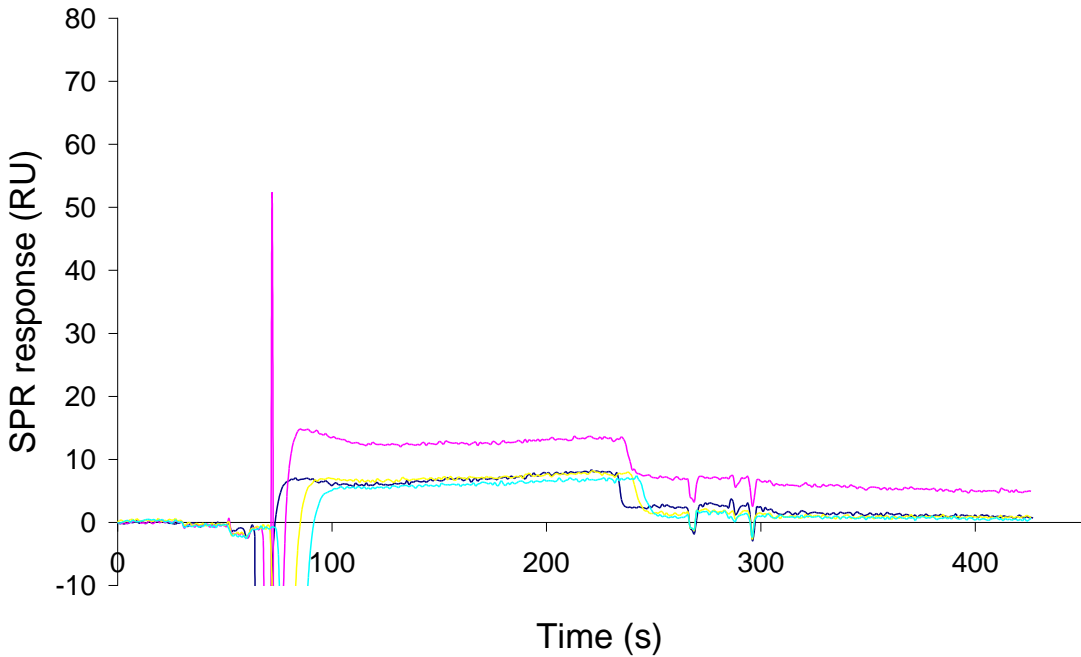


Figure 6

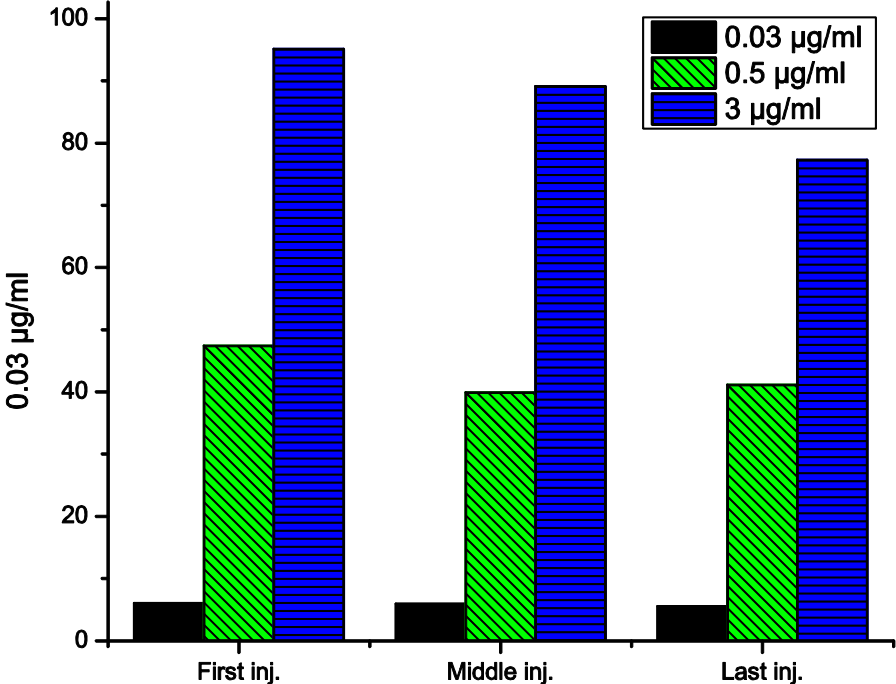


Figure 7

