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Nonregeneration Protocol for Surface Plasmon Resonance: Study of High-Affinity Interaction with High-Density Biosensors

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Abstract

Surface plasmon resonance (SPR) has been used in determining kinetics and thermodynamics of biological interaction in the past decades. One difficulty encountered in this technology is the need for a proper regeneration, which means the removal of analytes from the bound complexes to regenerate the activity of the ligands. Regeneration is not always practical since the harsh regeneration reagents may destroy the bioactivity of the ligands. It is even more difficult for complexes with high affinity constants. In this paper, we report a nonregeneration protocol for SPR techniques in which subsequent ligand/analyte interactions can be measured without regeneration; thus ligand biological activity could be retained. Kinetics, binding models, and mathematics of this protocol are discussed in detail using rabbit IgG as the analyte and engineered recombinant antibody A10B single-chain fragment variables (scFv) as the ligand. The affinity constant of rabbit IgG binding with A10B scFv measured by using a nonregeneration protocol was $(2.5 \pm 0.2) \times 10^7 \text{ M}^{-1}$, which was comparable with the value determined with a conventional regeneration SPR method $((2.2 \pm 1.5) \times 10^7 \text{ M}^{-1})$ and quartz crystal microbalance $(1.9 \times 10^7 \text{ M}^{-1})$. A paradigm of streptavidin–biotin binding was analyzed to validate this protocol. The affinity constant for each binding subunit of streptavidin to the immobilized biotin was determined to be $(7.3 \pm 0.2) \times 10^6 \text{ M}^{-1}$, which was comparable with the solution-based value of $2 \times 10^7 \text{ M}^{-1}$. The nonregeneration protocol requires a relatively high ligand density on the biosensor surface so that more data points can be obtained before surface saturation. The small size of scFv enables them to be constructed in the biosensors for such purpose.

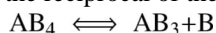
Surface plasmon resonance (SPR) is an optical phenomenon that takes place at the total internal reflection when a fraction of energy of the incident light is absorbed by surface delocalized electrons (plasmon), resulting in a decrease of the reflected light at a certain angle. The biosensor based on SPR was first introduced as a real-time, nonlabel technique for analysis of biological interaction in the early 1990s.¹ According to a recent review, SPR dominates the field of optical biosensors and Biacore AB is the major provider of SPR systems.² One application of SPR technology is to measure the k_{on} and k_{off} as well as the affinity K_A of ligand/analyte interactions. In doing this, the ligand (e.g., the antibody) is immobilized on the surface of an SPR gold chip and then aliquots of analyte (e.g., the antigen) solution at various concentrations are injected. The bindings between ligands and analytes are measured, which gives the k_{on} , k_{off} as well as K_A .

In a typical SPR analysis, unless for the extremely weakly bound analyte–ligand complexes,³ proper regeneration between consecutive analyte injections is always required to remove the bound analytes yet maintain the bioactivity of the ligand. Another reason for regeneration is

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to keep the ligand density the same for each analyte injection, which is required by conventional data analysis. Consequently, the surface usually is very close to saturation after one injection and needs the regeneration to conduct a second experiment. An excellent regeneration is a combination of proper chemicals, proper concentration, and proper contact time of the regeneration. The discovery of an acceptable regeneration is not always an easy task especially for stable ligand–analyte complexes, where k_{off} is relatively small, for example, in the order of 10^{-4} s^{-1} . The difficulties lie in two aspects. The first is that the regeneration may fail in removing all the bound analyte molecules; as a result, the SPR signal is not close enough to the baseline after the regeneration. The second is the concern that harsh regeneration reagents may somehow destroy the bioactivity of the ligand molecules and the consequence is unexpected. Even though Biacore SPR is quite user-friendly, to develop an accurate kinetic assay is challenging and often needs experienced personnel. To avoid these difficulties in finding an acceptable regeneration method, we have developed a protocol that does not require regeneration between successive analyte injections. This nonregeneration protocol is especially useful for the study of high-affinity antigen/antibody interactions in which finding a good regeneration method is especially difficult. High density of the immobilized ligands is required for better analysis as more data points can be obtained before surface saturation.

In this work, we tested this protocol by studying the kinetics and thermodynamics of a single-chain fragment variable (scFv) binding with its antigen rabbit IgG. The scFv is a mouse anti-rabbit IgG scFv (A10B) and binds to the constant region ($\text{C}_{\text{H}}1$) of the rabbit IgG. A cysteine-containing peptide linker, $\text{CGGGS}-(\text{GGGGS})_2$, was incorporated to the scFv to facilitate immobilization on the sensor surface by forming a Au–S self-assembly monolayer (SAM).⁴ Due to the small size of the scFv (i.e., 27 000 MW) and self-assemble immobilization processes, high density and good orientation of ligands can be achieved;⁵ as a result, this was an ideal system to test our protocol. The second system we selected to validate our protocol is the binding of streptavidin to biotin, which has been studied over 60 years.⁶ Being a tetrameric protein, streptavidin has four binding sites or four subunits for biotin molecules. The core structure of streptavidin with the four subunits has a D2 symmetry, and two subunits are on one side and the other two on the other side.⁷ When biotin is immobilized on the surface, at most two of the streptavidin binding subunits can be occupied.⁸ The binding on each subunit is independent and identical. There is no interaction between these binding sites.⁹ The high affinity between streptavidin and biotin is due to the combination of many factors such as multiple hydrogen bonding, van der Waals force, and structural quaternary changes.¹⁰ The high-affinity constant makes the streptavidin–biotin binding almost irreversible. The previously reported affinity constant was actually obtained from the dissociation process.⁹ The dissociation constant K_{D} was obtained from the following process, and the affinity constant K_{A} was assumed to be the reciprocal of the dissociation constant.



where AB_4 and AB_3 are the tetrameric streptavidin bound with four or three biotin units and B is the free biotin unit in solution. The K_{A} measured with nonregeneration SPR protocol is the direct analysis of the association process; therefore, the result can be complementary to what has been reported. In addition, biotin is a small unit, which makes it possible to construct a high-density biosensor using biotin units.

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA) fraction V (Catalog No. A-9418), rabbit IgG (Catalog No. I-5006), 2-aminoethanethiol hydrochloride (Catalog No. 12292-0), and (+)-biotin *N*-hydroxysuccinimide ester (Catalog No. H-1759) were purchased from Sigma, Inc. Goat anti-human IgG (Catalog No. 109-005-003) and goat anti-mouse IgG (Catalog No. 115-036-071)

were purchased from Jackson Immunolabs. Phosphate-buffered saline (PBS, pH 7.2, Catalog No. 20012-027) was purchased from Gibco. Streptavidin (Catalog No. 21122) was purchased from Pierce. Zwittergent 3-08 detergent (Catalog No. 693019) was purchased from Calbiochem. All chemicals were used without further purification.

A10B Anti-Rabbit IgG scFv and Monoclonal Antibody

The production of scFv is described in detail in an early work.⁵ Briefly, female Balb/c mice immunized with purified rabbit IgG were used as a source of B spleen cells to produce the A10B hybridoma cell line. The A10B hybridoma produces an IgG1 monoclonal antibody that specifically interacts with the CH1 domain on rabbit IgG. The Amersham QuickPrep mRNA Purification Kit was used to purify mRNA from $\sim 5 \times 10^6$ A10B hybridoma cells. The Amersham Recombinant Phage Antibody System (RPAS) was used to produce A10B ScFv according to the manufacturer's instructions, with the following modification. The (GGGGS)₃ linker peptide sequence used in the RPAS kit was changed to CGGGS (GGGGS)₂ (Cys linker). A10B scFv displaying the modified linker sequence retained antigen-binding activity.

Construction of scFv Biosensor

Gold chip (BIAcore SIA Kit Au, Catalog No. BR-1004-05) was cleaned by immersing it in the 1:1 concentrated nitric acid and sulfuric acid mixture. Copious biograded water (18 M Ω deionized water filtered by a 2- μ m filter and radiated by UV light) was used to rinse the acid-treated gold chip before it was set on the BIAcoreX instrument for the following real-time biomolecular interaction analysis. The instrument has two flow cells (FCs) in series. Working mode can be selected as FC1 only, FC2 only, or FC1 and FC2 concurrently. When the baseline was stable, the reference channel (FC1) was closed. A 10- μ L sample of A10B scFv (~ 2 μ g/mL) was injected and flowed through the sample channel (FC2). Duration of incubation, at least a few hours, is critical for the scFv self-assembled processes on the gold surface. Under continuous flow, the duration of a few hours would cost a large amount of reagents. A novel method was developed for long-time incubation using the BIAcore X SPR system. After a small amount of scFv was injected into the system, the connection block was dismantled so that the scFv was retained in the sample channel. The incubation was kept for 4 h. After incubation, the connection block was remounted and the buffer washed away the excessive scFv from the system. Next, 30 μ L of 0.1% BSA was injected and flowed through both reference and sample channels to block unoccupied areas¹¹ and prevent nonspecific adsorption on the scFv-modified gold surface. Both 2-mercaptoethanol and BSA were studied as blocking reagents. Even though BSA was much larger than 2-mercaptoethanol, there was no obvious difference in their effects as blocking reagents. This indicated that the immobilized scFv-Cys formed a dense layer leaving only small, unblocked bare gold. Rabbit IgG, with its large size (15 000 MW), was difficult to nonspecifically adsorb on the remaining small bare gold surfaces.

Rabbit IgG Detection

The flow rate of PBS buffer through the flow cells was set at 10–30 μ L/min. A series of 30 μ L of rabbit IgG solutions were injected and flowed through both reference and sample channels. The concentration of each injection varied from 6.25 to 200 μ L/mL. There was no regeneration between consecutive injections. There was no ligand on the reference channel FC1 surface; therefore, the response on the FC1 was due to nonspecific adsorptions. The sample channel FC2 was immobilized with scFv ligand so that the response on FC2 was due to specific binding between the scFv and the rabbit IgG as well as the nonspecific adsorptions. As the nonspecific adsorptions were expected to show no preference on FC1 or on FC2, the response difference between the sample channel FC2 and the reference channel FC1 was the real response due to the specific A10B scFv/rabbit IgG interaction. In the conventional SPR analysis, regeneration

was performed between the consecutive injections. Among various regeneration reagents tested (i.e., HCl, sodium dodecyl sulfate, glycine, and Zwittergent 3-08 prepared at various concentrations and pH values), 0.3 M Zwittergent 3-08 with pH of 2.0 was selected as the final regeneration reagent.

Construction of a Biotin Biosensor

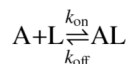
Gold chip was cleaned and mounted as described above. A 10- μ L sample of 10 mM 2-aminoethanethiol was injected and flowed through the sample channel (FC2). The connection block was dismounted, and the incubation was kept for 4 h. After incubation, the connection block was remounted and the buffer washed away the excessive 2-aminoethanethiol solution from the system. A 30- μ L sample of saturated (+)-biotin *N*-hydroxysuccinimide ester solution in PBS (less than 5 mM) was injected and flowed through the sample channel (FC2) at a flow rate of 1 μ L/min.¹² Last, 30 μ L of 0.1% BSA was injected and flowed through both reference and sample channels to block the unoccupied area to prevent nonspecific adsorption on the biotin-immobilized gold surface. The process of amine coupling is depicted in Figure 1.

Streptavidin Binding Study

The flow rate of PBS buffer through the flow cells was set at 30 μ L/min. A series of 30 μ L of streptavidin solutions were injected and flowed through both reference and sample channels. The concentration of each injection varied from 6.25 to 500 μ L/mL. There was no regeneration between consecutive injections. The response difference between sample channel and reference channel was the real response due to the specific biotin/streptavidin interaction.

RESULTS AND DISCUSSION

Figure 2 shows the SPR sensorgrams of scFv binding with the rabbit IgG. Association and dissociation took place while the rabbit IgG flowed through the gold chip immobilized with A10B scFv.



where A is the analyte (rabbit IgG), L is the immobilized ligand (A10B scFv), and AL is the complex of A binding to L.

The association rate equals to $k_{\text{on}}C(R_{\text{max}} - R)$, and the dissociation rate equals to $k_{\text{off}}R$, where k_{on} is the association rate constant, k_{off} is the dissociation rate constant, C is the concentration of rabbit IgG, R_{max} reflects the maximum amount of rabbit IgG that can be captured on the scFv surface, which theoretically means the maximum response that can be achieved when the concentration of rabbit IgG passing through the scFv surface and contact time are infinite, and R is the response to the binding of rabbit IgG. At equilibrium, the association rate equals to the dissociation rate; therefore

$$k_{\text{on}}C(R_{\text{max}} - R_{\text{eq}}) = k_{\text{off}}R_{\text{eq}}$$

or

$$\frac{1}{R_{\text{eq}}} = \frac{1}{R_{\text{max}}} + \frac{1}{K_A R_{\text{max}}} \frac{1}{C} \quad (1)$$

where the affinity constant, K_A , equals to $k_{\text{on}}/k_{\text{off}}$. The plot of $1/R_{\text{eq}}$ against $1/C$ is a straight line with the value of slope being $1/(K_A R_{\text{max}})$ and the intercept $1/R_{\text{max}}$. Consequently, the value of affinity constant K_A can be obtained from the slope and intercept.

Figure 3 indicates the linear relationship between $1/R_{eq}$ and $1/C$. The average affinity constant obtained was $(2.5 \pm 0.2) \times 10^7 \text{ M}^{-1}$, which is comparable with the value obtained with a quartz crystal microbalance $(1.9 \pm 10^7 \text{ M}^{-1})$.⁵ The R^2 value of 0.9852 indicates a good linear fitting as expected.¹³ The outlier in Figure 3 was due to a mass-transfer effect as well as an avidity effect and will be discussed in a later section. The assay had been repeated several times on different SPR gold chips. The values of R_{max} showed a little difference each time because the immobilized scFv surface density was not the same; however, the affinity constant obtained was always very close to what we report here.

Comparison with Conventional Regeneration SPR Analysis

The interaction of rabbit IgG with A10B scFv was first analyzed by SPR using a conventional regeneration protocol. Sensor surfaces were regenerated after each analyte injection to remove the bound analyte (rabbit IgG), from the A10B scFv–rabbit IgG complex on the sensor surface. A wide range of reagents (e.g., HCl, sodium dodecyl sulfate, glycine, and Zwittergent 3-08), adjusted to different concentrations and pH values, were used to regenerate the A10B scFv sensor surface. Although not perfect, we found 0.3 M Zwittergent 3-08 (pH 2.0–6.0) performed better than other reagents for dissociating rabbit IgG from the IgG–scFv complex. As a result, 0.3 M Zwittergent 3-08, pH 2.0, was used as the regeneration reagent. Figure 4A is the sensorgram with regeneration, and Figure 4B is the same sensorgram excluding regeneration after X- and Y-transform with BIAevaluation software (version 4.1, Biacore). The values of k_{on} , k_{off} , and K_A obtained from the conventional analysis were $(1.7 \pm 0.8) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $(7.7 \pm 5.3) \times 10^{-4} \text{ s}^{-1}$, and $(2.2 \pm 1.5) \times 10^7 \text{ M}^{-1}$, respectively. These results were comparable with the results obtained using the nonregeneration protocol (Table 1). The difference in k_{off} value was probably due to an avidity effect. In conventional SPR, regeneration kept the surface scFv density at a relatively high level. A dissociated rabbit IgG molecule might be captured by an available neighboring scFv immediately, decreasing the value of k_{off} significantly.

Mass-Transfer Effect

The analyte molecules have to diffuse to the vicinity of sensor surface before they could be captured by the ligands.



where k_m is the diffusion rate constant, A is the analyte in bulk solution, and A* is the analyte in the vicinity of the sensor so that it can be captured by the immobilized ligand (L). Under the constant-flow condition, the analyte concentration in the bulk solution is nearly a constant, C. The rate of diffusion from A to A* is, $r_{diff} = k_m C$ and the rate of binding to the ligand is $r_{bound} = k_{on}[A^*](R_{max} - R)$. The mass-transfer effect is significant when diffusion is a slow process or $r_{diff} \ll r_{bound}$. This should be avoided for the 1:1 binding model. Ideally, the concentration of A* is equal to that of A so that $r_{bound} = k_{on}C(R_{max} - R)$. In this case, it is not necessary to know the concentration of A*, which is difficult to obtain. To achieve the ideal condition, two factors need to be taken into consideration. First, a relatively high flow rate decreases the thickness of the diffusion layer and accelerates diffusion rate. Second, the concentration gradient is generated if A* complexes with the ligands rapidly. In our experiment conditions, r_{diff} only changes with the analyte concentration in bulk solution, while r_{bound} not only changes with the analyte concentration but also changes with the value of $(R_{max} - R)$, which is larger for the earliest injections. With more and more binding sites or ligands occupied, the value of $(R_{max} - R)$ is getting smaller. Therefore, r_{diff} is likely smaller than r_{bound} in the earliest injections, invalidating the 1:1 binding model. This accounts for the outlier point in Figure 3 for the first injection. (Note that the first injection had the lowest concentration; thus, it appears at the right side of the reciprocal axis.)

The high-density biosensor used for nonregeneration protocol is more prone to the mass-transfer effect because the high density of the ligand has a high possibility of capturing the analyte, producing a concentration gradient in the diffusion layer. To offset this adverse factor, the analysis has to be conducted at a flow rate higher than 30 $\mu\text{L}/\text{min}$. The flow rate may change the mass-transfer effect because higher flow rate reduces the thickness of the diffusion layer, increasing the value of k_m .¹⁴

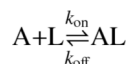
$$k_m = 0.98(D/h)^{2/3}(f/bx)^{1/3}$$

where D is the diffusion coefficient, h is the height of the flow cell, f is the flow rate, b is the width of the flow cell, and x is thickness of diffusion layer.

If a reaction is under mass-transfer control, the association rate and affinity constant would be larger at higher flow rate. In our study of scFv/IgG interaction, the result did not show much difference at various flow rates, indicating the association was not apparently affected by the mass-transfer effect under the experimental conditions.

Equilibrium Response

As the response at equilibrium (R_{eq}) is a function of the concentration of rabbit IgG (C), R_{eq} could be obtained either by the BIAevaluation software (Biacore) or a third-party SigmaPlot (SPSS, Inc.) to fit the curve. Our experience shows that the SigmaPlot is better to use to fit the real-time response curve with an exponential function based on a 1:1 Langmuir isothermal surface binding model without knowing the concentration of rabbit IgG. The increase of R , the response, expressed in resonance units (RUs), is the combined results of binding of IgG on the surface and the dissociation of bound IgG into the solution. For a model where mass-transfer effect is negligible,



where A is the analyte, L is the immobilized ligand, and AL is the complex of A binding to L .

The change of R over time, t , can be expressed in the following equation.

$$dR/dt = k_{on}C(R_{max} - R) - k_{off}R$$

where C is the concentration of A , R is the SPR response due to the formation of complex AL , and R_{max} is the maximum response when all ligands are transformed to the AL complex.

$$dR/dt = k_{on}CR_{max} - (k_{on}C + k_{off})R$$

or

$$dR/dt = k_w - k_{obs}R$$

where

$$k_w = k_{on}CR_{max} \quad \text{and} \quad k_{obs} = k_{on}C + k_{off}$$

The above equation can be transformed as follows.

$$\begin{aligned} -\frac{d(k_w - k_{obs}R)}{k_{obs}dt} &= k_w - k_{obs}R \\ -\frac{d(k_w - k_{obs}R)}{k_w - k_{obs}R} &= k_{obs}dt \end{aligned}$$

Integration of the equation gives

$$-\int_{R=R_0}^{R=R} \frac{1}{k_w - k_{obs}R} d(k_w - k_{obs}R) = k_{obs} \int_{t=0}^{t=t} dt$$

where the time of injection is set to be zero and R_0 is the response just before the capturing.

$$\begin{aligned} -\ln \frac{k_w - k_{\text{obs}}R}{k_w - k_{\text{obs}}R_0} &= k_{\text{obs}}t \\ \frac{k_w - k_{\text{obs}}R}{k_w - k_{\text{obs}}R_0} &= e^{-k_{\text{obs}}t} \\ \frac{k_{\text{obs}}R - k_{\text{obs}}R_0}{k_w - k_{\text{obs}}R_0} &= 1 - e^{-k_{\text{obs}}t} \end{aligned}$$

or

$$R = R_0 + (k_w/k_{\text{obs}} - R_0)(1 - e^{-k_{\text{obs}}t}) \quad (2)$$

Equation 2 indicates that the response R changes with time exponentially during the association time. The association curve therefore can be fitted to a three-parameter exponential-raise-to-max function in SigmaPlot, which takes a form of $y = y_0 + a(1 - e^{-bx})$, where $y_0 = R_0$, $a(k_w/k_{\text{obs}}) - R_0$, and $b = k_{\text{obs}} = k_{\text{on}}C + k_{\text{off}}$.

The value of R_{eq} can be obtained when $t \rightarrow \infty$. In fact

$$R_{\text{eq}} = k_w/k_{\text{obs}} = y_0 + a \quad (3)$$

A further investigation of eq 3 shows that the value of R_{eq} has the same relationship with C as described in eq 1.

Rate Constants

The constant b from the association curve fitting equals to the observed rate constant k_{obs} , or $b = k_{\text{obs}} = k_{\text{on}}C + k_{\text{off}}$. The plot of k_{obs} against C yields a straight line with a slope of k_{on} and an intercept of k_{off} as shown in Figure 5, in which the outlier has been excluded. The association rate constant measured thereafter is $(1.9 \pm 0.5) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and the dissociation rate constant is $(2.7 \pm 0.3) \times 10^{-2} \text{ s}^{-1}$.

Biotin/Streptavidin Interaction

Biotin binding to streptavidin has been well studied, and the association constant of $\sim 10^{13} \text{ M}^{-1}$ is widely accepted. This is the highest association constant known in natural biological systems. Because of geometric limitation, all subunits of a streptavidin tetramer cannot bind with the immobilized biotin. Liu et al. characterized the biotin/streptavidin interaction on a gold surface by electrochemical methods and SPR.¹⁵ They first constructed a SAM on the gold surface by immersing a gold chip in the solution of an engineered sDNA with a cysteine terminal (sDNA-Cys). A complementary DNA strand with a biotin terminal group was then hybridized with the immobilized sDNA-Cys. The biotin terminal then captured the streptavidin unit of ferrocene-streptavidin conjugates. Their study showed that each unit of biotinylated SAM bound only one streptavidin subunit. We constructed the biotin biosensor in a similar way; therefore, the affinity constant reported here is more likely to be the affinity constant for the binding between biotin and a single streptavidin subunit (Figure 6).

The calculation based on Figure 7 and Figure 8 gives the value of K_A of $(7.3 \pm 0.2) \times 10^6 \text{ M}^{-1}$, which is comparable with the solution-based value of $2 \times 10^7 \text{ M}^{-1}$ for biotin binding with a single streptavidin subunit.¹⁶ The leftmost point in Figure 8 looks to be deviating a little from the straight line. As this point represents the last injection with the highest streptavidin concentration, we assume that the deviation is due to the near-saturation status of the biosensor. In Figure 8, the intercept is $\sim 9.5 \times 10^{-4}$, which gives the value of R_{max} of 1.05×10^3 . For proteins, 10 000 RU is equivalent to 10 ng/mm² in coverage.¹⁷ Therefore, the maximum coverage of streptavidin is $\sim 1.05 \text{ ng/mm}^2$, or $1.2 \times 10^{12} \text{ molecules/cm}^2$. (The molecular weight of streptavidin is 52 800.) This value is consistent with the coverage reported by Liu et al., which was 0.9–1.5 molecules/cm².¹⁵

The apparent dissociation rate constant, k_{off} , is sometimes used to characterize the strength of binding. The value of k_{off} can be obtained based on the dissociation curve after each injection.

$$-dR/dt = k_{\text{off}}R$$

or

$$\ln R_0 - \ln R = k_{\text{off}}t$$

Each of the seven injections in Figure 7 contains a dissociation curve so that seven values of k_{off} can be obtained. The average is $(8.1 \pm 0.8) \times 10^{-5} \text{ s}^{-1}$. The comparison of nonregeneration protocol to the literatures is in the Table 2. The affinity constant and the apparent dissociation rate constant measured with the nonregeneration SPR protocol are comparable with what were reported by other researchers, indicating the reliability of the newly developed protocol.

It is interesting to find that the mass-transfer effect is not significant in the case of biotin/streptavidin interaction. We proposed the following explanations. Compared to streptavidin, rabbit IgG has (i) smaller k_m due to its relatively large mass (150 kDa for rabbit IgG vs 52.8 kDa for streptavidin), (ii) slightly larger R_{max} (1163 for A10B scFv chip vs 1050 for biotin chip), and (iii) probably smaller R because the dissociation rate of rabbit IgG from the A10B scFv is faster than that of streptavidin from the biotin sensor ($2.7 \times 10^{-2} \text{ s}^{-1}$ for rabbit IgG vs $8.1 \times 10^{-5} \text{ s}^{-1}$ for streptavidin). As a result, it is more likely that rabbit IgG has a significantly smaller diffusion rate r_{diff} than the binding rate r_{bound} . Because the rate of diffusion r_{diff} is equal to $k_m C$ and the rate of binding r_{bound} is equal to $k_{\text{on}}[A^*](R_{\text{max}} - R)$, the mass-transfer effect is significant for the IgG-scFv interaction ($r_{\text{diff}} \ll r_{\text{bound}}$) but not for the streptavidin/biotin interaction.

We also think that the avidity effect contributes to the outlier in the rabbit IgG/scFv interaction. The dissociation rate constant of rabbit IgG-scFv complex is larger ($2.7 \times 10^{-2} \text{ s}^{-1}$) than that of the streptavidin-biotin complex ($8.1 \times 10^{-5} \text{ s}^{-1}$); therefore, compared to dissociated streptavidin, there will be more dissociated rabbit IgG molecules in the vicinity of the SPR surface, which may be immediately recaptured by the neighboring ligands on the SPR surface.

CONCLUSIONS

Our study shows that the nonregeneration protocol for SPR technique is reliable and accurate in determining the binding kinetics and thermodynamics based on a 1:1 binding model, saving the efforts of finding a proper regeneration reagent that sometimes is impossible. The protocol reduces the risk and uncertainty as regeneration agents may cause biological damage to the immobilized ligands on the sensor chip. With no regeneration, the assay is much faster, but higher ligand density is required so that more data points can be obtained for a good linear regression in one assay. This is achieved by incubating a small ligand, i.e., recombinant scFv-Cys or biotin on a gold chip. Immobilizing a smaller ligand on the sensor surface achieves high density of ligand immobilization, which subsequently leads to an increase of sensitivity. For example, had we chosen streptavidin as the immobilized ligand on the surface, we would have lower density of ligand and smaller sensitivity as well as quick saturation of the ligands in our assay.

Even though kinetic and thermodynamic measurement performed on a surface may have a slight difference from those done in solution, it mimics surface interaction in biological systems, such as protein/receptor interaction on the cell membrane. Our results show that the immobilized ligand retains high specificity to the antigen, further confirming that the immobilization process is ideal and maintains the ligand bioactivity. The protocol developed in this work fits the gap for SPR technique where difficulty in regeneration is encountered and could be used by other affinity-based assay. Currently, we are applying the protocol developed

here to analyze kinetics and thermodynamics of γ -keotoaldehydes (IsoK) adducted proteins or peptides binding with IsoK adducts from biological fluids.

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References

1. (a) Karlsson R, Michaelsson A, Mattsson L. *J. Immunol. Methods* 1991;145(1–2):229–240. [PubMed: 1765656] (b) Jonsson U, Malmqvist M. *Adv. Biosens* 1992;2:291–336.
2. Karlsson R. *J. Mol. Recognit* 2004;17:151–161. [PubMed: 15137023]
3. Day YSN, Baird CL, Rich RL, Myszkka DG. *Protein Sci* 2002;11:1017–1025. [PubMed: 11967359]
4. (a) Mrksich M. *Chem. Soc. Rev* 2000;29:267–273. (b) Ulman A. *Chem. Rev* 1996;96:1533–1554. [PubMed: 11848802]
5. Shen Z, Stryker GA, Ray LM, Yu L, Yan H, Zeng X. *Anal. Chem* 2005;77:797–805. [PubMed: 15679346]
6. (a) Gyorgy P, Rose CS, Eakin RE, Snell EE, Williams RJ. *Science* 1941;93:477. [PubMed: 17757050] (b) Eakin RE, Snell EE, Williams RJ. *J. Biol. Chem* 1941;140:535.
7. Hendrickson WA, Pahler A, Smith JL, Satow Y, Merritt EA, Phizackerley RP. *Proc. Natl. Acad. Sci. U.S.A* 1989;86:2190–2194. [PubMed: 2928324]
8. Jung LS, Nelson KE, Stayton PS, Campbell CT. *Langmuir* 2000;16:9421–9432.
9. Green NM. *Adv. Protein Chem* 1975;29:85–133. [PubMed: 237414]
10. Weber PC, Ohlendorf DH, Wendoloski JJ, Salemme FR. *Science* 1989;243:85–88. [PubMed: 2911722]
11. Park J-W, Kurosawa S, Aizawa H, Wakida S-I, Yamada S, Ishihara K. *Sens. Actuators, B* 1997;43:217–223.
12. (a) Jordan CE, Frutos AGF, Thiel AJ, Corn RM. *Anal. Chem* 1997;69:4939–4947. (b) Jonsson U, Fagerstam L, Ivarsson B, Johnsson B, Karlsson R, Lundh K, Lafas S, Persson B, Roos H, Ronnberg I, Sjolander S, Stenberg E, Stahlberg R, Urbaniczky C, Ostlin H, Malmqvist M. *Biotechniques* 1991;11:620–627. [PubMed: 1804254]
13. (a) Myszkka DG. *Curr. Opin. Biotechnol* 1997;8:50–57. [PubMed: 9013659] (b) Morton TA, Myszkka DG, Chaiken IM. *Anal. Biochem* 1995;227:176–185. [PubMed: 7668379]
14. Karlsson R, Roos H, Fagerstam L, Persson B. *Methods: Companion Methods Enzymol* 1994;6:99–110.
15. Liu J, Tian S, Tiefenauer L, Nielsen PE, Knoll W. *Anal. Chem* 2005;77:2756–2761. [PubMed: 15859590]
16. Green NM, Toms EJ. *Biochem. J* 1973;133:687–700. [PubMed: 4748830]
17. (a) Karlsson R, Falt A. *J. Immunol. Methods* 1993;166:75–84. [PubMed: 8228289] (b) Stenberg E, Persson B, Roos H, Urbaniczky C. *J. Colloid Interface Sci* 1991;143:513–526.
18. Perez-Luna VH, O'Brien MJ, Opperman KA, Hampton PD, Lopez GP, Klumb LA, Stayton PS. *J. Am. Chem. Soc* 1999;121:6469–6478.

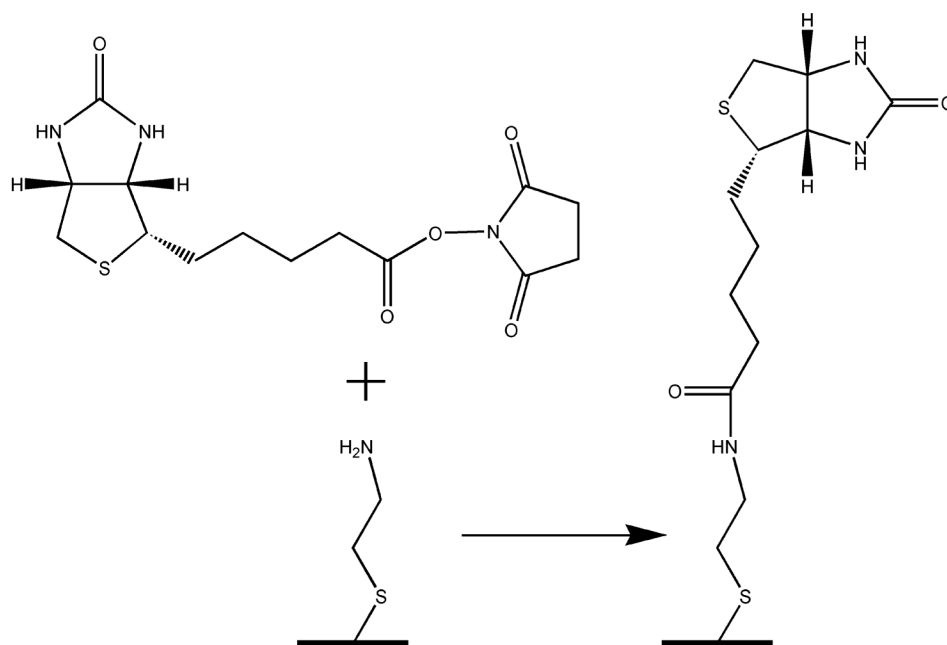


Figure 1. Schematic diagram of biotin sensor construction. 2-Aminoethanethiol formed a SAM on a gold chip. Biotin was immobilized on the surface after treatment with (+)-biotin *N*-hydroxysuccinimide ester.

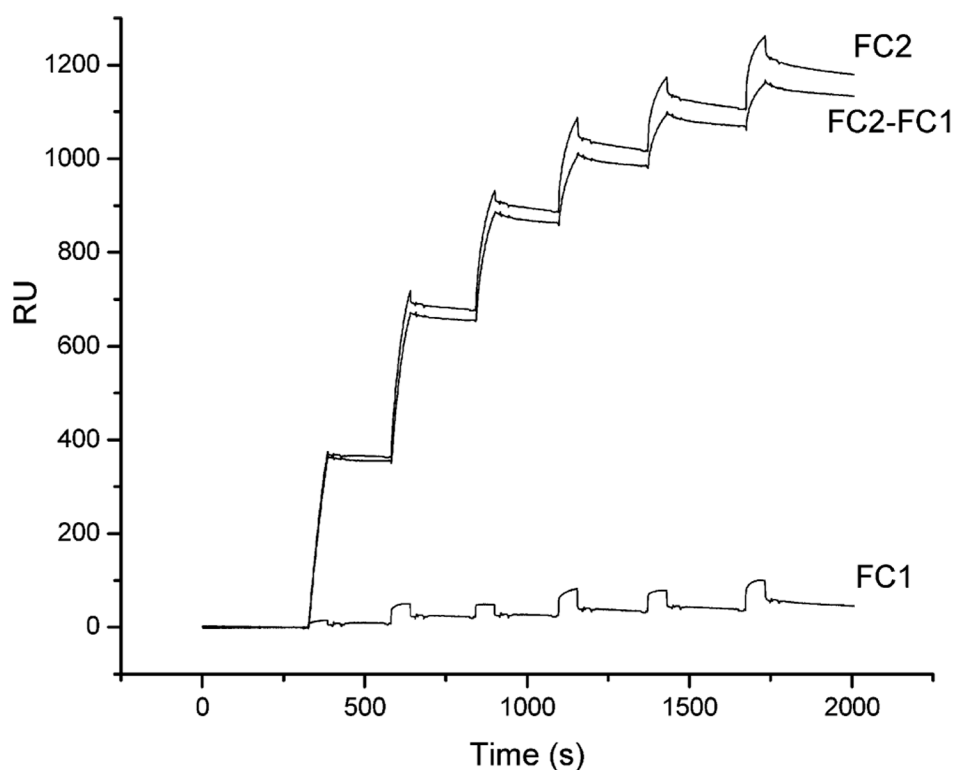


Figure 2. SPR response on A10B scFv-Cys biosensor reference channel (FC1) and sample channel (FC2). The graph contains six steps, where each step contains one uphill association curve and one downhill dissociation curve. The downhill curves are not very apparent because of the slow dissociation process. The six steps correspond to six different concentrations of rabbit IgG, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$ or 0.042, 0.083, 0.17, 0.33, 0.67, and 1.3 μM in that order.

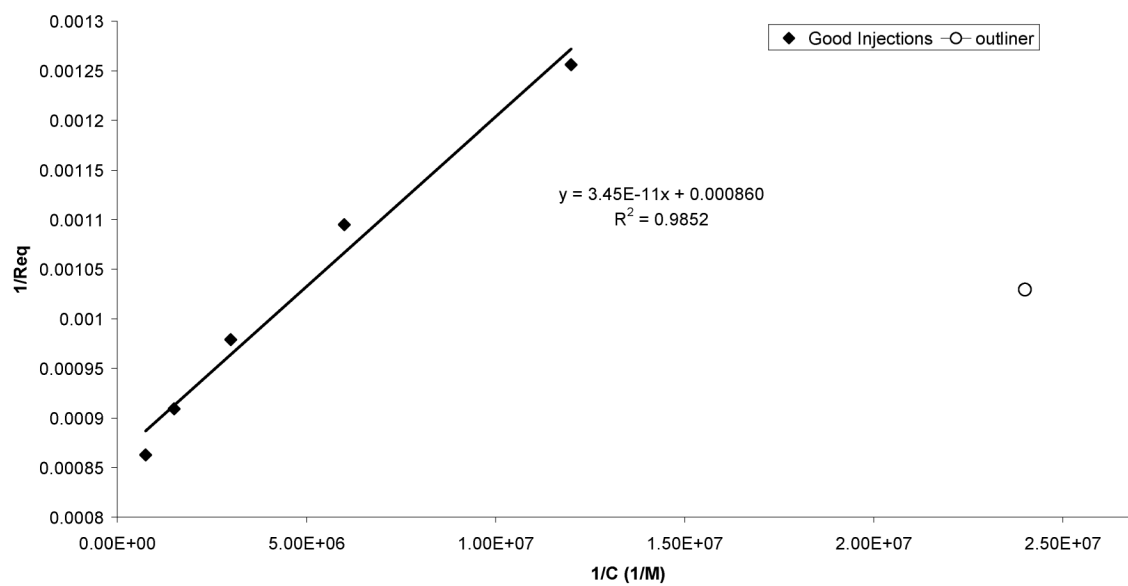


Figure 3. Plot of $1/R_{eq}$ against $1/C$. Rabbit IgG binding with A10B scFv on scFv-Cys biosensor. One outlier has been omitted in regression.

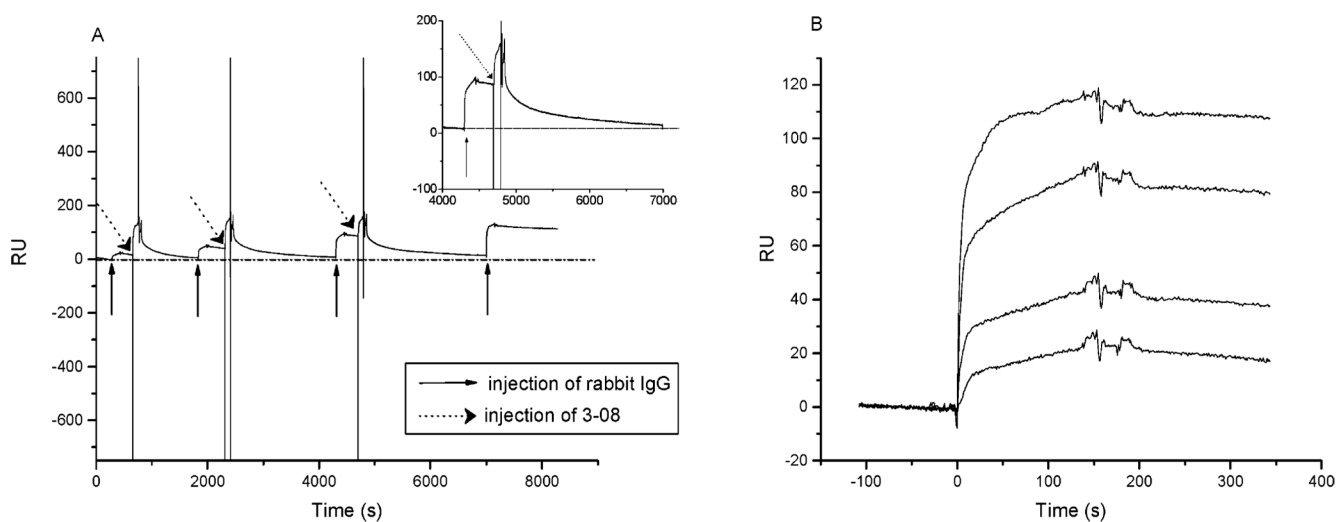


Figure 4.

(A) Conventional SPR analysis of rabbit IgG binding with A10B scFv. The concentrations of the four rabbit IgG injections are 10, 30, 80, and 200 µg/mL or 0.067, 0.20, 0.53, and 1.3 µM. The regeneration reagent was 0.3 M Zwittergent 3-08 pH 2.0. The inset shows the injection of 0.53 µM rabbit IgG. (B) After X- and Y-transform with BIAevaluation, excluding regeneration curves.

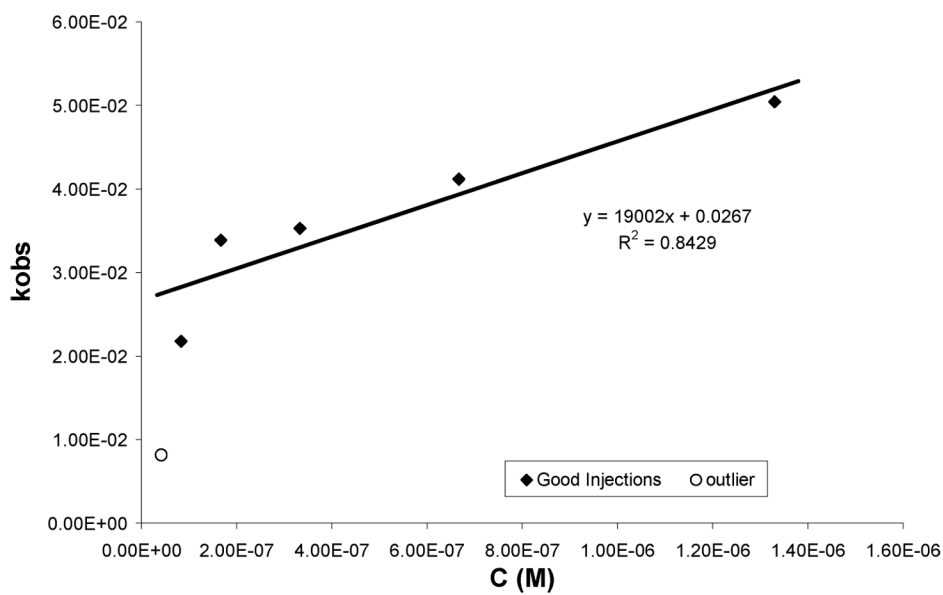


Figure 5. k_{obs} as a function of rabbit IgG concentration. Rabbit IgG binding with A10B scFv on scFv-Cys biosensor. One outlier has been omitted in regression.

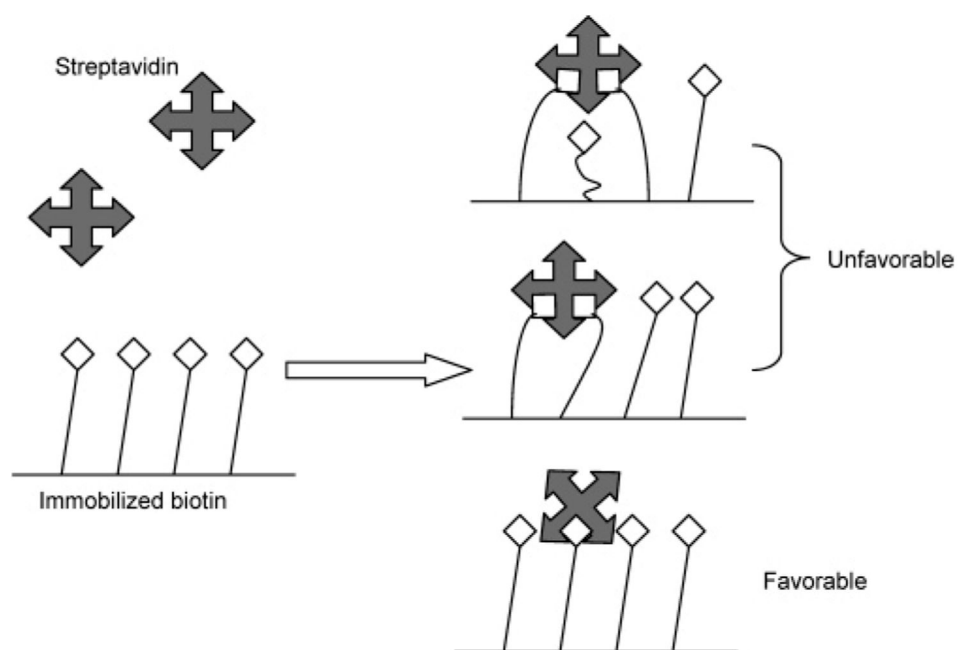


Figure 6.
Binding of streptavidin to the immobilized biotin units.

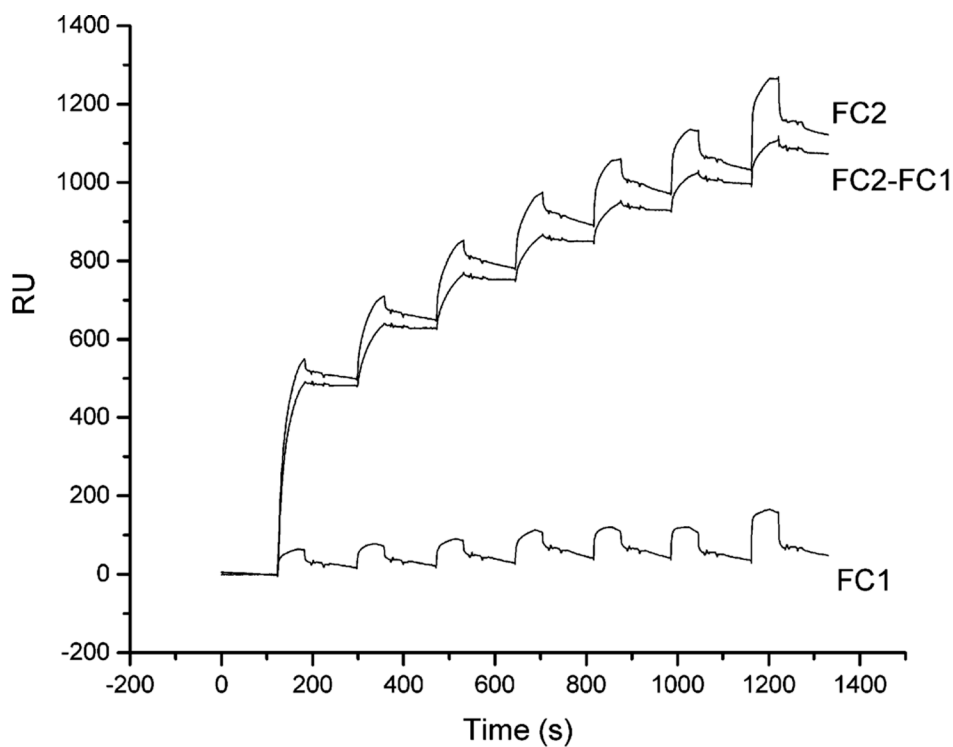


Figure 7.

SPR response on the biotin biosensor reference channel (FC1) and sample channel (FC2). The seven steps corresponds to seven different concentrations of streptavidin, 6.25, 12.5, 25, 50, 100, 200, and 500 $\mu\text{g/mL}$ or 0.12, 0.24, 0.47, 0.95, 1.9, 3.8, and 9.5 μM in that order. The molecular weight of streptavidin is 52 800.

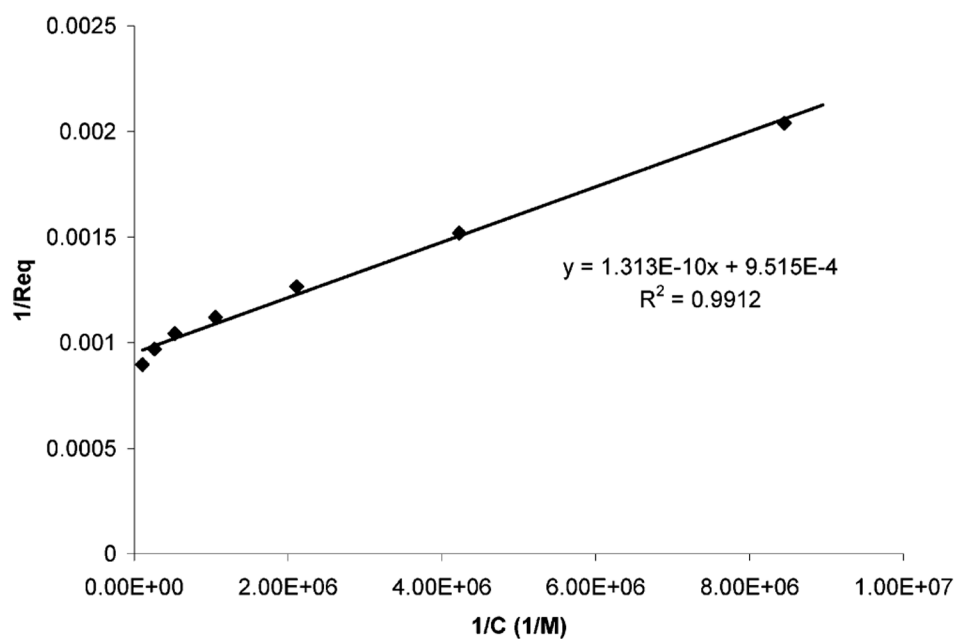


Figure 8.
Plot of $1/R_{eq}$ against $1/C$. Streptavidin binding with biotin biosensor.

Table 1 k_{on} , k_{off} , and K_{A} for Rabbit IgG Binding with A10B ScFv

| | k_{on} ($\text{M}^{-1}\text{s}^{-1}$) | k_{off} (s^{-1}) | K_{A} (M^{-1}) |
|------------------|--|--------------------------------------|------------------------------------|
| conventional SPR | $(1.7 \pm 0.8) \times 10^4$ | $(7.7 \pm 5.3) \times 10^{-4}$ | $(2.2 \pm 1.5) \times 10^7$ |
| nonregeneration | $(1.9 \pm 0.5) \times 10^4$ | $(2.7 \pm 0.3) \times 10^{-2}$ | $(2.5 \pm 0.2) \times 10^7$ |

Table 2

Association Affinities and Apparent Dissociation Rate Constants For Streptavidin–biotin Binding

| | single subunit K_A (M^{-1}) | apparent k_{off} (s^{-1}) |
|--------------------------|-----------------------------------|---|
| nonregeneration protocol | $(7.3 \pm 0.2) \times 10^6$ | $(0.81 \pm 0.08) \times 10^{-4}$ ($\chi_2 = 1$) ^b |
| literature | 2×10^7 ^a | $(1.37 \pm 0.08) \times 10^{-4}$ ($\chi_2 = 0.15$) ^c $(3.31 \pm 0.16) \times 10^{-4}$ ($\chi_2 = 0.007$) ^c |

^aReference 16.^b χ_2 is the fraction of biotin on the surface. For the nonregeneration protocol reported here, χ_2 is 1.^cReference 18.