Chapter 21

Capture of the Human IgG1 Antibodies by Protein A for the Kinetic Study of h-IgG/FcγR Interaction Using SPR-Based Biosensor Technology

Thierry Champion and Alain Beck

Abstract

Surface plasmon resonance (SPR) is a key technology to evaluate IgG effector functions in vitro involving binding to Fcgamma receptors (FcγR). We describe here the chemical coupling of protein A to a sensor chip. IgGs prepared either from purified antibodies or from crude cell media supernatants are captured by the protein A and are used as the ligand. Soluble forms of FcγRs are injected at different concentrations and are used as the analyte. This setup allows the definition of the kinetic rates of binding of the FcγRI (high affinity) or the FcγRIIIa (low affinity) on the Fc domain of IgGs.

Key words ADCC, Biacore X100, Fcgamma receptor, IgG effector functions, Protein A capture, Surface plasmon resonance

1 Introduction

Immune system effector cells actively lyse target cells that have been bound by specific antibodies. This mechanism of cell-mediated immunity is called Antigen Dependent Cell-mediated Cytotoxicity (ADCC). The effector cells express at their surface Fcgamma receptors (FcγR) which belong to the immunoglobulin superfamily (1, 2). This family includes several members such as FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD32), FcγRIIIA (CD16a), and FcγRIIIB (CD16b). All, except the FcγRI, have an extracellular portion composed of two immunoglobulin (Ig)-like domains. FcγRI presents a third Ig-like domain and binds to IgGs more strongly than FcγRII or FcγRIII. This property allows activation of FcγRI by a single IgG molecule, while the latter two Fcγ receptors have to bind multiple IgG molecules within an immune complex to be activated. Typically ADCC involves activation of natural killer (NK) cells, these cells express at their surface the FcγRIIIa Fc receptors which play a central role in this activation.

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For many therapeutic antibodies used in oncology such as rituximab, trastuzumab, alemtuzumab, cetuximab, and pertuzumab, ADCC is an integral part of their mechanism of action (2, 3). For others, ADCC may be responsible for some undesirable side effects. It is thus important to document the effector functions in the early stages of the research and development of therapeutic antibodies. Moreover, the affinity of the FcγRIIIa for human IgG is not only affected by the subclass nature of the IgG isotype but also by the glycan attached to the Asn 297 of the heavy chains (4) and by the aggregation tendency of the antibody. The glycan composition depends on the cell system and the culture conditions used for the production of the therapeutic antibody. The aggregation rate of an antibody depends on its primary sequence (5), the purification process and the storage condition of the final drug product. Therefore, it is mandatory to test for modifications to the effector potential of an antibody after any changes in the process to its production.

Easy to handle and low protein consuming SPR based experiments represent valuable surrogates to in vitro and in vivo ADCC assays to test any step of the production process for such potential modifications.

For this purpose, protein A is chemically coupled to a sensor chip. A solution of antibody (a purified solution or even a crude cell media supernatant) is captured by the protein A and is used as ligand. The soluble forms of FcγR are injected at different concentrations and are used as analyte. This setup allows the definition of the kinetic rates of binding of the FcγRI (high affinity) but also the FcγRIIIa (low affinity) on the Fc domain of IgGs.

2 Materials

1. The experiments were run on a Biacore X100.
2. CM5 sensor chips (Biacore).
3. HBS-EP + as the running buffer:
   - 10 mM HEPES [*N*-(2-hydroxyetyl) piperazine-*N*′-(2-ethanesulfonic acid)].
   - 150 mM NaCl.
   - 3 mM EDTA (Ethylenediaminetetraacetic acid).
   - 0.05% (v/v) surfactant P20 (Polyoxyethylene sorbitan) pH 7.4.
4. Protein A from *Staphylococcus aureus* (Sigma ref. P7837).
5. Trastuzumab (Herceptin), Roche.
6. *N*-hydroxysuccinimide (NHS).

7. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).
8. Sodium acetate.
9. Ethanolamine hydrochloride.
10. Sodium hydroxide.
11. Glycine HCl.
12. Soluble recombinant h-FcγRI (R&D Systems, ref 1257-FC-050).
13. Soluble FcγRIIIa (R&D Systems, ref 4325-FC-050).

3 Methods

3.1 Coupling of Protein A on the CM5 Sensor Chip

1. Protein A was coupled on both flowcells using the amine coupling kit.
2. The two flowcells were prepared successively as follow at a flow rate of 5 μl/min.
3. The carboxymethyldextran matrix was first activated by a 7 min injection of a 50/50 (v/v) mix of *N*-hydroxysuccinimide (NHS) 0.1 M and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) 0.39 M.
4. A 200 μg/ml solution of protein A was prepared in a 10 mM sodium acetate pH 5.0 buffer and injected for 7 min on the activated flowcell.
5. At the end of this injection, the non-reacting activated carboxylic functions were quenched by a 7 min injection of a 1 M ethanolamine hydrochloride, NaOH pH 8.5 solution.
6. 2,984 RU and 2,935 RU of protein A were chemically coupled to the Flowcells 1 and 2, respectively.

3.2 Single Cycle Kinetic with Trastuzumab (Herceptin®) and the h-FcγRI

1. A 2 μg/ml solution of antibody was prepared by diluting the trastuzumab stock solution (21 mg/ml) in the running buffer.
2. The solution was injected for 1 min at a flow rate of 30 μl/min on the flowcell 2 solely.
3. This injection allows the capture of around 700 RU of h-IgG1 (see Note 1).
4. A solution of the soluble recombinant h-FcγRI was prepared at 200 nM (assuming a molecular weight of 50 kDa) in the running buffer.
5. A range of five concentrations from 40 to 1 nM was prepared by a 2.5-fold serial dilution scheme.

6. The five concentrations were injected in the growing order for 90 s each at a flow rate of 30 µl/min.
7. After the last injection, a delay of 600 s was left for the dissociation phase study.
8. As a second reference, the running buffer was injected five times in the same conditions as the h-FcγRI concentration range.
9. At the end of each cycle, the antibody/hFcγRI complexes were discarded by a 30 s injection of 10 mM Glycine HCl pH 1.7 buffer at a flow rate of 30 µl/min.
10. The sensorgrams correspond to the flowcell 2 signal corrected by the reference flowcell 1 signal (without any captured antibody).
11. The blank sensorgram was then subtracted from the sensorgram obtained with the recombinant hFcγRI range.
12. The corrected data were fitted using the Biacore X100 evaluation software with the Langmuir (1:1) model (see Notes 2 and 7).

3.3 Single Cycle Kinetic with Trastuzumab and the h-FcγRIIIa

1. Except for the concentration range, the experiment was identical to 3.2.
2. A solution of the recombinant soluble FcγRIIIa was prepared at 2,000 nM (assuming a molecular weight of 45 kDa) in the running buffer.
3. A range of five concentrations from 2,000 to 125 nM was prepared by a twofold serial dilution scheme.
4. The data were analyzed with the Biaevaluation software using the Langmuir (see Note 3), the heterogeneous ligand (see Notes 4, 7 and 8), and the two state reaction models (see Notes 5–8).

4 Notes

1. Protein A–CM5 Sensor Chip captured the h-IgG1 very efficiently. The capture of trastuzumab is perfectly reproducible, very stable during the five injections of HBS-EP + buffer plus the 6 min delay at the end of each cycle, the antibodies are efficiently removed by a single 30 s injection of a 10 mM Glycine pH 1.7 buffer (see Fig. 1). As expected, (6) this capture enables the binding of the recombinant soluble h-FcγR (I and IIIa) (see Note 9). This setup enables testing antibody batches before the purification step. Even, a crude cell culture supernatant may be used. This useful property would not be reached with the inverse setup where the FcγR are used as ligand and the antibodies as analyte. Moreover, this last setup presents some other drawbacks (see Note 10).

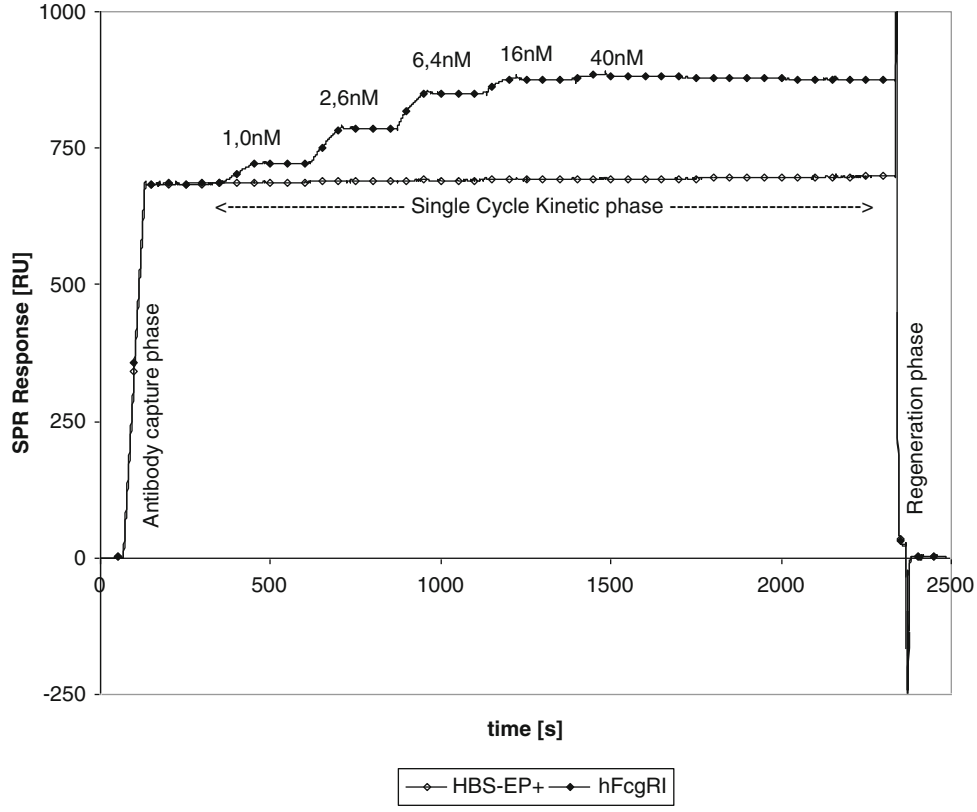


Fig. 1 Superposition of two sensorgrams corresponding to an experimental and the blank reference of a Single Cycle Kinetic experiment. IgG1 antibody (Trastuzumab) was captured on the flowcell 2 of a CM5–protein A grafted sensor chip (2,984 and 2,935 RU of protein A have been coupled, respectively, on the flowcells one and two). A growing range of five concentrations of h-FcγRI (from 1.0 to 40 nM) are injected during 1990s each after the capture phase. As a blank reference the running buffer was injected five times in the same conditions. After the fifth injection a 600 s delay was left to study the dissociation phase. At the end of each cycle the surface was regenerated by a 30 s injection of a 10 mM Glycine pH 1.7 buffer. The experiment was carried out on a Biacore X100 at a flow rate of 30 μl/min and at the temperature of 25°C

2. The kinetic binding of h-FcγRI on the h-IgG1 follows a Langmuir (1:1) model. The Langmuir model properly fits the single cycle kinetic experimental (see Fig. 2 and Table 1). The ratio $Chi^2/R_{max} = (1.41\ RU^2/186.4\ RU) = 7.6 \times 10^{-3}$ RU. This value is largely under the commonly used acceptable limit of 0.05 RU. The R_{max} value (186.4 RU) is in the range of the theoretical R_{max} value (229 RU) (see Note 11). The refractive index (RI) contribution is not significant: $-1.71\ RU < RI < 0.94$ RU (see Note 12). With these experimental conditions, the association kinetic rate k_a is equal to $(2.232 \pm 0.003) \times 10^6\ M^{-1}\ s^{-1}$ and the dissociation rate k_d is equal to $(6.82 \pm 0.13) \times 10^{-5}\ s^{-1}$. This latter value is close but above the $2 \times 10^{-5}\ s^{-1}$ limit. The dissociation

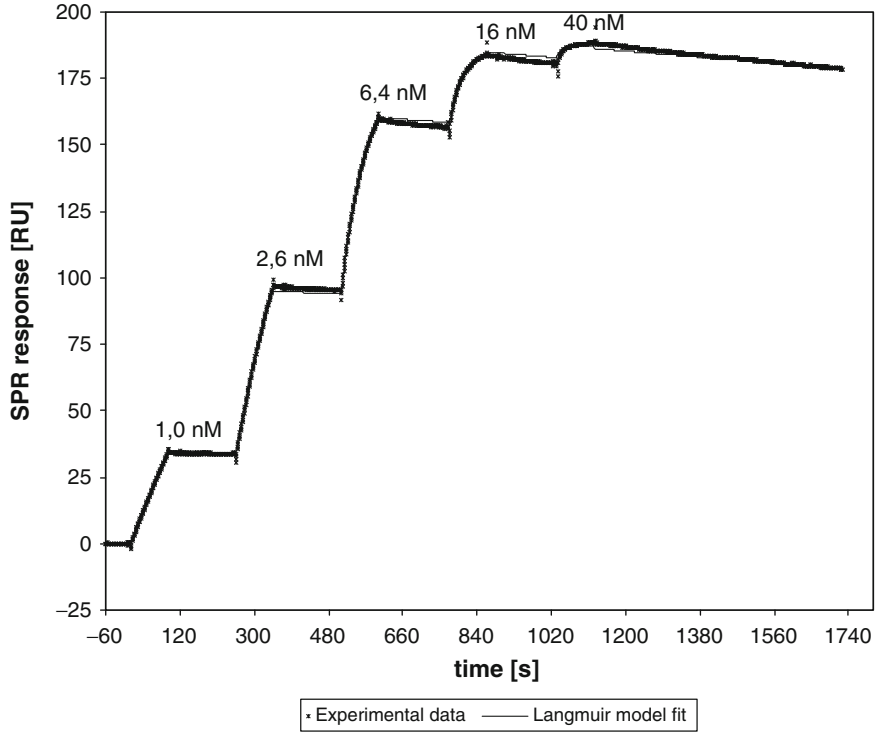


Fig. 2 Analysis of the single cycle kinetic experimental data corresponding to five injections in the growing range of concentrations (from 1 to 40 nM) of h-FcγRI on around 700 RU of captured h-IgG1 (Trastuzumab) corrected by the five HBS-EP + injections used as the double reference. These data are fitted with a Langmuir (1:1) model with the following parameters ($k_a = 2.23 \times 106$ M^{-1} s^{-1}, $k_d = 6.8 \times 10^{-5}$ s^{-1}, $R_{max} = 186.4$ RU, $Chi^2 = 1.14$ RU^2). The experiment was carried out on a Biacore X100 at a flow rate of 30 μl/min and at the temperature of 25°C

constant K_D corresponding to the k_d/k_a ratio is equal to 30.6 ± 0.7 pM.

3. Kinetic study of binding of h-FcγRIIIa on the h-IgG1: The kinetic with h-FcγRIIIa do not follow the Langmuir model. The Langmuir model improperly fits the single cycle kinetic experimental data (see Fig. 3 and Table 1). The ratio Chi^2/R_{max} = (23.4 RU^2/141.6 RU) = 0.165 RU. This value is largely above the 0.05 RU acceptable limits. The contribution of the refractive index is high too (–14.2 RU < RI < 3.6 RU). The calculated dissociation rate (k_d) seems to be overestimated (quicker than the experimental data). With this model, the dissociation constant K_D is equal to 421 ± 64 nM.
4. Kinetic study of binding of h-FcγRIIIa on the h-IgG1: The heterogeneous model This model considers the ligand (i.e., the antibody) as a mix of two components and defines for each one a k_a, a k_d, and a R_{max}. This model improves at least the fitting of the dissociation phase (see Fig. 4 and Table 1). With this

Table 1
Kinetic parameters of the binding of the two soluble recombinant h-FcγR at 25°C on Trastuzumab captured on a Protein A–CM5 sensor chip

	h-FcγRI/h-IgG1	h-FcγRIIIa/h-IgG1		
Mathematical models	**Langmuir**	**Langmuir**	**Heterogeneous ligand**	**Two state reaction**
k_{a1} [a]	$2.232 \pm 0.003 \times 10^{6}$ M^{-1} s^{-1}	$2.33 \pm 0.18 \times 10^{5}$ M^{-1} s^{-1}	$3.36 \pm 0.34 \times 10^{5}$ M^{-1} s^{-1}	$7.12 \pm 0.90 \times 10^{5}$ M^{-1} s^{-1}
k_{d1} [a]	$6.82 \pm 0.13 \times 10^{-5}$ s^{-1}	$98.1 \pm 7.4 \times 10^{-3}$ s^{-1}	$13.8 \pm 0.7 \times 10^{-3}$ s^{-1}	$254 \pm 32 \times 10^{-3}$ s^{-1}
K_{D1}	–	–	41.2 ± 6.2 nM	–
R_{max1}	–	–	18.8 RU	–
k_{a2}	–	–	$2.89 \pm 0.10 \times 10^{5}$ M^{-1} s^{-1}	$3.33 \pm 0.35 \times 10^{-3}$ s^{-1}
k_{d2}	–	–	$145.9 \pm 4.6 \times 10^{-3}$ s^{-1}	$14.7 \pm 1.0 \times 10^{-3}$ s^{-1}
K_{D} [b]	30.6 ± 0.1 pM	421 ± 64 nM	505 ± 33 nM	291 ± 12 nM
R_{max} [b]	186.4 RU	141.6 RU	107.0 RU	118.6
Chi^2	1.14 RU^2	23.4 RU^2	12.4 RU^2	14.3 RU^2

[a] k_a and k_d for the Langmuir model
[b] K_{D2} and R_{max2} for "Heterogeneous ligand" model

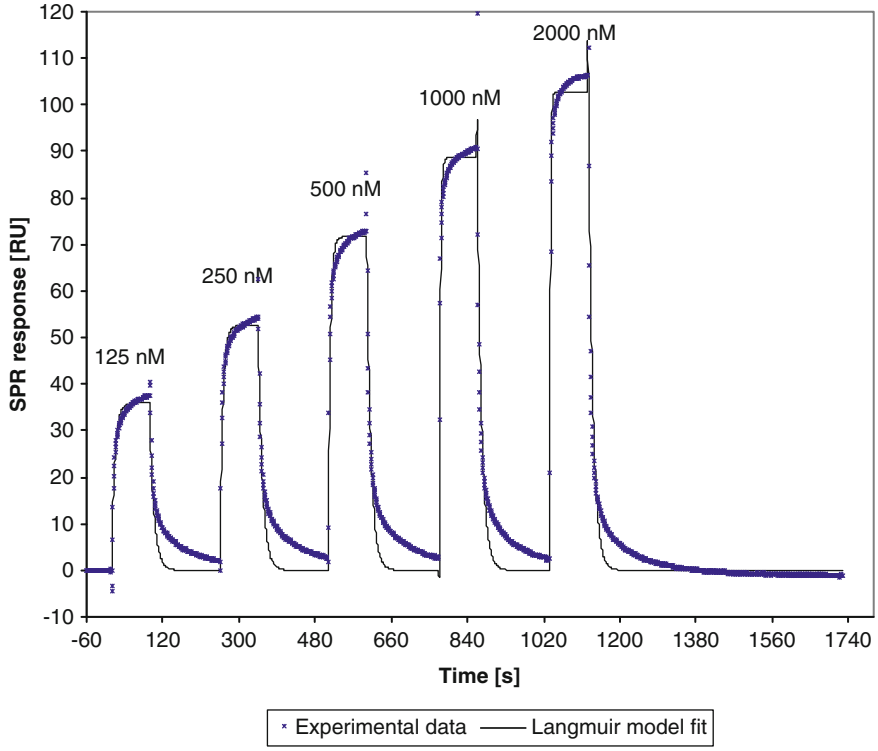
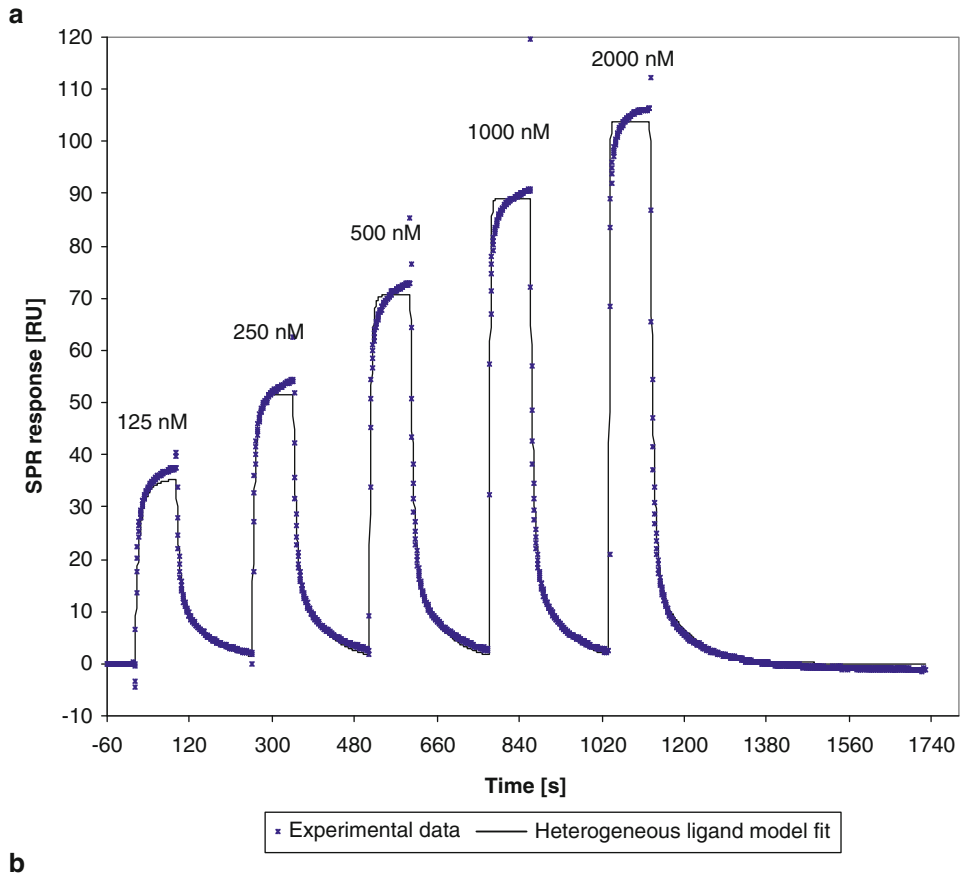
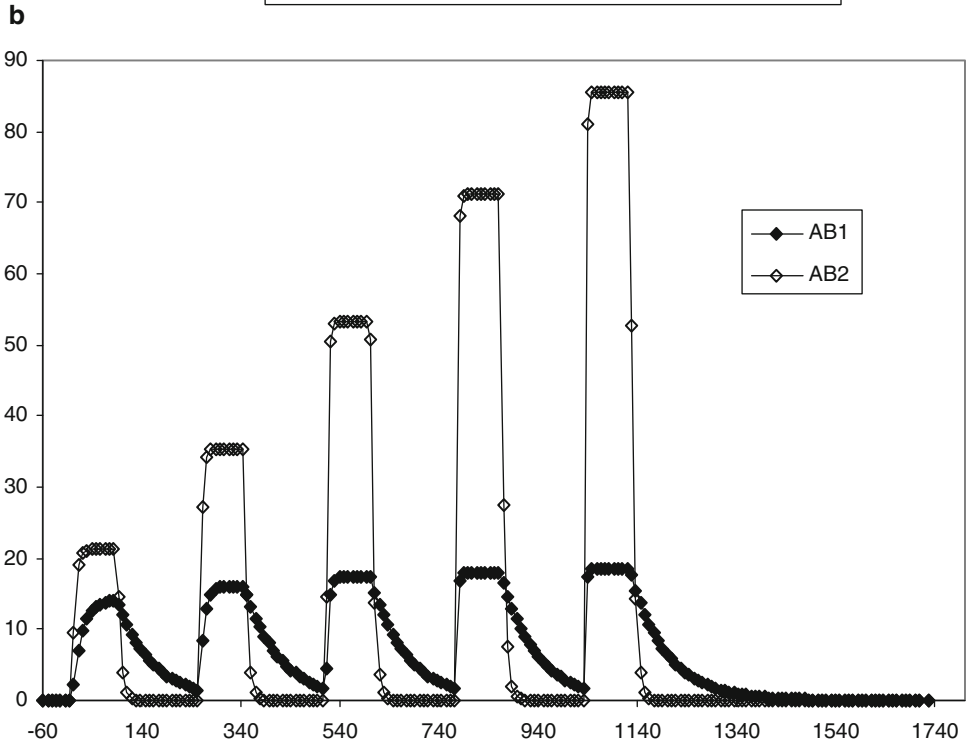


Fig. 3 Analysis of the single cycle kinetic experimental data corresponding to five injections in the growing range of concentrations (from 125 to 2,000 nM) of h-FcγRIIIa on around 700 RU of captured h-IgG1 (Trastuzumab) corrected by the five HBS-EP + injections used as the double reference. These data are fitted with a Langmuir (1:1) model with the following parameters ($k_a = 2.3 \times 105$ M^{-1} s^{-1}, $k_d = 98 \times 10^{-3}$ s^{-1}, $R_{max} = 141.6$ RU, $Chi^2 = 23.4$ RU^2). The experiment was carried out on a Biacore X100 at a flow rate of 30 μl/min and at the temperature of 25°C

model, the major (85.1%) and the minor (14.9%) component of the captured antibodies (see Note 13) present a dissociation constant K_D of 505 ± 33 and 41.2 ± 6.2 nM, respectively. The proportion defined by the kinetic analyses has to be compared with the published ratio of fucosylated (76%) and afucosylated (24%) glycan composition of trastuzumab (7) and the dissociation constant of a fully fucosylated (300 nM) and a fully afucosylated (7.2 nM) antibody with a soluble recombinant FcγRIIIa (4). So, this model makes sense even if the glycan heterogeneity is generally more complex than a simple binary component system (see Note 14).

Fig. 4 (**a**) Analysis of the same experimental data shown in Fig. 3 with the "Heterogeneous Ligand" model corresponding to the following parameters ($k_{a1} = 3.4 \times 105$ M^{-1} s^{-1}, $k_{d1} = 14 \times 10^{-3}$ s^{-1}, $R_{max1} = 18.8$ RU, $k_{a2} = 2.9 \times 105$ M^{-1} s^{-1}, $k_{d2} = 146 \times 10^{-3}$ s^{-1}, $R_{max2} = 107.0$, $Chi^2 = 12.4$ RU^2). The Bulk and Drift (RI) component was fixed to 0. (**b**) Decomposition of the fitted signal in its two components (AB1: binding of h-FcγRIIIa on the minor kind of antibody, and AB2: binding of h-FcγRIIIa on the major kind of antibody)


a
SPR response [RU]
Time [s]
125 nM
250 nM
500 nM
1000 nM
2000 nM
Experimental data
Heterogeneous ligand model fit


b
AB1
AB2

5. The two state reaction model supposes a conformational change induced by the binding of the FcγRIIIa to the Fc domain of the IgG1 antibody. This assumed mechanism is based on the 3D structure of the complex FcγRIIIa/Fc (PDB ID code 3SGJ) (4). This model, in accordance with other 3D models, shows that the FcγRIIIa makes close contact with both chains of the human Fc domain (see Note 15). At first, the Fc receptor may interact with one arm and then "lock" its position by slightly pushing aside to the second chain. Some glycan compositions (i.e., afucosylated structures) could facilitate this conformational change.
6. This model defined five kinetic parameters: two association rates, two dissociation rates and one R_{max} (see Table 1). The Chi² value of the fitting (14.3 RU²) is slightly higher than the Chi² of the heterogeneous model (12.4 RU²). Nonetheless this fitting is better than the fitting with the Langmuir model. The dissociation constant K_D, calculated with the four kinetic rates (see Note 16), is equal to 291 ± 12 nM.
7. The stable and easy to regenerate capture of the human IgG1 by protein A sensor chip is very efficient for the study of the binding of the Fcγ receptors. The high affinity FcγRI may be used as an assay to check the integrity of the antibody structure. The FcγRIIIa kinetic study is a little bit trickier. Therefore, the steady state analysis is generally used for the dissociation constant definition of the FcγRIIIa/Fc interaction. However, the kinetic rate definition gives more information than a sole K_D.
8. A kinetic study with different glycan compositions of a same antibody would be of great interest to understand how the carbohydrate portion of the Fc domain has this tremendous effect on the FcγRIIIa binding by finding a correlation between the glycan composition and the kinetic rates calculated at least by one of the two proposed models.
9. The protein A from *S. aureus* presents 4 IgG binding domains. Each of which is able to react with the "elbow" region between the CH2 and the CH3 domains of an IgG heavy chain (PDB ID code 1FC2). This interaction domain is apart from the binding domain of the FcγR.
10. There are at least two solutions for using the recombinant soluble FcγR as the ligand and the antibodies as the analyte. The first one is a chemical coupling of the soluble receptor. This approach needs to conjugate low quantities of protein to minimize the mass transport limit and to find a regeneration condition that does not unfold the coupled protein. When the device allows only two flowcells, one sensor chip per receptor is required. The second way is the capture of the receptor with an anti-Tag antibody (anti-6 His c-terminal Tag generally).

The capture allows assays with different receptors on the same sensorchip. Running the experiment, this capture seems very accurate: very stable capture and efficient regeneration. However, the h-FcγR interacts with the Fc domain of anti-Tag His antibody too (at least with mouse antibodies). This interaction complicates the analysis. NTA-sensor chip may be used to capture His-Tag protein to avoid this drawback. This surface has not been tested in house.

11. The theoretical R_{max} value is calculated with the following formula:

    Captured ligand level (RU) × $MW_{analyte}/MW_{ligand}$ (MW = molecular weight in Da).

12. SPR based technology measures changes in the refractive index at the surface of the sensorchip. The analyte solutions may have a refractive index different from the refractive index of the running buffer. These changes are responsible for the bulk effect represented in the mathematical models by the Refractive Index value. The use of a reference flowcell is supposed to cancel the bulk effect on the sensorgrams. So generally the RI values have to tend to 0.

13. The proportion of the two components is estimated by the R_{max1} and R_{max2} ratio.

14. For instance the published oligosaccharide profile of Trastuzumab (6) is:

    -N-N-M[M]-M[M]-M: 5%, -N[F]-N-M[M]-M-N: 2%.

    -N-N-M[M-N]-M-N (G0): 8.5%, -N[F]-N-M[M-N]-M-N (G0F): 43%.

    -N-N-M[M-N]-M-N-G (G1): 8%, -N[F]-N-M[M-N]-M-N-G (G1F): 28%.

    -N-N-M[M-N-G]-M-N-G (G2): 3%, -N[F]-N-M[M-N-G]-M-N-G (G2F): 2.5%.

    With N = *N*-acetylglucosamine, M = Mannose, F = Fucose, G = Galactose, and [X] represents a branched oligosaccharide sequence.

    Each glycosylation composition may have an impact on the binding of the FcγRIIIa.

15. The FcγRIIIa makes close contacts (<5 Å) with the residues belonging to the following sequences of one chain: PELLGGPS^{239} + KALPAPI^{332} and with the following sequences of the other chain: PELLGGPS^{239} + DVSHE^{269} + YN[NAG-FUCα1-6]ST^{299} + NKA^{327}.

16. With the two state reaction model (Fig. 5), the K_D is calculated with the following formula:

$$K_D = \frac{(k_{d1}/k_{a1})}{\left(1 + (k_{a2}/k_{d2})\right)}.$$

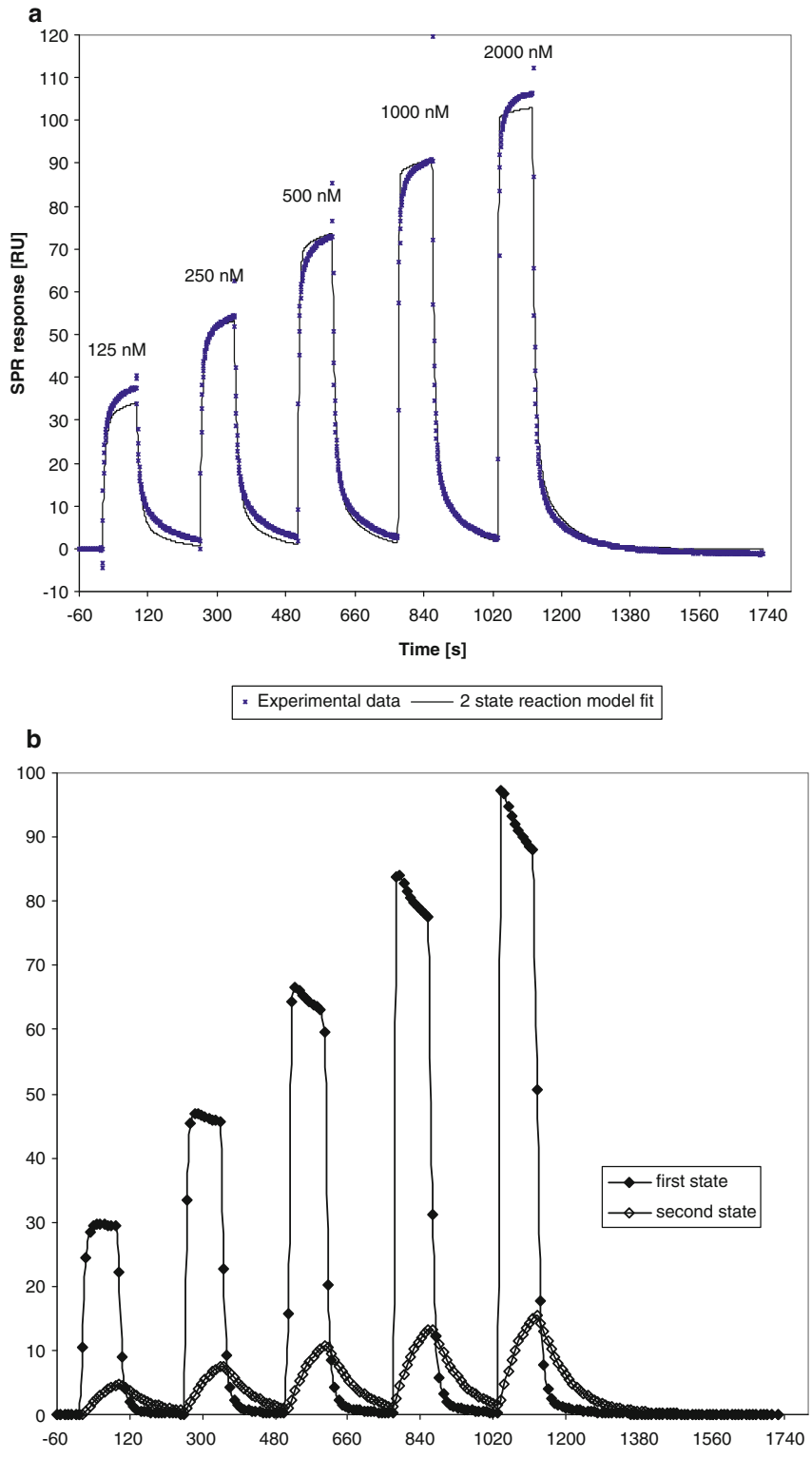


Fig. 5 Analysis of the same experimental data shown in Fig. 3 with the "Two state reaction" model corresponding to the following parameters ($k_{a1} = 7.1 \times 105\ M^{-1}\ s^{-1}$, $k_{d1} = 254 \times 10^{-3}\ s^{-1}$, $k_{a2} = 3.3 \times 0^{-3}\ s^{-1}$, $k_{d2} = 15 \times 10^{-3}\ s^{-1}$, $R_{max2} = 118.6$, $Chi^2 = 14.3\ RU^2$). The Bulk and Drift (RI) component was fixed to 0. (**b**) Decomposition of the fitted signal in its supposed two states of the binding of h-FcγRIIIa on the antibody

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