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Application of Optical Biosensor Techniques to the Characterization of PorA-Antibody Binding Kinetics

Janet Suker and Bambos M. Charalambous

1. Introduction

The design of novel vaccines and strategies to combat infectious disease requires an understanding of the interactions between pathogen and host. Biological interactions in vivo often rely on specific recognition mechanisms that begin with a binding step. The development of biosensor technology has allowed the real-time measurement of the binding characteristics of biomolecules and provides a powerful new tool for the analysis of molecular recognition. An optical biosensor comprises a detector linked to an optical transducer that generates a measurable signal from a biological interaction occurring at the detector surface. Evanescent optical biosensors have been available since the late 1980s, the most commonly known commercial systems being IAsys (which uses the resonant mirror sensor) ***(1,2)*** and BIAcore (which employs the optical phenomenon of surface plasmon resonance) ***(3)***. There is a multitude of different applications of biosensor technology including measurement of concentration, kinetic analysis, structural studies, fermentation monitoring, receptor-cell interactions, and equilibrium analysis. The most widespread applications have been to protein-protein interactions, in particular receptor-ligand and antibody-antigen binding. More recent studies have been extended to protein-carbohydrate, DNA-DNA, and DNA-RNA interactions. Examples of the diverse uses of biosensors are found in the field of meningococcal research such as in the study of transferrin binding pro-

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teins *(4,5)*, lipo-oligosaccharide (LOS)-antibody interactions *(6)* and serum responses to experimental vaccines *(7)*.

This chapter outlines one specific application of the IAsys resonant mirror biosensor to study the binding kinetics of PorA-antibody interactions. The method describes how the IAsys technology can be used to obtain both qualitative and quantitative information about the effects of antigenic variation on PorA-antibody recognition and also highlights the benefits and pitfalls of the use of biosensors in immunology. Firstly, the principles behind the IAsys technique and a general outline of the method will be given and then background information for each of the major procedures in the method will be explained.

1.1. Principles of the IAsys Technique

The IAsys biosensor consists of a resonant mirror structure *(1)* at the base of a disposable cuvette within which the interaction in question is carried out. One of the interacting moieties (the ligand) is immobilized on to the cuvette surface and subsequent interaction with another molecule (the ligate) alters the physical characteristics at the surface. The resonant mirror structure consists of two dielectric layers on glass. A high refractive index waveguide is separated from a high refractive index glass prism by a low index coupling layer. Monochromatic light is scanned on to the device and at a particular angle, the resonant angle, some of this light energy passes from the prism, through the spacer layer, to propagate in the waveguide as an evanescent wave. The light returns via the coupling layer and is incident upon a detector. Changes in refractive index owing to interactions at the sensor surface alter the angle at which the light can be made to propagate in the waveguide. This is recorded as an arc second response that is related directly to the concentration of ligate. Furthermore, the response data can be fitted to an appropriate mathematical equation to derive 'on' and 'off' rates of ligate binding and dissociation and hence determine the strength of the interaction by calculation of equilibrium constants. This latter facility has led to kinetic interaction analysis becoming the most widespread application of biosensor technology, particularly in the field of immunology.

1.2. Kinetics Analysis Using a Biosensor

For general descriptions of the use of biosensors in immunology, the reader is directed to the reviews in references *(8)* and *(9)*. Traditional immunoassays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) provide valuable information on antibody binding, but are time-consuming and have limitations such as that one of the reactants must be labeled and that the data obtained is from end-point measurements. Biosensors can overcome these restrictions and provide rapid measurements of real-time reaction kinetics. However, biosensor technology also has limitations because

certain assumptions in the interpretation of response data may not be fulfilled in every case. For example, data fitting usually assumes a 1:1 stoichiometry but interactions between ligand and ligate may deviate from first-order reaction kinetics owing to re-binding, mass-transport effects, or multivalency of ligand or ligate. Different mathematical strategies can be employed to take into account these factors ***(10)***, but the literature is inconsistent about the best interpretation of these data and many authors over-simplify or fail to state the model used.

In the study of antibody-binding kinetics it is important to distinguish between the 'affinity' or the 'avidity' of antibody-antigen interactions. The affinity of an antibody is the strength of binding of a monovalent ligand to a single antigen-binding site, whereas avidity (or 'functional affinity') refers to the overall strength of interaction between a complex antigen and a bi- or multivalent antibody. The strength of antibody (Ab)-antigen (Ag) interaction can be expressed as an equilibrium (or association) constant, K_A that is determined from the equation:

$$Ab + Ag \leftrightarrow Ab:Ag$$

and thus reflects the 'on' and 'off' rates of the interaction. The equilibrium constant may also be expressed as a dissociation constant (K_D). With small independent haptens binding is usually as rapid as diffusion allows, and the 'off' rates will determine differences in affinity constants. With larger haptens the interaction becomes more complex as the 'on' rate may vary as a consequence of diffusion rates. In the case of heterogeneous populations of antibodies or antigens, a combination of association constants will exist but an average equilibrium constant can be determined when 50% of the Ab:Ag complex is formed. Moreover, immunoglobulin molecules can interact via both their antigen binding sites with multiple epitopes that are present on the surface of bacterial pathogens. This increases the overall strength of binding, as both sites must be released simultaneously to dissociate. For example a decavalent IgM can have low-affinity binding sites but the avidity of the whole antibody to a bacterial surface displaying multiple identical epitopes can be very high. These considerations may make the interpretation of biosensor data more complex.

Another factor that complicates the interpretation of biosensor data is that the equations commonly used to calculate equilibrium constants assume that both binding partners are moving freely in solution. However, as the biosensor requires that the ligand is immobilized on the sensor surface, the design of experiments to measure true affinity constants must account for this, for example by using competition assays with free ligand ***(11)***. It is therefore important that the limitations of biosensor technology are recognized and that caution is exercised in the interpretation of rate constants. Nevertheless, with the use of appropriate experimental design, biosensor data may be used to provide new insights into the mechanisms of molecular recognition.

1.3. Outline of the Method

In order to study antibody interactions with PorA proteins in their native conformation and to allow screening of multiple variants of PorA, this experiment is carried out with purified MAb immobilized at the biosensor surface to which outer-membrane vesicle (OMV) preparations are added. The interaction could be carried out with PorA immobilized at the surface and the antibody added as ligate but this may alter the structure of PorA and would require making a fresh cuvette for every variant to be studied.

First, purified monoclonal antibody (MAb) is bound to a dextran-coated cuvette then membrane-vesicle preparations from meningococcal strains encoding different PorA variants are added. The amount of PorA in each membrane preparation is normalized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) densitometry and used to estimate the molar concentration of PorA protein in each preparation. The membrane vesicle suspensions are added to the biosensor cuvette and the interaction responses of different concentrations of PorA to the immobilized MAb are monitored. To show differences in the association rates and the amount of membrane vesicles bound to the immobilized MAb, the interaction profiles for each preparation of PorA at a single concentration are overlaid using the FASTplot software. This also illustrates whether or not a particular PorA variant is recognized by the MAb. The association and dissociation data at different concentrations of PorA are then collected and analyzed using the FASTfit software. From the FASTfit data, the apparent affinity constants of the different PorA-MAb interactions are calculated and reported as relative avidity.

1.4. Major Procedures Involved in the Protocol

1.4.1. Choice of Surface

The manufacturer (Labsystems Affinity Sensors) provides cuvettes with a choice of surfaces for immobilization of different ligands. If a large ligand is to be immobilized on to the cuvette surface, a thin coating matrix such as aminosilane is required to maintain the moiety within the evanescent-wave region. If a small protein ligand is used, it can be efficiently immobilized via its amino groups to a carboxymethyl-dextran surface using succinimide-ester chemistry. For nonprotein ligands, for example carbohydrates, the carbohydrate can be biotinylated and immobilized via a streptavidin bridge to a biotin-coated cuvette. In this application, the dextran surface is chosen for immobilization of antibody because: 1) the interacting ligate is the larger binding partner; 2) the chemistry of antibody immobilization to dextran is well-characterised; 3) the immobilized antibody is stable and binding activity is retained.

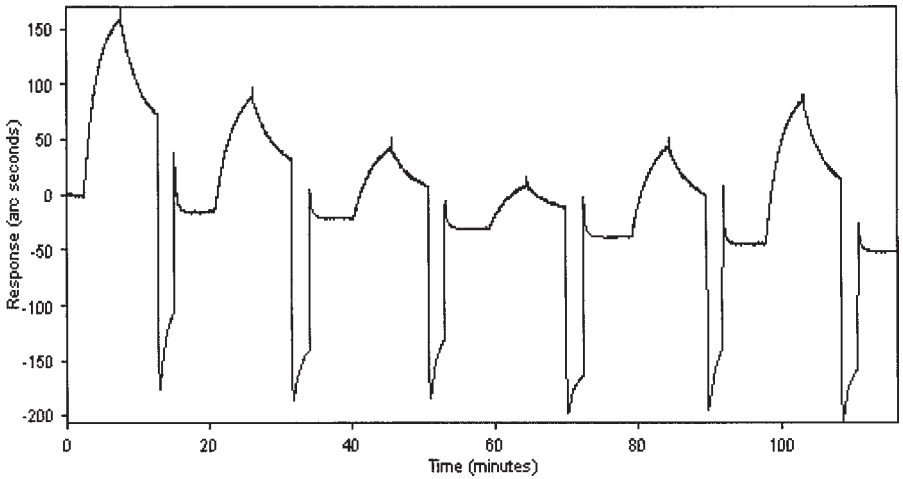

Fig. 1. Typical association and dissociation profiles at different ligate concentrations. Reproduced from IAsys tutorial software (Affinity Sensors).

1.4.2. Purification of OMVs Containing PorA and Estimation of PorA Molar Concentration

To ensure that the PorA proteins are as close as possible to their native conformation, ligate is prepared as OMVs. It is possible to carry out the interaction analysis with whole meningococcal cells and to determine which PorA variants bind to the MAb. However, for kinetic analysis, the relative concentration of PorA must be measured so that known concentrations of protein can be added to the cuvette for calculation of 'on' and 'off' rates. PorA preparations are made by a simple outer-membrane extraction procedure ***(12)*** and the presence of vesicles confirmed by electron microscopy (EM).

1.4.3. Interaction Analysis with MAb and Regeneration of the Sensor Surface

The cuvette coated with immobilized MAb is inserted into the IAsys instrument and aliquots of PorA vesicles are added manually. Data collection and experimental conditions are recorded using the IAsys software and the binding interaction is displayed as arc-second response plotted in real-time on the computer screen. Unbound ligate is diluted to zero concentration by washing and the dissociation is monitored in a similar manner. The surface is then regenerated before the next binding interaction by stripping any remaining bound ligate using a suitable buffer that maintains the integrity of the immobilized ligand. The cycle of binding–wash–dissociation–regeneration is repeated for different concentrations of ligate and the data is saved for analysis. **Figure 1** illustrates

a typical set of data showing the association and dissociation profiles of six different ligate concentrations.

1.4.4. Data Analysis Using the FASTfit and FASTplot Software

FASTfit is a program designed to analyze, and display the data collected from the IAsys biosensor running the IAsys software. This program fits the captured digital data from the biosensor by least squares analysis to either monophasic or biphasic interaction kinetics and calculates 'on' and 'off' rates. Because immunoglobulin molecules have two identical binding sites, the binding data in this application is fitted to a biphasic curve, with the occupancy of the first binding site representing the first reaction rate. However, complications arise from measuring the interaction kinetics of membrane vesicles because particulate ligate may not be freely diffusable in the reaction and binding is likely to be multivalent. Consequently, the association and dissociation rates cannot be used to calculate true affinity constants, but comparisons can be made between the apparent avidity of the different PorA variants.

2. Materials

2.1. IAsys Biosensor Requirements

IAsys instrumentation and software is supplied by Labsystems Affinity Sensors (Saxon Way, Bar Hill, Cambridge, UK). Further information can be obtained from: iasys.support@thermobio.com or the website http://www.affinity-sensors.com

1. Manual, single-cell model of the IAsys resonant mirror biosensor. (Dual cuvettes and robotic systems are now available for more rapid screening of multiple samples).
2. Desk-top PC with minimum system requirements of a 486 processor with 66 MHz clock speed and 32 MB of RAM running Windows 95/8.
3. Instrument running and data collection software IAsys version 2.1.8.
4. IAsys help manuals: Methods Guide (basic principles and experimental design); Users Guide (use of the IAsys instrument) and FASTfit Guide (data analysis and software).

2.2. Immobilization of MAb to a Carboxymethyl Dextran (CM-Dextran) Surface

1. CM-Dextran cuvettes (Labsystems Affinity Sensors code FCD-0101). Cuvettes are stored at room temperature before immobilization of ligand.
2. NHS coupling kit containing: N-hydroxysuccinimide (NHS); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1 *M* ethanolamine, pH 8.5

(Labsystems Affinity Sensors). The EDC and NHS reagents are stored at –20°C and the ethanolamine at room temperature (stable for several months).
3. Purified MAb MN20F4.17 ***(13)***. The MAb is available from National Institute for Biological Standards and Control (NIBSC) in the form of ascites fluid. It was purified by standard methods ***(14)***: ammonium sulphate precipitation followed by affinity chromatography with protein G using a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech).
4. Running buffer: Phosphate-buffered saline Tween (PBST): 138 m*M* sodium chloride, 2.7 m*M* potassium chloride, 4 m*M* sodium phosphate, 1.8 m*M* potassium phosphate, 0.05% (v/v) Tween 20.
5. Immobilization buffer: 10 m*M* sodium acetate, pH 4.3.
6. Regeneration buffer: 10 m*M* HCl.

Buffers 4, 5, and 6 are stored at room temperature and are stable for several weeks. During an experiment, the buffers are stored in the holder on top of the instrument to maintain them at the instrument running temperature.

Cuvettes with the immobilised antibody can be stored for several months at 4°C under PBST without significant deterioration. Prior to use, the cuvette must be equilibrated to the interaction temperature by placing it into the biosensor and washing once with regeneration buffer.

2.3. Purification of OMVs Containing PorA and Estimation of PorA Concentration

2.3.1. Meningococcal Strains

In this study NIBSC strains: 2151 (P1.5c,10); 2743 (P1.5c,10a); 2202 (P1.7d,10) and 2208 (P1.5c, VR2 deleted) were used. 2208 is a naturally occurring mutant containing an identical *porA* gene to the prototype P1.10 strain (2151) apart from an 87 base-pair deletion that entirely removes VR2. This was used as a negative control strain. Strains 2151, 2743, and 2202 contain different combinations of epitopes in VR1 and VR2.

2.3.2. Media and Buffers

1. 'Chocolate' agar plates: 10 % (v/v) horse blood, heated to 80°C in Blood Agar Base No. 2 (Oxoid, CM271).
2. Mueller Hinton broth (Oxoid, CM405).
3. TE buffer: 200 m*M* Tris-HCl/1 m*M* EDTA, pH 8.0.
4. TES buffer: 200 m*M* Tris-HCl/1 *M* sucrose, 1 m*M* EDTA.
5. Chicken egg-white lysozyme (1 mg/ml).
6. Bicinchoninic acid (BCA) protein assay kit (Pierce 23225).
7. Standard reagents and equipment for SDS-PAGE analysis ***(15)***.
8. Gel-scanning software (for example LabWorks Analysis Software, Ultra Violet Products).

2.4. Interaction Analysis with MAb and Regeneration of the Biosensor Cuvette Surface

1. Prepared membrane vesicles and cuvette with immobilized ligand (*see* **Subheading 2.2.** and **2.3.**).
2. PBST wash buffer: 138 m*M* sodium chloride, 2.7 m*M* potassium chloride, 4 m*M* sodium phosphate, 1.8 m*M* potassium phosphate, 0.05 % (v/v) Tween 20.
3. Regeneration buffer: 10 m*M* HCl.

2.5. Data Analysis Using the FASTfit and FASTplot Software

1. FASTfit version 2.01 and FASTplot version 3.0 software for data plotting and analysis.
2. GraphPad PRISM® V2.01, (GraphPad Software Inc.) or other standard graphics packages can be used to determine the linear correlation of the 'on' rates (K_{ON}) with the estimated PorA concentration and to plot the data with their respective errors.
3. Computer file in *.RMD format containing experimental data.

3. Methods

3.1. IAsys Biosensor Analysis

Some prior knowledge is required before attempting the procedures outlined in this method (for example, the operator should be familiar with the contents of the IAsys manuals listed in **Subheading 2.1.**). Hence, where detailed instructions are provided in the IAsys guides, they are not repeated here. Where a wash step is indicated, this means three changes with 200 μL buffer.

3.2. Immobilization of MAb to a Carboxymethyl Dextran (CMD) Surface

1. Switch on the instrument, insert a fresh CMD biosensor cuvette and start the data collection software.
2. Start a new experiment, record the experimental details in the notebook and alter the data-collection parameters: temperature to 25°C and data collection to 5 s (*see* **Note 1**).
3. Wash with PBST and leave for 10–20 min to obtain a steady baseline. During this time, check the integrity of the cuvette surface by running a resonance scan to ensure that it is satisfactory prior to immobilization (*see* **Note 2**).
4. Immediately before use, mix equal volumes of the NHS and EDC solutions. Add 200 μL of the mixed NHS/EDC solution to the cuvette and leave for 7 min to activate the carboxyl groups on the CMD.
5. Wash with PBST and leave for 2 min.
6. Wash with immobilization buffer (10 m*M* sodium acetate, pH 4.3) and leave for 2 min.
7. Add 200 μL purified MAb MN20F4.17 at 50 μg/ml in 10 m*M* sodium acetate, pH 4.3, to the cuvette and leave for 10 min to allow electrostatic uptake to occur (*see* **Note 3**).

8. Remove any free MAb by washing with PBST and leave for 2 min.
9. Replace the PBST with 200 μL ethanolamine at pH 8.5 and leave for 3 min.
10. Wash with regeneration buffer and leave for 2 min (*see* **Note 4**).
11. Wash with PBST and leave for 5 min.
12. Stop the experiment and save as a *.RMD file.
13. Proceed to interaction with antibody (**Subheading 3.4.**) or remove the cuvet (still containing PBST) wrap it in parafilm and store at 4°C. Coated cuvets can be stored at 4°C for several weeks provided that the surface is not allowed to dry out.

3.3. Purification of OMVs Containing PorA and Estimation of PorA Concentration

Appropriate safety precautions should be taken when handling live meningococci. Steps involving live meningococcal cells should be carried out in a class II safety cabinet (including centrifugation of broth culture).

1. Streak out the required strains on chocolate agar plates and incubate overnight at 37°C in a 5% CO_2 atmosphere.
2. Inoculate 5 mL of Mueller-Hinton broth with a large colony from the overnight growth and incubate the broth cultures at 37°C until OD_{600} ~0.6 (approx 6 h).
3. Harvest 4 mL of each culture and either freeze the pellets overnight or continue to **step 4**.
4. Resuspend cell pellets from 4 mL broth culture in 200 μL TE buffer.
5. Add 200 μL ice-cold TES buffer and mix by inversion.
6. Add 24 μL 1 mg mL^{-1} lysozyme and mix.
7. Add 400 μL sterile distilled water.
8. Shake the mixture on a shaker for 30 min.
9. Sediment the spheroplasts at 40,000*g* for 20 min.
10. Resuspend spheroplasts in 800 μL ice-cold distilled water (using a needle and syringe to aid resuspension).
11. Sediment the membrane fragments at 40,000*g* for 20 min.
12. Resuspend the membranes in 500 μL PBS/T water. The preparation can be frozen at –20°C for several months.
13. Before interaction analysis, the membrane preparations should be brought to room temperature and mixed well.
14. Estimate the total protein concentrations of the prepared membranes using the microtiter plate protocol included in the BCA assay kit (or other protein assay).
15. Run approx 20 μg total protein for each vesicle preparation on a 12% SDS-PAGE gel, according to standard procedures ***(15)***.
16. Scan the gel and integrate the peaks to estimate the percentage protein in each PorA band (Mr approx 44,000). The principles of densitometry on stained bands on an SDS protein gel are described in ***(16)***.
17. Calculate the total amount of PorA in each vesicle preparation.

3.4. Interaction Analysis with MAb and Regeneration of the Sensor Surface

1. If the cuvette has been stored at 4°C, allow it to warm up slightly before inserting it into the IAsys equipment (to avoid condensation forming on the cuvette, which will interfere with detection).
2. Switch on the data-collection software, start a new experiment and adjust data-collection rate to 5 s (*see* **Note 1**).
3. Wash with PBST and follow the response until a steady baseline is obtained (usually 30–40 min). Check the resonance scan again during this time (*see* **Note 2**).
4. While waiting for the baseline to stabilize, make up a range of dilutions of membrane preparations in PBST. For this method, the range of PorA concentrations to use is from 10 nM to 5000 nM for each strain (*see* **Note 5**).
5. When the baseline is steady, stop the experiment and start a new one, record experimental details in the notebook, set data collection to 0.3 s, and save as a new *.RMD file (NB: save data frequently during an experiment).
6. Wash with PBST and leave for 5–10 min.
7. Add 200 μL of the first dilution of vesicles.
8. Follow the binding for 10 min (*see* **Note 6**).
9. Wash unbound membranes with PBST.
10. Follow dissociation for 5 min.
11. Wash twice with regeneration buffer and leave for 2 min.
12. Wash with PBST for 2–5 min (*see* **Note 7**).
13. Repeat **steps 7–12** for 5–10 different concentrations of each PorA variant (*see* **Note 5**).
14. Stop the experiment and save the data to *.RMD file (*see* **Note 8**).

3.5. Data Analysis Using the FASTplot and FASTfit Software

3.5.1. Data Presentation Using FASTplot

The interaction profiles of the same concentration of different PorA variants with the immobilized P1.10 MAb are captured from the IAsys file of the experiment (*.RMD) and overlaid on to the same graph.

1. Open FASTplot and import the experimental data from the *.RMD file.
2. Manually define the baseline and binding interaction regions for each interaction to be displayed. A zoom function is available to allow the interaction profiles to be seen more clearly.
3. Set the coincidence time to specify the exact time of the binding phase (zero time-point for all curves to be overlaid).
4. Transfer the selected regions to the plot.
5. Repeat **steps 2–4** for the different PorA subtypes.
6. Add a title to the overlay plot and annotate the curve by using the plot properties dialog box (*see* **Fig. 2**).

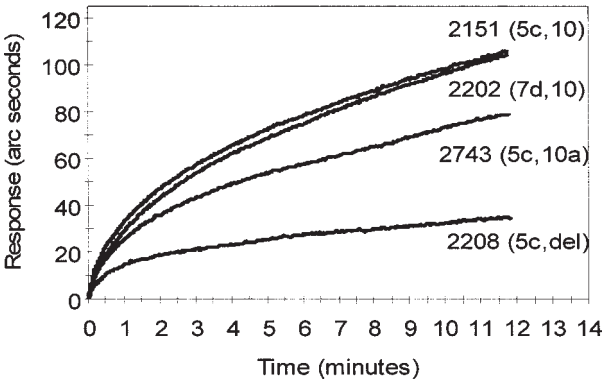

Fig. 2. Overlay plots of the interaction profiles of membrane preparations of different PorA subtypes with the P.10 specific MAb, MN20F4.17. The concentration of PorA is 100 nM for all the interaction profiles displayed. A single amino acid substitution present (R→P) in the VR2 domain of subtype 10a compared to 10 appeared to reduce the binding interaction of the mAb. In contrast, differences in the VR1 of strains 2151 and 2202 did not appear to alter the interaction profile of the MAb, suggesting that the interaction is VR2 specific. Figures are the strain number and figures in brackets are the PorA subtype.

3.5.2. Kinetics Analysis Using FASTfit

Data must be fitted in turn for each concentration of membrane vesicles added to the biosensor cuvette. A zoom function is available to enlarge the region of interest.

1. Open FASTfit and load the data file (*.RMD) generated by the IAsys software.
2. Correct the baseline drift if present.
3. Set the start marker of the first association data region at the point of addition of the membrane vesicles to the cuvette.
4. Set the start marker of the first dissociation region at the point of evacuating and filling the cuvette with wash buffer (dilution to zero concentration).
5. Specify the analysis regions for the baseline, association, and dissociation of the first interaction profile using the color-coded range selectors. Set the baseline range selector for approx 1 min of data before the association starts. Set the beginning of the association data range 5 s after the start of the association start marker (*see* **Note 9**) and the end of the association range for 10s from that point. Set the dissociation range for 5 min of data starting just after the dissociation start marker.
6. Choose the analysis required (*see* **Note 10**) and fit the data.
7. Record the association rates (k_{ON}) and dissociation rates (k_{OFF}) with their error values, and whether the data is fitted best to a monophasic or biphasic exponential (*see* **Note 10**). Also record the R_{max} value.

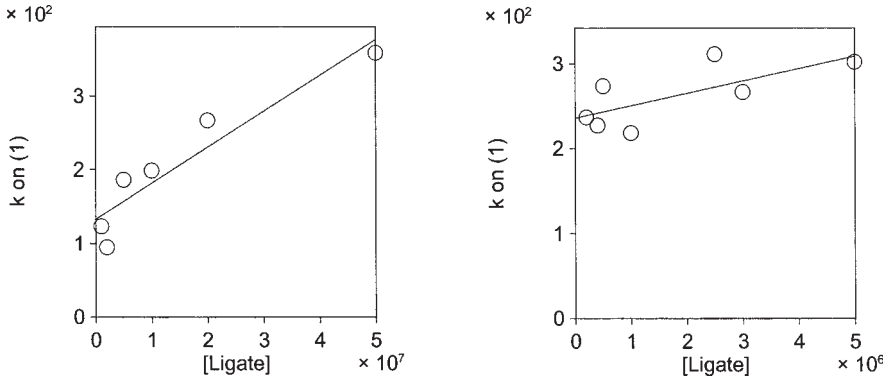

Fig. 3. The 'on' rates of two meningococcal PorA variants interacting with the immobilized MAb MN20F4.17. K_{ON} rates for the monophasic binding of meningococcal-membrane vesicles containing two PorA variants; left panel is P1.10, the right panel is P1.10a. The 'on' rates are fitted by least squares and the slope is the association constant (k_{ass}). However, because of the particulate nature of the ligate, these are only relative measurements and should not be considered as true rate constants. The P1.10 PorA variant has approx 60-fold greater relative avidity for MAb MN20F4.17 than the P1.10a variant.

8. Repeat **steps 5–7** using total association data ranges of 25s, 50s, 100s, 300s, and 600s and record the association rates (k_{ON}) with their error value for each data range (keep the dissociation range to 5 min).
9. Repeat **steps 3–8** for each interaction profile and for each different ligate.
10. Plot the association rate (k_{ON}) for each PorA concentration and perform a linear correlation on the data points (*see* **Note 11** for values to be excluded from this plot). The gradient of this line is the association rate constant (k_{ass}) (*see* **Fig. 3**).
11. The dissociation rate constant (k_{diss}) is calculated from the mean and standard deviation of the dissociation rates obtained from PorA concentrations from 200 nM to 5000 nM and should be close to the value of the intercept of the plot of k_{ON} versus ligate concentration (*see* **Note 12**).
12. The dissociation equilibrium constants of the different PorA proteins are calculated from the ratio of k_{diss}/k_{ass} (*see* **Note 12**).

4. Notes

1. During the association and dissociation stages the data should be collected as rapidly as possible (0.3 s intervals). Conversely, during the buffer or reagent incubations or ligand immobilization, the data-collection rate should be decreased to 5 s intervals. It is also important to save data files frequently during the experimental runs.
2. The resonance scan before immobilization and between bindings should show a single symmetrical peak indicating homogeneous coating of the surface. If the

scan is atypical at this stage, it may indicate a faulty cuvette and a fresh cuvette should be tested. The resonance scan can be checked at any stage during an experiment. However, the shape of the peak may change during binding and asymmetrical scans obtained during an experiment should recover after washing.

3. For efficient immobilization of the ligand, the pH and buffer conditions are optimized to obtain maximum interaction with the surface prior to coupling. The optimum pH for the immobilization buffer will vary depending on the pI of the biomolecule to be immobilized. This must be determined empirically by measuring the arc second response due to electrostatic interaction of the ligand in buffers of different pH.
4. The regeneration conditions must preserve ligate-binding activity and must be optimized for specific applications.
5. The ideal concentrations of ligate to use for calculations of equilibrium constants are from $10 \times K_D$ to $0.01 \times K_D$. However, in most cases, the K_D is not known in advance so the appropriate range of concentrations to use must be determined empirically. Dilutions can be made either by adding an appropriate amount of a concentrated stock solution to the correct amount of buffer in the cuvette or by making up the dilutions in advance and adding 200 μL of the pre-diluted solution to the cuvette. It is preferable to add the different dilutions of ligate in a random order and to repeat the first dilution again at the end of an experiment. This allows verification of the reproducibility of the cuvette surface.
6. The interaction data should be collected until binding is approaching saturation, i.e., when the interaction profiles are observed to be entering the plateau phase. The data can be analyzed in the FASTfit program while an experiment is running to check the progress of binding.
7. Allow at least 1–2 min for baseline between each interaction to facilitate setting data ranges within FASTfit.
8. It is possible to merge data from different data files during FASTfit analysis and it is a good idea to include data points repeated on different days.
9. Data for analysis by FAStfit is not captured immediately after the addition of membrane vesicles to the cuvette as changes in bulk refractive index give a biosensor response. Also data should not be captured and analyzed when the interaction profile begins to plateau. At this point the immobilized MAb is reaching saturation with the PorA antigen, which results in a reduction of the association rate.
10. The first stage is to decide whether the data is fitted best to a single exponential (monophasic) or a biphasic exponential. Most reactions, whether mono- or biphasic, can be fitted to a biphasic exponential because reaction velocities decrease with time as the reaction becomes limited by reactant depletion or product accumulation. The limiting factor in this experiment is the saturation of the MAb immobilized on the surface of the biosensor cuvette. Consequently, the data to be included for the calculation of the association rate constant should be from the early part of the binding-interaction profile, which is best fitted by a monophasic exponential.

The fit for the association data from the longest time periods (up to 600 s) will be poor initially because the software will be attempting to best fit the data to a monophasic exponential. At these time-points, the ligand is approaching the saturation of the immobilized MAb and consequently the binding kinetics are biphasic. The range of data to analyze at each concentration of ligate will depend on the association or dissociation rates. The object is to reduce the data range to be analyzed until good single-phase fits are obtained with small errors. This will become evident, as the errors will decrease dramatically as the range of data selected becomes monophasic. Several different ranges are selected and a record made of the rate constants and errors. You will find that as you decrease the data range the rate constant will increase without significant increases in error. If you continue to decrease the data range, the errors will increase and the rate constant becomes erratic.

Alternatively, the data can be fitted to a biphasic exponential and the software will determine the initial (k_{ON1}) and subsequent (k_{ON2}) rate constants. However, in our experience the more accurate method is to reduce the time period over which the data is captured so that it is best fitted by a monophasic exponential.

11. The plot of k_{ON} values against the PorA concentration to derive the association rate constant (k_{ass}) should not include concentrations of PorA that are approaching the saturation of the binding sites of the immobilized MAb. This will result in reduced association rates that deviate from linearity.
12. The affinity of a molecular interaction may be expressed as a K_D (dissociation equilibrium constant), which is the reciprocal of the association equilibrium (K_A) or affinity constant. The k_{diss} can be derived from the direct measurement of dissociation data ($k_{diss} = k_{OFF}$), or from the intercept of the plot of K_{ON} vs PorA concentration. However, as the k_{diss} approaches zero (slow off rates) the less accurate the value derived from the intercept of the k_{ON} vs ligate concentration becomes, and experimentally derived data is preferred. An approximate K_D can also be obtained from a plot of the total extent of binding vs ligate concentration and is used to confirm the values obtained.

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