Selection of protein therapeutic candidates using Biacore T100

A critical role for high-resolution kinetics in assessment of engineered human Fabs

- Protein interaction analysis of engineered human Fabs during development of a protein therapeutic against *P. aeruginosa* infections

- A unique level of protein interaction characterization ensures well-informed selection during *in vivo* development

- Fast, reliable high-resolution kinetic analysis for monitoring of affinity and kinetic profile changes during Fab development

- Extremely stable system performance at 37°C
  - physiological temperature analysis with optimal predictive value for *in vivo* performance

Figure 1. High-resolution kinetic analysis of engineered human Fabs using Biacore T100.

"Biacore provides the best available analysis of affinity and binding kinetics for our protein therapeutic candidates"

Peter Flynn, PhD, Director, Biochemistry Research, KaloBios Pharmaceuticals Inc., Palo Alto, California, USA

Introduction

Protein therapeutics, such as antibodies and antibody fragments, are becoming increasingly important. While these proteins offer unparalleled benefits in terms of targeting and specificity, issues such as the risk of immunogenic reactions in patients and the potentially high cost of the end product goods also present potential problems for pharmaceutical companies. To minimize these and other challenges, efficient selection and optimization of protein therapeutics is essential throughout the development process. Real-time protein interaction analysis is an invaluable characterization technique that contributes to all phases of protein therapeutic discovery programs, from basic research to selection and optimization of therapeutic candidates, to immunogenicity studies in preclinical and clinical trials.
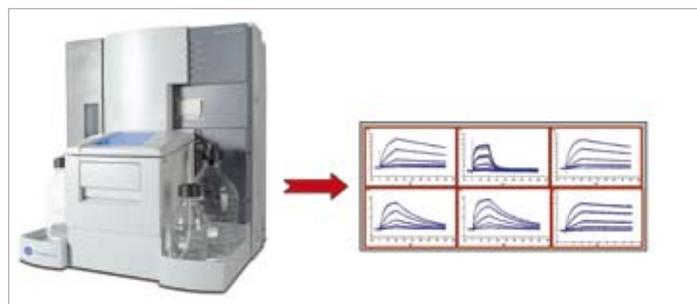
Purpose of study

Here we describe the role of high-resolution kinetic characterization using Biacore™ T100 in development of engineered human Fabs. KaloBios Pharmaceuticals Inc. employ a proprietary "Epitope Focused Selection" technology to convert high affinity mouse antibodies into engineered human antibodies with close-to-human germ-line sequences. These retain antigen specificity with even higher affinity, and are designed to show very low immunogenicity in clinical use.

Minimal essential antigen-binding sequences from the V-region of mouse antibodies are transferred to a proprietary human V-region library. Reconstructed V-regions are produced in a microbial cell expression system and antibody fragments are secreted into the culture medium. The resulting library of transformants is screened against the target antigen using a colony lift binding assay, in which the stringency of selection can be carefully controlled by antigen density. This approach enables rapid generation of a high-affinity, engineered human antibody from a mouse antibody within a matter of weeks.

The target antigen in this study was the PcrV protein, which forms part of the type III secretion system (TTSS) complex of the pathogenic bacterium, *Pseudomonas aeruginosa*. An anti-PcrV mouse antibody was shown to disrupt the needle-like TTSS complex required for injection of *P. aeruginosa* exotoxins into host cells and protect against bacterial cytotoxicity in cell and animal models[1,2]. A non-immunogenic engineered human antibody fragment against this protein would therefore provide a powerful new therapy against *P. aeruginosa* infections.

P. aeruginosa

This microorganism is the major causative agent in ventilator-associated pneumonia and is also associated with serious infections in patients with severe burns and cystic fibrosis. Following invasion of the lung epithelium, *P. aeruginosa* induces rapid systemic effects such as bacteremia & sepsis, often with high fatality rates. Treatment of such infections is often complicated by significant antibiotic resistance, making a protein therapeutic strategy an attractive alternative.

The library approach used to generate the engineered human antibody fragments, with screening at different stringencies and affinity maturation stages, results in a large number of candidate Fabs with different affinities. Biacore T100 was used to characterize a number of these different Fabs, with high-resolution kinetic analysis carried out at physiological temperature in order to provide the optimal predictive relevance for subsequent cell and animal experiments, and downstream clinical applications.

Results

High quality kinetic data for Fab binding to antigen at three surface densities

A total of 11 Fabs were analyzed: a high-affinity mouse Fab (A), four engineered human Fabs from a low-stringency screen (B1-B4), three from a high-stringency screen (C1-C3) and three affinity-matured Fabs (D1-D3) engineered from C2 by mutagenesis (see Table 1).

Fab	Details
A	mouse Fab
B1	low stringency selection
B2	low stringency selection
B3	low stringency selection
B4	low stringency selection
C1	high stringency selection
C2	high stringency selection
C3	high stringency selection
D1	affinity-matured
D2	affinity-matured
D3	affinity-matured

Table 1. Details of Fabs characterized in the study. With the exception of the high-affinity mouse Fab (Fab A), all other candidates are engineered human Fabs. The three affinity-matured Fabs were all derived from Fab C2.

Recombinant GST-PcrV antigen was immobilized on a sensor surface at three different densities (see Methods) and a dilution series of five different concentrations of each Fab was injected over the antigen. The label-free protein interaction analysis produces real-time plots of binding response against time (the sensorgram), which are then fitted to an appropriate binding model to derive kinetic rate and affinity constants (Figure 2).
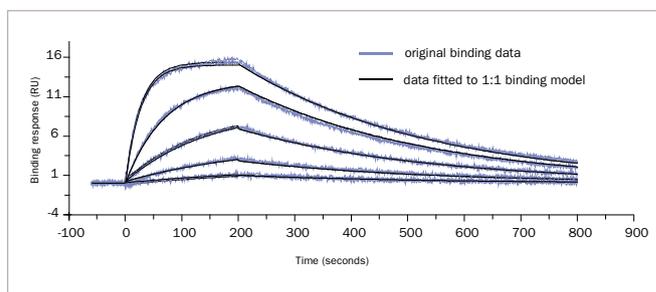

Figure 2. Kinetic analysis of Fab binding to the GST-PcrV antigen. The overlay plot shows sensorgrams of five different concentrations of one Fab binding to the highest of the three antigen surface densities. Binding response (changes in mass concentration detected as differences in refractive index by SPR) is shown in resonance units (RU).

Using different surface densities of the immobilized binding partner can be a helpful assay development step to determine optimal conditions, particularly when starting to work with a new experimental system. In this case excellent quality kinetic data was obtained from all three of the surface densities (Figure 3). Consequently, the final kinetic constants were obtained by globally fitting the total dataset for each Fab.

Figure 3. Kinetic analysis at three antigen immobilization densities. Overlay plots of 37°C kinetic assays for Fab C2 at PcrV immobilization densities of 10 RU (low), 15 RU (medium) and 30 RU (high). Excellent data fits were obtained for each of the three densities.

Kinetic profiles show the effects of selection and maturation during development of engineered human Fabs

An overview of the kinetic profiles for all eleven Fabs clearly showed the changes in binding properties occurring as a result of engineering the human Fabs. The dissociation profiles of the low-stringency screen Fabs (B1-B4) were significantly faster compared to the mouse Fab A, indicating a loss of binding stability and affinity (Figure 4). Screening at higher stringency selected human Fabs with profiles very similar to the mouse Fab in two out of the three examples examined (C2 and C3). Affinity maturation of Fab C2 resulted in Fabs with dissociation profiles that were significantly slower, even when compared to the mouse Fab.
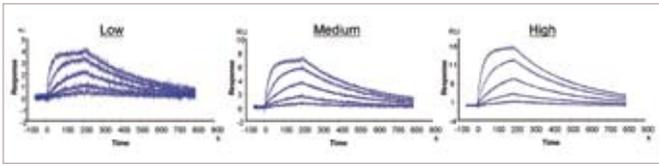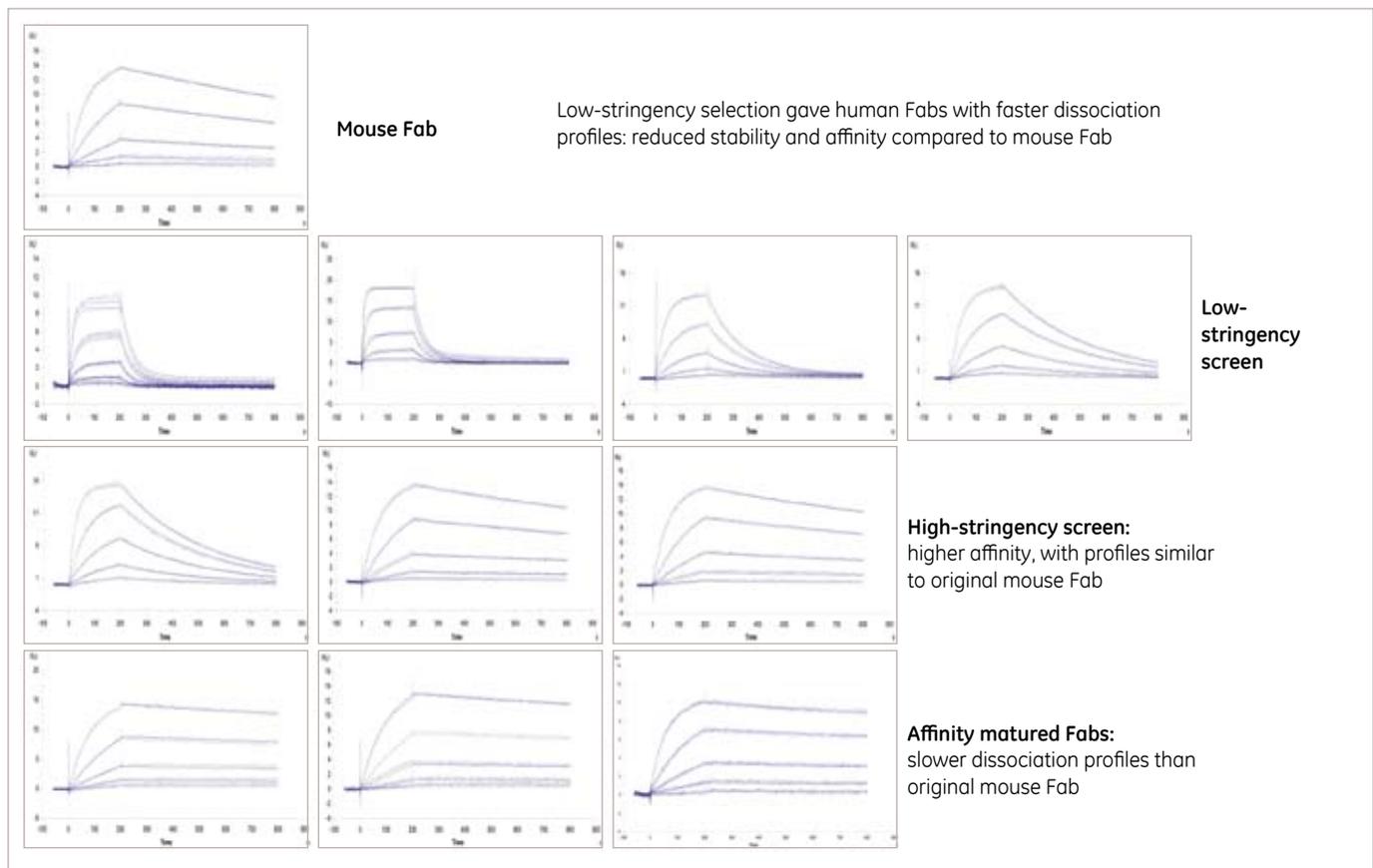

Mouse Fab

Low-stringency selection gave human Fabs with faster dissociation profiles: reduced stability and affinity compared to mouse Fab

Low-stringency screen

High-stringency screen: higher affinity, with profiles similar to original mouse Fab

Affinity matured Fabs: slower dissociation profiles than original mouse Fab

Figure 4. Kinetic profiles of PcrV-binding by all eleven Fabs. Overlay plots of 37°C kinetic assays for Fabs (high density surface examples shown).

Variations in binding affinities are primarily dependent on dissociation rate changes

Derivation of individual rate constants from the kinetic analyses (Table 2 and Figure 5) provided a detailed characterization of the binding properties displayed by the different Fabs. Affinities varied between 31.5 to 0.6 nM, with k_a values ranging from 1.8×10^5 to 1.5×10^6 $M^{-1}s^1$, and k_d values between 1.7×10^{-4} and 1.9×10^{-2} s^{-1}. Off-rates varied approximately 100-fold, compared to an on-rate variation of just 10-fold, showing that the affinity variations seen among the Fabs were principally driven by differences in off-rates.

The high-resolution kinetic characterization provided a clear picture of the changes in binding properties that occurred during development of the engineered human Fabs. The low-stringency screen selected Fabs with a significantly reduced affinity for PcrV compared to mouse Fab A, overwhelmingly driven by increased dissociation rates. Increasing the stringency of selection identified Fabs with affinities close to, or better than, the mouse Fab, with the improved selection focused heavily on reduced dissociation rates. Selection stringency appeared to be essentially a dissociation rate-dependent property, probably reflecting the incubation and wash conditions employed in the colony lift binding assay. This provides a useful selection criterion for therapeutic proteins since, while association rates may be accounted for, to some degree, by controlling dosage levels, stable binding to the therapeutic target *in vivo* cannot.

Affinity maturation produces Fabs with slow kinetic profiles

Kinetic resolution of binding properties was particularly revealing for Fabs D1, D2 and D3, which were affinity-matured derivatives of Fab C2. While the affinities of these Fabs were similar to, or lower than, Fab C2, their kinetic profiles were significantly slower (Figure 5). The most important observation from these analyses was that the affinity maturation process produces Fabs with the slowest off-rates of all those examined, thereby kinetically fine-tuning the high affinity Fabs obtained by screening at high stringency.
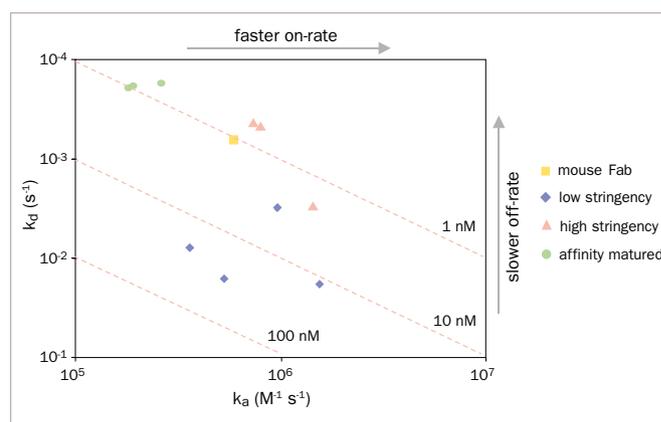

Figure 5. Off/on rate map showing resolution of binding affinities into rate constants. Log-scale plot of k_d (reversed scale, y-axis) against ka (x-axis), with the direction of off- and on-rate changes indicated by the arrows. In this plot, affinity (k_d/k_a) increases from bottom left to top right, resulting in iso-affinity diagonals.

Fab	Details	k_a	k_d	K_D (nM)
A	mouse	5.9E+05	6.2E-04	1.1
B1	low stringency	5.3E+05	1.7E-02	31.5
B2	low stringency	1.6E+06	1.9E-02	12.3
B3	low stringency	3.6E+05	8.0E-03	22.3
B4	low stringency	9.6E-05	3.2E-03	3.3
C1	high stringency	1.4E+06	3.0E-03	2.1
C2	high stringency	7.2E+05	4.4E-04	0.6
C3	high stringency	7.8E+05	4.7E-04	0.6
D1	affinity maturation	1.8E+05	1.8E-04	1.0
D2	affinity maturation	1.8E+05	1.9E-04	1.0
D3	affinity maturation	2.6E+05	1.7E-04	0.7

Table 2. Affinity and rate constants derived from kinetic analysis of Fabs. Rate constants for each Fab were derived from a global fit of binding data from all three antigen surface densities. Affinity values were derived from the ratio of the rate constants (k_d/k_a). Note that Fabs D1, D2 & D3 were affinity-matured from Fab C2.

High-resolution kinetics provides unique evaluation of Fab maturation process

The comparison of binding properties for the high-stringency screen and affinity matured Fabs provides a strong example of the value of kinetic analysis compared to affinity-based methods. Fabs with very similar affinities were readily differentiated on the basis of significantly different kinetic properties. This information enabled the selection of candidates with the most desirable kinetic properties for the intended therapeutic application.

A specific example of this resolving power is highlighted by Fabs C2 and D3. These have an almost identical affinities, differing by less than 10% (Figure 6A). Based on this information alone, the conclusion would be that affinity maturation of Fab C2 had little effect on the PcrV-binding properties of its derivative, D3. Kinetic resolution clearly shows that affinity maturation resulted in a significantly slower binding profile with reduced on- and off-rates (Figure 6B). The rate constant data for Fabs D1 and D2 showed a similar pattern and emphasize that the affinity maturation process produces a highly desirable off-rate reduction in the final engineered human Fabs. This important conclusion regarding the maturation process was therefore dependent on the kinetic resolution provided by Biacore T100.
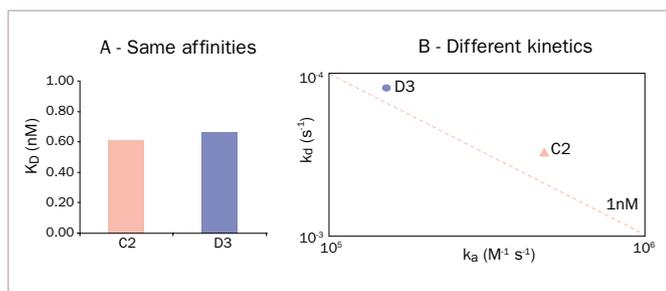

Figure 6. Kinetic resolution of affinities reveals essential information regarding binding properties of candidate Fabs. A) Affinity measurements indicate almost identical binding behavior of Fabs C2 and D3, with K_D values differing by less than 10% B) Off/on rate map resolution shows that these very similar affinities derive from distinctively different kinetic profiles, with Fab D3 exhibiting significantly slower on- and off-rates.

Excellent correlation of kinetic properties with cell assays

The PcrV-binding properties of the Fabs were compared with their therapeutic performance in a cell-based assay. Mouse myeloma cells were incubated with *P. aeruginosa* for 3 hours and cytotoxicity was assessed by the proportion of permeabilized cells (propidium iodide dye uptake, quantified by flow cytometry). Antibody potency was judged by the ability to reduce cytotoxicity levels, after co-incubation of different concentrations of Fabs with the myeloma cells and bacteria. In these assays, Fabs exhibited significant inhibition of the cellular toxicity induced by *P-aeruginosa*, with a range of different potencies (Figure 7A).

Since protein interaction analysis was carried out at 37°C, affinity and kinetic data could be directly compared to cell assay performance. There was excellent correlation when ranking Fabs by cytotoxicity protection potency and Fab-PcrV affinity values derived from interaction analysis (Figure 7B). Moreover, cytotoxicity protection correlated much more closely with Fab off-rates than on-rates. This shows that the dissociation rate constant is the best predictive parameter for biotherapeutic Fab activity, and that the off-rate bias in the Fab selection procedures used was entirely successful in producing protein therapeutics with increased efficacy in these cell assays.
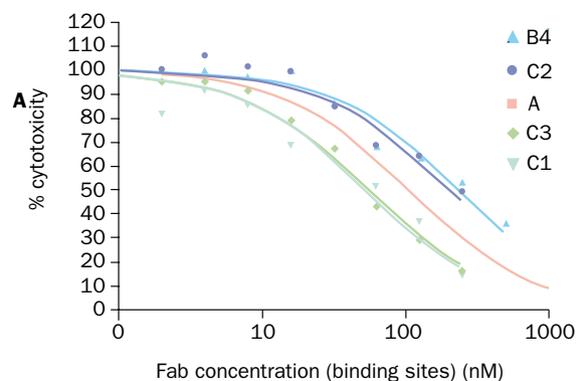
Rank	Highest affinity	Fastest on-rate	Slowest off-rate	Cell assay
1st	C2/C3	C1	C2	C2
2nd	-	B4	C3	C3
3rd	A	C3	A	A
4th	C1	C2	C1	C1
5th	B4	A	B4	B4

Figure 7. Correlation of Fab therapeutic function in cell-based assay with Biacore-derived binding parameters. A) Data representative of a cytotoxicity protection assay. B) Comparative ranking of Fabs based on inhibition of cytoxicity (cell assay) and PcrV-binding properties (Biacore T100 assays).

Variations in Fab binding properties at 25°C and 37°C

Despite the obvious relevance of characterizing proteins intended for therapeutic use at physiological temperature, measurements of biomolecular binding properties are frequently carried out at ambient temperature. To see if assay temperature had any major influence on the assessment of therapeutic candidates, three of the engineered human Fabs were chosen for direct comparison at 37°C and 25°C. As shown in Figure 8, all Fabs displayed similar properties with respect to assay temperature. The k_a values were around 1.5-fold higher at 37°C, with k_d values slightly more temperature-dependent, at around 2-fold higher. As a result, the affinities for Fab-PcrV binding were very similar at the two temperatures, being marginally lower at 37°C due to the slightly larger effect on dissociation rate.
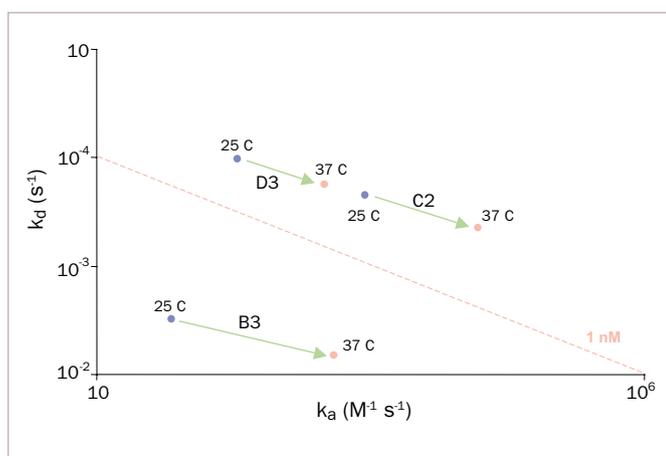

Figure 8. Off/on rate map of Fab binding properties at 25°C and 37°C. Note the difference in scale on the two axes, reflecting the ten-fold higher variation in off-rates seen among the Fabs.

While no major difference in thermodynamic properties were apparent for these Fabs, this predictability cannot simply be assumed when selecting from candidate protein therapeutics. Off-rates, which were the dominant property selected for during Fab development, were twice as fast at 37°C, emphasizing the value of analysis at physiological temperature in order to obtain clinically relevant data.

pH-dependence of Fab affinity is determined by on-rates

Protein therapeutics that show similar properties under closely controlled assay conditions may not necessarily show an equal degree of robustness in varied biochemical/biophysical environments. This could be a consideration when selecting among candidates intended for use in the more complex and variable environment of clinical treatment.

Using the buffer selector of Biacore T100, Fabs could be analyzed using assay buffers with four different pH values within the same run. Fabs C2, D1 and D2 were analyzed at pH 5.5, 6.5, 7.5 and 8.5 and all showed a marked decrease in affinity from pH 5.5 to 7.5, but a minimal change from pH 7.5 to 8.5 (Figure 9). Resolution of these affinity changes into their kinetic components revealed that the effects of pH were predominantly mediated by on-rates, which increased significantly at lower pH. The effects of pH on binding properties were similar for all three Fabs tested, however, indicating that robustness of binding behavior in different biochemical environments was not a relevant selection consideration for the conditions tested.
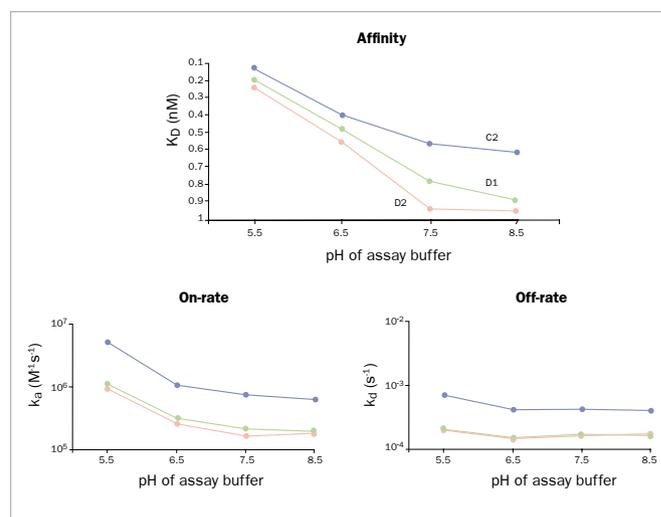

Figure 9. Effect of buffer pH on binding properties of Fabs. Each of the Fabs, C2, D1 and D2 was analyzed in a single run, using assay buffers with four different pHs.

Summary

- Good candidate protein therapeutics were identified for treatment of *P. aeruginosa* infections
  - engineered human Fabs against PcrV with superior affinities to starting mouse antibody
  - affinity maturation of Fabs from high-stringency selection further reduces off-rates

- Biacore T100 was invaluable in the assessment & selection of these protein therapeutic candidates
  - highest quality kinetic data
  - excellent performance at 37°C for physiologically relevant, predictive data
  - excellent correlation of k_d values with therapeutic performance in cell assays
  - assay flexibility for analysis of additional properties (temperature & pH effects)

- The vital role of high-resolution kinetics for characterization and selection of protein therapeutics was demonstrated:
  - discrimination among equal-affinity Fabs based on physiologically relevant binding properties
  - significantly better selection criteria than provided by affinity alone
  - better-informed assessment of the development process (e.g. slower off-rates following maturation of Fabs)

Methods

GST-PcrV and Fabs were provided by KaloBios Pharmaceuticals Inc. (Palo Alto CA, USA). Protein interaction analyses were carried out by David Myszka, Joe Papalia (University of Utah) and Helena Nordin (GE Healthcare), during development of Biacore T100.

GST-PcrV was immobilized by amine coupling to Series S Sensor Chip CM5, using three flow cells at different antigen densities (10, 15 & 30 RU). Main kinetic experiments were run at 37°C, using HBS-EP buffer supplemented with 200 µg/ml BSA. Regeneration of the sensor surface between analysis cycles was achieved using a 1:200 dilution of phosphoric acid. For the temperature and pH experiments, conditions were altered as detailed in Results. For evaluation, data was fit globally to a 1:1 interaction model with mass transport. The basic assay setup is shown in Figure 10.
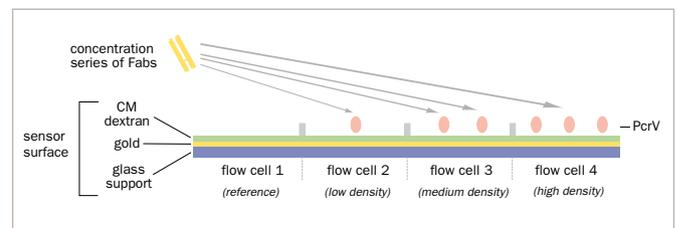

Figure 10. Assay setup for kinetic characterization of Fabs using Biacore T100. The four flow cells are formed by the contact of the sensor chip surface with the microfluidics system of the instrument. Immobilization of PcrV was controlled individually in each flow cell, while Fabs were then injected over all four, using flow cell 1 as a reference.

References

1. Faure, K. et al. Effects of monoclonal anti-PcrV antibody on *Pseudomonas aeruginosa*-induced acute lung injury in a rat model. J. Immune based therapies and vaccines 1, 2 (2003).

2. Frank, D.W, et al. Generation and characterization of a protective monoclonal antibody to *Pseudomonas aeruginosa* PcrV. J. Infect. Dis. 186, 64-73 (2002).

More information about protein interaction analysis can be found at
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GE Healthcare
Biacore AB
Rapsgatan 7
754 50 Uppsala
Sweden

www.biacore.com

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