Research paper

Anti-peptide antibody screening: Selection of high affinity monoclonal reagents by a refined surface plasmon resonance technique

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ABSTRACT

A refined surface plasmon resonance method was developed to measure the kinetics of peptide binding to rabbit monoclonal antibodies (RabMAbs). Optimized amounts of RabMAbs were captured onto sensor chips from hybridoma supernatants followed by binding of free peptides from solution. This allowed kinetic measurement of monovalent interactions of peptides with single antigen binding sites on the antibodies and determination of affinity constants without complications contributed by avidity considerations. Peptide-binding responses were normalized for the amount of antibody present in each sample and a simple interaction model was fit to all of the binding responses simultaneously. As a result, the kinetic rate constants ka and kd, and the affinity constant KD (kd/ka), could be determined for each antibody interaction under identical conditions. Higher-resolution studies involving multiple concentrations of peptide antigens were performed to validate the reliability of single-concentration measurements. By combining data on affinity, activity and concentration, ranking of the antibody-containing supernatants was performed, allowing selection of high quality RabMAbs for binding of peptides in solution.

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1. Introduction

Worldwide, the need for specific antibodies will grow rapidly in the post-genome era as there will be increasing and extensive demand for specific affinity reagents for research on the proteome of a multitude of microbes, plants and animals, including humans (Berglund et al., 2008). Indeed, an initiative to make antibodies to all human proteins has been underway for some time (see the Human Proteomics Organization Human Antibody Initiative; http://www.hupo.org/research/hai/). No particular use for these antibodies has been set in stone, although an application to immunohistochemistry for mapping the expression of human proteins in human tissues, cancer cells and cell lines has been established (see the Human Protein Atlas; http://www.proteinatlas.org/). It is clear however, that antibodies will be required for use in immunoassays other than immunohistochemical staining. In future antibody production projects, great effort will be required to select affinity reagents that are suited for use in specific applications. For example, peptide-binding ligands are already in demand for peptide enrichment in immuno-proteomics methods such as immuno-Matrix Assisted Laser Desorption Ionization (iMALDI; Jiang et al., 2007) and Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA; Anderson et al., 2004a,b) for quantitation of peptides in complex biological materials. In addition, there is a need for antibodies for research on antimicrobial peptides

(host defense peptides) (Mookherjee and Hancock, 2007) and for their quantification in biological fluids. These applications require anti-peptide antibodies for detection and/or enrichment and they must work in the format required for each application.

Even when probes for intact proteins are needed, anti-peptide antibodies are often made, especially when insufficient quantities of natural or recombinant proteins are available for immunization, screening and affinity purification. Since much effort, time and expense are involved in proper characterization of antibodies, it is desirable to ensure that they are of high quality (usually meaning high specificity and affinity) and that there is a large, guaranteed supply. Derivation of monoclonal antibodies solves the supply problem and provides chemically defined, thus highly reproducible, "reagents" that can be selected for their desired specificity, affinity and function. It is not always easy to find monoclonal antibodies with the desired characteristics and often large-scale screening of the supernatants from many different hybridoma clones is required. This problem is exacerbated when measuring anti-peptide antibodies since the assays are not as straightforward as are those using larger antigens. Several methods employing surface plasmon resonance (SPR) for measuring monoclonal antibody binding to protein antigens have been published (Canziani et al., 2004; Safsten et al., 2006). These methods allow screening of substantial numbers of hybridoma supernatants and have been used successfully to select monoclonal reagents with high affinities. However, using similar capture techniques in order to screen antibodies that target small molecules (such as peptides) presents a challenge. Because the magnitude of the signal obtained in SPR assays is proportional to molecular mass, it can be difficult to obtain adequate SPR binding responses when a large discrepancy in size exists between ligand and analyte. When this is the case, it has been the usual practice to immobilize the smaller molecules on the sensor chip surface. Thus when the larger molecules are subsequently injected over the surface as analyte, a strong signal is observed. When examining antibody–peptide interactions, this would entail immobilization of the peptide (small molecule) followed by injection of the antibodies (large molecule). However, to determine antibody affinities, this will not work since multivalent binding of the antibody can occur (increased cooperative binding interactions can occur with bivalent IgG and multivalent IgM) thus measuring avidity, not affinity. Methods allowing affinity determination of anti-peptide monoclonal antibodies (Gomes et al., 2000; Gomes and Andreu, 2002) by SPR have been developed for analysis of a single monoclonal antibody and have not been designed for high throughput screening.

Here we describe a method for screening of multiple hybridoma supernatants for anti-peptide antibodies that allows kinetic analysis and subsequent ranking of antibodies by their affinity, activity and concentration. The method is designed to compensate for different concentrations of antibodies in supernatants and is amenable for use with antibodies from any species either naturally secreted or produced by recombinant DNA techniques. In principle, the method can be applied to any peptide-binding ligand that can be captured on a sensor chip surface by an immobilized capture reagent.

2. Materials and methods

2.1. Peptides

Synthetic tryptic peptides were used throughout and were selected on the basis of parameters important for antibody derivation and behaviour in the mass spectrometer (Anderson et al., 2004a,b). For example, peptides that are unique to a protein and that give strong multiple reaction monitoring (MRM) signals in the mass spectrometer were selected, thus allowing their identification and quantitation by the SISCAPA technique (Anderson et al., 2004b). Although the peptides for this work were chosen as surrogates of biomarkers, any peptide of interest that can be bound by an antibody or any other ligand can be used with the method described here.

Peptides were synthesized by solid-phase methods by either the Chinese Peptide Company (Hangzhou, China) or by the UVic-Genome BC Proteomics Centre (Victoria, BC) and were tested for the correct mass by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry and for purity by high performance liquid chromatography. All peptides were of greater than 80% purity and their quantitation was performed by amino acid analysis. Peptides were first synthesized with C-terminal cysteines for thiol-coupling to keyhole limpet hemocyanin (KLH) carriers for immunization and were also used as antigens in enzyme-linked immunosorbent assays (ELISA) for detecting anti-peptide monoclonal antibodies. The same peptides synthesized without C-terminal cysteines were used in SPR assays for measuring antibody–peptide binding without interference from the linker cysteine. This is an important consideration since we have observed that many monoclonal antibodies and affinity purified polyclonal antibodies bind to the terminal cysteines as part of the recognized peptide epitopes (unpublished data). Ten different tryptic peptides ranging in length from 10–17 amino acids (1062–1855 Da) were synthesized and used for immunization of rabbits and production of monoclonal antibodies. All 10 of these peptides were also used in SPR assays for selection of high affinity antibodies in hybridoma supernatants. Approximately 1000 supernatants were screened in the work reported here. All of the peptides were completely soluble in phosphate-buffered saline, perhaps reflecting their charged nature as tryptic peptides.

2.2. Anti-peptide antibodies

Monoclonal antibodies were produced by Epitomics Inc. (Burlingame, CA) using their proprietary, stabilized rabbit plasmacytoma cell line derived from the original parental myeloma 240-W (1) as the parental myeloma fusion partner. All hybridoma supernatants tested by SPR were first shown to bind the relevant peptides by peptide ELISA using peptides (without carrier) dried onto ELISA plates. The rabbit hybridoma supernatants (usually obtained in small volumes of 400 μL) were stored at 4 °C before use and were never frozen and thawed.

2.3. Equipment and reagents for SPR analysis

Screening of anti-peptide RabMAbs was performed using a Biacore 3000 optical biosensor (Biacore, Uppsala, Sweden).

Research-grade CM5 chips (Order Code BR-1003-99) were used for all experiments and were obtained from Biacore Life Sciences (Piscataway, NJ). For immobilization of capture antibody onto CM5 chips, an Amine Coupling Kit (Biacore; Order Code BR-1000-50) was used, according to the instructions of the manufacturer. Affinity-purified goat anti-rabbit IgG, Fc fragment specific (Jackson ImmunoResearch Laboratories, West Grove PA; Cat No. 111-005-008) was used for capture of rabbit monoclonal antibodies. HBS-EP buffer was purchased from Biacore (Order Code BR-1001-88), carboxymethyl-dextran Sodium salt (CM-dextran) was purchased from Fluka Chemical Corp. (No. 86524, Milwaukee, WI) and BSA Fraction V (No. A-2153) was purchased from Sigma-Aldrich (Oakville, On).

2.4. Immobilization of anti-rabbit IgG capture antibodies

Prior to covalent immobilization of capture antibodies, Biacore CM5 chips were pre-conditioned in order to clean and hydrate the dextran layer by priming three times with HPS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) and then making two 10 s (100 μL/min) injections of each of the following solutions: 100 mM HCl, 50 mM NaOH, and 0.5% SDS. Affinity-purified anti-rabbit IgG (Fc fragment specific) was immobilized via amine coupling using the "Aim for Immobilization" procedure within the Biacore Control Software (version 3.2). The immobilization was performed at 32 °C using running buffer HBS-EP. Capture antibodies on the chip's four flow cells were immobilized separately and an "aim for" level of 20,000 response units (RU) was specified. At a flow rate of 20 μL/min, flow cells were activated with a 7-minute injection of freshly prepared 1/2 dilution of Biacore Coupling buffer (100 mM N-hydroxysuccinimide; 390 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride). Immediately before use, the capture antibody was diluted in 10 mM sodium acetate buffer, pH 5.0 to a final concentration of 75 μg/mL and the solution was centrifuged at 16,000 ×g for 5 min in a microcentrifuge to remove aggregates. The antibody solution was then injected (in repeated short injections) at a 10 μL/min flow rate until the chip was completely saturated. Finally, an injection of 1.0 M ethanolamine (pH 8.5) at a flow rate of 20 μL/min for 4 min was used to deactivate the surface and wash away any non-covalently bound capture antibody. Using this method, approximately 13000–16000 RU of capture antibody were immobilized on each of the four flow cells. When not being used, CM5 sensor chip supports were removed from their cassette and stored wet at 4 °C in a 50 mL polypropylene conical tube containing HBS-P buffer with 0.05% sodium azide added as preservative.

2.5. Capture of monoclonal antibodies and development of a "Smart Capture Method"

Capture of monoclonal antibodies from hybridoma supernatants was performed at 25 °C and data were collected at a rate of 1.0 points/s. The screening buffer (HBS, 1 mg/mL BSA, 1 mg/mL CM-dextran salt, 0.005% v/v surfactant P20) was prepared immediately before use from a 10×HBS stock (0.1 M HEPES, 1.5 M NaCl) by diluting in distilled H$_2$O. The CM-dextran salt was then added to a final concentration of 1 mg/mL and the pH adjusted to 7.4 before degassing for 1 h

using a vacuum pump. BSA was added from a 50 mg/mL stock to bring the final concentration in the buffer solution to 1 mg/mL and P20 surfactant from a 10% stock to bring the final concentration to 0.005%. Importantly, both BSA and P20 surfactant were stirred gently into solution to minimize the reintroduction of air. Finally the screening buffer was gravity-filtered through a 0.22 μm bottle-top filter. Two separate RabMAb-containing hybridoma supernatants were screened during each binding cycle, one on flow cell 2 (FC 2) and one on FC 4, with the other two flow cells functioning as reference surfaces used to control for non-specific binding, instrument artifacts and drift. Prior to injection, all RabMAb supernatants were diluted 1/2 in screening buffer and centrifuged at 16,000 ×g for 5 min to remove aggregates. Screening of supernatants was performed in one of two ways, depending on whether or not the concentration of RabMAbs was known (see 2.5.1 and 2.5.2 below). Antibody concentrations in supernatants are not usually known but anti-peptide antibody screening using SPR is simplified with known mAb concentrations.

2.5.1. Screening of supernatants containing known mAb concentrations

Using the Biacore 3000 Control Software, a customized application wizard was used to carry out RabMAb screening. At the beginning of each cycle a sample of screening buffer was injected using INJECT (30 μL/min, 2-min injection) across all four flow cells in order to monitor baseline stability and to ensure that any bulk refractive index changes observed were consistent between the four flow cells. For antibody capture, each supernatant was injected using INJECT at 10 μL/min for 1 to 14 min depending on the concentration of the RabMAbs in the supernatant, in order to achieve a target capture level of approximately 1600 RU. An EXTRACLEAN command was executed for each antibody injection in order to minimize carryover.

2.5.2. Screening of supernatants containing unknown mAb concentrations

To screen supernatants with unknown concentrations we developed a new "Smart Capture Method" (SCM) to capture optimal levels of RabMAbs in order to maximize the quality of peptide-binding curves and screening throughput. SCM uses conditional command blocks during antibody injection and accomplishes several objectives: 1) eliminates the need for an additional "scouting" run to determine antibody concentration, 2) assures consistent antibody levels, thereby obtaining better saturation kinetics when screening with a fixed concentration of peptide and 3) eliminates the screening of undesirable supernatants (that contain low antibody levels) by moving to the next cycle as soon as they are detected. Briefly, the SCM involves a programmed set of conditional steps as follows: first, a 1 min injection of supernatant is made in order to determine the effective-concentration of RabMAb in the supernatant. Based on the level of bound antibody, the supernatant is categorized as level A, B, C, D, or "low concentration". Category A is defined as a high concentration supernatant where more than 1600 RU of antibody is bound during the effective-concentration injection and the cycle progresses to the next stage. "Low concentration" supernatants are those in which less than 200 RU of antibody are

bound during the 1 min injection. If the supernatants being screened on FC 2 and 4 are both found to be "low concentration", the flow cells are immediately regenerated, no blank or peptide injection is made and the run is advanced to the next cycle. Categories B, C, and D are intermediate-concentration supernatants where between 200 RU and 1600 RU of antibody are bound during the effective-concentration injection. These supernatants are subsequently re-injected for between 1 and 11 min (this range may need to be varied for different mAbs) to bring the capture level up to the desired target level of 1600 RU. This method requires that all supernatants be present in volumes required for the maximum length injection (approximately 200 μL of diluted supernatant). It is important to note that supernatants, which fall into the "low concentration" category, are required in large volumes in order to capture the desired amount of RabMAb. For this reason, these supernatants are screened at a later time to keep the required starting volume of all supernatants low. If the "low concentration" supernatants were injected in the volume required to capture the target level of 1600 RU using the SCM, an unacceptably large volume of the higher density supernatants would be left unused and thus wasted. Each user must decide how many supernatants they want to screen and how the concentration of antibody and availability of supernatants affects their experimental design.

2.6. Peptide capture

Following antibody capture on flow cells 2 and 4, all flow cells were cleaned with several needle washes, IFC washes and buffer injections in order to optimize baseline stability. After the cleaning steps, a "blank" injection of screening buffer (using the same conditions to be used for the peptide injection; KINJECT, 2-min injection, 10-min dissociation at a flow rate of 30 μL/min) was made over all antibody and control surfaces for double-referencing purposes. Peptide antigens were diluted to an empirically determined concentration within the range of 30 to 250 nM in screening buffer and the solutions were centrifuged at 16,000 ×g for 5 min prior to injection. Peptides were injected at 30 μL/min over all antibody and reference surfaces using KINJECT (2-min injection, 10-min dissociation).

2.7. Regeneration of flow cells

Flow cells were regenerated together using conditional command blocks in order to ensure accurate regeneration and to eliminate carry-over into subsequent cycles. At the end of each cycle all surfaces were regenerated using one 30-second (30 μL/min) injection of "regeneration solution" (10 mM glycine/0.1% P20 surfactant, pH 1.7) and then up to 10 conditional injections of the same acidic solution. In detail: a 30 second injection of regeneration solution is made using INJECT and a report point called "Regen1" is inserted 50 s after the injection is completed. A conditional command is then executed where a subsequent identical injection is made if the "absolute response" at the previously mentioned report point on flow cell 2 or flow cell 4 is greater than +15 RU relative to the "absolute response" at a BASELINE report point on flow cell 2 or flow cell 4 inserted at the beginning of the cycle before antibody injection. If this conditional command

is executed, a new report point, called "Regen2", is inserted 50 s after the injection is completed. Following this report point, but still within the conditional command block, is a nested conditional command identical to the first, except that instead of inserting a "Regen2" report point it inserts a "Regen3" report point. By progressively nesting 7 additional conditional command blocks in the same way, each with its own unique report point, the chip will ultimately receive between 1 and 10 injections to return it to its original baseline level. It is important to note that only rarely are more than two acid injections needed to regenerate the chip, but when carrying out a multi-cycle analysis it is comforting to know that measures are in place to minimize carry-over, a concern more pronounced with the high capture levels of RabMAbs necessary for our method. Following regeneration, screening buffer is pumped through all four flow cells at a rate of 30 μL/ min and the surfaces are allowed to equilibrate for 8 min using a WAIT command to allow all flow cells to stabilize before continuing to the next cycle. Although this at first appears to be a cumbersome process, it ultimately saves time and increases accuracy during screening of multiple peptide-binding ligands.

2.8. Analysis of peptide-binding data

In order to rank the anti-peptide RabMAbs in terms of their binding kinetics, SPR data were first processed using Scrubber software (version 2.0, BioLogic Software, Campbell, Australia). In Scrubber, data from the reference cell were subtracted from binding data in order to remove instrument artifacts, drift, and non-specific binding. The referenced "blank" and peptide-binding response data were then zeroed on the y-axis, and aligned on the x-axis. These data were next imported into BIAEval software (version 4.1, Biacore Ab, Sweden) for further processing and kinetic modeling. Any artifacts due to the dissociation of captured RabMAb were subtracted from peptide-binding data by subtracting the "blank" response from the peptide-binding response to double-reference the data. These responses were then normalized by dividing each double-referenced antigen response curve by the level of antibody captured from the supernatant, which was measured 5 s prior to peptide injection for each antibody screened. RabMAb-peptide binding responses which were significantly above background (>0.004 normalized RU) were then fit locally using a 1:1 Langmuir binding model in order to determine kinetic rate constants. The affinity constant (KD) was calculated from the rate constants ka and kd.

2.9. Kinetic ranking of RabMAbs

To choose those antibodies deemed most useful for peptide capture from solution, presumably those with highest affinities and more specifically, the lowest off-rates, RabMAbs were ranked based on the following criteria: 1) affinity (KD), 2) activity (based on % of theoretical binding capacity) and 3) antibody concentration in the hybridoma supernatants. When the KDs were similar, those RabMAbs with higher activity were ranked higher. When the KDs and activities of RabMAbs were similar, those at higher concentrations were ranked higher since the hybridoma clone will usually continue to produce more antibody, a serious consideration if

that antibody is selected for further use. It is important to note that RabMAbs with relatively high activities were required for our purposes and in general antibodies that were found to have low activities (<30% theoretical activity) were eliminated from the selection process even if they had high affinity.

2.10. Multiple concentration analysis of selected mAbs

Several monoclonal antibodies were analyzed using a series of different antigen (peptide) concentrations for high-resolution kinetic analysis. In this way, we were able to assess whether the values obtained were similar to those originally determined by single pass, single concentration screening. Each selected antibody supernatant was diluted 1/2 in a volume of screening buffer sufficient to reach the targeted capture level of approximately 1600 RU in each cycle and centrifuged at 16,000 ×g for 5 min prior to injection. Peptides were serially diluted in screening buffer (usually in the range of 1 nM to 125 nM) and centrifuged at 16,000 ×g for 5 min immediately prior to use. Cycles for high-resolution analysis were identical to those for single-concentration screening, except the antibody was kept constant while the peptide concentration was varied. Buffer (0 nM peptide) injections were also interspersed for the purpose of double referencing. Each data set was processed in the same way as the single-concentration screening data, and fit globally using a 1:1 Langmuir binding model in order to determine rate constants.

3. Results and discussion

3.1. Strategy for ranking anti-peptide monoclonal antibodies

Our primary goal was to develop a method for kinetic analysis and utility ranking of a large number of peptide-binding monoclonal antibodies. The method was designed to allow screening and selection of antibodies of sufficiently high affinity and activity for binding peptides from solution. As a model system we developed the method using rabbit monoclonal antibodies since they tend to be, on average, of much higher affinity than the more commonly used monoclonal antibodies from rats and mice (Rader et al., 2000; Rossi et al., 2005; Mage et al., 2006). Thus, a strategy employing SPR was devised that allows universal capture of RabMAbs from hybridoma supernatants, followed by kinetic measurement of peptide binding to the captured RabMAbs. Since the peptides we use are small and in most cases likely comprise a single epitope, each antigen binding site on an antibody will only bind one peptide, allowing measurement of antibody affinities (i.e. binding of a single epitope to an antigen binding site), without avidity considerations contributed by multivalent binding. This strategy and method of true affinity ranking can be used for antibodies from any animal species and theoretically can be used for any peptide-binding ligand. A schematic overview of the method is shown in Fig. 1.

3.2. Selection and immobilization of capture antibodies

It is important that the ligand chosen for covalent attachment to the Biacore CM5 chip binds the captured antibody strongly to minimize the dissociation of the captured antibody during subsequent peptide binding and washing steps.
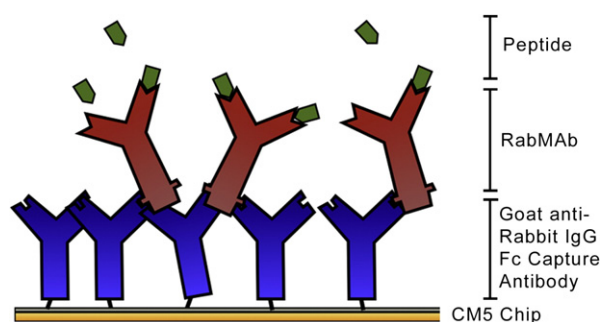
Fig. 1. Schematic overview of the SPR method for kinetic analysis of anti-peptide antibodies. Step 1. Selected, high affinity goat anti-rabbit IgG (Fc fragment specific) capture antibodies (illustrated in blue) are immobilized on the CM5 chip via amine coupling. Any capture ligand of sufficiently high affinity can be used here, thus allowing capture of antibodies from other species. Step 2. RabMAbs (illustrated in red) from hybridoma supernatants are captured by their Fc regions in the correct orientation for peptide antigen binding. Step 3. Peptides (illustrated in green) are bound to a single (univalent) antigen binding site of an antibody, thus allowing affinity measurements. After the kinetic analysis is completed for a given RabMAb, the chip is regenerated using an acid solution to dissociate the RabMAb and peptide, leaving the capture surface intact and ready for capture of the next RabMAb-containing hybridoma supernatant to be screened. This method depends on selection and covalent immobilization of a first capture reagent that has a very high affinity, primarily a low dissociation constant, since we are interested in measuring the kinetics of peptide-binding and release without error introduced by dissociation of the anti-peptide antibody itself. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, selection of high quality, affinity purified antibodies with low dissociation constants is crucial. In the model system reported here, we measured the binding kinetics of a selected affinity-purified anti-rabbit IgG (Fc fragment specific) to several RabMAbs. An example of a multi-concentration analysis with one RabMAb is shown in Fig. 2. Off rates (kd) in the range of 10^{-6} to 10^{-7} s^{-1} were observed (see discussion in "3.6 Data processing"). In contrast, the off-rates observed for RabMAb-peptide binding were always much faster, in the range of 10^{-3} to 10^{-5} s^{-1}. The slow off-rates observed with the captured RabMAbs can perhaps be explained by a combination of high affinity, and especially avidity, of the capture antibody since multiple epitopes on the Fc region of the RabMAbs are available for binding.

The low molecular mass of the peptides was the determining factor when calculating how much anti-rabbit capture antibody needed to be immobilized in order to have the capacity to bind a sufficient amount of peptide for kinetic modeling. We aimed to capture enough RabMAb to bind approximately 20 to 50 RU of peptide. We applied the Biacore standard analyte-binding capacity equation to our antibody–peptide capture method:

$$R_{max}(RU) = \left(\frac{\text{Peptide MW}}{\text{RabMAb MW}} \right) \times \text{Captured RabMAb(RU)} \times \text{Stoichiometric ratio*}$$
(*in this case, the stoichiometric ratio is 2 since the antibodies are bivalent)

Based on this equation, the required level of captured antibody can be calculated to be in the range of approximately 1100 RU to 2500 RU for a peptide of 1400 Da molecular mass. To capture this much RabMAb within a reasonable time frame, we empirically determined that immobilization levels of the covalently coupled anti-rabbit IgG needed to be greater than
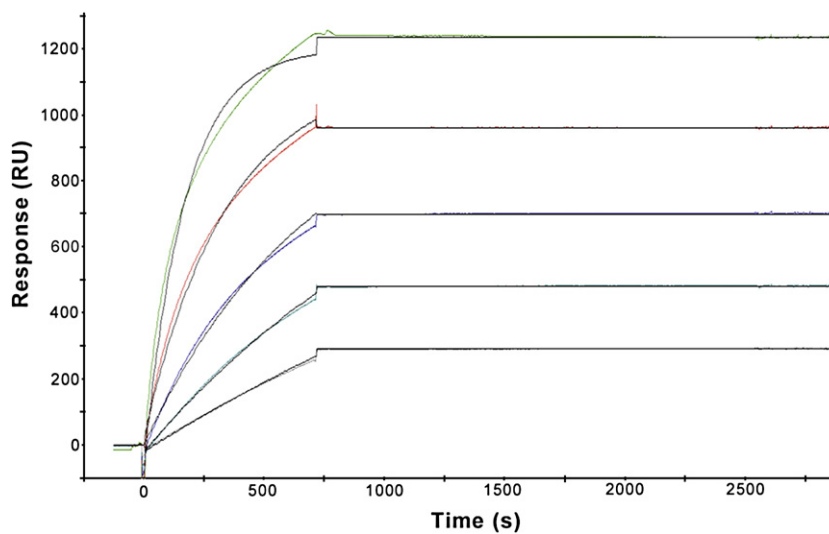
Fig. 2. Kinetic characterization of capture antibody. Kinetic rate constants of the selected goat anti-rabbit IgG (Fc) capture antibody that we used throughout this work were accurately determined in a multi-concentration analysis using a representative RabMAb. Five different concentrations of RabMAb (0.42 μM to 6.7 μM) along with a blank for double-referencing purposes, were injected over the capture surface for 2 min at a flow rate of 10 μL/min, and allowed to dissociate for 40 min. The referenced data were then fit globally using a 1:1 Langmuir binding model.

12,000 RU. Presumably, a high density capture surface is required to capture 1100–2500 RU of RabMAb due to the random orientation of the matrix-bound capture reagent which leaves only a fraction available for binding. Alternatively, it is possible that only a fraction of the commercial capture antibody is active. Since the quality of many commercial antibodies is often unknown it is important that several be tested empirically before settling on one for larger-scale use.

Several strategies were employed to obtain a dense capture antibody surface. First, we raised the biosensor's temperature to 32 °C for the immobilization procedure to increase the rate of the amine-coupling reaction. We also "scouted" different concentrations of the capture antibody and pH's of the coupling reaction and found that maximum binding of our batch of selected affinity purified anti-rabbit IgG was achieved using 75 μg/mL at a pH of 5.0, in 10 mM acetate buffer. Centrifugation of the diluted capture antibody at 16,000 ×g for 5 min immediately prior to immobilization ensured an aggregate-free antibody and resulted in higher immobilization levels. The "Aim for Immobilization" wizard (Biacore software) was used because aiming for a higher than expected level of binding ensured surface saturation and was found to give consistent levels of binding across flow cells. Our bound ligand levels ranged from 13,000 to 16,000 RU (mean= 14475.15 RU, $n=8$), with a variation ranging from 0.65% to 5.89% (mean=2.63%, $n=8$) between the flow cells on a given chip. We observed that immobilizing all flow cells at once with a single injection of capture antibody resulted in a gradual decrease in bound level from Fc1 to Fc4 (data not shown) whereas when each flow cell was immobilized separately and directly, uniform levels of bound ligand were achieved.

### 3.3. Anti-peptide antibody capture considerations

Given that a fixed concentration of peptide is injected over different RabMAbs, it is important to capture similar amounts of functional antibodies from each RabMAb supernatant to permit the resolution and comparative ranking of peptide binding. If the levels of captured RabMAbs are too low, then the peptide can saturate the RabMAb too quickly, leading to a steep saturation curve; consequently, not enough data are obtained during the saturation phase for accurate kinetic modeling. In contrast, if too much RabMAb is captured, the injected peptide may not saturate the abundant antibody, thus giving a linear peptide-binding response and uninformative data for kinetic characterization. In addition, when peptide-binding data are sub-optimal, the entire cycle needs to be repeated, thereby wasting time and reagents and compromising the effective lifespan of the sensor chip. For these reasons we developed a new Smart Capture Method (SCM) to capture consistent levels of each RabMAb, leading to increased throughput, and higher-quality peptide-binding data. A schematic overview of the SPR workflow, including a representation of the Smart Capture Method is shown in Fig. 3.

We empirically determined that diluting hybridoma supernatants 1/2 in screening buffer allows enough RabMAb to be captured within a reasonable time frame (1 to 14 min). Undiluted hybridoma supernatants were not used in order to reduce microfluidic contamination, which may arise over time due to the high protein concentration of fetal bovine serum present in the tissue culture supernatants. Using a SPR capture method with a much larger antigen (65 kDa), murine hybridoma supernatants were optimally diluted 1/10 or 1/20 (Myszka, 1999). The higher concentration of supernatant required for RabMAb binding can be explained by the fact that rabbit hybridomas typically secrete fairly low amounts of monoclonal antibody (0.2 μg/mL to 5 μg/mL; Spieker-Polet et al., 1995) when compared to mouse hybridomas (1–100 μg/ mL; Peterson and Peavey, 1998).

3.4. Antigen response optimization

We have often observed that both affinity purified poly-clonal antibodies and monoclonal antibodies bind to linker
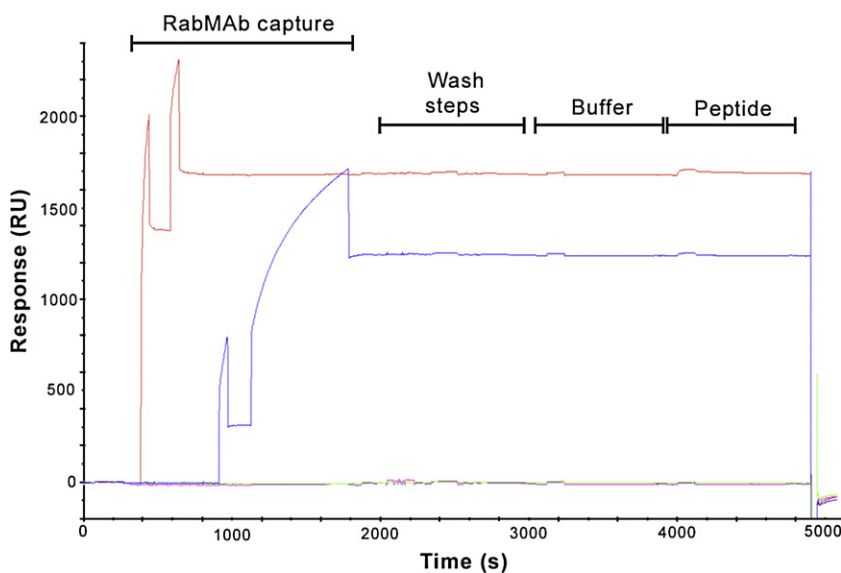
Fig. 3. Overview of a typical anti-peptide RabMAb screening cycle, showing the Smart Capture Method (SCM), blank injection, peptide injection and flow cell regeneration. Data from all four flow cells (two sample cells – red and blue lines, and two reference cells – pink and green lines) are shown. In this particular cycle, both a high concentration (red line) and a lower concentration (blue line) RabMAb supernatant were screened. It can be seen that during the one minute effective-concentration injection a greater amount of antibody from the high concentration supernatant binds relative to the antibody from the lower concentration supernatant. The supernatant containing a higher concentration of RabMAb was categorized as "Density A" and was injected for 1 min, while the supernatant containing less RabMAb was categorized as "Density C" and was injected for 11 min. Consequently, both flow cells have captured similar levels of RabMAb. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

moieties, including the terminal cysteines used to couple peptides to carriers for immunizations. Thus it is important to stress that in the work reported here, peptides without C-terminal cysteines were used in SPR assays for measuring antibody-peptide binding. This ensures that the SPR results obtained truly reflect the correct antibody-peptide affinities, without influence of the artificially added C-terminal cysteines.

As discussed in Section 3.3, an ideal peptide-binding response must have enough curvature, as it approaches steady state, in order to provide useful information about the reaction kinetics of an antigen–antibody interaction. For each peptide, an appropriate rate of association, and thus response curvature, was empirically determined. We typically used a 125 nM peptide solution as a starting point and adjusted it as necessary (usually between 30 nM and 250 nM)
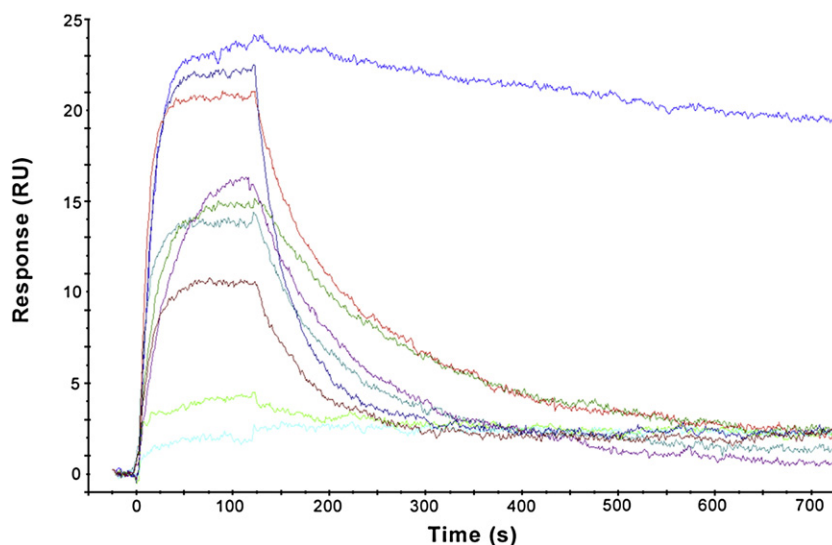
Fig. 4. Peptide-binding data from a representative set of rabbit monoclonal antibodies. Representative raw peptide response data overlaid to highlight the variety of responses observed during RabMAb screening. A 40 nM solution of a selected peptide was injected over nine different captured RabMAbs. The single-referenced data indicate differences in on- and off-rates but must ultimately be processed further to allow more accurate ranking.
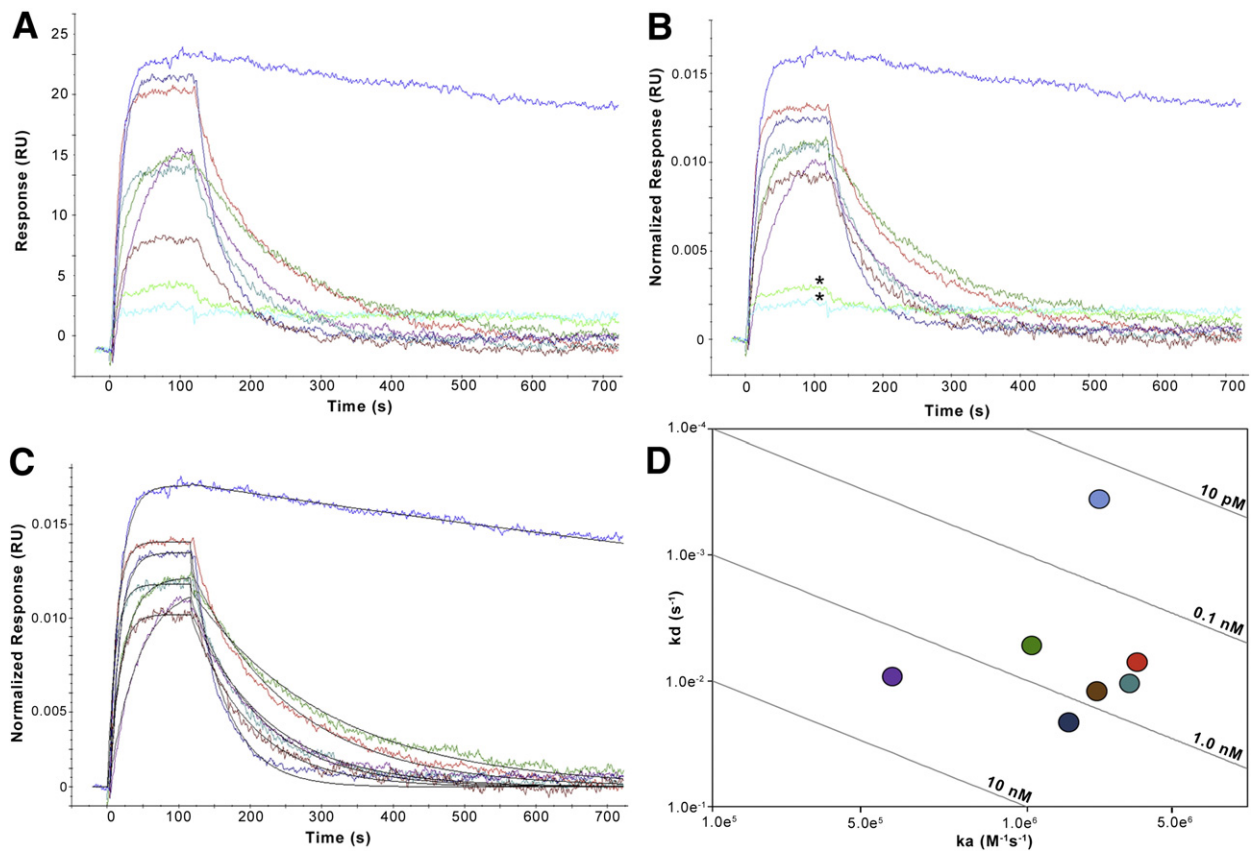
Fig. 5. Processing and ranking of peptide-binding data. Processing of peptide response data from RabMAb screening was performed in order to accurately rank RabMAbs captured from different supernatants. The peptide-binding data from Fig. 4 are carried forth here. A) The single-referenced data were double-referenced by subtracting the response of a blank injection. This eliminates any signal contribution made by the slow dissociation of captured RabMAb during the peptide-binding phase of the screening cycle. B) Double-referenced data were then normalized to account for differences in captured RabMAb levels, thus allowing the direct comparison of peptide-binding responses. Low responders (<0.004 normalized RU) were eliminated at this stage (marked with asterisks). C) Normalized data (coloured lines) were then fit locally using a 1:1 interaction model (black lines). Resulting values for ka and kd were then used for ranking purposes. D) Ranking is displayed as a kinetic distribution plot of ka versus kd. Diagonal lines represent affinity isotherms. Higher affinity RabMAbs are displayed closer to the upper-right hand corner of the plot (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

for each peptide. Peptide-binding responses were in the range of 6 to 40 RU. Representative examples of such responses are shown in Fig. 4. In order to obtain accurate dissociation constant (kd) measurements, we monitored the dissociation phase of the interaction for 10 min.

3.5. Optimization of flow cell regeneration

To prevent antibody carryover effects from cycle to cycle, it is important to regenerate each flow cell to its original baseline level at the end of each cycle. If flow cells are not sufficiently regenerated it is possible that erroneous kinetic data will be collected during subsequent cycles. Errors could be introduced by additive effects from any antibody that is carried over or by inhibition of antibody capture in the next cycle due to a fraction of the capture antibody being blocked by the RabMAb from the previous cycle. Conversely, if flow cells are regenerated too vigorously then the life of the chip may be compromised and/or the immobilized capture antibody may become less active. With these considerations in mind, we found that the best way to regenerate flow cells back to baseline levels at the end of each cycle was with a

10 mM glycine pH 1.7+0.1% P20 surfactant regeneration solution and by using the "conditional method" outlined above in Section 2.7. When using this conditional method the timing of the report point following the regeneration injection is an important consideration. Following a low-pH acid injection, the flow cell's signal will drift until it returns to equilibrium. With this in mind it may be necessary to empirically determine how long it takes to reach equilibrium, and to ensure that the report point is inserted after this time period.

3.6. Data processing

While peptide-binding curves must be single-referenced by subtracting reference cell data to eliminate instrument drift, instrument artifacts and non-specific binding, it is also necessary to double-reference the screening data since this method involves two independent and different capture events. To account for the dissociation of antibody from the capture ligand, we double-referenced our peptide response data by subtracting the association and dissociation phases of the "blank" injection (see Fig. 5A). Since RabMAb dissociation

from the capture surface would occur at a constant rate, independent of whether or not peptide is present, we placed the "blank" response used for double-referencing immediately prior to the peptide capture response in order to accurately represent the formation and decay of the peptide–RabMAb complex alone. The omission of this double-referencing step would produce kinetic data reflecting the sum of two separate interactions (i.e. capture ligand–RabMAb and RabMAb–peptide) and thus would not be a true representation of the interaction of interest (RabMAb–peptide). It follows that the capture ligand must hold on to the captured RabMAb with sufficient strength, through a combination of affinity and avidity, to enable the resolution of peptide dissociation.

Although the Smart Capture Method minimizes the differences in levels of different RabMAbs captured, in order to accurately rank the RabMAbs in terms of their activities all peptide-binding responses must still be normalized. Normalizing the responses permits direct comparison between RabMAbs, indicating precisely how much peptide is bound for a given level of captured RabMAb (see Fig. 5B). Comparing these responses highlights the differences in individual peptide-binding capacities (Rmax) in a population of RabMAbs. Experimental Rmax values, as determined by kinetic modeling, allow the calculation of RabMAb activity:

$$\text{Activity} (\%) = \left(\frac{\text{Experimental Rmax}}{\text{Theoretical Rmax}} \right) \times 100$$

Aside from being a key criterion in selecting antibodies, binding activity is also useful information to have when working with antibodies that have been subjected to purification techniques or storage conditions that may inactivate a significant portion of the antibody population.

For affinity determination, normalized peptide-binding responses were fit using a 1:1 Langmuir binding model (see Fig. 5C). Applying the model to the peptide-binding responses of each RabMAb individually (i.e. fitting a group of curves locally) gave good fits and allowed the measurement of kinetic rate constants (ka, kd) and individual binding capacities for each RabMAb. Kinetic rate constants derived from these fits were then used in RabMAb ranking (see Fig. 5D).

3.7. Multiple-concentration analysis of selected RabMAbs

After ranking of RabMAbs, a more detailed kinetic analysis was performed on selected supernatants in order to determine their kinetic parameters with more accuracy and to confirm that our single pass values were reliable. Thus, SPR measurement of peptide binding was performed using multiple peptide (antigen) concentrations (Fig. 6). The results obtained with three representative RabMAbs showed that the initial "single pass" affinity determinations at a single peptide concentration were clearly accurate when compared with the multiple antigen concentrations (Table 1).

3.8. Ranking of mAbs as peptide binding reagents

RabMAb–peptide binding responses were ranked based on three considerations. In order of importance, these factors were: affinity, activity and concentration in the hybridoma supernatants. Affinity (KD) was considered first because we were interested in choosing antibodies that bound strongest to peptides in solution. In choosing useful affinity reagents, we desired a fast on-rate to ensure rapid binding of peptides in solution but considered off-rate even more important since methods of particular interest to us such as iMALDI) (Jiang et al., 2007) and SISCAPA (Anderson et al., 2004a,b) demand very stable antibody–peptide antigen complexes to be formed. Antibody activity was also important because we wanted to ensure that maximum levels of peptides would be bound by a fixed amount of the capture reagent. Finally, the concentration of antibody in hybridoma supernatants was considered. As a rough indication of high-secreting clones, this criterion is important for future production of large amounts of selected antibody reagents. The compilation of ranking characteristics of some selected RabMAbs is shown in Table 1.

### 3.9. Antibody screening throughput, CM5 chip life and Biacore maintenance considerations

When screening multiple hybridoma supernatants, throughput is an important consideration. Using the technique outlined above on a Biacore 3000, we were able to screen approximately 40 hybridoma supernatants per day when using two flow cells as reference surfaces and up to 60 when using one flow cell as reference surface. Using a more advanced system such as a Biacore A100, which has 5 "spots" on each of the four flow cells of its Series S CM5 chips, it is possible to screen eight antibodies per cycle with each having a dedicated reference surface, allowing screening of 386 supernatants in a single 12 h run (Safsten et al., 2006).

We have found that signal quality from our CM5 chips starts to deteriorate after approximately 70 to 80 cycles. If using two reference surfaces per chip, it should be possible to screen 160 supernatants per chip, or up to 240 supernatants per chip if using only one surface as reference. With rabbit monoclonal antibodies our experience has demonstrated that these numbers are more than sufficient to obtain several antibodies of at least nanomolar affinity.

Our experience shows that it is vital to keep the microfluidics of the Biacore 3000 as clean as possible in order to obtain high-quality peptide-binding curves for kinetic analysis. In addition to the regular maintenance recommended by Biacore, we have found it necessary to perform weekly "sanitize" procedures when screening hybridoma supernatants. Additionally we perform monthly "super clean" and/or "super desorb" (Navritilova et al., 2007) procedures. The importance of regular cleaning and preventative maintenance when using a Biacore 3000 cannot be stressed enough, especially when regularly capturing antibodies from relatively large volumes of hybridoma supernatants and when measuring the binding of small ligands, such as peptides, that give SPR signals of low magnitude.

4. Conclusions

We set out to develop a method for measuring the kinetics of antibody–peptide interactions to allow selection of high affinity peptide-binding reagents. As peptide-binding ligands
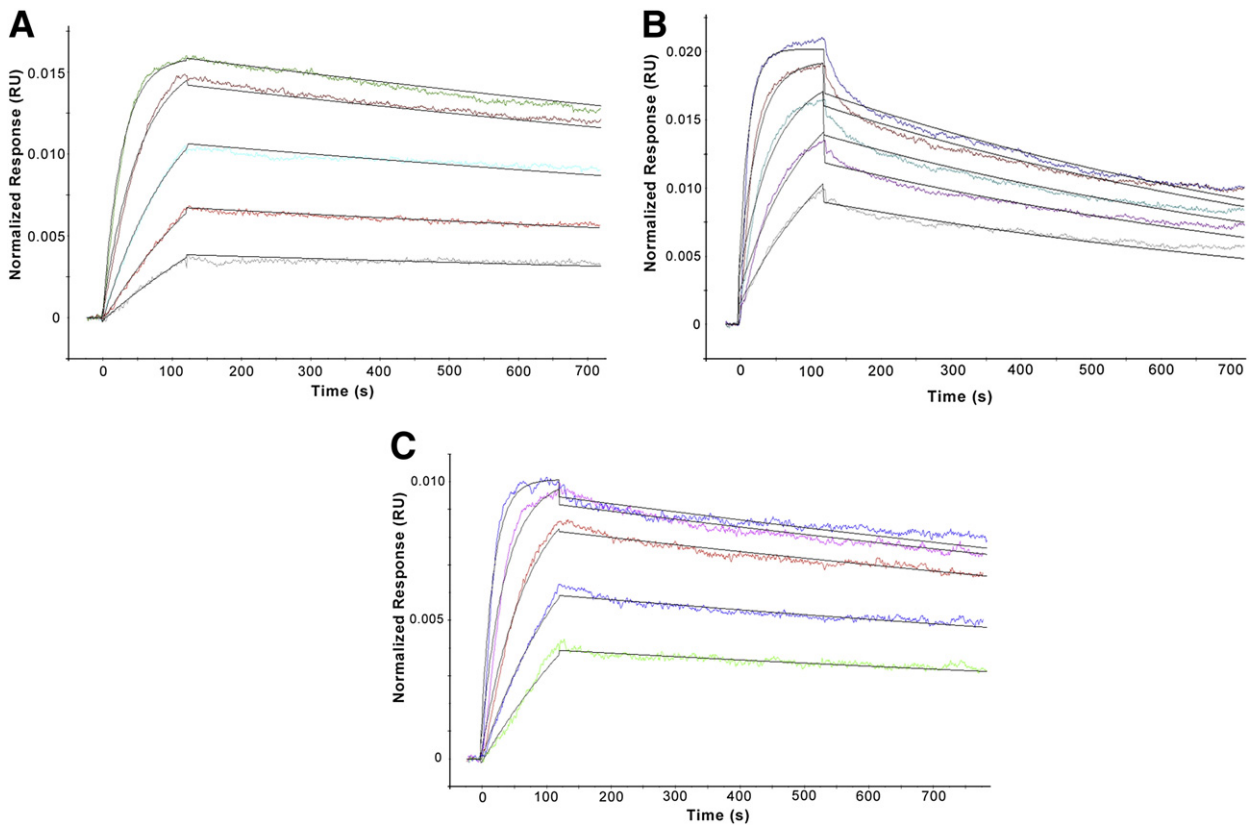
Fig. 6. Representative multiple-concentration analyses of peptide-binding kinetics of two selected RabMAbs. Normalized responses (coloured lines) were fit globally (black lines) using a 1:1 interaction model. A) Peptide 1 was injected over captured RabMAb 30 at 0, 1.63, 3.25, 7.5, 15, and 30 ηM. B) Peptide 2 was injected over captured RabMAb 108 at 0, 6.25, 12.5, 25, 50 and 100 nM. C) Peptide 3 was injected over captured RabMab 58 at 0, 4.7, 9.4, 18.8, 37.5, and 75 nM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we chose to use rabbit monoclonal antibodies (RabMAbs) since they are reported to be of higher average affinity than antibodies from other species (Rader et al., 2000; Rossi et al., 2005; Mage et al., 2006). Monoclonal antibodies are chemically pure glycoproteins so they can be truly considered as reagents, whereas, antisera or polyclonal antibodies, which are not chemically defined, cannot be exactly reproduced and thus may not be considered as reagents. To allow high throughput screening of peptide binding and to determine affinity constants it was necessary to first capture consistent and large amounts of RabMAbs from hybridoma supernatants in the correct orientation to allow monovalent binding of peptides from solution. This was accomplished using an affinity-purified, high-affinity/high-avidity antibody for specific capture of RabMAbs from culture supernatants. The off-rates for the captured antibodies were much lower than off-rates for captured peptides thus allowing accurate measurement of anti-peptide binding kinetics. For selection of quality antibodies, the peptide off-rate is of particular importance since this determines how long the antibody-peptide-complexes can be washed before appreciable losses occur. Peptide binding methods such as iMALDI and SISCAPA frequently involve 15 min of wash steps prior to analysis of bound peptides by mass spectrometry, hence at a minimum, peptide off-rates of less than 1.11×10^{-3} s^{-1} (corresponding to an off-time of 15 min) are necessary, unless washing can be substantially accelerated. The method we have developed clearly allows ranking of antibodies based on off-rate. In addition, peptide-binding activity and effective antibody concentration were measured by our optimized SPR method, thus allowing ranking and selection of the antibody reagents most useful for binding of peptides from solution.

Table 1
Kinetic analysis of RabMAb–peptide interactions

RabMAb	RabMAb target	ka (M^{-1}s^{-1})		kd (s^{-1})		KD (ηM)		Activity [a] (% theoretical)
		Single [Ag]	Six [Ag]	Single [Ag]	Six [Ag]	Single [Ag]	Six [Ag]	
30	Peptide 1	1.69E^6	1.2E^6	3.59E^{-4}	3.39E^{-4}	0.21	0.28	68.1
108	Peptide 2	7.46E^5	7.31E^5	2.65E^{-3}	1.03E^{-3}	1.04	1.41	97.0
58	Peptide 3	6.0E^5	6.97E^5	3.55^{-4}	3.28E^{-4}	0.59	0.47	91.4

[a] Activity based on single [Ag] runs.

Acknowledgments

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