

Selection of binders from phage displayed antibody libraries using the BIAcore™ biosensor

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Abstract

In this report we show that phage displayed antibodies can be selected based on dissociation rate constants, using a BIAcore™ biosensor. To demonstrate the principle, two Fab phage stocks displaying antibodies specific for hen egg lysozyme or phenylloxazone were mixed in a ratio of 1 : 10 and injected over the biosensor chip containing immobilized lysozyme. Antigen-specific bound phages were eluted and analysed for specificity and phage titer. This procedure enriched for phages carrying specific antibodies. Selection of high affinity binders from phage libraries was then demonstrated with the BIAcore™ when phages were eluted and collected at different time points. Soluble antibody fragments were subsequently expressed and their kinetic parameters were determined. The time of elution was directly proportional to the affinity, due to decreased dissociation rate constants. This procedure offers a rapid and simple approach for selecting binders from phage libraries differing in antibody dissociation rate constants.

Keywords: Phage display; Selection; BIAcore™; Dissociation rate constant

1. Introduction

Expression of antibody fragments on the surface of filamentous phages (Parmley and Smith, 1988; McCafferty et al., 1990) greatly facilitates the selection of specific binders from a large pool of different antibody specificities. The source of V-regions can be derived either from immunized (Burton et al., 1991) or non-immunized individuals (Marks et al., 1991), the latter providing so called naive antibody libraries. The selection is most commonly performed

by panning (Barbas III et al., 1991), although selection using magnetic beads has distinct advantages (Schier et al., 1996). Recently, a novel concept in phage selection was achieved by linking antigenic recognition and phage replication (Dueñas and Borrebaeck, 1994)

It has been possible to selectively isolate binders based on their kinetic behavior (Hawkins et al., 1992; Dueñas et al., 1996b) and we report here on the use of the BIAcore™ biosensor (Jönsson et al., 1991) for selection of phage displayed antibodies, based on binding kinetics. BIAcore™ is a surface plasmon resonance based system (Liedberg et al., 1983) for analysing biospecific interactions. Thus, one interacting molecule is immobilized to a sensor-

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chip and its counterpart is injected into a continuous buffer flow. The interaction is monitored in real time thereby offering the possibility of calculating the kinetic rate constants for the interaction (Karlsson et al., 1991). Our results demonstrate that the biosensor can be used for rapid and simple selection of phage antibodies with a predefined dissociation rate constant.

2. Materials and methods

2.1. Antigens

Lysozyme was purchased from Sigma Chemical Co. (St. Louis, MO). HM90-5, which contains the AD-2 epitope expressed by cytomegalovirus (CMV) glycoprotein B (Meyer et al., 1990), was a gift from Michael Mach and pB1, which contains the V3 loop on gp120 of HIV-1 (LAI) (Putney et al., 1986) was obtained from Repligen, Cambridge, MA.

2.2. Preparation of phage stocks

Anti-lysozyme (Fab), anti-phenyloxazolone (Fab) and anti-lipopolysaccharide (LPS) (scFv) phage stocks were prepared using the pEXmide 3 (Söderlind et al., 1993) and pEXmide 4 (Ohlin et al., 1996) vectors, according to Hoogenboom et al. (1991). Briefly, transformed *E. coli* XL1 Blue cells were grown overnight at 37°C in 2 × YT medium, supplemented with ampicillin (100 µg/ml), tetracycline (10 µg/ml) and glucose (1%). The cultures were diluted 1/100 and grown to an OD₆₀₀ ≈ 0.5. The cells were washed once and helper phages (VCSM13) were added at a ratio of 20 phages per cell. After growth for 1 h, kanamycin was added to a final concentration of 20 µg/ml and the cells were cultured overnight. Cells were removed by centrifugation for 10 min at 7740 × *g* and the phage-containing supernatant was filtered through an 0.45 µm filter. The anti-CMV library was prepared, using an anti-CMV heavy chain shuffled with a light chain library (Ohlin et al., 1996). This phage displayed library had twice been selected on antigen coated microtiter plates to enrich antigen-specific phage clones, but only 1/7 clones were specific for the antigen in question. The anti-pB1 library was pre-

pared from in vitro immunized cells (Dueñas et al., 1996a).

2.3. Analysis and selection of phages in BIAcore™

The BIAcore™ equipment was modified, using a stainless steel syringe needle coupled to the drain of the flow cells (BIAcore™ System Manual, 1991) to facilitate collection of samples (Malmqvist, 1993).

Lysozyme was immobilized to the sensorchip surface, as described (Borrebaeck et al., 1992), giving 800–1000 RU. All buffers used in the BIAcore™ experiments were as described previously (Malmborg et al., 1992).

40 µl of an anti-lysozyme Fab phage stock containing 3.1×10^8 , 1.2×10^9 , 1.0×10^{10} or 2.0×10^{10} cfu/ml were injected over the sensor chip carrying immobilized lysozyme at a flow rate of 1 µl/min for the analysis of antibodies specific for lysozyme.

To demonstrate the principle of specific phage selection, a 10% mixture of anti-lysozyme Fab phages with a non-specific phage stock (anti-LPS or anti-phenyl-oxazolone) was established. The mixtures were injected at either 3 µl/min (36 µl) or at 1 µl/min (40 µl) over the sensor chip carrying immobilized lysozyme. The titers were in the range 2.5×10^7 – 7.2×10^9 cfu/ml. After the association phase, the sensor chip was washed either by a continuous flow for 30 min or by using the 'rinse command', which allows washing of the sensor chip at a high flow rate for about 5 min (BIAcore™ System Manual, 1991). Regeneration was performed using 9 µl 100 mM HCl. The eluate was collected (totally 55 µl at 3 µl/min and 165 µl at 1 µl/min) and neutralized with 10 µl 2 M Tris base. The eluate was analysed, as described in Section 2.4, and injected in a second round of selection.

Selection of phages from the anti-CMV and the anti-pB1 library was performed by immobilizing either pB1 or HM90-5, respectively, to the sensor chip as described (Dueñas et al., 1996a; Ohlin et al., 1996), giving approximately 8000 RU and 2500 RU, respectively. The phage libraries were injected as described above. The dissociation, which was performed under a continuous buffer flow at 1 µl/min, was followed for several hours and 10 µl fractions were collected at five different time points (1.0 h,

1.3 h, 2.2 h, 3.5 h and 4.5 h for the anti-CMV library and 1.0 h, 1.7 h, 2.7 h, 3.5 h and 6.7 h for the anti-pB1 library).

2.4. Analysis of phage titer and specificity

The phage titer was determined by infection of 100 μ l *E. coli* XL1 Blue, harvested at log phase and frozen until use, with 10 μ l of sample. The samples were dilutions of eluted phage, as described in Section 2.3, as well as the original phage stocks and mixtures. After a 30 min incubation at room temperature the cells were plated on 2 $\times$ YT agar plates with ampicillin and grown overnight at 37°C. The number of cfu were counted and the cfu/ml was calculated for each of the various samples.

The specificity of selected phages was determined by the following methods.

1. For restriction enzyme analysis, individual infected cells were grown overnight and plasmid preparations were carried out using Wizard Minipreps DNA Purification System (Promega, Madison, WI). The plasmids were digested with *NotI*/*PstI* for 2 h in 37°C, and analysed by 1.5% agarose gel electrophoresis, giving two bands at 4006 and 1410 bp for the lysozyme specificity and one band at approximately 4700 bp for the LPS specificity.
2. PCR-based analysis was carried out using two set of primers, one which amplifies the V_H region on pEXmide 3 vector (5'-GGC CAT GGC CCA GGT GCA GCT GAA GGA GTC as 5'-primer and 5'-ATG GGC CCT TGG TAC CGG CTG CAG AGA CAG TGA CCA as 3'-primer), and one which amplifies the V_L region on anti-oxazolone plasmids (5'-CCG CGG CCG CAC CAG CGA TGG CCC AAA TTG TTC TCA CCC A as 5'-primer and 5'-TGG GTT ACT ACT CCA CTG CTG as 3'-primer). PCR profile: 30 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 3 min were used with colonies from infected *E. coli* XL1 Blue as template. Colonies from the ampicillin plates of *E. coli* XL1 Blue infected by phages eluted from the anti-CMV library were screened for insert by PCR using 5'-GTC CTC GCA ACT GCC CCA TGG CCC AGG TGC AGC TGG TGC AGT CTG G as 5'-primer and 5'-GAG TCA TTC TCG ACT GCT AGT CGA

CCT AAC GTT TGA TCT CCA CCT TGG TCC C as 3'-primer.

3. Mini phage stocks were prepared using colonies from *E. coli* XL1 Blue infected by eluted phages from the anti-pB1 library. Each colony was grown in 100 μ l 2 $\times$ YT, ampicillin and tetracycline overnight in 37°C. Subsequently helper phage at a 20:1 ratio were added and the cells were grown at 30°C. After 2 h kanamycin was added and growth was continued overnight. The plates were centrifuged at 1500 rpm for 30 min and the supernatants analyzed in a pB1-specific ELISA with anti-M13 antiserum according to Dueñas et al., 1996a. Clones were scored as positive if > mean background + 3 SD.

2.5. Expression of soluble antibody fragments

Plasmids were prepared from the anti-pB1 positive clones using Jetprep Plasmid Miniprep kit (Genomed, Research Triangle Park, NC, USA) and transformed into *E. coli* strain TOPP2, made competent with $RbCl_2$. Fresh transformants were grown in 10 ml overnight cultures at 37°C in LB medium supplemented with ampicillin, tetracycline and glucose. The glucose was washed away and the cells induced with 0.1 mM IPTG and grown for 20–24 h at 30°C. The soluble Fab fragments were rescued by 15 min centrifugation at 3500 rpm and the supernatants were sterile filtered. Soluble scFv fragments from the anti-CMV library were expressed directly in *E. coli* XL1 Blue.

2.6. Evaluation of soluble antibody fragments

The concentrations of soluble Fab fragments were determined by ELISA using Fab specific antiserum/peroxidase labelled antiserum (Sigma Chemical Co., St. Louis, MO). Purified human Fab (Organon Teknika-Cappel, Durham, NC) was used as standard. Fab-containing samples were concentrated in dialysis tubing with dextran T70 (Pharmacia Biotech Norden, Sollentuna, Sweden) to obtain concentrations in the range 1–10 nM. ScFv was not concentrated. The soluble antibody fragments were analysed in BIAcore™ and dissociation rate constants during the first 10 min were calculated (Malmberg et al., 1992) and compared for cycles

giving the same response i.e. around 100–200 RU. Clones obtained from the scFv anti-CMV library were sequenced using CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Bio-Labs, Beverly, MA).

3. Results and discussion

3.1. Specific binding of antibodies displayed on phage

The possibility to monitor binding of anti-lysozyme Fab fragments displayed on a filamentous phage to immobilized lysozyme was initially studied. Phage titers as low as about 3×10^8 cfu/ml were detected (Fig. 1). This is comparable to what has previously been reported for phage interactions in BIAcore™ (Lasonder et al., 1994). However, phage display vector systems containing amber suppressor codons, such as pExmide 3, are known to release soluble Fab fragments into the medium. Thus, the signal in Fig. 1 can also, in part, be due to soluble Fabs.

A 1:10 mixture of an anti-lysozyme and nonspecific phage stock was established to evaluate whether BIAcore™ could be utilized for the selection of specific phages. Analysis of the selection, after interaction with lysozyme at 3 μ l/min and washing for

30 min by continuous flow, gave 0/10 lysozyme specific phages in the starting mixture, 1/10 after the first round and 4/10 after the second round of selection. Injection at 1 μ l/min and a washing step using the 'rinse command' (5 min at a high flow rate) gave 1/16 lysozyme specific phages in the starting mixture and 9/16 after the first round of selection. Injection of eluted phages in a second round gave no recovered phages after washing and regeneration. This implied that the eluted phages, when washed by 'rinse command', must be reamplified before the second round. Furthermore, flow rate as low as possible should be used when working with phages in the BIAcore™ in order to counteract any diffusion limitations due to bulky molecules.

3.2. Selection of clones from a VL shuffled library

The system was tested on a light chain shuffled scFv library obtained using an anti-CMV clone specific VH sequence. The scFv-carrying phage library was allowed to interact with immobilized recombinant antigen. The number of clones containing insert of the correct size was examined by PCR. In the start mixture 3/10 were positive in the PCR assay, while the percentage of positive clones ranged from 10 to 67% throughout the dissociation. The clone with the fastest apparent dissociation rate ($5.5 \times 10^{-4} \text{ s}^{-1}$)

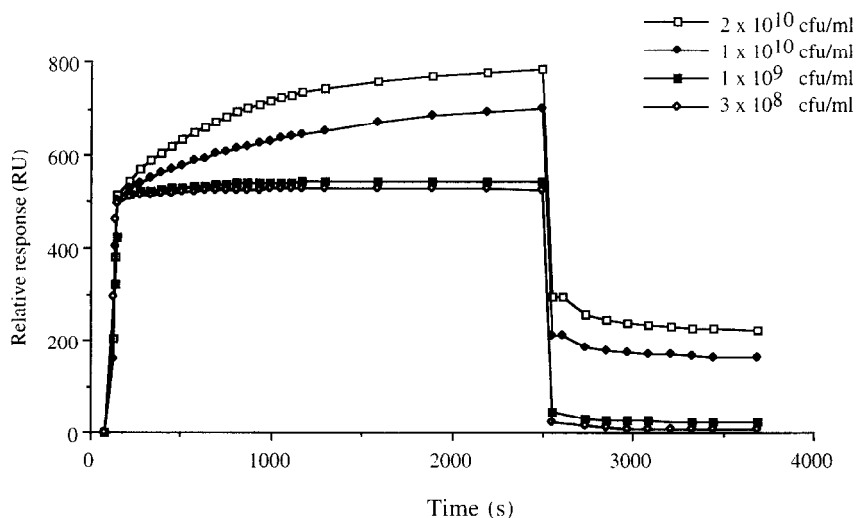


Fig. 1. Interaction between immobilized lysozyme (850 RU) and a lysozyme specific Fab phage stock at various concentrations: 2×10^{10} ($\square$), 1×10^{10} ($\blacklozenge$), 1×10^9 ($\blacksquare$) and 3×10^8 ($\diamond$) pfu/ml.

was obtained after 1 h and the clone with the slowest apparent dissociation rate ($1.1 \times 10^{-4} \text{ s}^{-1}$) was obtained after 4.5 h of dissociation (Fig. 2). Panning of the same library gave apparent dissociation rate constants in the range 1.8×10^{-4} – $1.0 \times 10^{-3} \text{ s}^{-1}$. The original antibody expressed as an scFv fragment gave an apparent $k_{\text{diss}} = 6.5 \times 10^{-4} \text{ s}^{-1}$ i.e. within the interval of the panned clones, but a faster apparent dissociation rate constant than any of the clones selected in BIAcore™. This technique will not discriminate between clones showing an increased apparent affinity constant as a consequence of a true increase in affinity for the antigen or an increased avidity occurring as a consequence of scFv dimerization (Pack et al., 1993). Nevertheless, our results clearly demonstrate that BIAcore™ based selection can be used to enrich for high affinity clones. In order to minimize the problem associated with avidity effects caused by dimer formation, Fab encoding expression vectors might be used instead. Sequenc-

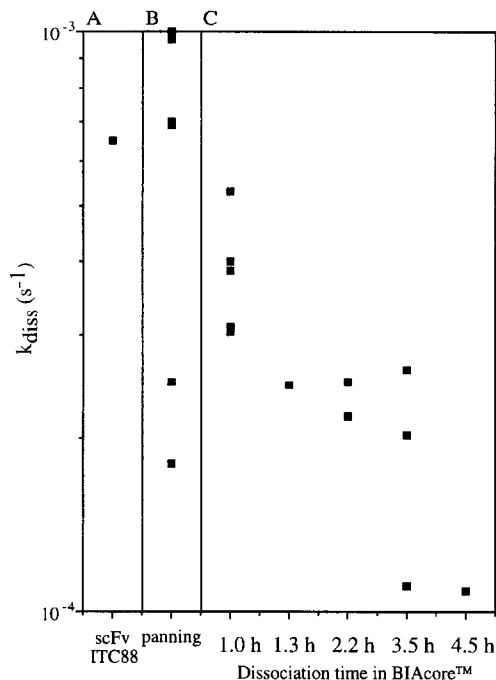


Fig. 2. Calculated apparent dissociation rate constants for scFv clones directed against CMV. A shows the original antibody (ITC88) expressed in bacteria as soluble scFv, B shows clones obtained by panning of a light chain library and C shows clones obtained by selection in the BIAcore™ at different time of dissociation from the same library as in B.

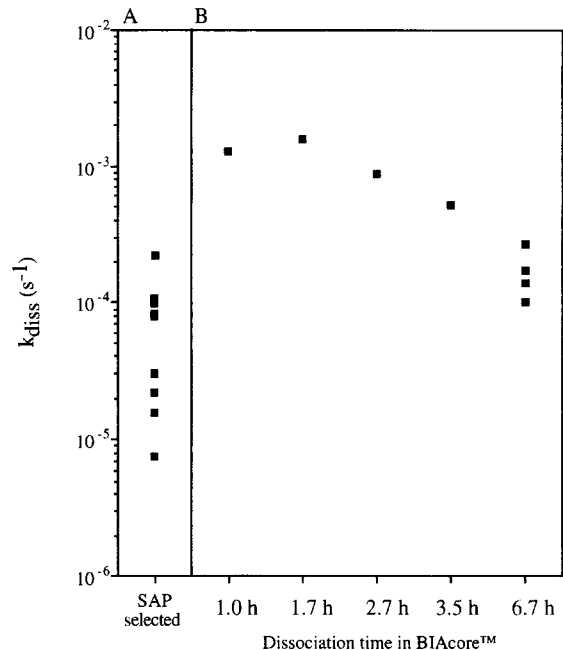


Fig. 3. Calculated dissociation rate constants for Fab clones directed against pB1. A shows clones obtained by SAP selection of an antibody library obtained from secondary in vitro immunized cells and B shows clones obtained by selection in BIAcore™ at different times of dissociation from the same library as in A.

ing of the panned clones gave V κ 3a for all the selected clones, while the BIA also permitted selection of V κ 1 clones (data not shown).

3.3. Selection of clones from a library obtained from secondary in vitro immunized cells

The model was also tested on a phage displayed Fab library established from in vitro immunized cells. The phage library was eluted from the BIAcore™, to which the antigen had been immobilized, and fractions of eluate were collected. Positive clones, 26/48 throughout the dissociation as determined in phage ELISA, were expressed as soluble Fab fragments and their dissociation rate constants were determined to be in the range 1.6×10^{-3} – $1.0 \times 10^{-4} \text{ s}^{-1}$, clearly showing a decreasing dissociation rate constant with increased time of dissociation (Fig. 3). The same library has undergone selection with the SAP system (Dueñas et al., 1996a) giving dissociation rate constants in the range 2.2×10^{-4} –

$7.8 \times 10^{-6} \text{ s}^{-1}$. SAP stands for selection and amplification of phages and is based on a gene III deleted helper phage that gives rise to non-infectious phages expressing antibody fragments. These are made infective by the use of a fusion protein consisting of the appropriate antigen and protein III. Thus, only specific phages infect the bacteria. As has been shown (Dueñas et al., 1996b) the SAP system preferably selects for clones of high affinity and slow dissociation rate, whereas the BIAcore™ based selection method selects clones with faster dissociation from the same library. This might be explained by the fact that SAP is a non-solid phase based system that immediately links the interaction to infection, whereas in the BIAcore™ system the phages bind to the solid phase and require dissociation before infection.

Acknowledgements

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