Determination of Phage Antibody Affinities to Antigen by a Microbalance Sensor System

BioTechniques 26:956-964 (May 1999)

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ABSTRACT

Over the past decade, phage display has maturated to be a frequently used method for the generation of monoclonal antibodies of human origin. The essential step of this method is the "biopanning" of phage carrying functional antibody fragments on their surface on an immobilized antigen. The screening of large combinatorial gene libraries with this method usually leads to a set of diverse clones specifically binding to the antigen that need to be characterized further. Beside its specificity, the key parameter to be determined is the affinity of the recombinant antibody fragment to its antigen. Here, we present a mass sensitive microsensor method that allows the estimation of antibody affinity directly from the phage supernatant. Binding of phage antibodies to the antigen immobilized on a quartz crystal microbalance (QCM) induced a mass dependent decrease in frequency. This principle was used to determine the apparent affinity of a single-chain (sc)Fv antibody against the RNA polymerase of Drosophila melanogaster *presented on the surface of a filamentous phage (M13) from its association and dissociation rates. The apparent affinity obtained is in accordance with the affinity of the scFv fragment as determined by conventional equilibrium enzyme-linked immunosorbent assay (ELISA) and plasmon resonance methods.*

INTRODUCTION

Within the last decade, a novel approach has been made available that allows the production of human antibodies to highly infectious, pathogenic or toxic agents. The key technology is phage display, i.e., the surface expression of antigen-binding fragments of the antibody on filamentous bacteriophage (2,4,10). The development of these methods is based on the finding that functional antibody fragments could be secreted into the periplasmic space (3,22). This finding opened the way for transferring the principles of the immune system, for producing specific antibodies, to a given antigen into bacteria (9). It was now possible to establish antibody libraries in *Escherichia coli* that could be directly screened for binding to the antigen. This was accomplished most successfully by the surface expression of functional antibody fragments on virions of the filamentous bacteriophage of the M13 family. Using this principle, extremely large antibody gene libraries containing at least 10^8 individual members can be screened. This selection system is as efficient as that of the immune system, in which the antibody receptor is bound to the surface of a lymphocyte. Moreover, phage display extends the ability of our body to generate antibodies by supplying a method to obtain human antibodies to highly pathogenic or toxic antigens that cannot be used for immunization.

The method requires three steps: (*i*) generation of an antibody gene library by polymerase chain reaction (PCR), (*ii*) packaging into phage particles that carry the antibody fragment on their surface and (*iii*) the selection of genes for specifically binding antibody fragments by an affinity purification. Because the phage body connects the function (antigen binding) with the structural information for this function (antibody genes), this method results in antibody clones to the antigen used for screening. To obtain this, a preparation of about 10^{12} phage antibody particles carrying antibodies will be incubated with a solid support coated with the antigen. After intensive washing, phage still bound to the solid phase are expected to specifically bind to the antigen. They can be eluted using pH shift or protease digestion. After elution, they can be reinfected into fresh *E. coli* cells to be amplified again. This "biopanning" process can be repeated up to five times. Usually achieved enrichment factors are around 100, so that after several rounds, almost all eluted clones are specifically bound to the antigen. The subsequent assay to check this specificity is usually a phage enzyme-linked immunosorbent assay (ELISA) (23). To achieve this, phage bound to the immobilized antigen are quantified using antibodies against the phage coat protein (18). For this type of ELISA, 96 separate clones can be conveniently cultivated in a microplate system, which allows the test for antigen specificity to occur directly from their supernatant (6). However, for the determination of affinity, to our knowledge, no convenient method has yet been available that has utilized phage-bound antibody fragments from culture supernatants directly. Usually, the recombinant antibodies had to be separated from the phage particle before the measurement. This has been achieved either by subcloning into bacterial secretion vectors (7,8,11) or by transfecting the expression phagemids into a different bacterial strain (suppressor strain), which allows the recognition of a stop codon between the antibody fragment

and the phage surface protein (10). To shorten this process, it would be desirable to measure not only specificity, but also affinity of the antibody fragment while it is still bound to the phage surface. Here, we demonstrate the feasibility of the determination of apparent affinity of single-chain (sc)Fv antibody fragments presented on phage using a mass-sensitive microsensor system.

MATERIALS AND METHODS

Measurement Setup

The measurement setup is shown in Figure 1. The quartz crystal microbalance (QCM) uses a 20-MHz quartz crystal with gold electrodes (diameter: 4 mm; thickness: 0.1 mm) driven by an oscillator circuit. A series resonance oscillator, which regulates its frequency at the minimum impedance of the quartz crystal is used, and a flow-injection analysis system for successive measurements supplies the QCM with a continuous liquid stream of 30 µL/min by a peristaltic pump. For each mesurement, 250 µL of sample solution were injected into the carrier stream with the help of an HPLC injection valve.

Biological Samples

The well-characterized, single-chain antibody 215 (scFv215) binding to RNA Polymerase II of *Drosophila melanogaster* was expressed on the surface of the filamentous phage (14). Briefly, the scFv215 antibody fragment was genetically fused to the aminoterminus of the gene III product (pIII, g3p) of the filamentous phage, transfected into an F^+ *E. coli* strain (Xl1blue) and packaged into phage particles after infection with the helper phage M13KO7 as described previously (4,23). Phage without the scFv portion were produced for negative control. Recombinant antigen was purified from inclusion bodies of *E. coli* carrying a plasmid encoding for the 215 epitope area of RNA polymerase II of *D. melanogaster* fused to β-galactosidase (β-gal) as described (14). Both antigen and phage antibody preparations were checked for function by phage ELISA (6,23) before immobilization to the QCM sensor.

Immobilization Procedure

An immobilization method based on nonspecific adsorption to the gold layer was applied. The immobilization procedure consists of a cleaning, an activation and two binding steps. First, the gold electrodes were rinsed with acetone to remove adsorbed components disturbing the coupling process. Then, the cleaned surface was incubated with dithiobissuccinimidyl-propionate (DSP) at a concentration of 0.4% in water-free dimethyl sulfoxide

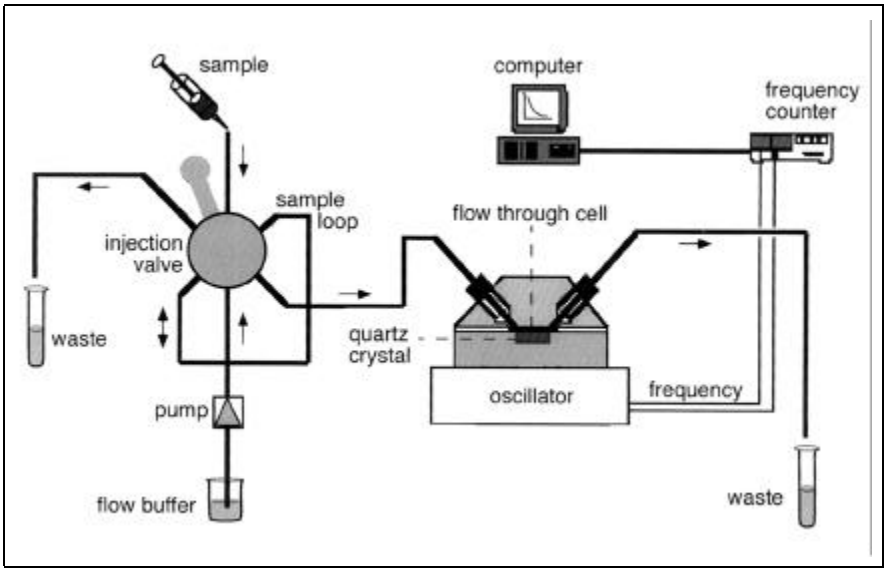

Figure 1. Setup of the QCM flow-through system.

(DMSO) at room temperature for 20 min. Another washing step with phosphate-buffered saline (PBS), pH 7.4, finished the activation procedure. Then, 10 µL of recombinant antigen (1 mg/mL) were added, and the quartz crystal was incubated at 4°C for at least 12 h. The quartz crystal was rinsed again with PBS and inserted into the clip holder. A blocking agent for the prevention of nonspecific binding was not applied.

Measurement and Regeneration Procedure

Measurements were performed in PBS as the incubation buffer. Phage preparations displaying single-chain antibodies in different dilutions were injected into the system, and the permanent frequency shift was determined. Each reaction cycle was completed by thoroughly rinsing the system with PBS. Preparations of wild-type phage at the corresponding concentrations were used as a negative control. After a variable number of measurements, the antigen layer was regenerated by incubation in 4 M sodium isothiocyanate, pH 11.0 (chaotropic reagent; Merck, Darmstadt, Germany).

Calculation of the Apparent Binding Constants

The frequency shift due to the adsorption of the antibody phage was calculated according to Equation 1.

$$\Delta F = -\frac{k_{on} \times C \times \Delta F_{max}}{k_{on} \times C + k_{off}} \times [1 - e^{-(k_{on} \times C + k_{off}) \times (t - t_0)}] \quad \text{[Eq. 1]}$$

where F is frequency change, C is concentration of analyte, F_{max} is signal of complete covered surface, t is time, t_0 is time at start of reaction and F_0 frequency at t_0. The desorption of the antibody phage was fitted by Equation 2:

$$\Delta F = -\Delta F_0 \times e^{-k_{off} \times (t - t_0)} \quad \text{[Eq. 2]}$$

RESULTS

To test whether a QCM microbalance system could be used to assess the apparent affinities of scFv antibodies

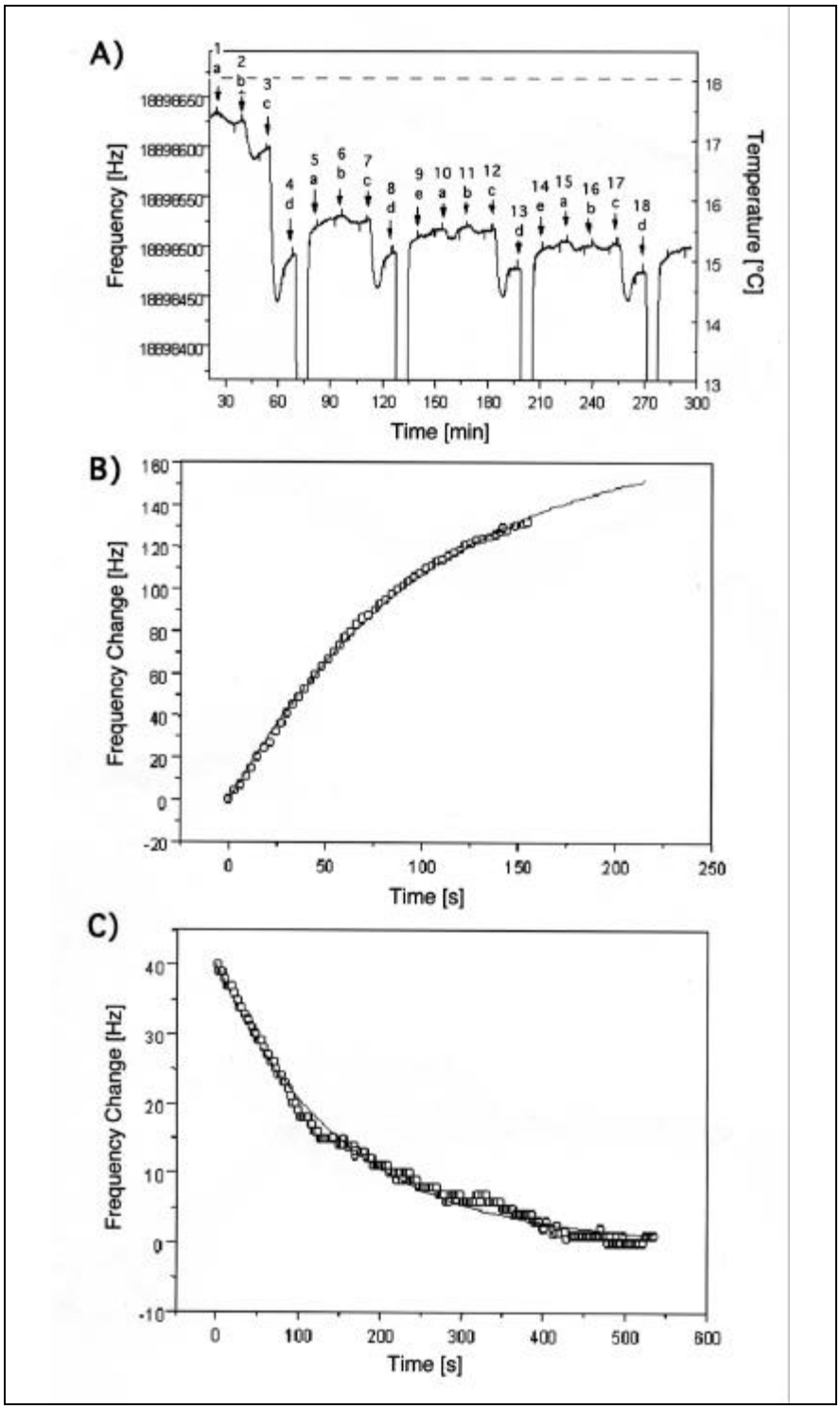

Figure 2. Determination of apparent affinities of scFv215 phage antibodies to recombinant 215 antigen using QCM. (A) Raw data set of frequency changes (solid line) resulting from subsequent addition of 18 samples containing either scFv215 phage antibodies, controls (wild-type phage) or regeneration buffer. Each sample application is indicated by an arrow and is numbered. The content of each sample injection is indicated below the sample number by letters: a, 10^{11} phage; b, 10^{12} phage; c, 10^{13} phage; d, regeneration and e, 10^{13} wild-type phage. The dotted line shows the temperature. (B and C) Examples of curves fit to frequency change data for the determination of k_{on} rate and k_{off} (measurement No. 3). (B) Frequency modulation in Hz due to specific adsorption of scFv 215 page antibody particles to the quartz crystal coated with recombinant 215 antigen. (C) Frequency modulation in Hz due to desorption of scFv 215 page antibody particles from the quartz crystal coated with recombinant 215 antigen.

Table 1. Sequential QCM Measurements of Binding Constants of phAb215 to Recombinant 215 Antigen Determined on the Same Gold Chip

Sample No.	2	3	7	12	17
Regenerations	none	none	1	2	3
Number of phage	10^{12}	10^{13}	10^{13}	10^{13}	10^{13}
C[M] of phage	1.7×10^{-9}	1.7×10^{-8}	1.7×10^{-8}	1.7×10^{-8}	1.7×10^{-8}
F_{max}[Hz]	569	569 ± 41[a]	190	190	190
k_{on}[$M^{-1}s^{-1}$]	2.3 (±0.01) $\times 10^5$	1.7 (±0.1) $\times 10^5$	2.3 (±0.1) $\times 10^5$	3.6 (±0.2) $\times 10^5$	2.0 (±0.02) $\times 10^5$
k_{off}[s^{-1}]	4.0 (±0.07) $\times 10^{-3}$	6.6 (±0.05) $\times 10^{-3}$	8.3 (±1.0) $\times 10^{-3}$	1.2 (±0.05) $\times 10^{-2}$	9.8 (±1.1) $\times 10^{-3}$
k_d[M]	3.1×10^{-3}	3.9×10^{-8}	3.8×10^{-8}	5.3×10^{-8}	5.2×10^{-8}

The sample numbers were taken from Figure 2A. The variations given in brackets for k_{off} and k_{on} are derived from the fitting process.

[a]F_{max} was determined from the fitting process of Sample No. 3. The given variation is derived from the fitting process. Because F_{max} describes the maximum frequency change, it should be constant throughout the measurements. However, because a loss of two-thirds of signal, indicating a loss of two-thirds of antigen function, was observed after the first chaotropic treatment (see Figure 2A), the measurements were calculated using a F_{max} reduced to one-third of the original value.

presented on the surface of filamentous phage, a flow-through setup was used (Figure 1). A well-analyzed model antibody/antigen pair was used, thus allowing a comparison of the obtained apparent affinities to the binding constants generated by other methods. The single-chain antibody, scFv215, binds to a defined epitope of RNA polymerase II of *Drosophila* (14,17). Its binding constant to recombinant antigen has been determined by equilibrium ELISA (12,14) and a kinetic method [Biacore® plasmon resonance (Biacore, Piscataway, NJ, USA)] (13). For this study, it was presented on the surface of filamentous phage particles. After injection of 10^{12} or 10^{13} phage particles displaying scFv215 into a flow cell containing the gold-chip sensor coated with recombinant 215 antigen, a concentration-dependent decrease of frequency was observed (Figure 2A). Upon washing the cell with buffer, the frequency increased again, indicating dissociation of the phage. Similar measurements with wild-type phage (Figure 2A, samples 9 and 14) did not result in any significant frequency shifts (Figure 2A). This further indicates that the reactive gold surface of the resonator chip was sufficiently saturated by the antigen layer, and unspecific binding did not occur. A substantial decrease in the rate of frequency changes was observed after the first regeneration with chaotropic 4 M sodium isothiocyanate (Figure 2A, sample 4). This indicates irreversible damage or a washing away of one-third of the coated antigen. However, after this solitary decrease, the F_{max} did not change significantly over the three subsequent binding and regeneration cycles, comprising of 3 samples each (Figure 2A). Due to the lower F_{max} after the first regeneration, the sensitivity dropped to one-third of the non-regenerated chip, so that only measurements using 10^{13} phage (1.7×10^{-8} mol/L) were considered in the three further repeats of the measurements. After each further regeneration with chaotropic reagent, the baseline shifted slightly but remained constant during the subsequent measurements.

Based on the kinetics of the frequency decrease during specific adsorption of the phage particles (Figure 2B), k_{on} values were calculated for five subsequent applications (Table 1). To determine k_{off}, the frequency increase caused by desorption was monitored after reaching the absolute minimum frequency (Figure 2C). From these association and dissociation rates, the dissociation constants (K_d) were calculated (Table 1). The calculated apparent affinities determined from subsequent measurements using 10-fold different concentrations of phage (Figure 2A, samples 2 and 3, containing 10^{12} or 10^{13} phage, i.e., 1.7×10^{-9} or 1.7×10^{-8} mol/L, respectively) were remarkably similar (Table 1). If corrected for the decreased F_{max} after the first chaotropic treatment of the chip, the calculated apparent affinities of all five determinations were in good accordance (Table 2), having a mean K_d of 3.7×10^{-8} M with a standard deviation of 1.3×10^{-8} M (35%).

DISCUSSION

Due to a lack of sensitivity, affinity determinations of phage particles carrying recombinant antibodies have not yet been possible with the established methods, such as equilibrium ELISA or plasmon resonance. Here, we present QCM as a new method to directly monitor the association and dissociation of phage antibodies to their antigen, thus allowing the determination of an appar-

ent binding constant. The QCM is an acoustic sensor based on a piezoelectric crystal as suggested by Sauerbrey (21), which can be used for the measurement of specific interactions between immobilized molecules and analytes in solution. The binding of a soluble analyte causes a frequency shift in the resonance frequency that can be recorded by a frequency counter with high resolution. This frequency shift is correlated with the concentration of the added analyte (1,5,15,16,19,20). The binding constant of the secreted form of the scFv antibody chosen for this study has been extensively studied by equilibrium (12,14) and kinetic methods (13). The apparent affinity of the phage-bound scFv antibody, as determined in this study, is well inside the variations observed between the different measurement methods applied to date (Table 3). A partial explanation for the variation between the K_d values measured in the cited studies could be given by the fact that scFv antibodies frequently form noncovalent bivalent dimers. However, in some studies, these dimers had been separated from monomers. Further, the significantly lower apparent affinity that was measured by plasmon resonance might be due to diffusion effects in the three-dimensional dextran layer used to couple the antigen in the Biacore system. In contrast, QCM applies an antigen monolayer immobilized on a flat, gold surface with an antigen monolayer that is not influenced by mass transport effects at the liquid-solid interface. Taking this into account, the apparent K_d of phage antibodies measured by QCM is in close conformity with the affinities reported in the literature for the original antibody or the recombinant derivatives of it (Table 3). Interestingly, the QCM value is closest to the monovalent forms of the antibody. However, this does not suggest the conclusion that each phage carries only one scFv, because the fraction of total phage carrying scFv fragments might not be 100%. Rather, a mixture of phage might be present, also taking into account that a constant fraction of pIII surface-expressed scFv antibodies might be shedded by proteolysis. In some phage-display systems, soluble scFv fragments can be present in the phage supernatants because of the existence of an amber codon between scFv and pIII. Thus, for the determination of the true affinities of the recombinant antibody fragments, a more accurate determination of true molarities would be necessary. Nevertheless, for a ranking of antibody fragments from a screening experiment, the apparent affinities as determined by the presented method are sufficient. Future experiments assessing the true number of presented antibodies might allow for an extension of the scope of this method for the determination of true affinities.

A drop of sensitivity to approximately one-third was observed after the first regeneration with chaotropic reagent. This buffer is expected to denature part of the protein's tertiary structure, whereas large portions of the secondary structure can remain intact. The epitope of the scFv215 used in this study was recently determined to be an oligopeptide composed of 12 amino acids, which form a rigid α-helical structure resistant to the usual denaturation agents, e.g., sodium dodecyl sulfate (SDS) gel electrophoresis (1% SDS at 100°C) (17). Therefore, the observed decrease in sensitivity after the regeneration might be attributed to a partial elution of the antigen coated to the gold chip, rather than to the denaturation of the epitope structure. In accordance, further regeneration cycles did not result in an additional significant decrease in sensitivity.

In summary, QCM can be considered a new, fast and accurate method for the determination of apparent affinities of recombinant antibody fragments obtained from phage-display libraries. The amount of phage particles required for a set of measurements can easily be generated in a small-scale laboratory

Table 2. Kinetic Constants of phAb215 Binding to Recombinant 215 Antigen Calculated from Five Subsequent QCM Measurements

Constant	Mean	SD	Relative SD
k_{on}	2.4×10^5 $M^{-1}s^{-1}$	0.06×10^5 $M^{-1}s^{-1}$	2.5%
k_{off}	8.1×10^{-3} s^{-1}	3.1×10^{-3} s^{-1}	38%
K_d	3.7×10^{-8} M	1.3×10^{-8} M	35%

Table 3. Comparison of the Apparent Affinities of phAb215 to Recombinant 215 Antigen to Affinities Determined by Different Methods for the Original Antibody and Recombinant Forms Not Bound to Phage

Antibody	K_d	Method	Study
MAb215	$2.7 \pm 1.3 \times 10^{-9}$ M	ELISA	Reference 14
MAb215	$1.28 \pm 0.17 \times 10^{-8}$ M	ELISA	Reference 12
scFv215	$3.8 \pm 0.9 \times 10^{-9}$ M	ELISA	Reference 14
scFv215[a]	$3.54 \pm 0.67 \times 10^{-8}$ M	ELISA	Reference 12
scFv215[a]	4.4×10^{-8} M	ELISA	Reference 13
scFv215[a]	1.07×10^{-7} M	Plasmon Resonance	Reference 13
phAb215	$3.7 \pm 1.3 \times 10^{-8}$ M	QCM	This study

[a]Monomeric fraction from a molecular sieve column.
Abbreviations: MAb, monoclonal antibody and phAb, phage antibody.

culture, thus making possible a high-throughput comparison of a large number of specifically binding clones, as is frequently obtained from phage panning. We have also shown that repeated measurements are possible from the same gold chip. In the future, the observed decrease in performance, as a result of the regeneration step, might be avoided with the use of more sophisticated regeneration procedures or the improvement of the antigen immobilization. Further, the measurement system is not restricted to the determination of antibody/antigen interactions. This has already been demonstrated for the characterization of a human pancreatic secretory trypsin inhibitor mutant from a phage library (J. Decker and C. Kösslinger, data not shown), but could also be used for any other receptor/ligand pair presented on the surface of large viral particles such as filamentous phage, adenoviral vectors targeted for gene therapy or retroviral particles carrying respective domains.

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Received 19 October 1998; accepted 17 February 1999.

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