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A practical kinetic model for efficient isolation of useful antibodies from phage display libraries

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Abstract

To isolate phages displaying a practical and useful antibody with a high k_{on} value and/or a low k_{off} value from phage display antibody libraries, we developed a rational strategy based on a kinetic model. In the model, the recovery of a phage displaying an antibody after a round of biopanning is expressed as a function of five parameters, the apparent association rate constant of the phage antibody to the immobilized antigen (k'_{on}), the apparent dissociation rate constant of the phage antibody from the immobilized antigen (k'_{off}), the effective antigen concentration (C), the time for the binding process (t_b) and the time for the washing process (t_w). An optimum set of operating parameters (C, t_b and t_w) for isolating phages displaying an antibody with a high k_{on} value was designed based on the model. Three rounds of biopanning were carried out under the designed conditions, against a phage library in which the hypervariable regions of an original antibody were randomized. All isolated phages displayed an antibody with a higher k_{on} value and one displayed an antibody with a 30-fold greater k_{on} value than that of the original antibody. Experimental conditions which improve the efficiency of conventional off-rate selections are also described.

Keywords: Phage display; Kinetic model; Association rate constant; On-rate selection; Antibody

1. Introduction

Since antibodies recognize their antigens with a high specificity and high affinity, antibody–antigen interactions have been applied to detection, quantification, and therapy. When an antibody reacts with its antigen in solution, the change in the concentration of the antibody–antigen complex is expressed as:

$$\frac{d(Ab\,Ag)}{dt} = k_{on} Ab^{free} Ag^{free} - k_{off} Ab\,Ag \quad (1)$$

where $Ab\,Ag$ is the concentration of the antibody–antigen complex, Ab^{free} and Ag^{free} the concentrations of the free antibody and the free antigen, respectively, k_{on} and k_{off} the true association rate constant and the true dissociation rate constant, respectively. (In this study, we handle monovalent antibodies such as single-chain Fvs or Fabs.) When the reaction time is sufficient to achieve a state of equilibrium ($d(Ab\,Ag)/dt = 0$), Eq. (1) can be rewritten as:

$$K_A = \frac{1}{K_D} = \frac{k_{on}}{k_{off}} = \frac{Ab\,Ag}{Ab^{free} Ag^{free}} \quad (2)$$

where K_A is the equilibrium association constant and K_D the equilibrium dissociation constant which represents the free antigen concentration at which half of the antibody molecules form the complex ($Ab^{free} = Ab\,Ag$).

The usefulness of an antibody (sensitivity of the antibody) has often been evaluated by its K_D or K_A value. Even in the practical applications of antibodies, the kinetic parameters of antibody, k_{on} and k_{off} have not been considered seriously. However, the K_D and K_A are static parameters at equilibrium. Since the equilibrium is not achieved in most of the practical applications in which the reaction time is limited, the usefulness of antibodies should be evaluated based on

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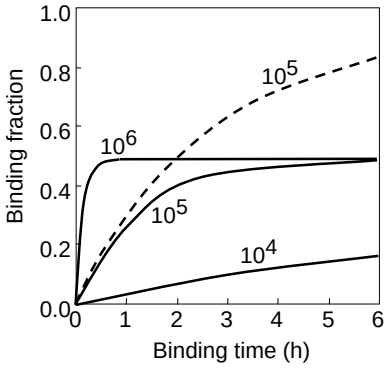

Fig. 1. Time courses of antibody–antigen reaction. Time courses of the binding fraction of the assumed antibodies with the K_A value of $10^9\,M^{-1}$ (solid lines) and $10^{10}\,M$ (broken line) were simulated based on Eq. (8) ($C = 10^{-9}\,M$, $k'_{on} = k_{on}$, $k'_{off} = k_{off}$). The numbers represent the k_{on} values in units of $M^{-1}\,s^{-1}$.

the kinetic parameters, k_{on} and k_{off}. It is known that the k_{on} value of natural antibodies ranges from 10^4 to $10^6\,M^{-1}\,s^{-1}$ [1,2]. When three assumed antibodies with the same K_A value of $10^9\,M^{-1}$, but with different k_{on} values of 10^6, 10^5 and $10^4\,M^{-1}\,s^{-1}$, react with an antigen at a concentration of $10^{-9}\,M$, these antibodies requires 19, 192 and 1920 min, respectively, to reach 90% equilibrium (Fig. 1, solid lines). When the reaction time is 30 min, the binding fractions of these antibodies are 0.49, 0.15 and 0.018, respectively. When these antibodies are used to detect the antigen, this difference reflects the detection sensitivity. Even in the case of an antibody with a K_A value ($10^{10}\,M^{-1}$) that is 10 times greater than that of these antibodies, if the k_{on} value is $10^5\,M^{-1}\,s^{-1}$, the binding fraction at 30 min is only 0.16 (Fig. 1, broken line). Speed is required for clinical assays, and the neutralizing antibodies should react rapidly with their antigens. Furthermore, reducing the reaction time increases the operating rate of the auto-analyzers used for routine laboratory tests. Therefore, the k_{on} is more important parameter than K_A (or K_D) when the reaction time is limited, and antibodies with high k_{on} values are preferable for practical applications.

Phage display technology [3–5] is one of the most powerful tools for isolating the specific ligands for a given target. This technology involves the display of an antibody or a peptide on the surface of a filamentous phage by fusing the gene of the antibody or peptide to one of the coat protein genes of the phage. Highly diverse antibody libraries have been constructed by inserting antibody genes into one of the coat protein genes, and many antibodies have been isolated from the libraries. The screening of phage libraries, referred to as “biopanning”, is based on a specific interaction between a given target and an antibody displayed on the phage. Biopanning consists of five steps: the immobilization of a target molecule on a solid support, the reaction of a library solution with the immobilized target (binding process), the washing of the solid phase to remove unbound phages (washing process), the elution and recovery of the bound phages, and finally the amplification of the bound phages by infecting host cells with the phages.

To isolate phages displaying an antibody with a high affinity against an antigen from phage antibody libraries, “off-rate selection” has been employed [5,6]. This method involves eliminating those phages that display an antibody that dissociates quickly from the immobilized antigen by extending the washing time. As a result, phages displaying an antibody with a high K_A value through their having a low k_{off} value are enriched. In the off-rate selection, however, the association rate of the antibodies is not taken into account. Instead, we found that the off-rate selection eliminates phages displaying an antibody with a high k_{on} value (see discussion for details).

To obtain practical antibodies enabling rapid and sensitive assay, it is necessary to isolate phages displaying an antibody with a high affinity against an antigen by their possessing a high k_{on} value. It would seem obvious that shortening the binding time in biopanning would enrich these phages. It was found in this study, however, that not only the binding time but also the effective antigen concentration and the washing time affect the enrichment of the phages displaying the desirable antibody. The purposes of this study are: (1) the establishment of a dynamic model describing the recovery of a phage antibody in a round of biopanning; (2) the design of an appropriate set of experimental conditions for isolating phages displaying antibody with a high k_{on} value from a phage antibody library; and (3) the isolation of phages displaying an antibody with a high k_{on} value from a customized library in which two hypervariable regions of an original antibody are randomized.

2. Experimental

2.1. Strains and reagents

Escherichia coli strain TG1 was used to express the phage antibody as well as for phage rescue. *E. coli* HB2151 was used to prepare soluble single-chain Fv. The helper phage, M13KO7, was purchased from Amersham Pharmacia Biotech. Bovine pancreatic ribonuclease A (RNase A), purchased from Sigma–Aldrich (code R-5500), was dialyzed against distilled water prior to use. Polystyrene paramagnetic microparticles (2.6% suspension, diameter 1–2 μm) were obtained from Polysciences Inc. Fluorescein succinimidyl ester was purchased from Panvera Co. To block the polystyrene beads, Block Ace (Yukijirushi, Tokyo), a blocking protein solution made from milk and designed for immunoblotting, was used.

2.2. Construction of the customized phage library

Anti-RNase A single chain Fv gene [7] was subcloned into pCANTAB5E (Amersham Pharmacia Biotech) at the *Sfi*I and *Not*I sites (pCANE/3A21). Since it is known that

the H3 region of antibodies plays an important role in antigen recognition, and we found that the H1 region of the anti-RNase A antibody forms a salt bridge with RNase A but an amino acid replacement in the L3 region causes no effect on the antigen recognition [8], two random sequences were introduced into the H1 and H3 regions of the gene to construct a customized library. An *Apa*LI site and a stop codon were introduced into the gene at the 5′ flanking region of the H1 region by using a standard in vitro mutagenesis technique with oligonucleotide 5′-CCCTCAC**G**TGCACTGTCACTGGCTA**A***TCAATCACCAGTGATTAT*GCC (the *Apa*LI site and the stop codon are underlined, the H1 region is italicized, mismatched bases are indicated by bold characters). An *Apa*LI–*Eco*T14I fragment, which contains degenerated sequences in the H1 and H3 regions, was amplified from the resulting phagemid by using a standard PCR technique with 5′-TCACGTGCACTGTCACTGGCTAC$(XXX)_7$TGGAACTGGATCCGCCAATTTCCAG and 5′-GACCAGAGTCCCTTGGCCCCAGTAAGC$(XXX)_5$CCCTCTTGCACAATAATATGTGG acting as primers (the *Apa*LI and *Eco*T14I sites are underlined), where XXX is NNK or RNS (N = A + C + G + T, K = G + T, R = A + G, S = G + C, $(XXX)_7$ and $(XXX)_5$ correspond to the entire regions of H1 and H3, respectively). The NNK fragment and the RNS fragment were each ligated with the large fragment of pCANE/3A21 digested with *Apa*LI and *Eco*T14I and then introduced into *E. coli* TG1 by means of electroporation. The resultant NNK library was estimated to contain 5×10^7 individual clones, while the RNS library was estimated to contain 2×10^7 individual clones. The resulting libraries were mixed and then used for this study.

2.3. Preparation of the phage solutions

Phage solutions of isolated clones and the library were prepared using the methods described in the instructions for the recombinant phage antibody system (Amersham Pharmacia Biotech), except that phage propagation was performed at 30 °C and those phages precipitated by polyethylene glycol were dissolved in PBS (0.2 g of KH_2PO_4, 2.9 g of $Na_2HPO_4{\cdot}12H_2O$, 8.0 g of NaCl, and 0.1 g of KCl in 1 l of deionized water, pH 7.2 at 30 °C).

2.4. Determination of the display ratio

The original phage antibody (3A21) was affinity purified to eliminate phages displaying no antibody molecule as described previously [9]. The purified and unpurified phage solutions were each reacted with the antigen-coated beads for 1 h at room temperature. After washing the beads three times with PBS, the bound phages were eluted in 0.1 M triethylamine and then titrated. The display ratio (R) was calculated by using the following equation:

$$\frac{\text{intput}_{\text{purified}}}{\text{output}_{\text{purified}}} = \frac{R \times \text{input}_{\text{unpurified}}}{\text{ouput}_{\text{unpurified}}}$$

2.5. Preparation of the fluorescence labeled antibody

The original phage antibody was constructed from monoclonal antibody 3A21 (IgG) recognizing RNase A [10]. The IgG from the supernatant of the hybridoma cell was purified by RNase A Sepharose 4B [9] and dialyzed against PBS. The monoclonal antibody (1×10^{-5} M) was reacted with fluorescein succinimidyl ester (5×10^{-5} M) for 2 h at 37 °C, after which the reaction was quenched by the addition of glycine solution to give a final concentration of 0.1 M. The labeled antibody was purified using a Sephadex G25 column equilibrated with PBS. The concentration of the labeled antibody (B) was calculated based on the absorbencies at 280 and 492 nm, using the following equation:

$$B = \frac{A_{280} - 0.26 \times A_{492}}{\varepsilon}$$

where ε is the molar absorbance of the monoclonal antibody, the value of which was determined to be $2.3 \times 10^6\ \text{M}^{-1}\ \text{cm}^{-1}$ based on the amino acid composition and molecular weight deduced from the DNA sequence.

2.6. Determination of the effective antigen concentration

The antigen on the beads effective for antibody binding was quantified by the fluorescent labeled antibody prepared as described earlier. The antigen-coated beads were incubated in the labeled antibody solution (2×10^{-6} M) for 2 h at 30 °C. Since the K_D of the labeled antibody for the antigen was estimated to be 2×10^{-8} M, 99% of the effective antigen was calculated to bind to the labeled antibody when the effect of steric hindrance of the labeled antibody was assumed to be negligible. The beads were washed five times with PBST and then re-suspended in PBST at 30 °C. The beads in an aliquot of the suspension were collected and the labeled antibody bound to the beads were eluted by 0.2 M glycine–HCl (pH 2.7). The supernatant was neutralized and diluted with PBS. The amount of the bound antibody was calculated based on the fluorescence intensity at 30 °C in PBS. During the washing, however, the labeled antibody dissociates from the antigen immobilized on the beads. To revise the dissociation, the residual suspension was incubated at 30 °C. Every 10 min, the washing buffer was exchanged and the amount of the bound antibody was measured in the same manner as described earlier. The logarithm of the amount of the bound antibody was plotted against the washing time (incubation time in the washing buffer) and the amount of the bound antibody at time zero was then extrapolated.

2.7. Beads ELISA

The time courses for the association of the phage mixture from the output in the third round of biopanning to the antigen were compared with that of the original antibody. The solution of the original phage antibody or the phage mixture was added to the beads coated with RNase A and the suspension was rotated gently at 30 °C. At different time intervals, an aliquot of the suspension was withdrawn and the beads were quickly washed with PBS three times. The HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was added to the beads and then incubated at room temperature for 1 h. After washing the beads with PBST three times, 100 μl of BM blue (Roche Applied Science) was added. After incubation for 20 min at room temperature, the reaction was stopped by adding 50 μl of 1 M H_2SO_4. The beads were removed by filtration and the absorbance of the solution at 450 nm was measured.

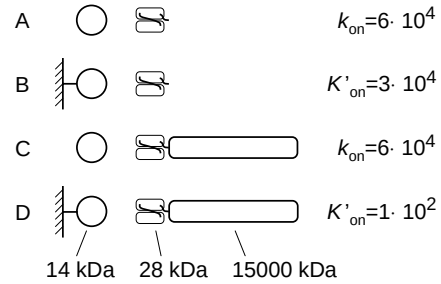

Fig. 2. Association rate constants. (A) The association rate constant of the antibody in soluble form to the antigen in solution (determined by ELISA [21]). (B) The apparent association rate constant of the antibody in soluble form to the antigen immobilized on the solid phase (determined by the Biacore [9]). (C) The association rate constant of the phage antibody to the antigen in solution (determined by ELISA [21]). (D) The apparent association rate constant of the phage antibody to the antigen immobilized on the solid phase (determined in the present study). The numbers represent the k_{on} values in units of $M^{-1}\,s^{-1}$ (at 30 °C in PBS).

3. Results

3.1. The kinetic model

To construct a kinetic model of antibody–antigen interaction in biopanning, we considered the following three points. First, when an antigen is immobilized on a solid support, only part of the antigen molecules that are immobilized on the solid phase are effective for phage binding because access to part of the epitope is hindered by the solid phase, the antigen molecules themselves, or blocking proteins [9]. In addition, in the case of the antigen being biotinylated and immobilized on streptavidin beads, part of the antigen molecules might be biotinylated at the epitope, and in the case of the antigen being immobilized on a dish or a bead by passive adsorption, part of the antigen molecules on the solid phase are denatured [9,11,12]. Thus, the concentration of the antigen used in the model must be that which is effective for antibody binding but not the total concentration of antigen. Second, the association rate of antibody molecule to antigen molecule depends mainly on the diffusion rate of each molecule [13,14]. When an antibody displayed on the surface of a phage, a huge filamentous molecule, reacts with its antigen that is immobilized on a solid support, the apparent association rate of the reaction should be much lower than that when the antibody and/or the antigen exist in solution (Fig. 2). Thus, the association rate constant used in the kinetic model must be that shown in Fig. 2D (see Sections 3.3 and 4.2 for details). Third, the dissociation rate of phage antibodies from the solid phase is affected not only by the dissociation rate constant of the antibody from the antigen but also by non-specific interactions between the entire molecule of the phage antibody and the solid phase. One phage particle has more than 2000 molecules of gene 8 product [4] and the density of the antigen is very high on the solid phase. If there is a non-specific interaction between the gene 8 product and the antigen (or the solid support), even if the interaction is weak, the overall interaction between the entire molecule of the phage antibody and the solid phase cannot be ignored because of its avidity effect. Consequently, the apparent dissociation rate of the phage antibody from the immobilized antigen might be lower than the dissociation rate in the event of the antibody reacting with the antigen in solution.

Considering the three points discussed earlier, when a phage antibody reacts with an immobilized antigen, the change in the concentration of the phage antibody–antigen complex is expressed as:

$$\frac{dX}{dt} = k'_{on} P^{free} C^{free} - k'_{off} X \tag{3}$$

where X is the concentration of the phage antibody–antigen complex, P^{free} the concentration of the free phage antibody, C^{free} the concentration of the free antigen that is effective for the phage antibody binding, k'_{on} the apparent association rate constant of the phage antibody to the immobilized antigen, and k'_{off} the apparent dissociation rate constant of the phage antibody from the immobilized antigen. Given the mass balance of the phage antibody and the effective antigen, the following equations can be devised:

$$P = P^{free} + X \tag{4}$$

$$C = C^{free} + X \tag{5}$$

where P is the total concentration of the phage antibody and C the total concentration of the effective antigen. Since C is much higher than P in biopanning, X is negligible in Eq. (5). Thus, C^{free} can be approximated to C. Therefore, Eq. (3) can be rewritten as:

$$\frac{dX}{dt} = -(k'_{on} C + k'_{off}) X + k'_{on} PC \tag{6}$$

The concentration of the complex at time t is given by integrating Eq. (6) (when $t = 0$, $X = 0$):

$$X = \frac{k'_{on}CP\{1 - \exp[-(k'_{on}C + k'_{off})t]\}}{k'_{on}C + k'_{off}} \quad (7)$$

Therefore, the binding fraction of the phage antibody (ratio of the bound phage to the total phage) after the binding process is given as:

$$F = \frac{X_b}{P} = \frac{k'_{on}C\{1 - \exp[-(k'_{on}C + k'_{off})t_b]\}}{k'_{on}C + k'_{off}} \quad (8)$$

where t_b is the time for the binding process. In the washing process, the complex begins to dissociate because the washing buffer contains no phage antibody ($P^{free} = 0$ in Eq. (3)). Therefore:

$$\frac{dX}{dt} = -k'_{off}X \quad (9)$$

The concentration of the complex after the washing process is given by integrating Eq. (9):

$$X = X_b \exp(-k'_{off}t_w) \quad (10)$$

where t_w is the time for the washing process (when $t = 0$, $X = X_b$). Therefore, the final binding fraction of the phage antibody after the washing process is given as:

$$F_f = \frac{X}{P} = \frac{k'_{on}C\{1 - \exp[-(k'_{on}C + k'_{off})t_b]\}}{k'_{on}C + k'_{off}} \exp(-k'_{off}t_w) \quad (11)$$

When the apparent rate constants (k'_{on} and k'_{off}) and the effective antigen concentration (C) are given, one can calculate the binding fraction of the phage antibody at the binding time (t_b) and the washing time (t_w) based on Eq. (11).

3.2. Preparation of the antigen-coated beads and estimation of the effective antigen concentration

The polystyrene paramagnetic beads (2.6 mg) were washed three times with 0.1 M sodium carbonate buffer (pH 9.6) and were then incubated in 0.1 ml of 4.5 μM RNase A in the same buffer at 4 °C overnight with gentle rotation. The beads were washed three times with PBS and then incubated in PBSM (PBS containing 2% Block Ace) at 30 °C for 3 h with gentle rotation. Under these conditions, most of those antigen molecules in the reversibly adsorbed form are removed from the solid phase such that the loss of the phage antibody–antigen complex by desorption from the solid phase during binding and washing becomes negligible [9]. When the resultant beads are suspended in 1 ml, the effective antigen concentration was estimated to be 5×10^{-7} M.

3.3. Estimation of the apparent rate constants of the original phage antibody

The affinity-purified original phage antibody (5×10^6 cfu) was incubated in a suspension of beads coated with the antigen ($C = 5 \times 10^{-7}$ M) at 30 °C for 2 h ($t_b = 7200$ s). The beads were washed with PBS every 2 min during the first 10 min and then every 10 min. The bound phage was titrated every 10 min, after which each binding fraction (bound phage/input phage) was calculated. The k'_{off} value and the binding fraction of the original phage antibody at $t_w = 0$ s were determined to be 2×10^{-4} s^{-1} and 1.8×10^{-1} by regressing the time course of the binding fraction to Eq. (10). Since the binding fraction of the original phage antibody at $t_w = 0$ s is the same as that at the end of the binding process ($t_b = 7200$ s), and as C and k'_{off} are given, the k'_{on} value was calculated as being 1×10^2 M^{-1} s^{-1} (at 30 °C in PBS) based on Eq. (8).

3.4. Design of an appropriate set of experimental conditions for on-rate selection

To isolate phages displaying an antibody with a high k_{on} value from the customized library, an appropriate set of the operating parameters, t_b, t_w and C was determined by simulations based on the kinetic model.

3.4.1. Determination of the binding time (t_b)

A customized phage antibody library constructed by introducing random amino acid sequences into the hypervariable regions of the original phage antibody should contain phages displaying an antibody with a range of k_{on} and k_{off} values for its antigen. We assume a desirable antibody with the same k'_{off} value as the original antibody but with a k'_{on} value that is 30 times higher than the original antibody. When the k'_{on} and k'_{off} values for the original phage antibody are given and C, t_b and t_w are set, the final binding fractions of the original and the desirable phage antibodies (F_{org} and F_{des}, respectively) can be calculated based on Eq. (11). To isolate phages displaying an antibody with a higher k_{on} value than that of the original antibody, the enrichment of the desirable phage antibody to the original phage antibody that is defined as:

$$E = \frac{F_{des}}{F_{org}} \quad (12)$$

should be as high as possible. Fig. 3 shows the effects of the binding time on the enrichment for different effective antigen concentrations. The enrichment decreases with the binding time, with the decrease being rapid when there is a high concentration of effective antigen. Considering the time required for the experimental operations of the binding process, the binding time was determined to be 1 min.

3.4.2. Determination of the washing time (t_w)

In the washing process, the degree of dissociation of bound phages from the solid support depends on the incubation time of the solid support in the washing buffer even though its efficacy in removing unbound phages depends on the number of washing operations (removal and addition of the washing buffer). In conventional off-rate selection,

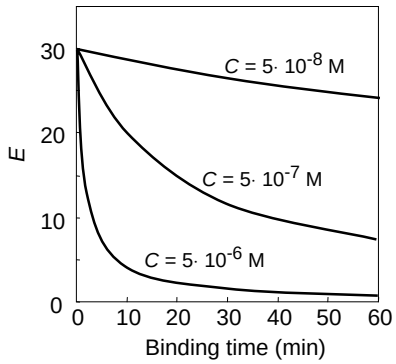

Fig. 3. Effect of binding time on enrichment. The enrichments of the phage displaying the desirable antibody (the k'_{on} value is 30 times higher than that of the original phage antibody) was simulated based on Eq. (8).

phages displaying an antibody with a low k_{off} value are enriched by the extension of the washing time because phages displaying an antibody with a higher k_{off} value dissociate more from the solid support during the washing process. Putting this another way, however, it can be said that extending the washing time eliminates phages with high k_{on} values. Fig. 5 shows the effects of the washing time on the final binding fractions of phages displaying an antibody with the same K_A value but with a different k_{on} value. The final binding fraction of the phages displaying an antibody with a high k_{on} value decreases with the washing time. The decrease is also observed when the binding time is long (Fig. 4, broken lines). This means that off-rate selection (when both the binding and the washing time are long) eliminates phages displaying antibodies with a high k_{on} value. These results indicate that the washing time should be as short as possible for the on-rate selection. However, non-specific phages should be removed as far as possible by the washing operation. To reconcile the short washing time with the removal of non-specific phages, we changed the washing buffer five

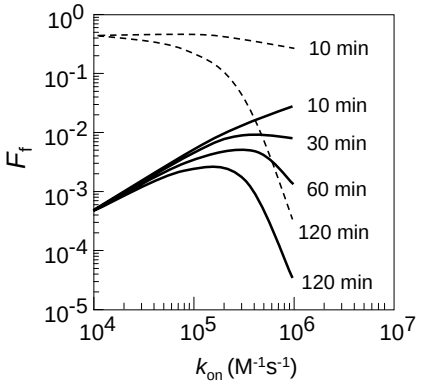

Fig. 4. Effects of washing time on final binding fraction of phage antibodies with different k_{on} values. The final binding fractions of phages displaying an antibody with a K_A value of $10^9\,M^{-1}$ were simulated based on Eq. (11). The k'_{on} values of each phage were assumed to be 1/600 of the k_{on} value of each antibody in soluble form. Solid lines: $t_b = 1$ min; broken lines: $t_b = 15$ h. $C = 5 \times 10^{-7}$ M. Numbers represent the washing time.

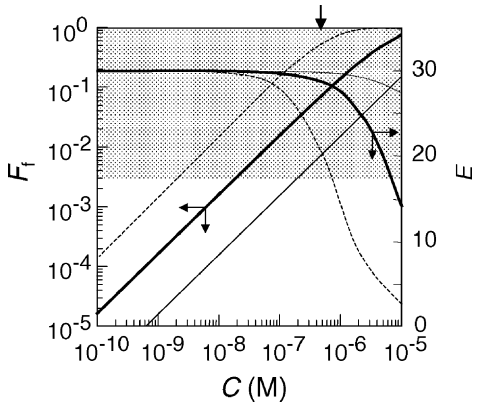

Fig. 5. Effects of effective antigen concentration on enrichment and final binding fraction of phage displaying desirable antibody. The shaded area represents $F_f > 3 \times 10^{-3}$. $t_b = 1$ min, $t_w = 10$ min, $k'_{off} = 2 \times 10^{-4}\,s^{-1}$, $k'_{on} = 1 \times 10^1\,M^{-1}\,s^{-1}$ (thin lines), $k'_{on} = 1 \times 10^2\,M^{-1}\,s^{-1}$ (bold lines), $k'_{on} = 1 \times 10^3\,M^{-1}\,s^{-1}$ (broken lines).

times in 10 min (It is possible to recover the magnetic beads within 1 min by laying the sample tube on a magnet).

3.4.3. Determination of the effective antigen concentration (C)

Since it is known that not all the phages display an antibody in the phage antibody libraries [15], the number of each phage clone involved in an antibody library is expressed as:

$$N_0 = I\frac{R}{D} \tag{13}$$

where I is the input phage number, R the ratio of a phage displaying an antibody molecule to the phage encoding the antibody gene, and D the diversity of the library. The purpose of biopanning is the isolation of phage clone(s) with desirable properties. In other words, at least one phage molecule must be recovered for each clone. Thus, the unequal equation:

$$N_0 F_f \geq 1 \tag{14}$$

must be satisfied. The diversity of the present library was determined to be 7×10^7, and the display ratio of the original phage antibody was determined to be 0.025. Since we input 10^{12} phages in the biopanning experiment, the quantity of each clone was calculated to be 4×10^2, assuming the display ratio of the phage library to be the same as that of the original phage antibody. In our case, therefore, the final binding fraction must be greater than 3×10^{-3} (the shaded area in Fig. 5) and should preferably be greater than 10^{-2} to enable the reproducible recovery of the desirable phage clones. As shown in Fig. 5, the final binding fraction increases with an increase in the effective antigen concentration, but the enrichment decreases considerably at an effective antigen concentration of more than 10^{-6} M. When the antigen-coated beads were suspended in the library solution at a concentration of 2.6 mg/ml, the effective antigen concentration was estimated to be 5×10^{-7} M as described

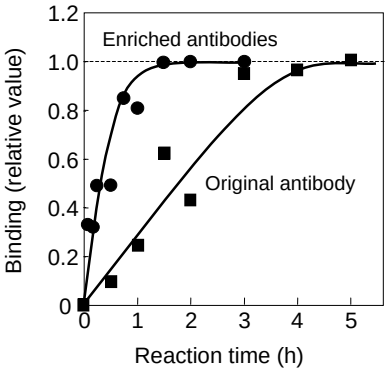

Fig. 6. Comparison of reaction rates. The mixture of the enriched antibodies were amplified directly from the output of the third round. The time courses of the relative binding fraction of the mixture and the original phage antibody to the antigen immobilized on the beads were compared. $C = 5 \times 10^{-7}$ M; phage concentration $= 10^{11}$ cfu/ml.

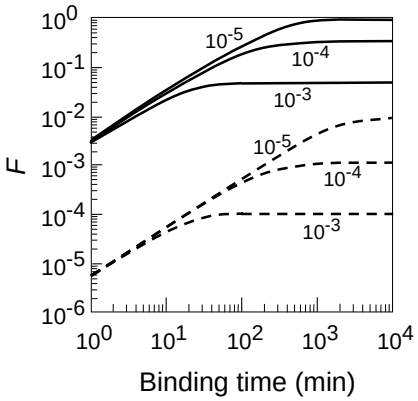

Fig. 7. Time course of binding fraction of assumed phage antibodies during binding. The numbers represent the k'_{off} values. $k'_{on} = 1 \times 10^2\ M^{-1}\ s^{-1}$, $C = 5 \times 10^{-7}$ M (solid lines), $C = 1 \times 10^{-9}$ M (broken lines).

earlier. Since this concentration provided both a high level of enrichment and sufficient recovery of each phage clone, we used the coated beads as is for the on-rate selection

3.5. Results of on-rate selection

Three rounds of biopanning were performed for the customized library under the conditions determined rationally as described earlier ($t_b = 1$ min, $t_w = 10$ min and $C = 5 \times 10^{-7}$ M). The reaction rate with the antigen of the phage mixture amplified from the output of the third round was compared with that of the original phage antibody by the beads ELISA method described in the methods section. As shown in Fig. 6, the phage mixture amplified from the output of the third round reacted with the antigen faster than the original phage antibody. Although the antigen concentration and the phage quantity were identical in this experiment, the quantity of antibody could differ because the display ratio of each phage could differ. Therefore, we isolated 10 phage clones and estimated the equilibrium association constant (K_A) and the association rate constant (k_{on}) of each phage antibody to the antigen in solution. As shown in Table 1, all the isolated phage antibodies had higher K_A values because they possessed higher k_{on} values than the original phage antibody.

Table 1
Kinetic parameters of isolated antibodies

Clone	K_A ($\times 10^8\ M^{-1}$)	k_{off} ($\times 10^{-3}\ s^{-1}$)	k_{on} ($\times 10^5\ M^{-1}\ s^{-1}$)	Fold
1	6.4 ± 1.0	1.4 ± 1.2	9.0 ± 3.3	15
2	2.2 ± 0.3	1.7 ± 1.0	3.8 ± 1.7	6
3	4.0 ± 0.5	3.3 ± 0.6	13.2 ± 0.9	22
4	1.4 ± 0.1	2.9 ± 0.4	3.9 ± 0.2	7
5	3.2 ± 0.3	1.2 ± 0.3	3.8 ± 0.5	6
6	2.3 ± 0.3	0.7 ± 0.1	1.6 ± 0.1	3
7	2.6 ± 0.2	0.8 ± 0.3	2.1 ± 0.5	4
8	3.9 ± 0.3	2.4 ± 0.5	9.4 ± 1.4	16
9	3.2 ± 0.3	5.5 ± 1.2	17.6 ± 2.4	29
10	2.7 ± 0.3	6.3 ± 1.1	16.8 ± 1.3	28
Original	0.24 ± 0.07	2.5 ± 0.9	0.60 ± 0.06	1

K_A and k_{on} were determined by ELISA at 30 °C in PBS and k_{off} was calculated based on Eq. (2). Error represent the 95% confidence limit of each regression.

4. Discussion

4.1. The present kinetic model

Mandecki et al. established the first model for a biopanning process in 1995, describing the relationship between the binding fraction of a phage antibody and the total concentration of its antigen based on the Langmuir adsorption isotherm [16]. That same year, Kretzschmar et al. introduced a simple relationship between the binding fraction and the total concentration of the antigen based on mass balance under a state of equilibrium [17]. Based on these two studies, Levitan established a stochastic model and calculated the relative output of a clone assuming an affinity distribution in a library [18]. In our previous paper [9], we proposed a kinetic model that considered the effective antigen concentration and desorption of antigen from the solid phase. In our previous model, we assumed equilibrium in the binding process and we used an apparent dissociation constant, calculated based on the apparent association and dissociation rate constants of the antibody in soluble form for the antigen immobilized on a Biacore sensor chip. However, the apparent association rate constant of the phage antibody to the immobilized antigen was found to be 300 times lower than that of the antibody in soluble form to the immobilized antigen (Fig. 2). As shown in Fig. 7, when the k'_{on} value is $10^2\ M^{-1}\ s^{-1}$, a phage antibody with a k'_{off} value of $10^{-5}\ s^{-1}$ requires 10 h in the case of $C = 5 \times 10^{-7}$ M and 63 h in the case of $C = 1 \times 10^{-9}$ M to reach 90% equilibrium, whereas a phage antibody with a k'_{off} value of $10^{-3}\ s^{-1}$ requires less than 40 min in both case. Therefore, we can conclude

that equilibrium is not achieved in most cases of biopanning. Here, we propose a fully dynamic model for the entire biopanning process using the apparent kinetic constants of the phage antibody for the immobilized antigen (k'_{off} and k'_{on}) and the effective antigen concentration (C). When one determines experimentally (or assumes) the apparent rate constants of a phage antibody and sets C, one can simulate the time course of the binding fraction of the phage antibody based on the model. To simplify this model, however, the dissociation of the antigen (or the antibody–antigen complex) from the solid phase is not considered because of the reasons described further, even though it was incorporated into our previous model. In biopanning, the antigen is immobilized on the solid phase by passive adsorption or by using a biotin–streptavidin system. In the former case, desorption is negligible when the blocking time is sufficient [9]. In the latter case, since the dissociation rate constant of biotin from streptavidin is extremely low ($2.4 \times 10^{-6}\,s^{-1}$) [19], the dissociation of the biotinylated antigen from streptavidin is negligible when streptavidin is immobilized covalently on a solid phase.

4.2. Apparent association rate constants of the phage antibody to the immobilized antigen

Since antibody–antigen interactions are usually diffusion-limited interactions [13,14], the association rate of the reactions depends mainly on the diffusion rate of the smaller molecule of the partner. When an antibody is displayed on the phage, the diffusion rate of the phage antibody should be much lower than that of the antibody in soluble form because the phage is a huge filamentous molecule with a molecular mass of over 10^7 Da [4]. In those cases in which an antibody reacts with its antigen in solution, the association rate of a phage antibody does not differ so much from that of the same antibody in soluble form because the antigen diffuses freely (here we assume a soluble globular protein with a typical size as the antigen). In our case, the true association rate constant of the original antibody (as a soluble single chain Fv form, 28 kDa) to the antigen (RNase A, 14 kDa [20]) in solution and that of the original phage antibody were both estimated to be $6 \times 10^4\,M^{-1}\,s^{-1}$ (Fig. 2A and C, respectively) [21]. In contrast, in those cases in which an antibody reacts with its antigen immobilized on a solid support, the association rate depends on the diffusion rate of the antibody. In our case, the apparent association rate constant of the original phage antibody to the immobilized antigen (Fig. 2D) was estimated to be $1 \times 10^2\,M^{-1}\,s^{-1}$. This value was 300 times lower than that of the antibody in soluble form to the immobilized antigen (Fig. 2B). Similar results were reported in the case of biotinylated DNA binding to streptavidin immobilized on a particle. Huang et al. observed that the apparent association rate constant of 5000 bp DNA is about 20 times lower than that of 100 bp DNA [22].

The k'_{off} value of the original phage antibody from the immobilized antigen was estimated to be $2 \times 10^{-4}\,s^{-1}$, which was one order of magnitude lower than the k_{off} value of the original phage antibody from the antigen in solution. This difference could be caused by electrostatic interaction between the antigen that is immobilized on the solid support at a high density and the major coat protein, g8p, of the phage. This may be because the antigen, RNase A, is a basic protein (isoelectric point = 9.6) [20] and the N-terminus region of g8p that is exposed on the surface of the phage contains three acidic amino acid residues [4].

4.3. The effects of the error of the k'_{on} on the quality of the designed experimental conditions

Although the association rate constants should first be determined under various concentrations of their antigens, we estimated the apparent association rate constant of the original phage antibody to the immobilized antigen under a concentration of the antigen. In addition, although we assume that all molecules of the affinity-purified phage display the antibody molecule, it is difficult to confirm that all the antibody molecules displayed on the phage have the same affinity with the original antibody. Considering the probability that the estimated apparent association rate constant might contain a significant error, we examined the effects of the error on the quality of the designed experimental conditions. We assumed two cases in which the actual k'_{on} value is 10 times lower and higher than the estimated k'_{on} value. In both cases, as shown in Fig. 5, the designed experimental conditions ($t_b = 1$ min, $t_w = 10$ min and $C = 5 \times 10^{-7}$ M) were found to give both a sufficiently high enrichment and an acceptable recovery of the desirable antibody. In conclusion, biopanning under the designed experimental conditions enriches the desirable phages even if the estimated k'_{on} value contains a significant error.

4.4. Properties of the isolated phage antibodies

After three rounds of on-rate selection, 10 independent phage clones were isolated and the K_A and the k_{on} of each phage antibody to the antigen in solution were determined according to the methods devised by Stevens [23] and Zhuang et al. [21], respectively. The derived K_A and k_{on} are the true association constant and the true association rate constant, respectively, because the antibody and the antigen react in solution (neither of them is immobilized on solid support) in these methods. As shown in Table 1, the K_A values of the isolated phage antibodies were 6–37 times (average 13 times) higher than that of the original phage antibody. The k_{on} values of the isolated phage antibodies were 3–30 times (average 14 times) higher than that of the original phage antibody. Since the k_{off} values of the isolated phage antibodies were almost the same as those of the original phage antibody, the isolated phage antibodies have higher K_A values by possessing higher k_{on} values than the original antibodies. The phage antibody with the highest k_{on} value among the isolated phage antibodies has a

k_{on} value of $(1.76 \pm 0.24) \times 10^6\,M^{-1}\,s^{-1}$ which is 29-fold higher than that of the original phage antibody. Since it is known that the k_{on} value of natural antibodies ranges from 10^4 to $10^6\,M^{-1}\,s^{-1}$ [1,2], it can be concluded that we succeeded in isolating one of the phage antibodies with the highest k_{on} value from the phage library. It was reported that electrostatic interaction between antibody and antigen accelerates the association [24]. However, some isolated antibodies have no acidic amino acid residue in the randomized region while a few isolated antibodies have one or two acidic amino acid residue (RNase A is a basic protein). To explain the causes of increase in the association rate constant, therefore, increase in the degree of accessibility of the antigen to the binding site of the antibody and/or other factors should be also considered. Although the specificity of the isolated antibodies to the antigen should be further examined, the present results demonstrate that the designed experimental condition were appropriate for the on-rate selection. Duenas et al. reported a selection of phage antibodies based on kinetic constants from a model library consisting of six different phage antibodies [25]. They showed experimentally that a short incubation (less than 30 min) of the model library solution with an antigen enriches a phage antibody with a high association rate constant. In their SAP selection system, however, since the phage antibodies react with a fusion protein of the antigen and g3p in solution, the apparent association rate constants should be much higher than those in the case that phage antibodies react with an antigen immobilized on a solid support. In addition, they did not show the absolute concentration of the antigen used for the selection. Therefore, their experimental conditions would not be applicable to conventional selection systems in which an antigen is immobilized on a solid support.

4.5. Applicability of the on-rate selection to universal phage antibody libraries

In the present study, we applied on-rate selection to the customized library constructed for an antigen, RNase A, and succeeded in isolating the phages displaying a practical and desirable antibody. The on-rate selection should be also applicable to the universal libraries constructed for various antigens. If, as has been proposed, a library of 10^5–10^6 had sufficient shape space to recognize all the antigens, one would expect 10^3–10^4 different binding clones per antigen from a universal library with a diversity of 10^9 [26,27]. There would be phages displaying an antibody with a high k_{on} value among the clones. Therefore, it should be possible to isolate phages displaying the practical and useful antibody from universal phage antibody libraries like the Griffiths library [28]. The same set of experimental conditions with the present study should be applicable for biopanning of universal libraries. In the first round of biopanning for universal libraries, however, it must be noted that the numbers of each phage displaying an antibody are small. Even when one inputs 10^{12} cfu of a phage antibody library with a diversity of 10^9, N_0 is calculated to be only 10^2 (in the case of $R = 0.1$) or less. To ensure the reproducible recovery of positive clones, the final binding fraction should be above 10^{-1} in this case. Thus, as shown in Fig. 7, C should be high ($\geq 10^{-6}$ M) and t_b should be long (overnight). Although, in the present study, we immobilized the antigen by passive adsorption, it was reported that the immobilization of antigen by a biotin–streptavidin system is much better than that attained by passive adsorption to gain the effective antigen concentration [9]. In the second and subsequent rounds of biopanning, one can set the appropriate set of the experimental conditions because N_0 is expected to be much higher than that in the first round.

4.6. Comparison between on-rate and off-rate selection

Since $K_A = k_{on}/k_{off}$ at the equilibrium, we can say that off-rate selection reduces the denominator and on-rate selection increases the numerator. For nine kinds of assumed phages, each of which displays an antibody with a k_{on} value of 10^4, 10^5 or $10^6\,M^{-1}\,s^{-1}$ and with a k_{off} value of 10^{-3}, 10^{-4} or $10^{-5}\,s^{-1}$, the final binding fractions after one round of a typical "off-rate selection" and our "on-rate selection" were compared (Fig. 8). As expected, the final binding fractions of the phages displaying an antibody with k_{on} value of $10^6\,M^{-1}\,s^{-1}$ were high in the case of on-rate selection, while those with a k_{off} value of $10^{-5}\,s^{-1}$ were high in the case of off-rate selection. If the phage displaying the antibody with the highest affinity constant of 10^{11} (which associates fastest and dissociates slowest) among the nine antibodies is contained in a universal library, it would be isolated both by the on-rate and off-rate selection. However, it is known that the frequency of an antibody against an antigen in an antibody library decreases with an increase in the affinity. Lancet et al. estimated the frequencies of antibodies with K_A values of 10^9, 10^{10} and $10^{11}\,M^{-1}$ are 10^{-7}, 10^{-9} and 10^{-11}, respectively, based on their model [29]. Assuming that this affinity distribution can be applied to the universal library with a diversity of 10^9, one could expect the library to contain 10^{-2}, 10^0 and 10^2 kinds of antibodies with K_A values of 10^{11}, 10^{10} and $10^9\,M^{-1}$, respectively. Therefore, most of phage antibodies involved in the output of a round of biopanning of the library are expected to have K_A values of $10^9\,M^{-1}$ (or less). Among these phage antibodies (the shaded bars in Fig. 8), the on-rate selection enriches the phage displaying the antibody with the k_{on} value of $10^6\,M^{-1}\,s^{-1}$ while the off-rate selection eliminates this antibody. When one wishes to obtain antibodies for an application for which the reaction time is limited, one should employ on-rate selection.

In contrast, when one wishes to obtain antibodies for an application that requires an extensive washing operation, off-rate selection should be employed to avoid a loss of antibodies during the washing operation. It is notable that the efficiency of the off-rate selection is improved by extending the time for the binding process. As shown in Fig. 7, the ratio of the binding fraction of a phage displaying antibody

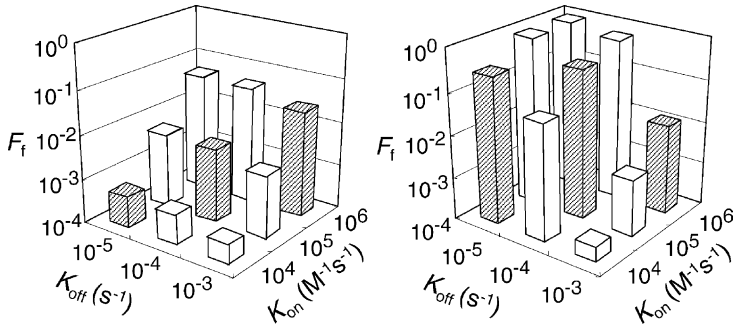

Fig. 8. Comparison of on-rate and off-rate selection. Left, on-rate selection ($t_b = 1$ min, $t_w = 10$ min); right, off-rate selection ($t_b = 15$ h, $t_w = 1$ h). The shaded bars represent the final binding fractions of the phages displaying an antibody with a K_A value of $10^9\,M^{-1}$. $C = 5 \times 10^{-7}$ M. The k'_{on} values of each phage were assumed to be 1/600 of the k_{on} value of each antibody in soluble form. The k'_{off} values of each phage were assumed to be the same as the k_{off} value of each antibody in soluble form.

with a high k_{off} value to that with a low k_{off} value is higher when the time for the binding process is longer. The ratio is also higher when the effective antigen concentration is lower while the binding fraction of the phage displaying antibody with a high k_{off} value decreases. Therefore, the effective antigen concentration should be high in the first round of biopanning because N_0 is low but it should be low in the further round of biopanning if $N_0 F_f \geq 1$ is satisfied. Simply, the N_0 value can be calculated by dividing the input of the present round by the output of the previous round.

4.7. Importance of kinetics in chip technology

As shown in Fig. 1, an increase in the concentration of the antibody–antigen complex depends on the k_{on} value of the antibody. When an antibody with a low k_{on} value is immobilized on a chip, the reaction rate is low, resulting in a low sensitivity for a fixed reaction time, even when the antibody has a sufficiently high K_A value. On the other hand, the sensitivity is high in the case of those antibodies with a high k_{on} value. In addition, the amount of antigen trapped on an antibody with a low k_{on} value is affected directly by the error in the reaction time, which could cause a problem in reproducibility. In contrast, the amount of the antigen trapped on an antibody with a high k_{on} value is less affected by the error in the reaction time (for example, compare the slopes of the antibodies at $t = 1$ h in Fig. 1). In conclusion, in most applications of antibodies, including chip technology, the kinetic parameters of the antibodies must be considered and those antibodies with high association rate constants, as isolated by the present method, should be much more useful than those prepared by conventional methods.

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