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The application of magnetic force differentiation for the measurement of the affinity of peptide libraries

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Abstract

A new method has been developed for measuring the binding affinity of phage displayed peptides and a target protein using magnetic particles. The specific interaction between the phage displayed peptides and the target protein was subject to a force generated by the magnetic particle. The binding affinity was obtained by analyzing the force–bond lifetime.

Keywords: Magnetic tweezers; Phage displayed peptide; Bond lifetime; ELISA; Force measurement; Biotin–PEG; Streptavidin; Protein assay

1. Introduction

The application of ultrasensitive force measurement techniques to single molecule has provided a fundamentally new way to characterize the physical and chemical properties of biological macromolecules [1–3]. Magnetic microparticles are exciting candidates for characterizing the biological bond since they can generate piconewton scale force under an external magnetic field. In the previous measurements, the magnetic particles were used to stretch and twist DNA double helix [4–6]. They have also been used to enhance the specificity and sensitivity of immunochemical assays for proteins, viruses and bacteria using force differentiation [7]. In the assay, the weakly adhesive, nonspecific interactions were identified by displacement of the magnetic particles under an external magnetic field, but the force was not strong enough to rupture the specific interactions.

Peptides serve a crucial role in living systems as hormones, enzymes, neurotransmitters and signal transduction initiators that regulate physiological functions as diverse as growth, reproduction, digestion, blood pressure, inflammation and immunity. These wide range biological activities of peptides make them ideal candidates for new therapeutic drugs. The phage display technique

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has been widely used in finding high affinity peptides for therapeutic purposes. It is a very effective way to produce a large number of diverse peptides and to isolate the peptides with specific affinity [8]. Phage display libraries containing several billion mutants can be constructed simultaneously. These libraries can be easily used to select the phage bearing the peptide with desired binding specificities. Enzyme-linked immunosorbent assay (ELISA) is the most widely used method to determine the enrichment of phage bearing the peptide of interest. However, this method does not give the quantitative measurement of the affinity, such as bond lifetime.

In this paper, we expand the application of magnetic tweezers to the measurement of the binding affinity between phage displayed peptides and a target protein. The use of magnetic particles potentially permits higher resolution screening and the measurement of the binding affinity of several million ligand–receptor pairs in a single experiment. As shown in Fig. 1, each particle can stretch one ligand–receptor pair. A large number of particles can be stressed at the same time such that many ligand–receptor pairs can be stretched simultaneously. We have developed a surface chemistry for the modification of the magnetic particles and polystyrene surface to minimize the nonspecific adsorption. The bond lifetime for each phage displayed peptide was obtained.

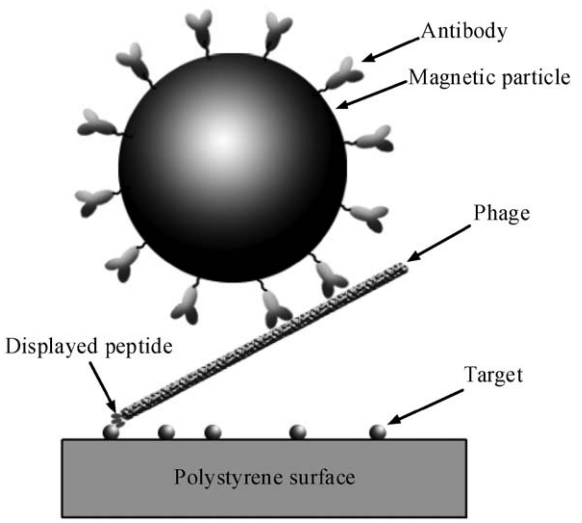

Fig. 1. Schematic presentation of experimental principles.

2. Materials and Methods

2.1. Phage preparation

The phage library was acquired from New England BioLabs Inc. (Beverly, MA) and carried peptides consisting of 12 random amino acids fused to the end of the pIII protein. The library was screened against a target protein following the manufacturer's instructions. The target protein was composed of 27 amino acids with the sequence of SGSWLRDVWDWICTVLTDFKTWLQSKL-K(biotin)-NH_2, which is the amino-terminal 27 amino acids of the hepatitis C NS5A protein [9]. After three rounds of screening, 10 single phage plaques were selected and amplified. The DNA sequences of the phage were determined using dye-terminator method to deduce the sequence of the displayed peptides.

2.2. Surface modification

Commercially available iron oxide–polymer composite microparticles (Seradyn Inc., Indianapolis, IN) were used in this study. These particles have a nominal diameter of 0.78 μm, a magnetic saturation of 30.3 EMU/g, and a density of 1.4 g/cm^3 [10]. The surface of the particles was modified with carboxyl groups that were readily available for covalent coupling of proteins and other molecules. Magnetic particles were functionalized with the antibody against phage major coat protein (anti-pVIII, Amersham Biosciences, Piscataway, NJ) by covalent bond through a poly(-ethylene glycol) (PEG) (Shearwater, Huntsville, AL) monolayer as described previously [7]. The 1 mm height polystyrene reactors or 8-well microtitor strips (Corning Inc., Corning, NY) were modified with biotin–PEG following a similar approach. An antibody against phage pIII protein (Exalpha Biologicals Inc., Watertown, MA) was conjugated with streptavidin using an approach described previously [11].

The surface coverage of antibody on magnetic particles was measured by micro-BCA assay (Pierce, Rockford, IL). A sulfone group, which can be hydrolyzed under basic conditions, is between the PEG linker and the antibody [12]. 1.16×10^9 magnetic particles were suspended in phosphate buffered saline (PBS). The pH of the particle solution was adjusted to 11.6 by adding NaOH. The particles were incubated at 37 °C for 2 h. The solution was separated from the particles by a magnetic separator. The magnetic particles were washed once with PBS and the solutions were combined together. The pH was adjusted to 7 before the BCA assay.

An ELISA assay for different concentrations of streptavidin–anti-pIII conjugate was performed to assess the property of the biotin–PEG surface. The conjugate was diluted in PBS at 1:50, 1:100 and 1:200 ratio, respectively. Each dilution was added to a biotin–PEG modified well and incubated for 1 h followed by rinsing with PBS. The phage was diluted in PBS to 10^9, 10^8, and 10^7 PFU/ml. Each diluted phage was added to an appropriate well, incubated for 1 h, and rinsed with PBS. The anti-pVIII-HRP (Amersham Biosciences, Piscataway, NJ) was diluted in PBS by 1:100 ratio before being added to each well and incubated for 1 h followed by PBS washing. The colometric assay was carried out by following a previous method [13].

2.3. Force assay

A permanent ring magnet composed of neodymium iron boron (NdFeB 35) was used in the experiment. The magnet is 0.375 in. in diameter and 0.060 in. in thickness with a hole of 0.075 in. in diameter at the center of the magnet. The magnetic flux was measured with a teslameter (DTM-133 digital teslameter, GMW associates, San Carlos, CA) with a Hall probe as a function of perpendicular distance from the center of the magnet.

The target protein was diluted in PBST (PBS with 0.05% Tween 20) to 1 μg/ml and added to the shallow polystyrene reactor modified with PEG–streptavidin. The target protein was incubated in the reactor for 1 h at room temperature followed by copious washing with PBST. The phage was diluted in PBST to 1×10^9 PFU/ml. The phage solution was added to the reactor and incubated for 2 h at room temperature followed by copious washing with PBST. The anti-pVIII modified magnetic particles were diluted to 1×10^8 particles/ml with PBS prior to use. The diluted particles were added to each reactor. The reactor was placed 2 cm above a block magnet for 25 s and further incubated for 20 min at room temperature. The magnetic particles were tracked by an inverted optical microscope (Nikon TE300 with $40 \times$ ELWD lens) equipped with a video CCD camera (Sony, DXC-107A, Japan) and an XYZ motorized stage (Prior, Rockland, MA). Images were captured before and after the magnetic force was applied. The ring magnet, controlled by a micromanipulator, was placed 3 mm above the reactor surface. As soon as the magnetic force was applied, the images were captured at 1 s interval for 60 s and at 10 s interval for another 15 min using digital acquisition software (MetaVue, Universal Imaging, Downingtown, PA) with a viewing area of $220 \times 165\,\mu m^2$. The number of the magnetic particles was counted by Scion Image (Scion Corporation, Frederick, MA).

3. Results and Discussions

3.1. Force differentiation assay technology

Fig. 1 presents the experimental principles of force assay. Magnetic particles were used to apply a force to the bond between the phage and the target protein. This assembly was constructed from three layers. First, the target protein was immobilized on the PEG modified polystyrene surface through a streptavidin-biotin bond. Second, the phage was incubated with the target modified surface to allow bond formation. Third, the magnetic particles with densely coated anti-pVIII antibody were added to the surface. The phage can form multiple bonds with the particle due to the multiple binding sites of anti-pVIII on the surface of the phage. The weakest point along this chain was the bond between the phage displayed peptide and the target because the single bond was formed at this point. As a result, when the magnetic force was used to lift the particle, the

bond between phage displayed peptide and the target was most likely to be ruptured.

Fig. 2 shows the ELISA results. The ELISA assay shows that the streptavidin–anti-pIII conjugate was able to bind the phage specifically, and the PEG modified well had excellent ability to resist nonspecific binding [14,15]. Several trends are of interest. First, the 1:50 and 1:100 conjugate dilution produced a similar color intensity, which is indicative of the amount of phage bound to the surface, suggesting that the maximum binding capacity has been reached at 1:100 dilution. Second, the color intensity decreased as the phase was diluted, which suggests a single bond is formed between the anti-pIII antibody and the phage. For the blank row, in which no streptavidin–anti-pIII was added, the wells had color intensity of 60, which appeared clear to the naked eye. This result shows the PEG layer resists the nonspecific binding of phage and proteins.

The antibody density on the magnetic particles was measured to be 1.57×10^4 antibody/particle, which was equivalent to $106\,\mathrm{nm}^2$/antibody. Structural analysis of an antibody suggests each molecule can occupy approximately $40\,\mathrm{nm}^2$. Simple geometric analysis suggests that the antibody density reached 40% of a monolayer on the surface.

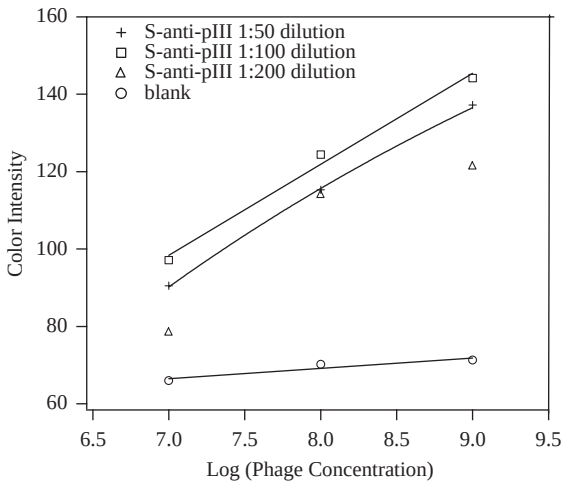

Fig. 2. Result of phage ELISA. S-anti-pIII represents streptavidin–anti-pIII conjugate. The scanned image was processed in Adobe Photoshop. The average color intensity in each well was measured on a scale of 0–255.

3.2. Magnetic force

The relationship of distance and the force generated by the magnetic particles in the magnetic field was calculated by

$$F = VM\,\frac{\mathrm{d}B}{\mathrm{d}x}, \tag{1}$$

where F is the force, V is volume of the magnetic particle, and M is volumetric magnetization of the magnetic particle and B is the magnetic induction. It is well known that the magnetic flux along the axial of a ring magnet has the analytical form

$$B = \frac{B_r}{2}\left\{\left[\left(\frac{L+x}{\sqrt{R^2+(L+x)^2}}\right)-\left(\frac{L+x}{\sqrt{r^2+(L+x)^2}}\right)\right] - \left[\left(\frac{x}{\sqrt{R^2+x^2}}\right)-\left(\frac{x}{\sqrt{r^2+x^2}}\right)\right]\right\}, \tag{2}$$

where B_r is the residual induction ($B_r = 12,300$ Gauss for NeFeB 35), L is the thickness of the magnet, x is the distance away from the magnet surface along the axial where B is calculated, r is the inner radius of the ring, and R is the outer radius of the ring. The calculated and measured magnetic flux gradient are presented in Fig. 3. The maximum force generated by the ring magnet on a

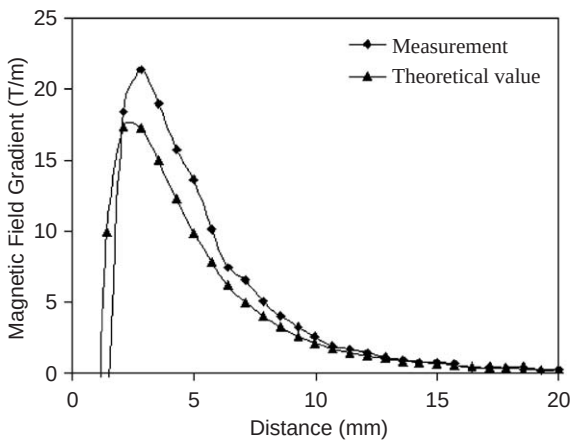

Fig. 3. Magnetic field gradient vs. distance of the ring magnet.

single magnetic particle is about 0.1 pN at 3 mm above the magnet.

3.3. Bond lifetime under magnetic force

Fig. 4 shows the typical optical images of a reactor surface before and after the magnetic force is applied to the magnetic particle–phage–target protein assembly. Most of the particles leave the surface after a 0.1 pN magnetic force has been applied for 15 min. Only the particles bound by strong interactions still remain on the surface.

If all the particles have the same bond lifetime, we would expect that the number of bound particles decreases exponentially with time to have the form

$$N(t) = N_{\text{bound}}(0)\exp\left(-\frac{t}{\tau}\right), \tag{3}$$

where $N(t)$ is the bound particle number at time t, $N_{\text{bound}}(0)$ is the bound particle number at time zero and τ is the bond lifetime [16]. The time zero is defined as the point when the magnet force is applied. At time zero, some of the particles bind to the surface by the specific interactions, while other particles sit on the surface without binding. We cannot differentiate these two types of particles from the images. As a result, both bound and unbound particles were counted. The parameter a is introduced and defined as initial binding fraction, which is the ratio of initial number of bound particles to the total number of particles on the surface, $a = N_{\text{bound}}(0)/N(0)$. The parameter a is proportional to the phage number on the surface and is characteristic of the binding affinity. This equation can be expressed in terms of the fraction of bound particles

$$\ln(f) = -\frac{1}{\tau}\times t + \ln(a), \tag{4}$$

where $f = N(t)/N(0)$ is the ratio of the particle number at time t to the particle number at time zero.

The results were plotted as $\ln(f)$ against t in Fig. 5. The slope and intercept were obtained from the linear least square fit. The bond lifetime and initial bind fraction were calculated. The results are summarized in Table 1. The bond lifetime analysis indicates that the p1 phage has a 62% longer lifetime than p2 phage. However, the significant variation was observed in the bond lifetime of a given phage. A t-test confirmed that the bond lifetime for p1 phage was longer than that of p2 phage with a $p = 0.026$.

The bond lifetimes measured in this work are significantly longer than the previous measurements [16,17]. We believe that the difference may result from the size of the phage. That is, the phage is an object approximately 930 nm in length and 6.5 nm in diameter, which will result in a significant damping effect that is not accounted for in the bond rupture model implicit to equation (3).

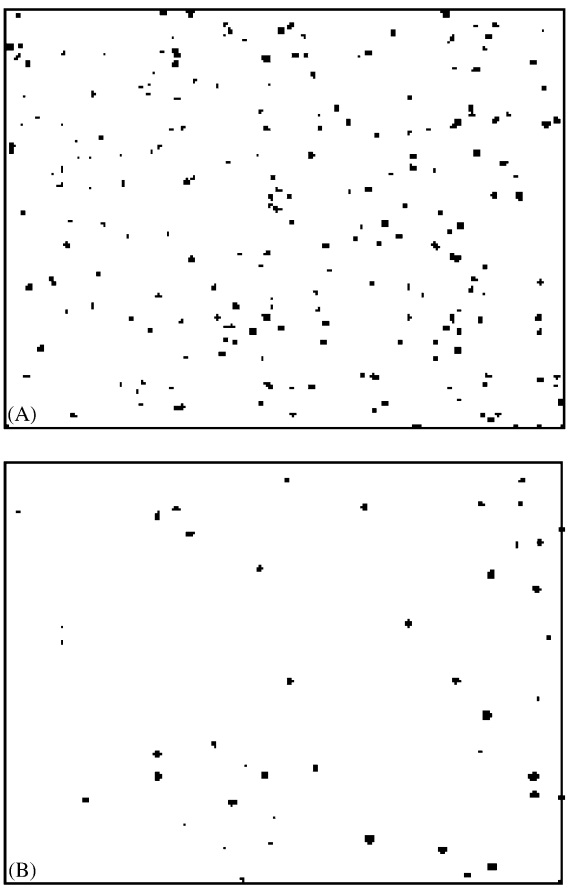

Fig. 4. Images of the reactor surface before (A) and after (B) the magnetic force was applied. The images were taken at the same position.

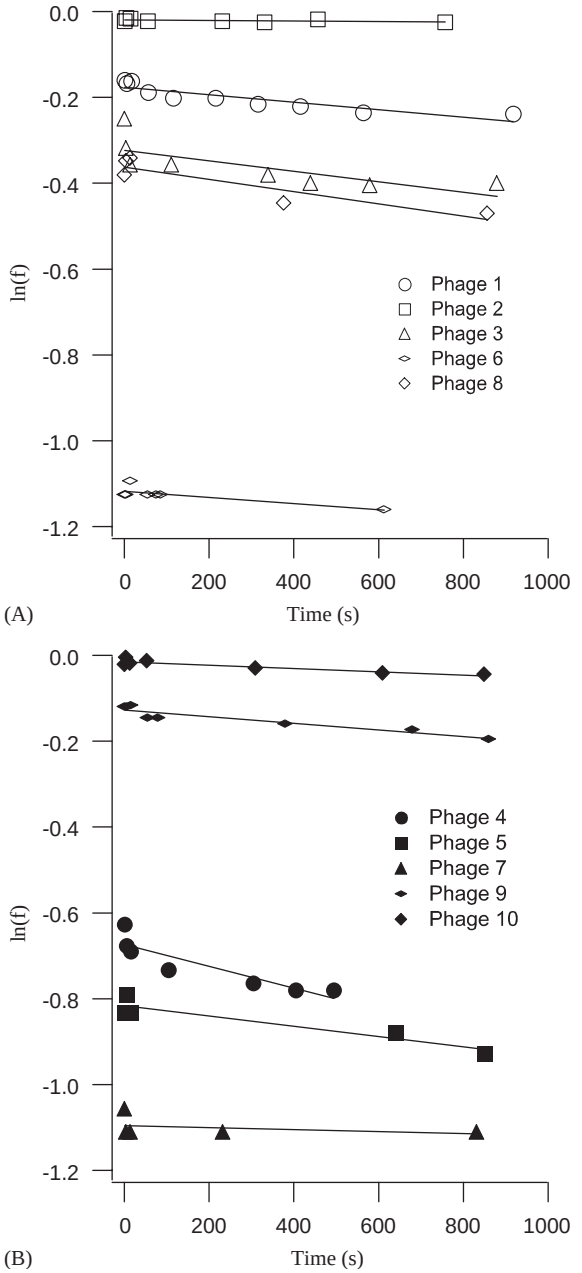

Fig. 5. The ln(f) vs. Time curve. Phage 1, 2, 3, 6, and 8 carry the same peptide, which is named p1 (A). Phage 4, 5, 7, 9, and 10 carry another peptide, which is named p2 (B).

The initial binding fraction is proportional to the number of phage on the surface. The phage concentration used in these assays was uniform, thus we would expect the same coverage of phage on the surface if the peptide–target protein affinity was constant. In Table 1 and Fig. 5, the p1 phage has on average a 10% higher initial binding fraction than p2 phage, which also suggests that p1 phage forms a stronger bond than p2 phage. The initial binding fraction result is consistent with the analysis of the bond lifetime and proves that the p1 phage can form stronger bond than p2 phage. However, the difference in the initial binding fractions of the phage was found to vary significantly, which we attribute to the variation in the coverage of phage across the reactor surface due to hydrodynamic factors.

Table 1
The bond lifetime and initial binding fraction of p1 phage and p2 phage. s is standard deviation

	Bond Lifetime ($\times 10^{-4}$s)	s	Initial Binding Fraction (%)	s
p1	1.59	1.05	71.2	7.3
p2	0.98	0.41	61.3	4.6

Although these results clearly demonstrate that force assay can be applied to screening phage libraries, there was significant variation in the bond lifetime. Other single molecule techniques suggest the force used in this experiment is weak compared with the strength of the bond [17]. These studies indicate that the antibody–antigen interactions can produce forces as strong as 40 pN. Because of the weak pulling force, the rupture of the bond is dominated by thermal fluctuations and falls in the slow-loading regime [18]. In this regime, the variations of the rupture force and bond lifetime are large, which increase the time and noise in the assay. The resolution of this technique could be greatly enhanced by using higher forces which may be obtained through improved magnetic materials or magnet sources. Another source of noise in this technique is variation in the magnetic content of the magnetite particles that we have used.

This research demonstrates the application of magnetic particles in parallel massive measurement of the binding affinity between phage displayed peptides and the target protein. In contrast to the previous research, in which the

particle was observed individually and only a limited number of particles could be examined, we can track the behavior of a large amount of particles. Our research shows the magnetic tweezers as a powerful tool in measuring the binding property of peptide libraries. Future research would include the optimization of magnetic field and preparation of magnetic particles with higher magnetization to produce larger magnetic force.

References

[1] G.U. Lee, L.A. Chrisey, R.J. Colton, Science 266 (1994) 771.
[2] M.D. Wang, H. Yin, R. Landick, et al., Biophys. J. 72 (1997) 1335.
[3] S.M. Block, L.S.B. Goldstein, B.J. Schnapp, Nature 348 (1990) 348.
[4] S.B. Smith, L. Finzi, C. Bustamante, Science 258 (1992) 1122.
[5] T.R. Strick, J.F. Allemand, V. Croquette, et al., J. Stat. Phys. 93 (1998) 647.
[6] S.H. Leuba, M.A. Karymov, M. Tomschik, et al., Proc. Natl. Acad. Sci. USA 100 (2003) 495.
[7] G.U. Lee, S. Metzger, M. Natesan, et al., Anal. Biochem. 287 (2000) 261.
[8] G.P. Smith, Science 228 (1985) 1315.
[9] A. Grakoui, D.W. McCourt, C. Wychowski, et al., J. Virol. 67 (1993) 2832.
[10] Seradyne Sera-Mag Magnetic Microparticles Technical Data.
[11] S.W. Metzger, M. Natesan, C. Yanavich, et al., J. Vac. Sci. Technol. 17 (1999) 2623.
[12] G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, 1996.
[13] J.B.D. Green, A. Novoradovsky, G.U. Lee, Langmuir 15 (1999) 238.
[14] J.M. Harris, PolyEthylene Glycol Chemistry, Biotechnical and Biomedical Applications, Plenum Press, New York, 1992.
[15] J.M. Harris, N.E. Martin, M. Modi, Clin. Pharmacokinet. 40 (2001) 539.
[16] T. Nishizaka, R. Seo, H. Tadakuma, et al., Biophys. J. 79 (2000) 962.
[17] D.A. Simson, M. Strigl, M. Hohenadl, et al., Phys. Rev. Lett. 83 (1999) 652.
[18] E. Evans, K. Ritchie, Biophys. J. 72 (1997) 1541.