

New method for determination of average scFv fragment number displayed on the M13 phage surface*

Peng Zhao¹, Guijie Zhu¹, Lihua Zhang^{1,‡}, Zhen Liang¹, Zonghai Li², and Yukui Zhang¹

¹Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R. & A. Center; Dalian Institute of Chemical Physics, The Chinese Academy of Science, Dalian 116023, China; ²Shanghai Cancer Institute of Shanghai JiaoTong University, Shanghai 200240, China

Abstract: Single-chain-Fv (scFv) display M13 phage library has been regarded as a powerful tool for screening specific antibodies via binding with target proteins. Generally, the library quality is evaluated through detecting gene fragments by molecular biology methods, which is not only time- and labor-consuming, but also impossible to obtain quantitative information about the binding capacity of the phage library. In our recent study, a new method to calculate the average scFv number displayed on the M13 phage surface was proposed by capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection. By this method, enhanced green fluorescent protein (EGFP) and scFv phage clones that could specifically bind with EGFP were mixed with different ratios, followed by analysis by CE-LIF. With the dilution of EGFP by phage solution, the peak areas of scFv phage clones and free EGFP were decreased continuously, while that of the EGFP-M13 phage complex was found to decrease initially, then trend to be stable, and finally decrease further. When the volume ratio of the M13 phage to EGFP reached 660:1, corresponding to the molecule number ratio as 1:2.6, no more EGFP was found to bind with the M13 phage, which demonstrated that, by average, 2.6 scFv fragments that could bind with EGFP were displayed on the M13 phage surface. All these experimental results demonstrated that, by such a method, the quantitative evaluation of the phage library could be achieved with high throughput and accuracy.

Keywords: capillary electrophoresis with laser-induced fluorescence; enhanced green fluorescent protein; quantitative evaluation; single-chain-Fv display M13 phage library.

INTRODUCTION

Phage display is one of the most important and efficient techniques to study interaction between proteins, DNA/RNA and proteins, as well as small molecules and proteins. Among various phage display techniques, scFv display M13 phage library has been regarded as a powerful tool to screen antibodies against specific targets [1,2], by which complex immunization and hybridoma techniques could be obviated, thus allowing the rapid screening of antibodies in a prokaryotic system [3–5]. For such a phage

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‡Corresponding author: Tel.: 86-411-84379720; Fax: 86-411-84379779; E-mail: lihuazhang@dicp.ac.cn.

library, scFv fragments are displayed on the M13 phage surface as fusions to a protein. Therefore, it could be regarded as a direct physical link between phenotype and genotype [6]. The expected scFv display M13 phage library should include enough kinds of scFv fragments (up to 10^6), and enough copies (up to 5) for each kind of scFv fragment [7].

To obtain all potential antibodies against specific targets, it is important to screen with a well-established scFv display M13 phage library. Therefore, the evaluation of the phage display library is prerequisite before screening [8,9], which is usually performed by detecting external gene fragments with molecular biology methods, such as restriction endonuclease digestion, DNA sequencing, and enzyme-linked immunoabsorbent assay (ELISA), by which only information about the existence or loss of external gene fragment could be obtained. The actual number of scFv fragments displayed on the M13 phage, which determines the binding capacity of phage library, could not be determined. Furthermore, usually such procedures are time- and labor-consuming, not suitable for high-throughput evaluation of phage library quality.

Enhanced green fluorescence protein (EGFP) is a unique variant of green fluorescent protein (GFP), which contains a chromophore mutation [10,11], resulting in improved detection sensitivity compared to GFP [12]. Furthermore, the recombinant EGFP can keep its fluorescence and stability even when produced as a fusion protein with foreign genes [13]. Therefore, as an auto-fluorescent tag, EGFP is ideal for labeling protein ligands [14], tracking living cells [15], detecting tumors [16], studying the distribution and function of cell factor receptors [17] and screening drugs [18]. Recently, specific scFv fragments that could bind with EGFP were screened by phage display technology, and were successfully expressed by gene clone technology to detect EGFP [14].

In this paper, a capillary electrophoresis with laser-induced fluorescence (CE-LIF)-based method was developed to quantitatively evaluate the phage library by calculating the average external scFv fragment number displayed on the M13 phage surface, according to the changes of peak areas of EGFP-M13 phage complex by diluting the mixture of EGFP and M13 phage library with phage solution. The procedure was not only fast, but also could provide more accurate information compared to the traditional biological methods. All these results demonstrate that such a method has promise to be used as a regular means for evaluating the quality of phage library.

EXPERIMENTAL

Instrumentation and materials

P/ACE System 5010 with an LIF detector (Beckman Culture, Fullerton, CA, USA) was used to perform CE operation. The water purification system was bought from Millipore Corporation (Billerica, MA, USA). Capillaries (50- μm i.d., 375- μm o.d.) were purchased from Sino Optical Fiber Co. Ltd (Tangshan, China).

EGFP (*pI* around 6) and scFv display M13 phage library were kindly donated by Prof. Zonghai Li (Shanghai Jiaotong University). M13K07 helper phage and *E. coli* JM109 cells were ordered from New England Biolabs, Ltd. (Ipswich, MA, USA). Ampicillin, kanamycin sulfate, and thrombin (from bovine plasma) were bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Tween-20 was purchased from Sino-American Biotechnology Company (Beijing, China).

Growth of specific M13 phage binding with EGFP

E. coli JM109 cells were inoculated in 5 mL $2 \times \text{TY}$ medium (16 g/l tryptone, 10 g/l Bactoyeast extract and 5 g/l NaCl, pH 7.0) with shaking at 37 °C until the OD value reached 0.4 at 600 nm. Five microliter-specific M13 phages that could bind with EGFP (8×10^{11} pfu/ml) were mixed with 1 μl thrombin (1 unit/ μl) in 94 μl autoclaved water, and incubated at 37 °C for 1 h. Five-milliliter JM109 culture (containing 10 % glucose) was infected with the mixture, and incubated at 30 °C for 30 min without shak-

ing. Then the culture was infected with 2×10^{11} M13K07 helper phage, and incubated at 37 °C for 30 min without shaking, followed by 30 min with shaking at 250 rpm/min. The cells were subsequently harvested by centrifugation for 10 min at $4000 \times g$, and the pellet was resuspended in 50 ml of 2 $\times$ TY containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin. Finally, the infected JM109 cells were incubated at 37 °C at 250 rpm/min overnight.

Precipitation of specific scFv display M13 phages

Bacteria were pelleted by centrifugation at $4000 \times g$ for 10 min. scFv display M13 phages in supernatant were precipitated with 0.20 volume of poly(ethylene glycol) (PEG)/NaCl solution (20 % w/v PEG 8000, 2.5 M NaCl) for 1 h on ice with gentle shaking, and pelleted for 20 min at $12000 \times g$ at 4 °C. Then scFv display M13 phages were resuspended in 1-ml sterile cold phosphate-buffered saline (PBS), and the residual cell debris were removed by centrifugation at $10000 \times g$ for 10 min. Finally, phages were filtered by 0.45- μ m filter membrane, and stored at 4 °C for usage.

Measurement of specific M13 phage stock titre

One microliter phage stock was diluted with 100- μ l PBS, and further diluted with 900- μ l PBS, which was further diluted to 10^6 , 10^8 , and 10^{10} folds in turn with PBS. Serial 100- μ l 100-fold dilutions were mixed with 900- μ l suspension of JM109 cells ($OD_{600} = 0.4$), and the mixture was incubated at 37 °C for 30 min. Subsequently, 3-ml top agarose (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1g/l $MgCl_2 \cdot 6H_2O$, and 7 g/l agarose) was added into 1 ml incubated mixture before spotted onto a TYE plate (15 g/l Bacto-Agar, 8 g/l NaCl, 10 g/l tryptone, and 5 g/l yeast extract), containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin and 1 % (w/v) glucose, following by growing overnight at 37 °C. Finally, the titer of phage stock was calculated by multiplying the recorded plaque in each TYE plate and dilution folds.

CE operation

EGFP (4 mg/ml) and scFv display M13 phage clone (8.62×10^{12} pfu/ml), confirmed to be able to specifically bind with EGFP, were mixed with different volume ratios ranging from 1:100 to 1:740 with constant M13 phage concentration and decreased EGFP concentration. After incubation at 20 °C for 30 min, the mixture was separated by CE with three consecutive runs.

All experiments were performed on a P/ACE 5010 instrument with an LIF detector (Ex/Em = 488/520 nm). The total and effective lengths of capillary were 27 and 20 cm. The incubation buffer was 1 $\times$ PBS (pH 7.4), and the electrolyte buffer for separation was 1 $\times$ PBS (pH 7.4) containing 1 % (v/v) Tween 20. Sample was injected for 5 s at 0.5 psi, and separated under 10 kv voltage at 20 °C.

RESULTS AND DISCUSSION

In this paper, a new CE-LIF based method to calculate the average number of external scFv fragments displayed on the M13 phage surface was proposed. The specific scFv display M13 phage that could bind with EGFP was screened from the scFv display M13 phage library [14], with amino acid sequence shown in Fig. 1. Furthermore, it should be pointed out that no other scFv display phages which did not bind to EGFP were used, since the fluorescence property of EGFP was used to determine the displayed scFv number.

MAQIQLLQSGPELVKPGASVKISCKASGYTFTNYYLNWVKQKPGQGLEWIGWIYPGN
 GKTEYNEKFKGKATLTVDTSSNTAYIQLSSLTSEDTAVYFCARLITTAIDVWGAGTTV
 TVSSGGGGSGGGGSGGGGSDIVMSQSPAIMASAPGEKVTMTCSASSSVSYMHWFQQK
 PGTSPKLWIYSTSNLASGVPARFSGSGSGTSYSLTISRMEAEDAATYYCQQRSSYPPTF
 GSGTRLEIKR

Fig. 1 Amino acid sequence of scFv fragment displayed on the specific M13 phage that could bind with EGFP.

In our studies, EGFP and the mixture of EGFP and specific M13 phages were separated by CE, respectively. To avoid the nonspecific adsorption of EGFP on the specific M13 phage surface, 1.0 % Tween-20 was added into the buffer. From Fig. 2, it was found that in both cases, two peaks appeared, respectively, with similar peak shapes and migration times. In our paper, because the analyte (EGFP) and detector (LIF) are fixed, the R-factor correction for fluorescence should be unchangeable. To evaluate the effect of operation parameters on R-factor, the reproducibility of peak areas in 3 consecutive runs was studied. It was found that the relative standard deviation (RSD) values of the peak areas of peaks 1 and 2 were 2.98 and 3.86 %, respectively, demonstrating good reproducibility of CE operation.

According to previous work for CE-based GFP analysis [19], peaks shown in Fig. 2 represented autofluorescent media components (1) and free EGFP (2) respectively. Since the molecular weight of the M13 phage was much larger than that of EGFP, the appearance migration time of the EGFP-M13 phage complex should be faster than that of free EGFP due to the opposite direction of electroosmotic flow and electrophoretic migration. Thus, the complex should be co-eluted with autofluorescent media components in peak 1. Furthermore, it was observed that the fluorescent intensity of EGFP dissolved in specific M13 phage solution was obviously stronger than that dissolved in $1 \times$ PBS buffer, caused possibly by the structure change of EGFP diluted by phage solution, which demonstrated no fluorescence quenching happened.

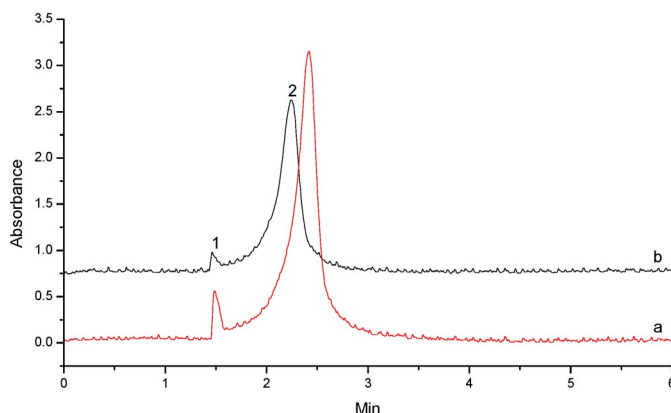
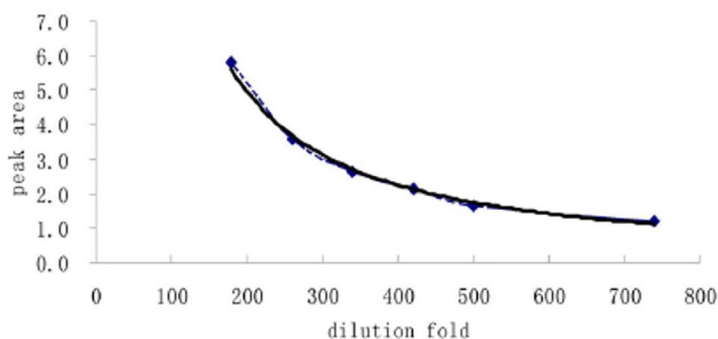
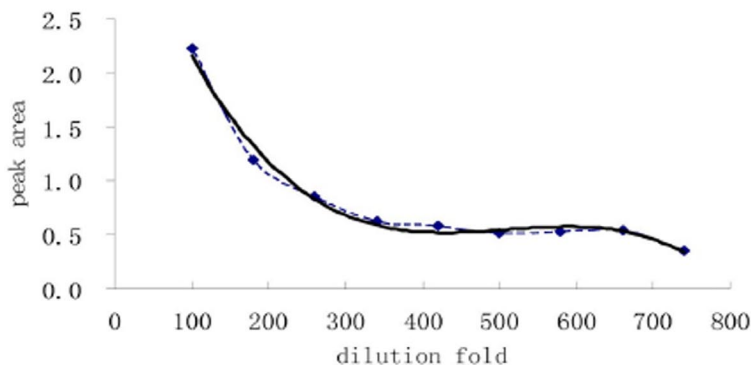


Fig. 2 Electropherograms of EGFP and the mixture of EGFP and specific M13 phage. Experimental conditions were the same as those shown in “Experimental” section. (a) EGFP (80 μ g/ml) dissolved in the specific M13 phage solution with titre of 8.62×10^{12} pfu/ml; (b) EGFP (80 μ g/ml) dissolved in $1 \times$ PBS (pH 7.4).

EGFP was respectively diluted by $1 \times$ PBS (pH 7.4) and specific M13 phage solution with different ratios, and separated by CE for comparison. Obviously, in the first case, peak areas of 1 and 2 were decreased gradually with increased volume dilution fold, and when it was diluted over 500-fold, both peaks were disappeared. However, for the latter case, even when the volume dilution time was over 700-fold, both peaks could still be seen. Thus, further study on peak area changes of the mixture of EGFP and M13 phages diluted by phage solution was performed. From Fig. 3a, it could be seen that the peak area of free EGFP decreased continuously with increased dilution fold. As shown in Fig. 3b, similar phenomenon was observed for peak 1 when the volume dilution fold was less than 500, which should be contributed to the dilution of autofluorescent media components and EGFP-M13 phages complex. However, in ranges from 500 to 660, the peak area of 1 was almost kept constant, after which it continued to decrease. Since in such dilution range, the signal of autofluorescent media components was already disappeared, as shown in the control experiments when EGFP was diluted by PBS, peak 1 should only represent the complex of EGFP and M13 phages. Because under such situation, there were still much more EGFP than specific scFv display M13 phages in solution, the concentration of EGFP-M13 phage complex was determined by phage concentration, which was fixed at



(a)



(b)

Fig. 3 Peak areas of free EGFP (a) and the mixture of EGFP-M13 phages complex and autofluorescent media components vs. dilution fold (v/v) by phage solution. The broken line was drawn according to experimental results; the black curve was a fitting curve.

8.62×10^{12} pfu/ml. With the volume dilution fold over 660, not enough EGFP existed to bind with M13 phages, leading to decreased peak area of the complex. From the above data, it could be concluded that EGFP in solution, diluted by 660-fold from 4 mg/ml, was completely bound with the M13 phages with titre of 8.62×10^{12} pfu/ml, corresponding to 2.6 scFv fragments displayed on each M13 phage to bind with EGFP.

CONCLUSIONS

A novel CE-LIF based method to quantitatively evaluate the scFv display phage library was proposed, by which the average scFv fragment number displayed on each phage could be obtained according to the completely binding between specific scFv fragments and target proteins. Compared to traditional biological evaluation methods, not only the analysis time could be shortened to several hours from several days, but also accurate quantitative information describing binding capacity of phage library could be obtained. Therefore, it might be promising to be used as a regular means to evaluate the newly built phage library and detect the loss of external fragments displayed on the phage surface in phage display technology.

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