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Talanta 66 (2005) 181–187
Talanta
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Selection of phage antibodies with GPX activity by combination of phage displayed antibody library with chemical modification and their characterization using a surface plasmon resonance biosensor

Ying Mu[a,b,1], Daqian Song[b], Ying Li[a], Han-Qi Zhang[b], Wei Li[c], Gui-Min Luo[a,**], Qin-Han Jin[b,*]

[a] *Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, Jilin University, 119 Jiefang Road, Changchun 130023, PR China*
[b] *College of Chemistry, Jilin University, Changchun 130023, PR China*
[c] *College of Life Science, Jilin University, Changchun 130023, PR China*

Received 10 July 2004; received in revised form 8 November 2004; accepted 10 November 2004
Available online 24 December 2004

Abstract

Glutathione peroxidase (GPX) is an important antioxidant enzyme, which plays an important role in scavenging reactive oxygen species. To obtain humanized GPX catalytic antibodies, the phage displayed human antibody library on the surface of the filamentous bacteriophage was used to select novel antibodies by repetitive screening. Phage antibodies B8, H6 and C1 with the GSH-binding site were obtained from the library by enzyme-linked immunosorbent assay (ELISA) analysis with four rounds of selection against three haptens, *S*-2,4-dinitrophenyl *t*-butyl ester [GSH-S-DNP-Bu (B)], *S*-2,4-dinitrophenyl *t*-hexyl ester [GSH-S-DNP-He (H)] and *S*-2,4-dinitrophenyl cycle-hexyl ester [GSH-S-DNP-cHe (C)], and characterized using surface plasmon resonance (SPR) biosensor. The gold layer was modified by dithiodiglycolic acid (DDA) and three haptens were easily attached to DDA by self-assembling to form a biosensor membrane. The membrane bounds specifically corresponding antibodies. The kinetic process of the reaction between phage antibodies and their haptens was studied by SPR biosensor. In order to improve selectivity, chemical modification was used to incorporate directly catalytic group selenocysteine (Sec) into selected phage clone B8, H6 and C1 to form Se-B8, Se-H6 and Se-C1, respectively. The GPX activities of Se-B8, Se-H6 and Se-C1 were found to be 3000, 2000 and 700 units/μmol, respectively. Compared with conventional ELISA analysis, the proposed method based on SPR biosensor is much more rapid and simpler.

Keywords: Selection; Glutathione peroxidase; Phage displayed antibody library; Catalytic antibody; Chemical modification; Selenium; Surface plasmon resonance; Biosensor

1. Introduction

Glutathione peroxidase (GPX) is an important antioxidant enzyme, which plays an important role in scavenging reactive oxygen species. GPX is a selenoenzyme composed of four identical subunits of 21,000 Da, which catalyzes the reduction of H_2O_2 and other hydroperoxides. Glutathione (GSH) is utilized as a cofactor, supplying the electrons for the following reductive reaction:

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

The mechanism by which GPX catalyzes breakdown of hydroperoxides has been extensively studied. The selenol of a reduced selenocysteine (Sec) molecule (ESeH) is oxidized

* Corresponding author. Tel.: +86 431 8499805; fax: +86 431 8499805.
** Co-corresponding author. Tel.: +86 431 8980440; fax: +86 431 8980440.
E-mail addresses: ymu100@yahoo.com.cn (Y. Mu), gmluo@mail.jlu.edu.cn (G.-M. Luo), qhjin@mail.jlu.edu.cn (Q.-H. Jin).
[1] Tel.: +86 431 8499805; fax: +86 431 8499805.

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doi:10.1016/j.talanta.2004.11.014

by hydroperoxides to generate a selenenic acid (ESeOH). Tripeptide GSH then reacts with the selenenic acid, affording the corresponding water and selenenyl sulfide (ESeSG). A second molecule of GSH attacks the sulfur atom of the latter species, producing GSH disulfide and regenerating the selenol (ESeH) to complete the catalytic cycle [1].

Complementarity between enzyme and transition state is the essence of the biological catalysis, which has been used to generate catalytic antibody [2–4]. In previous works, our group has developed a new strategy for generating catalytic antibody: the monoclonal antibody (mAb) with substrate binding site is first prepared and then a catalytic group is incorporated into the mAb's binding site by chemical mutation. Using the substrate analogue GSH-S-DNP (4A4) [5,6], GSH-S-DNP-Me (3G5), GSSGMe (5C9) or GSH-S-DNP-Bu (2F3) [7] as haptens, we obtained respective mAbs, which are able to bind substrate GSH, respectively. These mAbs could be converted into catalytic antibodies when the selenocysteine molecules are incorporated into the binding sites of the antibodies. In this way, we have generated several catalytic antibodies that can efficiently catalyze the breakdown of hydroperoxides by GSH [5–13]. Among them, several antibodies (Se-4A4, Se-3G5 and Se-2F3) have been found to display remarkably high catalytic efficiency, which surpasses some of native enzymes.

In order to overcome the shortcomings of the mAb's, such as less availability and large mass, we have also prepared some single-chain variable region fragments of antibodies (scFv) with smaller size. The scFv protein is one of the smallest antibody fragments that retain the ability to bind antigen. Compared with mAb, scFv has several advantages including (a) it can be expressed by gene engineering, (b) its molecular weight is smaller than mAb and its structure is easy to be analyzed by NMR or X-ray crystallography, and (c) it is more appropriate for use as drug candidates.

Phage antibody library is now one of the ways for producing human antibodies and antibody fragments [14]. Antibody library displayed on phage offers a new kind of antibody that binds specific substrate without antigenicity in vitro [15,16]. In general, the phage M13 has two coated proteins, pIII and pVIII. Phage repertoires are generated by fusion of DNA encoding antibodies at the ends of pIII and pVIII that display the expressed proteins on their surfaces. Binding antibodies are often selected by repetitive panning [17,18]. In this study, combination of phage displayed antibody library with chemical modification was used to directly select phage scFv catalytic antibodies.

Three substrate analogues (GSH-S-DNP-Bu, GSH-S-DNP-He and GSH-S-DNP-cHe) were designed and synthesized in order to use them as haptens to select antibodies from a semi-synthetic phage displayed antibody library and the phage antibody clones capable of binding the haptens were obtained. Phage clones B8, H6 and C1 were chosen by their highest signals of enzyme-linked immunosorbent assay (ELISA) and some catalytic groups Sec's were incorporated into them.

Surface plasmon resonance (SPR) sensors are becoming useful tools in studying the interactions among macromolecules and determining the concentrations and masses of macromolecules. This technique has two obvious advantages over traditional methods. First, labeling of molecules is not necessary. Second, real-time observation of the interaction among macromolecules can be realized and, therefore, kinetic constants of reactions can be obtained. Such instrumentation is to be used in fields such as on-line process monitoring, environmental, medical science and many other life science disciplines [19–24]. Since early 1990s, commercially available SPR-based biosensor equipment has attracted the interest of pharmaceutical companies as a tool for both selective and sensitive in vitro screening of promising novel pharmaceutical products from combinatorial libraries. Compared with ELISA or RIA procedures, which only produce meaningful data at the end-point, SPR biosensor offers real-time measurement of the binding events. The change in the resonant wavelength is correlated with the amount of analyte bound to the sensor surface. The corresponding analyte (such as antibody or antigen) in samples could be detected very rapidly by the specific reaction between biomolecules.

A novel optical biosensor based on surface plasmon resonance has been developed and improved [25,29] for biomolecular interaction study and determination. The sensor is designed on the basis of fixing angle of incidence and measuring the reflected intensities in the wavelength range of 400–800 nm simultaneously. On the sensing element of a right angle glass prism, a replaceable glass sensor chip was added. The chip was coated with a surface plasmon active gold layer (thickness, 50 nm) by vacuum vapor deposition. The chip was attached to the bottom of the prism with cedar oil. A flow cell with a volume of 180 μL was installed under the chip. The sensing membrane with different function groups on the gold substrate was formed by molecular self-assembling in solution in the flow cell. The system has been used for the determination of some chemical species, such as dimethylamine, alcohol, glucose and antibody–antigen recognition [25–32]. This paper presents an improved device that can be used as both a molecular recognition device for detecting related biomolecules and a tool for studying macromolecular interactions including measuring antibody–antigen and ligand–receptors kinetic association constants. The sensor was shown to be of simplicity, sensitivity, selectivity, rapid response and cost effectiveness.

2. Materials and methods

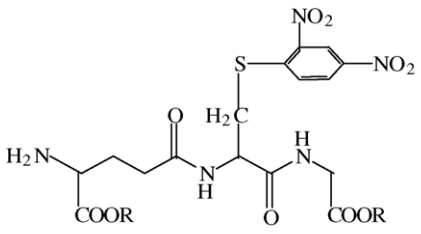
2.1. Materials

Phage displayed human antibody library; *E. coli* TG1 and TG1 (pHEN 2) were generous gifts from Medical Research Council (MRC). Haptens (GSH-S-DNP-Bu, GSH-S-DNP-He and GSH-S-DNP-cHe) were synthesized in our laboratory

[5–7]. M13K07 helper phage and vector were purchased from Phamarcia Biotech. Dithiodiglycolic acid (DDA; $C_4H_6O_4S_2$) and mouse anti-M13 phage antibody labeled with horseradish peroxidase were purchased from Sigma. All other chemicals were of analytical grade. All solutions were prepared with ultra-pure water (>18 MΩ cm^{-1}) supplied by an EASYpure RF compact ultra-pure water system (USA).

2.2. Selection of phage clones with GSH binding activity against GSH-S-DNP-Bu (B), GSH-S-DNP-He (H) and GSH-S-DNP-He (C)

Phage particles displayed antibodies were rescued with helper phage M13K07 on a 50 mL scale [33,34] and purified from bacterial supernatant through precipitation with 20% polyethylene glycol 6000/2.5 mol/L NaCl (PEG6000/NaCl). About 10^{11} phage particles were preincubated with *E. coli* TG1 containing 3% bovine serum albumin (BSA) at 37 °C for 2 h in order to eliminate the affinity of absorbed phages. The supernatant containing non-specially selected phages was incubated overnight at 37 °C. Fifty micrograms hapten B (or H, or C) (shown in Fig. 1) was dissolved in 1 mL methanol and coated on culture plate (Nunc). The plate was washed three times with phosphate buffer saline (PBS) and blocked with 3 g/L BSA at 37 °C for 2 h. Phages that were not bound with BSA were added to the plate and incubated at 37 °C for 2 h. The plate was washed 10 times with PBS buffer containing 3% BSA and 0.5% Tween 20, and 10 times with PBS buffer. Then, bound phages were eluted with 0.1 mol/L triethylamine and immediately neutralized with 0.5 mol/L Tris-hydroxymethyl aminomethane (Tris)–HCl buffer (pH 7.4). Eluted phages were used to infect the exponential growing *E. coli* TG1 cells. Recovered phages were amplified, put on the 2× YT plates containing 100 μg/mL ampicillin and 1% glucose and incubated overnight at 30 °C. The bacterial cells were scraped into 2× YT broth. Fifty microliters bacterial cells were added to 50 mL 2× YT broth containing 100 μg/mL ampicillin and 1% glucose and incubated at 37 °C with shaking until reaching an optical density of 0.5 at 600 nm. The biopanning was repeated three times. The remaining bacterial cells were obtained by centrifugation at 3300 × *g* for 10 min, and then resuspended in 2× YT broth, into which 15% glycerol was added, and then stored at −70 °C.



R：-$CH_2(CH_2)_2CH_3$ (hapB); -$CH_2(CH)_4CH_3$ (hapH); -C_6H_6 (hapC)

Fig. 1. Structures for haptens used for selecting phage antibodies.

2.3. Identification of phage clones and measurement of their dissociation constants for GSH

From the plate incubated overnight (as described above), one bacterial colony was picked up, added to 1 mL 2× YT broth containing 100 μg/mL ampicillin and 1% glucose and incubated overnight at 37 °C. The culture of 20 μL was incubated in 1 mL 2× YT broth containing 100 μg/mL ampicillin and 1% glucose with shaking at 37 °C until reaching an optical density of 0.5 at 600 nm. Eight microliters M13K07 helper phage was added at ratio of 1:20 (phage particles:helper phage) and infected at 37 °C for 30 min. Phage particles were recovered by centrifugation at 3300 × *g* for 10 min, and then resuspended in 2 mL 2× YT broth containing 100 μg/mL ampicillin and 50 μg/mL kanamycin and incubated overnight at 30 °C. Infected bacterial cells were precipitated by centrifugation at 3300 × *g* for 15 min. The pellets were resuspended in 1/5 volume of PEG6000/NaCl and centrifuged at 10,800 × *g* for 30 min. The precipitates were phage antibodies.

The phage antibodies were tested with ELISA. In this experiment, 50 μg/mL B, H or C, M13K07 helper phage (used as positive control), TG1 containing pHEN2 (used as negative control), PBS, 2× YT, TG1 and 3% BSA were used. Each of them was coated in one well of ELISA plate and then incubated overnight at 4 °C. Plate was washed three times with PBS and blocked with PBS containing 3 g/L BSA at 37 °C for 2 h. After washing three times with PBS, into the plate was added the phage antibodies and incubated at 37 °C for 2 h. The plate was washed five times with PBST (PBS buffer containing 0.5% Tween-20); an anti-M13 monoclonal antibody coupled to horseradish peroxidase was added and incubated at 37 °C for 2 h. The plate was washed three times again with PBS and the substrate solution [1 mg/mL 3,3′,5,5′-tetramethyllbenzidine (TMB) and 0.3% hydrogen peroxide, 0.11 mol/L citrate buffer, pH 5.8] was added. The reaction was stopped with 1.0 mol/L sulfuric acid in 20 min. The absorbances (at 450 nm) were measured with a microplate reader. Sixty phage clones were randomly picked up and B8, H6 and C1 with the highest ELISA signals were chosen for further study. The dissociation constants were measured by the same experiment as ELISA according to Prism software [35].

2.4. Immobilization of haptens on to the sensor chip

SPR biosensor [25] used was developed and improved by the Institute of Miniature Analytical Instrumentation, College of Chemistry, Jilin University, China. First, the gold film was modified using 6.7 mmol/L DDA for 30 min, and then the activated gold film was incubated with 50 μg/mL B, H or C (pH 7.4 PBS) for 30 min; finally, the excess binding sites were blocked by 5 g/L BSA in PBS buffer (pH 7.4).

2.5. Characterization of phage antibodies B8, H6 and C1 with SPR biosensor

The kinetic analysis of the reaction of biological molecules with SPR biosensor was as follows: flow cell was washed with three injections of PBS for 10 min and the resonant wavelength at this point was recorded as baseline. Association—100 μmol/L phage antibodies was diluted 10-fold with pH 7.4 PBS and injected into flow cell and incubated for 30 min and the change of the resonant wavelength was monitored every 2 min for 30 min during an analytical run. Dissociation—flow cell was washed with three injections of 200 μL PBS buffer and the change of the resonant wavelength was monitored every 2 min for another 30 min during washing. Regeneration—0.3 mol/L citrate (pH 2.7) was used to rinse the sensing surface for 2 min to remove the antibody molecules bound to the sensing membrane and make SPR biosensor regenerated. Return to baseline—flow cell was washed with PBS for 10 min until the resonant wavelength returns to the baseline, and then the biosensor is ready for a new run.

2.6. Preparation of Se-B8 (H6 and C1) and determination of GPX activity

One liter phage antibodies (B8, H6 or C1) approximate to 10^{13} phage particles were reacted with PMSF (20 μL of 0.2 mg/mL in acetonitrile) at 25 °C for 3 h. The mixture was flushed with purified N_2 for 20 min, and then 20 μL of 1 mol/L NaHSe solution was added [36] and incubated at 37 °C for 40 h under the nitrogen atmosphere. Se-B8 (H6 and C1) was separated from the reaction mixture by using Sephadex G-25 column, eluted with 20 mmol/L Tris/HCl (pH 8.0) and freeze-dried.

A phage antibody GPX activity was measured according to the literature [1]. The reaction mixture was in 250 μL 50 mmol/L potassium phosphate buffer (pH 7.0) containing 1 mmol/L EDTA, 50 μL 1 mmol/L GSH and 10–50 nmol/L Se-B8 (Se-H6 and Se-C1), and then incubated at 37 °C for 10 min. One unit of GSH reductase and 50 μL 0.25 mmol/L NADPH were added and incubated at 37 °C for 3 min. Then, the reaction was initiated by adding 50 μL 0.5 mmol/L of H_2O_2. The activity was measured by the change of NADPH absorption at 340 nm. The same experiment was carried out with the phage antibodies without B8, H6 and C1 and the absorbance values of them were used as blank control. The activity unit is defined as the mole number of Se-B8 (Se-H6 and Se-C1), which consumes 1 μmol of NADPH per min.

3. Results

3.1. Screening of GSH binding phage antibodies

Both the concentrations of the haptens and the ratios between the haptens and the phage particles were varied in each round. The concentrations were 50 μg/mL in the first round and 1 μg/mL in the last round. The ratios between the haptens and the phage particles were ranged from 1:1 to 1:50. During the selection step of each round, the concentrations of the haptens were decreased while the washing times with PBST buffer were increased. The recovery ratios become higher and higher when the rounds of selection were increased. This indicated that the bound phage antibodies were enriched while the selections were carried out one after another. ELISA results (not shown in this paper) also showed that above 70% clones randomly chosen from the fourth round were positive ones. It was found from the ELISA signals that B8, H6 and C1 had good ability to bind GSH. Therefore, when catalytic group Sec was incorporated into the B8, H6 and C1, the GPX activities of Se-C1, Se-H6 and Se-B8 (shown in Table 1) reached 700, 2000 and 3000 units/μmol, respectively. These results indicate that the catalytic antibody with higher GPX activities could be obtained by incorporating Sec into the phage mAb with GSH binding site [5–13].

As shown in Table 1, the dissociation constants of the B8, H6 and C1 are in the order of magnitude of μmol/L, indicating that the binding between the phage antibodies and GSH analogues was firm.

3.2. Kinetic determination

The DDA monolayer sticks firmly on the gold surface with its disulfide bonds. To observe the DDA assembling on gold substrate, a solution of DDA was injected into the flow cell. The changes of resonant wavelength were determined in real time. The shift of the resonant wavelength ($\Delta\lambda$) reaches about 99% of its total shift within 15 min in the 6.7 mmol/L DDA solution. When the assembling time increases, the $\Delta\lambda$ keeps almost constant. That means the self-assembly has completed. Because the disulfide bond of DDA easily reacts with Au to form S–Au bond, a good monolayer is formed.

A series of hapten (B, H and C) solutions were prepared and injected into the flow cell, respectively. After the reaction of hapten (B, H or C) with DDA on the Au film was completed, a large excess of 5% BSA was used to block the non-specific binding sites on the sensor surface and no wavelength shift was observed in this process. Then, 10 μmol/L of the phage antibodies was injected into flow cell and kept to immerse the sensing membrane for 30 min. SPR spectra of molecule assembling on the sensor surface are shown in Fig. 2. The optimum determination conditions were shown to be in PBS buffer (pH 7.4) at 20 °C for 30 min. Room temperature (20 °C) was chosen for molecular assembling. Repeating the determination of 10 μmol/L phage antibodies for 11 times, a relative standard deviation of 1.5% is obtained.

As shown in Fig. 3, the SPR resonance wavelength shift of the phage B8 on the sensor is much larger than that of H6 and C1. This result coincides with that detected via ELISA (Table 2). For SPR, resonance wavelength shift showed the affinity between hapten and phage. For ELISA, OD_{450} was used to measure the affinity between hapten and phage. By

Table 1
GPX activity comparison between the phage abzymes and other abzymes

Species	Phage (Cfu/mL) before binding Se	Protein (μg/mL) before binding Se	Protein (μg/mL) after binding Se	GPX activity (U/μmol)	K_D (μmol/L)
Se-B8	10^{13}	260	220	3012	14
Se-H6	10^{13}	271	260	2102	12
Se-C1	10^{13}	253	241	694	12
Se-2F3-scFv				3394 ± 68[a]	
Native GPX				5780[a]	

Note: [GSH] = 1 mmol/L; [H_2O_2] = 0.5 mmol/L.
[a] [13].

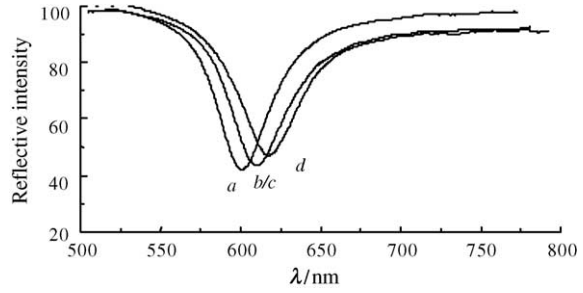


Fig. 2. SPR spectra of molecule assembling on the surface of Au Film: (a) with DDA; (b) with hapten (B, H or C), t = 30 min; (c) with BSA; (d) with phage, t = 30 min.

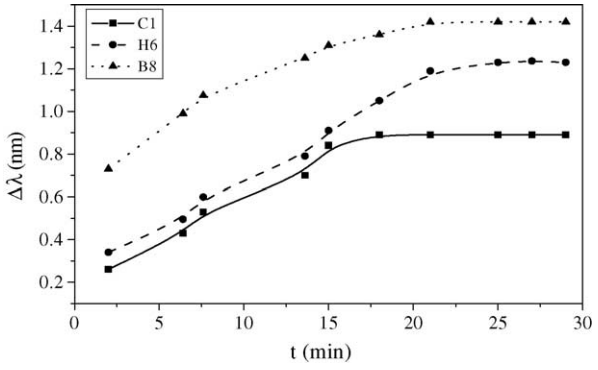


Fig. 3. The kinetic adsorption curves of B8, H6 and C1 phages on the surface of biosensor's film.

using either ELISA or SPR, it was found that the affinity obtained between phage B8 and hapten B is highest. The affinity between phage C1 and hapten C is the lowest. The kinetic process could be studied in real time by monitoring the change of the resonant wavelength with SPR biosensor. The whole assay can be well done within 2 h. Compared with ELISA method, SPR biosensor was shown to be much simpler, more rapid and cost-effective, as well as without the need to label antibodies with HRP.

Table 2
The comparison between ELISA and SPR in molecular interaction analysis

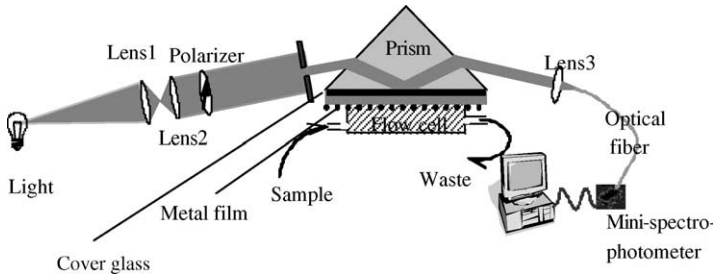
Phage	μmol/L	ELISA		SPR	
		OD_{450}	t (h)	Δλ (nm)	t (h)
B8	10	1.5	>24	1.4	<2
H6	10	1.3	>24	1.2	<2
C1	10	0.6	>24	0.8	<2

4. Discussions

In this paper, several kinds of GPX catalytic antibodies were screened from phage displayed antibody library and detected by ELISA and SPR biosensor. GPX activities of the phage clones (Se-B8, Se-H6 and Se-C1) were directly determined on the basis of ratio relationships among the mole fraction of Se-B8, Se-H6, Se-C1, phage particles, the titer of phage particles and the concentrations of proteins when the Sec group was incorporated into B8, H6 and C1. This method relies on: (1) unique enzyme is highly specific for the substrate, GPX activity is mainly ascribed to the phage clones with GSH binding site; (2) the structure and the genome of the phage are well known. A phage is considered as a bio-macromolecule in protein level and its molecular mass is approximate to 3000 protein pVIII (1.6×10^4 kDa). Furthermore, there is a specific ratio between titer of phage particles and the concentration of protein. The protein concentration of 10^{13} phage particles ($10^{13} \times 1.6 \times 10^7 / 6.02 \times 10^{23}$) equals to 2.7×10^{-4} g/mL in theory. Not only the protein concentration of freeze-dried Se-B8 (H6 and C1), but also the phage titer of Se-B8 (H6 and C1) and the mole fraction can be accurately calculated according to this ratio. On the other hand, the expressed proteins are always fused at the end of protein pIII of the phage particles. In general, every phage has five pIII proteins, of which two or three are fused V_H-Linker-V_L genes. So, every phage is fused 2.5 expressed proteins on average. The ratio between the phage titer and the mole fraction of expressed protein is thus obtained. The equation for calculating GPX activity of scFv is as follows:

$$\frac{\text{GPX activity of phage with B8 (H6 and C1)} - \text{GPX activity of phage without B8 (H6 and C1)}}{\text{expressed protein of every phage on the average}} = \text{GPX activity of ScFv}$$

A newly developed SPR optical biosensor based on simultaneous multi-wavelength detection is suitable for monitoring phage clone binding (Scheme). The SPR spectra are shown in terms of reflected light intensity versus wavelength of



Scheme. The SPR design for simultaneous multi-wavelength detection.

incident light, while the SPR spectra of BIAcore instruments (Pharmacia) are shown in terms of light intensity versus angle of incident light. The intensity of the reflected light is the minimum at the resonant wavelength. Molecular self-assembling in solution is used to form the sensing membrane on the gold substrate. The processes of sensing monolayer formation were studied in real time through observing the change of resonant wavelength. Compared with ELISA, SPR biosensor could be used to study the kinetic process in real time by monitoring the change of the resonant wavelength. It was shown that the method is simple, rapid as well as without the need to label antibodies with HRP. In addition, the SPR apparatus is rather cost-effective compared with BIAcore systems.

In summary, a novel combination of phage displayed antibody library with chemical modification was developed for rapidly selecting phage antibodies. Three phage antibodies with GPX activity were successfully selected by using this method, demonstrating that the method can be used to prepare rapidly humanized single chain antibodies with GPX activity. Here, we present an improved device that can be used as both a molecular recognition device for detecting related biomolecules and a tool for studying macromolecular interactions including measuring antibody–antigen, and ligand–receptors kinetic association and dissociation constants. Under optimum experimental conditions, the SPR biosensor has a good repeatability, sensitivity, reversibility and selectivity.

Acknowledgments

We thank the National Natural Science Foundation of China (Project No. 20272016, 30200050) and Jilin University for financial support and MRC for generously providing phage antibody library.

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