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Construction of Single Chain Variable Fragment (ScFv) and BiscFv-Alkaline Phosphatase Fusion Protein for Detection of *Bacillus Anthracis*

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This paper describes an attempt for convenient and sensitive detection of *Bacillus anthracis* with single chain variable fragment (scFv)-based protein chip. Phage display technology was employed to generate scFv by using the protective antigen (PA) of *B. anthracis* for immunization. V_H and V_L genes of the scFv were amplified separately by reverse transcriptase-PCR from mRNA of immunized mice and then assembled into scFv gene with a linker DNA sequence. The scFv gene was inserted into a phagemid vector pCANTAB-5E and then transformed into *Escherichia coli* TG1 to yield recombinant phages after infection with helper phage M13KO7. After six rounds of panning with PA, the phage clones displaying scFv fragments of the antibody were selected by ELISA. One phage clone scFv-6w10 showing the strongest positive signal in ELISA was selected. To enhance the affinity of the scFv-6w10, a recombinant bivalent single-chain Fv antibody (biscFv-6w10) directed against PA was constructed and tested in functional assays. The affinity of the biscFv-6w10 was much higher than that of scFv-6w10 and reached 6.5×10^9 M^{-1}. An expression system was constructed for the production of *E. coli* alkaline phosphatase (EAP) labeled biscFv-6w10 (biscFv-6w10-EAP) in *E. coli* cells. The expressed fusion protein retained both antigen-specific binding and enzymatic activity and thus directly served as an enzyme-labeled antibody. Detections of PA and bacterial cells of *B. anthracis* using biscFv-6w10-EAP and Cy3-labeled biscFv-6w10 were performed on a protein chip. The fusion protein (biscFv-6w10-EAP) chip could detect 10 pg of PA and 500−1000 bacterial cells in ~2 h, while the sensitivity of Cy3-labeled protein chip reached 1 pg of PA and 50−100 cells within 2 h.

Bacillus anthracis is a Gram-positive aerobic bacillus responsible for anthrax, a severe death disease. Its potential spread in recent years has caused worldwide concern. Therefore, rapid and sensitive detection of *B. anthracis* is critical to prevent human and wild stock suffering from the disease.

B. anthracis can be identified by comparing its phenotype with the rest of *Bacillus cereus* group or distinguished from closely related *Bacillus* by specific target gene assay or immunoassay,[1] among which the immunoassay, especially ELISA using enzyme-labeled antibody, is well established and widely applied for clinical, field, and spot detection.[2] So far, the main target protein of *B. anthracis* is its protective antigen (PA), which is known secreted from rapidly growing *B. anthracis* cells and binds to a specific host cell surface receptor and leads to anthrax.[3]

Single-chain antibody fragment (scFv) is a small engineered antibody, in which the variable heavy chain (V_H) and light chain (V_L) of the antibody molecule are connected by a short, flexible polypeptide linker. Using scFv for detection of antigen has several advantages. First, it retains the specific affinity to the antigen, though usually lower than its original antibody; second, scFv can be produced in large quantity in bacterial expression system at low cost;[4] and third, it is easy to be manipulated to adapt different applications, for example, fusion with protein drug to target and kill pathogens or with marker molecules for detection purposes.[5,6]

To increase the affinity of scFv, multimeric forms were suggested.[7] The multimeric size of a scFv functional unit can be

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controlled by selection of the linker length. The most commonly used linker contains a 15-combination of glycine and serine residues $(GGGGS)_3$ that provides flexibility and enhances the hydrophilicity of the peptide backbone.[8] ScFvs are predominantly monomeric when the linker is at least 12 residues.[9] When V_H and V_L domains are joined with a linker of 3−12 residues, it cannot assemble into a functional Fv domain, and instead, the V_H and V_L domains of one scFv associate with those of a second scFv molecule to form dimers (diabodies). Reducing the linker length below three residues promotes the assembly of scFvs into trimers (triabodies).[9,10] The increased binding valency in these scFv multimers results in high avidity. Bivalent antibody fragments can be generated by the dimerization of two scFv fragments. They were usually produced by heterodimerization or homodimerization of two fragments of the structure scFv expressed in the same cell. These molecules also possess an increased functional affinity and improved binding specificity compared with monomeric scFv.[11,12]

Analyte detection in immunoassays is carried out with primary or secondary antibodies that are labeled with sensitive reporter molecules, like fluorescent dyes or enzymes. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are commonly used enzymes for labeling antibodies by means of established chemical coupling procedure.[13] However, conventional labeling procedure is a random cross-linking chemical reaction; the resulted labeling reagent is a mixture of various conjugates, many of which are unwanted, e.g. enzyme−enzyme conjugates, antibody−antibody conjugates, conjugates with the active sites being blocked, etc. So, to obtain the reactive antibody-enzyme conjugates, a subsequent separation procedure must be needed. An attractive alternative to the chemical coupling is the construction of fusion protein consisting of an enzyme and an analyte-specific antibody moiety by genetic technology,[14] by which the enzyme-labeled antibody could be produced easily with a well-controlled quality.

We had obtained a PA-specific scFv-6w10 through phage display procedure and used it for detection of PA. To improve the affinity of scFv-6w10, a recombinant bivalent scFv antibody (biscFv-6w10) directed against PA was constructed and tested in functional assays. This biscFv-6w10 was fused with EAP by gene fusion and largely expressed in *Escherichia coli*. A protein chip format was built using the biscFv-6w10-EAP fusion protein for detection of PA and bacterial cells of *B. anthracis*. In addition, the detection of PA and cells with Cy3-labeled biscFv-6w10 was also performed. The experiment results are reported herein.

EXPERIMENTAL SECTION

Materials. PCR primers were synthesized by Sangon (Shanghai, China). Mouse scFv DNA construction kit, phage-displayed scFv expression and detection kits, anti-M13 tag antibody, and pCANTAB-5E were purchased from Pharmacia. DNA restriction enzymes, mRNA isolation kit, reverse transcriptase, Taq DNA polymerase, and T4 DNA ligase were bought from Promega. *E. coli* AD494 (DE3) and *E. coli* SM547, plasmid pET32a (+), and pASK75 were all stored in our laboratory. Plasmid pOPE101-215 (Yol) was kindly denoted by Professor Mi-Fang Liang (Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China). Ampicillin, kanamycin sulfate, bovine serum albumin (BSA), isoproyl β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl (BCIP), and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co. HRP-labeled goat anti-mouse IgG was from Sino-American Biotechnology Co. PCR purification minikit and gel extraction minikit were purchased from Shanghai Huashun Co. Protective antigen gene (*pag*) of *B. anthracis* was kindly donated by Dr. Beyer (University of Hohenheim, Institute for Environmental and Animal Hygiene, Stuttgart, Germany). Ni^{2+} metal affinity resins were from Novagen. All the oligonucleotides listed in Table 1 were synthesized by Shanghai Bioengineer Co. All other reagents used were of analytical-reagent grade.

Expression, Purification, and Western Blot Assay of the Recombinant Protective Antigen. The primer oligonucleotides P-1 and P-2, spanning the PA encoding gene *pag*, were synthesized to amplify *pag* gene using virulent *B. anthracis* genome as template.[15] Restriction enzymatic sites *Bgl*II and *Xho*I were introduced to the N- and C-terminal primers, respectively. PCR products were purified by a PCR purification kit and then cloned into the pGEM-T vector and confirmed by sequencing. The *Bgl*II/*Xho*I DNA fragment was cloned in-frame into the *Bgl*II/*Xho*I linearized expression vector pET32a(+) using the *E. coli* strain AD494 (DE3) for expression. Recombinant clones were grown overnight at 37 °C in liquid LB medium containing 100 μg/mL ampicillin and 25 μg/mL kanamycin sulfate. The cultures were diluted 100-fold in the same medium and grown until an OD_{600} of 0.3. Recombinant PA expression was induced by adding 1 mmol/L IPTG (final concentration) and analyzed by SDS−PAGE.

A PA-producing AD494 (DE3) clone was scaled up to 1 L. Three hours after induction, the bacteria were pelleted. The cells were washed and resuspended in PBS buffer. After being sonicated, the supernatant was purified with Ni^{2+}-chelation affinity resin. The purified PA was identified by Western blot assay.[16]

Immunization of Mice and mRNA Preparation. Six female mice were immunized with purified PA. PA was first injected subcutaneously using 50 mg of PA with complete Freund's adjuvant, and the later injections were taken every 20 days for a further three times with incomplete Freund's adjuvant. The anti-PA antibody titer was determined by ELISA.

Seven days after the last boost, spleen cells were prepared for mRNA extraction using the mRNA kit according to instruction. The spleen was cut roughly up into small pieces with sterile scissors in a sterile Petri dish, and the pieces were transferred into a 2-mL tube. After adding 1 mL of Trizol to the tube, the spleen tissue was homogenized with an mircohomogenizer on ice for ∼5 min. The homogenate was transferred to a 1.5-mL Eppendorf tube and 0.1 mL of chloroform was added. After shaking vigorously for 15 s, the homogenate was centrifuged at 12000*g* at 4 °C for 15 min. The clear aqueous upper layer was

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Table 1. List of Oligonucleotides

oligonucleotide[a]	sequence
P-1	AGAGATCTGATGAAAAAACGAAAAGT
P-2	TCACCTCGAGTTACCTTATCCTATC
P-3 (pCANTAB5-R1)	CCATGATTACGCCAAGGTTTGGAGCC
P-4 (pCANTAB5-R2)	CGATCTAAAGTTTTGTCGTCTTTCC
P-5	CAATTCCTTTAGTTGTTCCTTTCTATGC
P-6	CACCGTCGACTGAACCGCCTC
P-7	TGGAGTCGACGACATTGAGC
P-8	ACGGCACCGGCGCAC
P-9	CGCCGTCGACTGAGGAGAC
P-10	CAATTCCTTTAGTTGTTCCTTTCTATGC
P-11	CTGCGTCGACCCGTTTGATTTC
P-12	CCAGGAATTCATGGCCCAGG
P-13	ACGGCACCGGCGCAC
L-1	TCGACGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGAGGATCGG
L-2	AATTCCGATCCTCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCG
LAp-1	CCTGGCCCAGCCGGCCCATGGCGAGATCTGCGGCCGCTAGCGGCTCAGGATCTGGATCA TATCTGGCGGCGACGGATCCCTGCAGCATCATCACCATCATCATA
LAp-2	AGCTTATGATGATGGTGATGATGCTGCAGGGATCCGTCGACGCCAGATCCTGATCCAGAT CTGAGCCGCTAGCGGCCGCAGATCTCGCCATGGGCCGGCTGGGCCAGG
EAP-1	CGGACGTCGACACACCAGAAATGCCTG
EAP-2	TGCGCTGCAGTTTCAGCCCCAGAG

[a] Text in parentheses represents other names assigned to oligonucleotides.

carefully pipetted off from the lower organic layer, and the upper layer were transferred to a fresh Eppendorf tube. An equal volume of 2-propanol was added to the tube, and samples were centrifuged at 12000*g* at 4 °C for 15 min. The pellets were dried and dissolved in 100 μL of DEPC-treated water. This product was total RNA, and poly-(+)-mRNA was further purified using a Fast Track 2.0 kit from Invitrogen (Carlsbad, CA) according to standard procedure.

Construction of Phage-Displayed Anti-PA ScFv Library. The phage-displayed scFv library was prepared using a recombinant phage antibody system (RPAS) including mouse scFv and expression modules according to the protocols supplied by the manufacturer. Briefly, 5 μL of poly-(A)-mRNA was used for the cDNA synthesis of either the V_H or V_L with murine reverse transcriptase. The amplified V_H and V_L genes were assembled into a scFv gene using a linker sequence. The assembly was reamplified to incorporate flanking *Sfi*I and *Not*I cloning sites at its 5′- and 3′-ends, respectively, and the amplified products were purified, digested with *Sfi*I and *Not*I, and cloned into the *Sfi*I/*Not*I linearized phagemid pCANTAB-5E containing E-tag sequence in frame. The recombinant phagemid was then introduced into competent *E. coli* TG1 cells by electroporation. The transformed cells were inoculated for 1 h at 37 °C with shaking at 250 rpm. A 100-μL aliquot of the diluted, transformed cells was spread onto the SOBAG plates that contained 2% glucose and 100 μg/mL ampicillin. A 100-μL aliquot of untransformed competent TG1 cells was also spread in a separate plate as a negative control. The plates were incubated at 30 °C for overnight. The colonies on the plates were counted for calculation of the library size. The library size = (clone numbers × transformed rate)/mg of target DNA.

The recombinant phagemid was rescued with helper phage M13KO7, and recombinant clones were selected on a SOBAG medium, containing 2% glucose and 100 μg/mL ampicillin. Each transformed *E. coli* TG1 clone was transferred into a separate microtiter well for phage rescue. The supernatant containing the recombinant antibody phage was collected by centrifugation.[17]

Panning of Phage Display Library. A 96-well microtiter plate was coated overnight at 4 °C with 100 μL of 10 μg/mL PA solution in PBS and blocked with 200 μL of 4% nonfat milk in PBS (PBSM). A control panning was done in parallel in an uncoated, blocked well. Phages were added and incubated for 2 h at 37 °C. Unbound phages were removed by washing 20 times with PBS and 20 times with PBS + 0.05% Tween. Antigen-bound phages were eluted by adding 100 μL of triethylamine (1.0 M) for 10 min and neutralized with 100 μL of Tris-HCl (1.0 M, pH 7.4). Eluted phages were used to reinfect *E. coli* TG1 and plated on SOBAG medium to determine the titer.[18]

Screening Specific Binding Clones by Phage-ELISA. Monoclonal phage stocks were prepared in microtiter plates according to the RPAS protocol. For each phage clone, one well was coated with 100 μL of 5 μg/mL PA and blocked with 4% PBSM and another uncoated well blocked as a control. Phages were added, incubated for 2 h at 37 °C, unbound phages were washed away, and bound phages were detected with anti-M13 antibodies conjugated to HRP. The OD_{405} was measured after 30 min of 2,2-azino-di-3-ethylbenz-thiazoline sulfonate (ABTS) reaction.

Sequence of ScFv Gene. For each ELISA-positive clone, PCR and *Sfi*I/*Not*I enzyme digestion analysis were used for the further assay. After the further identification, the positive clones were sequenced with primers P-3 (pCANTAB5-R1) and P-4 (pCANTAB5-R2). Individual V_H and V_L sequences were searched against the Genbank/EMBL database and Kabat database for sequence homology analysis with known murine genes.

ScFv Expression. The selective positive scFv gene was digested from the pCANTAB-5E vector and subcloned into the *Nco*I/*Not*I linearized expression vector pOPE101-215 (Yol) fused to the C-terminal His-tag and C-myc-tag sequence in frame. The

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recombinant expression plasmid, designated as pOPE-6w10, was identified by restriction enzyme digestion and PCR.

Analysis of Soluble ScFv Fragments and Affinity Detection. For each analyzed scFv fragment, a well was coated with 100 μL of 10 μg/mL PA in PBS and blocked with 4% PBSM whereas an uncoated well was blocked as control. To both wells, 1 μg of each expressed scFv was added and incubated for 2 h at 37 °C. The plates were washed and bound scFv fragments were detected using HPR/anti-c-*myc* 9E10 monoclonal congjugate. The reaction, using ABTS as substrate, was detected at 405 nm.

The expressed scFv proteins were analyzed by SDS−PAGE. The affinity constant (K_{aff}) of scFv against PA was determined using the protocol described. Calculation of K_{aff} was according to the mathematic formula as follows: $K_{aff} = (n - 1)/(n[Ab2] - [Ab1])$, where [Ab1] and [Ab2] represent the respective scFv concentrations required to achieve 50% of the maximum absorbance obtained at two different concentrations of coated antigen ($[Ag1] = n[Ag2]$) and n is the dilution factor between concentrations of antigen used.[19]

Construction of Expression Vector of scFv Multimers. To construct an expression vector of scFv multimers, the selected positive plasmid scFv-6w10 (scFv monomer) having a 15-residue linker was used as the PCR template. The experiment protocols for the vector construction of each multimers expression are given below.

Construction of Diabody. The coding sequence of V_H of the diabody was generated by using primers P-5 and P-6 and digested with *Nco*I and *Sac*I to yield PCR fragment V_H comprising the linker DNA. The coding sequence of V_L of the diabody was generated by using primers P-7 and P-8 and digested with *Sal*I and *Not*I to yield PCR fragment V_L. *Nco*I/*Not*I-linearized vector pOPE101-215 (Yol), V_H DNA, and V_L DNA were purified by PCR purification kits and then ligated to form the diabody expression vector pOPE-diabody. The linker DNA between V_H and V_L coding sequences codes for a five-amino acid residue (GGGGS).

Construction of Triabody. The coding sequence of V_H of the triabody was generated by using primers P-5 and P-9 and digested with *Nco*I and *Sal*I to yield the PCR fragment V_H having no linker. The coding sequence of V_L of the triabody was the same as that of the diabody. After purification with PCR purification kits, the coding sequences of V_H and V_L and *Nco*I/*Not*I-linearized vector pOPE101-215 (Yol) were ligated to form the triabody expression vector pOPE-triabody, in which V_H and V_L was ligated directly.

Construction of Bivalent Antibody (BiscFv). The coding sequence of ScFv1 of biscFv was generated by using primers P-10 and P-11 and digested with *Nco*I and *Sac*I to yield the PCR fragment scFv1. The coding sequence of ScFv2 of biscFv antibody was generated by using primers P-12 and P-13 and digested with *EcoR*I and *Not*I to yield the PCR fragment scFv2. The synthesized oligonucleotides linkers L-1 and L-2 were annealed at 70 °C for 5 min to form a dsDNA fragment containing a 19-aa linker coding sequence named fragment L with *Sal*I and *EcoR*I at its N- and C-terminal, respectively. *Nco*I/*Not*I-linearized vector pOPE101-215 (Yol), fragment L, and scFv1 and scFv2 coding sequences were purified and then ligated to form the bivalent antibody expression vector pOPE-biscFv.

Figure 3 is an illustration of these multiforms. All these constructs were expressed using *E. coli* AD494 (DE3), and the scFv multimers were formed automatically after the proteins expressed in the host cell. The affinities of the expressed and purified products were determined using the above method.[20]

Atomic Force Microscop (AFM) Experiment. Purified proteins were concentrated by ultrafiltration to ~1.5 mg/mL in PBS buffer (pH7.4). A 10-μL protein sample was spotted on the mica surfaces. After 5 min, the mica surfaces were washed 20 times using MiniQ water to remove the protein that were not been adsorbed. The AFM images were obtained by using a Picoscan (Molecular Imaging Co., Tempe, AZ) operating under a magnetic ac mode with a Maclever type I. The AFM tip had a force constant of 0.6 N/m and resonant frequency of 75 kHz in air.[21]

Construction, Expression, Purification, and Activity Assays of Fusion Protein BiscFv-EAP. The adaptors LAp-1 and LAp-2 were mixed to introduce new multiple cloning sites into pASK75. After heating for 10 min at 94 °C, they were cooled slowly to room temperature and ligated (10:1 molar ratio) into the *Stu*I and *Hin*dIII digested vector fragment of pASK75. This introduced new multiple cloning sites. The resulting vector (pASKL) was transfected to *E. coli* SM547. The purified pASKL vector was isolated from pooled transformed bacteria and digested with *Sal*I and *Pst*I. The primers EAP-1 and EAP-2 were used to amplify *E. coli* EAP gene from the vector pASKII+L[22] by PCR.The amplified product (1.3 kb) was purified on agarose gel and digested with the restriction enzyme *Sal*I and *Pst*I. The digested EAP fragment was inserted into the *Sal*I/*Pst*I-digested pASKL to form the vector pASKLEAP. The scFv plasmid was digested with *Sfi*I and *Not*I, ligated into *sfi*I/*Not*I-digested pASKLEAP vector, and transfected to *E. coli* SM547. Transformed bacteria were picked and tested for the expression of biscFv-EAP fusion proteins. The fusion protein was purified by osmotic shock method, and the bifunctional activity assay was performed by standard methods.

Preparation of Protein Chips. Protein chip was prepared according to Wang et al.[23] The glass slides (25 mm × 72 mm) were treated with 5% K_2CrO_4 and 98% H_2SO_4. To create cell arrays on the chip, a mask was placed on the surface of the chip, and the rest of the glass chip was covered with olefin, allowing the cell surface to be exposed to 20% hydrofluoric acid. The etching was performed at room temperature for 30 min to generate four arrays, each containing 4 × 4 reaction wells. The etched slides were washed with Milli-Q water to remove the hydrofluoric acid. The next steps were referred to Rogers et al.[24] Briefly, the cleaned slides were immersed in 25% ammonia solution overnight and rinsed with Milli-Q water for 10 min, followed by rinsing with anhydrous ethanol. The slides were then immersed in a mixture of 1% (3-mercaptopropyl)trimethoxysilane (Sigma), 95% ethanol, and 16 mmol/L acetic acid (pH4.5) for 30−60 min. The silanized slides were immediately rinsed with a solution containing 95%

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ethanol and 16 mmol/L acetic acid (pH 4.5) and cured under dry nitrogen overnight at room temperature. PA sample was diluted to a series of concentration. One microliter of diluted PA sample was added to each well of the chip. The chips were then incubated in a humid chamber for 12−16 h, followed by blocking with 4% PBSM for 2 h. After being washed with PBS, the silanized protein chips coated with PA were ready for use. Each well of the protein chip was added with 1 μL of diluted biscFv-EAP, allowing interaction with the coated PA. The chip was incubated at room temperature for 1 h to perform the binding reaction.

Detection of PA with Protein Chips. The unbound biscFv-EAP were removed by washing three times with PBS sequentially. After adding 1 μL of the enzyme substrate BCIP/NBT solution to the wells, the reaction was allowed to perform at 37 °C for 20 min or up to 1 h. The purple color developed in the spots area during incubation indicated the existence of the PA antigen.

Cy3 labeling of biscFv was performed at room temperature in a 1-mL reaction mixture containing biscFv and Cy3 biofunctional dye. BiscFv to be labeled was dissolved at a concentration of 5 mg/mL, and this solution was added to the dye vial. The vial was then capped and mixed thoroughly for 30 min at room temperature. Cy3-labeled biscFv can be prepared from the excess, unconjugated dye by gel filtration chromatography. It is convenient to preequilibrate the column with phosphate-buffered saline and to elute using the same buffer. Two pink bands developed during elution. The faster moving band was Cy3-labeled biscFv, and the slower was free dye. Cy3-labeled biscFv was spotted onto the wells. After the unbound Cy3-labeled biscFv was removed, a GenePix 4000B (Axon Instrument) fluorescence scanner was used to obtain the Cy3 fluorescence images. All images were analyzed using GenePix Pro 4.0 analysis software (Axon Instruments).[25]

Detection of PA in Bacteria with Protein Chips. Four *B. anthracis* isolates, 8 *Bacillus* strains from other species, and 12 bacterial strains of the genera other than *Bacillus* were tested using both types of protein chip. All the spore-forming strain cultures were from the Institute of Microbiology and Epidemiology (IME), and the rest strains were home institute stocks. Bacteria culture and treatments were performed in the P3 laboratory of IME. The cultures were treated in boiling water for 30 min. After centrifugation, the supernatants were diluted and spotted onto the silanized protein chip wells. The subsequent steps were the same as for the detection of PA. At the same time, the cultures were diluted and spread onto the plates. Concentration of the bacterial sample solution was obtained by counting the clones on the plates.

EXPERIMENTAL RESULTS

Expression and Western Blot Assay of the Recombinant PA. To obtain a sufficient amount of protein for immunization, PA-mediated recombinant phage selection, and subsequent scFv fragment characterization, PA was overproduced in *E. coli* AD494 (DE3) using the pET32a (+) vector system. The expressed PA was actually a fusion structure, with a thioredoxin in its N-terminal and a His-tag in its C-terminal. The thioredoxin facilitate soluble yields of the expressed protein to overcome the "inclusion body" problem, while the His-tag was used to purification of the desired protein. This fusion structure has a molecular mass of 96 kDa and formed ~15% of the total cellular proteins (Figure 1A).

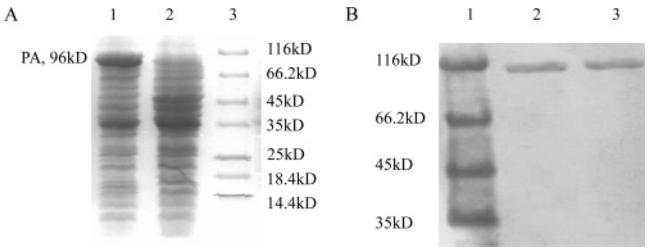

Figure 1. SDS−PAGE and Western blot assay of *E. coli* AD494-(DE3)/pET32a-*pag* expressed products. (A) SDS−PAGE assay. Line 1, the expressed products of AD494 (DE3)/pET32a-*pag* after induction for 4 h; line 2, the expressed products of AD494 (DE3)/pET32a; line 3, low molecular weight marker 116. (B) Western blot assay. Line 1, low molecular weight marker 116; lines 2 and 3, purified products of AD494 (DE3)/pET32a-*pa*g.

Western blot assay (Figure 1B) showed that the purified PA fusion protein retained its activity against antibodies.

Construction of Mouse Anti-PA Display Library. Total mRNA extracted from the spleens of two Balb/c mice that had high anti-PA antibody titer (~1:8000) was used to generate cDNAs. The cDNAs were then used to amplify the V_H (340 bp) and V_L (325 bp) chains. ScFv DNA fragments with an expected length of ~750 bp were obtained successfully (data not show). The phage-displayed scFv library was constructed with a transformed rate of 5.5×10^6. Fifty clones were randomly selected from the plates for plasmid extraction and analysis. Result showed that the recombinant rate was 90%, which indicated the size of the phage-displayed antibodies library was $\sim 5 \times 10^6$.

Isolation of PA-Binding ScFv Fragments. The recombinant phages with high affinity to PA were selected by six rounds of biopanning with 10 μg/mL PA per cell. As a control, panning was carried out against BSA. One hundred individual phage stocks were prepared both from the original library and from the enriched libraries after each panning step and screened for PA binding by ELISA. We were able to isolate two different PA-binding scFv fragments from the phage display library consisting of 5×10^6 recombinant clones. One phage, which gave stronger positive signal designated the clone scFv-6w10, was chosen for further study.

Sequence of the Anti-PA ScFv Gene. The nucleotide sequence of the anti-PA scFv-6w10 clone is presented in (Figure 2). The scFv has 741 nucleotides encoding 247 amino acids including a flexible amino acid linker of $(Gly_4Ser)_3$ and an E-tag polypeptide. Amino acid numbering and complementary determining region (CDR) of the V_H and V_L domains were determined according to Kabat et al.[26]

Anti-PA scFv-6w10 gene sequence blast using all GenBank + RefSeq Nucleotides + EMBL + DDBJ + PDB sequence showed that V_H and V_L sequences of scFv-6w10 belonged to the mouse antibody variable region genes. There were 97% homologues between V_H sequences of scFv6w10 and Mus musculus clone 3F9 anti-alpha02 integrin I-domain, and 95% homologues between V_H sequences of scFv6w10 and Mus musculus clone YZ-6G5 anti-VIPase.

Expression and Affinity of the ScFv Clone 6w10. Expression of the selected scFv in *E. coli* HB2151 (which usually used

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1   ATG GCC CAG GTG CAA CTG CAG CAG TCT GGG GCT GAA CTG GCA AGA CCT GGG GCC TCA GTG   60
1    M   A   G   V   Q   L   Q   Q   S   G   A   E   L   A   R   P   G   A   S   V   20
61  AAG ATG TCC TGC AAG GCT TCT GGC TAC ACC TTT ACT AAC TAC ACG ATG CAC TGG GTA AAA  120
21   K   M   S   C   K   A   S   G   Y   T   F   T   N   Y   T   M   H   W   V   K   40
                                            VH-CDR1
121 CAG AGG CCT GGA CAG GGT CTG GAA TGG ATT GGA TAC ATT AAT CCT AGC AGT GAT TAT ACT 180
41   Q   R   P   G   Q   G   L   E   W   I   G   Y   I   N   P   S   S   D   Y   T   60
                                                VH-CDR2
181 AAT TAC AAT CAG AAG TTC AAG GAC AAG GCC ACA  TTG ACT GCA GAC GAA TCC TCC AGC ACA 240
61   N   Y   N   Q   K   F   K   D   K   A   T   L   T   A   D   E   S   S   S   T   80
            VH-CDR2
241 GCC TTC ATG CAA CTG AGC AGC CTG ACA TCT GAG GAC TCT GCA GTC TAT TAC TGT GCA AGA  300
81   A   F   M   Q   L   S   S   L   T   S   E   D   S   A   V   Y   Y   C   A   R  100
                                                                        VH-CDR3
301  TTC CCC CAT CTC GAG GAC TAC TGG TAC TTC GAT GTC TGG GGC CAA GGG ACC ACG GTC ACC 360
101  F   P   H   L   E   D   Y   W   Y   F   D   V   W   G   Q   G   T   T   V   T  120
                    VH-CDR3
361 GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGA GGA TCG GAC ATT 420
121  V   S   S   G   G   G   G   S   G   G   G   S   G   G   G   G   S   D   I  140
                                    Linker
421 GAG CTC ACC CAG TCT CCA ACA ATC ATG TCT GCA TCT CTA GGG GAG AAG GTC ACC ATG ACC 480
141  E   L   T   Q   S   P   T   I   M   S   A   S   L   G   E   K   V   T   M   T 160

481 TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TAT TTG CAC TGG TAC CAG CAG AAG TCA GGT  540
161  C   R   A   S   S   S   V   S   S   S   Y   L   H   W   Y   E   E   K   S   G  180
                        VL-CDR1
541 GCC TCC CCC AAA CTC TGG ATT TAT AGT ACA TCC AAC CTG GCT TCT GGA GTC CCA GCT CGC  600
181  A   S   P   K   L   W   I   Y   S   T   S   N   L   A   S   G   V   P   A   E  200
                                VL-CDR2
601 TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGC ATG GAG GCT GAA  660
201  F   S   G   S   G   S   G   T   S   Y   S   L   T   I   S   S   M   E   A   E  220

661 GAT GCT GCC ACT TAT TAC TGC CAC CAG TAT CAT CGT TCC CCA CGG ACG TTC GGT GGA GGC  720
221  D   A   A   T   Y   Y   C   H   Q   Y   H   R   S   P   R   T   F   G   G   G  240
                                    VL-CDR3
721 ACC AAG CTG GAA ATC AAA CGG                                                    741
241  T   K   L   E   I   K   R                                                     247
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Figure 2. Nucleotides and deduced amino acid sequences of the scFv-6w10 fragment. Amino acid numbering and complementary determining regions of the V_H (CDR H) and V_L (CDR L) domains were determined.

for expression of scFv in phage display) still remains a rather low level that is not sufficient for clinical application. Furthermore, purification of the soluble scFv via the C-terminal E-tag is severely restricted by the high cost for anti-E-tag antibody. A generic strategy for transferring scFv-encoding variable regions from pCANTAB-5E into a highly efficient expression vector was reported to produce antibody fragments commercially.[27] In the present study, anti-PA scFv gene from the recombinant phagemid scFv-6w10 was subcloned into pOPE101-215 (Yol) with a c-*myc* sequence and expressed with a hexahistidine tail fusion protein to overcome the limitations of RPAS. The resulted expression vector pOPE-6w10 was expected to express a fusion protein of 29 kDa containing His-tags at C-terminal. The expression plasmid was introduced into *E.coli* AD494 (DE3) and induced by 1 mmol/L IPTG (final concentration) at 30 °C. SDS−PAGE revealed that a protein of ~29 kDa was strongly expressed after 3-h incubation with IPTG. By ELISA, the affinity constant of scFv-6w10 to the purified PA was determined to be 1.3×10^9 M^{-1}.

Formation of Multimeric ScFv Fragments and AFM Image of Triabodies. Figure 3 is an illustration of the multiform of the scFv-6w10. They formed through interactions between the monomers when expressed in the cells. With a linker of 15 amino acid residues $(GGGGS)_3$, V_H and V_L domains of scFv-6w10 associate and form a stable Fv fragment. The construct with the linker of 5-aa did not form a stable Fv fragment, but each two could form a diabody. If the constructs contain no linker, they are unable to fold to form an Fv fragment, but have a chance to form triabodies through interactions between their V_H and V_L domains. Each

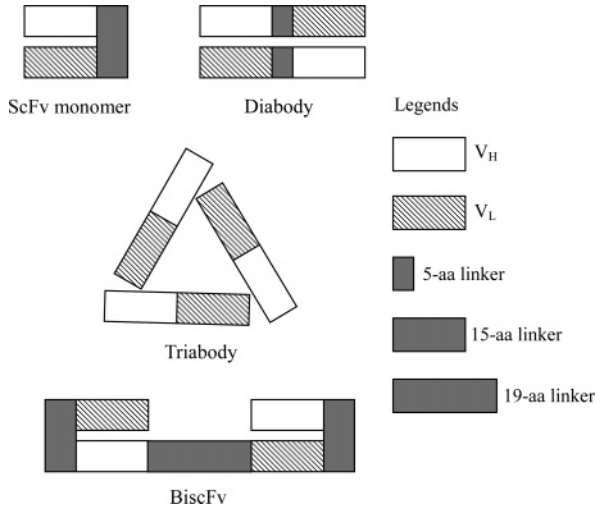

Figure 3. Carton of the formation of multimer proteins. scFv-6w10 having a 15-residue linker segment formed monomeric scFv; scFv-6w10 having 5-residue linker formed between V_H and V_L diabodies; scFv-6w10 having no linker between V_H and V_L promoted the assembly of scFvs into trimers (triabodies); bivalent antibody (BiscFv) was formed when two scFvs jointed by a 19-aa length linker.

pOPE-scFv construct was expressed in 400 mL and purified with Ni^{2+}-chelation affinity resin. The purified proteins were checked by SDS−PAGE. The bands of 29-kDa scFv-6w10 and 60-kDa band biscFv-6w10 on SDS−PAGE were seen, but there was no appearance of the 60-kDa band of diabodies and 90-kDa band of triabodies. Instead, they comprised only a single component with apparent molecular mass 27−29 kDa on reducing SDS−PAGE

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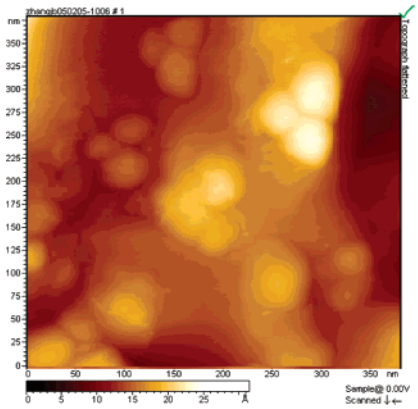

Figure 4. AFM 2-D images of the triabodies of scFv-6w10 immobilized on the mica surface.

Table 2. Affinity Constants of the Parent scFv-6W10 and the Multimeric Forms

form of scFv-6w10	affinity constant (M^{-1})[a]
monomer	1.3×10^9
diabody	2.5×10^9
triabody	3.8×10^9
BiscFv	6.5×10^9

[a] Average of three duplicated measurements.

(data not shown). We thus turned to use the AFM method to observe the formation of the multiform. The AFM 2-D image in Figure 4 provides evidence of existing triabodies of scFv-6w10. Many triabodies were seen on the mica surface. Each triabody was composed by three monomers to form a "Y" shape. Similar results were demonstrated in other investigations with electron micrographs[10,28] and X-ray crystal structure.[29] Bivalent antibody fragments were generated by the dimerization of two scFv fragments with a linker of 19 residues, and this biscFv-6w10 was the homodimerization of two fragments of the scFv-6w10. All these constructs should have more antigen binding sites than their parent scFv-6w10. The affinity constants of the parent scFv-6w10 and the multimeric forms are listed in Table 2. Obviously, these multimeric molecules possess an increased functional affinity, among them the bisFv-6w10 had the highest affinity constant, 6.5×10^9 M^{-1}, ~5 times higher than the monomer, which enhances the detection sensitivity.

Bifunctional Assay of BiscFv-EAP and Detection of PA. After expression and purification of the fusion protein, the EAP enzyme activity and antigen binding affinity of the biscFv-6w10-EAP fusion were carried out, and the result is shown in Figure 5A. On the microplate, both well 2 and well 3 were negative controls, well 3 was coated with PA without adding biscFv-6w10-EAP fusion protein, while well 2 was coated with BSA with adding the fusion. Well 1 showed a positive reaction, where the PA was coated and the fusion protein was added. All wells had two duplicates. The results clearly showed that the constructed fusion protein biscFv-6w10-EAP retained both EAP enzyme activity and antigen binding activity.

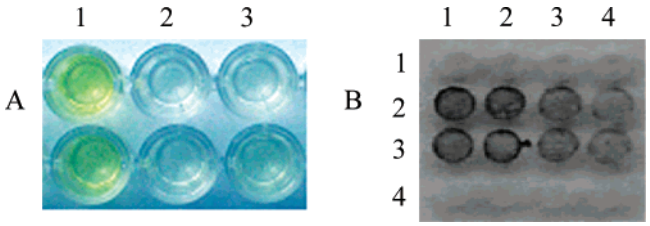

Figure 5. Evaluation of biscFv-6w10 and detection of PA. (A) Bifunctional activity assay of the fusion protein biscFv-6w10-EAP. 1, positive reactions, the wells that developed yellow show positive reactions; 2, negative control, the wells coated with BSA; 3, negative control, the wells coated with PA without adding enzyme; each loaded two duplicates. (B) PA detection on the protein chip. (2, 1), (3, 1), (2, 2), and (3, 2) are the positive results, other spots were all negtive controls; the quantities of PA on each spot: (2, 1) and (3, 1), 100 ng/mL PA; (2, 2) and (3, 2), 10 ng/mL PA; (2, 3) and (3, 3), 1 ng/mL PA; (2, 4) and (3, 4), 10 ng/mL BSA, respectively. The rest were all negtive controls.

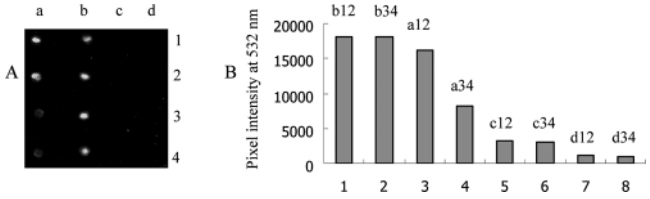

Figure 6. Detection of PA by Cy3-labeled scFv-6w10 on glass chip. (A) Scanning images. a3 and a4, 1 ng/mL PA; a1 and a2, 10 ng/mL PA; b1 and b2, 100 ng/mL PA; b3 and b4, 1000 ng/mL PA; c1 and c2, 1 ng/mL BSA; c3 and c4, 10 ng/mL BSA; d1−d4, blank control. (B) Pixel intensity analysis. Each column was the average of two duplicates, sample numbers correspond to those of (A).

To detect PA on the glass chip, BiscFv-6w10-EAP was spotted into the wells coated with PA. After the binding reaction, the unbound biscFv was removed by washing with PBS. A 1-μL sample of the enzyme substrate BCIP/NBT solution was added to each well. After 30-min incubation, a purple color developed in the spots area, indicating the existence of the PA antigen. Figure 5B shows a gradient detection of PA on the glass chip. About 10 ng/mL PA could be identified confidently in 2 h. To detect *B. anthracis* samples, the detection limit was ~500−1000 cells (by counting colonies on the culture plates).

Detection of PA with Cy3-Labled BiscFv. Cy3-labeled biscFv-6w10 was spotted into the wells coated with PA. After the binding reaction, the unbound biscFv-6w10 was removed by washing with PBS. A GenePix 4000B scanner was used to obtain the Cy3 fluorescence images. As shown in Figure 6, the detect limit was ~1 ng/mL, or ~50−100 bacterial spores, which is 10 times higher that the biscFv-6w10-EAP system.

Detection of Bacterial Samples. The specificity of protein chips was tested against four *B. anthracis* isolates and eight *Bacillus* strains from other species using biscFv-6w10-EAP and Cy3-labeled biscFv-6w10. All four of *B. anthracis* produced positive results (Table 3). In addition, 12 strains of other bacteria (genera other than *Bacillus*) were detected using this method, and all gave negative results.

DISCUSSION

Since the pioneer work of Smith 20 years ago, the phage antibody technique has become one of the most remarkable achievements in antibody technology. With the technique, several

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Table 3. Detection of Bacterial Samples

strain[a]	PA content	detection result
Bacillus anthracis (Sterne)	+	+
B. anthracis 170044	+	+
B. anthracis 170045	+	+
B. anthracis 17003−25	+	+
Bacillus subtilis	−	−
Bacillus pumilus	−	−
Bacillus cereus	−	−
Bacillus mycoides	−	−
Bacillus thuringiensis	−	−
Bacillus licheniformis	−	−
Bacillus sphaericus	−	−
Bacillus megaterium	−	−
Yersinia pestis EV76	−	−
E. coli JM109	−	−
E. coli DH5a	−	−
Pseudomonas aeruginosa	−	−
Shigella flexneri	−	−
Proteus mirabilis	−	−
Staphylococcus aureus	−	−
Burkholderia cepacia	−	−
Salmonella choleraesuis	−	−
Salmonella typhimurium	−	−
Corynebacterium pekinese	−	−
Lactobacillus lactis	−	−

[a] Each strain sample had two duplications.

scFv antibodies against PA of *B. anthracis* were obtained in this study (data not shown), among which the clone 6w10 showed highest affinity. The repertoire of V_H and V_L genes were amplified and joined together by PCR and were finally inserted into phagmid. The critical step in cloning scFv DNA was the assembly of V_H and V_L DNAs with linker DNA. The 93-base linkers have homology with the 3′-end of the V_H gene and the 5′-end of the V_L gene. A total of 24 bases on either end of the linkers are complementary to the ends of the V_H and V_L. The central 45 bases of the linkers encode the flexible $(GGGGS)_3$ linker that joins the V_H and the V_L to form a scFv fragment. The assembly reaction ultimately produces a small amount of the scFv gene where the V_H region is linked to the V_L region via a linker that maintains the correct reading frame. The scFv DNA fragment was −750 base pairs in length. The assembled antibody scFv DNA fragment was amplified with a set of oligonucleotide primers that introduce restriction sites for cloning into the pCANTAB 5E vector. *Sfi*I and *Not*I sites were added to the 5′- and 3′-ends of the scFv gene, respectively. These particular restriction sites occur with very low frequency in antibody genes and should allow most scFv genes to be cloned as a single *Sfi*I/*Not*I fragment. In the assembly and fill-in reaction, an exact quantification of the purified V_H and V_L products and the linker DNA is very essential. Even slight deviations of the equimolar ratio V_H/V_L/linker lead to either no visible scFv product or to the formation of V_H linker or V_L linkerimers, apparently 450 bp in size.[30]

Enzyme-scFv fusion is another point of the study. Compared with the conventional ELISA technique, the advantage of the biscFv-EAP fusion is obvious: the fusion structure is a ready enzyme-labeled antibody when expressed in *E. coli* cells, no subsequent labeling step is needed, and so it is very ease of use. This idea was realized using gene manipulation. The carefully designed linker peptides helped the fusion proteins fold correctly to form reasonable orientations that contained activities of the EAP catalytic center and scFv binding site. In Contrast, in the conventional ELISA, the conjugation reaction between the enzyme and the antibody is a random chemical labeling procedure, which results in a mixture of labeled, unlabeled, and inactivated coupled reagents and thus the reagents need further purification. Therefore, our protocol allows stable production of high-quality enzyme-labeling reagent from batch to batch. Besides, since the EAP protein is a dimmer, any fusion structure with EAP should have doubled antigen-binding sites, which benefits the detection sensitivity. However, EAP is an enzyme with low catalytic activity, its specific activity is about ∼40 units/mg, and this results in a low detection sensitivity. To solve this problem, we had previously obtained an evolved EAP with 30-fold increased catalytic activity through error-prone PCR and DNA shuffling.[22] Using this engineered EAP and an engineered divalent form of scFv to construct scFv-6w10-EAP, fusion resulted in a protein chip with sensitivity comparable to the conventional ELISA. The detection results could be seen by the naked eye, with no need of an expensive machine.

Cy3 dye is a bifunctional NHS-ester fluorescent, containing free amino groups. It is widely used for labeling DNA and proteins. ScFv labeled with Cy3 has two main advantages over the biscFv-EAP system: high fluorescence intensity and no cross-talking between dots of the array, so having higher sensitivity and resolution. These features make it suitable for high-density array detection in future study, in spite of the requirement of an expensive laser scanner for to read the results.

Supersonic and some lyses buffer treatment methods were used to extract protein from bacterial samples, and we obtained almost the same result (data not shown). The boiled water method is simpler than other methods, and this treatment can well denature bacterial proteins and could expose more protein binding sites. ScFv identifies a linear epitope rather than a conformational epitope. The boiled water method cannot destroy a linear epitope, So we chose the boiled water method to extract proteins from samples.

In conclusion, we had obtained a scFv-6w10 to PA through a phage display procedure and successfully constructed a number of scFv-6w10 multimoners and biscFv-6w10-EAP fusion. The protective antigen of *B. anthracis* was identified with high fidelity using the reagents with protein chip formats, reading either an enzymatic produced signal or a fluorescent signal. The proposed protocols provide alternatives for quick and sensitive detection of PA and *B. anthracis* cells and thus could be further exploited for real sample applications.

ACKNOWLEDGMENT

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