Analytical Letters, 39: 2153–2167, 2006

ISSN 0003-2719 print/1532-236X online
DOI: 10.1080/00032710600748905

Taylor & Francis
Taylor & Francis Group

SENSORS

Construction of Three Single-Chain Antibody Fragment Variable Fusion Structures for Sensitive Detection of Nucleocapsid Protein of Hantaan Virus

Jia Chen
Joint Research Group for Analytical Pathogen Microbiology, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, State Key Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China and Graduate School of the Chinese Academy Sciences, Beijing, China

Wei-Hong Xie, Zhi-Ping Zhang, Xian-En Zhang, Wei Li, Shi-Hua Wang, and Ya-Feng Zhou
State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China

Mi-Fang Liang
National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

Ke-Min Wang
State Key Laboratory of Chemical and Biological Sensing & Chemometrics, Hunan University, Changsha, China

Zhan-Qiu Yang
Institute of Virology, Medical College, Wuhan University, Wuhan, China

Received 20 November 2005; accepted 20 March 2006
Address correspondence to Xian-En Zhang, Joint Research Group for Analytical Pathogen Microbiology, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Tel./Fax: +86 10 64888464; E-mail: zhangxe@mail.most.gov.cn

Abstract: Three single-chain fragment variable (scFv) fusion structures were constructed for use in rapid and sensitive detection of nucleocapsid protein (NP) of Hantaan virus. The detection of NPs on glass chips was signalized by enzyme labeling or fluorescence dye Cy3, or Cy5 cluster nanoparticles. The sensitivity of the methods with different signal systems was evaluated and compared. The detection limits of scFv-alkaline phosphatase fusion, fluorescence labeling (scFv-Cy3), and nanoparticles labeling (scFv-SBP-streptavidin-nanoparticle) were 0.1 μg/mL, 1 ng/mL, and 0.1 ng/mL NP, respectively, which were all lower than that in a conventional enzyme-linked immunosorbent assay (ELISA) (1 μg/mL). Twenty Hantaan virus isolates were detected using the proposed methods.

Keywords: Hantaan virus, single-chain antibody fragment variable (scFv), fusion protein, alkaline phosphatase, fluorescence dye, nanoparticles

1. INTRODUCTION

Hantavirus (a genus of the *Bunyaviridae* family) is a causative agent of human infection of hemorrhagic fever with renal syndrome (HFRS). More than 50,000 cases have been found annually in China since 1981 (Song 1999) and the global cases are estimated at 100,000 to 200,000 every year (Hart and Bennett 1999). Due to its ease of becoming widespread and causing serious harm to people's health, the spread and infection of Hantavirus has become a growing public concern. Identification of the virus is thus a critical point in controlling the disease.

There have been some methods for Hantavirus detection, such as plaque reduction neutralization test (PRNT) (Koch et al. 2003), immunoassay (Brummer-Korvenkontio et al. 1980; Elgh et al. 1997; Takakura et al. 2003), and gene identification (Ahn et al. 2000; Tang et al. 2001; Moreli et al. 2004; Aitichou et al. 2005). PRNT is a conventional method for virus assay. It requires cell culture, and therefore is time consuming and not suited for rapid detection. Enzyme-linked immunosorbent assay (ELISA) can detect antigens or antibodies in serum; however, its sensitivity is too low to diagnose an early infection. To identify the specific gene or genotype of Hantavirus, reverse polymerase chain reaction (PCR) is normally required, because Hantavirus is a RNA virus. The method is very sensitive but sometimes produces false-positive results. Hantavirus nucleocapsid protein (NP), a major antigen inducing an early and long-lasting humoral immune response during Hantavirus infection (Zoller et al. 1993), has been used as the main antigen target in immunoassay. With enzyme or fluorescent dyes, labeling the sensitivity and reliability of immunoassay has been greatly improved.

Single-chain antibody fragment variable (scFv) is an engineered antibody of small molecule, in which the variable heavy chain (VH) and light chain (VL) of the antibody molecule are connected by a short, flexible polypeptide linker. Although scFv is only a part of a monoclonal antibody (mAb), it retains the complete antigen binding site, which allows it to maintain its specificity

for the antigen. In addition, it can be produced in large scale in *Escherichia. coli* at low cost. In particular, it is only 1/6 the size of the monoclonal antibody and this feature enables to be easily to fused by genetic manipulation with the labeling enzyme or other entities for detection, site-directed immobilization, and purification purposes (Ward 1993; Kortt et al. 2001; Kriangkum et al. 2001; Worn and Pluckthun 2001).

The aim of this study is to develop more sensitive and convenient methods by using recombinant scFv technology for the sensitive, fast, and early Hantaan virus detection. Three scFv fusion structures were constructed: scFv-alkaline phosphatase fusion, fluorescence labeling (scFv-Cy3), and nanoparticle labeling (scFv-SBP-streptavidin-nanoparticle). Sensitivity comparisons of the proposed methods were made with conventional ELISA as a reference. The results are presented herein.

2. MATERIALS AND METHODS

2.1 Antigen Preparation

Recombinant nucleocapsid protein (NP) expression was performed with *E. coli* AD494 (DE3) harboring pET32a (+) plasmid (see Fig. 1A). *E. coli* AD494 (DE3) and plasmid pET32a (+) were from Novagen Company. After preincubation overnight in LB medium (1 liter H_2O containing 10 grams Trypton, 5 grams yeast extract and 10 grams NaCl, autoclave to sterilize) containing kanamycin sulphate (15 μg/mL) and ampicillin (100 μg/mL), cells were inoculated with 1% into fresh LB medium, and further shaken at 37°C. For induction of expression, isopropylthio β-D-galactoside (IPTG) was added to a final concentration of 1 mmol/L at an optical density (OD_{600}) of 0.6 and cells were shaken for another 3 h at 28°C. Cells were harvested by centrifugation at 4,000 g for 10 min, then were resuspended in binding buffer (5.0 mmol/L imidazole, 0.5 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.9) and finally was disrupted by sonication. The lysate were centrifugated at 10,000 g for

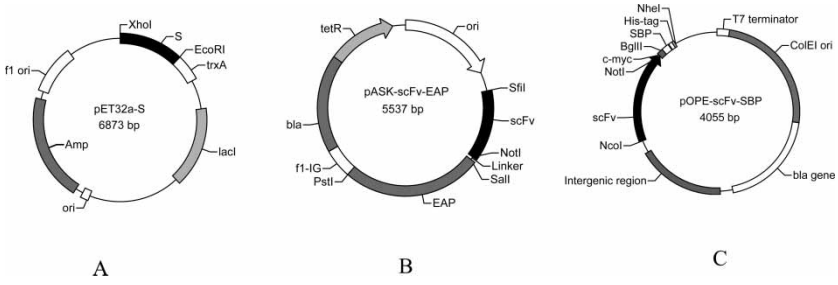

Figure 1. Expression vector of fusion proteins. (A) Trx-NP fusion protein; (B) scFv-EAP fusion protein; (C) scFv-SBP fusion protein.

15 min at 4°C. The clear supernatant was collected and processed on a nickel-nitrilotriacetic acid (NTA) resin. The chromatography procedure was performed according to the manufacturer's protocol (Qiagen, Merck, German). NP antigen concentration was identified using a bicinchoinic acid (BCA) kit (Pierce, USA). Streptavidin (SAV)-modified Cy5 cluster fluorescent nanoparticles were prepared according to the procedure reported previously (Tapec et al. 2002).

2.2 Single-Chain Fragment Variable Preparation and Modification

2.2.1 ScFv Preparation

Anti-NP scFv was acquired by phage-display technology. scfv was expressed after subcloned to pOPE101-215 (Yol) vector in *E. coli* (Li et al. 2003). The scFv gene and vector pOPE101-215 (Yol) were kindly donated by Prof. Mi-Fang Liang (National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention).

2.2.2 Preparation of scFv-*E. coli* Alkaline Phosphatase (EAP) Fusion Proteins

The adaptors *LAp*1 (CCT GGC CCA GCC GGC CCA TGG CGA GAT CTG CGG CCG CTA GCG GCT CAG GAT CTG GAT CAG TAT CTG GCG GCG ACG GAT CCC TGC AGC ATC ATC ACC ATC ATC ATA) and *LAp*2 (AGC TTA TGA TGA TGG TGA TGA TGC TGC AGG GAT CCG TCG ACG CCA GAT CCT GAT CCA GAT CCT GAG CCG CTA GCG GCC GCA GAT CTC GCC ATG GGC CGG CTG GGC CAG G) were mixed to introduce new multiple cloning sites into pASK75 (Shao et al. 2000). 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 *Hind* III digested vector fragment of pASK75. The resulting vector (pASKL) was transformed to *E. coli* SM547. (*E. coli* strain SM547 was kindly donated by professor Kantrowitz and pASK75 was purchased from Biometra.) The primers *phoA* 1 (CGG ACG TCG ACA CAC CAG AAA TGC CTG) and *phoA* 2 (CGG ACG TCG ACA CAC CAG AAA TGC CTG) were used to amplify the EAP coding gene *phoA* by PCR (Shao et al. 2000). The amplified product (1.3 kb) was purified and digested with the restriction enzyme *Sal* I and *Pst* I. The digested *phoA* fragment was ligated with the *Sal* I/*Pst* I-digested pASKL, and the ligation product was transformed to *E. coli* SM547. Then, positive cultures grew overnight at 37°C on selective LB plates (containing 100 μg/mL ampicillin). scFv gene was introduced enzyme sites *Sfi* I/*Not* I by PCR. Primer sequences were as followed: *Sfi* I-ATA TGG CCC AGC CGG CCC ATG GCG GAGG and *Not* I-TCA AGC GGC CGC GGA TAC AG. The following program was run: 5 min at 95°C followed by 30× (1 min at 94°C,

1 min at 55°C, and 1 min at 72°C). The purified PCR products were digested with *Sfi* I and *Not* I and ligated into *Sfi* I/*Not* I-digested pASKL + EAP vector (see Fig. 1B) and then transformed to *E. coli* SM547. Transformed bacteria were picked and tested for the expression of scFv-EAP fusion proteins according to references (Skerra et al. 1994; Shao et al. 2000).

For induction of expression, tetracycline (Tet) was added to a final concentration of 0.4 μM at an optical density (OD_{600}) of 0.5 and cells were shaken for another 6 h at 22°C. Bacteria were collected and then resuspended in 1/20 of the original culture volume of 50 mmol/L Tris/HCl buffer (pH 8.0), containing 20% sucrose and 1 mmol/L ethylenediaminetetraacetic acid (EDTA). After incubation for 5 min at 0°C, the bacteria were pelleted and subjected to osmotic shock by the addition of 1/20 the original culture volume of 5 mmol/L $MgSO_4$ and incubated for 45 min at 0°C and then centrifugated by 16,000 rpm for 30 min at 4°C. The clear supernatant containing the $(His)_6$-tagged scFv-EAP fusion proteins was purified with immobilized metal affinity chromatography (IMAC) using Ni^{2+}-charged chelating Sepharose. Bound proteins were eluted with binding buffer containing 250 mmol/L imidazole. ELISA was used to evaluate the bifunctional specificity of the scFv-EAP fusion proteins. The wells of a 96-well microtiter plate were coated with the NP antigen (10 μg/mL) for 2 h at 37°C or overnight at 4°C. After blocked with PBS containing 5% skimmed milk powder (PBSM; PBS containing 4 mmol/L KH_2PO_4, 16 mmol/L Na_2HPO_4, 115 mmol/L NaCl, pH 7.4) for 1 h at 37°C and washed with PBST (PBS containing 0.05% Tween-20), the wells were incubated with scFv-EAP proteins for 1 h at 37°C. After being washed, the wells were incubated with *p*-nitrophenyl phosphate (*p*NPP).

2.2.3 Preparation of scFv-SBP (Streptavidin Binding Protein) Fusion Proteins

The primers *SBP*1 (CCGC AGA TCT TTC GAG CTC AGG AGG TAG) and *SBP*2 (TT CACAG GCT AGC TTA GTG ATG ATG) were used to amplify SBP-tag coding sequence *sbp* by PCR. The following program was run: 5 min at 95°C followed by 30× (1 min at 94°C, 45 sec at 55°C, and 40 sec at 72°C). The amplified product (200 bp) was purified and digested with the restriction enzyme *Bgl* II and *Nhe* I. The digested *sbp* fragment was ligated with the *Bgl* II/*Nhe* I-digested pOPE101-215 (Yol) (containing scFv gene) (see Fig. 1C) and transformed to *E. coli* XL1-Blue. The transformed cells were grown overnight at 37°C on selective LB plates (containing 100 μg ampicillin/mL).

For induction of expression, IPTG was added to a final concentration of 0.1 mmol/L when the optical density (OD_{600}) reached 0.5 and the cells were shaken for another 6 h at 22°C. The expressed scFv-SBP fusion proteins were purified according to the above description.

ELISA was used to evaluate the specificity of the scFv-SBP fusion proteins. The wells of a 96-well microtiter plate were coated with NP antigen (10 μg/mL) for 2 h at 37°C or overnight at 4°C. After blocking

with 5% PBSM (200 μL/well) for 1 h at 37°C, the wells were incubated with scFv-SBP proteins for 1 h at 37°C, followed by washing with PBST. The washed wells were incubated with streptavidin conjugated with AP for 1 h at 37°C and washed again. The AP's substrate *p*-nitro phenyl phosphate (*p*NPP) was finally added for enzyme activity assay.

2.3 Preparation of Antigen-Coated Chips

The antigen-coated chips were prepared according to Wang et al. (2004). The glass slides (25 mm × 72 mm) were treated with 5% K_2CrO_4 and 98% H_2SO_4 and then etched with 20% hydrofluoric acid for 30 min at room temperature (glass slides for fluorescence scanning omit this step) to form arrays. After being immersed in a 25% ammonia solution and rinsed, the slides were immersed into a mixture of 1% 3-mercaptopropyl trimethoxysilane (MPTS), (Sigma), 95% ethanol, and 16 mmol/L acetic acid for 30–60 min. The silanized slides were immediately rinsed with the solution containing 95% ethanol and 16 mmol/L acetic acid and then cured under dry nitrogen overnight at room temperature. The antigens NP were coated as an array on the slides by pipetting (1 μL/well for EAP detection and 0.1 μL/spot for fluorescence scanning). The NP chips were then incubated in a humid chamber for 12–16 h, followed by washing and blocking with 5% PBSM at 37°C for 2 h. After washing with PBST, the silanized slides coated with serial ten-fold dilution NP antigen were ready for use, as described below.

2.4 Detection of NP with Fusion Proteins scFv-EAP

scFv-EAP fusion proteins were spotted onto the wells coated with gradient dilution NP antigen (1 μL/well). The chips were incubated for 1 h at 37°C to perform the binding reaction. The unbound scFv-EAPs were removed by washing three times with PBST. The enzyme substrate BCIP/NBT solution (5-bromo-4-chloro-3-indolyl phosphate [BCIP] and nitro blue tetrazolium [NBT]) was added to the wells. The reactions were allowed to perform at 37°C for 20 min or up to 1 h (Wang et al. 2004).

2.5 Detection of NP with Cy3-Labeled scFv

scFvs were purified on NTA resin according to Dolezal et al. (2000). The purified scFv proteins were then labeled with Cy3. The Cy3 dye is a bisfunctional NHS-ester for the labeling of compounds containing free amino groups and excited at 532 nm. The labeling of scFv was performed for 30 min at room temperature in a 1-mL reaction mixture containing scFv and Cy3 biofunctional dye. scFv to be labeled was dissolved in sodium carbonate–sodium

bicarbonate buffer (pH 9.0), and this solution was added to the dye vial. The vial was then capped and mixed thoroughly. Care was taken to prevent foaming of the fusion protein solution. The reaction mixture was incubated with additional mixing approximately every 10 min for 30 min at room temperature. Cy3-labeled scFv can be separated from the excess, unconjugated dyes by gel filtration chromatography. It is convenient to pre-equilibrate the column with phosphate-buffered saline and to elute the scFv proteins using the same buffer. Two pink bands developed during elution. The faster moving band was Cy3-labeled scFv and the slower band was free dyes. Cy3-labeled proteins were stored at -20°C (product specification, AmershamBioscience).

Cy3-labeled scFvs were added onto the glass chips coated with NP antigens (0.1 μL/spot). The chips were incubated for 1 h at room temperature to perform the binding reaction. The unbound scFv-Cy3 was removed by washing three times with PBST. A GenePix 4000B (Axon Instrument) fluorescence scanner with a sensitivity of 0.1 fluorophore/μm^2 for Cy3 was used to obtain the Cy3 fluorescence images. All images were analyzed using GenePix Pro 4.0 analysis software (Axon Instruments) (Bi et al. 2003).

2.6 Detection of NP with scFv-SBP Fusion Proteins and Nanoparticles

The scFv-SBP fusion proteins were added onto the slide coated with NP (0.1 μL/spot). The chips were incubated for 1 h at 37°C to perform the binding reaction. The unbound scFv-SBP proteins were removed by washing three times with PBST. Streptavidin (SAV)-modified Cy5 cluster fluorescent nanoparticles (excited at 635 nm) were added and incubated for 1 h at room temperature. The slides were washed three times (10 min each) with PBST to remove unbound nanoparticles. A GenePix 4000B was used to obtain the nanoparticle fluorescence images. All images were analyzed using GenePix Pro 4.0 analysis software (Axon Instruments).

2.7 Detection of NP with ELISA

The method was referred to by Ausubel et al. (1995). ELISA plates (Costar) were coated overnight at 4°C using gradient diluting NP antigen (100 μL/well). Wells were washed four times with PBST. PBSM was used as a blocking buffer (200 μL/well). Plates were incubated for 2 h at 37°C and then washed four times with PBST. Then, anti-NP mAb L133 was added to wells (100 μL/well). Plates were incubated for 2 h at 37°C and washed four times with PBST. Secondary goat anti-mouse IgG antibody conjugated with calf alkaline phosphatase (CAP) was added to each well (100 μL/well). Plates were incubated for 2 h at 37°C and washed four times with PBST. Finally, AP substrate *p*-nitrophenyl phosphate (*p*NPP) solution

(*p*NPP, 20 mM in 1*M* Tris-base, pH 10.0) was added to all wells. Absorbance was read at 405 nm. ELISA detection limit was determined by serial titration.

2.8 Detection of Clinical Isolates

Twenty Hantaan virus isolates from different patients' blood were provided by the Institute of Virology, Medical College of Wuhan University (China). scFv-SBP and nanoparticles were employed for the detections. By cell culturing, virus isolates were collected and treated innocuously. After being centrifugated, the suspension was diluted (1:1000) and pipetted onto the slides. After blocking and washing, the chips coated with antigen protein were prepared. Remaining procedures were the same as those described above. Additionally, the prototype strain of Hantaan virus strains 76-118 and A9 were used as positive controls and conventional ELISA (1:100 diluting samples) was carried out in parallel.

3. RESULT AND DISCUSSION

3.1 Expression of Recombinant Antigen

Figure 2A shows the expression of the recombinant NP. The expressed recombinant was a fusion structure, containing a thioredoxin in the N-terminal and a His-tag at the C-terminal. The former may increase the NP soluble yield and the latter facilitates the purification (Jiang et al. 2002; Wang and Wang 2004). The purified fusion protein was identified by Western blot assay, and the result showed that the fusion structure maintained its bioactivity against anti-NP antibodies (Fig. 2B). The recombinant NP was then used to prepare the protein chips.

3.2 Preparation of the scFv Fusion Proteins

The optimal temperature for *E. coli* growth is 37°C. Expression of the recombinant protein at this temperature is often preferred to keep growth periods as short as possible, because a number of recombinant proteins are primarily produced in an inclusion body at 37°C (Bi et al. 2005). The yield of soluble, functional protein can be substantially increased by lowering the expression growth temperature. Consequently, lower temperature was chosen to perform the soluble expression of the fusion proteins. Both scFv-EAP and scFv-SBP fusion proteins were functionally expressed in quantities of 20% of total cellular proteins (data not shown). The mature fusion scFv-EAP contains two EAPs and two scFvs, because functional EAP is a dimmer. So, the fusion structure is actually an enzyme-linked bivalent

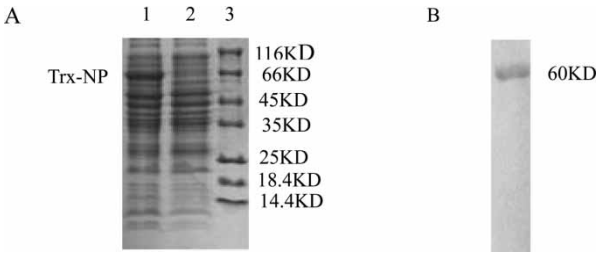

Figure 2. SDS-PAGE and Western blot assay of *E. coli* AD494 (DE3)/pET32a-Trx/NP expressed products. (A) Lane 1, the expressed products of AD494 (DE3)/pET32a-Trx/NP after induction for 3 h at 28°C; lane 2, the expressed products of AD494 (DE3)/pET32a; lane 3, low molecular weight marker 116. (B) Western blot assay of the crude extract of NP fusion protein.

antibody. The SBP-tag is a 38-amino acid–long streptavidin-binding peptide with an equilibrium dissociation constant (K_D) of 2.5 nM (Keefe et al. 2001). As the surface of the nanoparticle was modified with streptavidin, the specific interaction of SBP and streptavidin ensures the binding of the nanoparticles to scFv-SBP on the chip surface.

3.3 Detection of Hantaan Virus NP

The Hantaan virus NP was coated on the chip surfaces with a series of dilutions (antigen chips can be stored for about 2 weeks at 4°C). Constructs of scFv-EAP, scFv-Cy3, and scFv-SBP-streptavidin-nanoparticle were applied to bind the coated NP and to generate signal, respectively.

The specific activity of the wild-type EAP is about 40 U/mg, which is too low to be used for signaling. The EAP used in this study is an engineered one from our previous work through error-prone PCR and DNA shuffling, having catalytic activity over 1000 U/mg (Xu et al. 2003). Figure 3 shows the result of the protocol. The fusion partner EAP catalyzed the substrate to produce color precipitation. The result could be read either by the naked eye or quantitatively analyzed with the Adobe PhotoShop software. Statistic revealed that the detection limit was about 0.1 μg/mL of NP (signal-to-noise >3.0).

The scFv-Cy3 protocol is straightforward. No additional step and no additional reagent are needed. The Cy3 dye is an orange sulfoindocyanine dye with intense fluorescence and high water solubility, providing significant advantages over many other existing fluorophores (Mujumdar et al. 1993). Figure 4 shows the fluorescence images for NP detection. Four concentrations of Hantaan virus NP (0.1 μg/mL, 0.01 μg/mL, 1 ng/mL, and 0.1 ng/mL) were tested on the chips, and the limit of detection was 1 ng/mL of NP (signal-to-noise >3.0).

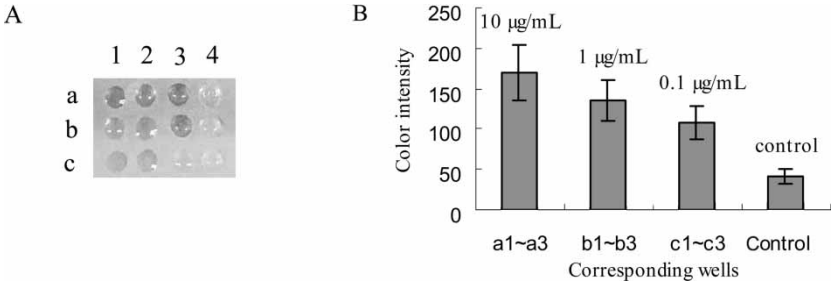

Figure 3. NP detection results using scFv-EAP on protein chips. (A) Enzymatic signal by the fusion protein scFv-EAP. Concentrations of NP in each row were: a1–a3, 10 μg/mL; b1–b3, 1 μg/mL; c1–c3, 0.1 μg/mL, respectively. Negative controls are made with BSA and concentrations were: a4, 10 μg/mL; b4, 1 μg/mL; c4, 0.1 μg/mL. Sample size was 1 μl/well. (B) Color intensity analysis. Columns 1, 2, 3, 4 correspond to the average of a1–a3, b1–b3, c1–c3, the control (a4, b4, c4) of (A), respectively.

In the nanoparticle protocol, scFv-SBPs were first bound to the NPs that were coated on the chips; streptavidin nanoparticles were then added. Labeling of the signal molecules onto the detection antibodies was achieved through SBP-streptavidin interaction. The nanoparticle is an organic dye-doped silica component. One nanoparticle encapsulates thousands of Cy5 molecules in the silica matrix, enabling significant amplification of the response signal. It therefore can remarkably enhance the detection sensitivity and meets the requirements of detection of low abundant proteins (Santra et al. 2001; He et al. 2004; Lian et al. 2004; Tan et al. 2004; Zhou and Zhou 2004).

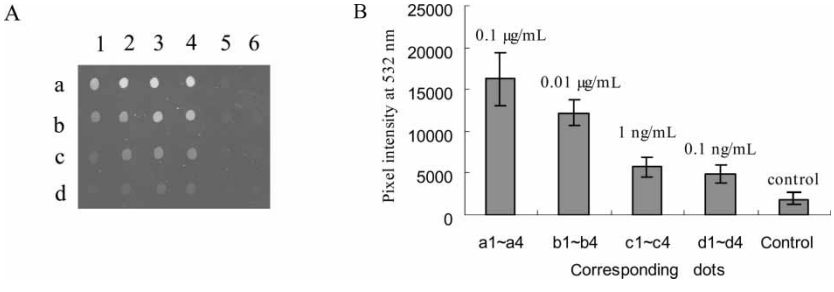

Figure 4. NP detection results using scFv-Cy3 on protein chips. (A) Fluorescence signal on the protein chip. Concentrations of NP in each spot were: a1–a4, 0.1 μg/mL; b1–b4, 0.01 μg/mL; c1–c4, 1 ng/mL; d1–d4, 0.1 ng/mL. The negative controls were also made with BSA and concentrations were: a5–a6, 0.1 μg/mL; b5–b6, 0.01 μg/mL; c5–c6, 1 ng/mL. The sample size was 0.1 μL/spot. (B) Pixel intensity analysis at 532 nm. Columns 1, 2, 3, 4, 5 correspond to the average of a1–a4, b1–b4, c1–c4, d1–d4, the control of (A), respectively.

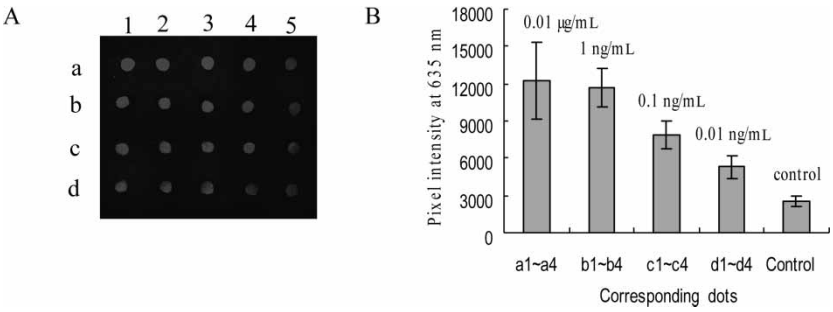

Figure 5. NP detection results based on scFv-SBP & nanoparticle. (A) Nanoparticle fluorescence signal based on scFv-SBP. Concentrations of NP in each spot were: a1–a4, 0.01 μg/mL; b1–b4, 1 ng/mL; c1–c4, 0.1 ng/mL; d1–d4, 0.01 ng/mL. The negative controls were also made with BSA and concentrations were: a5, 0.01 μg/mL; b5, 1 ng/mL; c5, 0.1 ng/mL; d5, 0.01 ng/mL. The sample size was 0.1 μL/spot. (B) Pixel intensity analysis at 532 nm. Columns 1, 2, 3, 4, 5 correspond to the average of a1–a4, b1–b4, c1–c4, d1–d4, the control of (A) respectively.

In this study, the detection limit with nanoparticles was 0.1 ng/mL of NP (Fig. 5) (signal-to-noise >3.0).

Table 1 summarizes the performances of all three protocols plus the conventional ELISA method. Each method has its distinct features in terms of sensitivity and detection time cycle, as well as operation convenience. All the proposed protocols are compared with the conventional ELISA. Among them, the scFv-EAP system produces results directly seen by the naked eye; the scFv-Cy3 protocol is sensitive and the simplest; and the nanoparticle protocol is obviously the most sensitive.

3.4 Detection of Hantaan Virus Isolates

Detection results of 20 Hantaan virus isolates using scFv-SBP and nanoparticle protocols are shown in Table 2. As predicted, all Hantaan virus isolates gave positive results. The sensitivity of detection for the viral isolates was lower than purified NPs due to interference arising from

Table 1. Comparison of different methods for detection of Hantaan virus NP

Method	Signal molecules	Recognizing molecules	Sensitivity
Direct ELISA	CAP	mAb	1 μg/mL
scFv-EAP	EAP	scFv	0.1 μg/mL
scFv-Cy3	Cy3	scFv	1 ng/mL
scFv-SBP	Nanoparticles	scFv	0.1 ng/mL

Table 2. Detection of NP in Hantaan virus isolates

Sample no.	Isolate	Detection results
1	*HV*-92	+
2	*HV*-102	+
3	*HV*-178	+
4	*HV*-238	+
5	*HV*-53	+
6	*HV*-100	+
7	*HV*-101	+
8	*HV*-114	+
9	*HV*-375	+
10	*HV*-376	+
11	*HV*-430	+
12	*HV*-431	+
13	*HV*-435	+
14	*HV*-553	+
15	*HV*-558	+
16	*HV*-654	+
17	*HV*-674	+
18	*HV*-689	+
19	*HV*-379	+
20	*HV*-204	+
21	BSA	–

+ means positive detection; – means negative detection result.

heterogeneous proteins of the samples. When NPs of the samples were immobilized on the slides, other irrelevant proteins (mainly structure proteins of Hantaan virus, such as envelope glycoproteins) were also absorbed. These interfering proteins competed with NPs for open sites on the slides, which resulted in a poor protein surface performance. Still, the chip-based detections were more sensitive than the conventional ELISA. As results, the ELISA method could detect 1:100 diluting samples, while the proposed protocols could detect the target antigen after 1:1000 dilutions of the same samples.

4. CONCLUSION

Three scFv fusion structures were constructed for rapid and sensitive detection of Hantaan virus NP with chip formats. All three protocols share the advantages of high sensitivity and easy operation, as well as potential high-throughput detections. Nevertheless, each protocol has its own features and thus could be used for different purposes. Further studies include optimization of the detection systems and applications of the real samples and multi samples.

ACKNOWLEDGMENT

Support from the Chinese Academy of Sciences and the Ministry of Science and Technology is acknowledged.

REFERENCES

Ahn, C., Cho, J.T., Lee, J.G., Lim, C.S., Kim, Y.Y., Han, J.S., Kim, S., and Lee, J.S. 2000. Detection of Hantaan and Seoul viruses by reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) in renal syndrome patients with hemorrhagic fever. *Clin. Nephrol.*, 53 (2): 79–89.

Aitichou, M., Saleh, S.S., McElroy, A.K., Schmaljohn, C., and Ibrahim, M.S. 2005. Identification of Dobrava, Hantaan, Seoul, and Puumala viruses by one-step real-time RT-PCR. *Virol. Methods*, 124 (1–2): 21–26.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. 1995. *Short Protocols in Molecular Biology*, 3rd ed.; John Wiley & Sons, Inc.: New York, NY.

Bi, L.J., Zhou, Y.F., Zhang, X.E., Deng, J.Y., Zhang, Z.P., Xie, B., and Zhang, C.G. 2003. A MutS-based protein chip for detection of DNA mutations. *Anal. Chem.*, 75: 4113–4119.

Bi, L.J., Zhou, Y.F., Zhang, X.E., Deng, J.Y., Wen, J.K., and Zhang, Z.P. 2005. Construction and characterization of different MutS fusion proteins as recognition elements of DNA chip for detection of DNA mutations. *Biosens. Bioelectron.*, 21 (1): 135–144.

Brummer-Korvenkontio, M., Vaheri, A., Hovi, T., von Bonsdorff, C.H., Vuorimies, J., Manni, T., Penttinen, K., Oker-Blom, N., and Lahdevirta, J. 1980. Nephropathia epidemica: Detection of antigen in bank voles and serologic diagnosis of human infection. *J. Infect. Dis.*, 141: 131–134.

Dolezal, O., Pearce, L.A., Lawrence, L.J., McCoy, A.J., Hudson, P.J., and Kortt, A.A. 2000. ScFv multimers of the antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V_L to V_H orientation drives the formation of dimmers, trimers, tetramers and higher molecular mass multimers. *Protein Eng.*, 13 (8): 565–574.

Elgh, F., et al. 1997. Serological diagnosis of hantavirus infections by an enzyme-linked immunosorbent assay based on detection of immunoglobulin G and M responses to recombinant nucleocapsid proteins of five viral serotypes. *J. Clin. Microbiol.*, 35: 1122–1130.

Hart, C.A. and Bennett, M. 1999. Hantavirus infections: epidemiology and pathogenesis. *Microbes. Infect.*, 1 (14): 1229–1237.

He, X., Duan, J., Wang, K., Tan, W., Lin, X., and He, C. 2004. A novel fluorescent label based on organic dye-doped silica nanoparticles for HepG liver cancer cell recognition. *J. Nanosci. Nanotechnol.*, 4 (6): 585–589.

Jiang, S.T., Tzeng, S.S., Wu, W.T., and Chen, G.H. 2002. Enhanced expression of chicken cystatin as a thioredoxin fusion form in *Escherichia coli* AD494(DE3) pLysS and its effect on the prevention of surimi gel softening. *J. Agric. Food Chem.*, 50 (13): 3731–3737.

Keefe, A.D., Wilson, D.S., Seelig, B., and Szostak, J.W. 2001. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-tag. *Protein Expr. Purif.*, 23: 440–446.

Koch, J., Liang, M., Queitsch, I., Kraus, A.A., and Bautz, E.K. 2003. Human recombinant neutralizing antibodies against hantaan virus G2 protein. *Virology*, 308 (1): 64–73.

Kortt, A.A., Dolezal, O., Power, B.E., and Hudson, P.J. 2001. Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting. *Biomol. Eng.*, 18 (3): 95–108.

Kriangkum, J., Xu, B., Nagata, L.P., Fulton, R.E., and Suresh, M.R. 2001. Bispecific and bifunctional single chain recombinant antibodies. *Biomol. Eng.*, 18 (2): 31–40.

Li, J.D., Wang, T., Li, C., Ji, Y., and Liang, M.F. 2003. Construction and expression of anti-hantavirus N protein single chain Fv antibody in *Escherichia coli*. *Chin. J. Virol.*, 19 (2): 118–122.

Lian, W., Litherland, S.A., Badrane, H., Tan, W., Wu, D., Baker, H.V., Gulig, P.A., Lim, D.V., and Jin, S. 2004. Ultasensitive detection of biomolecules with fluorescent dye-doped nanoparticles. *Analytical Biochemistry*, 334 (1): 135–144.

Moreli, M.L., Sousa, R.L., and Figueiredo, L.T. 2004. Detection of Brazilian hantavirus by reverse transcription polymerase chain reaction amplification of N gene in patients with hantavirus cardiopulmonary syndrome. *Mem. Inst. Oswaldo. Cruz.*, 99 (6): 633–638.

Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C., and Waggoner, A.S. 1993. Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjugate Chem.*, 4 (2): 105–111.

Santra, S., Zhang, P., Wang, K., Tapec, R., and Tan, W. 2001. Conjugation of biomolecules with luminophore-doped silica nanoparticles for photostable biomarkers. *Anal. Chem.*, 73 (20): 4988–4993.

Shao, W.H., Zhang, X.E., Liu, H., Zhang, Z.P., and Anthony, E.G.C. 2000. Anchor-chain molecular system for orientation control in enzyme immobilization. *Bioconjugate Chem.*, 11 (6): 822–826.

Skerra, A. 1994. Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. *Gene*, 151 (1–2): 131–135.

Song, G. 1999. Epidemiological progresses of hemorrhagic fever with renal syndrome in China. *Chin. Med. J. (Engl).*, 112 (5): 472–477.

Takakura, A., Goto, K., Itoh, T., Yoshimatsu, K., Takashima, I., and Arikawa, J. 2003. Establishment of an enzyme-linked immunosorbent assay for detection of hantavirus antibody of rats using a recombinant of nucleocapisid protein expressed in *Escherichia coli*. *Exp. Anim.*, 52 (1): 25–30.

Tan, W., Wang, K., He, X., Zhao, X.J., Drake, T., Wang, L., and Bagwe, R.P. 2004. Bionanotechnology based on silica nanoparticles. *Med. Res. Rev.*, 24 (5): 621–638.

Tang, J.Q., Cao, M., Tang, T., Wang, C.J., Wei, C.B., and Lei, W. 2001. Genotyping and preparation of the recombinant nucleocapsid protein antigen of hantavirus. *Chin. Med. J.*, 114 (10): 1030–1034.

Tapec, R., Zhao, J., and Tan, W. 2002. Development of organic dye-doped silica nanoparticle for bioanalysis and biosensors. *J. Nanotechnol.*, 2: 405.

Wang, S.H., Wen, J.K., Zhou, Y.F., Zhang, Z.P., Yang, R.F., Zhang, J.B., Chen, J., and Zhang, X.E. 2004. Identification and characterization of *Bacillus anthracis* by multiplex PCR on DNA chip. *Biosens. Bioelectron.*, 20 (4): 806–812.

Wang, T.F. and Wang, A.H. 2004. High-throughput screening of soluble recombinant proteins. In *Purifying Proteins for Proteomics: A Laboratory Manual*; Simpson, R.J. (ed.); Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Ward, E.S. 1993. Antibody engineering using *Escherichia coli* as host. *Adv. Pharmacol.*, 24: 1–20.

Worn, A. and Pluckthun, A. 2001. Stability engineering of antibody single-chain Fv fragments. *J. Mol. Biol.*, 305 (5): 989–1010.

Xu, H.F., Zhang, X.E., Zhang, Z.P., Zhang, Y.M., and Gass, A.E. 2003. Direct evolution of *E. coli* alkaline phosphatase towards higher catalytic activity. *Biocatal. Biotransfor.*, 21 (1): 41–47.

Zhou, X. and Zhou, J. 2004. Improving the signal sensitivity and photostability of DNA hybridizations on microarrays by using dye-doped core-shell silica nanoparticles. *Anal. Chem.*, 76 (18): 5302–5312.

Zoller, L., Yang, S., Gott, P., Bautz, E.K., and Darai, G. 1993. Use of recombinant nucleocapsid proteins of the hantaan and nephropathia-epidemica serotypes of hantavirus as immunodiagnostis antigens. *J. Med. Virol.*, 39: 200–207.