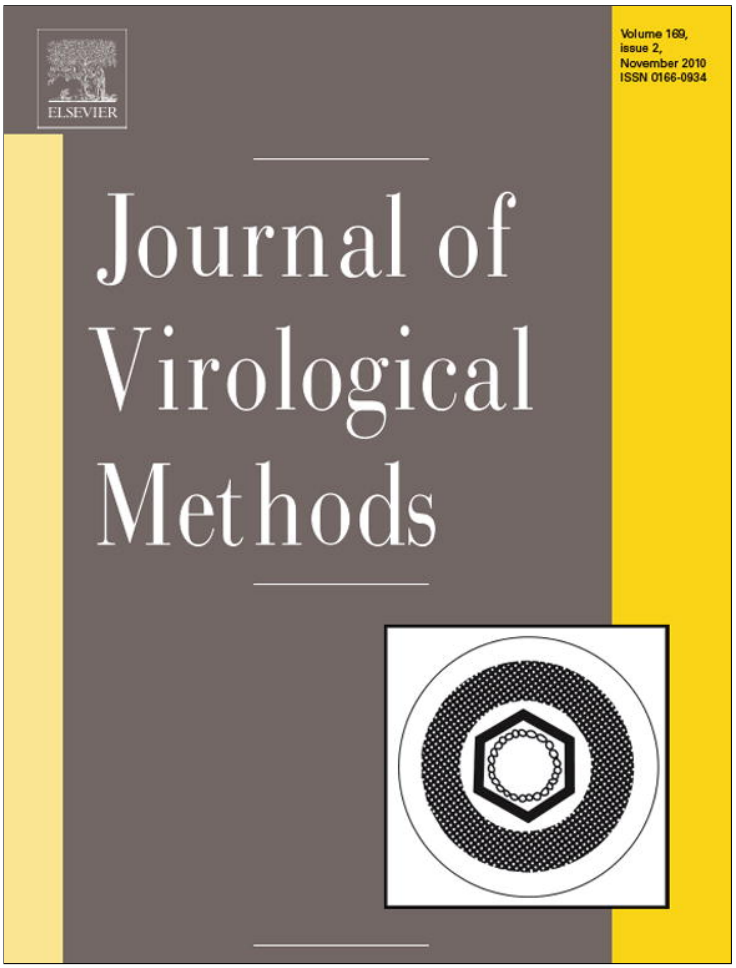

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Protocols

Influenza virus detection with pentabody-activated nanoparticles

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A nanoparticle-based immunoassay was developed for the rapid and sensitive detection of avian influenza virus (AIV). In this method, AIV-specific pentabody (pVHH3B) was conjugated to magnetic nanoparticles (MNPs) and used to capture AIV. Gold nanoparticles (GNPs), labelled with the anti-AIV mouse monoclonal antibody 3C8, were used as a detector. In the presence of target samples, the pentabody pVHH3B enriched AIV on the MNPs. Thereafter, mAb 3C8-labelled GNPs (GNPs-mAb3C8) bound to MNPs via AIV and were separated using a magnetic field. GNPs in the complex catalyzed the oxidation of hydroquinone to quinone, and the OD value of quinone was measured. The developed assay displayed substantial signal change after incubation in an AIV sample in a concentration-dependent manner. The detection limit was 10 ng/ml, which is 10 times more sensitive than conventional double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). In conclusion, by combining MNPs and a novel pentabody pVHH3B, this study provided a sensitive influenza viral detection assay that has the potential to become a rapid, sensitive and inexpensive diagnostic tool for infectious diseases.

1. Introduction

Influenza virus has become an increasingly severe threat to human health. The recent outbreak of H1N1 influenza and avian influenza virus H5N1 requires diagnosis and treatment of influenza virus infection. Early diagnosis of a viral infection can improve patient outcomes and decrease the possibility of viral transmission. For influenza virus detection, various antibody-based ELISA (Luo et al., 2008) or immunofluorescence (Madeley and Peiris, 2002) tests have been used widely in addition to conventional methods such as virus isolation and reverse transcription polymerase (RT-PCR) techniques (de Jong and Hien, 2006). Strategies have been developed to improve the specificity or sensitivity of diagnosis, including the development of more specific antibodies (He et al., 2007) and introducing microspheres in the immunoassay (Deregt et al., 2006). Despite these efforts, the development of more sensitive antibody-based assays for rapid detection of viruses remains a challenge.

Camelidae such as camels, llamas and alpacas develop heavy chain antibodies along with conventional IgGs as part of their humoral immune system (Hamers-Casterman et al., 1993). Unlike conventional IgGs, the heavy chain antibodies consist of only V_H, C_H2 and C_H3 domains. The V_H of heavy chain antibodies, responsible solely for antigen binding, is often referred to as the variable region of the heavy chains of heavy chain antibodies (V_HH) or single-domain antibodies (sdAb). Compared with other recombinant antibodies such as scFv or Fab, sdAb has been shown to have many advantages, including their small size, simple structure and high expression level in various expression systems (Sheriff and Constantine, 1996). In addition, the high surface hydrophilicity renders sdAbs more soluble (Joosten et al., 2005) and more stable with respect to thermally induced denaturation, even at 90 °C (van der Linden et al., 1999).

Different approaches, including *in vitro* affinity maturation (Friedman et al., 2008) and oligomerization (Zhang et al., 2004a, b), have been developed to improve the affinities or functional affinities of sdAbs. The affinity maturation method requires sdAbs library construction and the screening of new binders. As a alternative, production of oligomers, such as the formation of pentamers (Zhang et al., 2004b), can be used to generate high-avidity pentameric sdAbs (pentabodies) quickly.

Magnetic nanoparticles are of particular interest because they are used in the fields of biological imaging and separation (Taton et al., 2001; Wang et al., 2001). Several ultrasensitive protein detection methods that take the advantages of both the use of magnetic nanoparticles (MNPs) and the use of gold nanoparticles (GNPs) have been developed (Ao et al., 2006; Fan et al., 2008).

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Table 1
The primers used for library construction.

Primers for library	
First PCR	
CH2FORTA4	5′-CGCCATCAAGGTACCAGTTGA-3′
VHBACKA6	5′-GATGTCCAGCTGCAGGCGTCTGG (A\G) GGAGG-3′
Second PCR (with SfiI restriction site)	
F-PRCCAMEL	5′-CCTTTCTATGCA*GGCCCAGCCGGCC*GCATGGCCGA(G/T)G T(G/C)CAGCT-3′
R-PCRCAMEL	5′-GGCCGCAA*GGCCTCGGGGGCC*TGAGGAGACGGTGACCTG-3′

Nested PCR was used to exclude the contamination of V_H originating from conventional antibodies.

In this study, a novel immunoassay for the rapid and sensitive detection of AIV, combining pentabody-labelled MNPs and mAb 3C8-conjugated GNPs, has been developed. First, target antigens of AIV were enriched using a pentabody that was conjugated onto MNPs. Then, mAb 3C8-conjugated GNPs sandwiched AIV with the MNP-pentabodies and were separated using a magnet. The GNPs in the complex catalyzed the oxidation of hydroquinone, and the OD value of the generated quinone was measured at 390 nm.

2. Materials and methods

2.1. Plasmids, antibodies, virus and reagents

pET28a (+) vector was purchased from Novagen (Darmstadt, Germany). pCANTAB5E phagemid vector and horseradish peroxidase (HRP)-conjugated anti-M13 antibodies were purchased from Amersham Pharmacia (Buckinghamshire, England). HRP-conjugated anti-mouse IgG was obtained from Pierce (Rockford, IL, USA), and anti-His-Tag mAb was obtained from Novagen (Darmstadt, Germany). The pVT2 vector used for the formation of pentabody has been reported previously (Stone et al., 2007b). Pure, inactivated avian influenza virus A/Chicken/Henan/16/2004 (H5N1) was provided by Dr. Ze Chen from Wuhan Institute of Virology, Chinese Academy of Sciences. Pure, inactivated A/Turkey/England/N28/73 (H5N2) virus was provided by Beijing Veterinary Centre, and the AIV-specific mouse monoclonal antibody 3C8 was obtained from WANTAI Biological Pharmacy Enterprise (Beijing, China). The 1-ethyl-3-(3-dimethly-aminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and Silver Enhancement Kit solution were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Immunization of alpacas and generation of an immune single-domain antibody library

To obtain a good immunization effect and elicit antibodies having a broad spectrum against influenza virus, two types of highly purified influenza viruses were used. Two healthy young alpacas were immunized primarily with pure inactivated A/Chicken/Henan/16/2004 (H5N1) virus (1 ml, 500 μg) and A/Turkey/England/N28/73 (H5N2) virus (1 ml, 500 μg) mixed with an equal volume of Freund's incomplete adjuvant. Two boosts were given with same amount of antigen at days 30 and 45 after the first immunization. Following each boost, the antibody titre in the animal serum was measured. One week after the last boost, 10 ml of blood was taken from the two alpacas and used to construct the library.

The blood of immunized alpacas was collected and treated with red cell lysis buffer (TIANGEN Biotech, Beijing, China) for 30 min at room temperature, and the peripheral blood lymphocytes were prepared after centrifuging. Total RNA was isolated from peripheral blood lymphocytes using the RNAgent® kit from Promega (WI, USA). The concentration of mRNA was calculated from the absorbance at 260 nm. Approximately 2.5 μg of mRNA was used for preparing first-strand cDNA using the Superscript®III RT system (Invitrogen, Carlsbad, CA, USA).

To avoid contamination with V_H genes originating from conventional antibodies, sdAb genes were amplified by nested PCR (Arbabi Ghahroudi et al., 1997) using primers with the restriction site *SfiI*, as listed in Table 1. The 5′ part of the immuno-globulin heavy chains was amplified by PCR using the primers VHBACKA6 and CH2FORTA4. In this primary PCR, 2 μl of cDNA was mixed with 2 pmol of each of the two primers. For ExTaq (Takara, Otsu, Shiga, Japan), 5 μl of 10× reaction buffer, 2 μl of 2.5 mM dNTPs were added to a final volume of 100 μl. The PCR protocol consisted of an initial denaturation step at 94 °C for 5 min, followed by 25 cycles of (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min) and a final extension step at 72 °C for 7 min. The V_HH genes of the PCR products from the first PCR were reamplified with the primers F-PRCCAMEL and R-PRCCAMEL using the same PCR protocol.

After the sdAb genes were inserted into the phagemid pCANTAB5E, *Escherichia coli* TG1 cells were transformed with the ligation products by electroporation, and the transformants were plated onto 2× YT plates containing 1% glucose and 100 μg/ml ampicillin. All the colonies were collected by scraping and stored as the primary sdAb library. Before use, phage antibodies were rescued using M13KO7 helper phage (Duan et al., 2005).

2.3. Selection of phage sdAbs against avian influenza virus (AIV)

Pure inactivated A/Chicken/Henan/16/2004 (H5N1) (5 μg/ml) in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.5) was used as antigen to coat immunotubes (Nunc, Rochester, NY, USA). After blocking with 2% BSA in phosphate-buffered saline (PBS) and incubation with phage-displayed sdAbs in PBS for 2 h at room temperature, the bound phage sdAbs were eluted using 0.1 M glycine/HCl, pH 2.2, and immediately neutralized with 1.0 M Tris–HCl, pH 8.0. After infecting log phase *E. coli* TG1 with the eluted phages, helper phage M13KO7 were added to rescue the phages, and the generated phage particles were used in the next round of panning. After four rounds of panning, individual phages were generated as described above, but at a smaller scale.

2.4. Phage ELISA

Inactivated A/Chicken/Henan/16/2004 (H5N1) (5 μg/ml) were coated on 96-well microtitre plates overnight at 4 °C in coating buffer, and blocked with 2% BSA in PBS. After incubation with individual phage displaying sdAbs at room temperature for 2 h, bound phages were detected with HRP-conjugated anti-M13 antibody, using ortho-phenylenediamine (OPD) as substrate for colour development. The absorbance was measured at 490 nm using a Bio-Rad ELISA reader (Hercules, CA, USA)

2.5. Expression and purification of soluble sdAbs and pentabodies

The gene of selected VHH3B phage was subcloned into the pET28a (+) vector using the primers listed in Table 2, and was

Table 2
The primers used for subcloning to express the sdAb monomer and pentabody.

Primers for subcloning	
For pET28a vector	
FpET (with *BamHI*)	5′-CG*GGATCC*ATGGCC GAGGTCCAGCTGC-3′
RpET (with *XhoI*)	5′-CCG*CTCGAG*TGAGGAGACGGTG ACCTGGGTC-3′
For pVT2 vector	
FpVT (with *BspEI*)	5′-TAG*CTCCGGA*ATGGCCGAGGTCCAGCTGC-3′
RpVT (with *BamHI*)	5′-CG*GGATCC*TGAGGAGACGGTGACCTGGGTC-3′

expressed in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany). After inducing with 1 mM IPTG for 6 h at 37 °C, the cells were collected by centrifugation and lysed using lysozyme. Soluble sdAbs containing His-tags were purified from the cell lysate by Immobilized Metal Affinity Chromatography (IMAC) using a HisTrap HP metal affinity resin column (GE Healthcare, Piscataway, NJ, USA). The sdAb proteins were eluted using 300 mM imidazole (Sigma–Aldrich, St. Louis, MO, USA) and subsequently dialysed against PBS.

To improve the avidity, the gene of VHH3B was cloned into the pVT2 vector using the primers listed in Table 2 to express pentabodies, designated pVHH3B. *E. coli* TG1 cells from Novagen (Darmstadt, Germany) harbouring the plasmid pVHH3B were induced using 1 mM IPTG for 16 h at 28 °C and lysed using lysozyme. The recombinant pentabodies were purified using IMAC from the lysate supernatant as described above.

2.6. SDS-PAGE and Western blotting

VHH3B or pVHH3B were separated by electrophoresis using SDS-PAGE and transferred to a nitrocellulose blotting membrane (Amersham, Bucks., England) using a semi-dry transblot system obtained from Bio-Rad (Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in PBS for 1 h at room temperature and incubated for 1 h at room temperature with mouse anti-His-tag antibodies to detect VHH3B and with mouse anti-myc-tag antibodies (9E10) to detect pVHH3B. After washing three times with PBST (PBS with 0.05% Tween 20) and once with PBS (10 min each wash), the HRP-conjugated anti-mouse IgG was added and the membranes were incubated for a further hour at room temperature. The membranes were then washed three times with PBST and once with PBS, and then incubated with the luminescence substrate Super Signal West Dura (Pierce, USA) for 5 min. The specific protein bands were visualized by autoradiography on Kodak X-ray film for 10 s.

2.7. ELISA

2.7.1. Direct ELISA

To test the binding capacity of the isolated sdAb to influenza virus, 5 μg/ml of inactivated A/Chicken/Henan/16/2004 (H5N1) was coated onto 96-well microtitre plates overnight at 4 °C in coating buffer. After blocking with blocking buffer (provided by Wantai, Beijing, China) at room temperature for 2 h, the recombinant antibodies were added and the plates were incubated at room temperature for 2 h. Bound VHH3B and pVHH3B were detected with anti-His-tag mAb or anti-myc mAb, respectively, using an HRP-conjugated goat-anti-mouse antibody. Visualization was carried out by adding TMB as substrate. After stopping the reaction using 2 M H_2SO_4, the colour reaction was measured at 450 nm.

To determine the binding spectrum of the selected sdAb against influenza virus, 5 μg/ml of various inactivated influenza virus strains (provided by WANTAI Biological Pharmacy Enterprise, Beijing, China) were coated onto 96-well microtitre plates overnight at 4 °C in coating buffer. After blocking with blocking buffer at room temperature for 2 h, the coated plates were incubated with HRP-conjugated VHH3B (the conjugation of HRP was accomplished by Wantai, Beijing, China) for 1 h at 37 °C. Visualization was carried out by adding TMB as substrate. After stopping the reaction using 2 M H_2SO_4, the colour reaction was measured at 450 nm. The positive reactions were determined based on a cutoff value that was twice the value of the control (no antigen coating on the plate).

2.7.2. Double-antibody-sandwich ELISA (DAS-ELISA)

pVHH3B or mAb 3C8 (4 μg/ml) was coated onto a 96-well microtitre plates in coating buffer and blocked with blocking solution (Wantai, Beijing, China) for 2 h at 37 °C. The coated plates were incubated with various dilutions of influenza virus for 1 h at 37 °C and, subsequently, with 1 μg/ml HRP-conjugated mAb 3C8 (provided by Wantai Biological Pharmacy Enterprise, Beijing, China) for 1 h at 37 °C. Each incubation was followed by four washes with PBST and one wash with PBS. Finally, the colour was developed by adding TMB as substrate, and the results were recorded at 450 nm. The detection limits for A/Chicken/Henan/16/2004 (H5N1) were determined based on a cutoff value that was twice the value of the control (using PBS instead of diluted virus).

2.8. Surface plasmon resonance (SPR) analysis

A/Chicken/Henan/16/2004 (H5N1) (200 μg/ml) in 20 mM sodium acetate, pH 3.7, was immobilized on the surface of a CM5 sensor chip (BIACORE AB) docked into a BIACORE 3000 instrument (flow rate: 5 μl/min) using the amine coupling kit (BIACORE AB) following the manufacturer's instructions (Abad et al., 2002). Purified VHH3B or pVHH3B (500 nM) was injected at a flow rate of 5 μl/min, and the response was measured in RU (Resonance Units). A blank flow cell was prepared by injecting the same antibody sample with same flow rate over a blank sensor chip to obtain the baseline RU. The net RU reported was obtained by subtracting the baseline RU from the response RU obtained using the antibody.

2.9. MNPs-based immunoassay

Gold nanoparticles were provided by Dr. Fangqiong Tang from the Technical Institute of Physics and Chemistry, at the Chinese Academy of Sciences. The average size of the gold nanoparticles (GNPs) was 10 nm (data not shown). The detection antibody, 3C8, was conjugated to the GNPs. Briefly, 10 μg of purified 3C8 was added to a colloidal gold solution (pH 8.5). The mixture was allowed to stand for 10 min and then centrifuged at 12,000 × *g* for 30 min. After centrifugation, the gold pellets were blocked with 1% BSA for 15 min. The GNP probes were then suspended in PBS at an optical density of 5.0 at 520 nm.

Fifty microlitres of 50 mg/ml EDC, 50 μl of 50 mg/ml NHS, and 900 μl of deionized water were added to a vial and vortexed. Approximately 10 mg MNPs (provided by Dr. Fangqiong Tang of the Technical Institute of Physics and Chemistry, at the Chinese Academy of Sciences) was dissolved in this solution and the mixture was incubated at room temperature for 30 min. The nanospheres were collected using a magnet and washed twice with deionized water before they were added to a solution containing 100 μl pVHH3B (1 mg/ml) and 900 μl of NaAc (pH 6.0, 0.2 M). After vortexing, this mixture was incubated at 4 °C for 2 h. Antibody-labelled MNPs were separated using a magnet and washed twice with PBS before incubation in Tris (50 mM, pH 7.5) at room temperature for 30 min. The antibody-labelled MNPs were resuspended in 1 ml of PBS.

The pVHH3B-coated MNPs (20 μg) were transferred to a 1.5 ml centrifuge tube and washed with 200 μl of PB buffer (i.e., phosphate buffer without sodium). Subsequently, a series of dilutions of A/Chicken/Henan/16/2004 (H5N1) (from 10 μg/ml to 0.0001 μg/ml) were added, and the mixtures were incubated at room temperature for 15 min with gentle stirring. The MNPs that

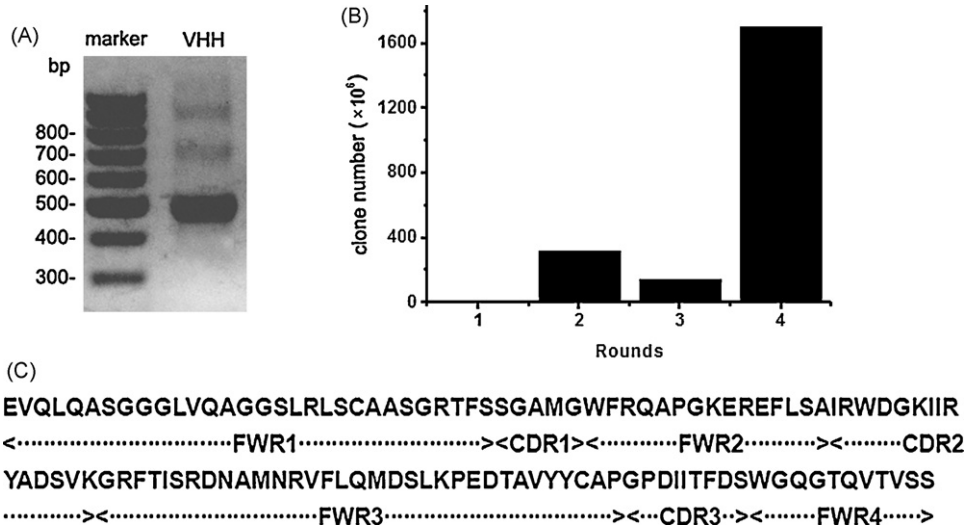

Fig. 1. AIV-immunized sdAb library construction and panning. (A) sdAb gene segments amplified from B cells of alpacas using nested PCR. (B) After four rounds of panning, influenza virus-specific binders were specifically enriched. (C) The amino acid sequence of VHH3B deduced from its DNA sequence. Framework regions 1–4 (FW1–4) and complementarity-determining regions 1–3 (CDR1–3) are shown.

had captured influenza virus were then separated using a magnet, and 2 nM mAb 3C8-coated GNPs were added. The reaction was carried out at room temperature for 15 min. The MNPs separated by a magnetic field were washed with 1 ml PBST, and 100 μl of Silver Reduction Kit (Sigma–Aldrich, St. Louis, MO, USA) solution (prepared by mixing solutions A and B 1:1) was added. The hydroquinone oxidation reaction was carried out in solution for 5 min without stirring. Finally, the optical absorption of the generated quinone was measured.

3. Results

3.1. Construction of an AIV-immunized alpaca sdAb library and selection of AIV-specific sdAbs

To construct an sdAb library with high diversity, the peripheral blood lymphocytes were separated from two immunized young alpacas with high antibody titre to influenza viral particles in their serum (data not shown). Using nested PCR, the sdAb gene fragments were amplified specifically (Fig. 1A) and inserted into the phagemid pCANTAB5E. *E. coli* TG1 was transformed by electroporation with the ligation products to construct an AIV-immunized sdAb library with a size of 2×10^6. Sequences of 30 randomly picked clones suggest a good diversity of the library, as each individual sdAb was unique (data not shown).

Table 3
The binding spectrum of VHH3B against avian influenza virus.

Virus strain (subtype)	$OD_{450\,nm}$	Binding[a]
A/Chicken/Henan/16/2004 (H5N1)	0.824	+
A/Chicken/HongKong/Yu22/2002 (H5N1)	0.554	+
A/Chicken/HongKong/Yu324/2003 (H5N1)	0.452	+
A/Swine/Guangdong/LM/2004 (H1N1)	0.713	+
A/Ruddy turnstone/DE/142/98 (H2N8)	0.600	+
A/Duck/Hong Kong/22A/1976 (H3N3)	0.639	+
A/Mallard/Zhalong/88/04 (H4N6)	0.539	+
A/Teal/Hongkong/W312/97 (H6N1)	0.037	–
A/Chicken/Shaanxi/3/2002 (H9N2)	0.287	+
A/Wild bird feces/Korea/ES1-17/03(H10N8)	0.089	–
A/Duck/Hokkaido/66/2001 (H12N5)	0.043	–

[a] The evaluation of the binding of VHH3B to different strains of virus depends on the comparison of $OD_{450\,nm}$ between experiment group and control group (coated with PBS only, $OD_{450\,nm} = 0.053$).

After rescuing phage-displayed sdAbs, they were bio-panned in stringent conditions to enrich for candidates with good binding capacity to A/Chicken/Henan/16/2004 (H5N1) particles.

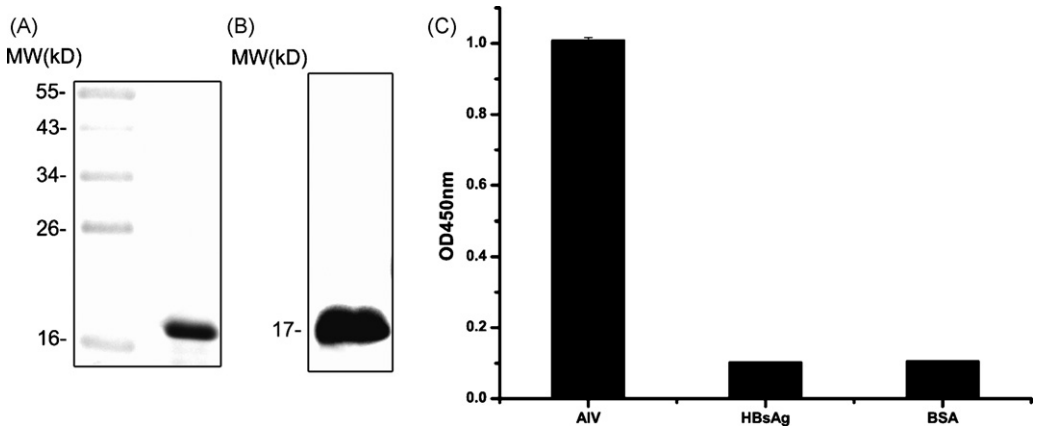

Fig. 2. The purification and binding capacity of VHH3B. (A) Purified VHH3B yields a single, 17-kDa band using SDS-PAGE. (B) Western blot analysis of VHH3B with an anti-His-tag antibody. (C) VHH3B shows good binding capacity towards coated A/Chicken/Henan/16/2004 (H5N1) at a low antibody concentration (100 ng/ml). There was no signal from control groups coated with BSA and HBsAg.

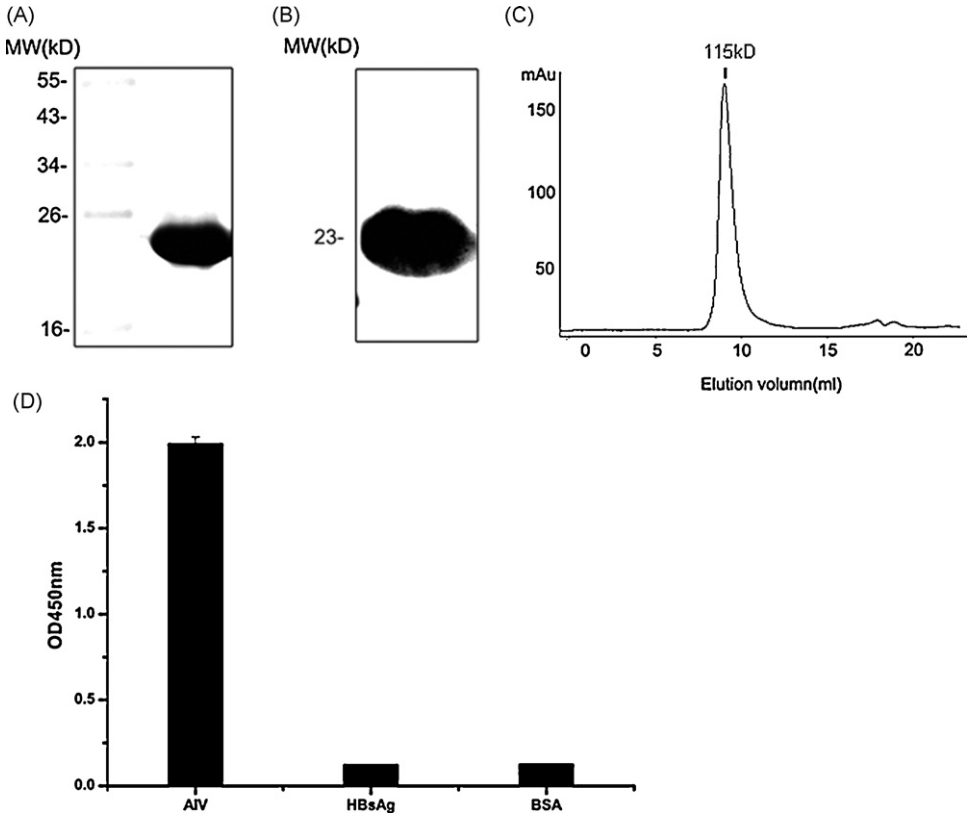

Fig. 3. The purification and binding capacity of pVHH3B. (A) Purified pVHH3B yields a single, 23-kDa band on SDS-PAGE, showing one subunit of the pentabody pVHH3B. (B) A Western blot of pVHH3B against the anti-myc-tag antibody. (C) A pure pentamer whose molecular weight is around 115 kDa (size exclusion) shows that the production of pentamer was successful. (D) pVHH3B still binds strongly to A/Chicken/Henan/16/2004 (H5N1) after pentamer formation.

After four rounds of panning, AIV-specific phages were enriched (Fig. 1B). Fifty randomly selected clones all showed high apparent binding to A/Chicken/Henan/16/2004 (H5N1) virus as measured using phage ELISA. The clone with the strongest binding to A/Chicken/Henan/16/2004 (H5N1) virus, referred to as VHH3B, was selected. Apparently, this sdAb (Fig. 1C) is derived from V_H of the heavy chain antibody because it has residues characteristic of camelid V_HH (Muyldermans, 2001).

3.2. Purification of VHH3B and the testing of its binding capacity towards influenza virus

The VHH3B gene was subcloned into pET28a and expressed in *E. coli* BL21(DE3). Approximately 10–20 mg of VHH3B was obtained from 1 l of bacterial culture. VHH3B sdAb was purified using a Ni^{2+} column, and the resulting purified protein gave a single 17-kDa band on an SDS-PAGE gel (Fig. 2A). The sdAb product was further detected by Western blot using an anti-His-tag antibody, again yielding a single band at 17 kDa (Fig. 2B). Overall, expression of sdAb using this method is simple and gives high yield. This contrasts with the purification of scFv, which suffers from low yield and aggregation problems.

Direct ELISA was used to test the binding activity and specificity of VHH3B to AIV. From the results, it is obvious that VHH3B can bind strongly to A/Chicken/Henan/16/2004 (H5N1), but not to Hepatitis B Surface Antigen (HBsAg) or to Bovine Serum Albumin (BSA) at a concentration of 100 ng/ml (Fig. 2C). To measure the influenza virus-binding spectrum of VHH3B further, 11 influenza virus strains were tested using ELISA with HRP-conjugated VHH3B. As shown in Table 3, VHH3B

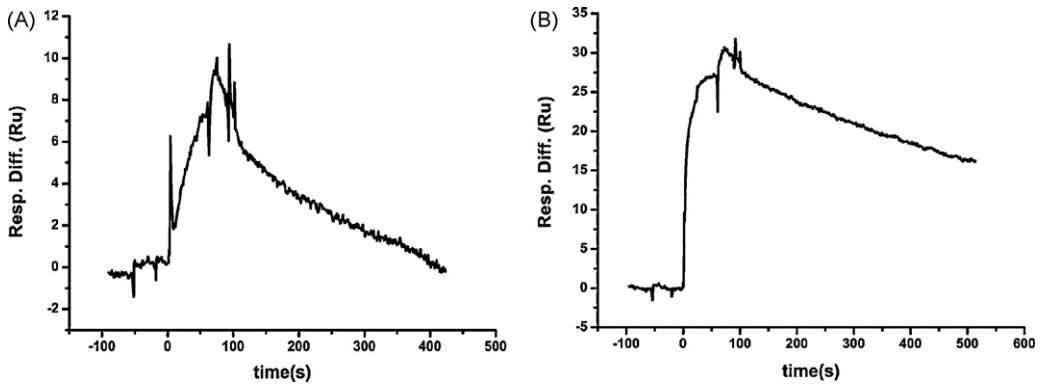

Fig. 4. Kinetic analysis of VHH3B and pVHH3B towards inactivated influenza virus. (A) VHH3B was detached from the immobilized influenza virus more rapidly than (B) pVHH3B.

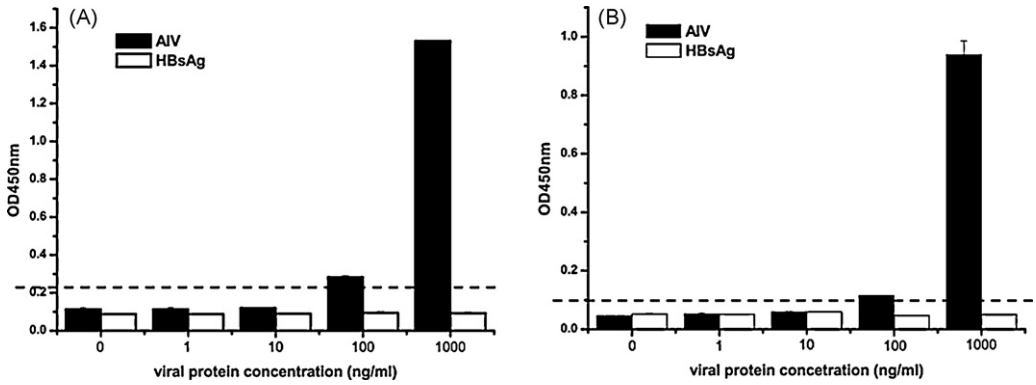

Fig. 5. The use of DAS-ELISA analysis for influenza virus detection. (A) DAS-ELISA using pVHH3B as the capture antibody and HRP-conjugated mAb 3C8 as the detector for A/Chicken/Henan/16/2004 (H5N1) detection. The lowest viral protein concentration detected was 100 ng/ml. (B) DAS-ELISA using mAb 3C8 as the capture antibody and 3C8-HRP as the detector can detect AIV at concentrations as low as 100 ng/ml. Dotted lines represent cutoff values.

showed a relatively broad spectrum to the influenza virus tested.

3.3. Improving the avidity of pVHH3B by forming pentamers of VHH3B

To improve the functional affinity, the VHH3B gene was spliced into vector pVT2, and a pentabody was formed by fusing VHH3B to B subunit of verotoxin, which has a self-assembled pentamer structure. The VHH3B pentabody was purified with a Ni^{2+} column after expression in *E. coli* TG1 cells and is referred to as pVHH3B. A band at 23 kDa (Fig. 3A), consistent with the deduced molecular weight of pVHH3B, was detected using an anti-myc-tag antibody in a Western blot assay (Fig. 3B), and the pentameric assembly was demonstrated using size exclusion chromatography (Fig. 3C). The strong binding activity and specificity of pVHH3B to inactivated A/Chicken/Henan/16/2004 (H5N1) demonstrated using ELISA, as shown in Fig. 3D, indicates that VHH3B pentamerization does not affect its binding.

To compare the binding capacity of VHH3B and pVHH3B quantitively, surface plasmon resonance (SPR) analysis was performed to determine the k_d's, which are 6.71×10^{-3}(1/s) for VHH3B and 1.30×10^{-3}(1/s) for pVHH3B. The results are shown in Fig. 4. It is obvious that pVHH3B (Fig. 4B) dissociates more slowly than VHH3B (Fig. 4A), indicating that pentamerization can improve the functional affinity of VHH3B effectively.

3.4. DAS-ELISA detection of influenza virus

Before setting up the novel MNPs-based detection system, the feasibility of using DAS-ELISA with pVHH3B and mAb 3C8 was evaluated. pVHH3B was coated onto microtitre plates to capture AIV, and HRP-conjugated mAb 3C8 was used for detection. On the basis of the cutoff value, A/Chicken/Henan/16/2004 (H5N1) was detected at 100 ng/ml (Fig. 5A). This result is consistent with the results of DAS-ELISA using conventional mAb 3C8, which has been used to develop an AIV detection kit (Fig. 5B), and is comparable with other reported methods using conventional mAbs (Luo et al., 2008; Zhang et al., 2006). Therefore, this novel pentabody is suitable for use in the DAS-ELISA system.

3.5. Magnetic nanoparticle-based hydroquinone oxidation immunoassay for rapid detection of influenza virus

MNPs and GNPs were first conjugated with pVHH3B and mAb 3C8, respectively. The pVHH3B-labelled MNP was then used to capture influenza viral particles. After separation using a magnet, GNPs-mAb 3C8 was added to detect AIV, which was captured by MNPs. With silver enhancement, GNPs catalyzed the oxidation of hydroquinone to quinone, and the OD value of the quinone generated was measured to analyze the immune reaction of AIV and the corresponding Abs.

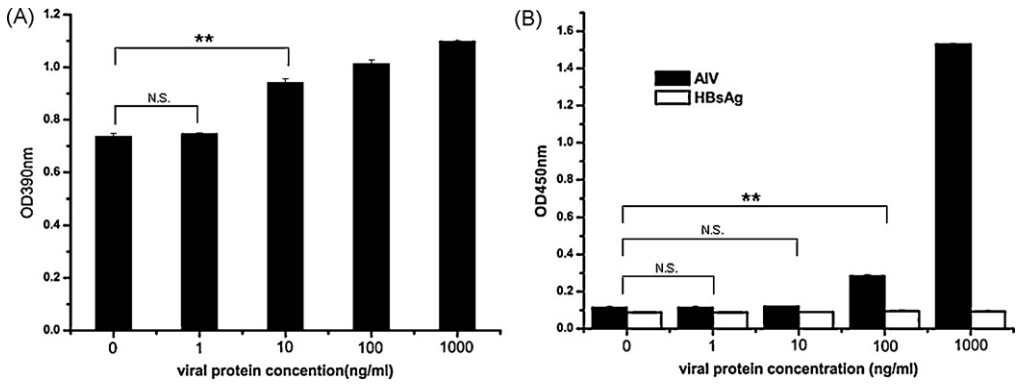

Fig. 6. Comparison of influenza virus detection between magnetic nanoparticle-based immunoassay and DAS-ELISA. (A) A/Chicken/Henan/16/2004 (H5N1) was detected at 10 ng/ml of viral protein with MNP-based immunoassay. (B) Conventional DAS-ELISA was used to detect as low as 100 ng/ml AIV using same antibodies. HBsAg was used as a negative control. Not significant (N.S.): $P > 0.05$, **: $P < 0.01$.

As shown in Fig. 6A, the OD value of the virus sample containing 10 ng/ml of AIV was significantly higher than the control without antigen ($P < 0.05$). However, 1 ng/ml of AIV did not show significant difference from the control group ($P > 0.05$). These results suggest that AIV can be detected at a concentration of 10 ng/ml, approximately 10 times more sensitively than when using DAS-ELISA with the same pair of antibodies (Fig. 6B).

4. Discussion

In this study, a sensitive AIV detection method was developed by combining a novel type of antibody, the pentabody, and a novel MNPs-based detection system.

To respond quickly to emerging pathogens such as AIV, rapid and simple detection methods are needed for pathogen identification in clinical samples. As a widely used convenient detection method, DAS-ELISA provides a platform for the screening of clinical samples and offers well-documented advantages over other traditional methods, such as RT-PCR (which has a high false-positive rate) or immunofluorescence assay (which relies on the inoculation of clinical samples into Madin Darby Canine Kidney cells. DAS-ELISA usually entails the use of conventional mAbs). In this study, a novel pentabody was used. sdAb has been widely used in antibody engineering ever since Hamers-Casterman described the heavy-chain-only antibody in camels in 1993. Functional antibody molecules have been generated using sdAb pentamers as tumour detection reagents (Mai et al., 2006; Zhang et al., 2004a), toxin neutralizing agents (Stone et al., 2007a) and antigen delivery reagents for immunization (Li et al., 2009). Here, the use of pentabodies for AIV detection has been described for the first time. The pentabody-based detection assay that we have developed has the following advantages: First, the entire antibody isolating process uses only *E. coli* cells, making it much more economic than hybridoma screening. Second, large quantities of soluble antibody are easily obtained. Third, the phage display method is more rapid and simple to implement than hybridoma technology.

It is also noteworthy that in the process of library construction and sdAb isolation, whole viral particles were chosen for immunization and screening, because the natural conformation of target antigen would elicit more useful antibodies, and antibodies targeting the natural status of antigen could be more suitable for the detection of AIV in clinical samples. The selected sdAb VHH3B has a relatively broad spectrum against AIV; it could be foreseen that subtype-specific sdAbs could be isolated if different types of AIV were used for screening. This would be more helpful for subtype identification in diagnosis.

Considering that enrichment of target antigen by antibody-conjugated MNPs could increase the sensitivity of detection, a novel nanoparticle-based detection system was developed that was based on a double-antibody-sandwich method with pentabody-labelled MNPs. Because of their multivalent structure, pentabodies were able to capture more binding sites on the surface of AIV, thereby providing strong capturing ability. After the hydroquinone oxidation reaction was catalyzed by GNPs in the complex, the difference in signal dependent on the presence or absence of AIV was evaluated by measuring the optical absorption of quinone generated from the oxidation reaction. Quinone can be generated continuously through Ag enhancement, which amplifies the detection signal. This makes this detection method more sensitive for AIV detection, beyond the enrichment effect of the MNPs. Fig. 6 shows that this novel detection system is 10-fold more sensitive than DAS-ELISA for AIV detection. The incubation time of every step can be shortened to half an hour, and therefore detection could be made even more efficient. As mentioned above, silver ions in the solution can be reduced to elemental silver by the oxidation of hydroquinone, and these ions can be measured electrochemically.

In summary, the study described above provides a sensitive influenza virus detection assay, and this assay has the potential to become a rapid, sensitive and inexpensive diagnostic tool for detecting infectious diseases.

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