ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Generation of a Single-Chain Variable Fragment Antibody against Feline Immunoglobulin G for Biosensor Applications

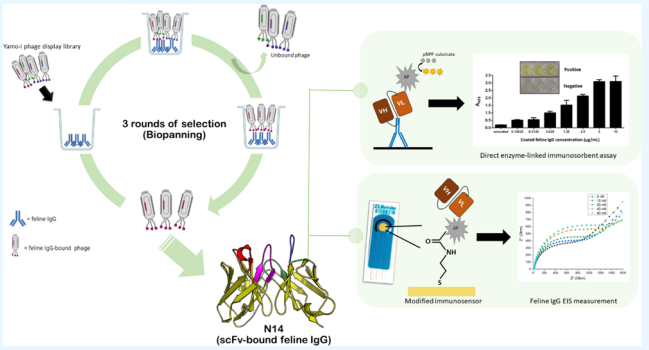
Natchaya Rasri, Lueacha Tabtimmai, Charoenkwan Kraiya, Montarop Yamabhai, Chomdao Sinthuvanich, Jatuporn Rattanasrisomporn, and Kiattawee Choowongkomon*

Cite This: *ACS Omega* 2023, 8, 27688−27696

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: For many decades, feline infectious disease has been among the most common health problems and a leading cause of death in cats. These diseases include toxoplasmosis, feline leukemia virus (FeLV), and particularly feline immunodeficiency virus (FIV) disease. Early diagnosis is essential to increase the chance of successful treatment. Generally, measurement of the IgG level is considered to be indicative of an individual's immune status for a particular pathogen. The antibodies specific to feline IgG are crucial components for the development of a detection kit. In this study, feline IgG-bound scFv was selected using phage display technology. Three rounds of biopanning were conducted against purified feline IgG. Through an indirect enzyme-linked immunosorbent assay (ELISA), two scFv clones demonstrating the best binding ability to feline IgG were chosen for biochemical characterization. In addition, the selected scFv (N14) was expressed and purified in a bacterial system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the size of the purified N14 was 29 kDa. A sandwich ELISA was used to evaluate the binding capacity of the purified scFv to feline IgG. As expected, the purified N14 had the capacity to bind feline IgG. Furthermore, N14 was modified to create a scFv-alkaline phosphatase (scFv-AP) fusion platform. The surface plasmon resonance (SPR) results revealed that N14-AP bound to feline IgG with an affinity binding value of 0.3 ± 0.496 μM. Additionally, the direct ELISA demonstrated the binding capacity of N14-AP to feline IgG in both cell lysate and purified protein. Moreover, N14-AP could be applied to detect feline IgG based on electrosensing with a detection limit of 10.42 nM. Overall, this study successfully selected a feline IgG-bound scFv and developed a scFv-AP platform that could be further engineered and applied in a feline infectious disease detection kit.



1. INTRODUCTION

Feline infectious disease has been the most common health problem and a significant cause of death in domestic cats for over a decade. Infected cats may not exhibit symptoms for years, making early diagnosis crucial for successful treatment. However, the lack of rapid detection leads to disease progression and transmission to other cats.[1] Therefore, the development of a rapid detection kit remains essential and challenging. The IgG level is typically targeted for feline infectious detection and has been incorporated into commercial detection kits available worldwide.[2] Monoclonal antibodies specific to feline IgG are vital components in feline detection kits. Some feline infectious disease detection kits utilize the IgG response for diagnosing diseases, such as feline immunodeficiency virus (FIV)[3,4] and toxoplasmosis.[5] Unfortunately, the production of monoclonal antibodies faces limitations such as their large size (~150 kDa), instability, and time- and cost-consuming production, which increase the overall cost of the kit. To address these concerns, phage display has emerged as an effective strategy for the rapid production of binder molecules, such as a single-chain variable fragment (scFv), which are time- and cost-effective and mainly animal-host-independent.[6−8]

scFv is a small fragment of an antibody (~25−30 kDa) that comprises the variable regions of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker. Due to their smaller size than those of full-length antibodies,[9] scFvs can bind tightly to the target, are easily expressed in the bacterial system, and exhibit greater stability than antibodies. Consequently, this molecule has been reported in commercialized for development in feline detection kits and biochemical assay to identify feline calicivirus disease (F1D7: Creative Biolabs)

Received: May 22, 2023
Accepted: July 5, 2023
Published: July 19, 2023

The current study aimed to develop a feline detection kit using phage display scFv antibody libraries to select appropriate scFv targeting feline IgG. The incorporation of selected scFvs can be beneficial in reducing the production cost of the detection kit.

2. MATERIALS AND METHODS

2.1. Materials. The Yamo-I phage display library, which was utilized in this study, was generously provided by Prof. Montarop Yamabhai laboratory, as described previously.[10] *Escherichia coli* TG1, HB2151, and KM13 helper phage were obtained from the Tomlinson libraries and propagated as described in the MRC phage display protocols.[11] Purified feline IgG was purchased from Bethyl. Purified human IgG was purchased from Abcam, U.K. pSANG14-3F was gifted by John McCafferty (Addgene plasmid #39265; http://n2t.net/addgene:39265; RRID:Addgene_39265)

2.2. Selection of Feline IgG-Bound scFv Using a Phage Display Library. *2.2.1. Biopanning.* A biopanning process was carried out using purified feline IgG as a target. The selection procedure was performed on a 96-well microtiter plate. To select the feline IgG-bound scFv-displayed phages, the Yamo-I phage display library[10] ($\sim 10^9$ pfu/mL) was added and incubated with the immobilized target. To eliminate unbound phages, extensive washing was performed with 0.1% PBST (PBS+Tween 20) 10 times. All bald phages were removed using trypsin digestion. The feline IgG-bound phages were eluted by adding a 200 mM glycine solution (pH 2.2) and neutralizing with 1 M Tris-HCl pH 9.2. For phage titer determination, the eluted phages were subjected to 10-fold serial dilution and incubated with mid-log phase *E. coli* TG1 for phage infection. The infected *E. coli* TG1 were grown on LB agar containing 100 μg/mL ampicillin. For phage propagation, superinfection or phage rescue was performed by incubating KM13 helper phages with *E. coli* TG1 harboring phagemid. The supernatant from the KM13 helper phage-infected *E. coli* TG1 was precipitated using PEG. The precipitated phages were suspended and subjected to the next round of biopanning. This process was repeated for a total of three rounds, following the same procedure described above.

2.2.2. Negative Selection. In total, three rounds of selection with feline IgG were carried out using the biopanning procedure, specifically targeting feline IgG. Negative selection was performed against purified human IgG. Feline IgG-bound phages ($\sim 10^{12}$ pfu/mL) obtained from the third round of biopanning were incubated with purified human IgG to remove any phages that bound nonspecifically. The unbound phages were subsequently incubated with feline IgG to retain those specifically binding to feline IgG. Phage titer determination was performed for each target using the previously described procedure above.

2.3. Binding Screening of Feline IgG-Bound scFv Clones Using an Indirect Enzyme-Linked Immunosorbent Assay. To prepare the soluble feline IgG-bound scFv, eluted phages from the negative selection process were infected with the nonsuppressor *E. coli* HB2151 strain. The infected *E. coli* HB2151 was grown on LB agar containing 100 μg/mL ampicillin. Individual phage clones were randomly picked and grown on LB broth containing 100 μg/mL ampicillin at 37 °C for 16−18 h. The soluble scFv production was carried out by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubating at 30 °C for 16−18 h. The pellet of each clone was harvested and determined for protein expression using western blot analysis. The expression of scFv was visualized using a 6×-histidine tag antibody. The supernatant of the expressed clone was subjected to binding screening based on the enzyme-linked immunosorbent assay (ELISA) against feline IgG, with BSA and human IgG used as the control antigen. The supernatant containing the soluble scFv was added to an antigen-immobilized well and incubated at 4 °C for 16−18 h. Detection was performed based on a hexahistidine tag antibody and HRP conjugated goat anti-mouse as the primary and secondary antibodies, respectively. The color of the reaction was developed using a TMB substrate. The reaction was quantified by measuring the absorbance at 450 nm (A_{450}). scFv clones with an A_{450} value of feline IgG 2-fold higher than that of the control antigen were chosen for further analysis.

2.4. Biochemical Characterization of scFv-Bound Feline IgG. Phagemid extraction of positive scFv clones was submitted for the polymerase chain reaction using a GF-1 plasmid DNA extraction kit (Vivantis; Malaysia) and used as a DNA template. The forward and reverse primers were LMB3 (5′- CAG GAA ACA GCT ATG AC-3′) and pHEN seq (5′-CTA TGC GGC CCC ATT CA-3′), respectively. Expression screening was carried out through western blot analysis using a hexahistidine tag antibody.

2.5. Expression and Purification of Recombinant scFv. The N14 scFv was synthesized into pET22b (+) by Genscript and then transformed into *E. coli* BL21 (DE3) cells. The transformed bacteria were grown in LB broth supplemented with 100 μg/mL ampicillin and 1% glucose until the optical density at 600 nm (OD_{600}) reached the mid-log phase. To induce the production of scFv, IPTG was added at a final concentration of 0.1 mM, and the culturing was continued at 30 °C for 16−18 h. After incubation, the cell pellet was harvested by centrifugation for protein purification. Each crude scFv sample was subjected to purification using a hybrid conditioning method through Ni-NTA resin. The cell pellet was suspended in denaturing lysis buffer (100 mM NaH_2PO_4 pH 8.0, 100 mM Tris-HCl, 6 M urea, 5% (v/v) glycerol, 0.5% Triton X, 1 mM PMSF). Cell disruption was achieved using an ultrasonicator. The cell lysate was centrifuged and passed through a 0.45 μM filter. The Ni-NTA column was equilibrated with denaturing buffer (100 mM NaH_2PO_4 pH 8.0, 100 mM Tris-HCl, 6 M urea, 5% (v/v) glycerol) followed by loading the filtrated supernatant. Nonspecific binders were washed out using denaturing buffer, followed by washing with denaturing washing buffer (100 mM NaH_2PO_4 pH 6.8, 100 mM Tris-HCl, 6 M urea, 5% (v/v) glycerol). To achieve refolding of the desired scFv, a native purification buffer (PBS pH 8.0, 5% glycerol) was applied. Stepwise elution of the histidine-tagged scFv was performed using imidazole. The purified scFv was confirmed using 12% SDS-PAGE and further validated through western blot analysis using a 6×-histidine tag antibody.

2.6. Determination of the Binding Capacity of Purified Recombinant scFv Using Sandwich ELISA. To perform the binding assay, the purified scFv N14 was immobilized onto a 96-well microtiter plate and incubated at 4 °C for 16−18 h and BSA was used as the control. After immobilization, the excess immobilized target was removed by washing with 0.05% PBST. Then, the coated well was blocked with 5% skimmed milk at 4 °C for 1 h. At the indicated time, the well was washed with 0.05% PBST. Next, feline IgG (10

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

μg/mL) was added and incubated at 4 °C for 1 h, allowing the feline IgG to bind to the immobilized scFv N14. For detection, a goat anti-feline IgG HRP conjugated antibody (1:5000) was used for detection. The colorimetric detection was developed using a TMB substrate and the reaction was quantified by measuring the absorbance at 450 nm (A_{450}).

2.7. Production of N14 scFv to Alkaline Phosphatase Fusion. The N14 scFv gene from the pET22b(+) vector was double-digested with NcoI and NotI restriction enzymes and subcloned into pSANG14-3F, an alkaline phosphatase (AP) fusion vector.[12] The pSANG14-3F_N14 vector was transformed into *E. coli* strain DH5α. The transformed bacteria were plated on agar containing the appropriate antibiotics. To confirm the presence of the recombinant plasmid, colony PCR was performed. The T7 promoter and the T7 terminator were used as forward and reverse primers, respectively. The empty pSANG14-3F plasmid was used as the negative control. The PCR products were then analyzed by gel electrophoresis (1% agarose gel).

For large-scale production, the pSANG14-3F_N14 vector was transformed into an *E. coli* BL21 (DE3) strain. The production of the scFv-AP protein was induced by adding IPTG at a concentration of 0.2 mM. The culture was then continued at 25 °C for 16−18 h to allow for protein expression. For protein purification, cell pellets were harvested and suspended in TES buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA, 20% sucrose, 1 mM PMSF) and incubated on ice for 30 min. The supernatant containing the scFv-AP fusion protein was collected by centrifugation at 4 °C and 3000*g* for 10 min. The collected supernatant was dialyzed against buffer A (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5% glycerol) at 4 °C for 16−18 h using a dialysis bag with 3000 Da MWCO. After dialysis, the supernatant was collected by centrifugation and filtered through a 0.45 μM membrane. The filtered supernatant was applied to Ni-NTA for affinity purification. Nonspecific proteins were washed out using buffer A. The histidine-tagged scFv-AP fusion was eluted from the column using stepwise elution with imidazole. Then, the eluted protein was dialyzed against buffer A and further purified through DEAE anion exchange chromatography. Finally, the purified protein was eluted using stepwise elution with increasing concentrations of NaCl. The result was visualized using SDS-PAGE and western blot analysis, as described above.

2.8. N14-AP Affinity Determination. The binding affinity of N14-AP and feline IgG was assessed using an OpenSPR 2-Channel Starter Pack R4.2 (Nicoya Lifesciences; Canada) at 20 °C. A two-fold dilution of scFv-AP was injected into a feline IgG-immobilized amine sensor chip (NECTEC, NSTDA; Thailand) through an injection port. First, the instrument was rinsed with the running buffer (10 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50 mM KCl). Then, the amine sensor chip was loaded into an instrument, and the baseline was determined using the running buffer. The feline IgG was immobilized on the sensor chip by injecting 200 μL of EDC/NHS/feline IgG (50 μg/mL) coupling at a flow rate of 20 μL/min in the running buffer. After eliminating nonspecific binding using a blocking buffer (1 M ethanolamine pH 8.5), a two-fold dilution of scFv-AP (4−64 nM) was injected with a flow rate of 20 μL/min and allowed to dissociate for 5 min. The binding affinity and kinetics were analyzed using Trace Drawer 1.9.1 software (Ridgeview Instruments; Sweden) with a one-to-one model.

2.9. Determination of Binding Capacity of Recombinant N14 scFv-AP Fusion Using Direct ELISA. A two-fold dilution of purified feline IgG was immobilized onto a 96-well microtiter plate and incubated at 4 °C for 16−18 h, and TBS (uncoated) was used as the control. The excess immobilized feline IgG was washed off by 0.05% TBST. The coated wells were then blocked with 5% skimmed milk at 4 °C for 1 h. At the indicated time, the wells were washed with 0.05% TBST. Cell lysate or purified N14 scFv-AP (10 μg/mL) was added and incubated at 4 °C for 1 h. To determine the binding of scFv, an anti-6×-histidine tag-HRP conjugated antibody (1:5000) was added and incubated at 4 °C for 1 h, followed by the TMB substrate for colorimetric detection. The reaction was quantified by measuring the absorbance at 450 nm (A_{450}). To determine the binding of scFv and AP activity, p-nitrophenyl phosphate (pNPP substrate) was added instead of the anti-6×-histidine tag-HRP conjugated antibody. Finally, the reaction was quantified by measuring the absorbance at 405 nm (A_{405})

2.10. Recognition of scFv-AP and Feline IgG Binding Using an Immunosensor. Electrochemical impedance spectroscopy (EIS) was applied to investigate the binding interaction between scFv and feline IgG using a PalmSens4 potentiostat (PalmSens; the Netherlands). The electrode preparation was modified and adapted from Klangprapan et al.[13] The functional gold sensor involved the use of cysteamine as a linker to immobilize scFv-AP on the gold surface. In brief, a screen-printed gold electrode (Methrohm; the Netherlands) was modified with 10 mM cysteamine for 16−18 h at room temperature. To immobilize N14 scFv-AP on the gold electrode, EDC/NHS/N14 scFv-AP (100 μg/mL) coupling was applied to the gold surface and incubated at 4 °C for 1 h. To minimize nonspecific binding, 100 mM ethanolamine was used as a blocking reagent. A schematic diagram of the immunosensor preparation is shown in Figure 5A. For the binding experiments, various concentrations of feline IgG, 5−50 nM, in PBS buffer were dropped onto the modified gold electrode for 15 min at room temperature. The electrode was then washed with ultrapure water to remove any unbound feline IgG. All measurements were performed by applying 200 μL of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl covering all three electrodes (modified gold as a working electrode, carbon as a counter electrode, and Ag/AgCl as a reference electrode) as the electrolyte. EIS was conducted within the frequency range of 0.1−100 kHz using the 5 mV potential amplitude through PSTrace 5.8 software. The EIS spectra were presented as Nyquist plots.

3. RESULTS

3.1. Selection of scFv Specific to Feline IgG by Biopanning. To select scFv against feline IgG, a naïve YAMO-I phage display library was utilized in the biopanning procedure, with an input titer of 10^9 pfu/mL. The phage enrichment rate (output/input phage concentration) was calculated to determine the binding capacity of each round of biopanning. The highest phage enrichment rate, approximately 10-fold, was achieved in the third round of biopanning (Supporting Figure S1). The number of phages bound to feline IgG was 10-fold higher than that for human IgG (Supporting Figure S2), from the screening of expression based on western blot analysis (Supporting Figure S3). Subsequently, eight scFv clones were evaluated for their binding capacity to feline IgG based on indirect ELISA. Surprisingly, N8 and N14 exhibited

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

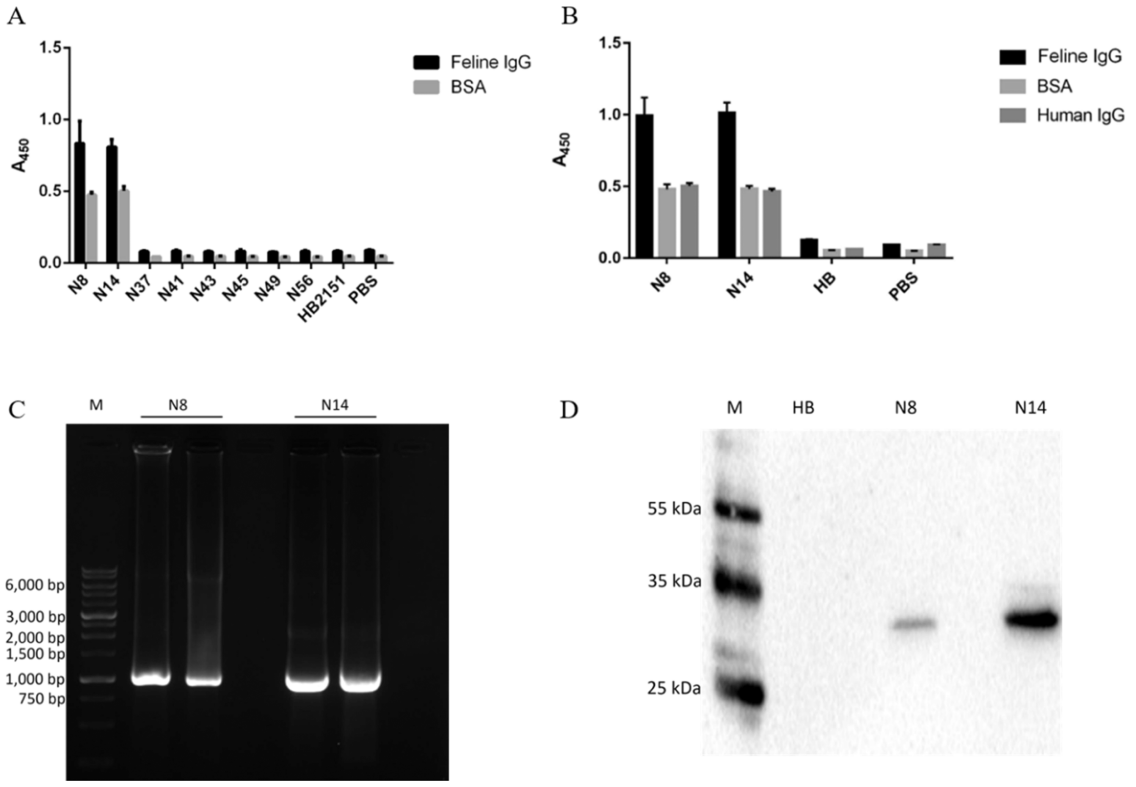


Figure 1. Screening and biochemical characterization of feline IgG-bound scF. (A) Indirect ELISA screening of feline IgG-bound scFv. (B) Specificity test of selected scFv using indirect ELISA with BSA and human IgG as the antigen control, and HB2151 and PBS as the negative control. N8 and N4 clones showed binding capacity to feline IgG. All data are expressed as mean ± SD of triplicates. (C) PCR amplification using a scFv-specific primer, showing positive bands at 1000 bp. (D) Western blot analysis of expressed scFv in *E. coli*, indicating that a protein band was at 29 kDa; M in (C) DNA ladder bp; M in (D) protein marker in kDa; HB in (D) HB2151 as the negative control; N8 and N14 are scFv clones.

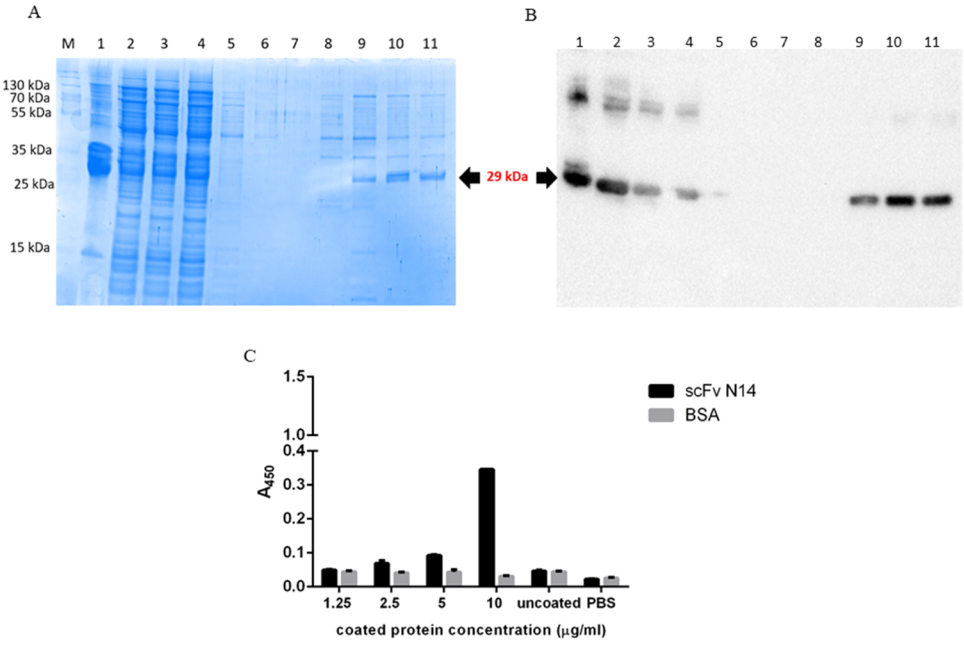


Figure 2. N14 scFv hybrid Ni-NTA purification and activity. (A) 12% SDS-PAGE stained with Coomassie Brilliant Blue (CBB). (B) Western blot analysis. Purified fractions from hybrid condition were collected and probed with the 6×-histidine tag antibody; M, protein marker; 1, pellet; 2, supernatant after cell lysis; 3, flow-through fraction; 4, washed with denatured buffer (pH 8.0); 5, washed with denatured buffer (pH 6.3); 6, washed with native buffer (pH 8.0); 7−11, eluted fraction with 25, 50, 75, 125, and 250 mM imidazole, respectively. Purified scFv was visualized at the expected 29 kDa molecular weight. (C) Binding capacity of purified scFv and feline IgG using sandwich ELISA. Determination of binding capacity of purified N14 scFv performed using sandwich ELISA with BSA as the antigen control, and PBS and uncoated as the negative control. Purified N14 scFv showed a binding capacity to feline IgG. All data are expressed as mean±SD of triplicates.

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

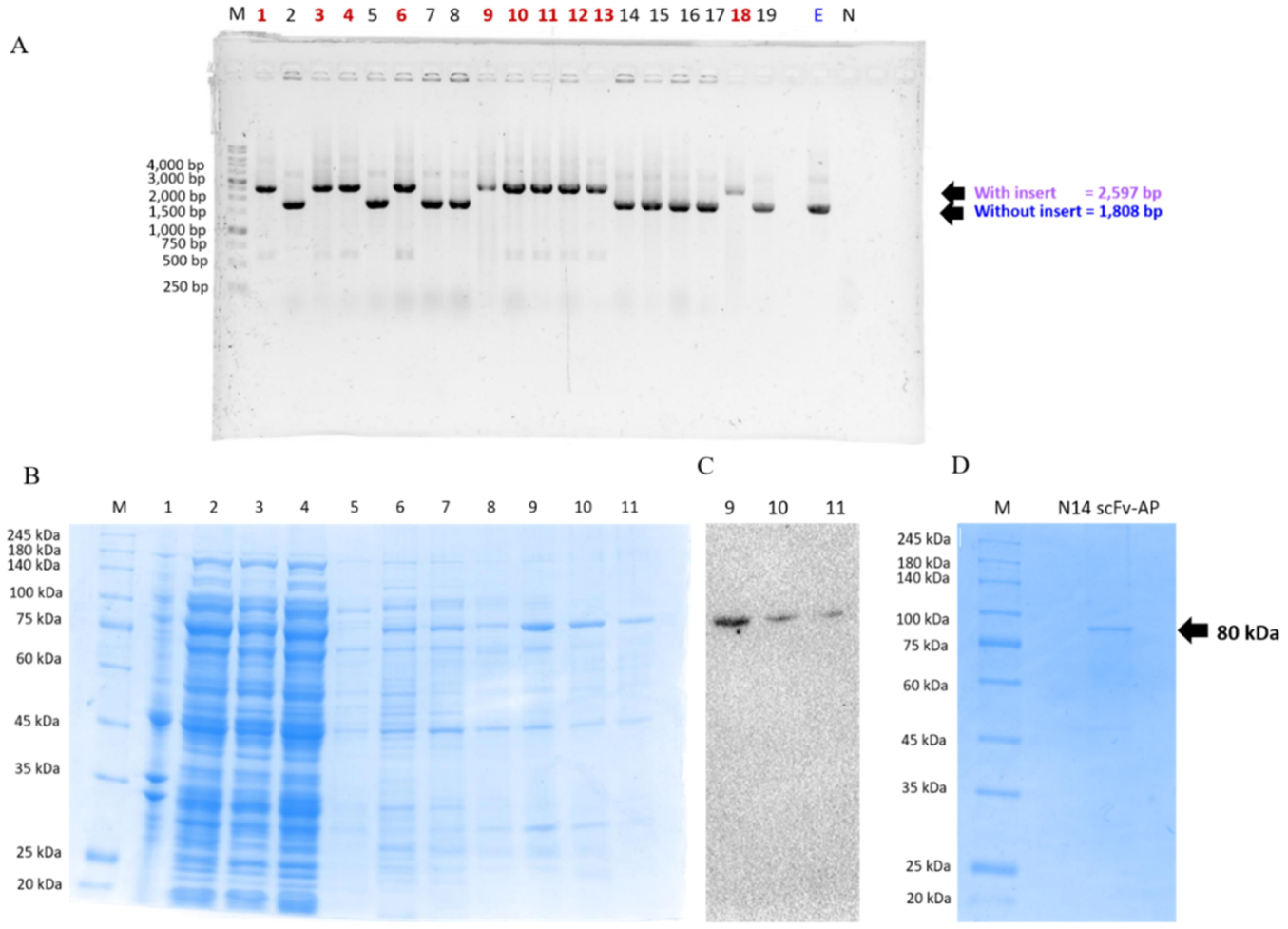


Figure 3. Production process for scFv-AP. (A) 1% agarose gel electrophoresis of colony PCR amplification of different clones. M, DNA ladder; 1−19, clones; E, empty pSANG14-3F vector; N, negative control. Clones 1, 3, 4, 6, 9, 10, 11, 12, 13, and 18 revealed a positive band at 2,597 bp. (B) 10% SDS-PAGE stained with CBB. M, protein marker; 1, pellet; 2, supernatant after cell lysis; 3, flow-through fraction; 4, wash fraction; 5−11, eluted fraction with 12.5, 25, 37.5, 50, 75, 125, and 250 mM imidazole, respectively. (C) Western blot analysis of his-tagged scFv-AP. (D) 10% SDS-PAGE of purified N14 scFv-AP after DEAE anion exchange chromatography. Purified N14 scFv-AP was eluted from the column at 75, 125, and 250 mM imidazole. The size of the eluted protein was approximately 80 kDa and identified as N14 scFv-AP.

the highest binding capacity among the eight clones, with their signals being 2-fold higher than the antigen control (Figure 1A). Furthermore, the specificity of these positive clones to feline IgG was assessed (Figure 1B) and both clones demonstrated specific binding to feline IgG with their signals being 2-fold higher than those for BSA and human IgG. The molecular sizes of N8 and N14 were confirmed through PCR and western blot analysis. Both N8 and N14 showed the expected size of approximately 1000 bp on agarose gel (Figure 1C). Additionally, western blot results confirmed the molecular weight of scFv at approximately 29 kDa, as depicted in Figure 1D, with *E. coli* HB2151 lysate not expressing any relevant protein bands. In summary, N8 and N14 would be promising clones for feline IgG detection.

3.2. N14 scFv Hybrid Ni-NTA Purification and Activity. Due to the inability to purify recombinant scFv under native conditions, a hybrid approach combining native and denatured conditions (hybrid) was employed. Since most of the expressed scFv accumulated in the insoluble fraction, a commonly used denaturing agent urea was utilized. In this study, 6 M urea was added to the buffer for cell lysate to unfold the insoluble protein. The unfolded protein was then subjected to purification on a Ni-NTA column, followed by refolding on the column. The histidine-tagged scFv was eluted using stepwise elution with imidazole under native conditions. The majority of scFv was successfully eluted from the column using the elution buffer containing 125 and 250 mM imidazole, as observed on a 12% SDS-PAGE gel (Figure 2A) at the expected 29 kDa molecular weight. The purified scFv was further confirmed through western blot analysis by detecting the 6×-histidine tag at the N-terminus of recombinant scFv with an anti-6×-histidine tag antibody (Figure 2B). Subsequently, sandwich ELISA was performed to assess the activity of purified N14 scFv. The purified N14 scFv at various concentrations (1.25, 2.5, 5, and 10 μg/mL) was immobilized onto a 96-microtiter well as a target, while BSA and an uncoated well served as controls. Feline IgG (10 μg/mL) was added and incubated at 4 °C for 1 h, followed by the addition of goat anti-feline IgG for detection. The sandwich ELISA results demonstrated that purified N14 scFv exhibited a binding capacity to feline IgG, with the signal increasing as the concentration of scFv N14 increased. The signal was 2-fold higher than that of the control well (Figure 2C).

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

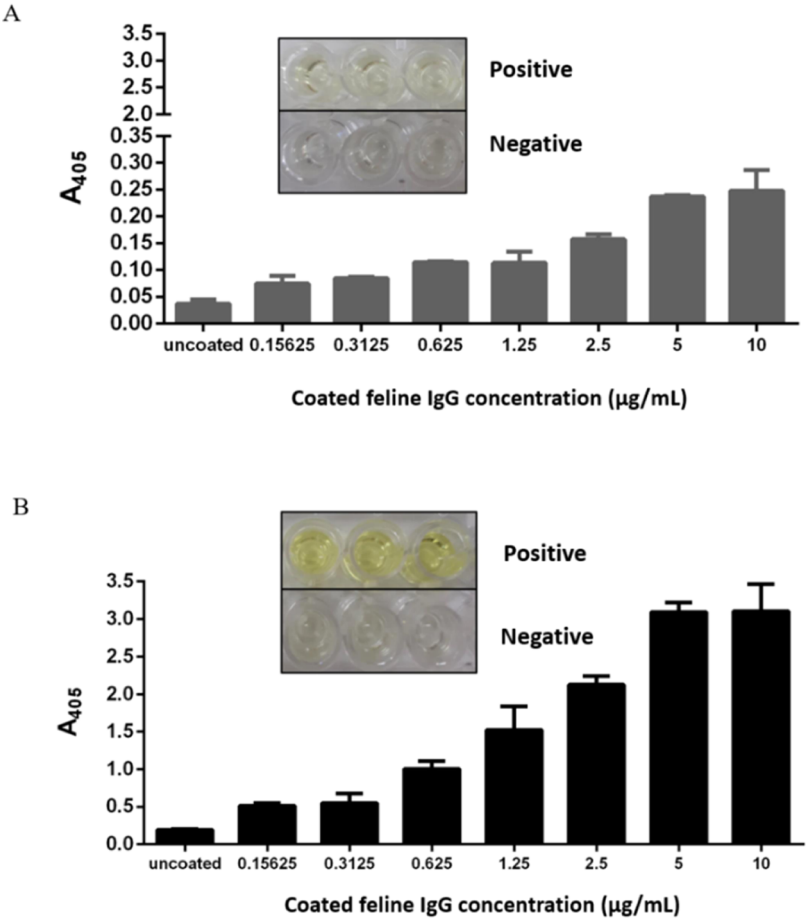


Figure 4. Binding capacity of recombinant N14 scFv-AP fusion using direct ELISA. Binding of scFv-AP cell lysate (A) and the purified protein (B) to feline IgG performed using direct ELISA with the AP substrate (pNPP). A two-fold dilution of feline IgG was immobilized as the target antigen, with BSA as the antigen control and PBS as the negative control. N14 scFv-AP cell lysate showed a binding capacity to feline IgG. All data are expressed as mean ± SD of triplicates. A yellow solution from the positive reaction could be observed with the naked eye compared to the negative reaction, which was colorless.

3.3. scFv N14-AP Production. The N14 scFv was subcloned into pSANG14-3F, followed by transformation into *E. coli* DH5α using heat-shock transformation. On the next day, *E. coli* DH5α cells containing the recombinant plasmid were identified through colony PCR using a T7 promoter and a T7 terminator as the forward and reverse primers, respectively. The results from 1% agarose gel (Figure 3A) revealed 10 positive clones (1, 3, 4, 6, 9, 10, 11, 12, 13, and 18) with a PCR product size of 2597 bp, compared to empty pSANG14-3F, which had a size of only 1808 bp. Therefore, the scFv N14-AP platform was successfully generated. Furthermore, pSANG14-3F_N14 was transformed into *E. coli* BL21 (DE3) for protein expression and purification. The histidine-tagged scFv-AP was eluted from the column using an elution buffer containing 75, 125, and 250 mM imidazole, and the size of the eluted protein was approximately 80 kDa, confirming it as N14 scFv-AP (Figure 3B). To further increase the protein's purity, the eluted fraction underwent purification using DEAE anion exchange chromatography. The scFv-AP was separated from the column by adding 100 mM NaCl, resulting in a purity of 90% (Figure 3D). Subsequently, this purified protein was evaluated for its binding to feline IgG using SPR, direct ELISA, and an electrochem immunosensor.

3.4. Affinity Determination Using SPR. The affinity of the purified scFv-AP to the feline IgG was assessed using an OpenSPR instrument. The binding kinetics between the immobilized feline IgG and the scFv-AP were investigated. A two-fold dilution of purified scFv-AP (4−64 μM) was injected onto the immobilized feline IgG on the amine sensor chip. The SPR sensorgram (Supporting Figure S4) revealed the binding affinity, which was determined by the dissociation constant (K_D). The K_D value between scFv-AP and feline IgG was 3×10^{-7} M and provided parameters with an association rate (k_a) of 1.5×10^2 M^{-1} s^{-1} and a dissociation rate (k_d) of 3.49×10^{-5} s^{-1}.

3.5. Binding Capacity of Recombinant N14 scFv-AP Fusion Using Direct ELISA. The N14 scFv-AP was expressed in *E. coli* BL21 (DE3) and tested for its binding to feline IgG using direct ELISA. For the binding assay, a two-fold dilution series of feline IgG was immobilized onto a 96-well microtiter plate as the target antigen, while BSA was used as the control. The cell lysate containing expressed N14 scFv-AP or the purified protein was added to the immobilized target and incubated at 4 °C for 1 h. The absorbance at 450 nm (A_{450}) demonstrated that the binding capacity of N14 scFv-AP from the cell lysate sample to feline IgG was 2-fold higher than those for BSA and PBS (Supporting Figure S5) The A_{450} value also varied depending on the concentration of immobilized feline IgG. In addition, p-nitrophenyl phosphate (pNPP) the AP substrate was used to investigate the binding of scFv and AP

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

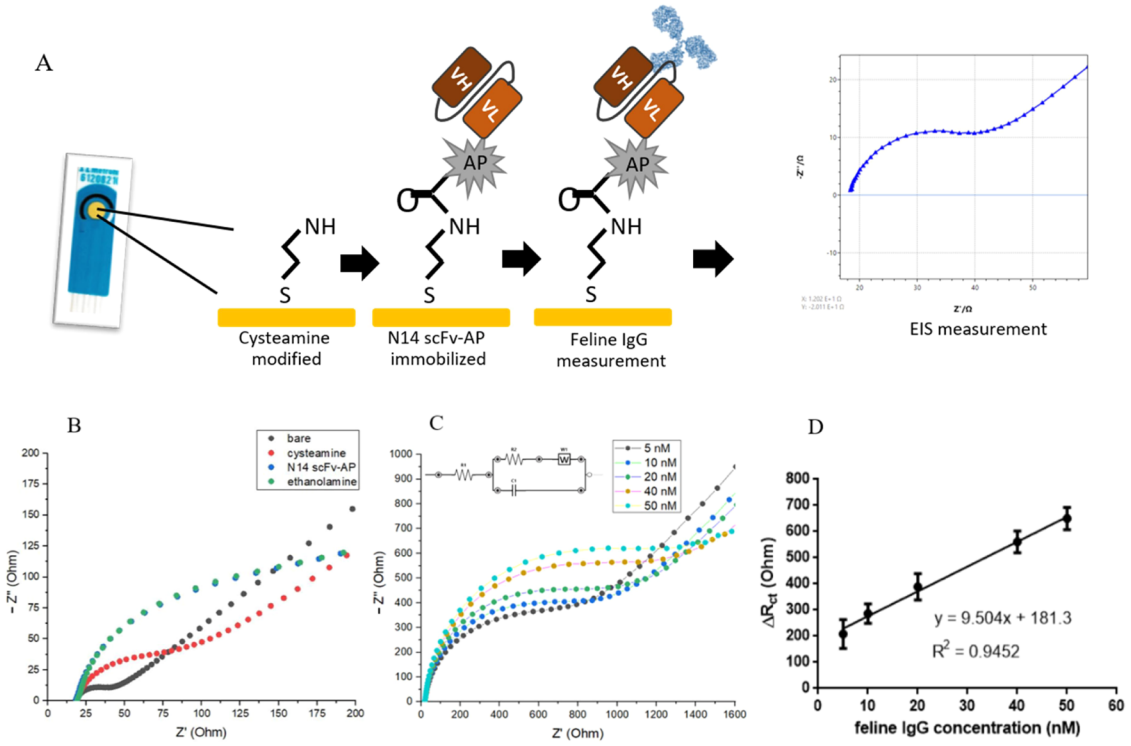


Figure 5. Detection of scFv-AP to feline IgG using an immunosensor. (A) Schematic illustration of the immunosensor preparation process and EIS detection. (B) EIS responses for characterization of a bare gold electrode (black), a cysteamine linker (red), scFv-AP (blue), and ethanolamine blocking (green). (C) EIS signal of immunosensor-measured feline IgG at different concentrations (5−50 nM). EIS spectra analyzed by fitting them to an equivalent electrical circuit model (inset C). (D) Linear correlation between relative resistance and feline IgG at 5, 10, 20, 40, and 50 nM (R^2 = 0.9452). EIS was conducted in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl within a frequency range of 0.1−100 kHz.

activity. The absorbance at 405 nm (A_{405}) was measured using a microplate reader. Surprisingly, both cell lysate (Figure 4A) and the purified scFv-AP (Figure 4B) showed that the signal was bound to feline IgG as the signal increased with an increase in the feline IgG concentration. These results suggest that the scFv-AP fusion format has potential for further modification, characterization, and development for use in a feline infectious disease detection kit.

3.6. Recognition of N14 scFv-AP and Feline IgG Binding Using an Immunosensor. Electrochemical impedance spectroscopy (EIS) was applied to demonstrate the recognition of N14 scFv-AP to feline IgG. The modification procedure of a gold electrode involved the sequential attachment of cysteamine, scFv-AP, ethanolamine, and feline IgG as illustrated in Figure 5A. The effectiveness of each modification step of the gold electrode was evaluated by monitoring the change in charge transfer resistance (R_{ct}) represented in the Nyquist plot (Figure 5B). The R_{ct} of the gold electrode gradually increased upon immobilization of cysteamine and N14 scFv-AP. Following the blocking step with ethanolamine, no significant change in the R_{ct} was observed, indicating that the N14 scFv-AP was completely immobilized onto the gold electrode surface. Then, the modified electrode was further incubated with various concentrations of feline IgG. The EIS responses (Figure 5C) exhibited a progressive increase corresponding to the incremental concentrations of feline IgG (5, 10, 20, 40, and 50 nM), with corresponding R_{ct} values of 208, 286, 389, 560, and 650 Ω, respectively. These R_{ct} values were further adjusted by subtracting the blank value obtained at feline IgG concentration = 0 nM, and the resulting values were reported as ΔR_{ct}. The plot of ΔR_{ct} versus different concentrations of feline IgG (5−50 nM) produced a linear relationship. The measurements were performed in triplicate with a correlation coefficient of 0.9452 and a limit of detection (LOD) of 10.4 nM (Figure 5D). These findings further corroborated the enhanced detection capability of N14 scFv-AP in the immunosensor setup for feline IgG detection.

4. DISCUSSION

There has been an alarming rise in mortality rates associated with infectious diseases in domestic animals, particularly in cats. The early diagnosis and development of rapid detection kits are necessary for successful treatment. Immunoglobulin G (IgG) is a class of antibodies produced by immune cells in response to infections. The level of IgG is commonly targeted for the detection of feline infectious diseases and is widely utilized in commercial detection kits available globally.[2] Herein, enrichment selection of a single-chain variable fragment was performed to select feline IgG-bound scFv for biosensor development. In the field of monoclonal antibody production, phage display technology has emerged as a highly effective method. When compared to hybridoma technology, phage display offers several advantages. First, it enables the rapid production of binder molecules, such as antibody fragments, in a time- and cost-effective manner. Second, the production process is consistent from batch to batch, ensuring reliability and reproducibility. Importantly, phage display technology does not involve the use of animal-based components, making it more ethical and suitable for various applications. Antibody fragments, including single-chain variable fragments (scFvs), have gained significant attention for their potential in biosensor development.[6,8,14] In the

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

present study, the successful three-round biopanning and negative selection resulted in an increased phage enrichment. A total of 65 clones were identified that exhibited binding capability to the target. Among them, N8 and N14 emerged as potential scFv candidates, demonstrating the highest binding capacity in comparison to the other clones and the antigen control. Furthermore, the PCR and western blot results confirmed the presence of scFv characteristics, including the DNA sequence and molecular weight, in N8 and N14.[15] The N14 scFv was chosen for pilot-scale production. However, purifying the N14 scFv under native conditions proved to be unfeasible. Therefore, a hybrid approach combining native and denatured conditions was employed. As the majority of the expressed scFv accumulated in the insoluble fraction, recovery using Ni-NTA beads was unsuccessful. Consequently, urea, a commonly used chemical reagent for protein denaturation, was utilized. To regain the functional protein, a native condition buffer was employed in the column, and the histidine-tagged scFv purification was conducted similarly to the previous study, which aimed to purify the scFv targeting adhesion to epithelial cell molecules in the extracellular domain.[16]

The production of immunoassay-based biosensors commonly involves the use of the enzyme immunoassay, which utilizes both primary and secondary antibodies. Typically, the antibodies are labeled with enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), which act as reporter signals. In previous studies, alkaline phosphatase (AP) has been frequently employed, as the enzyme fused with the scFv to enable one-step detection of various targets, such as aflatoxin-B1,[17] parvalbumins,[18] and *Bacillus anthracis*.[19] The current study successfully developed a pilot-scale production system for the scFv-AP fusion platform in a bacterial system. The results obtained from SPR analysis using Trace Drawer 1.9.1 software with a one-to-one model revealed the binding kinetics between scFv-AP and feline IgG. The findings demonstrated a strong and tight interaction between N14 scFv-AP and feline IgG, as indicated by a dissociation rate (k_d) of 3.49×10^{-5} s^{-1}, suggesting a gradual release. It is noteworthy that this study represents the first successful isolation of a scFv specific to feline IgG. Furthermore, the affinity value of our scFv (3×10^{-7} M) is comparable to that of another scFv isolated from the same library, which targeted the same type of antigen (protein). Eskafi reported affinities of 6×10^{-7} and 4×10^{-7} M for a scFv against Iranian Macrovipera lebetina snake venom.[20] In addition, phage display technology has been reported as a tool for identifying the specific antibody to human IgG by using an immunized phage display library and identifying the human IgG-specific nanobody with an affinity at 7.5 nM.[21] The results obtained from the direct ELISA, employing p-nitrophenyl phosphate (pNPP) as the substrate for alkaline phosphatase (AP), further validated the binding and activity of N14 scFv-AP with feline IgG. Additionally, the electrochemical immunosensor-based electrochemical impedance spectroscopy (EIS) indicated the promising potential of N14 scFv-AP in recognizing feline IgG within the nanomolar range. The level of IgG in feline depends on their age and immune status with the reference interval ranging between 2 and 200 mg/mL (13−1300 mM). In healthy cats, the typical value in serum is 2.145 mg/mL (14.3 mM) in adults (2−4 years) and 2.448 mg/mL (16.32 mM) in seniors (10−14 years).[22] However, the total IgG levels are higher in infected cats. For example, feline immunodeficiency virus (FIV)-infected cats exhibit IgG levels of 25−35 mg/mL (166−233 mM), while FIV-infected cats with oral lesions ranging from periodontitis to severe ulceroproliferative stomatitis show levels of 35−40 mg/mL (233−266 mM).[23−25] In feline leukemia virus (FeLV)-infected cats, the IgG level is around 18−19 mg/mL (120−126 mM).[26] Considering the LOD (limit of detection) of our immunosensor, which is 10.4 nM, it is lower than the IgG levels within the reference interval range in both healthy and infected cats. Therefore, the immunosensor is capable of effectively detecting IgG in the serum of cats.

In summary, the current study successfully selected a scFv against feline IgG using phage display technology. The developed N14 scFv-AP fusion platform exhibited promising potential for application in a feline infectious detection kit and other biochemical techniques. However, further study is required on IgG in infected cats.

5. CONCLUSIONS

This study demonstrated the efficacy of phage display technology in the production of recombinant antibodies. By employing this approach, the scFv capable of binding feline IgG was successfully selected and further developed into a scFv-AP platform. The scalability of production was achieved using a bacterial system, allowing for pilot-scale manufacturing. Importantly, the developed platform holds the potential to reduce the production costs associated with detection kits, offering a more cost-effective solution.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03581.

Enrichment of feline IgG-bound phages for three rounds of biopanning; phage enrichment from negative selection; screening of soluble scFv; affinity determination; and binding of scFv-AP cell lysate (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Kiattawee Choowongkomon − *Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*; orcid.org/0000-0002-2421-7859; Email: fsciktc@ku.ac.th

Authors

Natchaya Rasri − *Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

Lueacha Tabtimmai − *Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok 10800, Thailand*

Charoenkwan Kraiya − *Electrochemistry and Optical Spectroscopy Center of Excellence, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*; orcid.org/0000-0002-5660-7747

Montarop Yamabhai − *Molecular Biotechnology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*; orcid.org/0000-0003-2674-2419

Chomdao Sinthuvanich − *Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*; orcid.org/0000-0002-2432-2122

https://doi.org/10.1021/acsomega.3c03581
ACS Omega 2023, 8, 27688−27696

Jatuporn Rattanasrisomporn − *Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c03581

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by Kasetsart University, Bangkok, Thailand through the Graduate School Fellowship Program, the Kasetsart University Research, Development Institute (KURDI), KURDI(FF(KU)16.66) and the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation [grant number B36G650005].

■ REFERENCES

(1) Bendinelli, M.; Pistello, M.; Lombardi, S.; Poli, A.; Garzelli, C.; Matteucci, D.; Ceccherini-Nelli, L.; Malvaldi, G.; Tozzini, F. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin. Microbiol. Rev.* **1995**, *8*, 87−112.

(2) Lin, D. S. Feline immune system. *Comp. Immunol., Microbiol. Infect. Dis.* **1992**, *15*, 1−11.

(3) Westman, M. E.; Malik, R.; Hall, E.; Sheehy, P. A.; Norris, J. M. Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats using point-of-care antibody kits. *Comp. Immunol., Microbiol. Infect. Dis.* **2015**, *42*, 43−52.

(4) Lombardi, S.; Poli, A.; Massi, C.; Abramo, F.; Zaccaro, L.; Bazzichi, A.; Malvaldi, G.; Bendinelli, M.; Garzelli, C. Detection of feline immunodeficiency virus p24 antigen and p24-specific antibodies by monoclonal antibody-based assays. *J. Virol. Methods* **1994**, *46*, 287−301.

(5) Ybanez, R.H.D.; Kyan, H.; Nishikawa, Y. Detection of antibodies against Toxoplasma gondii in cats using an immunochromatographic test based on GRA7 antigen. *J. Vet. Med. Sci.* **2020**, *82*, 441−445.

(6) Bazan, J.; Calkosinski, I.; Gamian, A. Phage display–a powerful technique for immunotherapy: 1. Introduction and potential of therapeutic applications. *Hum. Vaccines Immunother.* **2012**, *8*, 1817−1828.

(7) Ledsgaard, L.; Kilstrup, M.; Karatt-Vellatt, A.; McCafferty, J.; Laustsen, A. H. Basics of Antibody Phage Display Technology. *Toxins* **2018**, *10*, No. 236.

(8) Paschke, M. Phage display systems and their applications. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 2−11.

(9) Ahmad, Z. A.; Yeap, S. K.; Ali, A. M.; Ho, W. Y.; Alitheen, N. B.; Hamid, M. scFv antibody: principles and clinical application. *Clin. Dev. Immunol.* **2012**, *2012*, No. 980250.

(10) Pansri, P.; Jaruseranee, N.; Rangnoi, K.; Kristensen, P.; Yamabhai, M. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol.* **2009**, *9*, No. 6.

(11) Lee, C. M. Y.; Iorno, N.; Sierro, F.; Christ, D. Selection of human antibody fragments by phage display. *Nat. Protoc.* **2007**, *2*, 3001−3008.

(12) Martin, C. D.; Rojas, G.; Mitchell, J. N.; Vincent, K. J.; Wu, J.; McCafferty, J.; Schofield, D. J. A simple vector system to improve performance and utilisation of recombinant antibodies. *BMC Biotechnol.* **2006**, *6*, No. 46.

(13) Klangprapan, S.; Weng, C. C.; Huang, W. T.; Li, Y. K.; Choowongkomon, K. Selection and Characterization of a Single-Chain Variable Fragment against Porcine Circovirus Type 2 Capsid and Impedimetric Immunosensor Development. *ACS Omega* **2021**, *6*, 24233−24243.

(14) Alfaleh, M. A.; Alsaab, H. O.; Mahmoud, A. B.; Alkayyal, A. A.; Jones, M. L.; Mahler, S. M.; Hashem, A. M. Phage Display Derived Monoclonal Antibodies: From Bench to Bedside. *Front. Immunol.* **2020**, *11*, 1986.

(15) Zhang, L.; Cao, Y.; Liu, M.; Chen, X.; Xiang, Q.; Tian, J. Functional recombinant single-chain variable fragment antibody against Agkistrodon acutus venom. *Exp. Ther. Med.* **2019**, *17*, 3768−3774.

(16) Javadian, F. S.; Basafa, M.; Behravan, A.; Hashemi, A. Solubility assessment of single-chain antibody fragment against epithelial cell adhesion molecule extracellular domain in four Escherichia coli strains. *J. Genet. Eng. Biotechnol.* **2021**, *19*, No. 26.

(17) Rangnoi, K.; Jaruseranee, N.; O'Kennedy, R.; Pansri, P.; Yamabhai, M. One-step detection of aflatoxin-B(1) using scFv-alkaline phosphatase-fusion selected from human phage display antibody library. *Mol. Biotechnol.* **2011**, *49*, 240−249.

(18) Bublin, M.; Kostadinova, M.; Fuchs, J. E.; Ackerbauer, D.; Moraes, A. H.; Almeida, F. C.; Lengger, N.; Hafner, C.; Ebner, C.; Radauer, C.; Liedl, K. R.; Valente, A. P.; Breiteneder, H. A Cross-Reactive Human Single-Chain Antibody for Detection of Major Fish Allergens, Parvalbumins, and Identification of a Major IgE-Binding Epitope. *PLoS One* **2015**, *10*, No. e0142625.

(19) Wang, S. H.; Zhang, J. B.; Zhang, Z. P.; Zhou, Y. F.; Yang, R. F.; Chen, J.; Guo, Y. C.; You, F.; Zhang, X. E. Construction of single chain variable fragment (ScFv) and BiscFv-alkaline phosphatase fusion protein for detection of *Bacillus anthracis*. *Anal. Chem.* **2006**, *78*, 997−1004.

(20) Eskafi, A. H.; Bagheri, K. P.; Behdani, M.; Yamabhai, M.; Shahbazzadeh, D.; Kazemi-Lomedasht, F. Development and characterization of human single chain antibody against Iranian Macrovipera lebetina snake venom. *Toxicon* **2021**, *197*, 106−113.

(21) Kazemi-Lomedasht, F.; Behdani, M.; Rahimpour, A.; Habibi-Anbouhi, M.; Poshang-Bagheri, K.; Shahbazzadeh, D. Selection and Characterization of Specific Nanobody Against Human Immunoglobulin G. *Monoclonal Antibodies Immunodiagn. Immunother.* **2015**, *34*, 201−205.

(22) Campbell, D. J.; Rawlings, J. M.; Koelsch, S.; Wallace, J.; Strain, J. J.; Hannigan, B. M. Age-related differences in parameters of feline immune status. *Vet. Immunol. Immunopathol.* **2004**, *100*, 73−80.

(23) Poli, A.; Giannelli, C.; Pistello, M.; Zaccaro, L.; Pieracci, D.; Bendinelli, M.; Malvaldi, G. Detection of salivary antibodies in cats infected with feline immunodeficiency virus. *J. Clin. Microbiol.* **1992**, *30*, 2038−2041.

(24) Poli, A.; Falcone, M. L.; Bigalli, L.; Massi, C.; Hofmann-Lehmann, R.; Lombardi, S.; Bendinelli, M.; Lutz, H. Circulating immune complexes and analysis of renal immune deposits in feline immunodeficiency virus-infected cats. *Clin. Exp. Immunol.* **2013**, *101*, 254−258.

(25) Ackley, C. D.; Yamamoto, J. K.; Levy, N.; Pedersen, N. C.; Cooper, M. D. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J. Virol.* **1990**, *64*, 5652−5655.

(26) Wernicke, D.; Trainin, Z.; Ungar-Waron, H.; Essex, M. Humoral immune response of asymptomatic cats naturally infected with feline leukemia virus. *J. Virol.* **1986**, *60*, 669−673.