Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2018, **10**, 2629

Production and characterization of a single-chain variable fragment-alkaline phosphatase fusion protein for glycocholic acid detection in a one-step enzyme-linked immunosorbent assay

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A single-chain variable fragment (scFv)-alkaline phosphatase (AP) fusion protein for glycocholic acid (GCA) was produced and characterized. The scFv gene with a 218 linker was generated by splicing by overlap extension (SOE)-polymerase chain reaction (PCR) and sequentially inserted into the expression vector pecan45 containing AP gene to express the scFv-AP fusion protein in *Escherichia coli* (*E. coli*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses revealed that the fusion protein showed the expected molecular weight of about 80 kDa. Both the antibody binding capacity and AP enzyme activity of the scFv-AP fusion protein were validated by colorimetric analysis. One-step competitive direct enzyme-linked immunosorbent assay (ELISA) based on the scFv-AP fusion protein indicated that the average concentration required for 50% inhibition of binding (IC_{50}) and limit of detection (LOD) for GCA were 216 ng mL^{-1} and 37.0 ng mL^{-1}, respectively, and the linear response range extended from 71.0 to 657 ng mL^{-1}. The cross-reactivity (CR) of the scFv-AP fusion protein was similar to those of its parental scFv antibody. The scFv-AP fusion protein was bifunctional, retaining both antibody binding specificity and AP enzyme activity. This work indicates that the production of the scFv-AP fusion protein in *E. coli* strain BL21(DE3)pLysS is feasible and suggests that it could be further used as convenient one-step detection probes for GCA.

Received 15th April 2018
Accepted 22nd May 2018

DOI: 10.1039/c8ay00848e

rsc.li/methods

Introduction

Glycocholic acid (GCA), the secondary bile acid (BA), is derived from steroid acid by intestinal bacterial flora. BAs play pivotal roles in many physiological functions, such as the elimination of cholesterol and the absorption of lipids in the intestine.[1,2] The concentrations of BAs in plasma, urine and faeces have been suggested to be used as important prognostic and diagnostic biomarkers of hepatobiliary and intestinal dysfunction. Many diseases, along with metabolic and liver disorders, are characterized by the increased concentrations of BAs.[1,3–5] Thus, diagnosis of pathological conditions of liver could be achieved by investigating BA levels in patients. Recently, GCA, as shown in Fig. 1, has been identified as newly sensitive and selective biomarker for the hepatocellular carcinoma (HCC).[6,7] Thus, the use of GCA analysis might represent a valuable tool screening for HCC and other hepatic diseases.

At present, several methods have been described for qualitative and quantitative determination of GCA in complex biological matrices, including plasma, urine and faecal samples. The limit of detection (LOD) of these methods is about 5.6 μg mL^{-1} for high-performance liquid chromatography (HPLC),[8] 1 ng mL^{-1} for liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS),[9] 4.2 μg mL^{-1} for matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS),[10] respectively. The instrumental techniques are accurate, precise and sensitive. However, the instrument and labor costs, as well as the length of time needed to employ the

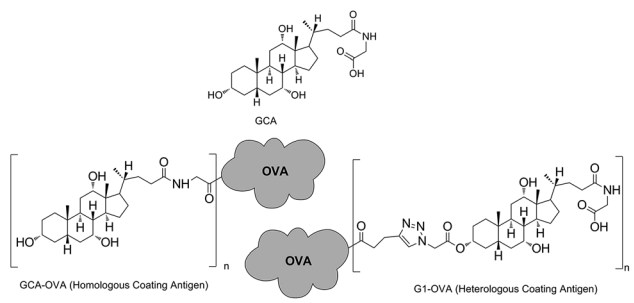

Fig. 1 Structures of GCA and coating antigens.

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instrumental methods have inhibited the scope of monitoring GCA.

In comparison, immunoassays, such as enzyme-linked immunosorbent assays (ELISA), can provide an attractive and promising alternative approach for the small molecule detection with the advantages of being rapid, convenient, cost-effective, and high-throughput. Generally, the antibodies are the key component that relate directly to the specificity and sensitivity of immunoassays. Immunoassays are commonly based on polyclonal antibodies (pAbs)[11,12] or monoclonal antibodies (mAbs)[13,14] or a combination of both.[15] However, the required conventional antibodies (pAbs or mAbs) used in ELISA always involve several drawbacks such as differences in quality from batch to batch for pAb or labor-intensive, long-period, high-cost, complex-procedure for mAb production.

Advances in the field of recombinant DNA technology provide an alternative method to produce recombinant antibodies (rAbs).[16,17] Unlike pAbs and mAbs, rAbs can be selected together with their DNA coding sequences from antibody gene libraries by phage display.[18] One of the most successful rAb formats is the single-chain variable fragment (scFv) antibodies.[19] The scFv antibodies can be expressed with a relatively high yield in the bacterial expression system,[20] and readily extracted from the periplasm space.[21] More importantly, its single chain makes it easy to fuse with other proteins, resulting in the formation of antibody with two or more desired functions.[22–24]

Study concerned about the determination of GCA by ELISA based on specific scFv antibody has been reported in our previous work.[25] However, this indirect competitive ELISA requires the addition of a secondary antibody chemically conjugated to an enzyme. The chemical conjugation method may cause random cross-linking of molecules, leading to heterogeneous molecules with the loss of enzyme activity or antibody binding ability. Also, the molecular ratios of antibody to enzyme are hard to control. In this report, the recombinant colorimetric immunotracer is composed of a scFv antibody fused with the alkaline phosphatase (AP) enzyme of *Escherichia coli* (*E. coli*), which can avoid the use of chemically produced antibody–enzyme conjugates. The production of the scFv-AP fusion proteins for the analysis of ractopamine,[22] clenbuterol,[26] aflatoxin-B1 (ref. 27) have been described. These scFv-AP fusion proteins function as combined target recognition and signal transduction molecules. Immunoassays based on scFv-AP fusion proteins can simplify the assay protocols and reduce the use of secondary antibodies, and therefore have been gaining increased attention in analytical methods. Until now, to the best of our knowledge, there are no reports about the production of the recombinant scFv-AP fusion protein for GCA.

As an extension of our previous work, the purpose of this study was to produce and characterize a recombinant colorimetric immunotracer. By inserting a DNA fragment encoding anti-GCA antibody into the expression vector pecan45 (ref. 28) containing AP gene, a recombinant plasmid pecan45-scFv-AP was constructed and the scFv-AP fusion protein was expressed in *E. coli* strain BL21(DE3)pLysS. After analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), both the antibody binding capacity and the AP enzyme activity of the scFv-AP fusion protein were determined by colorimetric analysis. Such a recombinant immunotracer made by the fusion of an antibody fragment with an enzyme is a key reagent in the development of a one-step competitive direct ELISA.

Materials and methods

Materials

All reagents were of analytical grade unless otherwise specified. Glycocholic acid (GCA) was obtained from TRC (Toronto, Canada). Taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurolithocholic acid (TLCA), glycochenodeoxycholic acid (GCDCA), and glycolithocholic acid (GLCA) were purchased from Steraloids (Newport, RI, USA). QIAquick Gel Extraction Kit, and QIApre Spin Miniprep Kit were obtained from Qiagen (Qiagen, Germany). *p*-Nitrophenyl phosphate (pNPP), isopropyl-β-D-thiogalactopyranoside (IPTG) and other common chemicals were purchased from Sigma (St. Louis, MO, USA). The restriction enzyme *Sfi*I and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA, USA). Phusion High-Fidelity DNA Polymerase, B-PER, HisPur Ni-NTA resin, NuPAGE® Bis-Tris Gels and chemically competent cells of *E. coli* BL21(DE3)pLysS were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The vector pecan45 (ref. 28) containing AP gene was a generous gift from Dr Jinny L. Liu and Dr Ellen R. Goldman (Naval Research Laboratory, Center for Bio/Molecular Science and Engineering, Washington, DC). The synthesis of hapten and coating antigens were described in our previous study.[25]

Construction of a plasmid encoding scFv-AP fusion protein

Previously, a specific scFv antibody against GCA was obtained from immunized chicken by phage display.[25] The recombinant colorimetric immunotracer was constructed by fusing the C-terminus of scFv antibody to the N-terminus of AP enzyme. Briefly, the scFv gene with a 218 linker nucleotide[29] was generated by splicing by overlap extension (SOE)-polymerase chain reaction (PCR) according to the previous reports[30,31] with minor modification (forward primer: GAG GAG GTG GCC CAG CCG GCC CTG ACT CAG CCG TCC TCG GTG; reverse primer 1: GCC AGG TTT ACC GGA GCC TGA GGT GGA GCC GGA GGA GAC GAT GAC TTC GG; reverse primer 2: GAG GAG CTG GCC CCC GAG GCC GCG CCC TTA GTT GAT CCC TCC CCA GGG CCA GGT TTA CCG GAG CCT G). The scFv gene products were purified with the QIAquick Gel Extraction Kit according to the manufacturer's instructions, followed digestion with *Sfi*I restriction enzyme. The purified scFv fragments were then ligated into the similarly digested expression vector pecan45 containing AP gene at a 10 : 1 molar ratio using T4 DNA ligase, followed by transforming into the chemically competent cells of *E. coli* strain BL21(DE3)pLysS by heat shock (42 °C, 90 s). The transformed cells were then seeded on Luria–Bertani (LB)-agar plates containing 50 μg mL^{-1} carbenicillin. The carbenicillin-resistant positive clones were selected and characterized by DNA sequencing (Division of Biological Sciences, Automated DNA Sequencing Facility, University of California, Davis).

Expression and purification of the scFv-AP fusion protein

A single clone was picked and cultured in 10 mL LB medium with 50 μg mL^{-1} carbenicillin at 37 °C overnight. Then, the overnight culture was inoculated 1 : 100 in 1 L of LB medium containing 50 μg mL^{-1} carbenicillin. When the cell density reach an A_{600} nm of 0.8–0.9, the bacterial cells were induced with 0.5 mM IPTG at 30 °C by shaking at 250 rpm overnight. The bacterial cells were collected by centrifugation at 10 000 g for 20 min, and the soluble fusion protein was extracted by B-PER according to the manufacturer's instructions. Briefly, the cell pellets were lysed with B-PER lysis buffer (5 mL g^{-1} pellet), followed by incubation at room temperature (RT) for 15 min. The bacterial lysate supernatants were collected by centrifugation at 10 000*g* for 10 min, followed by purification by Ni-NTA resin column. The column was equilibrated and washed with wash buffer (10 mM PBS containing 25 mM imidazole, pH 7.4). The captured scFv-AP fusion protein was eluted with elution buffer (10 mM PBS containing 250 mM imidazole, pH 7.4). After being dialyzed with PBS (10 mM PBS, pH 7.4) at 4 °C for 72 h, the obtained scFv-AP fusion protein was stored at −20 °C until use. The purity of the resulting scFv-AP fusion protein was evaluated by SDS-PAGE using the NuPAGE® Bis-Tris Gels according to the manufacturer's instructions.

Enzyme activity of the scFv-AP fusion protein

The AP enzyme activity of the scFv-AP fusion protein was measured using colorimetric analysis according to the reported methods.[32,33] Briefly, serially diluted scFv-AP fusion protein (50 μL) was added into 96-well microplates, followed by addition of 100 μL of pNPP substrate (1.0 mg mL^{-1} pNPP, 1 M glycine buffer, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4). The plate was mixed and incubated at 37 °C for 30 min. The reaction was stopped with 50 μL per well of 4 M NaOH. The well absorbance at 405 nm was measured on a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

One-step competitive direct ELISA based on fusion protein

The sensitivity and specificity of the scFv-AP fusion protein were determined by one-step competitive direct ELISA. The microplates were coated with 100 μL per well of G1-OVA (2 μg mL^{-1}) in coating buffer at 4 °C overnight. After discarding the buffer and washing the microplates five times with PBST (PBS containing 0.5% Tween-20), the non-specific binding was blocked with 250 μL per well of 5% skim milk power (w/v) in PBST at 37 °C for 1 h. After another washing step, 50 μL per well of the scFv-AP fusion protein diluted 1 : 200 in PBST was incubated together with 50 μL per well of standard GCA, or its analogs for 1 h. After washing again, 100 μL per well of pNPP substrate was added and incubated at 37 °C for 20 min. The reaction was stopped with 3 M NaOH, and then the absorbance $A_{405\ nm}$ was measured.

Determination of cross-reactivity

The selectivity of the scFv-AP fusion protein was evaluated by comparing the IC_{50} value of GCA with those of its structural analogues, including TCA, TCDCA, TLCA, GCDCA, and GLCA. Stock solutions of various bile acids were prepared in 100% (v/v) methanol and diluted using PBS containing 5% (v/v) methanol. The assays were performed following the one-step competitive indirect ELISA, as described above, for each of the bile acids tested. The CR was calculated by the following equation:

$$CR\ (\%) = (IC_{50}\ of\ GCA)/(IC_{50}\ of\ tested\ compound) \times 100$$

Results and discussion

Cloning and characterization of the scFv-AP fusion protein

In order to create a convenient one-step detection probes for diagnosis purpose, the plasmid pecan45-scFv-AP was constructed by inserting the scFv gene with a 218 linker nucleotide into the expression vector pecan45 containing AP gene and expressing it in *E. coli* strain BL21(DE3)LysS (Fig. 2). The sequences of the positive recombinant plasmids were verified by DNA sequencing, and matched the reported data.[25,28] The AP enzyme used in this study is 449 amino acids (~47 kDa) in length containing two mutations, D153G and D330N, exhibiting higher catalytic activity than the wild-type enzyme.[34] Due to the AP enzyme is a very stable colorimetric enzyme frequently used in immunoassays; it has been a highly attractive fusion partner. A flexible linker (218 linker) was used to link the scFv antibody and AP enzyme because it had been shown to promote the correct folding of the fusion protein for proper function.[29] The obtained scFv-AP fusion protein displayed a prominent band with an expected molecular size (~80 kDa) on the SDS-PAGE gel (Fig. 2C).

Enzyme activity and antigen binding capacity of the scFv-AP fusion protein

The AP enzyme activity of the scFv-AP fusion protein was evaluated using colorimetric analysis according to the reported method.[32,33] As shown in Fig. 3, the signal intensity increased as

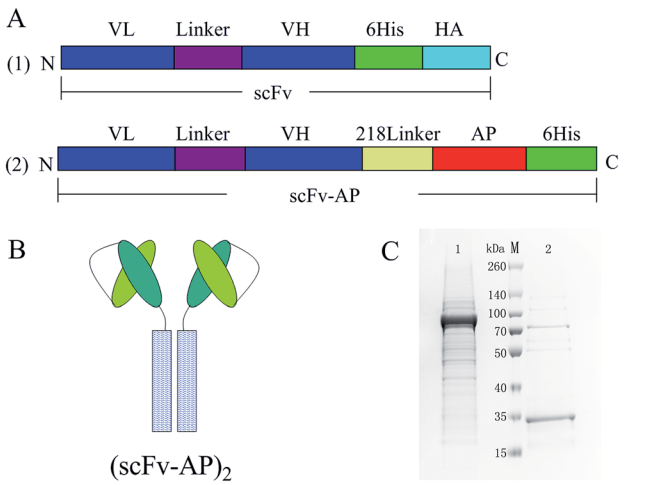


Fig. 2 Construct structures and SDS-PAGE of the scFv antibody and the scFv-AP fusion protein. (A) Construct structure of the scFv antibody (1), and the scFv fusion to alkaline phosphatase (AP) with a 218 linker (2); (B) schematic representation of the dimeric recombinant of the scFv-AP fusion protein; (C) image of a coomassie brilliant blue-stained SDS-PAGE gel of the scFv antibody and the scFv-AP fusion protein. M, protein ladder; Lane 1, scFv-AP fusion protein; Lane 2, scFv antibody.

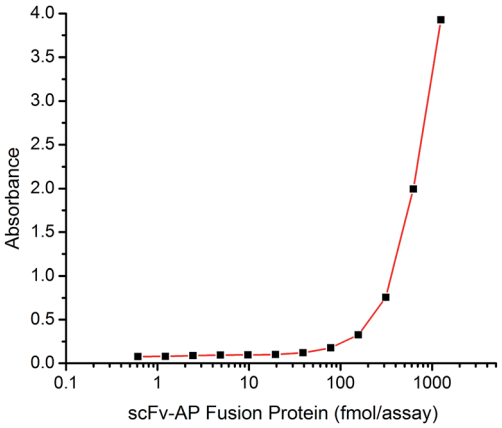


Fig. 3 Dose–response curves for AP enzyme activity of the scFv-AP fusion protein determined by colorimetric analysis. Error bars represent the standard derivation ($n = 3$).

the concentrations of the scFv-AP fusion protein increased. To evaluate the antibody binding capacity of the scFv-AP fusion protein, one-step competitive direct ELISA was carried out. Briefly, 50 μL of serial diluted concentrations of GCA standards was added into a 96-well microplate that had been coated with GCA-OVA (2 μg mL^{-1}), followed by addition of an equal volume of the scFv-AP fusion protein. The microplate was incubated at 37 °C for 1 h and washed five times with PBST. Subsequently, pNPP substrate (100 μL) was added and incubated at 37 °C for 30 min. The enzyme reaction was stopped by addition of 4 M NaOH (50 μL), and the absorbance of each well at 405 nm was measured. As shown in Fig. 4, it was obvious that the binding between the scFv-AP fusion protein and GCA–OVA conjugate could be inhibited by free GCA, showing the good anti-GCA reactivity of the scFv-AP fusion protein. The results suggested that both the specific binding capacity to GCA and the AP enzyme activity were retained in the scFv-AP fusion protein.

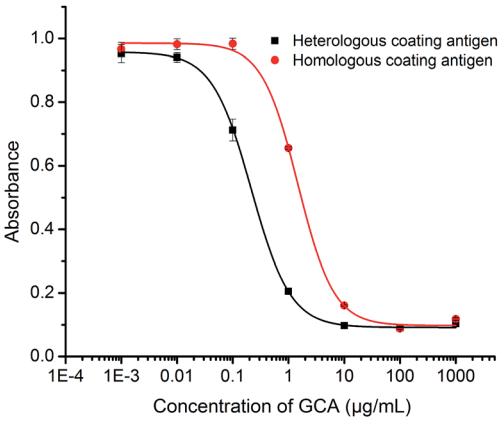


Fig. 4 Competitive binding curve based on the scFv-AP fusion protein by colorimetric analysis in homologous and heterologous coating antigen. The scFv-AP fusion protein and free GCA was added into the 96-well microtiter plate coated with appropriate coating antigen in 0.1 M carbonate-bicarbonate buffer (pH 9.6), and then the binding was detected by incubation with pNPP substrate. Error bars represent the standard derivation ($n = 3$).

One-step ELISA for GCA based on the scFv-AP fusion protein

The scFv-AP fusion protein provided reasonable affinity to the target in a competitive assay format. Among the coating antigen, as shown in Fig. 4, the heterologous coating antigen ($IC_{50} = 216$ ng mL^{-1}) resulted in a more sensitive assay than the homologous coating antigen ($IC_{50} = 1475$ ng mL^{-1}). In compared with homologous coating antigen, heterologous coating antigen usually provide weaker recognition of the antibody to the coating antigen, thus allowing the analyte to compete at low concentrations with the heterologous coating antigen, resulting in higher sensitivities for the target analyte.[35] So, in this study, the G1-OVA was selected for the GCA analysis because of the sensitivity, heterologous approach, and novelty of heterologous coating antigen structure.

In addition, reports show that water miscible organic cosolvent, ionic strengths, and pH values can influence the ELISA performance. Since GCA is highly lipophilic and may be slightly soluble in the assay buffer (about 3.3 mg L^{-1}, http://www.hmdb.ca), the water miscible organic cosolvents are often added in the assay buffer to improve the assay performance and sensitivity. Due to its smaller effect on the antigen–antibody binding, methanol (MeOH) is the most commonly used organic cosolvent in immunoassay.[36] In monitoring the MeOH effect, varying concentrations of MeOH in assay buffer were evaluated. There were no significant differences between the IC_{50} values obtained from 2.5 to 5% MeOH in assay buffer; however, when the final concentration of MeOH in assay buffer reached 20%, the A_{max} were greatly decreased (Fig. 5A). The different concentrations of the buffer ionic strength was investigated from 5 mM to 40 mM PBS. The IC_{50} and A_{max} values were significantly affected by higher ionic strength. According to the result in Fig. 5B, the IC_{50} values were almost equal in 5 mM and 10 mM PBS, but the value of the optimal intensity significantly decreased when the ionic strength values were 20 mM and 40 mM PBS. The influence of the assay buffer with the pH values between 6.2 and 8.0 was evaluated. As shown in Fig. 5C, there was no significant effect of pH values ranging from 6.2 to 8.0 in the buffer on the IC_{50} value. But, the maximum absorbance values were decreased as the pH values increased. Thus, the physiological pH, pH 7.4, was selected as the best one for the assay.

The optimized ELISA used heterologous coating antigen, G1-OVA, at a concentration of 2 μg mL^{-1} and scFv-AP fusion protein at a dilution of 1/100 prior to addition to microplate. The coated microplate was blocked with 3% skim milk. The assay buffer was 10% MeOH in 10 mM PBS, pH 7.5. As shown in Fig. 4, the average concentration of GCA required for 50% inhibition of binding (IC_{50}) and LOD were 216 ng mL^{-1} and 37.0 ng mL^{-1}, respectively, and the linear response range (IC_{20-80}) extended from 71.0 to 657 ng mL^{-1}.

Compared to the scFv-based ELISA ($IC_{50} = 706$ ng mL^{-1}),[25] the sensitivity was increased about 3-fold in the scFv-AP-based one-step ELISA. In this article, it was also shown that the conversion of the scFv antibody to fusion protein (scFv-AP fusion protein) could lead to slight improvement of sensitivity compared with its parental scFv antibody. These results were in

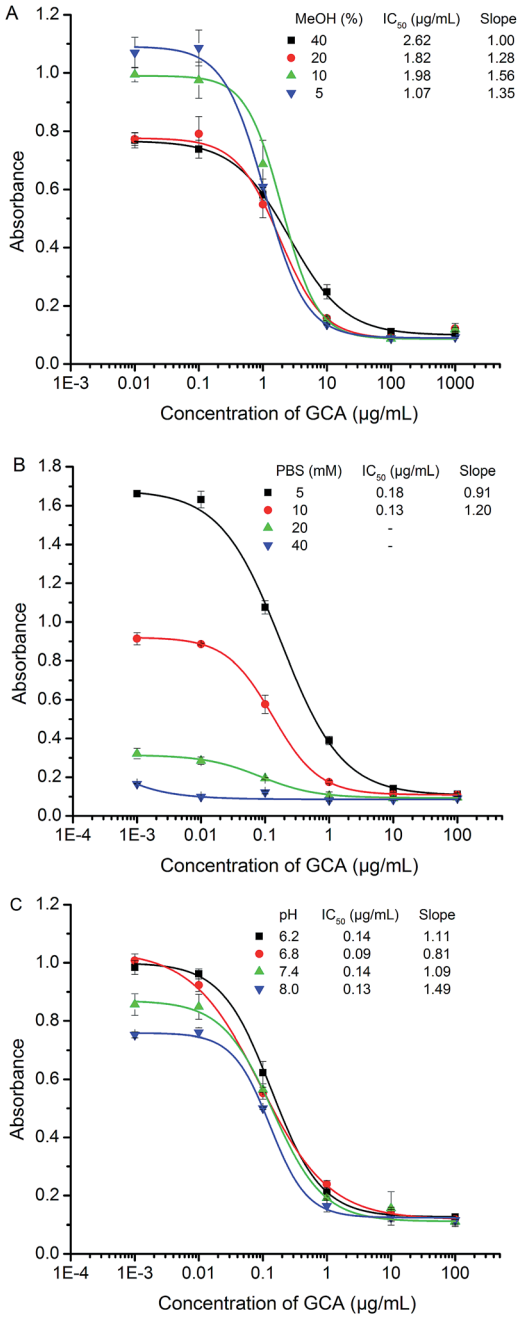
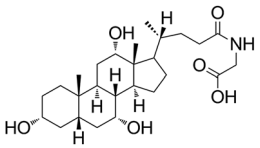
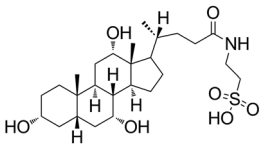
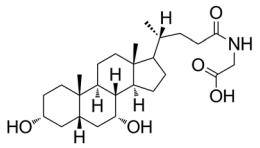
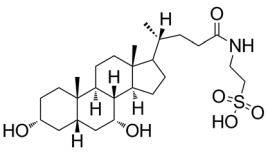
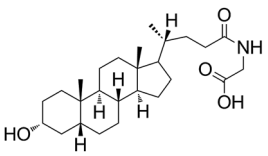
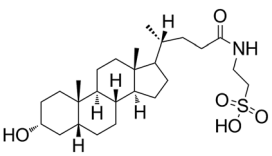


Fig. 5 Effects of MeOH (A), ionic strength (B), and pH (C) on the performance of one-step ELISA. Error bars indicate the standard derivation ($n = 3$).

accordance with previous reports that the assays based on the scFv-AP fusion proteins are often more sensitive than assays using the parental scFv antibody.[27] The AP protein itself normally forms homodimeric structures,[37] so that the scFv-AP fusion protein can mimic natural IgG molecule in which all the constant regions have been replaced with the dimeric form of AP protein. The dimerization of the AP fusion protein possibly alters the binding capacity of scFv antibody, giving rise to the slight change for small molecule binding. Maybe the dimerization of the scFv-AP fusion proteins could lead to a bivalent antigen-binding molecule, which would show higher apparent affinity (avidity) for its target as compared to monovalent scFv. This likely is an advantage for immunoassays.

To further evaluate the selectivity of the scFv-AP fusion protein, structurally related analogues of GCA were used for one-step competitive direct ELISA analysis. Percentage cross-reactivity (CR) values of the scFv-AP fusion protein to various bile acids are reported in Table 1. With both the homologous coating antigen and the heterologous coating antigen, the scFv-AP fusion protein showed a high degree of cross-reactivity to TCA. Negligible cross-reactivity toward other bile acids was observed. All together, these data showed that the characteristics of the scFv-AP fusion protein were similar to those of its parental scFv antibody. This observation is in agreement with other studies reported for other scFv-AP fusion proteins,[22,30] and therefore, the bifunctional scFv-AP fusion protein was suitable to develop a robust one-step immunoassay.

Table 1 Cross-reactivity of the scFv-AP fusion protein to GCA structurally related analogues

Analogues	Chemical structure	Homologous coating antigen CR (%)	Heterologous coating antigen CR (%)
GCA		100	100
TCA		82.7	89.5
GCDCA		<0.1	0.6
TCDCA		<0.1	0.7
GLCA		<0.1	<0.1
TLCA		<0.1	<0.1

Moreover, the scFv-AP fusion protein-based one-step ELISA can omit the use of an enzyme-labeled secondary antibody and reduce washing steps. Thus, the entire reaction time for this assay was only 90 min, while the reaction time would be 130 min or longer for the conventional assays that required the traditional pAbs or mAbs. The reduction in steps not only saves time, but it also reduces variability. In addition, this approach to design recombinant immunotracer has advantages over conventional chemical conjugation of whole IgG molecules, including the ability to produce large quantities of reagents quickly and cheaply in bacterial fermenters and to incorporate them into defined and standardized tests for use in different countries.

Conclusions

In this study, the plasmid pecan45-scFv-AP was constructed by inserting the scFv gene with a 218 linker nucleotide into the expression vector pecan45 containing AP gene. The scFv-AP fusion protein could be induced for expression in *E. coli* strain BL21(DE3)pLysS, and purified using affinity chromatography. Both the specific antibody binding capacity and the AP enzyme activity were retained in the scFv-AP fusion protein. The scFv-AP fusion protein-based one-step competitive direct ELISA showed a desired sensitivity with IC_{50} and LOD were 216 ng mL^{-1} and 37.0 ng mL^{-1}, respectively, and the linear response range extended from 71.0 to 657 ng mL^{-1}. This work demonstrated that the isolated specific chicken scFv antibody was able to be further engineered to create scFv-AP fusion protein, which could be further used to develop a one-step ELISA for the specific detection of GCA.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by the National Institute of Environmental Health Science Superfund Research Program (P42ES04699), the National Natural Science Foundation of China (41071176 and 41271340) and the Guangdong Natural Science Foundation (S2013030013338).

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