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Cui, Xiping
Vasylieva, Natalia
Shen, Ding
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Biotinylated Single-Chain Variable Fragment-Based Enzyme-Linked Immunosorbent Assay for Glycocholic Acid

Xiping Cui[a], **Natalia Vasylieva**[b], **Ding Shen**[a], **Bogdan Barnych**[b], **Jun Yang**[b], **Qiyi He**[a], **Zhengyun Jiang**[a], **Suqing Zhao**[a], and **Bruce D. Hammock**[b]

[a] Department of Pharmaceutical Engineering, Faculty of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, People's Republic of China.

[b] Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California, Davis, California 95616, United States

Abstract

Glycocholic acid (GCA) has been identified as a novel selective and sensitive biomarker for hepatocellular carcinoma (HCC). In this work, a recombinant antibody, scFv-G11, which was shown previously to have selective reactivity for GCA, was labeled with biotin using a chemical and an enzymatic method, respectively. The enzymatic method proved superior giving sensitive scFv-biotin preparations. Based on biotinylated scFv against GCA and biotin-streptavidin system for signal amplification, an indirect competitive biotin-streptavidin-amplified enzyme-linked immunosorbent assay (BA-ELISA) has been established for sensitive and rapid detection of GCA. Several physiochemical factors that influenced assay performance, such as organic cosolvent, ionic strength, and pH, were studied. Under the optimized conditions, the indirect competitive BA-ELISA based on the obtained biotinylated scFv antibodies indicated that the average concentration required for 50% inhibition of binding (IC_{50}) and the limit of detection (LOD) for GCA were 0.42 µg/mL and 0.07 µg/mL, respectively, and the linear response range extended from 0.14 to 1.24 µg/mL. Cross-reactivity of biotinylated scFv antibodies with various bile acid analogues were below 1.89 %, except for taurocholic acid. The recoveries of GCA from urine samples via this indirect competitive BA-ELISA ranged from 108.3 % to 131.5 %, and correlated well with liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS), which indicated the accuracy and reliability of biotinylated scFv-based ELISA in the detection of GCA in urine samples. This study also demonstrates the broad utility of scFv for the development of highly sensitive immunoassays.

Graphical Abstract
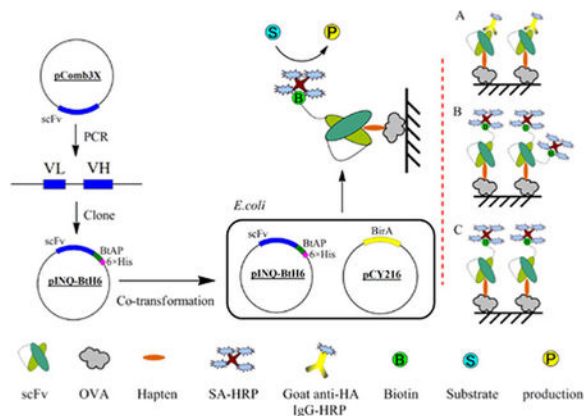
An indirect competitive ELISA was developed for GCA detection based on biotinylated scFv antibody.

Introduction

Enzyme-linked immunosorbent assay (ELISA) has been widely used for detection of small molecules in environmental contaminants,[1,2] food additives,[3,4] and biological metabolites,[5,6] due to its attractive advantages, such as high sensitivity, simplicity, high throughput, and low cost. The antibodies are the key determinant of specificity and sensitivity of immunoassay methods. Generally, ELISAs are based on polyclonal antibodies (pAbs) or monoclonal antibodies (mAbs) or a combination of both. To ensure quality and reproducibility of tests, the continuity of supply of high quality, well characterized antibodies are significant. However, for the individual pAb produced from immunized animals, the quantity is finite. In addition, because of variable immune response among animals, pAbs do not offer consistency from batch to batch. Each new batch must be optimized and validated prior to be used in testing. Thus, these limiting factors hampered pAbs in large-scale application or commercial production. On the other hand, although hybridoma cell lines secreting mAbs have the potential for being produced in consistent and unlimited supply, they must be stored at ultra-low temperature, and the cell lines may lose their viability during storage. Moreover, the mAbs preparations are time-consuming and costly. Recently, recombinant antibodies (rAbs) are being evaluated as an alternative to conventional antibodies (pAbs or mAbs).[7] Recombinant antibodies can be selected together with their DNA coding sequences from antibody gene libraries from any species by a phage-display method.[8] Furthermore, rAbs have the additional advantage in that the DNA coding for them can be stored for an indefinite period, allowing a potentially unlimited supply. Single-chain variable fragment (scFv) is the most popular rAb format. An antibody in scFv format consists of variable region of heavy (VH) and light chains (VL), which are joined together by a flexible peptide linker.[9,10] More importantly, its single chain makes it easy to fuse with other proteins, resulting in the formation of antibody molecules with two or more desired functions. Because of its reproducibility, ease of production in *Escherichia coli* (*E. coli*), and possibility of genetic engineering, scFv has gained widespread attention.

On the other hand, increasing the sensitivity of detection systems has always been considered as one of the major trends in development of immunoassay. In recent years, in order to further improve sensitivity, conventional ELISAs have been modified with a combination of chemiluminescence,[11] fluorescence,[12] or biotin-(strept)avidin system.[13] Among these strategies, the biotin-(strept)avidin amplified system, which is based on the extremely high specificity and strong affinity of (strept)avidin for biotin ($K_d = 10^{-15}$ M), offers the possibility of improving the sensitivity of immunoassay, which makes the ELISAs more effective. The schematic description of a conventional ELISA and biotin-(strept)avidin-amplified ELISA (BA-ELISA) are depicted in Scheme 1.

Typically, the biotinylation of antibodies is done by chemical method using activated derivatives of biotin, such as biotinyl-*N*-hydroxy-succinimide. However, the chemical method can modify the protein at random site, which could result in the inactivation lysine residues critical for protein function or disrupt the protein structure as well.[14] Also, the molar ratio of biotin to antibody is hard to control and costly reagents are required. In contrast to the chemical method of modification, enzymatic biotinylation of recombinant proteins guarantees site-specific attachment of only one biotin molecule per protein. In *E. coli*, there is only one protein known to be specifically biotinylated *in vivo*, namely, biotin carboxyl carrier protein (BCCP). The endogenous biotin ligase (BirA) of *E.coli* catalyzes a specific formation of an amino bond between the carboxyl group of biotin and the epsilon-amino group of the lysine residue from BCCP, which would be an appropriate enzyme for site-specific biotinylation of scFv antibodies.[15] More recently, a 15-mer acceptor peptide (BtAP) has been reported as the minimal substrate requirement in BirA-catalyzed biotinylation.[16] This biotinylated peptide provides a strong binding to the site to the acceptor (strept)avidin, which has given rise to a profusion of (strept)avidin bioconjugates for countless applications.[17-19] To explore the possibility of expanding the scope of this technology to the small molecules monitoring, in this study, we have developed a biotinylated scFv-based ELISA for glycocholic acid (GCA).

The secondary bile acid, GCA, is a derivative of steroid acids (http://www.hmdb.ca/). Recently, GCA has been identified as a new endogenous biomarker for hepatocellular carcinoma (HCC) in plasma and urine.[20,21] HCC is the fifth most common cancer worldwide and the second leading cause of cancer related mortality.[22] Early and accurate diagnosis of HCC is of central importance for timely treatment and five-year survival rate. For the HCC patients, the average concentration of GCA in urine (11.5 μg/mL) was found to be about three times as much as those in the healthy volunteers (3.9 μg/mL).[23,24] The urine has been shown to contain a wealth of metabolic information that may be altered due to diseases, and do satisfy the criteria of minimal invasiveness, reasonable cost, or minimal time demand.[25] Therefore, routine urinalysis would be of significant advantage and useful for primary diagnosis, surveillance and early detection of HCC. At present, the commonly used methods for analysis of GCA in human urine and serum are instrumental methods. The limit of detection (LOD) of these methods is about 5.6 μg/mL for high-performance liquid chromatography (HPLC),[26] 0.25 ng/mL for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS),[27] 1 ng/mL for liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS),[28] 4.2 μg/mL for matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS),[29] respectively.

It is well known that the instrument methods are effective, accurate, and reliable. However, these instrumental methods are generally expensive and time-consuming, require advanced infrastructure, as well as require a well-trained operator. So, it makes sense to develop a more highly sensitive, selective, high-throughput, and simpler analytical method. Immunoassays have emerged as an alternative to instrumental methods that can meet such demands. In an early work, we reported the selection of scFvs specific for GCA from immunized chickens and the development of scFv-based ELISA.[30] Compared with conventional ELISA, the BA-ELISA can improve the sensitivity of detection. This sensitive technique, to the best of our knowledge, has not been reported for GCA detection in urine samples.

In this study, a highly sensitive and selective indirect competitive ELISA, using a biotin-streptavidin-amplified system, was developed for detecting GCA. For establishing this proposed BA-ELISA, the biotinylated scFv antibodies were prepared primarily by a chemical and an enzymatic method, respectively. Several physiochemical factors that influence assay performance, such as organic cosolvent, ionic strength, and pH, were studied. Under the optimized conditions, this method was implemented to detect GCA in urine samples. The accuracy and sensitivity of the testing results were investigated. We believe that this proposed BA-ELISA will be useful for the screening of GCA in urine.

Results and discussion

Chemical and Enzymatic Biotinylated Single-Chain Variable Fragment

Biotinylated antibody was a key material in the BA-ELISA, which could bind to (strept)avidin-HRP. The most common and versatile approach is the chemical modification using activated derivatives of biotin.[13, 31, 32] In this work, by using the commercially available sulfo-NHS-LC-Biotin, three different molar ratios of biotin to scFv were used to modify scFv according to manufacturer's instructions. As shown in Fig. 1, the IC_{50} values obtained from three differentially biotinylated scFv conjugates (5:1, 10:1, 20:1) were 6.29 μg/mL, 5.14 μg/mL and 7.34 μg/mL, respectively, which were all higher than those obtained from traditional ELISA using the same scFv antibodies and homologous coating antigen (GCA-OVA, IC_{50}=5.08 μg/mL) described in our previous report.[30] Also, Fig. 1 indicated that the performance of BA-ELISA was significantly inhibited, while using the heterologous coating antigen (G1-OVA). However, in contract to the chemical biotinylated scFv, the enzymatic biotinylated scFv could increase the sensitivity of BA-ELISA, not only on the homologous coating antigen format, but also on the heterologous coating antigen format. Thus, based on the above data, it was possible that the significant loss of sensitivity was caused by alteration of binding properties of the scFv antibodies by biotinylation, since it is not possible to target precisely which lysine residue is linked to biotin during the *in vitro* chemical modification. In the case of anti-GCA scFv antibodies that contain lysine residues in the complementarity determining regions (CDRs), chemical biotinylation can substantially influence their activity and stability, so the choice was made to use enzymatic biotinylation *in vivo* by coexpression with BirA in *E.coli* cells.

Optimization of Biotinylated scFv-based Enzyme-Linked Immunosorbent Assay

In order to achieve better assay sensitivity, a series of experiments were performed. The effect of coating antigen on the assay sensitivity was evaluated first. Compared to homologous coating antigens, 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.[33,34] Except the carrier protein, the handle and the conjugation position should be different. Each hapten was then conjugated via different methods as descripted before.[30] As shown in Table 1, the heterologous coating antigen G1-OVA resulted in an IC_{50} value of 0.42 μg/mL, which had an improvement of > 4-fold compared to the IC_{50} value obtained from homologous coating antigen GCA-OVA (IC_{50} = 1.81 μg/mL). Thus, G1-OVA was selected as the optimal coating antigen for the further study.

Blocking Reagent Effect.—The blocking step was important to avoid nonspecific absorption in the assay. Otherwise, unoccupied sites of the plates may absorb the components, such as biotinylated scFv and SA-HRP during the subsequent steps, which may cause high background. In this work, a wide range of blocking reagents was tested, including gelatin, PEG 20 000, skim milk, and casein. The value of the *S/N* (the ratio between specific and nonspecific absorption) was used to indicate the degree of nonspecific absorption. The results showed that the signal intensity of the blank was from 0.10 to 0.31 when different blocking reagents were used. From Table 2, it is evident that 0.5 % skim milk had the highest *S/N* value, and was therefore chosen as the blocking reagent.

Organic Cosolvent Effect.—Because water miscible organic cosolvents are often added to the immunoassay to improve analyte solubility or as a part of the sample preparation procedure, it is desirable to assess the effect of organic cosolvent on immunoassay performance. The effects of four water miscible organic cosolvents (MeOH, DMSO, ACN, and acetone) in assay solution on the immunoassay system are presented in Fig. 2. The signal intensity did not change greatly when the concentration of MeOH was less than 20 %. Similarly, sensitivity varied slightly giving IC_{50} value ranging between 0.61 μg/mL (20 % MeOH), 0.45 μg/mL (10 % MeOH), and 0.54 μg/mL (5 % MeOH). However, the signal intensity significantly decreased with increasing concentrations of DMSO, ACN, and acetone, respectively. These organic cosolvents dramatically influenced assay performance. Thus, these data above demonstrated that MeOH caused the least negative effect in the performance of the immunoassay, which was in agreement with the results of several other studies.[35,36] Therefore, MeOH was the most suitable cosolvent for the assay. The optimum concentration of MeOH was 10 %, where A_{max}/IC_{50} was the highest.

Ionic Strength of the Assay Buffer.—Ionic strength of the buffer had a significant influence both on signal intensity and on sensitivity (Fig. 3). As the ionic strength increased, the A_{max} was generally significantly reduced. The A_{max} at the ionic strength of 20 and 40 mM PBS decreased by 48 and 78 %, respectively, from the A_{max} at the ionic strength of 10 mM PBS. 20 mM PBS had the lowest IC_{50} (0.18 μg/mL), followed by 10 mM PBS (0.37 μg/mL) and then 5 mM (1.38 μg/mL). These results assumed that the binding interaction between biotinylated scFv and analyte/coating antigen was gradually suppressed in solutions

with higher ionic strength. Therefore, the optimum concentration selected was 10 mM, at which A_{max}/IC_{50} was the highest.

pH Effect.—Under the acidic or alkaline condition, the antibody may contribute to the antibody unfolding, or change surface charge or the antibody paratope. In order to evaluate the effect of pH values on the biotinylated scFv-based ELSIA, the assay solution with pH values ranging from 6.2 to 8.0 were tested. As shown in Fig. 4, the effect of the pH (6.2 ~ 8.0) in the assay solution on immunoassay was not remarkable. This indicates that changes of pH in the tested range would not affect the accuracy for the quantitation of the target compound. Thus, the physiological pH, pH7.4 was selected as the best one.

The final assay conditions were as follows: the optimized BA-ELISA used a coating antigen of G1-OVA at a concentration 2 μg/mL and biotinylated scFv at a dilution of 1/500 in PBS before the competition. The plate coated with the coating antigen was blocked with 0.5 % skim milk. The standard analyte was loaded in assay buffer containing 10 % MeOH in 10 mM PBS, pH 7.4 (prior to antibody addition in the well). Under these conditions, the assay had a linear range ($IC_{20 \sim 80}$) of 0.14 ~ 1.24 μg/mL and an IC_{50} value of 0.42 μg/mL. The LOD in buffer was 0.07 μg/mL (Fig. 5).

Cross-reactivity

The relative specificity of the biotinylated scFv was evaluated by cross-reactivities (CRs). Compounds structurally similar to GCA, including TCA, TCDCA, TLCA, GCDCA, and GLCA, were investigated. Table 3 shows the CRs that were found by the biotinylated scFv-based ELISA, expressed as a percentage of the IC_{50} of GCA.

The antigen-antibody complex formation is a complicated interaction, and is easily affected by slight differences in chemical structure. The CR data may give some information about the binding preferences of the biotinylated scFv. As shown in Table 3, TCA was characterized by the highest percent of CR (43.75 %). For other bile acids, CR was negligible (<1.88 %). It is well-known that antibodies exhibit a preferential recognition to the part of the molecule that is the farthest from the conjugate site of immunogen.[37] In this case, because the antibodies were made to a hapten attached to the carrier protein through carboxyl group on the side chain, it was expected that the ability of the antibody to distinguish among bile acids would be less with substitutions in the side chain, but still quite good in the steroid nuclei. In addition, the antibody recognizes substitutions on the side chain as glycine > taurine as demonstrated by decreasing CR for GCA versus TCA. On the basis of these results, it is thought that the most antigenic determinants of the target analyte for the biotinylated scFv are the steroid nuclei.

Among the various bile acids, the biotinylated scFv showed relatively higher CR to TCA. This problem can be addressed by group separation of the glycine and taurine conjugated bile acids prior to analysis. [38,39] In addition, because glycine-conjugated bile acids represent a major fraction of the total metabolites of a particular bile acid, the biotinylated scFv still may be utilized for preliminary screening of urine samples for assessing the necessity for further inspection for individual bile acids. Alternatively, the TCA/GCA mixture may prove to be useful biomarker.

Analysis of Spiked Samples

Complexities of the samples are challenging for accurate analysis of biofluid samples. Sample extraction and cleanup procedures are effective ways to eliminate matrix effects; however, they are often time-consuming and laborious. Dilution and addition of the blank matrix to the calibration curve are also the valuable methods to account for decreasing interference. In this study, an artificial urine matrix was used in order to have an easy to access and easy to handle solution mimicking human urine samples.[40,41] The artificial urine samples diluted 2-, and 10-fold (50 ~ 10 % of matrix) were used to prepare serial concentrations of GCA standards containing 10 % MeOH for the BA-ELSIA. The signals of the standard curves prepared during the matrix dilution did not differ significantly from those in the matrix-free buffer, indicating that the biotinylated scFv fusion protein was resistant to matrix effects (data not shown). This is a big advantage compared to instrumental methods since the analysis can be performed without any sample treatment. It is of particular importance for the high-throughput screening of GCA in urine samples.

A spike-and-recovery analysis was carried out to evaluate the assay with the newly developed biotinylated scFv-based ELISA. The assay validation was performed in a blind fashion by direct dilution of urine samples, which were spiked with GCA concentrations ranging from 2.5 ~ 10 μg/mL. As shown in Table 4, the developed BA-ELISA had recoveries from 131.5 to 108.3 %. These data demonstrated that acceptable recoveries were obtained for this immunoassay. To evaluate the accuracy and applicability of the developed BA-ELISA, the spiked samples were analysed by LC-MS/MS. The average recoveries by LC-MS/MS for all samples ranged from 1.69 to 1.09 %. An excellent agreement between LC-MS/MS values and ELISA values was obtained from linear regression analysis ($y=1.195x - 1.487$ and $R^2=0.998$) (Fig. 6). The developed BA-ELISA and LC-MS/MS techniques yielded comparable results, indicating that the developed BA-ELISA was reliable and accurate. In addition, indicating the slope was 1.1, the ELISA gave slightly higher than the LC-MS/MS values. These results indicate that this BA-ELISA should be suitable for screening sample prior to LC-MS/MS.

Experimental

Safety.

All items coming in contact with bacteria were autoclaved before being discarded. The tubes containing urine samples were discarded as biological waste. GCA and its analogues were discarded as hazardous waste according to campus policies of the University of California, Davis.

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). Streptavidin PolyHRP80 conjugate (SA-HRP) originally produced by Stereospecific Detection Technologies (SDT) GmbH (Baes-weiler, Germany) was purchased from

Fitzgerald Industries International (Concord, MA, USA). Ovalbumin (OVA), Tween 20, 3,3',
5,5'-tetramethybenzidine (TMB), polyethylene glycol 20, 000 (PEG20 000), casine, D-
biotin, isopropyl-β-D-thiogalactopyranoside (IPTG), sulfo-NHS-LC-Biotin and other
common chemicals were purchased from Sigma (St. Louis, MO, USA). QIAquick Gel
Extraction Kit, and QIApre Spin Miniprep Kit was obtained from Qiagen (Qiagen,
Germany). Restriction enzyme S*fi*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 and chemically competent cells of *E.coli* BL21(DE3) were purchased from
Thermo Fisher Scientific (Rockford, IL, USA). The selection of anti-GCA scFv (G11) was
described in previous study.[30] The vector pINQ-BtH6 containing biotin-acceptor-peptide
(BtAP) gene and pCY216 containing BirA gene were generous gifts from Dr. Gualberto
Gonzalez-Sapienza (Universidad de la Republica, Montevideo, Uruguay).

Chemical Biotinylation of Single-Chain Variable Fragment

The biotinylated scFv antibodies were prepared by chemical method according to the
manufacturer's instructions with minor modifications. Briefly, the appropriate volume of
sulfo-NHS-LC-Biotin in dimethyl sulfoxide (DMSO) (10 mM) was added to the scFv at a
molar ratio of 5:1, 10:1, and 20:1, respectively. The reaction mixture was gently stirred for 1
hour (h) at room temperature (RT). Finally, the solution was dialyzed against 0.01 M
phosphate buffer saline (PBS) for 3 days at 4 °C, and the dialysis solution was changed
daily. Aliquots of the biotinylated scFv conjugates were stored at −20 °C until used.

Enzymatic Biotinylation of Single-Chain Variable Fragment

The scFv genes were amplified from phagemid pComb3X-G11 by polymerase chain
reaction (PCR) (Forward primer: 5′- GAG GAG GTG GCC CAG CCG GCC CTG ACT
CAG CCG TCC TCG GTG; Reverse primer 1: 5′- GCC AGG TTT ACC GGA GCC TGA
GGT GGA GCC GGA GGA GAC GAT GAC TTC GG; Reverse primer 2: 5′-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 PCR products were purified with the QIAquick
PCR purification kit according to manufacturer's instructions and then digested with S*fi*I
restriction enzyme. The purified genes were ligated into a similarly digested pINQ-BtH6
vector.[42] The recombinant vector was electroporated into *E. coli* BL21(DE3) cells
containing the pCY216 vector for overexpression of the *E. coli* biotin ligase BirA. The
transformed cells were then spread on Luria-Bertani broth (LB) agar plates (containing 35
μg/mL chloramphenicol and 50 μg/mL of kanamycin) for overnight incubation at 37 °C.
Single colonies were picked and inoculated into 10 mL of LB medium (containing 35 μg/mL
chloramphenicol and 50 μg/mL of kanamycin) at 37 °C overnight. On the next day, the
culture was transferred into 1 L of LB (containing 35 μg/mL chloramphenicol, 50 μg/mL of
kanamycin, 0.04 % of L-arabinose, and 100 μM D-biotin). The expression of fusion protein
was induced by 3 μM IPTG for 8 h at 30 °C with shaking. After centrifugation, the cell
pellets were resuspended in PBS containing 100 μM D-biotin, and lysed by three freeze-and-
thaw cycles. The mixture was purified by nickel-nitrilotriacetic acid (Ni-NTA) resin
according to the manufacturer's instructions to obtain the biotinylated scFv antibodies. The
size of biotinylated scFv antibodies were determined by sodium dodecyl sulfate-

polyacrylamide gel eletrophoresis (SDS-PAGE). The biotinylated scFv antibodies were collected and stored at −20 °C after dialysis with 0.01 M PBS.

Optimization of Assay Conditions

The influence of several experimental parameters on assay performance was evaluated to improve the sensitivity of the BA-ELISA.

Blocking Buffer.—In the immunoassay, the blocking step was important to avoid nonspecific absorption. Several blocking reagents were tested, including gelatin, PEG 20 000, skim milk, and casein.

Organic Cosolvent.—The tolerance of immunoassay to various organic cosolvents used to dissolve GCA was studied for assay optimization. The standard GCA solutions were prepared in methanol (MeOH), DMSO, acetonitrile (ACN), or acetone of various concentrations (5, 10, 20, and 40 % in PBS, which became 2.5, 5, 10, and 20 %, respectively, by combing with biotinylated scFv diluted with PBS).

Ionic Strength.—The influence of ionic strength of the assay solution on assay performance was evaluated using different concentrations (5, 10, 20, and 40 mM) of PBS.

pH.—The effect of buffer pH in a range of 6.2 ~ 8.0 on the assay performance was investigated.

Biotinylated Single-Chain Variable Fragment-Based Enzyme-Linked Immunosorbent Assay

The microtiter plate wells were coated with 100 μL/well of the coating antigen (G1-OVA, 2 μg/mL) in coating buffer followed by overnight incubation at 4 °C. After washing five times with washing buffer (PBS containing 0.05 % Tween 20, PBST), the unbound active sites were blocked by incubation with 250 μL of the blocking buffer (0.5 % of skim milk in PBS) for 1 h at RT. The solution was discarded, and the plates were washed five times by PBST. Then, 50 μL/well of the standard solution, or sample dilution, and an equal volume of the biotinylated scFvs diluted in PBS were added. The plates were incubated for 1 h at RT, and then washed five times with PBST. The SA-HRP (1:10 000 dilution, 100 ng/mL) was added at 100 μL/well, and the plates were incubated for 1 h at RT. The substrate solution was added at 100 μL/well after a final wash step, and the enzymatic reaction was incubated for 15 min at RT. The reaction was stopped by addition of 2 M H_2SO_4 (50 μL/well), and the absorbance of each well was measured at 450 nm.

Curve Fitting and Statistical Analysis

A four-parameter logistic equation as define below was used to fit the immunoassay data:

$$y = (A - D)/[1 + (x/C)^B] + D$$

where A is the response at high asymptote, B corresponds to the slope of the sigmoidal curve, C is the analyte concentration at 50 % of specific binding (IC_{50}), D is the response at low asymptote and x is the calibration concentration.

The sigmoidal curves were generated using OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA). The IC_{50} value, assay dynamic range ($IC_{20 \sim 80}$), and limit of detection (LOD) were determined as the analyte concentration corresponding to 50 %, between 20 % and 80 %, and 90 % inhibitory concentration, respectively.

Cross-reactivity

The selectivity of the biotinylated scFv-based assay was evaluated by determining the cross-reactivity (CR) of various bile acids with structural similarity to GCA, including TCA, TCDCA, TLCA, GCDCA, and GLCA. The CR was calculated by the following equation:

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

Analysis of Urine Samples

Artificial urine was synthesized according to a modified protocol published by Yetisen et al [41] with minor modification. The solution contained citric acid (2 mM), sodium bicarbonate (25 mM), urea (170 mM), uric acid (0.4 mM), creatinine (7 mM), calcium chloride (2.5 mM), sodium chloride (90 mM), magnesium sulfate (2 mM), sodium sulfate (10 mM), potassium dihydrogen phosphate (7 mM), dipotassium hydrogen phosphate (7 mM), and ammonium chloride (25 mM). The pH of the solution was adjusted to 7.4 by addition of potassium hydroxide solution (1 M).

Urine samples spiked with GCA (2.5, 5, and 10 μg/mL) were diluted with MeOH to reach a final percentage of 10 MeOH/urine (v/v). After completely mixing, the diluted samples were centrifuged, and the supernatants were subjected to the BA-ELISA using a calibration curve generated in PBS containing 10 % MeOH.

The biotinylated scFv-based ELISA was validated with LC–MS/MS (Agilent 1200SL coupled with AB Sciex 4000 QTRAP), which was carried out on a Supelco Ascentis Express C18 column (75×2.1 mm, 2.7 μm, Sigma). The analysis procedure was run with mobile phase consisting of water with 0.1 % acetic acid (solution A) and acetonitrile containing 0.1 % (v/v) acetic acid (solution B) at a flow rate of 0.2 mL/min. The injection volume of sample was 10 μL, and running time was 5 min. The mass spectrometry was performed in a negative ESI mode, transitions m/z 464.3>74 and 464.3>402.3 were used as quantitative and qualitative tracing of the target.

Conclusions

Recombinant scFv is a versatile alternative to the conventional antibody for the detection of small molecule, because of the cost-effective production using microbial systems and its capability of modification by creating gene fusions. For the *in vivo* biotinylation is highly specific for the BtAP lysine, it can be achieved without modification of critical lysine residues belongs to antibody recognition sequences and thus without functional loss of the recognition domains. In this study, a simplified and efficient method for production of site-specific biotinylated scFv antibody was developed. The recombinant fusion antibody functioned essentially in the same manner as the naive antibody in immunoassay for GCA

detection and also exhibited the streptavidin binding characteristics. This work, in terms of the biotinylated scFv antibodies, presents an innovative BA-ELISA for determining GCA. The developed BA-ELISA was used for the recovery study of the spiked samples and showed satisfactory recoveries. Collectively, the biotinylated scFv antibody-based immunoassay developed here could be a promising method for screening of GCA in human urine samples. Furthermore, this study also demonstrates the broad utility of scFv for the development of highly sensitive immunoassays.

Acknowledgements

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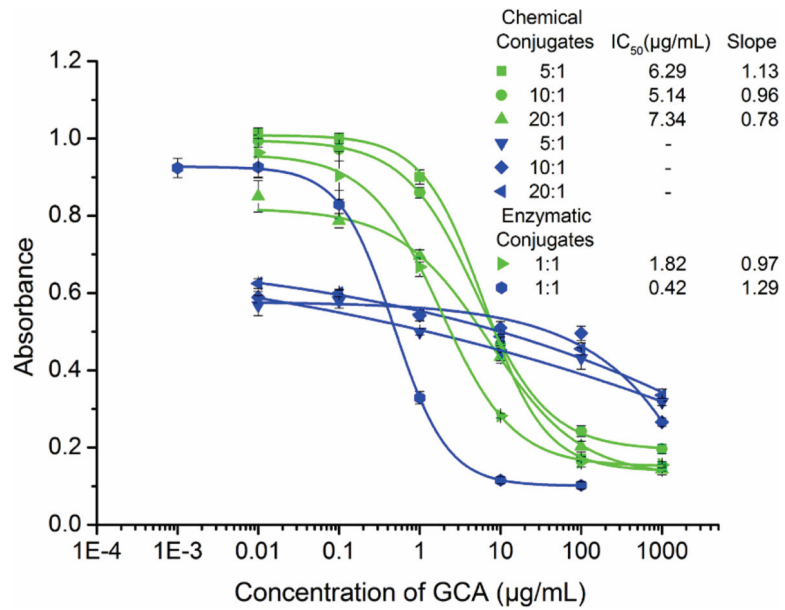
Fig. 1.
Effect of chemical and enzymatic biotinylated scFv conjugates on the inhibition curve for GCA. Assay conditions: coating antigen (GCA-OVA, green line; G1-OVA, blue line) (4 µg/mL); chemical biotinylated scFv antibody (125 ng/mL for GCA-OVA; 500 ng/mL for G1-OVA); enzymatic biotinylated scFv antibody (25 ng/mL for GCA-OVA; 100 ng/mL for G1-OVA);and SA-HRP (100 ng/mL). Each point represents the mean ± SD (standard deviation, $n = 3$).
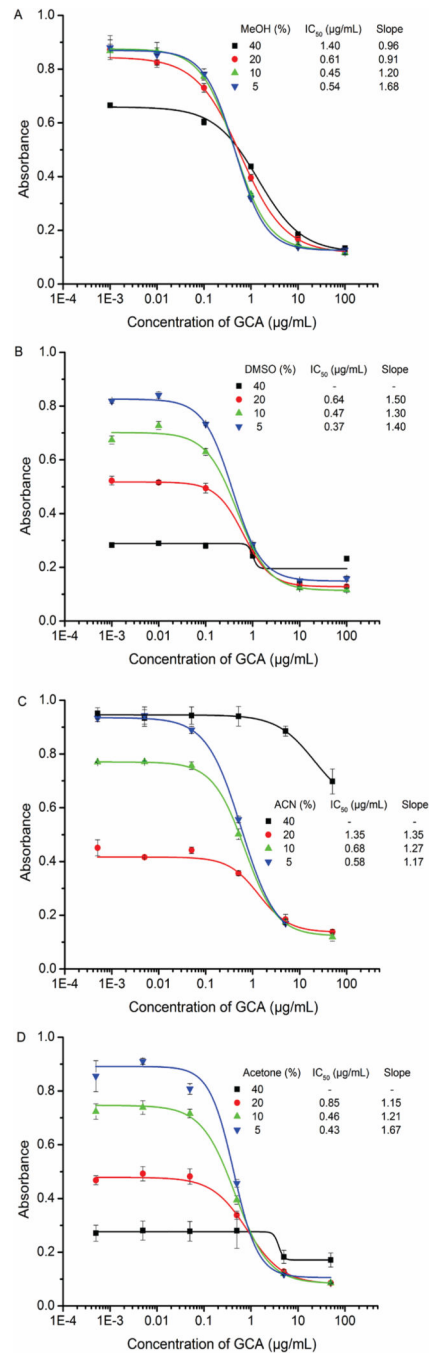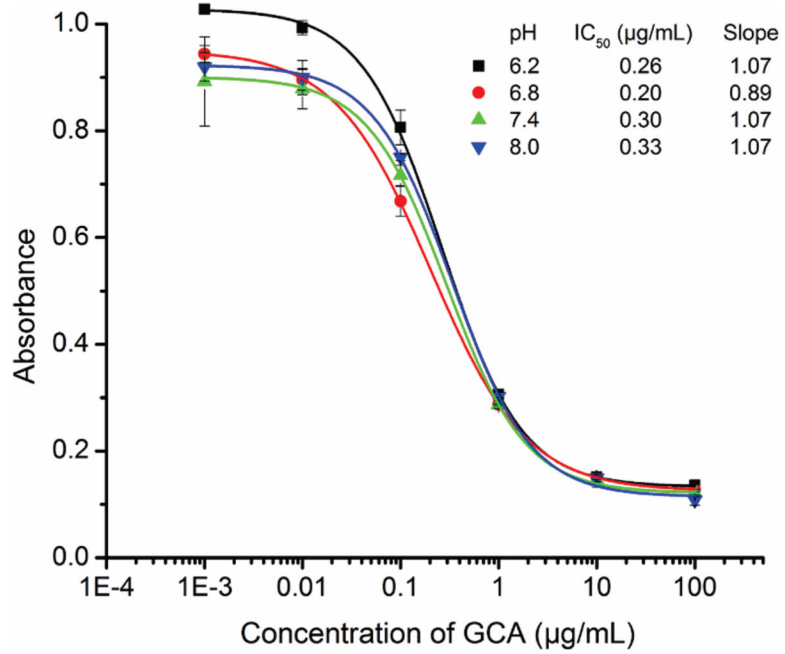
Fig. 2.
Influence of MeOH (A), DMSO (B), ACN (C), and acetone (D) on the assay sensitivity.
Assay conditions: coating antigen (G1-OVA, 2 μg/mL); biotinylated scFv antibody (1/500,
100 ng/mL); and SA-HRP (1/10 000, 100 ng/mL). Each point represents the mean ± SD (n =
3).
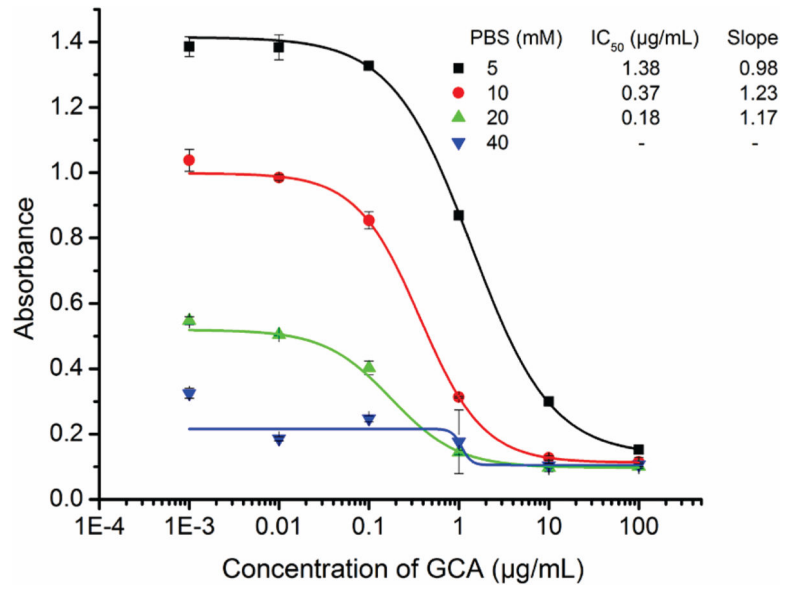
Fig. 3.
Influence of ionic strength on the assay sensitivity. Assay conditions: coating antigen (G1-OVA) (2 μg/mL); biotinylated scFv antibody (1/500, 100 ng/mL); and SA-HRP (1/10000, 100 ng/mL). Each point represents the mean ± SD ($n = 3$).

Fig. 4.

Influence of pH on the assay sensitivity. Assay conditions: coating antigen (G1-OVA) (2 μg/mL); biotinylated scFv antibody (1/500, 100 ng/mL); and SA-HRP (1/10 000, 100 ng/mL). Each point represents the mean ± SD ($n = 3$).
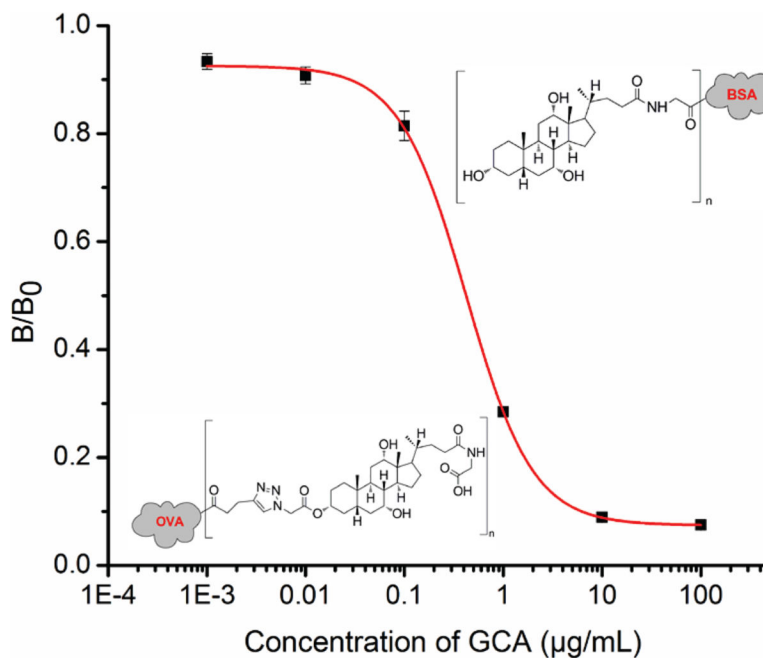
Fig. 5.
GCA competitive curve with biotinylated scFv. The microplate was coated with G1-OVA conjugate (on the left) at 2 µg/mL. Serial dilutions of GCA in PBS containing 10 % MeOH were mixed with an equal amount of the biotinylated scFv (1/500, 100 ng/mL) in PBS. The bound biotinylated scFv was detected with SA-HRP (1/10000, 100 ng/mL, 100 µL/well). The X axis is the log of the GCA concentration and the Y axis is %B/B_0 calculated from (absorbance of sample/absorbance of blank) ×100. Each point represents the mean ± SD (n = 3).
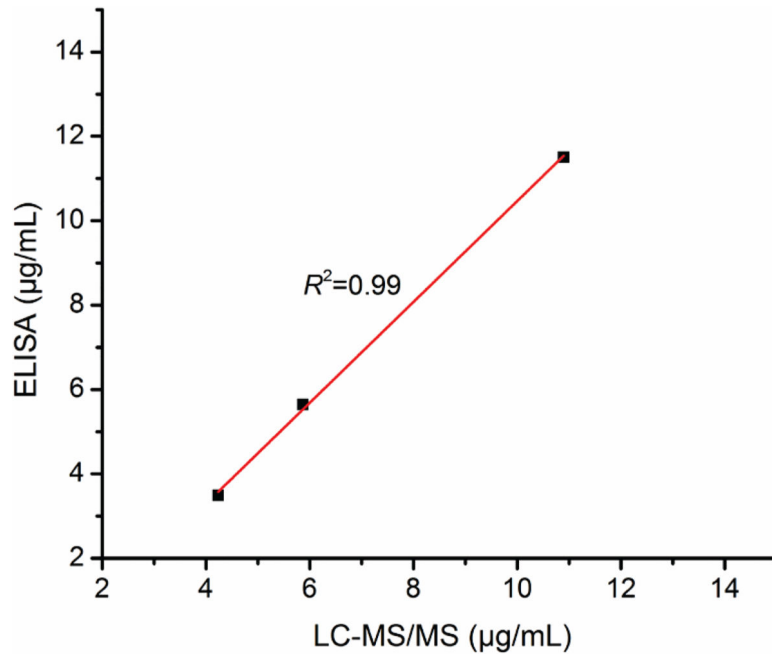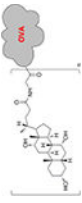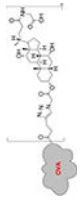
Fig. 6.
Correlation between BA-ELISA and LC-MS/MS results for the spiked urine samples. The regression equation for predicted BA-ELISA against LC-MS/MS values was y = 1.195 x – 1.487 and the correlation coefficient was 0.99.

Scheme 1.
Schematic description of the conventional ELSIA (A), the biotin-avidin-amplified ELISA using chemical biotinylated scFv (B), and the biotin-avidin-amplified ELISA using enzymatic biotinylated scFv (C).

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Table 1.

Standard Curve Characteristics of the BA-ELISA Using Homologous and Heterologous Coating Antigens[a]

Coating antigen (μg/mL)	Structure	Antibody (ng/mL)	A_{max}	IC_{50} (μg/mL)	LOD (μg/mL)	Linear Range (μg/mL)
GCA-OVA (2)		25	0.96	1.81	0.19	0.43 ~ 7.63
GI-OVA (2)		100	0.93	0.42	0.07	0.14 ~ 1.23

[a]The parameters of the assays were obtained from four-parameter sigmoidal fitting. Several rounds of optimization of assay conditions were used with each coating antigen before generating the above data.

Table 2.

The Effect of Different Blocking Reagents on the Nonspecific Adsorption ($n = 3$) [a].

Reagents	Total adsorption	Nonspecific adsorption	Specific adsorption	S/N
0.1 % Gelatin	0.46	0.19	0.27	1.42
0.5 % Gelatin	0.50	0.18	0.32	1.78
1.0 % Gelatin	0.49	0.18	0.31	1.72
0.5 % PEG 20, 000	0.97	0.21	0.76	3.45
1.0 % PEG 20, 000	0.92	0.31	0.61	1.97
2.0 % PEG 20,000	1.07	0.30	0.77	2.57
0.5 % Skim milk	0.72	0.10	0.62	6.20
1.0 % Skim milk	0.69	0.10	0.59	5.90
2.0 % Skim milk	0.62	0.10	0.52	5.20
1.0 % Casein	0.73	0.11	0.62	5.64

[a]Total adsorption was estimated as the absorbance of the control well at 450 nm; nonspecific adsorption (N) was estimated as the absorbance of blank well at 450 nm; specific adsorption (S) was estimated as the difference between total absorption and nonspecific absorption.

Table 3

Cross-reactivity (CR, %) of Various Bile Acids Structurally Related to GCA [a]

Analogues	Chemical structure	% CR
GCA		100
TCA		43.75
GCDCA		1.88
TCDCA		<0.1
GLCA		<0.1
TLCA		<0.1

[a]The microplate was coated with G1-OVA conjugate at 2 μg/mL. Serial dilutions of GCA or tested compounds in PBS containing 10 % MeOH were mixed with an equal amount of the biotinylated scFv in PBS (1/500, 100 ng/mL). The bound biotinylated scFv was detected with SA-HRP (1/10000, 100 ng/mL). CR is list as a percentage

Table 4

Recovery of GCA from Spiked Urine.

Spike (μg/mL)	Measured (μg/mL)	Mean Recovery (%)(SD)
2.5	2.8	110.8 (29.6)
5.0	6.3	125.6 (9.7)
10	12.4	124.4 (19.2)