Biotinylated single-chain variable fragment-based enzyme-linked immunosorbent assay for glycocholic acid

Xiiping Cui, Natalia Vasylieva, Ding Shen, Bogdan Barnych, Jun Yang, Qiyi He, Zhengyun Jiang, Suqing Zhao and Bruce D. Hammock

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 a biotin–streptavidin system for signal amplification, an indirect competitive biotin–streptavidin-amplified enzyme-linked immunosorbent assay (BA-ELISA) has been established for the 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$^{-1}$ and 0.07 μg mL$^{-1}$, respectively, and the linear response range extended from 0.14 to 1.24 μg mL$^{-1}$. Cross-reactivity of biotinylated scFv antibodies with various bile acid analogues was 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.

Introduction

Enzyme-linked immunosorbent assay (ELISA) has been widely used for the detection of small molecules in environmental contaminants, food additives, and biological metabolites, due to its attractive advantages, such as high sensitivity, simplicity, high throughput, and low cost. Antibodies are the key determinants of the 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 is 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 use in testing. Thus, these limiting factors hampered pAbs in large-scale applications 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 mAb preparations are time-consuming and costly. Recently, recombinant antibodies (rAbs) are being evaluated as an alternative to conventional antibodies (pAbs or mAbs). Recombinant antibodies can be selected together with their DNA coding sequences from antibody gene libraries from any species by a phage-display method. 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 the scFv format consists of a variable region of heavy (VH) and light chains (VL), which are joined together by a flexible peptide linker. 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 the development of immunoassays. In recent years, in order to further improve sensitivity, conventional ELISAs have been modified with a combination of chemiluminescence,14 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 (Kd = 10⁻¹⁵ M), offers the possibility of improving the sensitivity of immunoassays, which makes the ELISAs more effective. The schematic description of a conventional ELISA and biotin–(strept)avidin-amplified ELISA (BA-ELISA) is given in Scheme 1.

Typically, the biotinylation of antibodies is done by a chemical method using activated derivatives of biotin, such as biotinyl-N-hydroxy-succinimide. However, the chemical method can modify the protein at a random site, which could result in the inactivation of 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 the 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 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 small molecule 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 HCC patients, the average concentration of GCA in urine (11.5 µg mL⁻¹) was found to be about three times as much as those in the healthy volunteers (3.9 µg mL⁻¹).23,24 Urine has been shown to contain a wealth of metabolic information that may be altered due to diseases, and does 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⁻¹ for high-performance liquid chromatography (HPLC),26 0.25 ng mL⁻¹ for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS),27 1 ng mL⁻¹ for liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS),28 4.2 µg mL⁻¹ for matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS),29 respectively. It is well known that the instrumental methods are effective, accurate, and reliable. However, these instrumental methods are generally expensive and time-consuming, require advanced infrastructure, as well as 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 to conventional ELISA, 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, 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.

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

Chemical and enzymatic biotinylated single-chain variable fragment

Biotinylated antibody was a key material in BA-ELISA, which could bind to (strept)avidin–HRP. The most common and versatile approach is chemical modification using activated derivatives of biotin. 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 the manufacturer's instructions. As shown in Fig. 1, the IC50 values obtained from three differentially biotinylated scFv conjugates (5:1, 10:1, 20:1) were 6.29 µg mL−1, 5.14 µg mL−1 and 7.34 µg mL−1, respectively, which were all higher than those obtained from traditional ELISA using the same scFv antibodies and homologous coating antigen (GCA-OVA, IC50 = 5.08 µg mL−1) described in our previous report. Also, Fig. 1 indicated that the performance of BA-ELISA was significantly inhibited, while using the heterologous coating antigen (G1-OVA). However, in contrast to the chemically 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 assay sensitivity was evaluated first. Compared to homologous coating antigens, heterologous coating antigens 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. Except for the carrier protein, the handle and the conjugation position should be different. Each hapten was then conjugated via different methods, as described before. As shown in Table 1, the heterologous coating antigen G1-OVA resulted in an IC50 value of 0.42 µg mL−1, which had an improvement of >4-fold compared to the IC50 value obtained from homologous coating antigen GCA-OVA (IC50 = 1.81 µg mL−1). Thus, G1-OVA was selected as the optimal coating antigen for 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

![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−1); chemical biotinylated scFv antibody (125 ng mL−1 for GCA-OVA; 500 ng mL−1 for G1-OVA); enzymatic biotinylated scFv antibody (25 ng mL−1 for GCA-OVA; 100 ng mL−1 for G1-OVA); and SA-HRP (100 ng mL−1). Each hapten represents the mean ± SD (standard deviation, n = 3).](Image 68x323 to 268x478)

### Table 1 Standard curve characteristics of the BA-ELISA using homologous and heterologous coating antigens

<table>
<thead>
<tr>
<th>Coating antigen (µg mL⁻¹) Structure</th>
<th>Antibody (µg mL⁻¹)</th>
<th>Amax</th>
<th>IC₅₀ (µg mL⁻¹)</th>
<th>LOD (µg mL⁻¹)</th>
<th>Linear range (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA-OVA (2)</td>
<td>25</td>
<td>0.96</td>
<td>1.81</td>
<td>0.19</td>
<td>0.43–7.63</td>
</tr>
<tr>
<td>G1-OVA (2)</td>
<td>100</td>
<td>0.93</td>
<td>0.42</td>
<td>0.07</td>
<td>0.14–1.23</td>
</tr>
</tbody>
</table>

*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.*
of blocking reagents was tested, including gelatin, PEG 20 000, skim milk, and casein. The value of the $S/N$ ratio (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 ranged 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 cosolvents 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 an $IC_{50}$ value ranging between 0.61 $\mu$g mL$^{-1}$ (20% MeOH), 0.45 $\mu$g mL$^{-1}$ (10% MeOH), and 0.54 $\mu$g mL$^{-1}$ (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. $^{15,16}$ 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, $A_{max}$ 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 $\mu$g mL$^{-1}$), followed by 10 mM PBS (0.37 $\mu$g mL$^{-1}$) and then 5 mM (1.38 $\mu$g mL$^{-1}$). 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

### Table 2  The effect of different blocking reagents on the nonspecific adsorption ($n = 3$)*

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Total adsorption</th>
<th>Nonspecific adsorption</th>
<th>Specific adsorption</th>
<th>$S/N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% gelatin</td>
<td>0.46</td>
<td>0.19</td>
<td>0.27</td>
<td>1.42</td>
</tr>
<tr>
<td>0.5% gelatin</td>
<td>0.50</td>
<td>0.18</td>
<td>0.32</td>
<td>1.78</td>
</tr>
<tr>
<td>1.0% gelatin</td>
<td>0.49</td>
<td>0.18</td>
<td>0.31</td>
<td>1.72</td>
</tr>
<tr>
<td>0.5% PEG</td>
<td>0.97</td>
<td>0.21</td>
<td>0.76</td>
<td>3.45</td>
</tr>
<tr>
<td>20 000</td>
<td>0.92</td>
<td>0.31</td>
<td>0.61</td>
<td>1.97</td>
</tr>
<tr>
<td>1.0% PEG</td>
<td>1.07</td>
<td>0.30</td>
<td>0.77</td>
<td>2.57</td>
</tr>
<tr>
<td>20 000</td>
<td>0.69</td>
<td>0.10</td>
<td>0.59</td>
<td>5.90</td>
</tr>
<tr>
<td>1.0% skim milk</td>
<td>0.62</td>
<td>0.10</td>
<td>0.52</td>
<td>5.20</td>
</tr>
<tr>
<td>2.0% skim milk</td>
<td>0.73</td>
<td>0.11</td>
<td>0.62</td>
<td>5.64</td>
</tr>
</tbody>
</table>

*Total adsorption was estimated as the absorbance of the control well at 450 nm; nonspecific adsorption ($N$) was estimated as the absorbance of the blank well at 450 nm; specific adsorption ($S$) was estimated as the difference between total absorption and nonspecific absorption.
concentration selected was 10 mM, at which $A_{\text{max}}/IC_{50}$ was the highest.

**pH Effect.** Under acidic or alkaline condition, the antibody may contribute to the antibody unfolding, or change in surface charge or the antibody paratope. In order to evaluate the effect of pH values on the biotinylated scFv-based ELISA, 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 of 2 μg mL$^{-1}$ 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 ($IC_{20–80}$) of 0.14–1.24 μg mL$^{-1}$ and an $IC_{50}$ value of 0.42 μg mL$^{-1}$. The LOD in buffer was 0.07 μg mL$^{-1}$ (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 the immunogen. In this case, because the antibodies were made to a hapten attached to the carrier protein through the 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.\(^3\)\(^8\)\(^,\)\(^3\)\(^9\) In addition, because glycine-conjugated bile acids represent a major fraction of the total metabolites of a particular bile acid, the biotinylated scFv may still be utilized for preliminary screening of urine samples to assess the necessity for further inspection of 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 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.\(^4\)\(^0\)\(^,\)\(^4\)\(^1\) 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 BA-ELISA. 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 to 10 μg mL\(^{-1}\). 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 (\(\frac{y}{x} = 1.195x - 1.487\) 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 that the slope was 1.1, the ELISA gave slightly higher values than the LC-MS/MS values. These results indicate that this BA-ELISA should be suitable for screening samples 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

### Table 3 Cross-reactivity (CR, %) of various bile acids structurally related to GCA\(^a\)

<table>
<thead>
<tr>
<th>Analogues</th>
<th>Chemical structure</th>
<th>% CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA</td>
<td><img src="image1" alt="Image" /></td>
<td>100</td>
</tr>
<tr>
<td>TCA</td>
<td><img src="image2" alt="Image" /></td>
<td>43.75</td>
</tr>
<tr>
<td>GCDCA</td>
<td><img src="image3" alt="Image" /></td>
<td>1.88</td>
</tr>
<tr>
<td>TCDCA</td>
<td><img src="image4" alt="Image" /></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>GLCA</td>
<td><img src="image5" alt="Image" /></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>TLCA</td>
<td><img src="image6" alt="Image" /></td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

\(^a\) The microplate was coated with G1-OVA conjugate at 2 μg mL\(^{-1}\). 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\(^{-1}\)). The bound biotinylated scFv was detected with SA-HRP (1/10 000, 100 ng mL\(^{-1}\)). CR is listed as a percentage.

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### Table 4 Recovery of GCA from spiked urine

<table>
<thead>
<tr>
<th>Spike (μg mL(^{-1}))</th>
<th>Measured (μg mL(^{-1}))</th>
<th>Mean recovery (%) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>2.8</td>
<td>110.8 (29.6)</td>
</tr>
<tr>
<td>5.0</td>
<td>6.3</td>
<td>125.6 (9.7)</td>
</tr>
<tr>
<td>10</td>
<td>12.4</td>
<td>124.4 (19.2)</td>
</tr>
</tbody>
</table>

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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), glycochenodeoxycholic acid (GCDCA), and glycolithocholic acid (GLCA) were purified from Sigma (St Louis, MO, USA). Streptavidin pyranoside (IPTG), sulfo-NHS-LC-Biotin and other common chemicals were purchased from Sigma (St Louis, MO, USA). Ovalbumin (OVA), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), polyethylene glycol 20,000 (PEG 20 000), casein, β-D-thiogalactopyranoside (IPTG), sulfo-NHS-LC-Biotin and other common chemicals were purchased from Sigma (St Louis, MO, USA). Qiagen (Qiagen, Germany). Restriction enzyme SfiI 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 a previous study.10 The vector pINQ-BtH6 containing the biotin-acceptor-peptide (BtAP) gene and pCY216 containing the 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 a 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 buffered 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 TTC TCG GTG; reverse primer: 5′-GCC AGG TTT ACC GGA GCC TGA GTG GGA GCC GGA GGA GAC GAT GAC GTT TTC GG; reverse primer: 5′-GAG GAG GTG 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 the manufacturer’s instructions and then digested with the SfiI restriction enzyme. The purified genes were ligated into a similarly digested pINQ-BH6 vector.12 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−1 chloramphenicol and 50 μg mL−1 of kanamycin) for overnight incubation at 37 °C. Single colonies were picked and inoculated into 10 mL of LB medium (containing 35 μg mL−1 chloramphenicol and 50 μg mL−1 of kanamycin) at 37 °C overnight. On the next day, the culture was transferred into 1 L of LB (containing 35 μg mL−1 chloramphenicol, 50 μg mL−1 of kanamycin, 0.04% of L-arabinose, and 100 μM β-biotin). Expression of the fusion protein was induced by 3 mM IPTG for 8 h at 30 °C with shaking. After centrifugation, the cell pellets were resuspended in PBS containing 100 μM β-biotin, and lysed by three freeze-and-thaw cycles. The mixture was purified by nickel-nitrilotriacetic acid (Ni-NTA) resin according to the manufaturer’s instructions to obtain the biotinylated scFv antibodies. The size of biotinylated scFv antibodies was 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 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, skin milk, and casein.

Organic cosolvent. The tolerance of the immunoassay to various organic solvents 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 combining 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 per well of the coating antigen (G1-OVA, 2 μg mL⁻¹) 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 with PBST. Then, 50 μL per 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. SA-HRP (1:10 000 dilution, 100 ng mL⁻¹) was added at 100 μL per 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. SA-HRP (1:10 000 dilution, 100 ng mL⁻¹) was added at 100 μL per well, and the plates were incubated for 1 h at RT. The substrate solution was added at 100 μL per 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 H₂SO₄ (50 μL per well), and the absorbance of each well was measured at 450 nm.

**Curve fitting and statistical analysis**

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

\[ y = \frac{(A - D)}{1 + (x/C)^D} + D \]

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 (IC₅₀), 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 IC₅₀ value, assay dynamic range (IC₂₀–₈₀), 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, TCDDA, TLCA, GCDCA, and GLCA. The CR was calculated using the following equation:

\[ CR(%) = \frac{(IC_{50} \text{ of GCA})/(IC_{50} \text{ of tested compound}) \times 100}{} \]

**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⁻¹) were diluted with MeOH to reach a final percentage of 10 MeOH/urine (v/v). After complete mixing, the diluted samples were centrifuged, and the supernatants were subjected to 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 Scieix 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 a 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⁻¹. The injection volume of sample was 10 μL, and the running time was 5 min. 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 molecules, because of the cost-effective production using microbial systems and its capability of modifications by creating gene fusions. As the in vivo biotinylation is highly specific for BtAP lysine, it can be achieved without modification of critical lysine residues that belong 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.

**Conflicts of interest**

The authors declare that there is no conflict of interests to publish this paper.
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