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Noncompetitive immunoassays using protein G affinity capillary chromatography and capillary electrophoresis with laser-induced fluorescence detection

Qinggang Wang[a], Guoan Luo[a,*], Jianping Ou[b], William S.B. Yeung[b]

[a]*Department of Chemistry, Tsinghua University, Beijing 100084, China*

[b]*Department of Obstetrics and Gynaecology, University of Hong Kong, Hong Kong*

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Abstract

A new and simple approach to perform immunoassay using protein G affinity capillary chromatography and laser-induced fluorescence detection was described. A noncompetitive assay for monoclonal anti-bovine serum albumin (BSA) was used to test the performance of the system. Fluorescein isothiocyanate labeled BSA was used as a tracer to determine anti-BSA in p*M* level. Capillaries with inner diameter of 150 μm were packed with recombinant protein G-bound perfusive support. The packed capillary was used to capture the immunocomplexes, which were subsequently desorbed by 100 m*M* glycine (pH 9.0). Open tube capillary electrophoresis-based immunoassay (CEIA) for anti-BSA was also performed. Using standard samples, calibration curves for anti-BSA was established in both assays. Compared with CEIA, this system improved the concentration sensitivity for about 100-fold due to the pre-concentration of immunocomplex in the protein G column, while the mass sensitivity was similar in the two methods.

Keywords: Immunoassays; Proteins

1. Introduction

Immunoassays are the methods of choice for a wide variety of clinical, environmental, biochemical and pharmaceutical analyses due to their ability to determine minute amounts of analytes in complex matrices quantitatively. The high selectivity of the assay is based on the specific interaction between the antigen and the antibody, while the high sensitivity results from the use of labeling techniques such as radioactivity, fluorescence, chemiluminescence, or enzyme amplification [1]. The primary drawback of conventional immunoassays is that they are time consuming and labor intensive. Such assays are usually performed in batches to achieve high sample throughput. However, this is not desirable when rapid feedback is required. The problem has been partially solved by the use of expensive automated machines with their associated assay kits, but these machines usually do not allow users to customize the assay according to individual's needs.

To solve the above problem, researchers have investigated capillary electrophoresis-based immunoassay (CEIA) in recent years [2–16]. In CEIA, capillary electrophoresis (CE) is used to separate the immunocomplexes from the free ligands. When

*Corresponding author. Tel.: +86-10-6278-1688; fax: +86-10-6278-4764.

E-mail address: galuo@sam.chem.tsinghua.edu.cn (G. Luo)

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combined with fluorescent labels and laser-induced fluorescence (LIF) detection, CEIA can perform rapid separation with high mass sensitivity and simultaneous determination of multiple analytes. This method is also compatible with automation. The reported concentration limits of detection (LODs) of CEIA are usually in the n*M* levels [2–16], which are 2 to 3 orders of magnitude less sensitive than those of conventional immunoassays. Although the LODs of the currently developed CEIAs are low enough for some assays, they are not sensitive enough to detect the levels of many significant hormones, such as the luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol in human serum or plasma [17].

Flow-through immunological assays have also been investigated to improve the speed and automation of immunoassay [18–24]. In such flow systems, An antibody or an antibody binding protein, such as protein A or protein G, is immobilized in a column or cartridge that captures either a protein analyte or an antibody as it passed through the system. Some degree of analyte purification is generally associated with this capture step. Subsequent to capture, analyte is released directly to a detector [18] or transferred to a chromatography column for further separation and detection [19]. Kinetic and thermodynamic aspects of these interactions and the contribution of nonspecific interactions have also been studied [20]. Such flow-through immunological assays often encounter the problem of solvent interference with detection when UV absorbance detection mode is used. To solve this problem, Regnier et al. have developed a dual-column systems which consist of an affinity column coupled to an analytical column via a switching valve [19,21–23]. Although compatible with automation, the instrumentation of such dual-column immunoassays are rather complicated. Other solutions involve either competitive binding or sandwich immunoassays using immunological reagents labeled with enzymes or fluorophores. De Alwis and Wilson have used enzyme tags that generated an electroactive product [24,25]. Riggin and Regnier have demonstrated high-performance protein G affinity chromatographic immunoassay with fluorescence detection, Texas Red labeled human growth hormone was used as a fluorescence probe for detecting antibodies against human growth hormone [26]. Reinecke et al. have established a fast online flow injection analysis system for IgG monitoring in bioprocesses, and the IgG was detected without interference from other sample components by protein fluorescence [27].

In this paper, we describe a new and simple approach to perform immunoassay using protein G affinity capillary chromatography with LIF detection. Protein G is a bacterial protein that binds to immunoglobulins at the Fc region. Therefore, the binding of the antibody to protein G does not interfere with the antigen binding [28]. Protein G has the advantage of binding to a wider range of IgG species and subclasses than other bacterial IgG Fc binding proteins such as protein A [29]. A noncompetitive assay for monoclonal anti-BSA was used to test the performance of the system. The results are also compared to those of an open tube CEIA.

2. Experimental

2.1. Apparatus

All experiments were performed on an automated P/ACE Model 5010 capillary electrophoresis system (Beckman Fullerton, CA, USA) fitted with a LIF detector or a UV detector. The 488 nm line of a 5 mW argon ion laser was utilized as the excitation source of LIF detection, and the emitted fluorescence was collected at 520 nm. The instrument can supply a 138 kPa pressure rinse or separation. System control, data acquisition and analysis were accomplished with P/ACE Workstation software for Windows 95 using an IBM 586 personal computer. Polyimide-coated fused-silica capillary columns (75 μm or 150 μm I.D., 360 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Chemicals and reagents

Monoclonal anti-BSA (mouse IgG2a, 4.7 mg/ml or 32 μ*M* assuming the molecular mass of IgG2a is 146 000 [30]) were from Sigma (St. Louis, MO, USA). It was in mouse ascites fluid, and the total protein concentration was 22 mg/ml. BSA, glycine, tricine and sodium phosphate were from Sigma (St. Louis, MO, USA). Other chemicals were of ana-

lytical grade. All solutions were prepared with deionized water obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA), filtered through 0.45 μm filter, and degassed with vacuum for 20 min before use.

BSA was labeled with fluorescin isothiocyanate (FITC) using FluoroTag FITC conjugation Kit from Sigma. The labeled protein was purified with column chromatography (Sephadex G–25M), and the elution buffer was 10 m*M* phosphate-buffered saline (PBS; 10 m*M* sodium phosphate, 138 m*M* NaCl, 27 m*M* KCl, pH 7.4). The purified fraction was stored at 4°C until use. The fluorescein/protein (F/P) ratio and the final concentration were estimated to be 2.8:1 and 0.43 mg/ml according to absorbance measurements at 280 nm and 495 nm respectively. The corresponding molar concentration was 6.4 μ*M*, assuming that the molecular mass of BSA is 67 000.

2.3. Preparation of protein G affinity capillary column

The chromatographic packing material was Poros 20G (PerSeptive Biosystems, Cambrige, MA, USA). This is a perfusive support made of beads with a diameter of 20 μm that has recombinant protein G covalently bound onto the beads. The capillary with 150 μm I.D. was cut to a length of 20 cm, and a frit was made by packing 1–2 mm of 10 μm spherical silica particles at one end of the capillary under low pressure. The particles were then sintered in place by heating this section of the capillary in a flame for about 10 s. A water pressure of about 1 MPa was applied to the capillary with a syringe to test the stability of the frit. A slurry of the Poros 20 G particles was used to pack into the column using a bomb and a HPLC pump with a pressure of 6.9 MPa for over 15 min. The slurry was prepared by mixing the Poros beads with deionized water in a ratio of 1:80 (g/ml). The length of the packed bed was about 15 cm. The capillary was then cut to a length of 10 cm from the end with the frit. A second frit was made at the end of another 150 μm I.D. blank capillary with a length of 17 cm. The end of the packed capillary without the frit was connected to the end of blank capillary with the frit using a thin PTFE tubing. A detection window was made on the blank column by burning the outer coating of the capillary at a distance of 20 cm from the inlet. The connected capillaries were then fixed into the P/ACE capillary cartridge, and mounted into the CE instrument for analysis.

2.4. Immunoassay protocol

FITC–BSA and anti-BSA solutions were diluted to the appropriate concentrations with 10 m*M* PBS. Twenty microliters of each of these two diluted solutions were mixed in a 0.5 ml microcentrifuge tube and incubated at room temperature (23°C) for 20 min before injection.

2.5. Protein G affinity capillary chromatography procedure

A schematic diagram of the principle of the assay is shown in Fig. 1. The pressure separation function provided by the CE instrument was used for sample loading and elution. The sample loading buffer consisted of 50 m*M* sodium phosphate and 50 m*M* potassium sulfate (pH 7.0). Protein G column was first equilibrated with the sample loading buffer for 10 min. The sample was loaded onto the column for 5 min. Unbound FITC–BSA was removed by rinsing the capillary with the sample loading buffer for 20 min. The bound FITC–BSA was then desorbed from the column with 100 m*M* glycine buffer (pH 9.0).

2.6. Open tube CEIA procedure

Open tube CEIA was conducted in a 75 μm I.D. capillary with a length of 57 cm and an effective length of 50 cm (from inlet to the detection window). The incubated samples were injected for 5 s with a pressure of 3.4 kPa at the positive electrode. This resulted in an injection volume of about 30 nl. The applied voltage was 20 kV (351 V/cm), and 100 m*M* Tricine (pH 8.5) was used as the running buffer.

3. Results and Discussions

3.1. Optimization of protein G affinity capillary chromatography

In order to ensure that all the anti-BSA and

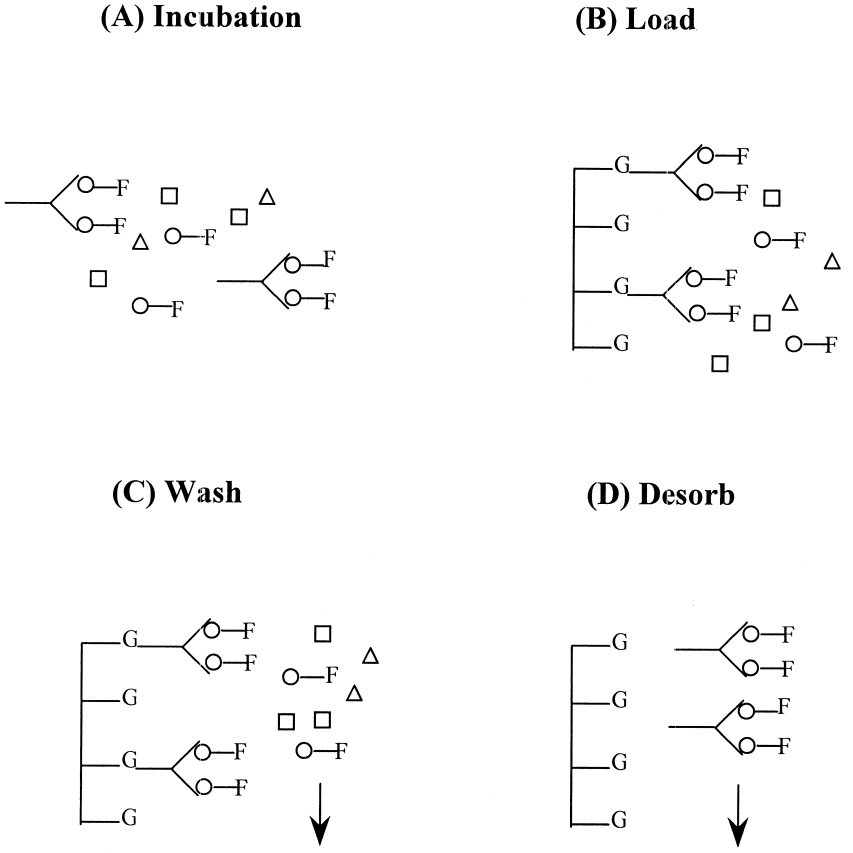

Fig. 1. Schematic diagram of the principle of protein G affinity capillary chromatographic immunoassay.

complexes injected were captured by the protein G column, two procedures were undertaken. First, the total amount of anti-BSA injected never exceeded 1 μg, which was less than one-twentieth of the estimated immunoglobulin binding capacity of the protein G column. Based on the manufacturer's information, the binding capacity of the column was about 21 μg. Second, a slow flow rate was used to allow the binding of the immunoglobulin onto the column. When 64 n*M* FITC–BSA was injected, and rinsed with sample loading buffer using the 138 kPa pressure separation function of the instrument, a peak came out at 3.1 min (RSD=1.0%, n=5). Thus the linear velocity was calculated to be 6.4 cm/min, and the flow rate was 1.1 μl/min. The chromatographic support used in this study is a perfusion type particle with large diameter that allows rapid sample loading. Protein G column with the same diameter was found to capture all the injected antibodies without loss even when the flow rate was 10 μl/min [31].

Elution of the adsorbed antibody from the protein G column under acidic conditions cannot be used in this study, because the fluorescence of FITC is quenched at low pH [32]. Akerstrom and Bjorck reported that the binding between mouse monoclonal IgG and protein G was strongest at pH 4 and 5, and weakened with increased pH value [28]. Therefore a series of 100 m*M* glycine buffers with pH value ranging from 8.0 to 9.0 were tested for their capability of desorbing the bound anti-BSA from the protein G column using UV detection at 280 nm. The results showed that the bound anti-BSA was totally desorbed at pH 9.0. This was supported by two observations. First, the peak for desorbed zone always returned to baseline after 10 min elution with

this glycine buffer. Second, the subsequent elution with an acidic buffer (100 m*M* glycine, 20% acetic acid, pH 2.5) did not produce any detectable peak.

3.2. Evaluation of FITC–BSA as the fluorescence probe for anti-BSA quantification

Two experiments were performed to evaluate FITC–BSA as the fluorescence probe for anti-BSA quantification in protein G affinity capillary chromatography. First, various concentrations of FITC–BSA were incubated with excess anti-BSA. The reaction mixtures were analyzed using the method described above. Typical chromatograms are shown in Fig. 2. When anti-BSA was in excess, all the FITC–BSA was in the complex form and retained by the protein G column. The bound FITC–BSA was eluted out only when the buffer was changed to 100 m*M* glycine (pH 9.0). The response of the instrument to the antibody bound FITC–BSA was linear (r^2= 0.996) in the tested range (8–32 n*M*) Second, various concentrations of anti-BSA were incubated with excess FITC–BSA. Typical chromatograms are shown in Fig. 3. The amount of free FITC–BSA decreased when the amount of anti-BSA in the sample increased. There was also a linear relationship between the peak area of bound FITC–BSA and the amount of anti-BSA in the sample (r^2=0.998) in the tested range (8–80 n*M*). These results suggested that the fluorescence response to FITC–BSA upon elution by basic buffer reflected the amount of FITC–BSA bound to anti-BSA. Thus, when excess FITC–BSA is used, the amount of anti-BSA injected onto the column is directly proportional to the peak area of bound FITC–BSA.

3.3. Determination of anti-BSA in pM level

Standard solutions of anti-BSA with concentration levels of 0–800 p*M* were prepared in triplicate, and FITC–BSA were added to a final concentration of 3.2 n*M*. After 20 min incubation, each sample was analyzed with protein G affinity capillary chromatographic method. Typical chromatograms are shown

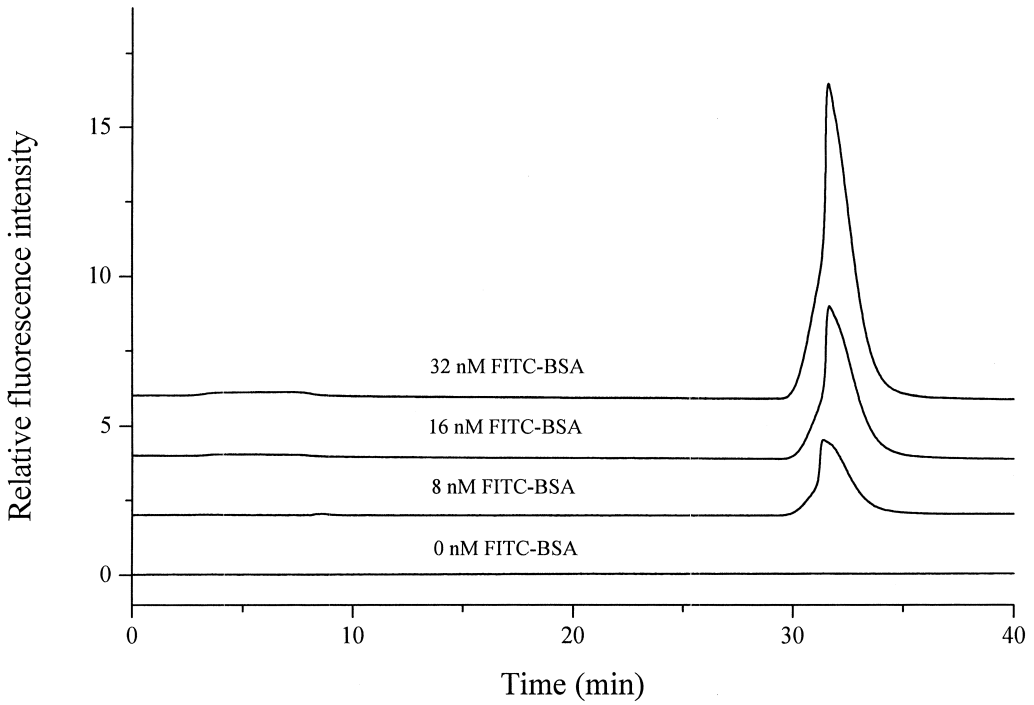

Fig. 2. Protein G affinity separation of free and anti-BSA complexed FITC–BSA in the presence of excess anti-BSA. Conditions are described in Experimental.

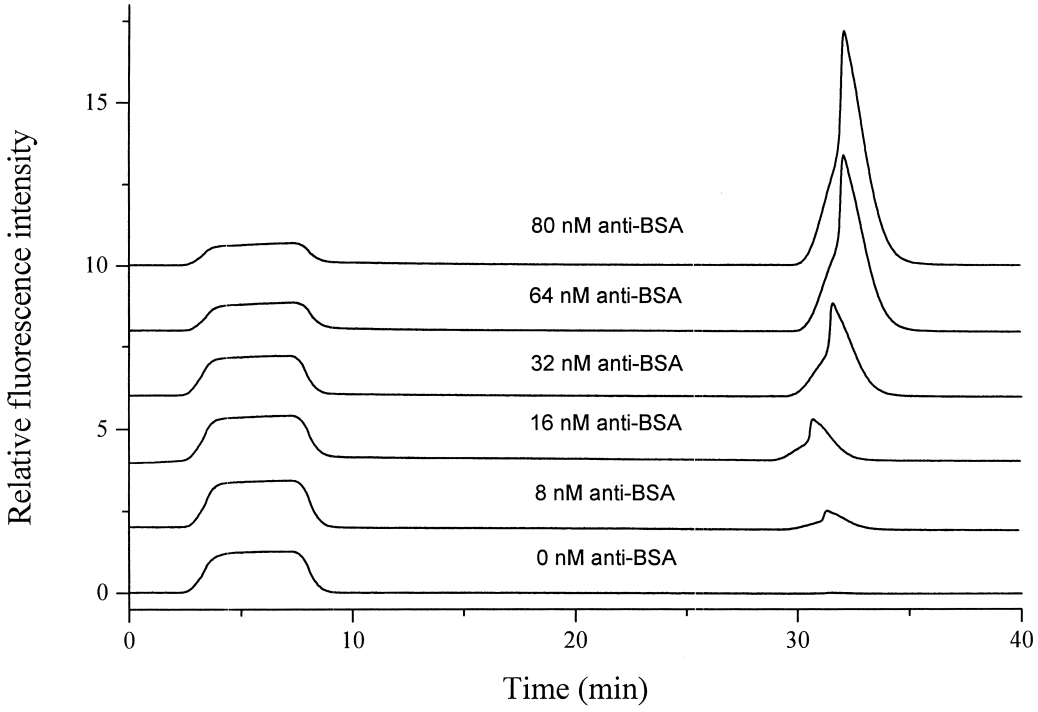

Fig. 3. Protein G affinity separation of free and anti-BSA bound FITC–BSA in the presence of excess FITC–BSA. Conditions are described in Experimental.

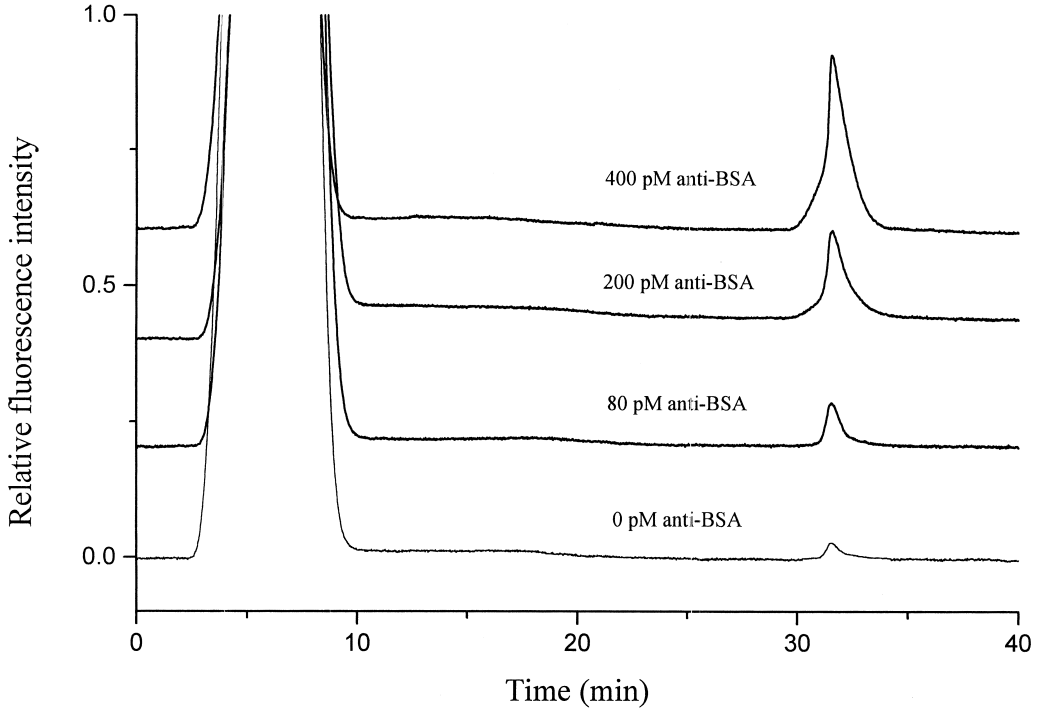

Fig. 4. Typical protein G affinity chromatograms of anti-BSA in p*M* level. Conditions are described in Experimental.

in Fig. 4. A calibration curve was obtained by plotting the concentration of anti-BSA versus the peak area upon basic elution (Fig. 5). The dynamic linear range for the calibration curve was from 80–800 p*M*. The sample without anti-BSA (negative control) was analyzed three times to determine the mean and standard deviation of the blank peak area. The concentration LOD was estimated to be about 80 p*M*, which was the mean plus 3 times its standard deviation. The injection volume was 5 μl considering the flow rate was 1.1 μl/min, and thus the mass LOD was about 0.4 fmol.

In the elution profile of the negative control, there was still a small background peak that was eluted with the basic glycine buffer. This was probably due to nonspecific binding of FITC–BSA to the protein G column since the amount of FITC–BSA used was in large excess. Although this background peak was less than 0.5% of the amount of FITC–BSA injected, it dictated the LOD of the method. The nonspecific binding can be minimized in two ways. First is by using longer washing time or high flow rate. Second is by rinsing the protein G column with sample loading buffer containing other proteins prior to the sample loading to saturate the nonspecific binding site of the column for FITC–BSA.

3.4. Noncompetitive open tube CEIA for anti-BSA

Noncompetitive assay for anti-BSA was also performed in open tube CEIA format. Standard solutions of anti-BSA with concentrations ranging from 0–320 n*M* were prepared in triplicate. FITC–BSA were added to a final concentration of 320 n*M*. After 20 min incubation, each sample was analyzed with open tube CE–LIF. Typical electropherograms are shown in Fig. 6. Results showed that the antigen–antibody complexes were well separated from the free FITC–BSA and the fluorescent impurities. Compared with the protein G affinity capillary chromatograms, there was an additional peak corresponding to complexes in the open tube meth-

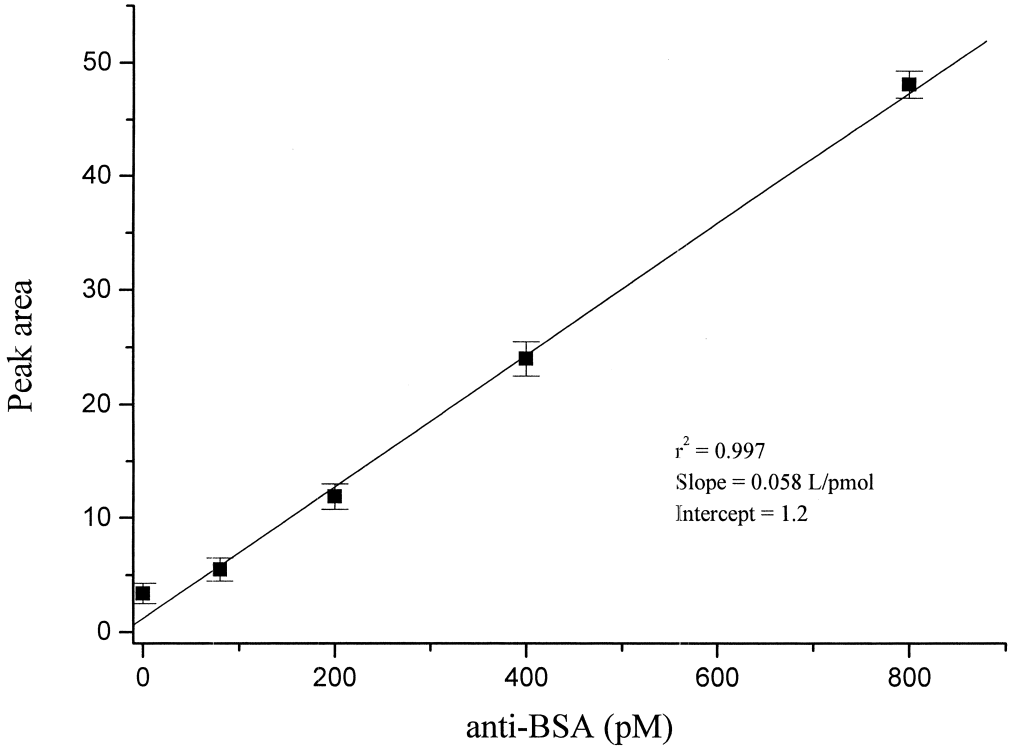

Fig. 5. Calibration curve for anti-BSA in p*M* level.

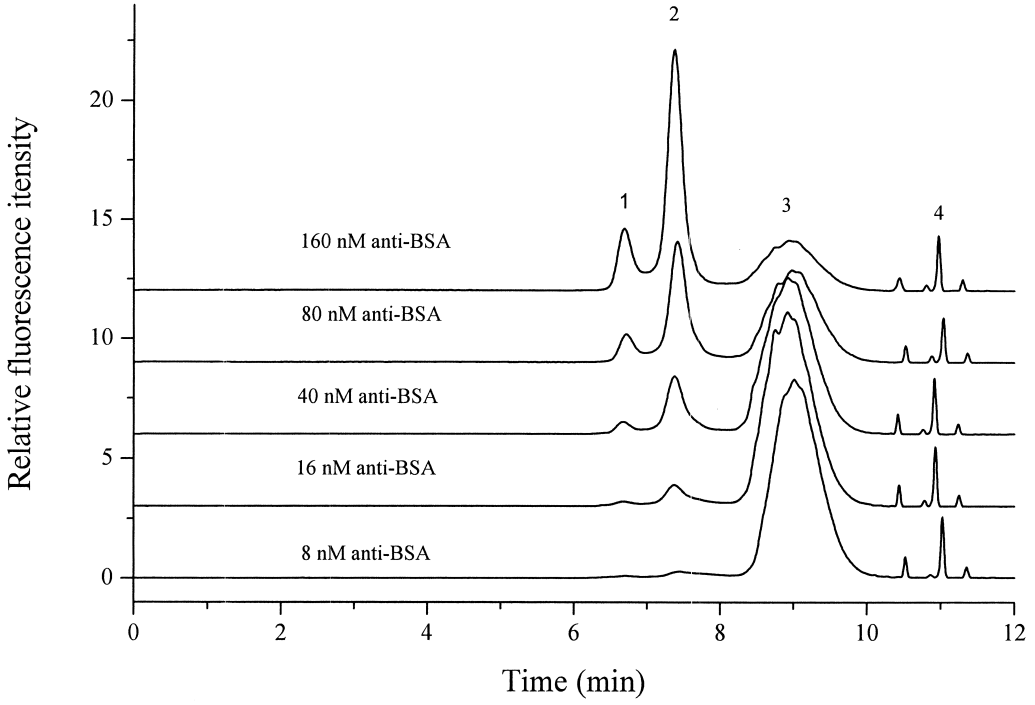

Fig. 6. Typical electropherograms of noncompetitive CEIA for anti-BSA. Conditions are described in Experimental. Peaks: (1) and (2) complexes, (3) free FITC–BSA, (4) fluorescent impurities.

od. This was probably due to the high resolving power of CZE. The antibody has two binding sites, and thus can form 1:1 and 1:2 two kinds of immunocomplexes. A calibration curve was obtained by plotting the concentration of anti-BSA versus the peak areas of the complexes (Fig. 7), which showed a linear relationship up to 160 n*M* of anti-BSA (r^2=0.999, slope=1.4 l/nmol, intercept=6.1). At higher concentration of anti-BSA, the curve gradually plateaued off, as would be expected if all the available binding sites on the FITC–BSA were saturated. The concentration LOD of this method was about 8 n*M*, and the corresponding mass LOD was about 0.3 fmol. When only FITC–BSA was injected onto the open tube, and analyzed with CE, the minimum detectable concentration was about 5 n*M* (S/N=3). So the sensitivity for open tube CEIA was dictated by the detector response, not the amount of FITC–BSA used. The higher concentration sensitivity of the protein G affinity capillary chromatography method is due to the pre-concentration of immunocomplex in the protein G column before elution.

4. Conclusions

Protein G affinity capillary chromatography and LIF detection was successfully combined to perform immunoassay with high concentration sensitivity. The system is compatible with automation, and only a few microliters of sample is required for the assay. Although each assay takes about 30 min to complete in this study, this time can be easily shortened by increasing the flow rate of sample loading and washing. A major advantage of this approach is that different immunoassays can be performed in the same system with the same operating conditions. The assay for anti-BSA was also performed in open tube CEIA format. Both methods are compatible with automation. The chromatographic method has a higher selectivity since it is a sandwich assay while

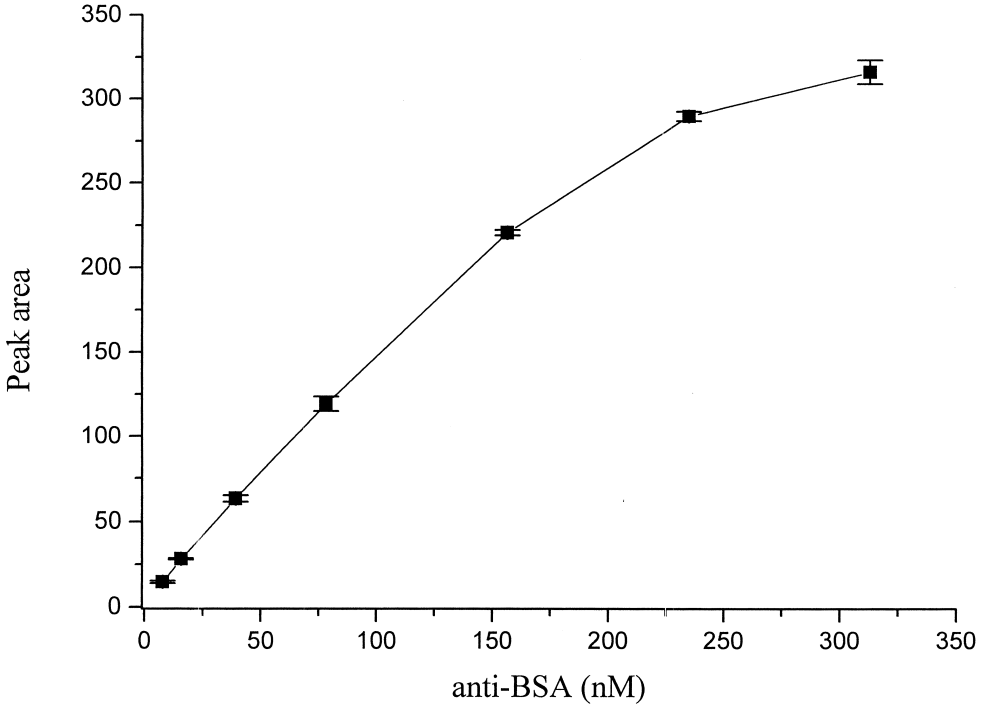

Fig. 7. Calibration curve of noncompetitive CEIA for anti-BSA.

the CEIA method has the advantage of determination of multiple analytes simultaneously. The chromatographic method improved concentration sensitivity about 100-fold due to the pre-concentration of immunocomplex in the protein G column, while the mass sensitivity was similar in two methods. The sensitivity for open tube CEIA was dictated by the detector response, while that of the chromatographic method was dictated by non-specific adsorption.

Acknowledgements

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