—Communication—

Electrochemical Flow Immunoassay Using Capillary Column and Ferrocene Conjugated Immunoglobulin G

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This paper describes a miniaturized flow immunoassay system. Ferrocene (Fc) conjugated with anti-HCG immunoglobulin G (IgG) antibody (Fc-IgG) was prepared and used as a novel analytical reagent. The system consists of the immunoreaction part, the capillary column packed with cation exchange resin, and the flow cell for electrochemical detection of Fc-IgG. The assay yielded a linear relationship between signal and HCG concentration in the range of 0-2000 mIU/mL (L = dm^3). This electrochemical flow immunoassay requires minute quantities of serum and generates highly reproducible results.

Key Words : Ferrocene Conjugated IgG, HCG, Electrochemical Flow Immunoassay, Capillary Column

1 Introduction

Genomics,[1] proteomics,[2] immunology,[3] neuroscience,[4] and drug investigation[5] are requiring smaller quantities of analytes to be separated from large sample numbers. Rapid development in microsystem technologies are producing miniaturized sensors. Integration of these new sensors require development of miniaturized systems capable of utilizing them.

Recently, we have constructed a new flow immunoassay system with packed capillary column for detection of anti-double-stranded DNA antibody in systemic lupus erythematosus serum.[6] Antigen-antibody complexes were separated from unreacted species by ion-exchange column on the basis of the difference in isoelectric points (pI).[7-9]

In this paper, we describe a miniaturized flow immunoassay system using electrochemical detector. This approach is simple and offers shorter assay times as compared with other immunoassay systems. Furthermore, it significantly reduces the quantity of reagents required. We report the diagnostic performance of the capillary electrochemical flow immunoassay system for detection of human chorionic gonadotropin (HCG).

2 Experimental

Ferrocenemonocarboxylic acid (4 mg) was dissolved in 800 μL (L = dm^3) of a 0.15 M (M = mol dm^{-3}) HEPES [N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid, soduim salt] buffer (pH 7.3). This solution was passed through the nitrocellulose membrane filter (pore size, 0.2 μm). EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, 10 mg) and immunoglobulin G (IgG ; anti HCG, 500 μg/mL, 90 μL) was added to the solution and then incubated for 4h at room temperature. Ferrocene conjugated IgG (Fc-IgG) prepared in this manner (Fig. 1) was homogeneous as determined by gel filtration using Superdex 200 column (Pharmacia, Uppsala, Sweden). Stock solutions of Fc-IgG prepared at 10 mg/ml in 150 mM HEPES pH 7.3 where stably stored for several weeks at 4℃. Analysis of the IgG solution by atomic absorption spectroscopy shows that prior to modification there are zero iron atoms per IgG molecule and

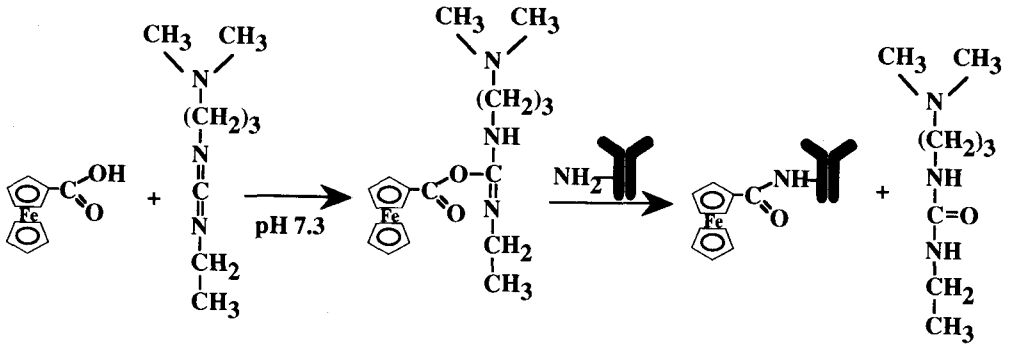

Fig. 1 Schematic diagram showing preparation of ferrocene conjugated IgG using EDC.

after modification there are 11 iron atoms per molecule.

A cation exchange capillary column (762 μm i.d. × 10 cm) was prepared using a SELF PACK kit (Perseptive Biosystems, Framingham, USA) as described previously.[6] Eluent flow was produced using a pump (P-500, Pharmacia, Uppsala, Sweden). Samples were applied to a injection valve (7725, Rheodyne, USA) fitted with a 10 μL loop. Fused silica tubing with an inside diameter of 100 μm was used to deliver reagents into the switching valves and acted as the waste line for the capillary electrochemical flow immunoassay. HCG (5 μL) was mixed with 5 μL of 5 μg/mL Fc-IgG. The mixture was loaded into an injection loop, incubated for 30 min, and passed into the cation exchange capillary column (sulfopropyl type, 762 μm i.d. × 10 cm), with malonate buffer (pH 6.0) as a binding buffer at a flow rate of 40 μL/min. The catalytic current of Fc-IgG in the eluent was measured using a three-electrodes flow cell (BAS Inc., Japan) equipped with a glassy carbon electrode, an Ag/AgCl reference electrode and a Pt counter electrode. Results were recorded on a chart recorder (SP-J 5 C, Riken Denshi, Tokyo, Japan).

3 Results and Discussion

The electrochemical properties of Fc-IgG in HEPES buffer was investigated by cyclic voltammetry. When cyclic voltammetry was performed in a Fc-IgG solution, a redox potential appeared around 395 mV and 300 mV vs. Ag/AgCl. The similar electrochemical behavior was observed in the case of ferrocenemonocarboxylic acid.[10] These results suggested that the electrochemical oxidation of ferrocenemonocarboxylate is catalyzed at a potential of 395 mV vs. Ag/AgCl and the applied potential of 395 mV is appropriate for detection of immunocomplex consisting of Fc-IgG and HCG.

We previously showed that antigen-antibody complex is successfully separated from unreacted antigen and antibody by the ion-exchange column on the basis of a difference in isoelectric points.[6-9] Analysis of isoelectric points of HCG (antigen), Fc conjugated IgG antibody and antigen-antibody complex was carried out by isoelectric gel electrophoresis using the Pharmacia Phastsystem™. Three species were found to have isoelectric points of 7.0 (Fc-IgG), 4.7 (HCG) and 5.6 (HCG-Fc-IgG complex). The antigen-antibody complex arising from immunoreaction between Fc-IgG and HCG was therefore separated from unreacted Fc-IgG under the desired conditions by cation exchange capillary column equilibrated at pH 6.0 with 50 mM malonate buffer. Using the capillary electrochemical flow immunoassay equipment described in the experimental section, various concentrations of HCG was injected into the system with malonate buffer eluent under optimal analysis conditions. The response obtained from a series of Fc-IgG and HCG complex injections is shown in Fig. 2. The signal from this complex increased with increasing HCG concentration. Furthermore, reversible, reproducible and sensitive responses were obtained, indicating the liquid-phase immunological sensing system appeared to be working. Injections of Fc-IgG alone gave no signal to indicate that the antibody was bound to the

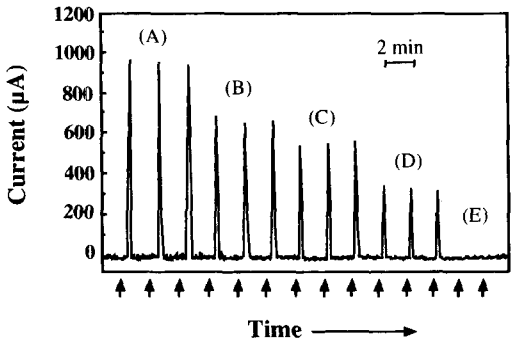

Fig. 2 Continuous electrochemical flow immunoassay analysis. (A) 2000, (B) 1200, (C) 1000, (D) 500 and (E) 0 mIU/mL HCG were reacted with 5 μg/mL Fc-IgG, and injected into the system. Arrows indicate sample injection.

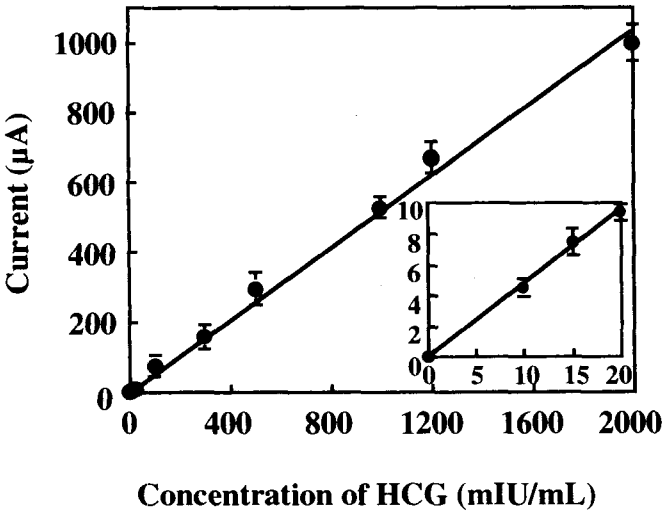

Fig. 3 Calibration for HCG concentration. Fc-IgG concentration; 5 μg/ml.

cation exchange resin.

A typical calibration response for HCG is shown in Fig. 3. A linear relationship was observed between the signal and HCG concentration in a range of 0-2000 mIU/mL. The correlation coefficient was 0.997 within this range. This method was faster (1.3 min), simpler to use, and gave better precision (<1.8%) compared with conventional ELISA using microtiter plates.[11-13] Reproducibility of the HCG measurement was examined using four different concentrations (20, 100, 500 and 1000 mIU/mL). Coefficients of variation were within 1.2-2.1%, and showed good correlation ($R = 0.996$, $n = 10$).

Miniaturization of a flow immunoassay system requires instruments that can deliver reproducible flow and sensitive detection. This paper describes HCG detection using a miniaturized microsystem. Analysis of serum from an ectopic pregnancy patient revealed less than 1000 mIU/mL of HCG.[14] These results indicate this capillary electrochemical flow immunosystem is suitable for diagnosis of pregnancy and/or gonadal tumors in clinical practice. This capillary electrochemical flow immunoassay requires minute quantities of serum and generates highly reproducible results. It is our hope to be able to develop

a totally miniaturized micro total analysis system which can detect other chemicals and drugs.

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