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# Glucose oxidase assisted homogeneous electrochemical receptor binding assay for drug screening

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**Abstract**

Although the idea of homogeneous electrochemical immunoassay using antibody and an electroactive modified antigen as a probe looks to be very useful for high-throughput drug screening, there have been few reports. One reason for this is the difficulty experienced making an electroactive probe, because the introduction of electroactive compounds to antigens often interferes with the antigen–antibody interaction. To apply a homogeneous electrochemical assay to drug screening, we have designed new probes referring to the information of immobilization on beads which could identify the drug receptor. FK506 (also called Tacrolimus), immunosuppressive agent is modified with ferrocene derivatives as an electron mediator between glucose oxidase and an electrode, at a non-obstructing part. One of the probes still indicated the electrochemical activity as a mediator and had the specific binding capability for FKBP12 (FK506 binding protein). The current decrease in response to the additional FKBP12, detected with constant voltage amperometry using the probe, was observed within 5 min. Then, free FK506 as a leader drug, rapamycin and cyclosporine A as unknown drugs were used as a model for drug screening. Since the order of response currents at the same concentration of each drug reflected their binding constants, it was shown that binding capacity of an unknown drug candidate could be estimated by comparison of response currents between the leader drug and the unknown drug candidate. Thus, this glucose oxidase assisted homogeneous electrochemical drug–receptor binding assay has been proved to be a useful tool for drug screening.


*Keywords:* Electrochemical detection; Homogeneous assay; Drug screening; Receptor binding assay; FK506; FKBP

## 1. Introduction

Pharmacological investigations of the mechanisms of both drugs and endocrine disruptor chemicals have revealed a wide variety of signal transduction pathways. Drug development based on the signal transduction pathways has become one of the major methods for discovering novel drugs. In particular, drug receptor identification is an important step in drug development, since receptors are considered to be promising target biomolecules of novel drugs. With the advance of combinatorial chemistry, it is now possible to make a large number of drug candidates for identifiable receptors as well (Gray, 2001; Geysen et al., 2003; Tochtrop and King, 2004; Koehn and Carter, 2005). Therefore the development of an easy method for drug screening, which estimates the binding capacity between small molecules such as these drug candidates and the identified receptor, is desired.

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To estimate the binding capabilities of these drug candidates with the receptor for drug screening, we focused on electrochemical methods since electrochemical detectors are capable of making measurements in turbid samples and the devices are relatively inexpensive and easy to operate. Also we considered that a homogeneous assay, a simplified method owing to the lack of the separation procedure, is preferred over a highly sensitive heterogeneous assay requiring troublesome separation steps for achieving an automatic high through-put screening system. Immunoassay methods have been developed and introduced for the quantitative analysis of molecules of particular biological interest and clinical endocrinology. Homogeneous electrochemical immunoassays which are performed using electroactive modified antigens have been developed as well (Heineman et al., 1979; Broyles and Rechnitz, 1986; Athey et al., 1993; Suzawa et al., 1994; Lin and Ju, 2005). Although, in particular, the idea of glucose oxidase assisted homogeneous electrochemical immunoassay using antibody and an electroactive modified antigen (Di Gleria et al., 1986) looks to be very useful, there have been few reports. There are also very few homogeneous electrochemical receptor binding assay studies (Sugawara et al., 1995, 2001; Kuramitz et al., 2002). One reason for this is the difficulty experienced making an electroactive probe for an antigen–antibody binding immunoassay, since the introduction of electroactive compounds to antigens often interferes with the antigen–antibody interaction. In case of receptor binding, it is considered more difficult to design a probe for a drug–receptor binding assay, since more strict regulations in attaching electroactive molecules are needed. Today, however, progress of beads technology is remarkable and it is possible that target receptors are identified by drugs immobilized on beads (Shimizu et al., 2000). Therefore, if the electrochemical probe could be designed with the appropriate connecting part referring to the immobilization method, a homogeneous electrochemical drug–receptor binding assay should prove to be a useful method. Moreover the flow, namely, first, target receptor identification with beads technology, then synthesize drug candidates with combinatorial chemistry, and electrochemical screening, these steps share the common information of drug modification, could be proposed for drug development.

In this report, we have proved feasibility of a homogeneous electrochemical drug–receptor binding assay with new designed probes referring to the information of immobilization on beads. FK506 (also called Tacrolimusan), immunosuppressive agent (Harding et al., 1989; Thomson and Woo, 1989), is modified with a well known electroactive molecule, a ferrocene derivative, as an electron mediator between glucose oxidase (GOD) and an electrode (Cass et al., 1984; Chaubey And Malhotra, 2002), at the 'non-obstructing' part where could have been linked with beads without lack of the binding capability for the target receptor FKBP12 (FK506 binding protein) (Shimizu et al., 2000). Then, we demonstrate that the glucose oxidase assisted homogeneous electrochemical receptor binding assay can be performed using the newly designed probe for estimating the binding capacities for FKBP12 (Siekierka et al., 1989; Maki et al., 1990; Standaert et al., 1990), as a model of drug screening.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Glucose oxidase (GOD; EC 1.1.3.4) was from Toyobo (Osaka, Japan). Ferrocene carboxylic acid was purchased from Aldrich (St. Louis, MO). FK506 was obtained from Calbiochem (La Jolla, CA). Rapamycin and Cyclosporine A were from Wako (Osaka, Japan). D-Glucose was from Nacalai Tesuque (Kyoto, Japan). Chemicals for bacterial culture medium were obtained from DIFCO Laboratories (Detroit, MI). All other chemicals were of analytical grade.

### 2.2. Synthesis of ferrocene-modified FK506 (Fec-FK506s)

Ferrocene-modified FK506s (Fec-FK506s) and feroccene carboxylic acid used in this report are shown in Fig. 1. Since the FKBP12 binding site of FK506 (Spencer et al., 1993; Pruschy et al., 1994) and a feasibility of the identification of FKBP12 with FK506 immobilized beads (Shimizu et al., 2000) have been already reported, Fec-FK506s (Fec-FK506A, Fec-FK506B, Fec-FK506C), the probe candidates for the homogeneous electrochemical receptor binding assay, were synthesized by connecting ferrocene ester and amides to *N*-hydroxysuccinimide ester of FK506 with referring to the previous reports. The synthetic routes to produce ferrocene amide (4), (9) and ferrocene ester (6) are outlined in Scheme 1. Treatment of ferrocenecarboxylic acid (1) with cyanuric fluoride provided the acid fluoride (2). (2) was coupled to mono-Fmoc ethylenediamine hydrochloride or Fmoc-glycinol in the presence of base to yield *N*-protected ferrocene amide (3) or ester (5). The *N*-Fmoc group was removed by treatment with 10% piperazine in dimethylformamide (DMF) to give compound (4) and (6). Aminoferrocene (7) was coupled to Fmoc β-alanine and subsequently Fmoc group was removed to give the compound (9). The synthesis of *N*-hydroxysuccinimide ester of FK506 (10) have been previously reported (Spencer et al., 1993; Pruschy et al., 1994). Compound (10) was treated with ferrocene derivatives (4), (6) and (9) to provide compounds (11)–(13) (Scheme 2). Removal of the hydroxy-protecting group (*tert*-butyl-dimethyl-silane (TBS)) with hydrofluoric acid in acetonitrile provided the three desired ferrocene modified FK506s (Fec-FK506A, Fec-FK506B, Fec-FK506C) respectively. The synthesis of Fec-FK506 compounds were characterized by the proton nuclear magnetic spectroscopy ($^1$H NMR) and the mass spectrometry (MS) (data not shown).