Sensors and Actuators B 139 (2009) 97–103

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Surface plasmon resonance sensor in the analysis of caffeine binding to CYP1A2 p450 monoxygenase in the presence and absence of NADPH

P.J.R. Roche [a], S.-M. Ng [a], K. Page [b], N. Goddard [a], R. Narayanaswamy [a,*]

[a] Centre for Instrumentation and Analytical Science, School of Chemical Engineering and Analytical Science, The University of Manchester, P.O. Box 88, Sackville Street, Manchester, M60 1QD, United Kingdom
[b] AstraZeneca, DMPK Group, Alderly Park, Macclesfield, Cheshire, SK10 4TF, United Kingdom

ARTICLE INFO

Article history:
Available online 9 October 2008

Keywords:
Caffeine
NADPH
Cytochrome p450 monoxygenases
SPR

ABSTRACT

An alternative to liquid chromatography–mass spectroscopic analysis of CYP catalysis of candidate drugs is proposed by a surface plasmon resonance method. By covalently linking microsomes containing human recombinant CYP1A2, NADPH reductase and cytochrome *b*5 to a dithiodipropionic acid self-assembly monolayer formed on a gold-coated glass slide, a surface plasmon resonance (SPR) waveguide sensor is investigated. Previously only the CYP monoxygenases have been immobilised for SPR experiments, which bring into question the accuracy of association and dissociation constants determined in the absence of the other essential components (NADPH, NADPH reductase, cytochrome *b*5) for CYP activity. Caffeine was used as a standard substrate of CYP1A2 and comparisons in the presence and the absence of NADPH were performed to investigate the influence of enabling catalysis upon kinetic constants. This has consequences for future CYP bioassay design as an alternative measure for CYP enzyme kinetics which is demonstrated. The effect of concentration on response time and angular change in the plasmon resonances was also observed, with potential applications for determining metabolism of compounds by this method.

1. Introduction

Binding and subsequent hydrolysis of xenobiotics by cytochrome p450 monoxygenases (CYPs) in the liver represents phase 1 of drug metabolism within the body. Failure of the CYPs to bind a compound or excessively oxidation a drug, results in problems with efficacy and other metabolism related problems in-vivo. With this in mind the pharmaceutical industry has to test each new candidate compound for its potential turnover by the CYPs. The established method is to quantify the production of oxidised compound by LC–MS analysis by sampling at regular intervals during a 30 min incubation of a drug compound with CYP microsomes and NADPH. Determination of which CYP isoform is responsible for the majority of oxidisation (primarily hydroxylation) of compound is achieved by incubation with single isoform microsomes.

At present the majority of drug determination studies involve the use of LC–MS methods to determine the hydroxylated products. Interest has grown in recent years to investigate new methods to determine rate constants, clearance rates and standard kinetic constants such as K_m (Michaelis–Menten constant) and V_{max} (maximum velocity). Novel approaches have include the use of monoclonal antibodies to determine clearance rates and relative activity factors [1], SPR sensors to determine k_{ass} and k_{diss} [2] and a collection of luminescence (fluorescence (BioRad Ltd.)), and chemiluminescence (Progema Ltd.) assays for investigation of drug–drug interactions. All these presently available methods have drawbacks. LC–MS analysis is time consuming, expensive in material and instrumentation costs, and is not readily convertible to high through put methods. Luminescent assays provide lower costs and comparable detection limits to LC–MS analysis but require the metabolism of a compound modified to produce a measurable signal, and are only applicable to analysis of drug–drug interaction. The monoclonal antibody study represents an alternative inhibition methodology but still requires HPLC/LC–MS instrumentation to determine rates and constants. The SPR methodology [2] presents a most potent alternative to LC–MS, but at present few examples have been tested. In this method, CYP enzymes immobilised by covalent attachment to a self-assembly monolayer on a gold chip. Surface plasmons interact with enzymes during binding and removal of substrate, resulting in changes in the angular reflectance measured by the linear CCD device. The difference between association and dissociation of substrate and the time required to reach equilibrium allow the calculations of k_{ass} and k_{diss}. Pearson et al. [2] demonstrated how these constants can be determined and that changes

* Corresponding author. Tel.: +44 161 306 4891; fax: +44 161 306 4399.
E-mail address: ramaier.narayanaswamy@manchester.ac.uk (R. Narayanaswamy).

0925-4005/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.snb.2008.09.043

in the haem structure of CYP occur, but the validity of constants has to be challenged. The basis of this rests with the singular immobilisation of CYP without the NADPH reductase, Cyt b5 and NADPH without which full catalytic function is not possible. Ivanov and Archakov [3] demonstrate that CYPs are not active without the other protein components and co-factor, without them effective complexation with compounds is not demonstrated. This brings the nature of the SPR results of Pearson and Zhao into question. A possible answer lies with the CYPs allosteric nature. The binding of NADPH reductase, cytochrome *b*5 and NADPH cause conformational changes in the enzyme fundamentally changing the binding kinetics [4]. While constants can be determined for binding and changes in the haem structure demonstrated by UV spectroscopy [2] without these essential catalytic components that are integral to the enzyme function and allosteric binding properties, constants will bear little relation to those determined in the enzymes native state.

With regard to the previous work [2] it was important to appreciate that in the sensor design stage CYP enzymes can only be included in the SPR methodology if they are in association with other biochemical factors in a microsome as this more accurately reflects the situation in vivo. In this manner the determination of k_{ass} and k_{diss} could be more realistic, as the assay maintains close similarity to the standard incubation used in the pharmaceutical industry and to the in-vivo environment.

2. Experimental

2.1. Solutions for assay

Phosphate buffer (PBS) was prepared from K_2HPO_4 and adjusted with NaOH and HCl to pH 7.4. NADPH was prepared to 20 mM stock solution in PBS and caffeine solutions were prepared in PBS from a stock solution of 0.2 M. Solutions for assay were premixed prior to types 1 and 2 assay (see later) and preheated to 37 °C. All chemicals were obtained from Sigma Aldrich (UK) and microsomes where kindly supplied by AstraZeneca Ltd.

2.2. Preparation of self-assembly monolayer (SAM) with covalently linked microsomes

The SAM method used in this project involves the self-assembly of a monolayer of 3,3′-dithiodipropionic acid molecules. Sulphur or thio groups have high binding affinities for gold allowing the carboxylic acid groups of the molecules free for modification. Direct covalent attachment to biomolecules is achieved after creation of the carbodiimide group by treatment with 0.5 M hydroxysuccinimide/0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

The SAM protocol was developed from the literature examples describing SAM immobilisation of biomolecules [5–7]. Care was taken to develop a method that ensures a high degree of binding of biomolecules to the SAM. In this case the sulphur group is provided by 3,3′-dithiodipropionic acid. The monolayer structure and shape of molecule attached is shown in Fig. 1.

The acid splits at the disulphide link and the sulphur binds to gold atoms on the surface of the gold slide in a monolayer structure. The carboxylic acid groups can undergo further reactions. The COOH group forms a carbodiimide coupling with EDC. This creates a urea derivative that is replaced by linkages through lysine residues when protein is administered. Urea derivatives that have not undergone this reaction after addition of protein are deactivated by addition of ethanolamine. The main advantages of SAM are that they can be regenerated without changing the SPR chip. By flushing the flow cell with NHS/EDC mixture

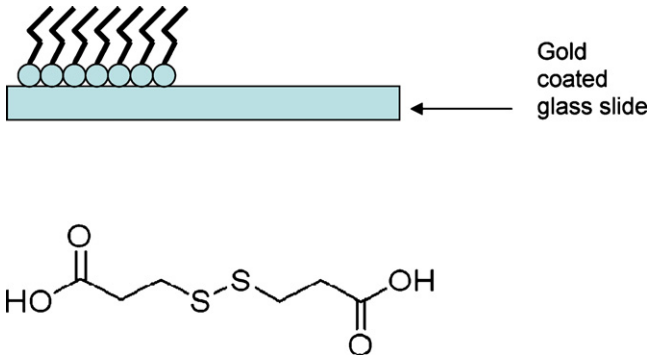

Fig. 1. Diagrammatic single monolayer of dithiodipropionic acid molecules. Individual acids represented as ball and stick structure. The chemical structure of 3,3′-dithio-dipropionic acid is included below the diagram for clarification.

the microsomes can be detached with reformation of the urea derivative. Fresh microsomes can be introduced with new covalent bonds being formed between the monolayer and the bilipid membrane.

Gold coated glass slides were immersed and incubated in a sealed Petri dish overnight at 25 °C in 1 mM 3,3′-dithiodipropionic acid in ethanol. Slides were then rinsed with ethanol and dried in 99.9% pure nitrogen. Slides were immersed in a solution of 0.5 M hydroxysuccinimide (NHS) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 20 min to activate the carboxylic acid group and then placed in Petri dish and covered with microsome containing CYP enzymes (protein concentration 50 ng μl). Slides were left for a period of 24 h to ensure efficient binding. CYP-SAM slides were rinsed with phosphate buffer (PBS) at pH 7 and immersed for 15 min in ethanolamine (pH 8.5) to deactivate remaining carbodiimide groups on SAM. Prior to use in assays, CYP-SPR slides were stored at −18 °C, and removed and preheated to 37 °C prior to experimental application.

2.3. Protocols for assays

Two types of assay are performed using the CYP-SPR chips. Descriptions for both assays are included below.

2.3.1. Type 1—spot assays

Type 1 involves the pipetting of PBS, β-NADPH (20 mM stock solution), drug compound (0.2 M stock) onto the chip and observing the binding response. The purpose of this assay is to determine the initial validity of the method, in terms of concentrations of reagent used and instrumentation configuration. As the enzymes are capable of catalysis determination of k_{ass} and k_{diss} is not appropriate as they refer to Langmuir binding kinetics. Enabling catalysis by inclusion of the NADPH, NADPH reductase and cytochrome *b*5 the constants determined will be referred to as the pseudo rate constant (PRC) for the reaction. The angular change induced by the enzymes catalysis is a product of positive and negative processes. Positive processes move the SPR angles in one direction and negative in the other. The positive processes such as substrate binding change the enzyme's haem structure to a trans conformation in addition to whole enzyme structural shifts commensurate with a bound state, following the induced fit hypothesis of substrate binding. Whereas negative processes such as substrate removal and product formation return the enzymes to the cis formation and the enzyme's gross structure to an unbound. The sum of the positive and negative process results in the PRC.