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A novel reagentless glutamate microband biosensor for real-time cell toxicity monitoring

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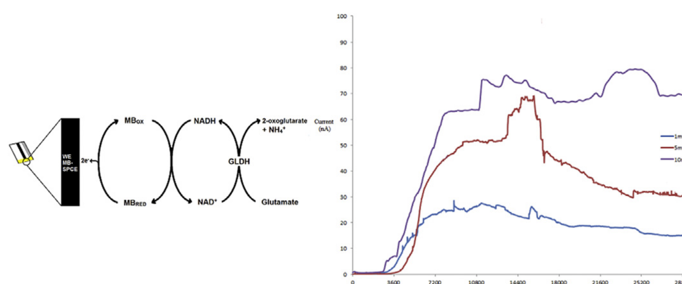
HIGHLIGHTS

- A simple method of fabricating a new microband glutamate biosensor was developed.
- The biocomponents, GLDH and NAD^+ were immobilised onto the electrode surface using chitosan.
- The biosensors operated continuously over an 8 h period in culture medium.
- The release of glutamate from HepG2 cells was monitored following toxic challenge.

GRAPHICAL ABSTRACT



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ABSTRACT

A reagentless glutamate biosensor was applied to the determination of glutamate released from liver hepatocellular carcinoma cells (HepG2) in response to toxic challenge from various concentrations of paracetamol. A screen printed carbon electrode (SPCE) containing the electrocatalyst Meldola's Blue (MB-SPCE) served as the electron mediator for the oxidation of NADH.

A mixture of the enzyme glutamate dehydrogenase (GLDH), cofactor nicotinamide adenine dinucleotide (NAD^+) and the biopolymer chitosan (CHIT) were drop-coated onto the surface of the transducer (MB-SPCE) in a simple one step fabrication process.

The reagentless biosensor was used with amperometry in stirred solution at an applied potential of +0.1 V (vs. Ag/AgCl). All experiments were carried out at the following conditions: pH 7, temperature 37 °C, atmosphere 5% CO_2.

The linear range of the device was found to be 25–125 μM in phosphate buffer (75 mM, containing 0.05 M NaCl) and 25–150 μM in cell culture medium. The limits of detection (LOD) were found to be 1.2 μM and 4.2 μM based on three times signal to noise, using PBS and culture medium respectively. The sensitivity was calculated to be 106 nA μM^{-1} cm^{-2} and 210 nA μM^{-1} cm^{-2} in PBS and cell medium respectively. The response time was ~60 s in an agitated solution.

HepG2 cells were exposed to various concentrations of paracetamol (1 mM, 5 mM and 10 mM) in order to investigate the drug-induced release of glutamate into the culture medium in real time. Two toxicity studies were investigated using different methods of exposure and analysis.

The first method consisted of a single measurement of the glutamate concentration, using the method of standard addition, after 24 h incubation. The concentrations of glutamate were found to be 52 μM, 93 μM and 177 μM, released on exposure to 1 mM, 5 mM and 10 mM paracetamol respectively.

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The second method involved the continuous monitoring of glutamate released from HepG2 cells upon exposure to paracetamol over 8 h. The concentrations of glutamate released in the presence of 1 mM, 5 mM and 10 mM paracetamol, increased in proportion to the drug concentration, ie: 16 μM, 28 μM and 62 μM respectively. This result demonstrates the feasibility of using this approach to monitor early metabolic changes after exposure to a model toxic compound.

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1. Introduction

Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system [1] and also has a central role in cell metabolism and function [2]. Glutamate is associated with processes such as protein synthesis/degradation, ammonia disposal, energy production and neurotransmission [3], thus glutamate plays a significant role in fundamental metabolic processes. As a result, glutamate is present in many cells such as skeletal muscle, liver, intestine and kidneys. Specifically, glutamate plays vital roles in hepatic metabolic pathways such as ureagenesis, gluconeogenesis and glutathione synthesis [4].

The concentration of intracellular glutamate is typically higher than that of extracellular glutamate. For example, within the brain, extracellular glutamate concentrations are normally within the 1–10 μM range [5,6] whilst intracellular glutamate concentrations are typically in the micromolar range [7]. Similar micromolar levels have been observed intracellularly in rat livers [8,9].

Drug discovery is a costly and extensive process, with product development comprising up to 30–35% of the cost of bringing a new pharmaceutical compound to the market [10]. Early candidates for drug development are selected based on their pharmacological and toxicological properties which are investigated through early-stage *in vitro* cell-based assays [11]. Cytotoxicity assays such as neutral red, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), LDH (lactate dehydrogenase) leakage, and protein assays are commonly used to evaluate the effects of drugs on cell metabolism and death [12]. However, these are employed by sampling off-line, or as end-point assays, and do not provide information on early toxicity events in real-time. In an approach using electrochemical biosensors to address the issue of continuous analyte monitoring *in vitro*, Pemberton et al. [13,14]. have described the development and application of a screen-printed microband biosensor to follow glucose uptake by HepG2 cells in the presence of different concentrations of paracetamol.

Some reports have described the use of chitosan for the immobilization of glutamate dehydrogenase on glassy carbon [15–18], gold [19] and platinum [20] electrodes. Both reagentless and non-reagentless conventional sized glutamate biosensors have previously been developed by drop-coating the required biomolecules onto the working electrode of a screen-printed carbon electrode [21–23]. We have recently reviewed electrochemical glutamate biosensor construction and their applications in food and clinical analysis [24]. Modified Chitosan has been utilised for the covalent immobilization of glutamate oxidase [25] and lactate oxidase [26], however, as far as we are aware, there are no examples of the use of chitosan to fabricate screen-printed microband glutamate biosensors. In this paper we explore this latter possibility and demonstrate the application of the proposed device for real-time monitoring of toxicity, through the metabolic changes (glutamate release) caused by the addition of paracetamol.

2. Materials and methods

2.1. Chemicals and reagents

Foetal bovine serum (FBS), Minimum Essential Medium Eagle (EMEM) and all other chemicals were purchased from Sigma-Aldrich. All chemicals were of analytical grade, purchased from Sigma Aldrich, UK, except glutamate dehydrogenase (CAT: 10197734001) which was purchased from Roche, UK. The 75 mM phosphate buffer (PB) was prepared by combining appropriate volumes of tri–sodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dihydrate and disodium hydrogen orthophosphate anhydrous solutions to yield the desired pH. Glutamate and NADH/NAD^+ solutions were dissolved directly in 75 mM PB. Chitosan was dissolved in 0.05 M HCl (pH < 3.0) to produce a 0.05% solution following up to 10 min sonication. A 25 mM stock solution of glutamate (monosodium glutamate) was utilised for glutamate calibrations. A 500 mM stock of paracetamol in 100% ethanol was diluted with cell culture media and filtered sterilised (0.2 μm) before use.

2.2. Apparatus

All electrochemical experiments were conducted with a two electrode system consisting of a carbon working electrode containing MB (MB–SPCE, Gwent Electronic Materials Ltd; Ink Code: C2030519P5), and a Ag/AgCl reference electrode (The difference between the screen-printed Ag/AgCl electrode and the conventional Ag/AgCl (3 M NaCl) was +80 mV) (GEM Product Code C61003P7); both printed onto PVC (poly vinyl chloride). The electrodes were connected to the potentiostat using gold clips. Microband electrodes/biosensors and reference electrodes were held by gold clips attached to cork lids which were push-fitted into 6-well plate wells (Corning®). Electrodes were connected to a PG580RM 5-channel potential (Uniscan Instruments Ltd, Buxton, UK; www.uniscan.com) which was controlled using UiEChem software (Version 2.02), for both single and multiple parallel well experiments. All cell culture work and dosing with paracetamol was conducted under a sterile conditions. The cork lid electrode holders was sterilised with 70% ethanol spray. The electrodes themselves were not sterilised as the ethanol would potentially damage the enzyme and cofactor. Experiments utilising culture medium and HepG2 cells were carried out in an incubator with a 5% CO_2 atmosphere at 37 °C in order to maintain the correct pH. Sonications were performed with a Devon FS100 sonicator (Ultrasonics, Hove, Sussex, UK).

2.3. Principle of operation of the biosensor

The overall principle of operation of the biosensor is shown in Fig. 1. Glutamate in solution is oxidised to form 2-oxoglutarate in the presence of the immobilised enzyme glutamate dehydrogenase (GLDH) and NAD^+; the products NADH and NH_4^+ are formed during this reaction. NADH chemically reduces Meldola's Blue which

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