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A novel functionalisation process for glucose oxidase immobilisation in poly(methyl methacrylate) microchannels in a flow system for amperometric determinations

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ABSTRACT

Different materials like glass, silicon and poly(methyl methacrylate) (PMMA) are being used to immobilise enzymes in microchannels. PMMA shows advantages such as its low price, biocompatibility and attractive mechanical and chemical properties. Despite this, the introduction of reactive functional groups on PMMA is still problematic, either because of the complex chemistry or extended reaction time involved. In this paper, a new methodology was developed to immobilise glucose oxidase (GOx) in PMMA microchannels, with the benefit of a rapid immobilisation process and a very simple route. The new procedure involves only two steps, based on the reaction of 5.0% (w/w) polyethyleneimine (PEI) with PMMA in a dimethyl sulphoxide medium, followed by the immobilisation of glucose oxidase using a solution containing 100 U enzymes and 1.0% (v/v) glutaraldehyde. The reactors prepared in this way were evaluated by a flowing system with amperometric detection (+0.60 V) based on the oxidation of the H_2O_2 produced by the reactor. The microreactor proposed here was able to work with high bioconversion and a frequency of 60 samples h^{-1}, with detection and quantification limits of 0.50 and 1.66 μmol L^{-1}, respectively. Michaelis–Menten parameters (V_{max} and K_M) were calculated as 449 ± 47.7 nmol min^{-1} and 7.79 ± 0.98 mmol. Statistical evaluations were done to validate the proposed methodology. The content of glucose in natural and commercial coconut water samples was evaluated using the developed method. Comparison with spectrophotometric measurements showed that both methodologies have a very good correlation ($t_{calculated,\ 0.05,\ 4} = 1.35 < t_{tabled,\ 0.05,\ 4} = 2.78$).

1. Introduction

Microfluidic devices have received considerable attention from researchers in recent years. Since the early 1990s, when Manz et al. [1] developed the first microfluidic device, these systems have become more popular as a result of their advantages over larger scale systems. Among these, their small dimensions, large surface-to-volume ratio, well-defined reaction times and small sample quantities requirements make these systems very promising with regard to their use for analytical procedures [2–4].

In this context, the use of microchannels for enzymatic applications has also gained support. Different materials like glass [5], polystyrene [6], silicon [7,8] and poly(methyl methacrylate) (PMMA) [9], have already been used to immobilise enzymes. PMMA shows some advantages compared to other materials, such as its low price, biocompatibility, excellent optical transparency and attractive mechanical and chemical properties [10].

The most common way to immobilise enzymes onto a microreactor is by using a support that can covalently attach to the enzyme or by a cross-linking agent, which can act as a spacer-arm molecule [11]. Immobilisation of enzymes provides many advantages such as their reuse, lower consumption of reagents and samples, higher analysis rate, extended lifetime and greater stability. However, for this purpose, the substrate of the reactor must have some reactive functional groups that can interact with the enzymes. A few approaches have already been made to introduce amino groups onto PMMA sheets [12–14].

Amino groups are well-known functional groups for enzyme immobilisation, but these modifications require long reaction times and/or the consumption of a large amount of reagents. The method most commonly used to add amine functionality to the surface of PMMA is a reaction with a lithiated diamine [14], but this reaction is air sensitive and the lithiated amines have limited lifetimes. In this context, Brown et al. [15] found a faster way to

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react PMMA with NH_2 groups. In their study, an ethylenediamine solution in dimethyl sulphoxide (DMSO) solvent was used and yielded 2-fold more amino groups on PMMA surface, when compared to other methodologies. The use of different amino reactants can also enhance the number of amino groups on PMMA surface. Bai et al. [16] observed that polyethyleneimine (PEI) gave the highest number of amino groups on PMMA due to its higher density of amino groups on the polymeric surface area after modification, resulting in a greater number of binding sites. Another advantage that can be associated with the use of PEI in relation to other amino groups containing reactants is the creation of a suitable microenvironment for enzyme immobilisation, improving its stability [17].

In this work, a rapid and very efficient process for GOx immobilisation on PMMA microreactors is described. In comparison with a previous study presented by our group [9], the new process requires less time and enhances the enzymatic immobilisation rate. This is based on PMMA functionalisation with PEI (using DMSO as solvent), followed by GOx immobilisation on the microchannel walls, using glutaraldehyde as the cross-linking agent. The performance of the enzymatic reactor prepared in this way was evaluated for glucose by differential amperometric determinations.

2. Material and methods

2.1. Reagents and solutions

All reagents were of analytical grade. Sodium dihydrogen phosphate, sodium hydrogen phosphate and isopropyl alcohol were purchased from Synth (Diadema, Brazil). Glutaraldehyde 25% and D-(+)-glucose were purchased from Merck (Darmstadt, Germany). Polyethyleneimine (MW 750,000, 50% in water), dimethyl sulphoxide and 4-aminoantipyrine were acquired from Sigma-Aldrich (St. Louis, MO, USA). Glucose oxidase from *Aspergillus niger* (124 U mg^{-1}) was purchased from Toyobo (Osaka, Japan) and horseradish peroxidase (115 U mg^{-1}) from Seppim (Puteaux, France).

Solutions were prepared with deionised water obtained from a Milli-Q water purification system (Dubuque, IA, USA). The resistivity measured after this treatment was not less than 18.2 MΩ cm. Coconut water samples were purchased from a local store. Four bottled coconut water samples, some of them extracted directly from the fruit, were evaluated.

2.2. Microchannel manufacture

Laser ablation has been widely employed to fabricate PMMA microdevices [18]. In this process, the beam of a high-energy laser is used to break bonds in polymer molecules and remove the decomposed fragments from the ablated regions [10].

In order to engrave PMMA plates with microchannels, a CO_2 laser-engraving machine (L-Solution 100, from Gravograph Industry International La Chapelle-St Luc, France) was used. The microchannel design was made using Corel Draw 12 software (Corel Corporation-version 12.0.0.458-2003). Engravings with 500 μm width, 200 μm depth and different lengths were crafted into an 8.5 cm × 5.5 cm × 2.0 mm PMMA plate. Reactors with 61.5 cm length were utilised for the majority of the measurements.

After digging the microchannel, the PMMA plate was washed with deionised water, dried, and then thermally sealed onto a 2.0 mm thick PMMA plate with the same dimensions. The sealing process was carried out using a heat press (model HT3020) operated at 110 °C under 590 kPa for 30 min.

2.3. Flow system and electrochemical configuration

The main constituents of the proposed method are depicted in Fig. 1. The flow arrangement was composed of two distinct propelling systems: an aquarium pump (A) was utilised to pneumatically propel the carrier electrolyte [19], and a programmable peristaltic pump (D) (Reglo MS Digital, Ismatec), used to introduce reproducible sample volumes into the microchannel (F). Injection of samples by the peristaltic pump was performed using 0.3 mm internal diameter Tygon® tubing. A throttle (B), located between the electrolyte reservoir and the pneumatic pump, allowed control of the flow rate into the microchannels.

Electrochemical measurements were performed using a μ-Autolab type III potentiostat (EcoChemie, Netherlands) in the chronoamperometric mode. Hydrogen peroxide (H_2O_2) generated by the microreactor was detected using a three-electrode system. A commercial dual platinum electrode (G_1) was employed as working and counter-electrodes and a miniaturised $Ag/AgCl_{(sat)}$ (G_4) was used as the reference electrode. A thin polypropylene layer (200 μm thickness—G_2) was used as a spacer to limit the electrochemical cells bound. A PMMA cover (G_3) was used to seal the cell. This seal was fixed using four screws located at the edges of the structure. In addition, three holes were drilled on the top of the PMMA cover. Two of them were used as the input and output of the flowing solution. The connections were made with 0.3 mm i.d. Tygon® tubing. The third hole was made exactly in front of the working electrode as a way to adapt the miniaturised $Ag/AgCl_{(sat)}$ reference electrode [20].

The arrangement depicted in Fig. 1 was explored to determine the kinetics parameters of the immobilised enzymes and to quantify glucose in coconut water samples. All samples were diluted in 0.10 mol L^{-1} phosphate buffer solution (pH 7.0) just before each analysis. Sample dilutions ranged from 1:100 to 1:2000.

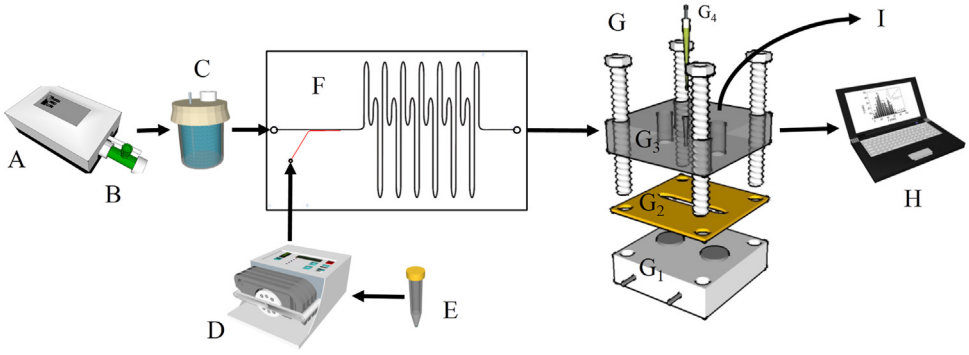

Fig. 1. Configuration of the applied micro flow system: (A) one-way pneumatic propellant aquarium pump, (B) throttle, (C) electrolyte reservoir, (D) programmable peristaltic pump, (E) sample, (F) microreactor, (G) electrochemical cell, (G_1) commercial dual platinum electrode, (G_2) polypropylene layer (200 μm thickness), (G_3) PMMA cover with holes, (G_4) miniaturised $Ag/AgCl_{(sat)}$ electrode, (H) microcomputer and (I) waste.