Microchemical Journal 118 (2015) 231–237

Contents lists available at ScienceDirect

Microchemical Journal

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

# Use of poly(methyl methacrylate)/polyethyleneimine flow microreactors for enzyme immobilization

Marcos Rodrigues Facchini Cerqueira [a,b], Mauro Sérgio Ferreira Santos [b], Renato Camargo Matos [a], Ivano Gebhardt Rolf Gutz [b], Lucio Angnes [b,*]

[a] Departamento de Química, Universidade Federal de Juiz de Fora (UFJF), Juiz de Fora, MG 36036-900, Brazil
[b] Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, 748, São Paulo, SP 05508-000, Brazil

ARTICLE INFO

*Article history:*
Received 7 August 2014
Received in revised form 10 September 2014
Accepted 21 September 2014
Available online 22 October 2014

*Keywords:*
Poly(methyl methacrylate)
Polyethyleneimine
Enzyme immobilization
Microbioreactor
Flow injection analysis

ABSTRACT

Recently our group has developed a simple and practical procedure to immobilize glucose oxidase (GOD) on poly(methyl methacrylate) (PMMA) microchannels. In the present study not only was the GOD microreactor preparation optimized but also, more important, it is shown that the functionalization of PMMA microchannels with polyethyleneimine (PEI) is a methodology of wider applicability to anchor enzymes with glutaraldehyde, with a potential for the development of microanalytical devices and systems. As a demonstration, the preparation and evaluation of microreactors with immobilized ascorbate oxidase (AAO), catalase (CAT), glutamate dehydrogenase (GDH) and a dual enzymatic system (GOD and horseradish peroxidase) is reported. Direct (GOD) and indirect (AAO and CAT) chronoamperometric measurements were made in order to evaluate the reactors' activity. GDH immobilization was confirmed photometrically through a thiazolyl blue tetrazolium bromide based reaction while a Trinder's reaction based method was used to evaluate the GOD/HRP microreactor. A small volume flow cell built in the lab was connected to two optical fibers to implement spectrophotometric measurements. After 15 days of use (~1000 injections), no change was observed in the efficiency of the CAT reactor (100% destruction of the analyte) while the GOD/HRP reactor that decayed to 25% of the original activity, was still useful despite the decrease in sensitivity.



## 1. Introduction

Supporting materials such as glass [1], polystyrene [2], polydimethylsiloxane (PDMS) [3,4] and poly(methyl methacrylate) (PMMA) [5], are increasingly explored for enzyme immobilization due to advantages such as the reuse of the enzymatic system, lower reactant consumption, extended enzyme lifetime and greater stability.

The possibilities to develop microfluidic devices made of polymers make them attractive for analytical applications. Those systems present some advantages in comparison with the direct use of biosensors such as their large surface-to-volume ratio, well-defined and greater reaction times and small sample quantities' requirements [6–8]. A large number of different applications involving the use of microchannel-immobilized enzymes are already presented. Those approaches are usually achieved by a) particle entrapment, such as the usage of 3-aminopropyltriethoxysilane–glutaraldehyde as a cross-linking agent on glass substrates [1] or the direct appliance of porous silica monoliths [9]; b) surface modification, by physically adsorbing a lipid biotin/avidin bilayer on $O_2$ plasma treated PDMS [10] or by disulfide bonds formed at silica surfaces [11]; and c) by membrane application, with the usage of a poly(vinyldenfluoride) membrane on PDMS or glass sheets onto which the enzymes are absorbed [8].

PMMA shows some advantages in comparison with other materials, such as its low price, biocompatibility, excellent optical transparency and attractive mechanical and chemical properties [12].

For enzyme immobilization in microfluidic systems the presence of reactive functional groups, which can interact directly with the enzyme or with a cross-linking agent (actuating as a spacer arm molecule) is required [13]. Different methodologies for PMMA functionalization, such as the direct addition of functional groups during polymerization (like acrolein monomer [14] or a water-soluble phospholipid polymer with an active ester group in the side chain [15]), were already reported. Another approach involving the attachment of active groups directly at PMMA surface can be achieved using N-lithiodiaminoethane, by exposure to UV radiation proceeded by N-(3-dimethylaminopropyl)-N-ethylcarbodiimide coupling of ethylenediamine addition [16], by incubation with 10% aminated solutions in alkaline medium (such as borate buffer pH 11.5, or 0.10 mol $L^{-1}$ NaOH solution) for 2 h [17] or by other even more laborious methodologies [12,18,19].

Amino groups are well-known functional groups for enzyme immobilization. In this context, Brown et al. [20] have reported a faster way of reacting PMMA with $NH_2$ groups. In his study,

* Corresponding author. Tel.: +55 11 3091 3828; fax: +55 11 3091 3781.
*E-mail address:* luangnes@iq.usp.br (L. Angnes).

http://dx.doi.org/10.1016/j.microc.2014.09.009
0026-265X/

an ethylenediamine solution in dimethyl sulphoxide (DMSO) solvent was used and yielded 2-fold more amino groups on PMMA surface, in comparison with other methodologies.

In a previous study, we proposed a simplified route for GOD immobilization with glutaraldehyde after a one step functionalization of PMMA microchannels with polyethyleneimine (PEI — a polymer which contains primary, secondary and tertiary amino groups), using DMSO as solvent [21]. In the present work we will not only present the optimization of this GOD microreactor, but also and more important, demonstrate that the PEI functionalized PMMA microchannels are well suited for efficiently anchoring other enzymes with glutaraldehyde as a crosslinker. The enzymes chosen to illustrate the wider applicability of the PMMA–PEI microreactor were ascorbate oxidase (AAO), catalase (CAT), glutamate dehydrogenase (GDH) and a dual enzymatic system (GOD and horseradish peroxidase) with the performances evaluated by amperometric and spectrophotometric detection. A brief literature revision of each immobilized enzyme and the potentialities of coupling our proposed microreactors with detectors and sensors are also presented.

## 2. Material and methods

### 2.1. Reagents and solutions

All reagents used along this study were of analytical grade. Sodium dihydrogen phosphate, sodium hydrogen phosphate, hydrogen peroxide and isopropyl alcohol were purchased from Synth (Diadema, Brazil). Glutaraldehyde 25%, D-(+)-glucose and L-(+)-ascorbic acid were from Merck (Darmstadt, Germany). Polyethyleneimine (MW 750000, 50% in water), dimethyl sulphoxide, 4-aminoantipyrine, (L)-glutamate dehydrogenase from bovine liver (14.7 U $mg^{-1}$), diaphorase from *Clostridium kluyveri* (4.5 U $mg^{-1}$), ascorbate oxidase from *cucurbit asp* (162 U $mg^{-1}$), catalase from bovine liver (2860 U $mg^{-1}$), (L)-glutamic acid monosodium salt monohydrate ≥98%, thiazolyl blue tetrazolium bromide and β-nicotinamide adenine dinucleotide hydrate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Glucose oxidase from *Aspergillusniger* (124 U $mg^{-1}$) was purchased from Toyobo (Osaka, Japan) and peroxidase from horseradish (115 U $mg^{-1}$) from Seppim (Puteaux, France).

All solutions were prepared with Milli-Q water (Dubuque, IA, USA). The resistivity measured after the purification of water was not less than 18.2 MΩ·cm. All standards and enzymatic solutions were prepared in 0.10 mol $L^{-1}$ phosphate buffer, pH 7.0.

### 2.2. Microchannel manufacture

Laser ablation has been widely used to fabricate PMMA microdevices [22]. 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 mainly by volatilization [23].

To engrave microchannels in the PMMA plates, 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 61.5 cm length were crafted into an 8.5 cm × 5.5 cm × 2.0 mm PMMA plate. After digging the microchannel, the PMMA plate was washed with deionized water, dried, and then thermally sealed onto a 2.0 mm thick PMMA plate with the same dimensions. The sealing process was made in a heat press (Ferragini model HT3020, São Paulo, Brazil) at 110 °C under 590 kPa for 30 min.

### 2.3. Flow system and detector configuration

Throughout this study, two main techniques were applied for analytical detection: chronoamperometry and UV–Vis spectrometry. The main constituents applied for these measurements are depicted in Fig. 1. Flow arrangement (Fig. 1-A) was composed of two distinct propelling systems: an aquarium pump ($a_1$) was utilized to pneumatically propel the carrier electrolyte [24] and a programmable peristaltic pump (Reglo MS Digital, Ismatec) ($a_2$) was used to introduce reproducible sample volumes into the microchannel ($a_3$). The injections of samples by the peristaltic pump was made using 0.3 mm internal diameter Tygon® tubing. A flow control pinch valve located between the electrolyte reservoir ($a_4$) and the pneumatic pump, allowed adjustment of the flow rate through the microchannels to the detector ($a_5$).

Electrochemical measurements were performed using a μ-Autolab type III potentiostat (EcoChemie, Netherlands) with a three electrode system operating in the chronoamperometric mode (Fig. 1-B). A commercial Kel-F block with dual platinum disk electrodes (EG & G. model MP 1303) ($b_1$) and a miniaturized $Ag/AgCl_{(sat\ KCl)}$ [25] ($b_4$) were employed as working, auxiliary and reference electrodes, respectively. A thin polypropylene sheet (200 μm thickness — $b_2$) was used as a spacer to limit the electrochemical cells bound. Cell sealing was accomplished by means of a PMMA upper block ($b_3$), which was fixed using four screws, each at one edge of the structure. Additionally, three holes were drilled on the PMMA cover top. Two of them were used as input and output of the flowing solution and the third hole was made exactly in front of the working electrode in order to adapt the miniaturized $Ag/AgCl_{(sat)}$ reference electrode. This arrangement was utilized for the selection of GOD, AAO and CAT immobilization parameters in the microreactors.

Spectrophotometric measurements were made in a lab made spectrophotometric cell (Fig. 1-C) coupled to an UB-4000 miniature fiber optics spectrometer (Ocean Optics, USA) with a Toshiba TCD1304AP3648-element linear CCD-array detector. A 50 mm × 30 mm × 10 mm PMMA plate ($c_5$) was used as cell "core". Three 0.8 mm diameter holes were drilled in this plate in order to define the inlet, outlet and optical path (1 cm) of the cell. Two 50 mm × 30 mm × 1.0 mm PMMA plates ($c_3$) were used as windows, to avoid direct contact of the flowing solutions with the optical fibers. The windows were attached to the "core" by screws after intercalation of a 100 μm thick silicone sealing lining on each side ($c_4$). A 200 μm wide central cut in the linings provide a flow path that interconnects the 3 holes of the "core". Finally, two more PMMA plates (3 mm thickness) ($c_2$) with a connector fixed in the middle of each ($c_1$) were used to connect the optical fibers.

This spectrophotometric arrangement was used to confirm the effectiveness of HRP immobilization in the microreactor. A Trinder's reaction based system, using a microreactor with both immobilized GOD and HRP was developed and applied for glucose spectrophotometric measurements.

All the acquired data were treated using Origin 8.6.0 software (OriginLab Corporation, USA). Michaelis–Menten constants ($V_{max}$ and $K_m$) were calculated using a nonlinear least square fitting (with weighting factors) to directly fit the obtained experimental data.

### 2.4. Microchannel modification

Microchannel modification procedure was based on our previous work [21]. All immobilization steps were performed under flow conditions and with the two PMMA plates already sealed together. The PEI solution was passed through the reactor for 20 min at 0.15 mL $min^{-1}$ flow rate. For all enzyme immobilizations, a 5 mL final volume mixture of enzyme and glutaraldehyde, in 0.10 mol $L^{-1}$ phosphate buffer (pH 7.00), was circulated for 30 min through the reactor. An Infinity video microscope (Infinity Photo-optical Company, Boulder, Colorado) connected to a Panasonic camera (Model GP-KR222) was used to obtain images of the microchannel surfaces, in order to verify the immobilization success.

## 3. Results and discussion

In this work, our goal is to extend our immobilization methodology to other enzymes such as AAO, CAT, GDH and HRP, demonstrating the