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Ceramic Microsystem Incorporating a Microreactor with Immobilized Biocatalyst for Enzymatic Spectrophotometric Assays

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Low-temperature cofired ceramics (LTCC) technology is a versatile fabrication technique used to construct microflow systems. It permits the integration of several unitary operations (pretreatment, separation, (bio)chemical reaction, and detection stage) of an analytical process in a modular or monolithic way. Moreover, because of its compatibility with biological material, LTCC is adequate for analytical applications based on enzymatic reactions. Here we present the design, construction, and evaluation of a LTCC microfluidic system that integrates a microreactor (internal volume, 24.28 μL) with an immobilized β-galactosidase from *Escherichia coli* (0.479 activity units) and an optical flow cell to measure the product of the enzymatic reaction. The enzyme was immobilized on a glyoxal−agarose support, maintaining its activity along the time of the study. As a proof of concept, the LTCC-β-galactosidase system was tested by measuring the conversion of *ortho*-nitrophenyl β-D-galactopyranoside, the substrate usually employed for activity determinations. Once packed in a monolithically integrated microcolumn, the miniaturized flow system was characterized, the operational conditions optimized (flow rate and injection volume), and its performance successfully evaluated by determining the β-galactosidase substrate concentration at the millimolar level.

The miniaturization of analytical systems for different applications is currently a very active field of analytical chemistry research. Ideally, all the tasks included in the analytical process would be scaled down and integrated in a whole system to develop a micro total analysis system (μTAS).[1] The inherent advantages of these microanalyzers are well-known: the increase of their analytical performance, for instance, exploiting new phenomena only available at a microscale, their low cost and portability, low consumption of sample/reagents, and the possibility to be operated by nonqualified personnel providing information in real time and in situ monitoring.[2,3] In early years, silicon and glass have been the most widely employed for analytical microfluidic device development.[4] However, the use of these materials is mainly limited to bidimensional devices and the integration of some element needed to carry out a whole analytical process (pretreatment step, reaction step, or detection device) provides an additional challenge.[5] In addition, the partial or total modification of the designed microdevice may take a long time, due to the complexity of the whole construction. Polymeric materials as poly(dimethylsiloxane) (PDMS)[6] or poly(methylmetacrylate) (PMMA) allow easy and fast construction of more complex structures that can also include elements of a different nature aimed at several analytical purposes.[5]

The low temperature cofired ceramics (LTCC) technology presents a good alternative due to some interesting advantages. LTCC permits a fast prototyping of devices and its multilayer methodology used simplifies the integration of materials of different nature into the ceramic body.[7,8] Among these materials, the integration of screen-printing conductors is one of the promising ones because they may be part of a circuitry needed to control the microanalyzer, i.e., for example, the detection system or the internal temperature control.[9]

LTCC is a microfabrication technology based on the use of green ceramic tapes (100−400 μm). The multilayer approach allows the design of 3D structures where mechanic, electronic, and fluidic components can be integrated in a single device. This microfabrication technology has been proved to be suitable to construct microanalyzers with complex three-dimensional fluidic inner structures and able to integrate different materials that, on

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the whole, permit the integration of all unitary operations of a classical analytical procedure.[9] Several detection systems based on electrochemical techniques have been integrated in an LTCC miniaturized analyzer as potentiometric detection based on crystalline membrane of $Ag_2S/AgCl$[10] for Cl^- determination in natural waters, solvent polymeric ion-selective electrodes[11] for NO_3^- analysis, and an amperometric detector[5] for pesticides analysis in environmental control. In spite of the multiple possibilities of these ceramics as microfabrication materials for microreactor construction, they have not been totally exploited.

A novel application of LTCC microfluidic systems is the incorporation of a microcolumn containing an immobilized biocatalyst. Because of their inert nature, LTCC systems are compatible with biological reactions, allowing the performance of enzyme-catalyzed reactions which evolution could be followed at the column outlet (by spectrometric detection in a flow cell, for example). Although there are some previous works concerning the use of enzymes in solution in LTCC microsystems, either for enzymatic inhibition detection[5] or electrochemical immunoassays,[12] only the use of polymer coated glass beads with immobilized enzyme (urease) loaded in one compartment of an LTCC system has been reported.[13,14]

The present work reports the construction and evaluation of the operational capabilities of a ceramic microsystem incorporating a microreactor with immobilized biocatalyst and an optical flow cell for enzymatic spectrophotometric assays. Enzymatic microreactors can be employed for analytical purposes as well as for substrates' screening in synthetic bioconversions.[15,16] For both applications, a miniaturized stable and robust system would be required, and the availability of stable immobilized enzyme is one of the key factors.[16]

Enzyme immobilization onto glyoxal-agarose by multipoint covalent attachment allows great improvement on the stability of immobilized derivatives compared to the soluble ones.[17] This enzyme immobilization method offers great possibilities for an intense but nondistorting, enzyme-support multi-interaction process by controlling immobilization conditions (pH, surface density of aldehyde groups, and temperature).[18]

The behavior of an LTCC system with a microcolumn containing immobilized β-galactosidase will be examined as a case example. This enzyme has been employed in analytical determinations of substrate concentration (i.e., lactose,[19]). On the other hand, the determination of its enzymatic activity has been also proposed as an indirect measure related to an analyte concentration. This is the case of tobramycin determination in serum.[20]

In addition, β-galactosidase is an enzyme which can be modified to exhibit allosteric responses. In particular, *Escherichia coli* β-galactosidase has been successfully tailored for the diagnosis of either foot-and-mouth disease[21] and human immunodeficiency virus (HIV) infection,[22,23] and conditions for assays in liquid and immobilized form have been completely established.[24,25]

As a proof of concept, the LTCC-β-galactosidase system will be tested by measuring the conversion of *ortho*-nitrophenyl β-D-galactopyranoside (ONPG), an usual substrate employed for β-galactosidase activity determinations. Immobilized *E. coli* β-galactosidase in glyoxal-agarose supports was introduced in a microcolumn monolithically integrated in a LTCC microfluidic system which also incorporates a flow cell for spectrophotometric detection. The colored product of the enzymatic hydrolysis (*o*-nitrophenol) can be continuously monitored at 430 nm.

EXPERIMENTAL SECTION

LTCC Devices Construction. Ceramic green tapes 951 (254 μm thickness) purchased from DuPont (El Segundo, CA) were used as described elsewhere.[11] The characteristic that makes this material so interesting for our purposes is the fact that the tapes can be easily mechanized by means of a standard milling machine or a laser in the green stage (before they are sintered), when they are still soft and pliable. Figure 1A shows all the layers involved in the fabrication procedure that, once overlapped, turned out to be the desired device. Layer A is the top one, and H is the bottom one. A total amount of 11 layers with 8 different mechanized structures were used: A, B, 2C, D, 2E, F, 2G, and H. The height of the device was ~2.2 mm after sintering. The design of each individual layer, by means of a CAD software, was done taking into account the shrinkage suffered by the ceramic tapes during the sintering step (~15% in each axis). The mechanization was performed by a LPKF Protomat C100/HF milling machine. The four holes in each corner of the layers were used to align them during the lamination process. Once the layers were overlapped, they were placed between two stainless steel sheets with four aligning marks and laminated following a thermocompression process, which was performed at ~3000 psi for 3−4 min at 100 °C. After that, the sintering was carried out in a furnace with a programmable software (Carbolite CBCWF11/23P16, Afora, Spain), so that a specific temperature profile could be applied. In order to compensate the shrinkage caused by the sintering process (12.3% in the $x-y$ axis and 15% in the z axis), the cavities

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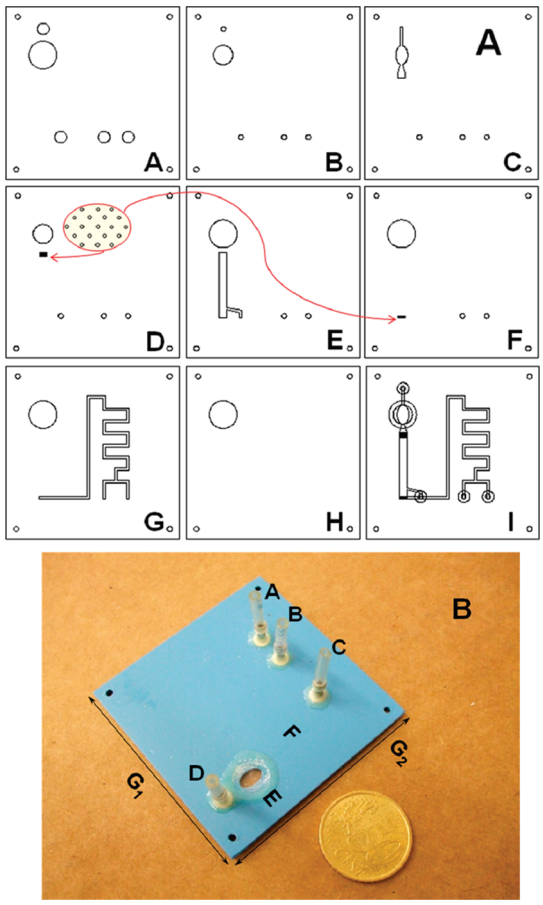

Figure 1. (A) CAD designs of the 11 overlapped layers (A−H) and microsystem inner structure of the final device constructed (I). The insets in the figure show the diffusers used to ensure the enzyme retention inside the microreactor. (B) Picture of the final enzymatic LTCC microanalyzer developed. Final dimensions of the device were G1 × G2, 6.2 × 6.2 cm; A and B, inlets; C, agarose with immobilized β-galactosidase inlet; D, waste (flow outlet and agarose with immobilized β-galactosidase outlet); E, optical detection cell; F, enzymatic microreactor.

designed to embed the flow connectors and optical flow cell were slightly bigger. The diameter of top and bottom diffusers was designed at optimal mechanizable size that ensured enzyme retention (bead diameter, 50−150 μM) inside the microreactor. The pore diameter of the two diffusers was set at 55 μm.

Apparatus and Reagents. Apart from the LTCC microanalyzer (Figure 1B), the rest of the hybrid flow system setup consisted of a peristaltic pump (Gilson, Wisconsin, US), a six-port distribution valve (Hamilton MVP, Reno, U.S.), 1.02 mm i.d. silicone tubing (Ismatec, Zurich, Switzerland), and 0.8 mm i.d. Teflon tubing (Scharlab, S.L., Cambridge, England).

Figure 2 shows the inner three-dimensional structure and the flow setup developed. Once the device was burnt-out, the final microreactor dimensions were 2.61 mm width, 23.26 mm length, and 0.40 mm height. The microreactor had a total volume of 24.28 μL. Flow rate and injection volume were optimized. Work temperature was set to 22 °C throughout all analysis. The microdevice was stored at 4 °C during nonworking periods filled with Z buffer (see the Experimental Section).

Detection system was specially designed for this application. The yellow-colored formed product was measured through the flow cell. A 430 nm light emitting diode (LED) (L-53MBC, Kingbright, Taiwan) was used as a light emitter and a photomultiplier (PMT) (Hamamatsu, H6780-03) was used as detector. The employed software used (based on Labview) was specially designed for our applications.

All the reagents used were of analytical grade and all solutions were prepared with Milli Q grade water. Z buffer[26] was prepared with Na_2HPO_4 97 mM, NaH_2PO_4 3 mM, KCl 20 mM, and $MgSO_4$ 1 mM at pH = 8.38, purchased from Panreac (Barcelona, Spain). *ortho*-Nitrophenyl β-D-galactopiranoside (ONPG) was purchased from Sigma (St. Louis, MO). ONPG standard solutions (0.5−30 mM) were prepared daily in buffer Z by dilution from a 30 mM stock.

Immobilization Procedure. The immobilization procedure included two steps: support activation[26] and immobilization process.[25] Glyoxal-agarose gels (agarose-O-CH_2-CHO) were prepared by etherification of 4BCL agarose beads (Hispanagar, Torrejón de Ardoz, Spain) with glycidol (2,3-epoxypropanol) (Sigma-Aldrich, St. Louis, MO) during 19 h at 20 °C and oxidized using $NaIO_4$ 0.1 M (Sigma, St. Louis, MO). The number of aldehyde groups available to link the ligand was calculated after 35 min by analyzing the consumed periodate through an enabled reaction with KI 10% and saturated $NaHCO_3$. This number resulted in 158 μmol of aldehyde groups per milliliter of support.

In the immobilization process, a solution of 100 mM $NaHCO_3$ at pH 9.5 was mixed to a solution of *E. coli* β-galactosidase (Sigma-Aldrich, St. Louis, MO) leading to a final concentration of 50 mM $NaHCO_3$. An appropriate volume of this solution (9 mL) was added to 1 mL of activated agarose, offering approximately 80 activity units per milliliter (AU/mL) of agarose support. A blank with water instead of agarose was performed in parallel. The process was carried out at 4 °C, under gentle stirring. The catalytic activity of the enzyme in the blank, supernatant and total suspension was measured at different times. When no activity was detected in the supernatant, the reduction of the Schiff bases was performed employing a 1 mg mL^{-1} solution of $NaBH_4$ during 30 min. Finally, the enzyme derivative was separated from the supernatant by filtration, washed with water and stored at 4 °C.

Enzymatic Activity Measurement. The catalytic activity of β-galactosidase was followed spectrophotometrically. The assay mixture comprised 20 μL of sample, 335 μL of 6 mM ONPG, and 645 μL of Z buffer[23] pH 8.4 in a 1 mL cuvette. In order to measure the immobilized enzyme activity, 40 μL of the suspension were mixed with equivalent double volumes of ONPG and Z buffer and gentle stirred in a 2 mL cuvette. Activity was calculated from the increase in absorbance at 28 °C and 420 nm, using an UV−vis Cary spectrophotometer (Varian, Palo Alto, CA). The molar extinction coefficient of the *ortho*-nitrophenol product at 420 nm was 4155 M^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of β-galactosidase which hydrolyzes 1 μmol of ONPG/min at 28 °C and pH 8.4.

RESULTS AND DISCUSSION

Preliminary studies allowed us to verify the immobilized enzyme activity and the correct operation of the developed setup. β-galactosidase from *E. coli* was immobilized by covalent attach-

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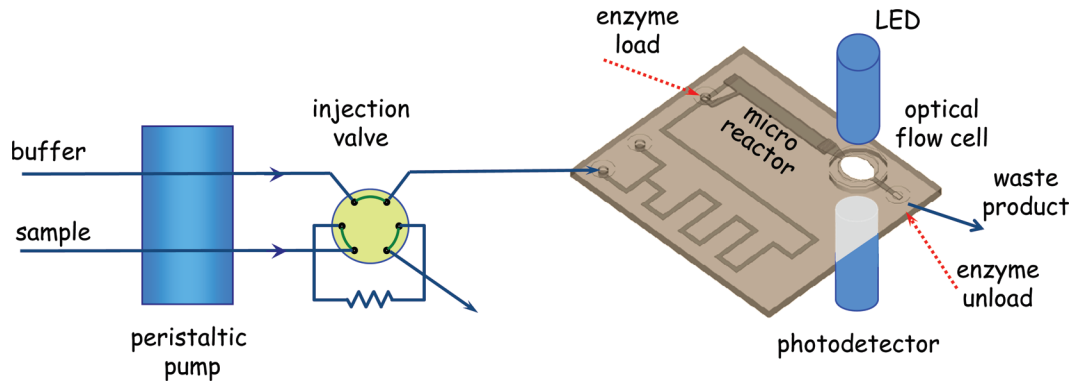

Figure 2. Developed flow setup and detail of inner three-dimensional structure with the microreactor.

ment on glyoxal-agarose support. After 48 h, almost all the offered activity was immobilized and the enzymatic retention of the final derivative was 28.1 U/mL of support. The loss of activity during the immobilization occurred in both suspension and blank, and it was due to the low stability of the enzyme at the immobilization pH 9.5.

A suspension of the agarose beads with immobilized β-galactosidase into buffer Z was injected manually into the microcolumn monolithically integrated in the LTCC microsystem until it was completely full. Microreactor loading was performed through inlet C (see Figure 1B). Microreactor unloading was carried out thought outlet D, introducing buffer Z in the opposite sense to the outlet flow.

The total load of the microreactor was determined by weight, and it was found to be 0.017 ± 0.001 g. Total retained enzymatic load in the microreactor was determined using a value of 1 g/mL for the specific gravity of agarose beads and it was found to be 0.479 AU ($U_{\text{microreactor}}$ = (0.017 g)(1 mL/1 g)(28.1 AU/mL)). Once filled, the microcolumn acts as an enzymatic reactor producing the hydrolysis of the substrate introduced as a sample in the microsystem. Flow of carrier solution through the microcolumn was performed without over pressure problems.

Operational conditions were studied in order to optimize enzymatic reaction and sensitivity of the developed miniaturized system. The first experiments were focused in the study of the outlet response (proportional to conversion) as a function of flow rate (0.26, 0.55, and 0.84 mL/min) and ONPG concentration (5, 10, and 30 mM). A high value for injection volume (100 μL) was selected because calibration at different substrate concentrations gave a linear response. Figure 3 shows the obtained results for all the analyzed concentrations at the three different flow rates. As expected, the obtained signals are higher as the flow rate decreases, considering that analysis time increases proportionally to the decrease in flow rate, the lower flow rate provides more reaction time and thus a higher percentage of substrate hydrolysis. Analysis time has been calculated as the time necessary for the analytical signal to recover 95% of the baseline value. A total of 4, 2, and 1.25 min analysis times were achieved for 0.26, 0.55, and 0.84 mL/min flow rates, respectively. If we consider the three obtained calibration curves for the three different studied flow rates it can be seen that in all cases the analytical response corresponds to a linear behavior in the studied concentration range and that sensitivity increases as the flow rate decreases.

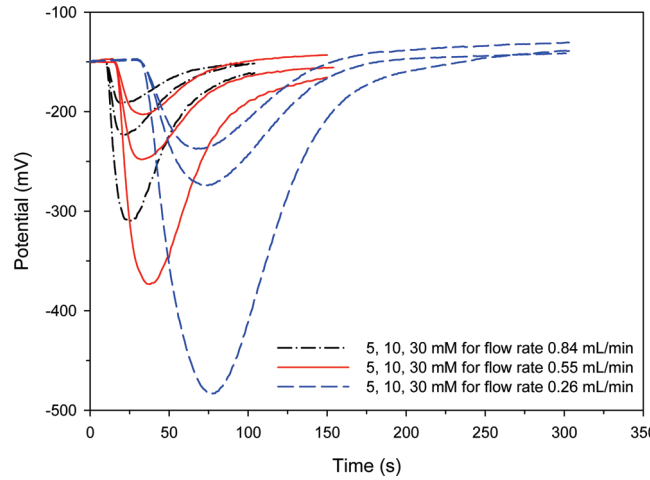

Figure 3. Microreactor response study as a function of substrate concentration and flow rate. Injection volume was set to 100 μL in all cases.

Obtained sensitivities were 8.986 mV/mM (r^2 = 0.999) for 0.26 mL/min, 6.600 mV/mM (r^2 = 0.997) for 0.55 mL/min, and 4.657 mV/mM (r^2 = 0.991) for 0.84 mL/min. The lowest flow rate was selected because of the higher substrate conversion resulting in an improvement of sensitivity.

Injection volume was studied in order to determine its effect on sensitivity and linear range response at different substrate loads, maintaining the previously found optimal flow rate of 0.26 mL/min. Six injection volumes were used: 25, 50, 75, 100, 125, and 200 μL at three consecutive different concentrations (5, 10, and 30 mM) of the substrate (ONPG). Figure 4A shows the proportional increase in analytical signal in relation to injection volume and substrate concentration until the 100 μL injection volume. It can be noticed that for volumes of 125 μL, the obtained response is practically equal to that obtained for 100 μL, but the response increases again for much bigger volumes (200 μL).

Apparently, our microreactor is able to hydrolyze high substrate loads (200 μL of 30 mM ONPG), but when looking deeply to the calibration curves (Figure 4B), it is clearly noticeable a decrease in the linear response range for the bigger injection volume (200 μL). Linear response is well maintained in the range of 25−125 μL injection volume. When comparing the obtained sensitivities in the range of 5−30 mM in relation to the injection volume, we achieved values of 5.07 mV/mM (r^2 = 0.999) for 25

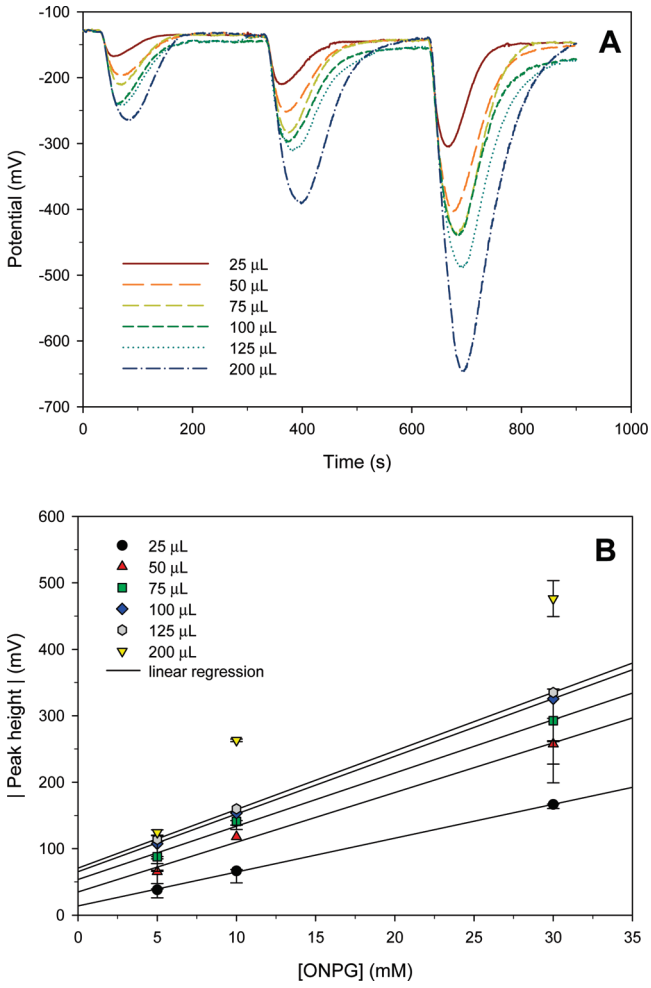

Figure 4. (A) Calibration curves at a flow rate of 0.26 mL/min using six injection volumes. (B) Calibration plots for each injection volume.

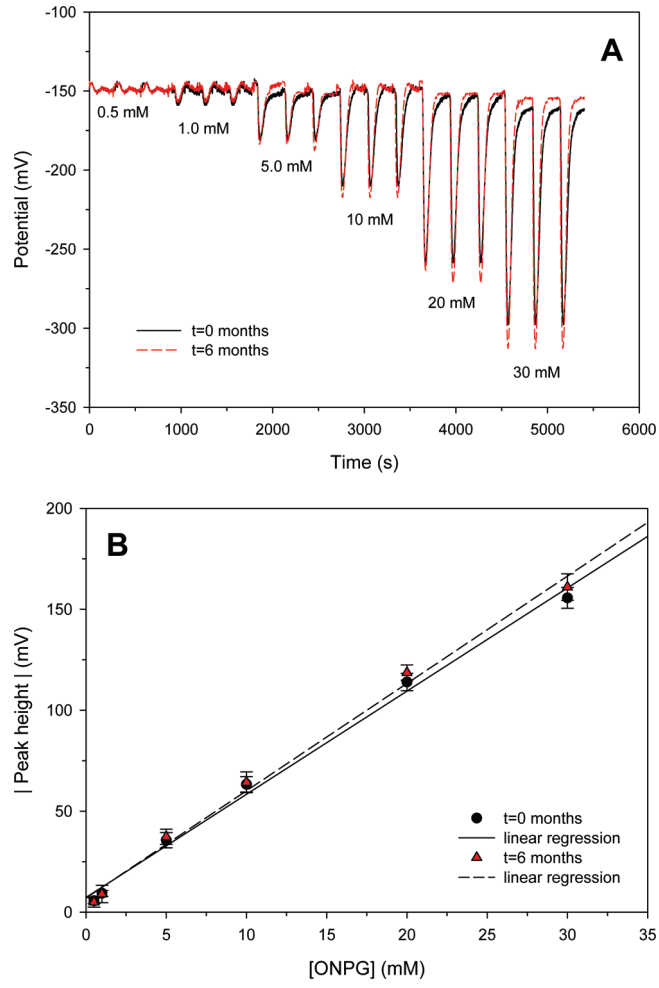

Figure 5. (A) Response curves obtained at a flow rate of 0.26 mL/min and V_{inj} = 25 µL after fresh enzyme is loaded into the reactor t = 0 and 6 months after. (B) Calibration plots of the two analysis times in part A.

µL, 7.54 mV/mM (r^2 = 0.998) for 50 µL, 8.11 mV/mM (r^2 = 0.998) for 75 µL, 8.70 mV/mM (r^2 = 0.999) for 100 µL, and 9.27 mV/mM (r^2 = 0.998) for 125 µL, observing again that the increase over values of 100 µL is lower.

There is a constant linear concentration range between 5 and 30 mM for injection volumes from 25 to 125 µL. Nevertheless, there is not a significant increase in sensitivity for the 125 µL case. Moreover, the linear range is lower for the 200 µL injection volume. This fact can be attributed to a response nonproportional to substrate concentration at the higher levels and fixed reaction time.

Although larger injection volumes led to higher sensitivity, 25 µL was selected as the sample volume in order to employ minimum volume and because of faster baseline recovery and linear response in the working range. On the other hand, the small microreactor volume (24.28 µL) justifies the use of small injection volumes.

Once the detection system had been tested and the enzymatic system had been optimized, the procedure as described in Figure 2B was used to perform the ONPG substrate analysis characterization. Extensive calibration curves by n = 3 for all concentrations were performed with an interval of 6 months (Figure 5A). The limit of detection (LD), repeatability, reproducibility (Figure 5B), and time of stability were determined.

The LD was calculated according to IUPAC.[27] The minimum detectable signal was estimated from a 20 blank series and its standard deviation, and it has been calculated as $\text{signal}_{LD} = S_b + ks_b$ (k = 3) by interpolating this value in the calibration curve, and the minimum detectable concentration was determined to be 0.56 ± 0.05 mM ONPG (n = 3, 95% confidence).

The repeatability of the system was estimated by means of the relative standard deviation obtained from 10 replicates of intermediate concentration in the range of study. Samples (10 mM) were used for this purpose, obtaining a good repeatability of the system as RSD was lower than 3.5%.

Microreactor reproducibility was evaluated comparing the obtained response in calibrations performed after 6 months of reactor enzyme load to those performed right after the enzyme load (Figure 5B). The storing conditions during nonworking periods are described in the Experimental Section. The immobilized enzyme stability after around 350 analyses has to be

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highlighted, and the data show that there are no significant differences in sensibilities when comparing both calibration curves. Calibration curves corresponding to Figure 5B are

$$H(\mathrm{mV})_{t:0\ \mathrm{months}} = 7(\pm 8) + 5.1(\pm 0.5) \times [\mathrm{ONPG}]\,(\mathrm{mM})$$
$$(n = 6;\ 95\%\ \text{confidence};\ r^2 = 0.994)$$

$$H(\mathrm{mV})_{t:6\ \mathrm{months}} = 7(\pm 9) + 5.3(\pm 0.6) \times [\mathrm{ONPG}]\,(\mathrm{mM})$$
$$(n = 6;\ 95\%\ \text{confidence};\ r^2 = 0.994)$$

CONCLUSIONS

A LTCC microreactor with immobilized biocatalyst and integrated optical flow cell was designed, manufactured, and positively tested. Results obtained in the optimization of hydrodynamic parameters like flow rate and sample injection volume and the operational features of the enzymatic microreactor were presented and discussed. These results have demonstrated the stability of the immobilized enzyme, the easy load of the enzyme derivative in the microreactor, and the robustness of the developed microdevice.

The LTCC microsystem has shown stable operation for 6 months. Only one load of immobilized biocatalyst was necessary for testing the microreactor capabilities during the study time, indicating that a minimum amount of enzyme was necessary because of its high stability. These facts represent attractive features for design and construction of "green" microreactors, due to their low maintenance requirements. In addition they are the starting point for further developments into fully integrated microsystems.

The presented system can be extended to different analytical applications of native or engineered β-galactosidases as well as to other immobilized enzymes. Other possible applications for this kind of enzymatic microreactors are their potential introduction into industrial-scale synthesis, applications in environmental protection or they could be used as microreactors for kinetic studies of immobilized enzymes using extremely small quantities of biocatalyst material. Enzymatic microreactors also facilitate characterization of enzyme activity as a function of substrate concentration and enable fast screening of new biocatalysts and their substrates.

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