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# Microcapsule-based biosensor containing catechol for the reagent-free inhibitive detection of benzoic acid by tyrosinase

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Abstract:
A biosensor based on the release of the enzyme substrate from its structure was developed for the inhibitive detection of benzoic acid. A polyurethane support comprising two perforated microcapsules (800 µm in diameter) filled with methylene blue as a model compound and covered with a conductive deposit of multiwalled carbon nanotubes, continuously released this stored dye for 24 h. An increase in methylene blue concentration of 0.5-0.75 µmol $L^{-1}$ $h^{-1}$ and 1.5-2 µmol $L^{-1}$ $h^{-1}$, in the presence and absence of the multiwalled carbon nanotube coating, respectively, was demonstrated by UV − vis spectroscopy in a 2 mL UV cuvette.
The same configuration with microcapsules filled with catechol was modified by a laponite clay coating containing tyrosinase enzyme. The resulting biosensor exhibits a constant cathodic current at -0.155 V vs AgCl/Ag, due to the reduction of the ortho-quinone produced enzymatically from the released catechol. The detection of benzoic acid was recorded from the decrease in cathodic current due to its inhibiting action on the tyrosinase activity. Reagentless biosensors based on different deposited quantity of tyrosinase (100, 200, 400 and 600 µg) were investigated for the detection of catechol and applied to the detection of benzoic acid as inhibitor. The best performance was obtained with the 400 µg-based configuration, namely a detection limit of 0.4 µmol $L^{-1}$ and a sensitivity of 228 mA L $mol^{-1}$. After the inhibition process, the biosensors recover 97-100 % of their activity towards catechol, confirming a reversible inhibition by benzoic acid.

Keywords: enzyme electrode, tyrosinase, benzoic acid, microcapsule, catechol

## 1. Introduction

An interesting alternative to current analytical methods is represented by biosensors which can circumvent conventional drawbacks such as low specificity, a long delay in receiving results, and a need for a high equipment cost. Biosensors are simple, highly sensitive, inexpensive and portable devices providing near real-time results in various fields such as medical, food and environmental monitoring. A main category of biosensors is based on electrochemical enzyme electrodes (Pereira da Silva Neves et al., 2018; Thévenot et al., 2001). Among them, an original approach consists in exploiting the inhibition of the enzyme immobilized on the electrode to detect an inhibitor or even to quantify its concentration (Amine et al., 2016; Harrad et al., 2018; Kurbanoglu et al., 2017). This concept has been widely used to detect drugs, food contaminants or pollutants like organic pesticides or toxic heavy metals. The detection of an inhibitor is carried out by measuring the evolution of the electroenzymatic activity of the biosensor in the presence of a substrate of the enzyme before and after the introduction of an inhibitor. However, the indirect monitoring of inhibitors requires the presence of the enzyme substrate in the sample solution.

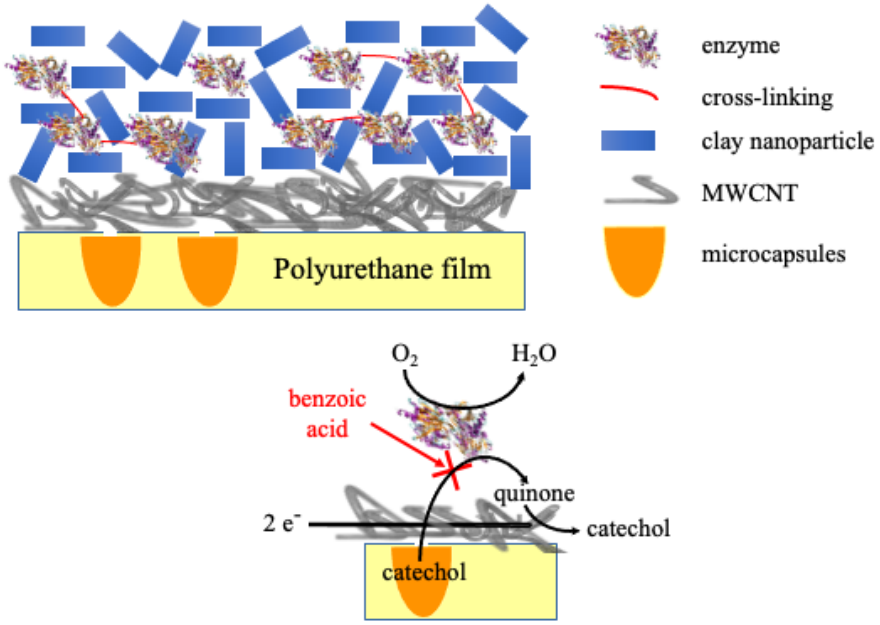
We report here an innovative enzyme-based biosensor configuration containing a reagent reservoir with continuous release of the enzyme substrate. The concept is to have a constant amperometric signal generated by the electrochemical oxidation or reduction of the product of the enzymatic reaction from the constant flux of substrate generated by the biosensor itself.

In order to illustrate this principle, an electrode was fabricated by creating a conductive multiwalled carbon nanotubes (MWCNT) deposit on a polyurethane support containing two microcapsules filled with catechol as substrate and pierced with a hole. The fabrication of the capsules uses top-down techniques and is based on the fabrication of a micro-cavity followed by a filling and closing of the capsule. The capsules produced for the biosensor were embedded in a polyurethane film allowing the nanotubes deposition (Ahangaran et al., 2019; Mc Murtry and El Mazria 2012).

A tyrosinase enzyme, which catalyzes the oxidation of phenols and *o*-diphenols into *o*-quinones with the concomitant reduction of oxygen into water, was entrapped in a clay matrix deposited onto the MWCNT electrode (Scheme 1). The functioning mechanism of the resulting

amperometric tyrosinase electrode was based on quinone reduction at -0.155 V vs AgCl/Ag (Cosnier et Innocent 1993). Therefore, this system can be dedicated to the direct detection of tyrosinase inhibitor without addition of the substrate of the enzyme in solution.

Benzoic acid, which is commonly used as a preservative for food and pharmaceutical products has been reported to be a reversible tyrosinase inhibitor (Casanova et al., 2020; Kochana et al., 2012; Li et al., 2010; López et al., 2011; Shan et al., 2008). In this context, the analytical characteristics of the catechol capsule-based tyrosinase biosensor and its performance towards the detection of benzoic acid as an inhibitor are therefore described.




**Scheme 1**. Schematic representation of the biosensor structure and its operating principle.

## 2. Material and methods

### 2.1. Materials and reagents

All reagents (analytical grade) and tyrosinase (7164 units/mg) were purchased from Sigma-Aldrich. Laponite was supplied by Laporte Inorganics. Multiwalled carbon nanotubes (MWCNTs) were acquired from Nanocyl (NC7000, ~90% purity, ⌀ = 10 nm, 1.5 µm length) and used as received. Electrochemical experiments were performed in stirred phosphate buffer

(0.1 M, pH 6.5). High purity $O_2$ and Ar were obtained from Air Liquide. UV−vis absorption spectra were recorded in quartz cuvettes using a PerkinElmer UV-Lambda 650 spectrophotometer.

### 2.2. Microcapsules

The polyurethane capsules were 800 micron in diameter and depth. The aperture allowing a slow diffusion of catechol was carried out mechanically, and has a 250 μm diameter. If we consider a sphere-shaped capsule with a diameter of 800 micron and a wall thickness of 80 μm, the filling ratio of the encapsulated active product can exceed 80% of the capsule internal cavity volume.

### 2.3. Bioelectrode preparation

Dispersion of MWCNT in ethylene glycol was carried out by 30 min sonication of 5 mg $mL^{-1}$ until a homogenous black suspension was obtained. Then, 60 μL of the MWCNT solution was spread on the two microcapsules included in a polyurethane support. Ethylene glycol was removed under vacuum for 2h, leading to a thick MWCNT film which was subsequently connected by an Ag-plated Cu wire bonded with carbon paste. A clay colloidal suspension of laponite, 1 mg $mL^{-1}$ was dispersed overnight under stirring conditions in deionized water. Tyrosinase was dissolved in various amounts of laponite dispersion and the resulting mixtures were spread on the surface of the MWCNT coating leading to different deposits varying between 100 to 600 μg of enzyme, the enzyme / laponite ratio (10/1) being kept constant. The inorganic biomatrix was placed in glutaraldehyde vapor for 40 min to allow protein crosslinking then the bioelectrode was dried at room temperature under vaccuum. Prior to electrochemical measurement, the bioelectrode is incubated for 45 min in buffer solution.

Electrochemical measurements were performed using a potentiostat AMETEK Princeton Applied Research PARSTAT PC1000. Experiments were performed at 25°C in 0.1 M phosphate buffer (pH 6.5) with a thermostated three-electrode cell containing the bioelectrode as the working electrode, a Pt wire counter electrode and an AgCl/Ag (sat. KCl) reference electrode.

## 3. Results and discussion

The concept of "reagentless biosensor" for the detection of inhibitors consists in an amperometric enzyme electrode whose functioning is based on a constant release over several minutes or even several hours of the enzyme substrate stored in microcapsules integrated into the biosensor structure. In order to illustrate the potential release of a solid molecular compound soluble in aqueous medium, methylene blue (MB) was stored in the polyurethane-capsule. The release of MB was recorded by following periodically its absorbance at 662 nm in stirred phosphate solution as a function of time for a polyurethane support containing 2 microcapsules filled with MB and pierced with a 250 µm diameter hole (Fig. 1). After an initial period of about 20 min, a stabilization of the release process occurs providing a continuous increase in MB concentration of 1.5-2 µmol $L^{-1}$ $h^{-1}$ in a UV cuvette containing 2 mL at least for 20 h.

With the aim to develop a bioelectrode for the detection of inhibitors, a conductive coating must be generated onto the polyurethane-capsule support while maintaining permeability for the trapped substrate as it dissolves in the capsule. For this purpose, the conductive coating was produced by adsorption of MWCNT dispersed in ethylene glycol, a solvent compatible with the polyurethane material.

The same experiment was carried out with a polyurethane support modified by a conductive MWCNT coating. Thanks to the permeability of the MWCNT deposit, it appears that the formation of an electrode onto the polyurethane support decreases slightly the MB release but does not block it. Similarly to the preceding behavior, after 30 min, a continuous almost linear increase of MB concentration of 0.5-0.75 µmol $L^{-1}$ $h^{-1}$ was observed over 24 h. This corresponds to the release in solution of 12.8 µg of MB after 24 h. Although the rate of release of MB decreases markedly for longer periods, a concentration of 85 µmol $L^{-1}$, corresponding to the dissolution of 54.4 µg of MB can be reached after 7 weeks.

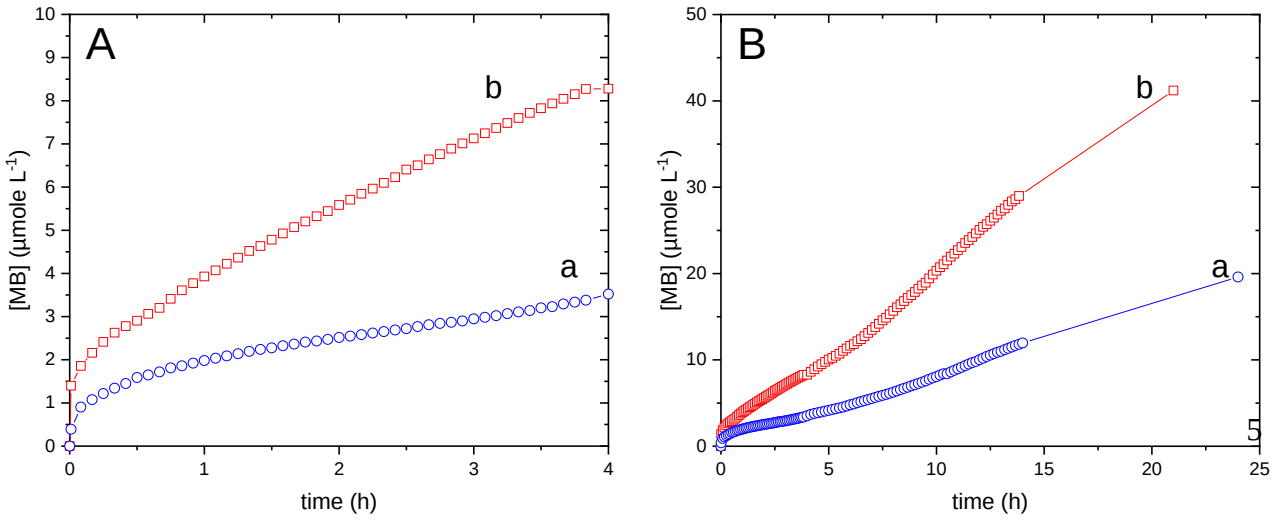


Fig. 1 : Evolution of methylene blue (MB) concentration as a function of time (A) spectrum recorded every 5 min, (B) spectrum recorded every 10 min, in 2 mL stirred phosphate solution for a polyurethane support containing 2 pierced microcapsules filled with MB (a) covered by a MWCNT deposit (b) without MWCNT.

For the construction of the tyrosinase-based biosensor, one of the best substrates of tyrosinase, the water-soluble catechol, was trapped in powder form into the capsule. After the formation of the MWCNT coating, the immobilization of tyrosinase was performed following a reported procedure by spreading and drying an aqueous mixture of enzyme and laponite on the conductive coating (Poyard et al., 1998). Then the proteins were crosslinked by glutaraldehyde vapor within the bioinorganic matrix. The effect of the variation of the deposited quantity of tyrosinase, namely 100, 200, 400 and 600 µg on the performance of the biosensor towards the release of immobilized catechol and the detection of inhibitor has been investigated. The analytical capabilities of the biosensors were first examined for the detection of added catechol concentration in air-saturated stirred phosphate buffer (0.1 mol $L^{-1}$, pH 6.5). The amperometric response of the biosensors was recorded at -0.155 V for successive increments of catechol concentration. The addition of catechol induces an increase in the cathodic current reflecting the reduction of the enzymatically produced quinone. The current responses were stable presenting a response time (determined as the time required to reach a new stable current value after a substrate injection) of 30-45 s, illustrating the successful entrapment of tyrosinase in the laponite matrix. The resulting calibration curves present a similar shape with a linear increase of the biosensor response with increasing catechol concentration up to 5-10 µmol $L^{-1}$ while a pseudo plateau was reached for higher concentrations (Fig. 2). As expected, the sensitivity of the biosensor increases continuously: 1.81, 3.97, 4.69 and 6.31 A L $mol^{-1}$ with the increase in the amount of the immobilized enzyme from 100 to 600 µg. In addition, the maximum current values at saturating catechol concentration increase linearly from 24 to 54 µA with the amount of tyrosinase.

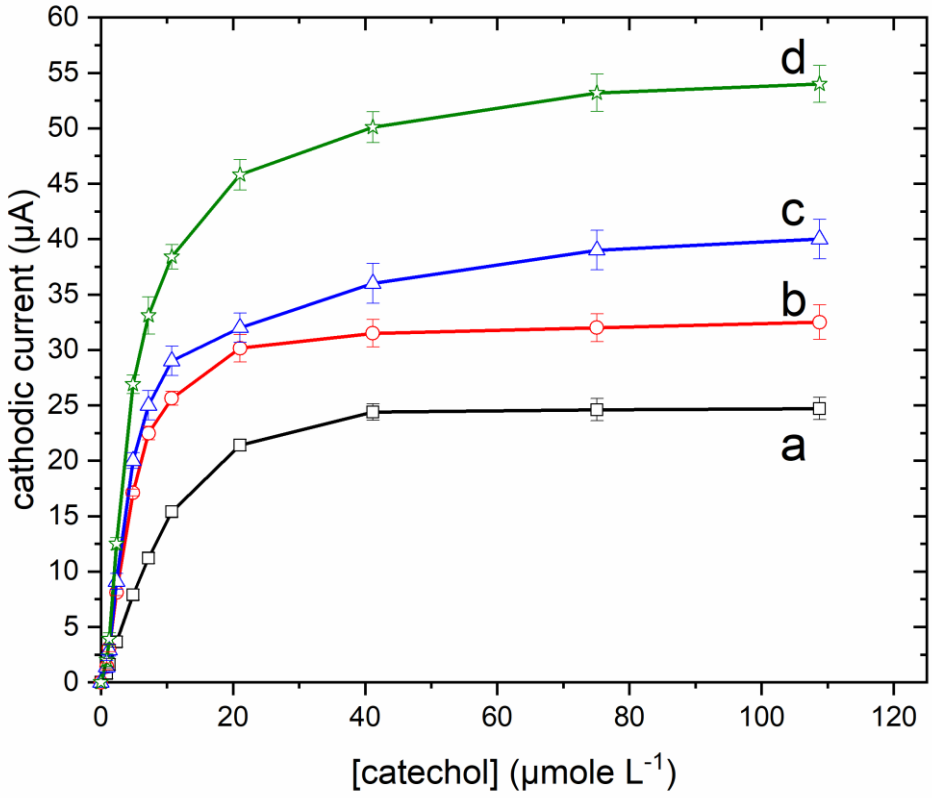



Fig. 2: Calibration plots for catechol in 0.1 mol $L^{-1}$ stirred phosphate buffer (pH 6.5) as a function of the amount of tyrosinase deposited on the MWCNT- polyurethane electrode (a) 100, (b) 200, (c) 400 and (d) 600 µg; the relative standard deviation was 3.3, 1.9, 2.4 and 2.1 %, respectively. Applied potential, −155 mV vs AgCl/Ag; current responses of biosensors are reported in absolute values.

Concerning catechol trapped in the microcapsules, it appears that all the biosensors actually exhibit a cathodic current in an electrolyte free of catechol. These cathodic currents reflect the reduction of enzymatically oxidized catechol coming from the capsules corroborating thus the expected process of release. Regarding the influence of the amount of tyrosinase immobilized on the cathodic current, the current intensity increases from -5 to -10 µA for biosensors based on 100 and 600 µg, respectively. It should be noted that the same experiment carried out with a similar electrode design but without tyrosinase in the laponite coating exhibits no cathodic current. To confirm the release of the entrapped catechol and its consumption by tyrosinase, the aqueous solution was purged with argon to remove oxygen necessary for the enzymatic

oxidation of catechol. As expected, a rapid decrease in cathodic current down to -1.5 µA was observed for all biosensors corroborating its origin due to the continuous release of the entrapped enzyme substrate.

For the four biosensors, the successive additions of benzoic acid induce a decrease of the steady-state cathodic current reaching a new stable current value with a fast response time (about 40 s). Figure 3 shows the biosensor response for the four configurations represented by the absolute value of the decrease in current intensity as a function of benzoic acid concentration. The resulting calibration curves show a similar behavior, i.e. an initial linear increase in absolute value up to 6-18 µmol $L^{-1}$ followed by a curve leading to a pseudo-plateau for concentrations above 250 µmol $L^{-1}$. These plateaus correspond to the maximum inhibition level of the biosensor response. The effect of benzoic acid on the amplitude of variation of the biosensor response increases sharply from the bioelectrode based on 100 µg to that based on 200 µg and then reaches a maximum for configurations based on 400 and 600 µg. The importance of the inhibitory effect thus increases with the increased performance of the biosensors for catechol detection.

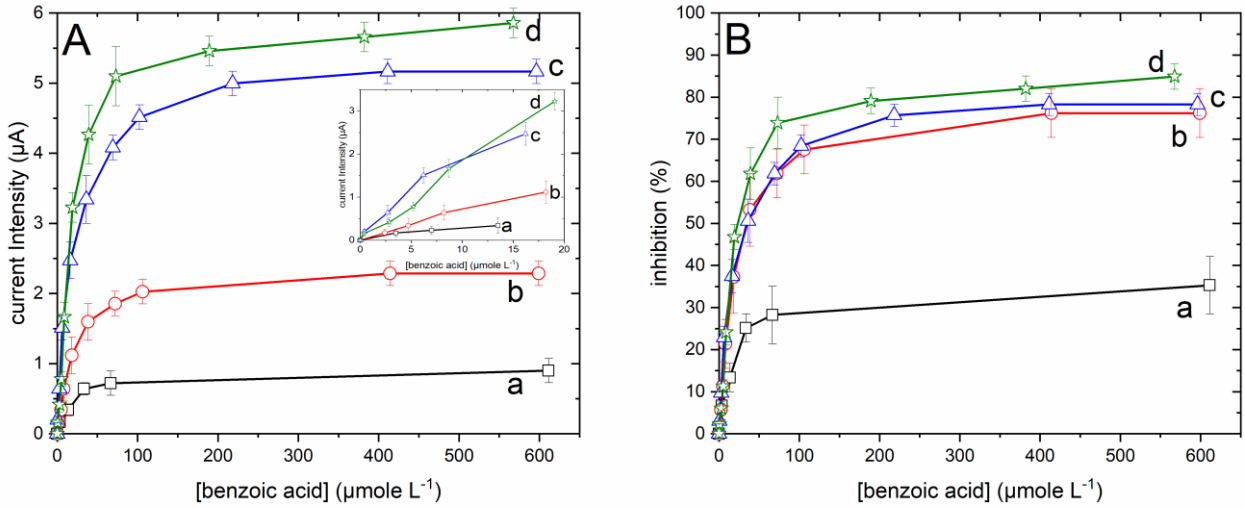



Fig. 3. (A) Decrease of cathodic response induced by increasing concentrations of benzoic acid at bioelectrodes elaborated with (a) 100, (b) 200, (c) 400 and (d) 600 µg of tyrosinase. Inset: initial part of the calibration curves. The relative standard deviation was 11.3, 16.0, 9.1 and 4.9 % for biosensor configuration based on 100, 200, 400 and 600 µg of tyrosinase, respectively. (B) representation in inhibition percentage of the amperometric response of bioelectrodes to catechol induced by increasing concentrations of benzoic acid. Experimental conditions as in Fig 2.

Biosensor sensitivity values for benzoic acid, defined as the slope of the linear portion of the calibration curve, are 17, 59, 170 and 228 mA L $mol^{-1}$ for configurations based on 100, 200, 600 and 400 μg, respectively. Surprisingly, it appears that the 400 μg-based configuration is more sensitive than the 600 μg-based configuration. This can be attributed to the thicker laponite clay deposit for the 600 μg-based configuration which increases the steric hindrance to benzoic acid penetration. The detection limit for the 400 μg-based biosensor is 0.4 μmol $L^{-1}$, which is similar to those already, observed for conventional electrochemical tyrosinase biosensors (Li et al., 2010; López et al., 2011; Sezgintürk et al., 2005; Shan et al., 2007), the equation of the linear part of the calibration curve being: I(μA) = 228 [benzoic acid] (μmol $L^{-1}$) +0.0774 with $R^2$ = 0.9953. The biosensor response to benzoic acid was also analyzed for all bioelectrodes as an inhibition percentage defined as the ratio of the decrease in current intensity (I°-I) to the initial current I° due to the reduction of the quinone produced. It appears that at saturation with benzoic acid above 500 μmol $L^{-1}$, the inhibition percentage of the biosensors is between 76 and 85% except for that based on 100 μg which has only 35% inhibition. After the inhibition process, biosensors were transferred in clean phosphate buffer and their sensitivity to catechol additions was again determined. It appears that the biosensors recover 97-100 % of their activity, corroborating thus the reversible character of the benzoic acid inhibition. This reversibility may also be facilitated by the immobilization matrix, the laponite nanoparticles being negatively charged, the matrix does not retain the inhibitor by electrostatic interactions (Ghadiri et al., 2013; Mousty 2004). Regarding the selectivity of the biosensor, the inhibitory effect of atrazine and 2-chlorophenol as representative of pesticides and disinfectants that inhibit tyrosinase electrodes was studied (Besombes et al., 1995a, 1995b; Campanella et al., 2006; Marino et al., 2011). It appears that successive additions of 2-chlorophenol or atrazine leading to increasing concentrations from 40 μmol $L^{-1}$ to 1.6 $10^{-4}$ mol $L^{-1}$ or 7 $10^{-3}$ mol $L^{-1}$ for atrazine and 2-chlorophenol, respectively, do not affect the biosensor response. In contrast, the subsequent addition of benzoic acid then catechol in presence of 2-chlorophenol (7 $10^{-3}$ mol $L^{-1}$) or atrazine (1.6 $10^{-4}$ mol $L^{-1}$), leads to a decrease and an increase of the cathodic current value, respectively. This corroborates the absence of inhibitory effect of these contaminants on the biosensor functioning.

## 4. Conclusions

The polyurethane support modified by two perforated microcapsules filled with catechol was easily transformed into a bioelectrode by the successive deposition of carbon nanotubes and a mixture of clay and enzyme. The resulting biosensor exhibits a cathodic current due to the reduction of the ortho-quinone produced by the enzyme and allowed the reversible detection of benzoic acid. This concept paves the way for the development of a new generation of reagentless inhibition-based biosensors, covering a wide range of environmental and medical applications. This design makes it possible to have ready-to-use portable biosensors using an enzymatic substrate which is in the form of a solid soluble in aqueous medium. Furthermore, the operation of the biosensor for several hours requires very small quantities of substrate. Moreover, modulation of the release rate of a substrate could be considered by varying the number of microcapsules and/or the diameter of the hole. In addition to the development of biosensors, this approach could thus be exploited in the design of biofuel cells self-fed in substrates.

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