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Enzymatic biosensor for the electrochemical detection of 2,4-dinitrotoluene biodegradation derivatives

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Abstract

In this work, we demonstrate for the first time that 4-methyl-5-nitrocatechol (4M5NC) and 2,4,5-trihydroxytoluene (2,4,5-THT), two compounds obtained from the 2,4-DNT biodegradation are recognized by polyphenol oxidase as substrates. An amperometric biosensor is described for detecting these compounds and for evaluating the efficiency of the 2,4-DNT conversion into 4M5NC in the presence of bacteria able to produce the 2,4-DNT-biotransformation. The biosensor format involves the immobilization of polyphenol oxidase into a composite matrix made of glassy carbon microspheres and mineral oil. The biosensor demonstrated to be highly sensitive for the quantification of 4M5NC and 2,4,5-THT. The analytical parameters for 4M5NC are the following: sensitivity of $(7.5 \pm 0.1) \times 10^5$ nAM^{-1}, linear range between 1.0×10^{-5} and 8.4×10^{-5} M, and detection limit of 4.7×10^{-6} M. The sensitivity for the determination of 2,4,5-THT is $(6.2 \pm 0.6) \times 10^6$ nAM^{-1}, with a linear range between 1.0×10^{-6} and 5.8×10^{-6} M, and a detection limit of 2.0×10^{-7}. Under the experimental conditions, it was possible to selectively quantify 4M5NC even in the presence of a large excess of 2,4-DNT. The suitability of the biosensor for detecting the efficiency of 2,4-DNT biotransformation into 4M5NC is demonstrated and compared with HPLC-spectrophotometric detection, with very good correlation. This biosensor holds great promise for decentralized environmental testing of 2,4-DNT.
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1. Introduction

Electrochemical biosensors have demonstrated to be extremely useful in different fields [1]. They present important advantages compared to other biosensors, connected to the sensitivity of electrochemical techniques, the possibility of portability and miniaturization, and the relative low-cost of electrochemical devices [1]. They have been successfully used for the determination of a wide range of analytes like glucose [2], neurotransmitters [3], alcohols [4], nucleic acids [5], and pollutants [6,7] among others.

Carbon materials have received special attention for the preparation of electrochemical biosensors [8]. In fact, glassy carbon, carbon fibers, and graphite in different forms have been used for the preparation of electrochemical biosensors [1]. However, just few examples of the application of glassy carbon composites for developing enzymatic biosensors have been proposed. Wang et al. [9] and Rodríguez and Rivas [10] have described the electrocatalytic properties of a glassy carbon paste electrode towards different analytes as well as the usefulness for developing enzymatic electrodes. Girault et al. [11] have also proposed a composite based on micro-glassy carbon particles and a polystyrene polymer.

The aim of this work is to propose an enzymatic biosensor based on the use of a glassy carbon composite and polyphenol oxidase (PPO) for the quantification of compounds derived from the biodegradation of 2,4-dinitrotoluene (2,4-DNT). These compounds have never been described before as substrates of PPO.

PPO is a cuproprotein widely distributed in nature, responsible for the oxidation of phenol and catechol to the corresponding quinones [12].

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The nitroaromatic compound 2,4-DNT is a precursor in toluene diisocyanate synthesis used for the production of polyurethanes. This compound is also an important intermediate in the manufacturing of the explosive 2,4,6-trinitrotoluene. Improper disposal of waste streams generated by the industrial activity and military operations have resulted in contamination of the environment with 2,4-DNT. The U.S. Environmental Protection Agency (EPA) includes 2,4-DNT into the priority pollutant list [13,14] because of its toxicity [15–17], carcinogenicity [17,18], and its widespread environmental occurrence. Therefore, manufacturing wastes are specifically regulated by the U.S. EPA.

Biological treatment for cleaning up polluted environments has became a potential alternative to conventional physical and chemical methods since it presents several advantages, such as low-cost and it provides a definitive solution. 2,4-DNT-degrading bacteria have been isolated from contaminated sites around the world [19–21]. All bacterial strains appear to follow the same degradation pathway (Scheme 1) [19,20]. This catabolic pathway has been extensively investigated at biochemical and genetic levels in the degrading bacteria *Burkholderia* sp. strain DNT and *Burkholderia cepacia* strain R34 [21–28]. The first step in the pathway involves a 2,4-DNT dioxygenase (DntA) that catalyzes the oxidation of 2,4-DNT to 4-methyl-5-nitrocatechol (4M5NC) with concomitant release of nitrite [21,28]. The second nitro group is removed from the aromatic ring by a monooxygenase (DntB) yielding 2-hydroxy-5-methylquinone (2H5MQ) that in turn is reduced to 2,4,5-trihydroxytoluene (2,4,5-THT) by a reductase (DntC) [22,23]. The resulting 2,4,5-THT undergoes a *meta*-cleavage catalyzed by a dioxygenase (DntD) producing 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA) [24,25]. A CoA-dependent methylmalonate semialdehyde dehydrogenase (DntE) and a bifunctional isomerase/hydrolase (DntG) catalyze the subsequent reactions to produce pyruvate and propionyl-CoA, which finally enter the central metabolic pathways of the cell [26].

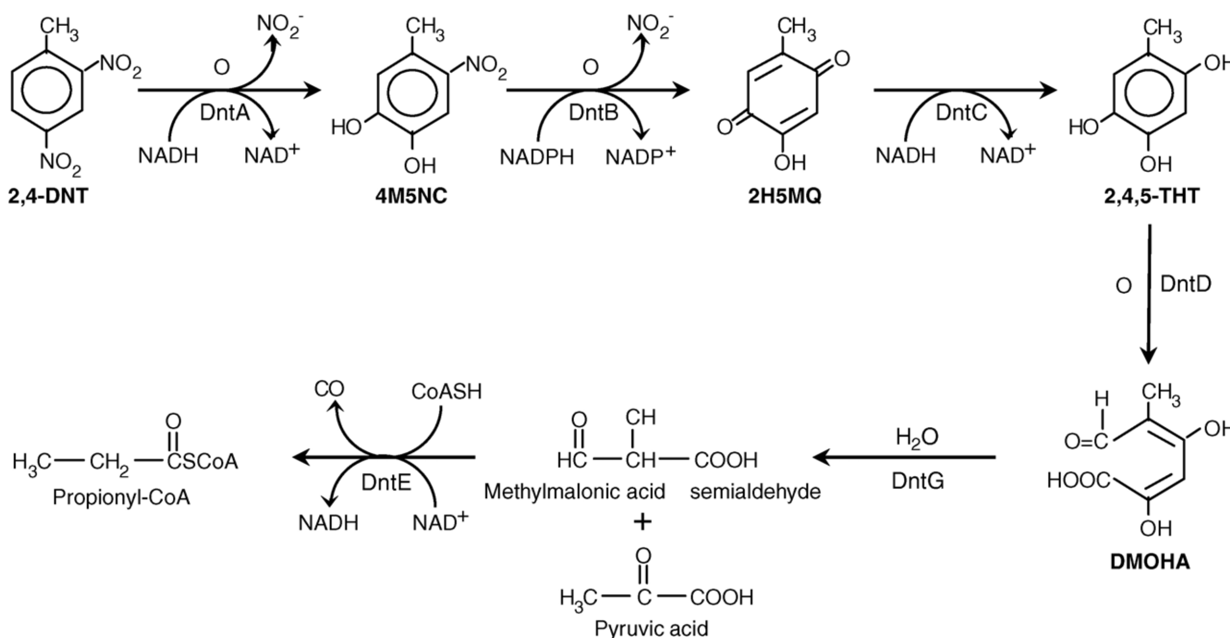
Previous reports have demonstrated the development of bioremediation strategies that exploit the ability of aerobic bacteria to completely degrade 2,4-DNT presents in polluted materials. Fluidized-bed and soil slurry reactors are effective ex situ methods for treatment of wastewaters and polluted soils, respectively [29–32]. To ensure the success of these bioremediation strategies, several factors must be optimized on the engineering design. Particularly, a real time monitoring of the biodegradation process is an important factor in the operation of the bioreactor system [32].

In the following sections, we demonstrate the affinity of PPO for two important compounds originated from the biological degradation of 2,4-DNT and propose the use of the glassy carbon paste electrode modified with PPO as an important tool to evaluate the efficiency of the biodegradation process.

2. Experimental

2.1. Reagents

Catechol and mushroom polyphenol oxidase (or Tyrosinase E.C. 1.14.18.1, 1030 U/mg) were purchased from SIGMA. 2,4-dinitrotoluene (2,4-DNT) (99% of purity) was obtained from Fluka. 4-Methyl-5-nitrocatechol (4M5NC) and 2,4,5-trihydroxytoluene (2,4,5-THT) were kindly provided by Jim C. Spain, Tyndall Air Force Base, FL. 2-Hydroxy-5-methylquinone (2H5MQ) was obtained by spontaneous oxi-



Scheme 1. Pathway for 2,4-DNT degradation in *B. cepacia* strain R34. The enzymes are: DntA, 2,4-dinitrotoluene (2,4-DNT) dioxygenase; DntB, 4-methyl-5-nitrocatechol (4M5NC) monooxygenase; DntC, 2-hydroxy-5-methylquinone (2H5MQ) reductase; DntD, 2,4,5-trihydroxytoluene (2,4,5-THT) oxygenase; DntG, 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA) isomerase/4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoate hydrolase; DntE, methylmalonate semialdehyde dehydrogenase. NO_2^-, nitrite; CoASH, coenzyme A.

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