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Physical and chemical immobilization of choline oxidase onto different porous solid supports: Adsorption studies

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ABSTRACT

This work carries out for the first time the comparison between the physical and chemical immobilization of choline oxidase onto aminated silica-based porous supports. The influence on the immobilization efficiency of concentration, pH, temperature and contact time between the support and choline oxidase, was evaluated. The immobilization efficiency was estimated taking into consideration the choline oxidase activity, which was assessed by using cadmium telluride (CdTe) quantum dots (QDs), obtained by hydrothermal synthesis, as photoluminescent probes. Hydrogen peroxide produced by enzyme activity was capable of quenching CdTe QDs photoluminescence. The magnitude of the PL quenching process was directly related with the enzyme activity.

By comparing the chemical process with the physical adsorption, it was observed that the latter provided the highest choline oxidase immobilization. The equilibrium data were analyzed using Langmuir and Freundlich isotherms and kinetic data were fitted to the pseudo-first-order and pseudo-second-order models. Thermodynamic parameters, such as Gibbs free energy and entropy were also calculated. These results will certainly contribute to the development of new sensing schemes for choline, taking into account the growing demand for its quantification in biological samples.

1. Introduction

Choline is a fundamental nutrient for the normal role of cells and its daily intake was recommended by the U.S. National Academy of Sciences [1]. It appears and plays highly important functions in different organs, such as kidney, lung, liver and placenta [2,3]. Choline is also the precursor and the metabolite of acetylcholine, a neurotransmitter implicated on cholinergic synapses [4], and consequently related with different neural disorders such as Alzheimer's disease [5,6], myasthenia gravis and schizophrenia [7,8]. Thus, the inhibition of the acetylcholinesterase activity is a general mechanism of action of the cholinergic drugs used in the treatments of this kind of diseases and also of the pesticides as organophosphates and carbamates [9]. Therefore, analytical methods for the determination of anti-acetylcholinesterase activity are necessary. However, choline and acetate, the enzymatic products of acetylcholine, are electrochemically and optically inert for common chemical analysis [10–12]. Thus, the development of biosensors for choline determinations and acetylcholinesterase inhibitory tests has been based on immobilized choline oxidase that converts choline into betaine and H_2O_2 that can be easily detectable.

For the immobilization of choline oxidase, some different supports and processes have been reported. Wang et al. [3] described the immobilization onto the multi-walled carbon nanotubes modified glass carbon electrodes, while Ricci et al. [13] described the immobilization on the surface of Prussian Blue modified screen-printed electrodes using Nafion and glutaraldehyde. There are also other works where the choline oxidase is immobilized: on a natural chitinous membrane obtained from the *Mictyris brevidactylus* soldier crab [14,15], on bovine serum albumin [4,16,17], on a pre-activated nylon membrane [18], on an oxygen sensitive layer formed by a luminescent Ru(II) complex, tris(4,-diphenyl-1,10-phenanthroline)ruthenium(II), dispersed in a silicone membrane [19], on sepharose beads [2], on an array using a water-based polyurethane/polyethylene oxide colloidal dispersion matrix [20], on a silica-monolith functionalized with polyethylenimine (PEI) polymer [21], and on a stainless steel column packed with alkylamino-bonded silica [22].

In this work it is intended, for the first time, to compare physical and chemical immobilizations of choline oxidase using aminated porous solid supports. Based on our previous experience [23,24],

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it is expectable that this kind of supports will allow the enzyme immobilization through physical adsorption and the establishment of chemical linking. The chemical process should be easy to perform and the physical one should also allow the reagentless immobilization, contributing to the current demands of Green Chemistry [25]. The performance of chemical and physical immobilizations is thoroughly studied as well as the influence on the immobilization efficiency of pH, concentration, contact time between the solid and the choline oxidase and temperature. These studies were based on the evaluation of enzymatic activity using cadmium telluride (CdTe) quantum dots (QDs) as photoluminescent probes, which were also synthesized and characterized during this work. Choline oxidase converts choline into betaine and H_2O_2 that can quench the QDs' photoluminescence [26]. Thus, the QDs photoluminescence quenching is directly proportional to the enzymatic activity.

Based on our previous experience in the immobilization of QDs onto aminated solid supports [27], adsorption isotherms, kinetic, and thermodynamic parameters for the adsorption procedure were calculated. The adsorption equilibrium was evaluated according Freundlich and Langmuir isotherms. Kinetic data were adjusted to the pseudo-first-order and pseudo-second-order models and Gibbs free energy and entropy were the calculated thermodynamic parameters. The stability of the immobilized choline oxidase was also evaluated.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared using high purity water (milli Q) with a specific conductivity <0.1 $\mu S\,cm^{-1}$. The reagents used were analytical grade.

Buffer solutions of Tris-HCl (2-Amino-2-(hydroxymethyl)-1,3-propanediol-HCl) with pH values between 7 and 9 were obtained by mixing appropriate volumes of 0.1 mol L^{-1} HCl (Panreac, Barcelona, Spain) and 0.1 mol L^{-1} Tris (Sigma-Aldrich, St. Louis, MO, USA). An acetate buffer solution (0.2 mol L^{-1}) with a pH of 6 was prepared by mixing adequate volumes of 2.0 mol L^{-1} sodium acetate (Sigma-Aldrich) and 2.0 mol L^{-1} acetic acid (Pronalab, Lisbon, Portugal) and diluting in water.

A glutaraldehyde solution (2.5% (w/w)) was obtained using an appropriate volume of a commercial solution (Sigma-Aldrich) diluted in water.

Choline oxidase and choline solutions were daily prepared by dissolving a proper amount of the commercial powders (Sigma-Aldrich) in buffer solution. QDs aqueous dispersions were daily prepared by dissolving in water, an appropriate amount of the dried nanocrystals.

2.2. CdTe quantum dots synthesis

The reagents used for the aqueous synthesis of MPA-capped CdTe QDs were cadmium chloride hemi(pentahydrate) ($CdCl_2{\cdot}2.5H_2O$, 99%), tellurium powder (200 mesh, 99.8%), sodium borohydride ($NaBH_4$, 99%) and 3-mercaptopropionic acid (99%), which were all purchased from Sigma–Aldrich (St. Louis, MO, USA).

These nanoparticles (MPA-CdTe QDs) were synthesized in accordance to the two-stage process method described by Zou et al. [28], but with some modifications wherein the molar ratio of Cd^{2+}:Te^{2-}:MPA was established at 1:0.1:1.7. In the first step, the complete reduction of tellurium with sodium borohydride in N_2 saturated water occurred thus producing NaHTe [29]. Then, the obtained solution was transferred into another flask containing 4.0×10^{-3} mol of $CdCl_2$ and 6.8×10^{-3} mol of MPA in 100 mL N_2 saturated aqueous solution with a pH previously adjusted with a 1.0 molL^{-1} NaOH solution to 11.5. The desired size of nanocrystals was obtained by controlling the refluxing time. Finally, the nanoparticles in crude solution were precipitated in absolute ethanol (Panreac, Barcelona, Spain) [30] in order to remove the contaminants. Thereafter, the precipitate was separated by centrifugation, dried under vacuum and held in amber flasks.

2.3. Instrumentation

In order to perform the characterization of the synthesized QDs it was used a luminescence spectrometer (Perkin Elmer LS-50B) (Waltham, MA, USA) to measure the QDs photoluminescence and a spectrophotometer (Jasco V-660) (Easton, MD, USA) to perform the absorption spectra between 400 and 700 nm of the QDs colloidal solutions. To evaluate the X-ray powder diffraction (XRD) studies it was used a Philips X'Pert X-ray MPD diffractometer (Cu Kα radiation). For XRD data collection a scan rate of 40.0 s for step at step intervals of 0.04° was used.

A refrigerated centrifuge (Thermo Electron Jouan BR4I) (Waltham MA, USA) was used to separate the precipitated QDs from the excess of reactants in the end of the synthesis process.

A shaking water bath (Clifton—NE 5) was used during the immobilization process in order to control the temperature and stirring.

A Synergy HT, BIO-TEK microplate reader was used for the measurement of QDs photoluminescence quenching caused by H_2O_2 that was resulted from the enzymatic reaction.

2.4. Choline oxidase immobilization process

In order to perform the immobilization of choline oxidase two aminated porous solid supports were selected. One of them was the aminoalkylated glass beads (aminopropyl-CPG-170 Å, 120–200 mesh, Fluka) (CPG) and the other one the aminopropyl-modified silica (Chromabond NH_2, 60 Å, Marcherey-Nagel) (CB).

Chemical and physical processes of immobilization were performed in order to verify which one is involved in the immobilization or even if the both could happen simultaneously. For the chemical approach it was used the pre-activation of the solid support with glutaraldehyde [31]. This procedure started with the incubation of 0.010 g of CPG or CB solid supports in 1.25 mL of a 2.5% (w/w) glutaraldehyde solution for 1 h. During the first 30 min, it was performed a nitrogen purging every 10 min. After this incubation, it was removed the unreacted glutaraldehyde, washing the solid support with water and Tris-HCl buffer solution. After the pre-activation it was promoted the contact between the solid support with 800 μL choline oxidase solution for 4 h. During the first 30 min, it was purged nitrogen every 10 min.

For the physical immobilization, the process was similar to the previously described for the chemical immobilization but without the pre-activation of the solid support with glutaraldehyde. Choline oxidase was in contact with the solid support for 4 h.

Both in chemical and in physical procedures, after the incubation of the choline oxidase with the solid support, the non-immobilized enzyme was removed using centrifugations and washes with water and Tris-HCl buffer solution.

2.5. Immobilization efficiency

The calculation of the immobilization efficiency was based on the measurement of the choline oxidase that remains in solution after the contact between the enzyme solution and the solid support, using the following equation (Eq. (1)).

$$\%Ads = ((C_0 - C_e)/C_0) \times 100 \quad (1)$$

where C_e and C_0 are the equilibrium and the initial concentrations (mg L^{-1}), respectively.