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Research Paper

Enzymatic DMFC device used for direct analysis of chloramphenicol and a comparison with the competitive immunosensor method

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ABSTRACT

This paper aims at investigating the possibility to use a catalytic "direct methanol fuel cell" (DMFC), previously optimized, for the analysis of chloramphenicol. The alcohol dehydrogenase enzyme was immobilized within it, for a direct analysis of the antibiotic. The analytical results confirmed the validity of this idea and highlighted the advantages, especially those associated with a remarkable decrease in the response time. A comparison with the use of the same device, but without the alcohol dehydrogenase enzyme (ADH), was made. The LOD was 8×10^{-7} M, while a 1.0–5.0 mM linear range was obtained in the presence of chloramphenicol. This also shows a high specificity for chloramphenicol. Finally, the main analytical data were compared and discussed with those obtained using a conventional amperometric immunosensor, assembled by us to this purpose, which uses a typical competitive format.

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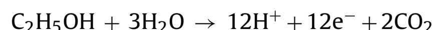
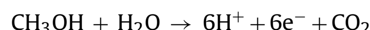
1. Introduction

Chloramphenicol is a drug with bacteriostatic antimicrobial properties, originally generated from the bacterium *Streptomyces venezuelae*. Its action is due to the protein inhibition effect, so chloramphenicol is effective in the treatment of several infectious diseases. Chloramphenicol was introduced into clinical practice in 1947 [1,2], however its ready availability and low cost has made it extensively used since the 1950s for treating animals all over the world. Nevertheless, it has the potential to cause serious toxic effects on humans and animals when given by mouth or injection [3]. Its use, therefore, should be limited to the treatment of serious infections [4], but, due to its low price and steady antibiosis effectiveness, illegal use of chloramphenicol in livestock and aquaculture still exists. Therefore, it is important to develop sensitive, analytical methods for determining residues of this antibiotics in foods and waters, including chromatography [5], e.g. liquid chromatography-tandem mass spectrometry [6,7], microbiological assay [8], enzymatic assay [9], SPR immunoassay [10], sensitive biotin-streptavidin amplified enzyme-linked immunosorbent assay (BA-ELISA) [11] and other new methods [12–15]. Lastly, in recent years more rapid sensor methods have also been proposed [16], such as aptameter-based colorimetric assay [17], optical sensing system using molecularly imprinted polymers (MIPs) [18], biotin-avidin-conjugated metal sulphide nanoclusters for electrochemical immunoassay [19], indirect competitive enzyme-linked immunosorbent assay (ELISA) with chemiluminescent (CL) detection [20], surface plasmon resonance (SPR) test [21], and a novel label-free folding induced aptamer-based electrochemical biosensor [22–26], however there are quite sophisticated. On the contrary, our approach to this analytical problem, described in the present paper, is quite simple.

In fact our team has recently investigated the opportunity to use, for analytical purposes [27], a small DMFC, originally built with the intent of obtaining energy from methanol or ethanol, and based on the following reactions:



$$C_2H_5OH + 3H_2O \rightarrow 12H^+ + 12e^- + 2CO_2$$



$$CH_3OH + H_2O \rightarrow 6H^+ + 6e^- + CO_2$$

Analytical applications to hydroalcoholic beverages showed good results in terms of precision data and recovery test [27]. The method was also validated by comparing the data obtained with method with data obtained by using two conventional enzymatic amperometric methods previously standardized by the authors [27]. However, research aimed at using the DMFC for analytical pur-

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poses, has further investigated this hypothesis [28], in particular about the possibility of improving the features, from an analytical point of view, of the catalytic fuel cell, by introducing an enzyme, immobilized into a dialysis membrane small bag, in the anodic area. This objective has been fully achieved, using the alcohol dehydrogenase enzyme, which has increased the sensitivity of the method and reduced dramatically the response time of the fuel cell to ethanol or methanol [28].

In addition, in previous paper [28], the response of the fuel cell to other types of alcohol, was also evaluated. It was experimentally established that, although the sensitivity to other more complex alcoholic molecules is not high, it is still enough for determining particular types of alcoholic compounds. For instance, real specific samples, such as pharmaceutical compounds containing an alcoholic group, are so accrued. In particular, the application to antibiotics containing (—OH) functional groups, some of which may also have poor alcoholic features, such as imipenem (a β-lactam antibiotic), or chloramphenicol, another type of not β-lactam antibiotic, showed interesting analytical results [28]. The only inconvenience that has already been observed in the previous paper [27] using the fuel cell for analytical purposes, was a longer measurement time, if compared, for instance, with ordinary enzymatic biosensors [29] or immunosensor methods to check other antibiotics [30]. In the present research, therefore, we tried to reduce the measurement time, for one of two antibiotics detection (i.e. chloramphenicol), also to reduce the response time of the fuel cell, by associating the alcohol dehydrogenase enzyme, to the catalytic device. In fact, according to trials, the alcohol dehydrogenase enzyme can contribute to speed up the oxidative process taking place in the fuel cell, also with chloramphenicol. In addition, in the present paper, we have also compared the obtained analytical features of the enzymatic fuel cell, with analogue features found using another biosensor, i.e. a conventional competitive amperometric immunosensor for chloramphenicol developed by us for this purpose. An analytical comparison of the two devices and their application to pharmaceutical formulation was therefore reported.

2. Materials

Alcohol dehydrogenase (from Saccharomyces cerevisiae E.C.1.1.1.1, CAS: 9031-72-5), was supplied by Sigma-Aldrich (Milan, Italy). The standard antibiotic solutions, used to measure the fuel cell, were obtained by diluting with distilled water, a known quantity of chloramphenicol (CAS: 56-75-7), supplied by Sigma Aldrich (Milan, Italy). The anti-chloramphenicol monoclonal antibody was provided by Acris (Acris Antibodies GmbH, Herford, Germany), while magnesium chloride, potassium chloride, dibasic and monobasic anhydrous potassium phosphate RPE were supplied by Carlo Erba Reagents (Carlo Erba, Milan, Italy). Ny+ Immobilon Affinity membrane (porosity 0.65 mm) was provided by Millipore (Millipore Corporation, Billerica, MA, USA). BiotinTag™ Micro Biotinylation Kit, consisting of a biotinylation reagent (BAC-SulfoNHS, namely biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinide ester), 5 M sodium chloride solution, micro-spin column (2 mL) (in practice, a small empty pre-packaged cylindrical vessel with Sephadex G-50), 0.1 M sodium phosphate buffer pH 7.2, 0.01 M phosphate buffer saline (PBS) pH 7.4 (reconstituted with 1 L of deionized water to give 0.01 M phosphate buffer, 0.138 M NaCl, 2.7 mM KCl, pH 7.4) and ExtrAvidin® peroxidase (containing 0.2 mL of ExtrAvidin Peroxidase conjugate at 2.0 mg mL^{-1}, with 0.01% thimerosal), dialysis membrane (art. D-9777), albumin from bovine serum (BSA), TRIS (hydroxymethyl-aminomethane) and TWEEN® 20 were all provided by Sigma-Aldrich, (Milan, Italy).

2.1. Samples

Both analysed drugs, which are injectable pharmaceutical formulations, containing chloramphenicol, have been purchased at a hospital drugstore.

2.2. Apparatus

2.2.1. Fuel cell apparatus

For fuel cell measurements, a DMFC H-TEC, model F111 (Fig. 1), weighing about 100 g, was obtained from the fuel cellStore (College Station, TX, USA). The electrode area was about 4 cm^2 and the maximum generated power was 10 mW. The fuel cell frame was made in Plexiglas®, while the electrode end plate was made in Pt-Ru black catalyst, assembled with Nafion™ membrane. For potentiostatic format measurement, Palmsens model EmStat potentiostat was used, connected to the fuel cell. The current supplied to the cell was so recorded and collected, with data interface by PSTrace Software (ver. 4.6) to Compaq Presario PC for data acquisition. For measurements in the presence of the alcohol dehydrogenase enzyme, a weighed quantity of the enzyme (5 mg of alcohol dehydrogenase) was placed on a small dialysis membrane cylindrical bag, with a drop of phosphate buffer. After positioning cautiously into the dialysis membrane bag a rigid plastic stick, a sort of cylindrical stiff bag is so held, sealed at the top, inside which the mush of the enzyme was contained. The bag was placed into the anode area of the fuel cell [28] (see Fig. 1) before the measurement. The successive measurement format was then the same as above described for the non-enzymatic fuel cell [27,28].

2.2.2. Apparatus for conventional amperometric immunosensor measurements

For chloramphenicol analysis a potentiostatic Biosensor Detector (model 3001 ABD) for amperometric measurement was used, connected to a hydrogen peroxide electrode (4006a model), both from Universal Sensor Inc. (New Orleans, USA), and a Omniscribe analogue recorder (d5126-2 model), Houston Instrument (Houston, USA). The test solution was contained in a cell thermostated at 25 °C and under constant magnetic stirring (291/lf), Amel Instruments (Milan, Italy).

3. Methods

3.1. Enzymatic fuel cell measurement and calibration curve

Fuel cell measurements and calibration curves were obtained by using the fuel cell working in potentiostatic format mode [27,31–33], the supplied current (SC) through the cell was therefore measured. The Palmsens potentiostat (EmStat model) was used connected to a PC, for data acquisition and processing. The fuel cell anode was connected to EmStat as a working electrode, while the fuel cell cathode was connected to EmStat as the reference and counter electrode. Before current measurement, EmStat automatically measured the open circuit voltage (OCV) [27,33,34] value, for about 200 s. Then, the anode potential was set to a value of the optimized applied potential (OAP) [32,33,35] (i.e., OCV minus 100 mV), experimentally fixed in a previous paper [27]. The fuel cell, before the measurement, was carefully washed several times with distilled water. Afterwards, the fuel cell was filled with the solution to be analysed (2 mL) and closed. Then the measurement could begin after conditioning the system for about 60 s. A calibration curve was built by using distilled aqueous standard solutions, containing increasing amounts of chloramphenicol, which were gradually added to the fuel cell. The current supplied by the cell was recorded each time [27,32,33,35] (see Fig. 2). When the (SC) had reached a stationary state value, its value was gradually read. The current

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