Biochemical Engineering Journal 126 (2017) 78–85

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Electrochemical detection of estrus specific phenolic compound *p*-cresol to assess the reproductive phase of certain farm animals

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ARTICLE INFO

Article history:
Received 26 January 2017
Received in revised form 31 May 2017
Accepted 14 June 2017
Available online 15 July 2017

Keywords:
Hydroxyapatite
p-cresol
Biosensor
Voltammetric method
Estrus
Buffalo
Artificial insemination

ABSTRACT

p-Cresol (4-Methylphenol) is one of the naturally occurring volatile phenolic compounds in the female mammalian animals urine during estrus, the period of time when the animals become sexually receptive and release specific odors that are induced by reproductive hormones. We have developed an electrochemical sensor for *p*-cresol detection using hydroxyapatite nanoparticles (HA NP) for the first time. The HA nanoparticles have been synthesized by microwave irradiation method using *plectranthus amboincus* plant-extract as solvent and its properties have been characterized by XRD, FTIR, and SEM methods The plant-extract-mediated HA NP modified glassy carbon electrode (GCE) exhibited improved electrocatalytic behavior towards *p*-cresol sensing compared to bare GCE and pristine HA NP/GCEs. Under optimized conditions, the proposed sensing platform permits the detection of *p*-cresol over a wide range of 0.275–23.5 × 10^{-6} M with the lowest detection limit of 116 × 10^{-9} M (S/N = 3) in phosphate buffer solution (pH 6.0).The fabricated sensor has high selectivity towards *p*-cresol in the presence of potential interferents such as nitrophenols, bisphenol A, catechol, hydroquinone, uric acid, glucose, folic acid, ascorbic acid and metal ions Na^+, K^+ and Mg^{2+}. The sensor has been successfully used to detect *p*-cresol in female buffalo urine which eventually indicates its readiness for artificial insemination. This device will be helpful to enhance the success rate of pregnancy in buffalo and other farm animals.

1. Introduction

The species specific signals, commonly called pheromones, are known to coordinate reproductive and social activities in mammals. The pheromone compounds secreted through urine, feces, vagina, saliva, and specialized scent glands in hair and wool during the estrous cycle indicates the timing of the physiological event of ovulation [1]. *p*-Cresol is one of the significant pheromone compounds and it is shown to be predominantly high in concentration in female buffalo urine during estrus period [2]. At this time, the female undergoes ovulation and exhibits several unique behaviors, releases specific odors that attract the male for natural coitus. This is the accurate time for insemination which results in high success rate of conception. The detection of estrus and diagnosing early pregnancy are important events in farm animals more particularly in buffaloes [3] for effective breeding. Buffaloes are the premier diary animals contributing more than 50% of milk production in India. It is estimated that $5.5 USD per animal per day is lost if one estrus event is missed [4].

The *p*-cresol has been identified as specific volatile compound in the urine of Alaskan deer [5] and rat [6] which showed attraction towards conspecific. Furthermore, the *p*-cresol is a lone pheromone compound identified in horse urine during estrus that provides information about the timing of ovulation in mare [7]. Since the *p*-cresol is present not only in the urine but also in other body fluids such as saliva [8] and faeces [9], this unique compound can be considered as a promising 'estrus indicator'. It is also involved in enhancing the bull libido through stimulating the neuroendocrine system of the male buffalo to increase the sperm quantity [10]. Hence, the identification of estrus period in such animals is an urgent need for effective use of mediated reproductive techniques like artificial insemination and in vitro fertilization/embryo transfer. Here, we propose to track estrus in buffalo by targeting *p*-cresol detection as one of the strategies for the purpose of enhancing livestock production.

Various techniques such as liquid chromatography [11], flow-injection analysis [12], and gas chromatography–mass spectrometry [13] have been evolved for detection of *p*-cresol. In

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http://dx.doi.org/10.1016/j.bej.2017.06.012
1369-703X/

recent years, electrochemical methods are readily available for determination of phenolic groups because of its huge advantage such as high sensitivity, long-term stability, good reproducibility, less time consuming, portability and low cost [14]. To date only a few modified electrodes have been reported for the electrochemical determination of *p*-cresol which includes MWNT-Nafion-Tyr/GCE [15], SWCNT/GCE [16], RGO-MWCNT Hybrid/GCE [17], MWCNT/DTDAB/Tyr-NCPE/CPE [18], Tyrosinase/BDND thin film [19], and PAB/PBO-Laponite electrode [20]. However, none of these electrodes have been used to detect *p*-cresol in real samples such as female buffalo urine. Here, we report the fabrication of a new electrode based on hydroxyapatite nanoparticles and its application for the detection of *p*-cresol in female buffalo urine.

Hydrxoyapatite [HA,$Ca_{10}PO_4(OH)_2$], one of the major components of bone, readily absorbs a large amount of impurity ions and is seldom found in its stoichiometric pure form. Matsunaga et al. [21] have demonstrated that Ca^{2+} vacancy in the crystal structure of HA occurs significantly more easily in the surrounding aqueous acidic solution. Especially, protons play role in stabilizing the Ca^{2+}vacancy in a lower pH condition as charge compensating defects. This ion exchange ability of HA favors the distribution of a number of divalent trace elements in place of Ca^{2+} ion which leads to significant changes in physical properties. In addition, HA NP have some unique advantages like high porosity, large surface area, and easy to synthesis in bulk quantities. Several novel routes such as wet, dry, hydrothermal, sol-gel and microwave methods have been developed to synthesize the HA NP [22].

Electrocatalytic behavior of HA NP can vary by size and shape which in turn depends on the synthesis process. Biosynthetic methods using plant-extract is known to influence the crystal size and morphology of HA NP [23,24]. Moreover, it is an eco-friendly and a less expensive procedure when compared to other chemical methods. *Plectranthus amboincus*, also known as karpooravalli, is a fleshy perennial plant in the family of Lamiaceae and it has been traditionally used for curing illness like as antipyretic, antioxidative, analgesic and anti-inflammatory activities of mankind. The plant-extract contains a large number of organic chemical compounds such as alkenes, amides, alkyl halides, aliphatic and aromatic groups [25] which have the potential to hyper-accumulate and biologically reduce the size of the nanoparticles during their growth at room temperature. Hence, we have synthesized HA NP by microwave irradiation method using *plectranthus amboincus* plant-extract as solvent and demonstrated its applicability for the detection of *p*-cresol by electrochemical method for the first time.

2. Experimental section

2.1. Synthesis of hydroxyapatite nanoparticles

$Ca(NO_3)\cdot 4H_2O$ (99.0%) and $(NH_4)_2HPO_4$ (98.0%) were purchased from Merk Private Limited, Mumbai. *p*-Cresol or 4-methyl phenol (C_7H_8O) was purchased from Sigma Aldrich. All other chemicals were of analytical grade and used without any further purification. 0.1 M phosphate buffer solution was prepared from Na_2HPO_4 and NaH_2PO_4 (Merck, Mumbai) and the pH was adjusted to 6.0 by adding 0.1 M HCl. Deionized water was used throughout the experiments.

The *plectranthus amboincus* plant-extract solution was prepared from a 70 g portion of thoroughly washed plant leaves which were finely cut and boiled in deionized water. The resulting extract was used as solvent for preparing HA. Initially, 0.24 M $(NH_4)_2HPO_4$ solution was added drop wise into 0.4 M ($CaNO_3\cdot 4H_2O$) with constant stirring for 1 h at room temperature. During the reaction, the pH of the solution was maintained at 10 by using ammonia solution. The mixture was then exposed to microwave radiation (600W) for 20 min, which resulted in a light brown color paste like product. This sample was washed with deionized water and dried at 80 °C in a hot air oven.The resultant powder is whitish brown in color. In order to investigate the influence of plant-extract-mediation on the synthesis and properties of Kv-HA NP, another series of HA NP were prepared under identical conditions, by using only deionized water as solvent. When compared with the whitish brown coloured Kv-HA NP from plant-extract solvent, water based HA NP were white in color.

2.2. Instrumentation

Electrochemical measurements were carried out using a three electrode cell system (CHI 609D workstation). Experiments were carried out at room temperature using a modified glassy carbon electrode as the working electrode, 3 M KCl containing saturated Ag/AgCl as the reference electrode and a platinum wire electrode as the counter electrode. Cyclic voltammograms (CVs) were acquired at the scan rate 50 mVs^{-1} in 0.1 M phosphate buffer containing 50 μM of *p*-cresol. The structure and morphology of the hydroxyapatite were characterized by powder X-ray powder diffraction (XRD) with XPERT-PRO diffractometer with CuKα radiation (1.5406 Å) at 40 kV and scanning electron microscopy were recorded using Philips XL 30-ESEM. Fourier transform infrared (FTIR) data were recorded from 4000 to 400 cm^{-1} at room temperature using Thermo Nichlolate system. Specimens were prepared by mixing HA NP (0.5 mg) and KBr (200 mg) powders, mixing with mortar and pestle and pressing under high pressure of about 400 bar for 1 min. Thermogravimetric (TG) analysis of the synthesized powder was performed in a NETZCH STA 4491 instrument between 30 and 1400 °C in air ambient at a heating rate of 10 °Cmin^{-1}.

2.3. Electrode preparation

Prior to modification, the GCEs (each 3 mm dia) were polished with 0.05 mm alumina powder on polishing cloth, rinsed thoroughly with deionized water between each polishing step, then sonicated and dried at room temperature. The modified electrodes were prepared by a simple drop casting method. 5 mg of HA NP were dispersed in 1 mL of deionized water and the mixture was ultrasonicated for about 30 min to get a stable and uniform suspension.10 μL of the above sonicated suspension was dropped on polished GCE surface and dried for about 2 h in room temperature.

2.4. Urine collection and confirmation of estrus

Urine samples were collected from heifers and lactating murrah buffaloes (*Bubalus bubalis*) (n = 6) at Krishi Vigyan Kendra veterinary farm, Karur (India). The animals were housed in sheds and paddocks, fed with standard diet and water *ad libitum*. The estrus phase of estrous cycle was assessed based on visual, behavioral and biochemical parameters in three consecutive cycles of each animal. Behavioral assay (frequent urination, swollen vulva, mucus discharge, raised tail, and restlessness) and sample collection were carried out under natural condition (from −10 to +10 day with day 0 corresponding to estrus) as per the protocol of Rajanarayanan and Archunan [26]. Male buffalo urine (n = 4) and human urine (n = 4) were also collected for the analytical purpose for cross checking and to rule out the possible presence of *p*-cresol in these samples.

3. Results and discussion

3.1. Characterization of hydroxyapatite nanoparticles

Fig. 1A shows the XRD patterns of the HA and Kv-HA NP synthesized by microwave irradiation method. The peak posi-