Biosensors and Bioelectronics 85 (2016) 226–231

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Use of 3,3',5,5' tetramethylbenzidine as new electrochemical indicator of DNA hybridization and its application in genossensor

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ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form
21 April 2016
Accepted 5 May 2016
Available online 6 May 2016

Keywords:
Biosensor
Electrochemical indicator
Tetramethylbenzidine
Diagnosis

ABSTRACT

Electrochemical tools are important biosensor platforms for disease diagnosis, due to their speediness, easiness, low cost and portability. However, for DNA detection, the use of indicators and/or intercalators is necessary to improve electrochemical sensitivity.

Currently, ethidium bromide (EthBr) is the cheapest and most used DNA intercalators, but presents carcinogenic and teratogenic properties. Other indicators may be important for DNA photonic detection, and besides being more expensive, they behave similarly to EthBr.

This investigation shows for the first time the use of tetramethylbenzidine(TMB) as a new remarkable non-carcinogenic DNA indicator for genosensing purposes, which may be used for nucleic acid detection of microorganisms, based on complementarity of base-pairing between probe and target molecules.

The results indicate that TMB can be used as a new electrochemical indicator readily applicable in genosensors, which is able to detect the hybridization of single stranded DNA probe with its complementary target strand. An additional advantage of TMB, beside its non-genotoxicity, is the electrochemical reduction property, which prevents interference of serum components and other oxidative samples in the electrochemical analysis.

1. Introduction

The sensor devices are important tools for relatively rapid assays, inexpensive and easy to use for diagnosis of disease. The use of electrochemical indicator in biological sensors is a tool that promotes the improvement of the sensitivity of the diagnostic system. Ethidium bromide (EthBr) is used like indicator in nanotechnology labs because it is cheaper, widely used, but its use is restricted due to its carcinogenic and teratogenic properties. Other indicators that do not have these features are expensive and difficult to import, preventing its use in routine diagnosis. Under these conditions the search for new electrochemical indicators is proposed for genosensors without the carcinogenic property placing researchers and users at risk and it is an affordable focus of numerous works on reagents industries (Hosseini et al., 2014; Bojdi et al., 2014a, 2014b, 2015a, 2015b, 2016).

The use of electrochemical indicators in genosensors shows the process of hybridization and improves the system sensitivity. For the DNA analyses are used suitable size and chemical nature compounds that bind to the propeller via sliding and interleaving between pairs of adjacent base portions in the structure. In the presence of single strand of DNA is shown definite signs, but it is certain that in the presence of double stranded DNA which may or may not greatly increases its signal strength (Le Pecq and Paoletti, 1967; Waring, 1965).

These compounds are mostly planar aromatic and so there are several ways in which the compounds may interact with DNA by electrostatic bond or merge (Richards and Rodgers, 2007).

Many studies report the Ethidium bromide (EthBr) as a useful DNA indicator (Iost et al., 2011; Balvedi et al., 2014), especially at affordable price. The EthBr intercalates in the double-stranded DNA (Waring, 1965). The Acridine Orange [3,6-bis (dimethyl) hemihydrochloride acridine] (AO) is an organic cationic compound and generally used for determining the cell cycle since it is a selective nucleic acid dye. The methylene blue [3,7-bis (dimethylamino) phenol-trazin-5-ium chloride] (MB) is the a photosensitive agent and widely used as an alternative to ethidium bromide. It is a flat heterocyclic compound and may interact with DNA and RNA, but the exact form of its binding to the duplex DNA is unknown (Kelly et al., 1987; Tran et al., 2011; Tuite and Kelly, 1995; Wong et al., 2005). The hematoxylin (H) is used in biological histology in heart procedures which gives a bluish color to affinity region, basic dyes react with anionic components of cells and tissues

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http://dx.doi.org/10.1016/j.bios.2016.05.016
0956-5663/

(Nasirizadeh and Zare, 2009; Zare and Nasirizadeh, 2010; Nasirizadeh et al., 2011; Zare et al., 2006).

Hoechst dye is a member of blue fluorescent dyes family used for staining the DNA (Latt and Stetten, 1976). These bis-benzimides are the most commonly used due to having both spectra/similar incitement and their signaling colors used like electrochemical indicator (Kerman et al., 2009; Zhao et al., 2012).

Under these conditions the search about new indicators for DNA hybridization that are electroactive is the objective of study. The tetramethylbenzidine (TMB) in addition to possessing the described properties is able to discriminate the process of DNA hybridization. It is a chromogenic substrate used in immunohistochemical staining procedures, besides it is a visualization reagent used in enzyme-linked immunosorbent assays, the ELISA (Liem et al., 1979).

The TMB is a white crystal powder degraded by sunlight and by fluorescent lights. Due to TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes such as horseradish peroxidase. The resulting diimine causes the solution to take on a blue color and can be read on a spectrophotometer at a wavelength of 650 nm. Studies report that the TMB is not mutagenic by the Ames assay and did not induce tumor formation in a study of 24 rats arm (Chung et al., 2000, 2006; Holland et al., 1974). Thus it has been used as a substitute for carcinogenic compounds, such as benzidine and phenylenediamine (Yang et al., 2008, 2014.).

The sensor technologies are important tools for relatively rapid assays, inexpensive and easy to use for diagnosis of disease. Describing the use of TMB and H_2O_2 as a mediator/substrate in an immunoassay based on amperometric detection (Volpe et al., 1998), use the same methodology for the detection of *Salmonella typhimurium* (Salam and Tothill, 2009). Dongneng et al. (2013) were designed to detect DNA of *E. coli*. upon DNA hybridization, the horseradish peroxidase (HRP) as substrate using H_2O_2/TMB, generates an electrochemical signal in conformity to the current target DNA. The optimization of mediated transduction system has determined the effectivity of TMB in a combination of H_2O_2 for obtaining a maximum response of DNA detection (Heurich et al., 2011).

This paper shows the application of only TMB as a new indicator for a genossensor by using reductive mode could constitute an interesting strategy to avoid the electrochemical interference, and the electrochemical reduction of dissolved oxygen the solutions were screened with N_2 prior to any detection electrochemical.

2. Materials and methods

2.1. Reagents

All used reagents were of analytical grade and used without further purification. Ultra-high purity water (Master System, Gehaka, Brazil) used for the preparation of aqueous solutions. 4-Aminophenol (Acros Organics, Belgium, France) was prepared in H_2ClO_4 solution (0.5 mol L^{-1}) immediately before use. Phosphate buffer solution 0.1 mol L^{-1}, pH 7.4. All experiments were conducted at room temperature (25 ± 1 °C). The TMB solution (Sigma Aldrich, St. Loius, MO, USA) was prepared in ultra-high purity water (5.0 mmol L^{-1}).

The selected DNA fragment was Epstein-Barr virus (EBV) because this virus is associated with mononucleosis, tonsillitis, leukoplakia, Burkitt and Hodgkin lymphomas, nasopharyngeal/gastric carcinomas and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Bhimrao, et al., 2015; Young and Rickinson, 2004).

The oligonucleotides (Invitrogen Life Technologies, Brazil) with the following sequences: EBV1:5′-AGGGATGCCTGGACACAAGA-3′, EBV2:5′-TCTTGTGTCCAGGCATCCCT-3′, NM2: 5′-ACAACCCGTTGGACTAAC-3′ and mm2EBV2:5'-TCTTGTGT**GG**AGGCATCCCT-3′ referred to as: probe, complementary target, non-complementary target and mismatch (two-base), respectively. Stock solutions and buffer components were prepared and conducted like Balvedi route (Balvedi et al., 2014).

2.2. Apparatus

Electrochemical polymerization and voltammetric measurements were performed using a potentiostat (CH Instruments, model 460 C, Austin, TX, USA), with a graphite disk (6 mm diameter- 99.99%, Alfa Aesar) as working electrode. Platinum was used as counter electrode. All potentials are referred to the silver-silver chloride reference electrode (Ag/AgCl). The graphite surface, prior to electropolymerization, was mechanically polished with alumina slurry (0.3 μm diameter), ultrasonicated, washed with distilled water and dried in the air. All solutions were degassed by nitrogen bubbling (Balvedi et al., 2014).

2.3. Electrochemical polymerization

Poly (4-aminophenol) or poly (4-AP) films were electrodeposited by cyclic voltammetry onto the graphite electrodes from a solution containing mono (4-AP) in H_2ClO_4 solution. The electrochemical experiments were conducted at room temperature (25 ± 1 °C), 50.0 mV s^{-1}, −0.4 and +1.0 V, 100 scans, three-compartment cell, like Vieira route (Vieira et al., 2006).

2.4. Use of 3,3′,5,5′-tetramethylbenzidine (TMB)

Then, a single-stranded DNA specific for EBV (EBV1) drip was immobilized on the modified graphite electrode, the incorporation confirmed by differential pulse voltammetry (DPV), indirect detection with TMB. The hybridization of complementary target (EBV2) using phosphate buffer (pH 7.4) as supporting electrolyte.

3. Results and discussion

3.1. The TMB in different concentrations in graphite electrodes

The same hydrogen donor and electrons can leave the electrochemical studies to redox the TMB and electrochemical reduction analysis prevents serum components or other samples, to be oxidative and interfere in the results electrochemical analysis (Fig. 1(A)).

In order to be able to choosing a better concentration, different concentrations of the indicator were available for electrochemical analysis of TMB in graphite electrode and graphite electrodes modified with poly (4-AP). Tests with TMB this confirmed the best electron transfer in the presence of polymeric film poly (4-AP) as a significant increasing of 45% in the current response (Fig. 1(B) and (C)).

The functionalization of electrode surfaces with conducting polymer films is usually applied in electroanalytical techniques aiming to optimize the electrochemical detection of analytes, non-ionic redox biomolecules immobilization, particularly enzymes, antibodies and DNA for the development of biosensors (Higgins et al., 2012).

Tests with TMB in genosensors this confirmed the biomolecules immobilization and best electron transfer in the presence of polymeric film poly (4-AP) as a significant increasing of 60% in current response was observed (Fig. 1 (D) and (E)).

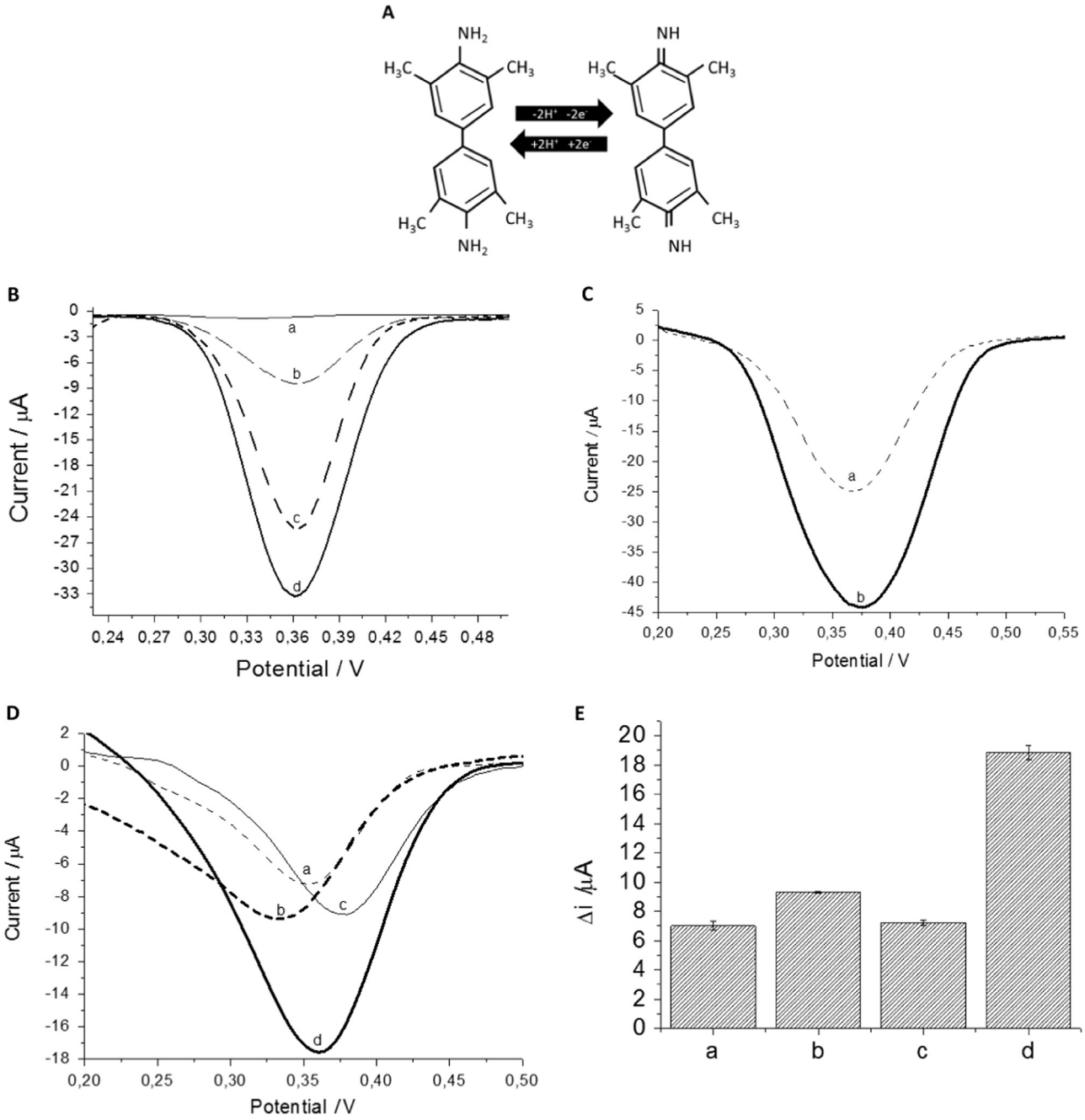

Fig. 1. : A. Redox of TMB. B. DPV of TMB in different concentrations in graphite electrodes (mmol L^{-1}): (a) 0.5, (b) 1.0, (c) 2.5, (d) 5.0, C. DPV of TMB in different concentrations in mmol L^{-1} onto graphite electrodes modified with poly (4-AP) : (a) 2.5, (b) 5.0. D. DPV using TMB: (a) probe, (b) probe- target, and graphite electrodes modified with poly (4-AP): (c) probe, (d) probe-target. E. Chart Bars shows the electrochemical sing of TMB in absence and presence of polymeric film in graphite electrode.

3.2. Hybridization time and specificity

The importance of the determining the best time for the best answer of TMB in genosensors is the study of hybridization (Fig. 2(A)).

After that, it has shown in the indirect detection, as monitored, if the peak reduction of TMB in +0.38 V and it was observed an modular increase in the current signal amplitude, caused by the accumulation TMB hybridized double-stranded DNA (EBV1: EBV2). The target (EBV1) recognized the complementary probe, the mismatch probe (mm2EBV2) and did not recognize no complementary probe (NM2) that were used in the specificity tests (Fig. 2(C)).

Study of hybridization time (EBV1:EBV2) for determined the best time in genosensors. The time of 30 min was shown as a better answer in 57 °C (Melt temperature).

In addition, the results showed an "increase" in the current signal amplitude, in presence of complementary target (EBV1: EBV2), indicating the accumulation of TMB on the surface of the modified electrode containing duplex. The current signal amplitude when the oligonucleotides containing two-base mismatch (EBV1: mm2EBV2) was fewer and has potential to differentiate mismatches, as required for detection of diseases caused by point mutations.

3.3. Calibration curve and stability study

The Fig. 3(A) showed the calibration curve in indirect detection evaluating the peak reduction of TMB of different concentrations of EBV2: 378nmol L^{-1}, 378.10^{-8} mol L^{-1}, 378.10^{-7} mol L^{-1},

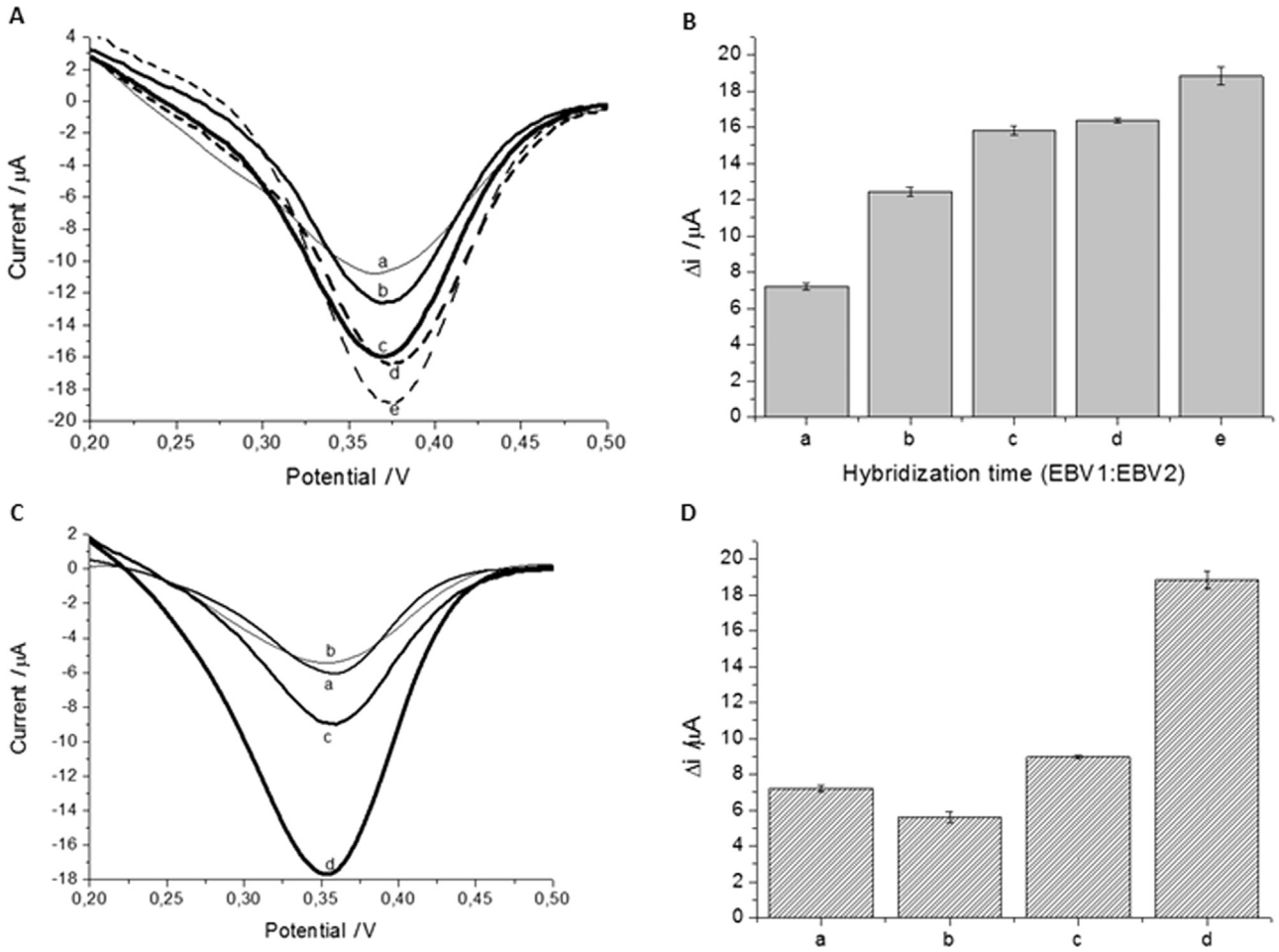

Fig. 2. : A. DPV for hybridization time (EBV1: EBV2): (a) EBV1, and EBV1: EBV2 in the times (b) 5, (c) 10, (d) 20, (e) 30 min (in 57 °C - Melt temperature). B. Bar chart of DPV. C. DPV of TMB (5.0 mmol L^{-1}) onto graphite electrode modified with poly(4-AP): (a) EBV1, (b) EBV1/NM2, (c) EBV1/mm2EBV2, and (d) EBV1/EBV2. D. Bar chart of DPV responses using the reduction signal from TMB in genosensor. Electrolyte: phosphate buffer (0.10 mol L^{-1}), pH 7.4. Modulation amplitude: 25 mV. Pulse interval: 0.2 s, Scan rate 13 mV s^{-1}.

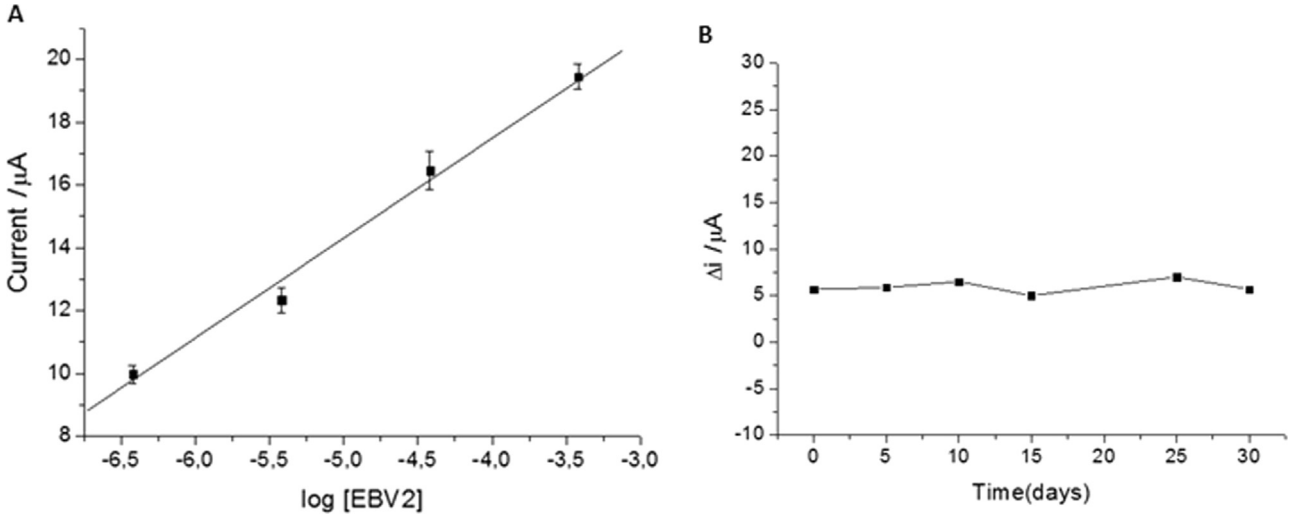

Fig. 3. : A. Electrochemical response for the reduction signal of TMB (5.0 mmol L^{-1}) obtained after the hybridization of modified electrode containing the probe EBV1 with log of different concentrations of EBV2 (378 nmol L^{-1} – 378 μmol L^{-1}). B. Storage stability profile of genosensor at 4 °C during 30 days.

378 μmol L^{-1} and graph showed a linear range of current peak vs. log(EBV2) concentration.

The calibration curve constructed from these results, showing that the system has linear correlation coefficient 0.995, a detection limit (LOD) 46.0 nmol L^{-1} and a quantification limit (LOQ) 153.318 nmol L^{-1}.

The stability study of the biological sensor poly(4-AP): EBV1 is shown in Fig. 3(B), the electrodes were at a temperature of 4 °C for 30 days, assays using TMB were performed. The results shows that the sensor response remains stable, without loss in biological

activity, indicating that the electrode modified contributed to this stability. The presence of amine groups favors the formation of covalent binding with oligonucleotides, indicating that a modification of the electrode surface, favors the stability and maintenance of biological activity of the device.

4. Conclusions

This work the sign of the oxidation that is not indicated for the experiments proposed, though, the use of TMB is only for reductions in electrochemical tests. Even being a limitation, a reduction signal limits others interference on the disturbance of the signal making that TMB to be an innovative electrochemical mediator.

The best timing used was 30 min, but that does not mean that it could be done in 20 min or less since other polymeric films could be used to amplify or reduce even more the response given. The nanoparticles are made from gold or silver those amplify the electrochemical results, such as graphene, carbon nanotubes (single wall and multiple walls) and other materials that biotechnology can develop to make a difference in the TMB sign. This study was done using DNA. In the future project other biomolecules can be tested/used.

Thus, the results indicate the use of 3,3',5,5' tetramethylbenzidine (TMB) exclusively can be termed as a new electrochemical indicator able to discriminate DNA in simple DNA ribbon on dual complementary strand (hybridized), so effective in its application in genosensors and more studies with other possibilities should be done to test others hypothesis.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful for the financial support from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (N°: APQ-04363-10), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (N°: 014/2010; 016/2012) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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