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Amperometric immunosensor for diagnosis of BLV infection

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Abstract

A new amperometric immunosensor for detection of antibodies against bovine leukemia protein (*gp*51) was designed. The detection of antibody–antigen complex formation was based on application of secondary antibodies labeled with horseradish peroxidase (HRP). Ferrocenecarboxylic acid (FCA) and *N*,*N*,*N*′,*N*′-tetramethylbenzidine (TMB) were selected as suitable mediators for this immunosensor. Optimal conditions for amperometric detection were found. Sensitivity of created system was compared with the results of enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) reaction, and was sufficient for detection of usual anti-*gp*51 antibody concentration present in the blood serum of BLV-infected cattle.
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1. Introduction

Bovine leukemia virus (BLV) still has a high impact in veterinary. Significant relationships between BLV-seropositivity and decreased milk production or mastitis have been reported, most in large herds with high seroprevalence (Pelzer, 1997). BLV is still common in Egypt, Pakistan, Canada, Cambodia and other countries (Meas et al., 2000a,b; VanLeeuwen et al., 2001; Zaghawa et al., 2002). BLV was registered even in some European countries (Ramanaviciene et al., 2004a,b). BLV belongs to a family of oncogenic retroviruses, which includes HTLV-1, HTLV-2, simian T cell leukemia virus 1 and 2 and these retroviruses share a common genomic and structural organization (Sagata et al., 1985). Bovine leukemia virus is associated with enzootic bovine leukemia (EBL), a disease characterized by a very extended course that often involves persistent lymphocytosis (PL) and culminates in B-cell lymphoma (Burny et al., 1988). Majority of retrovirus-induced diseases for a long incubational period show no clinical signs. One of the most effective steps to prevent spread of such infections is fast and effective strategy for detection. Agar gel immunodiffusion (AGID) reaction, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and immunoblotting are applied for the detection of retrovirus specific antibodies or DNA (Grover and Guillemain, 1992; Simard et al., 2000; Rola and Kuzmak, 2002).

At present, indirect (serological) detection methods are still dominating in the laboratory diagnosis of BLV infection. The AGID reaction and ELISA are predominantly used for routine BLV diagnosis. To diagnose BLV infection at the early stages polymerase chain reaction is applied. However, the main drawback of the traditional techniques are the following: (i) AGID is limited by extremely long detection time; (ii) ELISA is faster but requires a lot of manipulations; (iii) polymerase chain reaction-based techniques have long detection time limited by electrophoresis in agarose gel and the necessity to develop the electrophoreograms by highly poisonous ethidium bromide (Murtaugh et al., 1991). AGID reaction might be replaced by electrochemical detection system based on application of synthetic receptors, however

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such system is not sensitive enough to detect low concentrations of BLV proteins that are present in blood serum only at the early stage of BLV infection (Ramanaviciene and Ramanavicius, 2004a). Piezoelectric systems might be applied to simplify ELISA protocol but such systems are externally sensitive to external physical factors that are inducing drift in piezoelectric signal (Ramanaviciene et al., 2004b). Surface plasmon resonance technique can be also applied for label-free monitoring of antibody–antigen interactions in real-time (Kausaite et al., 2007). The PCR-based detection of target DNA can be significantly simplified if PCR coupled with DNA-sensors are applied (Ramanaviciene and Ramanavicius, 2004a). Quenching of nonspecifically adsorbed fluorescence agents by conducting polymer matrix might be exploited for increasing sensitivity of sensors devoted for diagnosis of BLV (Ramanavicius et al., 2007). In construction of all sensors mentioned here combination of electrochemistry and biochemistry (Ramanaviciene and Ramanavicius, 2002) was successfully exploited.

Construction of amperometric immunosensors based on registration of enzymatic reaction is a way that allows construction of sensitive bioanalytical systems. Horseradish peroxidase (HRP) is one of the mostly used enzymatic labels for the design of ELISA and amperometric immunosensors (Campas and Marty, 2007; Salinas et al., 2005). Usually amperometric immunosensors utilize redox mediators that are involved into electron transfer from redox active enzyme center towards the electrode (Ramanavicius et al., 2006). Major problems in the construction of amperometric immunosensors are the following: the choice of electrode suitable for immobilization of agents exhibiting affinity towards analyte and selection of suitable electrochemically active substrate able to transfer efficiently electrons from HRP.

The aim of this study was to develop an electrochemical immunosensor model for the amperometric detection of anti-*gp*51 antibodies that are present in the blood serum of BLV-infected cattle.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and used as received. The solutions were prepared by using HPLC grade water purified in a “Purator-B” Glas Keramic (Berlin, Germany). Hydrogen peroxide stock solution of 0.25 M has been used for preparation of aliquots and concentration of H_2O_2 was tested spectrophotometrically before each sequence of measurements. The BLV protein *gp*51, real BLV-not-infected bovine blood serum sample (BLV-not-infected serum) and BLV-infected bovine blood serum sample (BLV-infected serum) were obtained from ‘Biok’ (Kursk, Russia). The BLV-infected serum contained standardized concentration of antibodies against protein *gp*51 (anti-*gp*51). Horseradish peroxidase-labeled secondary antibodies (HRP-Ab*) for ELISA bovine leucosis serum screening test were received from Institute Pourquier (Montpellier, France).

Horseradish peroxidase activity 560 U/mg, 4-aminoantipyrine (AAP) and phenol-4-sulfonic acid (PSA) were received from ‘Fluka’ (Switzerland), *o*-phenylene diamine (OPD) from ‘Merck’ (Germany). Ferrocenecarboxylic acid (FCA), trimethyl hydroquinone, *N*,*N*,*N*′,*N*′-tetramethylbenzidine (TMB), *N*-cyclohexyl-(2-morfolinoethyl) *N*-cyclohexyl-(2-morfolinoethyl)carbodiimide meto-*p*-toluensulphonate (EDC), graphite rod electrodes (99.999% purity) 3 mm in diameter, were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). Biotin hydrazide was received from Pierce (Rockford, Il, USA).

2.2. Electrochemical setup

All electrochemical experiments were performed by potentiostat–galvanostat model – VoltaLab PGZ 402 ‘Radiometer Analytical’ (Villeurbanne Cedex, France) in conventional three-electrode system consisting of working graphite electrode (bare or modified as described), platinum plate as an auxiliary electrode and Ag/AgCl (in saturated KCl) as a reference one (Laurinavicius et al., 1999). The 0.05 M potassium phosphate buffer solution (PBS), pH 6.0 or 7.0, containing 0.1 M of KCl was used as default buffer for all experiments.

2.3. Spectrophotometric setup and spectrophotometric measurements

UV–visible measurements were performed with a spectrophotometer “Thermo Spectronic UV- 300” (Unicam Spectronic, Cambridge, UK) using a PVC spectrophotometric cuvette of 10 mm optical path length and 1 ml of working volume. Spectrophotometric measurements were carried out in 0.05 M phosphate buffer with 0.1 M KCl, pH 7.0 at 20 °C. In all spectrophotometric measurement solutions consisting of 0.29 μM HRP, and 0.5 mM the corresponding electron donor were used. To start the enzymatic reaction hydrogen peroxide was added to form initial 12.5 mM concentration of this substrate in a reaction vessel. Then subsequently the reaction rate and enzymatic activity of HRP were calculated from the changes in optical absorbtion at λ_{max} for each redox mediator used.

2.4. Electrode preparation and pretreatment

Graphite electrodes were prepared as follows: rods of spectroscopic graphite (Sigma–Aldrich, USA) were cut; then electrodes were polished on fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI, USA) and then polished with aluminum paste, following rinsing of the electrode surface with distilled water and drying at room temperature. Electrodes were sealed into silicon tubes to define constant geometric working area of graphite electrode. This area was of 0.071 cm^2. The graphite working electrodes after mechanical pretreatment (Ramanavicius et al., 2005a) were ultra-sonicated for 10 min in distilled water, washed with distilled water, air-dried and kept in the refrigerator at +4 °C.

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