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Electrochemical immunosensor for differential diagnostic of *Wuchereria bancrofti* using a synthetic peptide

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ABSTRACT

Lymphatic filariasis (LF) is a neglected tropical disease transmitted by mosquitoes and the second cause of permanent disability leading to a significant morbidity and mortality rate. Previously, we have identified epitopes of the filarial abundant larval transcript-2 (ALT-2) protein using a microarray mapping. In this study, one of the epitopes (Wb/ALT2-A5) was used to construct an electrochemical immunosensor. Electrochemical technique of cyclic voltammetry was performed for detecting the signal generated by the interaction between the (Wb/ALT2-A5) peptide and circulating antibodies of serum human samples. (Wb/ALT2-A5) epitope antigens were successfully immobilized on the working electrode of a screen-printed carbon electrode (SPCE) by their amine groups via chitosan film by coupling with glutaraldehyde as crosslinker. After the sensor ready, a pool of human sera infected with *Wuchereria bancrofti* was added to its surface. Electrochemical responses were generated by applying a potential of − 0.6 to 0.6 V, scan rate of 0.025 V/s. A detection limit of 5.0 $\mu g\,mL^{-1}$ for the synthetic peptides (Wb/ALT2-A5) and 0.002 $\mu g\,mL^{-1}$ for human serum, with a sensitivity of 1.86 μA. The performance of this assay was successfully tested in human serum samples from infected and healthy patients. Thus, this proposed immunosensor, which is able to identify circulating antibodies, can be applied to the diagnosis of the *W. bancrofti* parasitic disease.

1. Introduction

The lymphatic filariasis (LF) is a tropical disease considered a parasitic infection, known as elephantiasis, which is exclusive to humans and caused by the nematode worms *Wuchereria bancrofti* (Wb). Its transmission occurs by the bite of the *Culex quinquefasciatus* female mosquito infected with larvae of the parasite. The helminthic adult worm has as preferential locus the lymphatic vessels and lymph nodes, disfiguring and/or incapacitating thousands of infected individuals (Komoreng et al., 2017).

In endemic countries, about 120 million individuals are infected and more than 1.4 billion in 73 countries are at risk of acquiring the infection by this helminth (Komoreng et al., 2017). A great effort coordinated by the WHO is being implemented in several countries to eliminate LF (Wb, *Brugia malayi*, *B. timori*) by 2020 through mass drug administration (MDA) using diethylcarbamazine and albendazole. The rapid and early diagnosis of the disease however, remains a major problem (PAHO WHO, 2014; WHO, 2017; Small et al., 2014).

The parasitological diagnosis is still based on the research of microfilariae in peripheral blood, collected in one time compatible with the periodicity of the parasite in endemic regions (Chesnais et al., 2016; Rocha et al., 2004; Small et al., 2014). This the oldest test, it is extremely useful and can be performed by different techniques, such as a thick drop of blood, Knott's concentration and blood filtration in polycarbonate membrane. However, the interpretation of results is particularly difficult in patients with inflammatory symptoms, in chronic phase, or when the present a pulmonary disease (tropical pulmonary eosinophilia - TPE). In these cases, the microfilariae are normally absent from peripheral blood, requiring confirmation by a second testing. In attempting to circumvent the low sensitivity, a parasitological technique based on blood filtration in a polycarbonate membrane was developed for patients with microfilariae of *W. bancrofti*

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(Wb) (Chularerk and Desowitz, 1970). Nevertheless, this technique is still considered non-practice, when compared to others like the chromatographic assays or biosensors, requiring a skilled personal to collect large amount of blood by venipuncture and storage of biological material. Besides, this last technique presents a high cost and is time-consuming (Rocha, 2000; Small et al., 2014; Weil et al., 1997). The ultrasonography is another suitable tool to monitor the Wb infection (Amaral et al., 1994; Dreyer et al., 1994; Dreyer and Addiss, 2000; Norões et al., 1996), this technique appears to be limited for detection of adult worms and is not stable over time in humans, as is the case of the bancroftose. In patients with filariasis brugian, ultrasound examination cannot detect adult worms (Mand et al., 2006).

More advanced techniques based on the research of circulating filarial antigens, such as the immunoenzymatic assay (Og4C3-ELISA) and immunochromatography testing (ICT card test-AD12), showed superior sensitivity and specificity to parasitological methods (Rocha et al., 1996; Rocha, 2000; TropBIO, 1996). At present, antigen testing is most widely used, because it is more sensitive and convenient for detecting infection. ELISA tests are based on monoclonal antibodies AD12.1 or Og4C3 (Gass et al., 2012; Weil and Ramzy, 2007).

The rapid tests include the BinaxNOW® Filariasis card test (Alere, Scarborough, ME) (immunochromatography card test [ICT]), which has been used in GPELF since 2000, and the Filariasis Test Strip (FTS), introduced in 2013 by Alere, the successor company to Binax (Chesnais et al., 2016). However, a rapid and early diagnosis of the disease remains a major problem. The main limiting factor in the use of these tests is the high cost of kits, which restricts their application in health services where demand is high.

Recently, De-Simone's Research Group (FIOCRUZ-Brazil) using the peptide microarray technique have mapped the B linear epitopes of the abundant larval transcript-2 (ALT-2) protein of the worm and identified an apparent specific peptide by using bioinformatic tools and an indirect ELISA (data not published). Herein, one of these peptides (Ep5) was used to develop an electrochemical immunosensor for Wb. The Ep5 epitope was immobilized, on a chitosan film by covalent attachment (Holler et al., 2009) with a crosslinker agent, glutaraldehyde (GA) (Prado et al., 2017). This crosslinker was chosen to guarantee a non-denaturation of this peptide, i.e. activity loss, which commonly occur by entrapment of the protein of interest on polymer chains (Oliveira and Vieira, 2006).

Electroanalytical response was obtained by enzymatic reaction of anti-human IgG (anti-huIgG) conjugated to alkaline phosphatase (AP) that catalyzes the conversion of hydroquinone diphosphate salt (HQDP) to hydroquinone (HQ) (Michael et al., 2004; Mizutani et al., 1991). Electrical current signal was measured at a fixed potential of 25 mV applied to the working electrode vs. Ag/AgCl electrode, in this situation the hydroquinone is oxidized to quinone.

2. Experimental

2.1. Material and reagents

Hydroxymethyl aminomethane hydrochloride (Tris-HCl) (Sigma-Aldrich, Brazil), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) (Merck, Brazil) and glutaraldehyde (GA) (Sigma-Aldrich, Brazil) were all high purity grade. Phosphate buffered saline (PBS, pH 7.4) was obtained by mixing 0.1 mmol L^{-1} monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), potassium chloride (KCl) and sodium chloride (NaCl) 13 mmol L^{-1} in adequate proportions. Chitosan (Ch) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, USA). Goat anti-human immunoglobulin G labeled with alkaline phosphatase was purchased from Kirkegaard & Perri Laboratories (Dublin, USA), and the electrochemical substrate hydroquinone diphosphate (HQDP) was obtained from DropSens (Oviedo, Spain). All solutions were prepared with ultrapure water obtained from a Milli-Q system (Millipore, Billerica Inc, USA).

2.2. Human sera

Sera from patients with filariasis were provided by the Laboratory of Communicable Diseases of the Aggeu Magalhães Research Center, FIOCRUZ, PE, Brazil. The donor group was composed of residents of endemic areas (without a definition of health status, social group, age, sex or race), by obtainment of their signature, or their responsibles if menor or incapable, on the Free and Informed Consent Form (TCLE). Our study was approved by the Ethics Committee of FIOCRUZ (CEP, no. 559/10 and 23784114.8.0000.5248). Healthy human sera were obtained from the serum bank of the Protein and Peptide Biochemistry Laboratory (LBPP-IOC/FIOCRUZ), provided by HEMORIO, RJ, Brazil.

2.3. Apparatus and measurements

A cyclic voltammetry (CV) analysis was performed, with a Potentiostat/Galvanostat (Ivium Technologies, Fernandina Beach, USA) in an interface with a MEGATRON notebook, controlled by the Ivium Soft software. Screen-printed carbon electrodes (SPCE/110) formed by: a carbon working electrode with area of 4.0 mm diameter, a counter carbon electrode and a silver reference electrode, were obtained from DropSens (Oviedo, Spain). All electrodes were screen-printed on a ceramic substrate (3.4 × 1.0 × 0.05 cm) reference electrode and silver electrical contacts.

Electrochemical measurements were conducted by cyclic voltammetry (CV) technique in solution of 3.0 mmol L^{-1} HQDP prepared in 0.1 mol L^{-1} Tris-HCl, 20 mmol L^{-1}, $MgCl_2{\cdot}6H_2O$, pH 9.8; by applying a scan rate of 0.025 V S^{-1} and a potential ranging from − 0.6 to 0.6 V.

2.4. Epitope mapping and determination of the peptide concentration

The B-cell epitope mapping of the ALT-2 (UniParc: UPI0001C4B44D) protein from Wb was determined by the microarray peptide assay. Six IgG epitopes were identified using human patient's sera infected with Wb (non-published data). The epitope Wb/ALT2-A5 (**89SWTDKGCFCE98**) was chosen for this study. The specificity of the Ep5 was previously determined by an enzyme-linked immunoassay (ELISA) that was standardized by De-Simone's Research Group (FIOCRUZ-Brazil)

2.5. Solid phase peptide synthesis and determinations

The synthetic peptide **NENQSWTDKGCFCEG** containing the epitope Wb/ALT2-A5 was synthesized by the solid-phase chemical method using the 9-fluorenyl methoxy carbonyl (F-moc) strategy using an automatic synthesizer (PSSM-8, Shimadzu, Kyoto, Japan) as described previously (Souza et al., 2016). Benzotriazole 1-yl-oxy-tris-pyrrolidine phosphonium hexafluorophosphate (PYBOP) was added to the F-moc amino acid. The reaction was run in the reactor with a sintered glass filter containing Wang-Fmoc-Arg resin (Pmc). The F-moc moiety was removed with 25% 4-methylpyridine (Sigma-Aldrich, Brazil) and the F-moc amino acid coupling reagents were 0.1 mmol L^{-1} oxime (Sigma-Aldrich, Brazil) in dimethylformamide (DMF) and 8% N-methyl morpholine in DMF. The resin-bound peptide was deprotected and cleaved using trifluoroacetic acid (Sigma-Aldrich) and triisopropylsilane (Sigma-Aldrich). These peptides were precipitated with diethyl ether and lyophilized. Peptide concentration was determined by measuring the optical density using the molar extinction coefficient generated by the PROTPARAM software package [http://www.expasy.ch]. The peptide sequence was confirmed by mass spectrometry (MALDI-TOF MS; Matrix Assisted Laser Desorption Ionization Time-of-Flight).

2.6. Preparation of SPEs with a chitosan film containing the peptide Wb/ALT2-A5

Chitosan films were prepared as previously described (Prado et al.,