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Electrochemical DNA base pairs quantification and endonuclease cleavage detection

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ABSTRACT

A new electrochemical approach for an accurate quantification of DNA base pairs in genomic human DNA amplified by polymerase chain reaction (PCR) is described. The method is based on the immobilization of the sample (a thiolated DNA fragment) on the surface of a screen-printed gold electrode through the –SH group at the 5′-end and the subsequent intercalation of a ruthenium pentaamin complex as a redox indicator. The determination of the base pair number in the sequence is achieved by measuring the changes in the electroactivity of the ruthenium complex using Differential Pulse Voltammetry. Calibration curves correlating current intensity with the base pair number allow determining the size of DNA samples, even when very large (over 100 base pairs) sequences are assayed. The method has been successfully applied to detect the DNA cleavage by a site-specific restriction enzyme.

The electrochemical approach developed offers the advantage of ease of performance in comparison to other previously described approaches, which are time-consuming and require sophisticated and expensive instrumentation.

1. Introduction

Recent advances in molecular biology are enabling the diagnosis of genetic diseases and the identification of pathogens through the analysis of DNA. As the demand of clinical applications for molecular diagnosis increases, rapid, accurate, reliable and low cost methods for DNA analysis are necessary. The identification of DNA fragments resulting from sequence-specific amplification techniques (polymerase chain reaction-amplified DNA sequences (Innis et al., 1990)) has opened the way for carrying out the molecular diagnosis of genetic diseases, the prediction of genetic susceptibility to the occurrence of tumoral processes, and the earlier detection of infections induced by different types of pathogens and other organisms. In addition, the determination of the size of oligonucleotide fragments (i.e., the number of nucleotide units present in a DNA or RNA sample) is essential for many of these procedures and experimental techniques employed in molecular biology and, increasingly, in clinical, environmental, agricultural and forensic sciences applications. For all of these applications, as well as also for the proper interpretation of DNA-based information obtained from the current programs to map and sequence the human (or other organisms) genome, it is necessary to develop new analytical techniques capable to determining the DNA fragment size.

DNA products generated by amplification reactions such as the polymerase chain reaction (PCR) are typically analyzed by a number of gel electrophoresis techniques, employing either slab gels or gel-filled capillaries (Cedervall and Radivoyevitch, 1996; Chen and Ren, 2004; Wall, 2007). However, the gel electrophoresis procedures are, in general, limited by several factors such as, the large analysis time, low resolution, the requirement of label or staining steps, and the waste disposal of the stained or radioisotopically labelled materials. Mass spectrometry (MS) has been proposed as an alternative approach that offers the possibility to automated determination of DNA fragment size (Doktycz et al., 1995). Using this technique, both the lengthy separation step and the labelling requirement of electrophoresis-based methods can be eliminated. Continuing improvements of the sensitivity and resolution capabilities of mass spectrometry may make it suitable for differentiation of DNA fragments with minor structural or base number differences. High performance liquid chromatography (HPLC) and mass spectrometry (MS) represent established analytical techniques for the characterization and structural elucidation of single- and double-stranded nucleic acids, ranging in size from a few nucleotides to several thousand base pairs. Although both

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techniques are independently applicable for nucleic acid analysis, the on-line hyphenation significantly enhances their potential for the robust and fully automated routine analysis of minute amounts of DNA samples (Huber and Herbert, 2001). Capillary electrophoresis is an emerging technology in DNA fragment size analysis. Micro-fabricated single channel and multichannel electrophoresis chips are a new paradigm in the high-speed and high-throughput DNA analysis (Becker and Gartner, 2000; Dolnik et al., 2000; Gao et al., 2001). The use of this microchip-based technology enables the sequencing analysis of a DNA fragment of 600 bases in only 20 min (Liu et al., 2000). The main disadvantage of these techniques is the need of very sophisticated and expensive instrumentation that is not available to all research laboratories.

In this context electrochemical DNA biosensors have been proposed as means of providing rapid results from a sample employing a simple, handily, rapid response, portable, low manpower requirements and simple microfabrication technology instrumentation. However, although these devices have been widely used for the detection of a specific single-stranded DNA using immobilized DNA probes, and it has been also described the electrochemical detection of triplet repeat expansion (Fojta et al., 2004; Yang and Thorp, 2001), to the best of our knowledge, there has been no report on the application of these devices to the determination of DNA fragment size.

Our efforts over the past years have been focused to design DNA-sensing electrochemical systems. As part of our research goals, we have recently synthesized a redox indicator for detection of hybridization, general binding and interactions with DNA (García et al., 2008b). This redox indicator consists on a new pentaamin ruthenium [3-(2-phenanthren-9-yl-vinyl)-pyridine] complex in which the Ru centre provides a redox probe and the ligand may enhance the interaction with DNA via intercalation.

In the present work we take a step forward developing a new and rapid electrochemical method for the determination of DNA fragment size besides to endonuclease DNA cleavage detection directly in human genomic DNA. The method involves a disposable screen-printed gold electrode (AuSPE) modified with the sample (a thiolated double stranded DNA). The determination of the base pair number included in the strand is achieved by changes in the metal redox centre electroactivity of the ruthenium complex using Differential Pulse Voltammetry. Thus, the current intensity measured is expected to be proportional to the amount of these molecules intercalated between the base pairs of the strand and subsequently to the DNA fragment size. Calibration curves correlating current intensity with the base pair number allow determining the size of DNA samples, even when very large (over 100 base pairs) sequences are employed. The different signals obtained for different size sequences make possible to detect the DNA cleavage of sequences (from exons of genes) with more than 450 base pairs. The method has been applied to detect the DNA cleavage by a site-specific restriction enzyme.

2. Experimental

2.1. Chemicals

The complex pentaamin ruthenium [3-(2-phenanthren-9-yl-vinyl)-pyridine] (mentioned in the text as Ru**L**; Fig. S-1, Supporting information) was prepared *in situ*, as previously described (García et al., 2010). Potassium nitrate, sodium phosphate and sodium chloride were obtained from Scharlab Company. Tris–borate–EDTA buffer (TBE), $K_4Fe(CN)_6$, $K_3Fe(CN)_6$ and all other chemicals used in this work were reagent grade quality, obtained from Sigma–Aldrich Co., and used as received without further purification. Restriction enzymes MspI and EcoRI were purchased from New England Biolabs. All solutions were prepared just prior to use. Water was purified with a Millipore Milli-Q-System.

Different size synthetic thiolated sequences of exon 21 of gen MRP3 (see Table S-1, Supporting information) were purchased from Sigma Genosys Chemical Company.

PCR thiolated samples employed were different size double stranded DNA from exons 22 and 23 of gene MYH7 (see Table S-2, Supporting information). They were supplied and characterized by the Genomics Medicine Group.

2.2. Apparatus and electrodes

Electrochemical measurements were carried out at room temperature using an Autolab PGSTAT 30 potentiostat from Eco-Chemie (KM Utrecht, The Netherlands) using the software package GPES 4.9 (General Purpose Elec. Experiments) or FRA 4.9 (Frequency Response Analyzer) and were performed in a home made one compartment electrochemical cell. Integrated screen-printed gold electrodes (4 mm diameter, AuSPE) from DropSens S.L. (Oviedo, Spain) including a silver pseudoreference electrode and a gold counter electrode were used. The electrodes were connected using a SPE Connector (DropSens S.L.) as interface. For electrochemical impedance spectroscopy experiments gold-disk electrodes (1.5 mm diameter) from Bioanalytical Systems were used as working electrodes. A coiled gold wire served as auxiliary electrode and the potentials were reported against a mercury/mercurous sulphate electrode (MSE).

Prior to use, electrodes were activated as it was previously described (García et al., 2010; Revenga-Parra et al., 2011).

2.3. PCR amplification

PCRs were performed in 25 μL of final volume, with 1× Titanium PCR Buffer, 0.2 mM dNTPs, 2× Titanium Taq Polymerase (Clontech Laboratories Inc.) and 0.4 μM of each primer. The same 5′-thiolated forward primer was used for all amplicons. Different reverse primers were used to generate PCR fragments of different sizes (Table S-3, Supporting information). The PCR involves an initial denaturation step at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 30 s and polymerization at 68 °C for 90 s with a final elongation step at 68 °C for 3 min. PCR products were purified with MultiScreen $PCR_{\mu 96}$ Plate (Millipore Corporation) following manufacturing protocol.

2.4. Hybridization, immobilization and measurement procedures

Synthetic oligonucleotide (25–110 bp) hybridization was carried out in solution mixing 16 μL of 100 μM thiolated probe and 16 μL of 100 μM corresponding fully complementary sequence in a 80 μL final volume of 10 mM phosphate buffer (pH = 7) solution containing 0.4 M NaCl. The mixture was incubated for 1 h at 40 °C.

For DNA immobilization, 10 μL of thiolated synthetic duplex or 10 μL of 4.1 ng μL^{-1} thiolated PCR of human genomic DNA were transferred onto the clean gold electrode surface. The electrode was protected and kept for 72 h in humid environment at room temperature. Then, it was immersed in sterile water for at least 30 min before to be used. Two different electrochemical techniques were employed: Differential Pulse Voltammetry (DPV) and electrochemical impedance spectroscopy (EIS). When DPV was used, the DNA modified electrodes were immersed in a 5.0 mM KNO_3 solution containing 20 μM of the Ru**L** and the potential was cycled (200 times) from −0.5 to 0.1 V at 100 mV s^{-1}. Then, the electrodes were rinsed with sterile water, placed in 5.0 mM KNO_3 and differential pulse voltammograms (DPVs) were immediately recorded. EIS measurements were carried out directly in a 0.1 M phosphate buffer solution (pH = 7.0) containing 10 mM $K_3Fe(CN)_6$/10 mM $K_4Fe(CN)_6$.