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A novel diagnostic biosensor for distinguishing immunoglobulin mutated and unmutated types of chronic lymphocytic leukemia

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ABSTRACT

In chronic lymphocytic leukemia (CLL), the immunoglobulin heavy-chain variable (IgVH) region may be mutated (Ig-mutated CLL) or unmutated (Ig-unmutated CLL); and the presence or absence of mutations in this region of CLL cells distinguishes two clinically distinct forms. It is important for physicians to distinguish between patients with Ig-unmutated CLL, where typically have more indolent disease with median survivals close to 25 years, and Ig-mutated CLL, where have more aggressive disease with median survivals around eight years. In this work, a biosensor capable of diagnosis and distinguishing between these two types of CLL was reported. The biosensor was fabricated by modifying a gold electrode with gold nanoparticles (AuNPS) followed by coating of ZAP70 oligonucleotide probe on the surface to detect specific sequence of ZAP70 gene. ZAP70 could predict the IgVH mutation status and is a good marker for differentiating Ig-mutated and Ig-unmutated CLL and serve as prognostic marker. First, we focused on achieving hybridization between probe and its complementary sequence. Hybridization between probe and target was determined with electrochemical impedance spectroscopy (EIS). Then, our efforts turned to optimize the conditions for the detection of any point mutation and also to maximize the selectivity. Under optimal conditions, the biosensor has a good calibration range between 2.0×10^{-14} and 1.0×10^{-9} mol L^{-1}, with ZAP70 DNA sequence detection limit of 4.0×10^{-15} mol L^{-1}. We successfully detect hybridization first in synthetic samples, and ultimately in blood samples from patients. Experimental results illustrated that the nanostructured biosensor clearly discriminates between mutated and non-mutated CLL and predict the IgVH mutation status, which it has been considered as the single most informative stage independent prognostic factor in CLL.

1. Introduction

Cancer research is one of the most important research areas in the medical sciences. Cells become cancer cells largely because of changes in their genes that called mutations. Mutations play an important role in the development of cancer. The main conventional methods for detecting mutation are polymerase chain reaction (PCR) followed by restriction enzyme analysis (Zhang et al., 2015), ligase chain reaction (Bia et al., 2015) and nick translation PCR with fluorogenic DNA probes (Lee et al., 1993). These detection schemes are mostly based on the coupling of gel electrophoresis and radioisotopic labeling and are thus not suitable for automation (Ozsoz et al., 2003). The possibility of using minimally invasive analytical instruments to monitor genomic mutation provide great advances in cancer research. Biosensors have emerged as a new technique for monitoring mutation. The real success in the development of a reliable sensor depends on the ability to design powerful instrumentation that will facilitate efficient signal transduction from the biological process that occurs in the cellular environment (Sadik et al., 2009).

Modern electrochemical DNA sensors, have recently demonstrated great potential for monitoring cancer-related DNA mutations (Zhu et al., 2015; Akhavan et al., 2012). Electrochemical methods, when compared to the conventional methods for DNA analysis (e.g., fluorescence microscopy, radioactivity measurements, quartz crystal microbalance and surface plasmon resonance) are straightforward and sensitive and do not require sophisticated instrumentation. Consequently, they are appropriate for the development of inexpensive and portable devices for disease diagnoses (Alfonta. et al., 2000). To construct the DNA based biosensor for detection of specific DNA sequence; the single-strand functional nucleic acid that called probe is immobilized on the solid support and coupled with specific single strand DNA (the target) within the analyte. The complementary strands anneal to one another in Watson–Crick base pairing. The specific and selective detection of DNA sequences, with single-base mismatch

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detection ability, is a major challenge to address in DNA biosensing (Keller and Manak, 1993).

One of the human diseases caused by mutations is chronic lymphocytic leukemia (CLL). CLL is a biologically heterogeneous disease of the lymphoid system that is characterized by the progressive and irreversible growth of mature deficient B-cells and various genomic aberrations and mutations (Scupoli and Pizzolo, 2015). It is a disease of aged populations and the most common adult leukemia in the Western world, with an estimated incidence of 5.8 cases per 100,000 men per year and 3.0 per 100,000 women per year (Campregher and Hamerschlak, 2014; Strefford, 2015). It is estimated that there will be ~14,620 new cases of CLL and ~4650 deaths due to the disease in the United States alone in 2015 (Esencay and Hamad, 2015). It has been recently proven that 50–60% of patients with CLL have somatic mutations in the immunoglobulin heavy chain variable region (IgVH) gene (Šoljić et al., 2013). Patients with CLL, who express mutated IgVH genes as defined above, typically have more indolent disease and a better prognosis with median survivals close to 25 years, while those with unmutated IgVH genes often have more aggressive disease and a poorer prognosis with median survivals around 8 years (Moreno and Montserrat, 2008; Montserrat, 2006). Unlike other prognostic factors, IgVH gene mutation status does not change during the disease, but, due to the complexity of the analysis method, it is unsuitable for routine clinical practice (Rassenti et al., 2004).

Although the IgVH gene mutation status is a powerful prognostic indicator, much attention has focused on finding surrogate markers for the mutation status that could be more rapidly detected than the time-consuming sequencing that is necessary to determine the mutation status (Tobin and Rosenquist, 2005). Rosenwald et al. (2001) found more than 100 genes differentially expressed between the Ig-mutated and Ig-unmutated CLL subtypes with high statistical significance. The most differentially expressed gene was ZAP-70, which encodes a tyrosine kinase. The association of the IgVH gene mutation status, revealing that ZAP-70 can predict the IgVH gene mutation status with a high accuracy in CLL (Crespo et al., 2003). These studies suggest that ZAP-70 could function as a surrogate marker for IgVH gene mutation status in CLL, which would enable a faster analysis to predict prognosis than the IgVH gene analysis.

There are several methods to quantify ZAP-70 including: polymerase chain reaction (PCR), immunoblotting, immunohistochemistry, fluorescence in-situ hybridization (FISH) analysis and flow cytometry (Slack et al., 2007, Wang et al., 2012, Put et al., 2009, Vroblova et al., 2012, Matthews et al., 2004, Moreno and Montserrat, 2010). Use of flow cytometry for ZAP-70 detection seems to be advantageous as this technique enables us to assess the presence of ZAP-70 separately on CLL clone, T-cells and NK-cells. But the remarkable thing is that, in general, the above methods are costly and time consuming methods compared to simpler methods such as label free electrochemical method. Also, while the presence and absence of IgVH mutation state is currently the gold-standard prognostic factor, but this distinction is labor-intensive and costly (Wang et al., 2012). Generally, analysis of IgVH mutation state is a relatively expensive and time-consuming test with restricted availability. Thus providing a simple, reliable and low cost method that can distinguish between the Ig-mutated and Ig-unmutated CLL subtypes within the shortest possible time has particular importance.

In this paper, a label-free electrochemical DNA biosensor, based on ZAP70 probe, has been fabricated to detect specific sequence of ZAP70 gene, which is highly associated with CLL cancer and IgVH gene mutation status. The biosensor was fabricated by modifying a gold electrode with gold nanoparticles (AuNPs) followed by coating of ZAP70 oligonucleotide probe on the surface to detect specific sequence of ZAP70 gene. A probe ss-DNA (25–mer) modified with –SH is self-assembled onto the surface of the modified electrode. The target DNA, which contains complementary sequence to the probe DNA, would be captured by the probe DNA through hybridization. The ZAP70 oligonucleotide, which is complementary with another part of the target DNA would be hybridized. We also exploit the impedance spectroscopy as a platform for reagent-less DNA sensing assay. The biosensor was employed for the diagnosis and distinguishing of Ig-mutated and Ig-unmutated CLL conditions and the IgVH mutation status in patients. To the best of our knowledge, up to the present time, there is no electrochemical study dealing with sensing of ZAP70 gene level and the distinction between Ig-mutated and Ig-unmutated CLL conditions.

2. Experimental

2.1. Chemicals

All solutions were prepared using reagent grade chemicals and doubly distilled water was used through the work. Hydrogen tetrachloroaurate(III) ($HAuCl_4$, $4H_2O$) and potassium nitrate (KNO_3) were purchased from Merck (Darmstadt, Germany). All oligonucleotides were synthesized and purified in Takapu Zist Institute (Tehran, Iran). All oligonucleotides were diluted with ultrapure water and stored as a stock solution in a freezer. The following five oligonucleotides sequences were used in this study:

ZAP70 probe (p_{25})	5′-SH-CCCTGCGCCAGCACGCATAACGT-3′
ZAP70 target (C_{25})	5′-ACGTTATGCGTGCTGGCGCAGGG-3′
Target with longer sequence (C_{40})	5′-ACGTTATGCGTGCTGGCGCAGGGGTGTATCCATCT-GAGTT-3′
Mismatch sequence (M_{25})	5′-ACGTGATGCGTGCTGGCGCAGGG-3′
Mismatch with longer sequence (M_{40})	5′-ACGTGATGCGTGCTGGCGCAGGGGTGTATCCATCT-GAGTT-3′

All solutions were prepared with Milli-Q water (18 MΩ cm resistivity) from a Millipore system.

2.2. Apparatus

Electrochemical measurements were performed using Vertex (Potentiostat/galvanostat with optional FRA/EIS) from Ivium, connected to a standard one-compartment three-electrode cell. The reference electrode was Hg/Hg_2Cl_2 (3 mol L^{-1} KCl) and the counter electrode was a platinum wire. The modified gold electrode was used as a working electrode through the work. Metrohm pH–meter (Model 827) with a glass electrode (Corning) was used to adjust the pH of solutions.

2.3. Preparation of the modified gold electrode with AuNPs (AuNPs/