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Label free and amplified detection of cancer marker EBNA-1 by DNA probe based biosensors

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ABSTRACT

Epstein-Barr virus (EBV) is a human herpes virus that has been associated with several malignancies as Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease. All EBV associated malignancies showed a distinct viral gene expression pattern, while Epstein-Barr nuclear antigen 1 (EBNA-1) is constitutively expressed in all such disorders. Here, the development of a biosensor to detect EBNA-1 protein is reported, which was based on a nucleic acid bioreceptor and a quartz crystal microbalance with a dissipation monitoring (QCM-D) transducer. The DNA probe for EBNA-1 detection was designed and synthesized to mimic its palindromic target sites in the EBV genome. This DNA probe was immobilized on the Au-surface of a QCM-D electrode, followed by the blocking of the accessible Au-surface with 6-mercapto-1-hexanol (6-MHO). The system showed a limit of detection of 50 ng/mL in direct detection of EBNA-1, however, the sensitivity was improved by 2 orders of magnitude (0.5 ng/mL) when an amplification cascade, employing antibodies labeled with alkaline phosphatase (AP), was applied to the system.

1. Introduction

EBV is a human herpes virus that infects the majority of World's population (>90%). The virus is spread via saliva and the primary infection usually takes place asymptomatically during childhood. After infection, EBV immortalizes a portion of host B-lymphocytes and establishes a latent infection that persists through the whole host's lifetime. EBV is considered to be the causative agent of infectious mononucleosis and has been associated with several malignancies including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NFC), Hodgkin's disease, lymphoproliferative disorders in immunodeficient individuals, as post-transplant lymphoproliferative diseases (PTLDs), and to a subset of T and NK cell lymphomas (Middeldorp et al., 2003). Each type of EBV$^+$ tumor shows a characteristic viral gene expression pattern, usually limited to latent gene products, which are expressed in different transcription profiles (latency type I, II or III). However, EBNA-1 is the only viral protein consistently expressed in all EBV related cancers (Middeldorp et al., 2003).

EBNA-1 is a nuclear DNA binding protein that it is necessary for the maintenance, replication and transcription of the viral genome (Young and Murray, 2003). In latently infected cells the viral chromosome persists as a low copy number episome which replicate once per cell cycle (Yates and Guan, 1991). The replication of the viral chromosome involves the binding of EBNA-1 to specific sequences of the viral genome, located in the latent origin of replication (oriP) (Ambinder et al., 1990), and the recruitment of eukaryotic replication initiation factors (Chaudhuri et al., 2001). Afterwards, to ensure the maintenance of the episome in progeny nuclei, EBNA-1 cross-links the EBV genome with the host-cell chromosome (Sugden, 2002). Furthermore, EBNA-1 is a transcription factor that promotes the expression of other latent viral genes (Gahn and Sugden, 1995) and imposes changes in host gene expression by binding to cellular promoters, which may have evolutionary implications. Hence, EBNA-1 plays an important role in generating a cellular environment to support EBV's life cycle (Canaan et al., 2009). In addition, several studies have suggested the oncogenic potential of EBNA-1. Wilson et al. (1996) reported the development of lymphomas in transgenic mouse that express EBNA-1 specifically in B-cells. While Lu et al. (2011) suggested that EBNA-1 can contribute to the oncogenic process by up-regulating the apoptosis suppressor protein survivin in EBV associated B-lymphoma cells.

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In spite of the relevance of EBNA-1 to EBV related cancers, we are yet to see proper diagnostic systems using rapid methodology for its direct quantification. Antibodies against EBNA-1 had been suggested as biomarkers, such as IgA immunoglobulins raised against EBNA-1 in NFC diagnosis (Chang et al., 2008; Ayadi et al., 2009). In addition, the detection of anti-EBNA-1 IgG finds use in monitoring past infections and can play a crucial role for EBV routine diagnosis of blood samples prior to transfusions or transplantations. However, the detection of anti-EBNA-1 antibodies cannot be used as biomarkers in all EBV^+ cancers, as in the case of PTLDs or BL tumors. PTLDs are complications in transplant recipients whose immune system has been compromised due to a treatment with immunosupresive drugs. In the case of BL, it was suggested that the high levels of EBNA-1 produced by those tumors could downregulate the IFN-γ T cell responses due to the T cell exhaustion. So, no significant differences in EBNA-1 antibody levels were detected between BL patients and healthy controls (Moormann et al., 2009). Hence, it is highly desirable to develop a fast, sensitive and economic system to detect and quantify EBNA-1 for purposes of monitoring the appearance of EBV associated tumors.

In this work, we report, for the first time, a method for the direct quantification of EBNA-1. The detection system is based on an EBNA-1 binding DNA probe immobilized on the Au-surface of a QCM-D electrode. The biosensor we have developed enables the label-free detection of EBNA-1. However, systems with higher sensitivity are desirable for tumor biomarker detection. Thus, a signal amplification method based on the AP catalyzed oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate (Pavlov et al., 2004) was applied to EBNA-1 signal amplification. The oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate leads to the accumulation of an insoluble indigo derivative on the surface of the electrode providing an amplification route for the detection of EBNA-1. The reported biosensor was successfully used to quantify EBNA-1 concentration in buffer solution.

2. Materials and methods

2.1. Chemicals

5-bromo-4-chloro-3-indolyl phosphate, 6-mercapto-1-hexanol (6-MHO), rabbit serum and anti-mouse IgG-alkaline phosphatase were purchased from Sigma-Aldrich-Fluka (Spain). Anti-EBNA-1 mouse monoclonal antibody was supplied by LifeSpan BioSciences (USA). Functional $EBNA\text{-}1_{461–607}$ (lacking most Gly-Ala region) was produced according to the previously published procedure (Oddo et al., 2006). All water used was nanopure grade. Experimental details of biosensors manufacturing are given in Supplementary Information.

2.2. EBNA-1 detection

As shown in Scheme 1A, functionalized electrodes were used for the label-free sensing of EBNA-1. Different standard solutions of EBNA-1 (from 50 ng/mL to 2 μg/mL) were injected into flow cells. The analytical data was expressed as the difference obtained in the frequency shifts (Δf) or dissipation (ΔD) of the electrode before the addition of EBNA-1, and after washing (with binding buffer) subsequent to the biomolecular affinity interaction.

For the study of the non-specific binding of EBNA-1, the same standard solutions were added to the electrodes blocked with 6-MHO only, without DNA. At least 3 independent experiments were carried out in order to calculate the average values and standard deviations.

To amplify the Δf produced by the binding of EBNA-1 to the DNA probe, we used the biocatalytic deposition of an insoluble product on the gold electrode, as shown in Scheme 1B. After the binding event between EBNA-1 and the DNA probe, anti-EBNA-1 monoclonal antibody was injected into the flow chamber. When the resonance frequency of the electrode reached a stable value the secondary antibody (anti-mouse-IgG-AP), was added and left to incubate until the resonance frequency reached a stable value. Finally, the electrodes were incubated for 30 min in the biocatalytic deposition solution containing 2 mM 5-bromo-4-chloro-3-indolyl phosphate.

3. Results and discussion

3.1. DNA probe immobilization

The modification of the quartz crystal electrode surface with nucleic acids was the first step in the fabrication of the EBNA-1 biosensor. The 5′-thiolated DNA probe was immobilized on the Au-surface. The immobilization resulted in a frequency change of -42.17 ± 3.8 Hz that corresponds to an estimated surface coverage of 2.74×10^{-11} mol/cm^2. The DNA probe immobilization was performed in high ionic strength buffer (binding buffer containing 1 M KCl) in order to avoid the repulsion between DNA chains that occurs normally under low ionic strength buffer conditions, which may have resulted in a poorer surface coverage (Balamurugan et al., 2006). Our results do, however, confirm the reproducibility and efficiency of the immobilization procedure.

6-MHO was used to block the unreacted Au-surface, which showed the ability to prevent the non-specific binding of EBNA-1 to the electrode surface (data not shown). Furthermore, when 6-MHO was injected into the flow cell, the frequency of the electrode increased by 9.96 ± 2.94 Hz. This means that the blocking agent competed with the DNA probe for the Au-surface and was able to displace some part of the previously immobilized DNA probe. It was estimated that during incubation with the blocking agent, 20.4% of the immobilized DNA probe was released from the electrode and was subsequently replaced by 6-MHO. Thus, the 6-MHO surface coverage was calculated to be 1.88×10^{-10} mol/cm^2. Similar surface coverage was previously reported (Pavlov et al., 2004). The partial displacement of the DNA probe by 6-MHO could be advantageous in the case of EBNA-1 sensing, as it is well known, that in order to achieve an optimal interaction between DNA and its corresponding target binding protein, the DNA monolayer should not be too densely packed on the Au-surface (Steel et al., 1998).

3.2. Label free detection of EBNA-1

After the functionalization of Au-electrode with the DNA probe and subsequent blocking with 6-MHO, the label-free detection of EBNA-1 was performed (Scheme 1A). Solutions containing different concentrations of EBNA-1 were applied to the DNA probe based biosensor and Δf and ΔD were monitored real time by a QCM-D. As shown in Fig. 1, when EBNA-1 concentration increased from 50 ng/mL to 2 μg/mL the frequency shifts varied from -1.2 ± 0.21 Hz to -25.08 ± 1.19 Hz. A slight decrease of dissipation, from $-0.41 \pm 0.1 \times 10^{-6}$ D to $-0.90 \pm 0.3 \times 10^{-6}$ D, was also observed in the highest concentrations of EBNA-1 (0.75–2 μg/mL). Finally, control experiments were performed in which Au-electrodes were modified only with 6-MHO, without DNA probe. In this case, the injection of varying concentrations of EBNA-1, from 50 ng/mL to 1 μg/mL, did not result in a measurable frequency shift (data not shown).

The decrease of the resonance frequency along with the increase of the rigidity of the system when EBNA-1 was added, demonstrates the specific binding between EBNA-1 and the DNA probe. These results also indicate that the decrease of the resonance