Biosensors and Bioelectronics 52 (2014) 50–55

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ion sensing (EIS) real-time quantitative monitorization of isothermal DNA amplification

Bruno Veigas [a,b], Rita Branquinho [b], Joana V. Pinto [b], Pawel J. Wojcik [b], Rodrigo Martins [b], Elvira Fortunato [b,*], Pedro V. Baptista [a,**]

[a] CIGMH, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal

[b] CENIMAT/I3N, Departamento de Ciência dos Materiais, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa and CEMOP-UNINOVA, Campus de Caparica, 2829-516 Caparica, Portugal

ARTICLE INFO

Article history:
Received 9 July 2013
Received in revised form
15 August 2013
Accepted 16 August 2013
Available online 24 August 2013

Keywords:
DNA
LAMP
Isothermal amplification
EIS
Ion sensitive field effect sensors
Tantalum pentoxide
qRT-PCR
Real time
c-Myc
Cancer
Label free

ABSTRACT

Field-effect-based devices are becoming a basic structural element in a new generation of microbiosensors. Reliable molecular characterization of DNA and/or RNA is of paramount importance for disease diagnostics and to follow up alterations in gene expression profiles. The use of such devices for point-of-need diagnostics has been hindered by the need of standard or real-time PCR amplification procedures. The present work focuses on the development of a tantalum pentoxide (Ta_2O_5) based sensor for the real-time label free detection of DNA amplification via loop mediated isothermal amplification (LAMP) allowing for quantitative analysis of the *cMYC* proto-oncogene. The strategy based on the field effect sensor was tested within a range of 1×10^8–10^{11} copies of target DNA, and a linear relationship between the log copy number of the initial template DNA and threshold time was observed allowing for a semi-quantitative analysis of DNA template. The concept offers many of the advantages of isothermal quantitative real-time DNA amplification in a label free approach and may pave the way to point-of-care quantitative molecular analysis focused on ease of use and low cost.

1. Introduction

Molecular diagnostics based on DNA detection has increased tremendously over the past few years, in particular towards pathogen identification, drug screening and diagnosis of genetic diseases (Sadik et al., 2009). Most standard technologies showing high sensitivity and low detection limits are usually performed via optical methods, with emphasis on fluorescence intensity measurement from a reporter molecule (Espy et al., 2006). Still, these methods can be expensive and complex to implement, and the majority of these methodologies rely on the enzymatic amplification of DNA via polymerase chain reaction (PCR), generally regarded as an essential method in molecular genetics (Aoi et al., 2006). Real-time monitoring of enzymatic DNA polymerization reaction is of paramount relevance in molecular diagnostics, in particular quantitative DNA amplification real-time PCR (qRT-PCR) is highly effective for monitoring gene expression. Following reverse transcription (conversion of mRNA to cDNA), the amplification reaction can then occur allowing for comparison between samples and/or comparative quantitation (Ginzinger, 2002; Sakurai and Husimi, 1992; VanGuilder et al., 2008; Wong and Medrano, 2005).

Loop-mediated isothermal DNA amplification (LAMP) has emerged as a powerful amplification technique to be used as simple and rapid diagnostics tool (Notomi et al., 2000; Parida et al., 2008). LAMP relies on auto-cycling DNA synthesis performed by a DNA polymerase with strand displacement activity and a set of two specially designed inner and two outer primers. Because of the strand displacement capability, the reaction can be performed at the same temperature without the need for temperature cycling. The final products are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence. Because LAMP is performed under isothermal conditions and at a relatively low temperature, reverse transcription can simultaneously

* Corresponding author.
** Corresponding author. Tel.: +351 212 948 530.
E-mail addresses: emf@fct.unl.pt (E. Fortunato), pmvb@fct.unl.pt (P.V. Baptista).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.08.029

occur when the reaction mixture contains reverse transcriptase (Notomi et al., 2000; Aoi et al., 2006). However, thus far, the effectiveness of LAMP to quantitatively monitor gene expression has not been extensively used, probably due to the lack of automated equipment (Aoi et al., 2006; Maeda et al., 2005; Mori et al., 2004; Parida et al., 2004). The potential to quantify template DNA or RNA by the real-time monitoring of LAMP reaction has already been investigated using standard fluorescence real-time methodologies and apparatus (Mori et al., 2004).

Since the introduction of the ion sensitive field-effect transistor in the 70's by Bergveld (2003), the use of these devices and the number applications has significantly grown and field effect devices (FEDs) are now a promising alternative for label free DNA analysis. A change in DNA content, either due to hybridization or amplification reaction, yields a local pH variation and a rearrangement of ionic species near the sensor surface that modulate the sensor's response. Examples include DNA sequencing equipment – IonTorrent™ (Rothberg et al., 2011), real-time monitoring of PCR amplification (Branquinho et al., 2011) and electrochemical DNA microarray technologies (Audrey et al., 2008; Sadik et al., 2009). Despite the increasing use of electrochemical DNA detection approaches, only a few have been directed towards gene expression analysis. Kivlehan and co-workers developed an electrochemical method to monitor in real-time the isothermal helicase-dependent amplification of nucleic acids (Kivlehan et al., 2011). Still, these methods rely on the indirect detection of an intercalating redox probe during DNA amplification and/or electrode functionalization with a recognition probe (Priano et al., 2007; Sadik et al., 2009). Very recently, an integrated chip for real-time amplification and detection of nucleic acid using pH-sensing complementary metal-oxide semiconductor (CMOS) technology has been reported (Toumazou et al., 2013).

Here, we report on the development of a Ta_2O_5 electrolyte–insulator–semiconductor sensor for label free real-time quantitative LAMP DNA amplification towards gene expression profiling, without the need for additional labeling and/or reporter molecules. The accumulation of the reaction by-product of polymerization (protons) is detected by the Ta_2O_5 EIS sensor (Fig. 1). We demonstrate the potential to quantify in real-time *cMYC*, a proto-oncogene amplified and overexpressed in most human cancers (Dang et al., 2006). Quantitative comparison of tumor vs. normal tissue can be easily monitored. Optimization and integration of this sensing technology into a suitable platform could significantly lower the costs associated with gene expression analysis and consequently allowing for the molecular diagnostics of cancer at point-of-need.

2. Experimental details

2.1. Sensor fabrication and characterization

The electrolyte–insulator–semiconductor sensors were fabricated, characterized and optimized as previously described (Branquinho et al., 2011, 2012). Devices with a Ta_2O_5 sensing membrane were fabricated on p-type (100) Si wafers with a thermally grown SiO_2 film (100 nm). A 100 nm Ta_2O_5 film was deposited at room temperature on the SiO_2/p-Si substrate by radio frequency (rf) magnetron sputtering (AJA ATC-1300 sputtering system) from a 3″ Ta_2O_5 ceramic target (SCM, USA). The deposition pressure was 0.3 Pa with an Ar/O_2 ratio of 14/1 sccm and no intentional substrate heating was applied. The wafer's backside was etched with a buffered HF solution in order to remove the SiO_2 film prior to the Al (200 nm) back-contact deposition by electron beam assisted evaporation in a homemade system. The sensors were annealed at 200 °C for one hour in air with a 10 °C/min heating ramp using a Barnstead Thermolyne F21130 tubular furnace (Germany). The fabricated Ta_2O_5 sensor's structure is the following: back-contact Al/Si/SiO_2/Ta_2O_5/electrolyte. The measurement cell shows a sensitive area of 4 mm, which is inserted into an aluminum housing to allow for uniform heating and temperature control. A temperature sensor is connected to the cell and a thermoelectric power generation Peltier module (200 °C, 6.4 V, 3.1 W, Thermovoltaik) is pasted to the back of the aluminum housing with thermal paste and assembled to a small fan; these are then combined to a temperature controller (TC-XX-PR-59, Supercool AB) to allow for computer management of heating/cooling of the cell and temperature monitoring.

The Ta_2O_5 films were analyzed by XRD and results show an absence of diffraction peaks that indicate the existence of an amorphous phase, which is maintained after post-deposition annealing. RBS analysis showed that the Ta_2O_5 films present a quasi-stoichiometric oxygen proportion (2:4.8 vs. 2:5) – $Ta_2O_{4.8}$. Surface morphology was studied by SEM and AFM and results show that the Ta_2O_5 films a very homogeneous and smooth surface, with a root mean square roughness of less than 0.5 nm (Supplementary information – Table S1).

2.2. PCR amplification of the C-MYC gene fragment

A 229 base pair (bp) fragment of the human *cMYC* proto-oncogene (Ac. no. NM_002467) was PCR amplified using the primers MYCforward and MYCreverse (see Supplementary information – Table S2). PCR amplification was performed in triplicate on a Bio-Rad MyCycler Thermocycler (Bio-Rad, CA, USA) in 100 μl using 1 μM of the specific primers, 2.5 mM dNTPs with 1 U Taq DNA Polymerase (GE Healthcare Europe, Germany), with the following thermal cycling conditions: initial 5 min denaturation at 95 °C, followed by 24 amplification cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation at 72 °C for 5 min (Supplementary information – Table S2).

2.3. LAMP amplification primer design

The loop mediated isothermal amplification of a 207 base pair (bp) fragment of the human *cMYC* proto-oncogene requires four specific primers; forward outer primer (FP), backward outer primer (BP), forward inner primer (FIP), backward inner primer (BIP) (Supplementary information – Table S2). LAMP primers for *cMYC* were designed using Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/) and all primers were synthesized by STAB-Vida, Portugal.

2.4. LAMP amplification of the cMYC gene fragment

LAMP was performed as described by Notomi et al. (Notomi et al., 2000). The reaction was carried out in a 200 μl reaction mixture containing 1 μM of each inner primer FIP and BIP, 0.1 μM of each outer primer FP and BP, 0.3 mM of dNTP mix, 0.5 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 2 mM $MgCl_2$, 0.8 × of the supplied buffer, and (10^6–10^{11}) target copy number per 50 μl of reaction solution. LAMP reaction was performed by subjecting the reaction mixture to an initial 10 min denaturation step at 95 °C on a Bio-Rad MyCycler Thermocycler (BioRad, CA, USA); after which it was cooled down to 4 °C for one minute. The addition of 8U of Bst DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA) followed, and then the final reaction mixture was transferred to the LAMP measurement cell, covered with mineral oil to prevent evaporation, and allowed to react for 90 min at 65 °C. As a control, a sample solution submitted to the same reaction procedure without any template DNA was used, i.e. negative