Biosensors and Bioelectronics 26 (2011) 2012–2017

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Portable optoelectronic biosensing platform for identification of mycobacteria from the *Mycobacterium tuberculosis* complex

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ARTICLE INFO

Article history:
Received 9 June 2010
Received in revised form 27 August 2010
Accepted 31 August 2010
Available online 9 September 2010

Keywords:
Gold-nanoparticles
DNA
Mycobacterium tuberculosis
Tuberculosis
Optoelectronic platform
Amorphous thin film silicon color sensor

ABSTRACT

In this paper we report on the fabrication and performance of a portable and low cost optoelectronic platform integrating a double color tuned light emitting diode as light source, an amorphous/nanocrystalline silicon photodetector with a flat spectral response in the wavelength range from 520 nm to 630 nm and integrated electronic for signal acquisition and conditioning constituted by current to voltage converter, a filter and an amplification stage, followed by an analog to digital converter, with appropriate software for full automation to minimize human error. Incorporation of the double color tuned light emitting diode provides for a simple yet innovative solution to signal acquisition independently from the light intensity and/or solution concentration, while considerably decreasing production costs. Detection based on Au-nanoprobes constitutes the biorecognition step and allowed identification of specific sequences of *Mycobacterium tuberculosis* complex, namely *Mycobacterium bovis* and *M. tuberculosis* in biological samples.

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1. Introduction

Tuberculosis (TB) is a global health epidemic and every year there are nearly nine million new active TB cases and nearly two million TB deaths worldwide, most in the poorest communities of the developing world. This disease is caused by members of the *Mycobacterium tuberculosis* complex (MTC), a group of closely related pathogenic species that includes, namely, *M. tuberculosis*, the main agent of human TB, and *Mycobacterium bovis*, associated to bovine TB and representing a high potential for zoonotic transmission to humans. The MTC members are genetically very similar but differ in their epidemiology and antimicrobial susceptibility characteristics. A key challenge for the public health community is to be able to effectively diagnose TB patients, including the specific identification of the respective etiological agents, so that valuable resources and medicines are not wasted on misdiagnosis and repeated treatments (WHO, 2009a,b). The most commonly used diagnostic method (smear microscopy) used in the underdeveloped regions fails to efficiently and accurately diagnose TB. Patients must travel at great cost and time to microscopy centers to receive insensitive tests requiring trained technicians and repeated clinic visits, which may lead to significant death numbers, suffering, and the continued spread of disease (Uys et al., 2009). Serious effort has been directed to make available a robust, yet simple and portable, diagnostic platforms for use at peripheral laboratories and/or point-of-care (Buijtels et al., 2008; Csako, 2006; McGlennen, 2001; Mishra et al., 2005; Park et al., 2009; Pinsky and Banaei, 2008; Soo et al., 2006, 2009).

Previously, we demonstrated the potential use of gold-nanoparticles (AuNP) functionalized with specific oligonucleotides for identification of the most important members of the MTC, which relies on the colorimetric differentiation of Au-nanoprobes in presence and/or absence of specific target hybridization (Baptista et al., 2006; Costa et al., 2009). In solution, monodisperse AuNPs appear red and exhibit a relatively narrow surface plasmon resonance (SPR) absorption band centered around 525 nm. In contrast, a solution containing aggregated AuNPs appears blue, corresponding to a characteristic red-shift of the SPR (Fig. S1 in Supplementary information). Aggregation, upon salt addition, can be prevented by the presence of a specific complementary target hybridized to the Au-nanoprobe (Baptista et al., 2006; Costa et al., 2009; Doria et al., 2007). The increasing ionic strength of the solution, due to the addition of salt, provides a screening effect to the electrostatic repulsion between NPs derived from the charges of the exposed bases of ssDNA, leading to the aggregation of nanoprobes. Also, hybridization to the complementary target increases the steric hindrance

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doi:10.1016/j.bios.2010.08.078

between Au-nanoprobes, thus decreasing aggregation. The aggregation profile, and thus identification of MTC members, is achieved via the evaluation of the spectra acquired by traditional UV/Vis spectrophotometry. We also proposed the integration of this biodetection method into a sensing platform comprising a broad band color sensitive amorphous/nanocrystalline silicon photodetector and a laser (high intensity light source) (Portugal Patent no. 103561; South Africa Patent no. 2009/01612, Ref.: PTI-ZA 40004/09; Europe: PCT/IB2007/053614; Martins et al., 2007). This way, we can take advantage of the exceptional photoconductive behavior of amorphous/nanocrystalline silicon technology and the possibility to tailor the spectral response to the adequate range of the visible light spectrum (Martins et al., 2009; Zhang et al., 2005), ideal to be used in colorimetric applications. The integration of these technologies together with the possibility of miniaturization are of utmost importance for the development of an integrated biosensor suitable for peripheral laboratories and/or point-of-care diagnostics, providing a new tool in the fight against TB.

Here, we report on the fabrication and development of an integrated biosensing prototype platform based on the Au-nanoprobe biodetection and the optoelectronic sensor towards a low-cost portable system for the specific identification of MTC members and the consequent improvement of the laboratorial diagnostics algorithms of TB (Fig. 1).

From previous work (Martins et al., 2007; Martins et al., 2008; Silva et al., 2008), the detection platform has been modified so as to incorporate: (i) a low cost, controlled, tunable double color light emitting diode RGBA-LED (LZ4-00MA10 RGBA LED), with a specific narrow light emission in the red and in the green region (half width light band spectrum: $\Delta\lambda \cong 30$ nm), with the same light intensity as the light source, thus greatly reducing the production costs without compromising sensitivity, besides improving the platform detection reliability and decreasing the time required for the experiment; (ii) a broad band color sensitive amorphous/nanocrystalline silicon photodetector, with an almost flat spectral response in the wavelength range from 520 nm to 630 nm, allowing for integration of a wider region of the spectral profile and so a clear color detection discrimination, leading to increased robustness and platform reliability. Also, measurements are no longer dependent on one reference sample, as it happened in the previous reports; (iii) a sample holder in a dark box to reduce the effect of ambient light (background noise); and (iv) signal acquisition and processing electronics (as depicted by the printed circuit board, PCB, in Fig. 3) with associated software for direct data evaluation via USB port for PC connection and an user friendly interface. Portability and reliability for identification of MTC members were evaluated using reference and clinical isolates.

2. Experimental details

2.1. Sequence analysis and Au-nanoprobe synthesis

Comparative analysis of *gyrB* gene sequences from mycobacteria was performed through sequence alignment using CLUSTAL X software (Larkin et al., 2007). Gene sequences were retrieved from GenBank with accession numbers as follows: AB014184 for *M. bovis*; AJ276122 for *Mycobacterium. caprae*; AB014205 for *M. microti*; Z80233 for *M. tuberculosis*; AB014202 for *Mycobacterium. gastri*; AB014189 for *Mycobacterium. avium* subsp. *avium*; and EU029114 for *M. avium* subsp. *paratuberculosis*. Probe specificity was tested in silico using the BLAST tools from GenBank. Specific thiol-modified oligonucleotides for MTC, *M. tuberculosis* and *M. bovis* were used to functionalize the AuNPs and produce the Au-nanoprobes: MTC probe (gComplex) – 5′-thiol-CCGAGGACACAGCCTTGTTC-3′, *M. tuberculosis* probe (gMTub) – 5′-thiol-TTTGAAGCCAACCCCACCGACG-3′, and *M. bovis* probe (gMBov) – 5′-thiol-CGTTTGTGCAGAAGGTCTGTAAT-3′, all synthesized by STAB Vida Lda, Portugal.

AuNPs were prepared by the citrate reduction method described by Lee and Meisel (Lee and Meisel, 1982). Briefly, 250 ml of 1 mM $HAuCl_4$ were brought to boil while vigorously stirring. Twenty-five milliliters of 38.8 mM sodium citrate were quickly added and the mixture was refluxed for 15 min with continuous stirring. The solution was left to cool down to room temperature and stored in the dark until use.

Thiolated oligonucleotides were suspended in 1 ml of 0.1 M dithiothreitol (DTT), extracted three times with ethyl acetate and further purified through a desalting NAP-5 column (Pharmacia Biotech, Sweden). Specific Au-nanoprobes were synthesized by derivatizing the previously prepared aqueous solution of AuNPs with the specific thiolated oligonucleotide mentioned above. AuNP–DNA conjugates were prepared as previously described (Baptista et al., 2006; Doria et al., 2010). Briefly, an appropriate volume of 10 μM thiol-modified oligonucleotide was initially incubated with 6 ml of an aqueous solution of AuNPs (~8.55 nM) for at least 16 h. After centrifugation (20 min at 14,500 × *g*), the oily precipitate was washed with 5 ml of 10 mM phosphate buffer (pH 8.0), 0.1 M NaCl, recentrifuged and redispersed in 5 ml of the same buffer to a final concentration in AuNPs of 8.5 nM. The resulting AuNP–DNA conjugate was stored in the dark at 4 °C.

2.2. Au-nanoprobe assay

The colorimetric assays were performed as described in Costa et al. (2009) and Doria et al. (2007). Briefly, the Au-nanoprobe (final concentration 2.5 nmol/l) was mixed with the sample DNA at a final concentration of 30 μg/ml. After heat denaturation for 10 min at 95 °C, the vial was cooled down to room temperature for 30 min and $MgCl_2$ was added to a final concentration of 0.02 mol/l. Measurements were performed after 15 min of salt addition. The assay consists on comparison of samples and a blank containing an equivalent volume of 10 mM phosphate buffer (pH 7) instead of DNA. For sensitivity assays, each Au-nanoprobe was mixed with a synthetic complementary oligonucleotide in concentrations ranging from 0 fmol/μl to 300 fmol/μl. For biological samples, a 1020 bp specific fragment of the *gyrB* gene, suitable for differentiation of MTC members, was PCR amplified from reference and clinical mycobacteria isolates using a procedure adapted from Niemann et al. (2000) and used for the biological sample detection. Mycobacteria strains used are maintained at Laboratório Nacional de Investigação Veterinária (LNIV, Portugal), and/or American Type Culture Collection (ATCC, USA): LNIV 7257, LNIV 7638, LNIV 13027 and LNIV 8016 for *M. bovis*; LNIV 17320 for *M. caprae*; ATCC 25177 and LNIV 9605 for *M. tuberculosis*; ATCC 25291 for *M. avium* subsp. *avium*; LNIV 39888 for *M. avium* subsp. *paratuberculosis*; and LNIV 12352 for *Corynebacterium striatum* (see Table S1 in Supplementary information).

2.3. Photodetector fabrication

The sensor was produced as previously described (Fortunato et al., 2006; Martins et al., 2007). In brief, 4 cm × 4 cm glass substrates are coated using rf-sputtering techniques with a transparent conductive oxide (Gallium doped Zinc Oxide – GZO), followed by the growing of a hydrogenated amorphous silicon (a-Si:H) p/i′/i/n structure (p-boron doped a-Si:H and n-phosphorous doped a-Si:H) at 200 °C by plasma enhanced chemical vapor deposition (PECVD) using an exciting frequency of 13.52 MHz for the doped layers and of 27.12 MHz, for the i-layers. In this structure, the i′-layer works as a buffer layer, while the i-layer is the main photosensitive element of the device. The final electrical contacts (Ag/Al metal sandwich) are deposited by electron gun thermal evapora-

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