Biosensors and Bioelectronics 79 (2016) 294–299

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Designing anthraquinone–pyrrole redox intercalating probes for electrochemical gene detection

Yu-Jen Lin [a], Yung-Chao Wu [b], Veerappan Mani [a,b], Sheng-Tung Huang [a,b,*], Chih-Hung Huang [b], Yi-Chiuen Hu [c], His-Chi Peter Shan [d]

[a] Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, Taiwan, ROC
[b] Graduate Institute of Biomedical and Biochemical Engineering, National Taipei University of Technology, Taipei, Taiwan, ROC
[c] National Applied Research Lab, Hsinchu, Taiwan, ROC
[d] WinSense Biotech Corporation, Hsinchu, Taiwan, ROC

ARTICLE INFO

Article history:
Received 1 October 2015
Received in revised form
30 November 2015
Accepted 15 December 2015
Available online 17 December 2015

Keywords:
DNA sensor
Polymerase chain reaction
Electrochemistry
Biosensor
DNA amplification
Molecular design

ABSTRACT

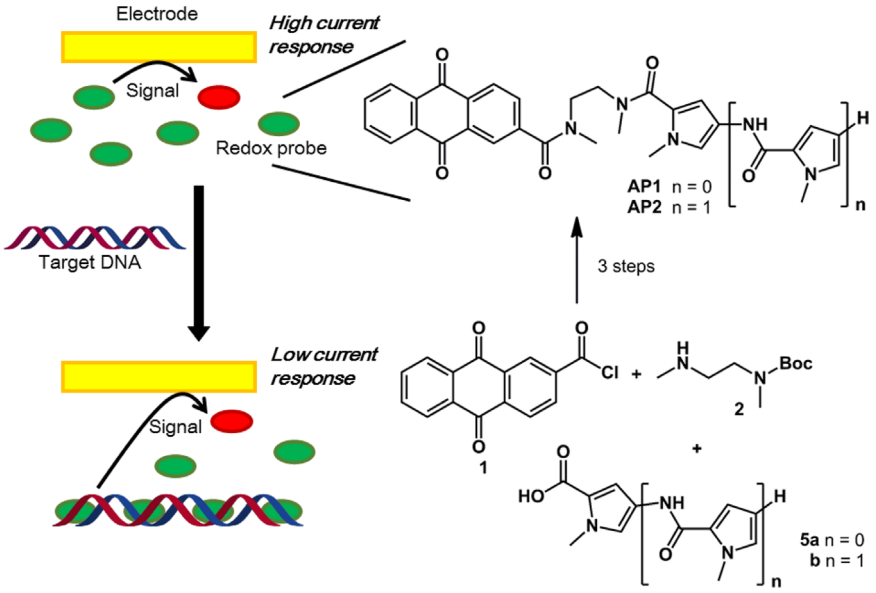
The real-time quantitative electrochemical monitoring of nucleic acid amplification through PCR is a promising renowned methodology to detect pathogenic DNAs. In this work, anthraquinone–pyrrole derivatives based redox intercalating probes (AP probes: AP1, AP2) have been designed, synthesized, characterized and successfully demonstrated in real-time like quantitative PCR. The rationally designed AP probes exhibited excellent DNA binding abilities and electrochemical behaviors. The binding parameters such as binding constant, binding site size and diffusion coefficient were estimated which were comparable to literature reports. Besides, the AP probes are highly stable under PCR thermal conditions and did not inhibit PCR. Therefore, a real-time like quantification of DNA amplification was demonstrated to quantify the initial copy number of target genes. The probe AP2 has excellent ability to detect $\sim 10^3$ copies of target *tpc* DNA with good sensitivity. The AP probes are metal-free, easily synthesizable, non-toxic, thermally stable and feasible for miniaturized PCR chips.

1. Introduction

The sensitive real-time quantitative gene analysis is extremely important in clinical diagnostics, food safety testing and environmental monitoring (Hsieh et al., 2015). The point-of-care use in clinical diagnostics requires simple and portable devices for rapid monitoring of pathogenic genes directly from untreated samples. In this regard, real-time polymerase chain reaction (PCR) amplification strategy is the most successful approach attributed to its accurate quantification of initial copy numbers of target DNA (Defever et al., 2011; Won et al., 2011). Current quantitative PCR (qPCR) instruments are based on the optical detection methods incorporated with fluorescent reporters such as intercalating dyes (Gašparič et al., 2010), primer-based chemistries (Nazarenko et al., 2002) and sequence specific oligonucleotides based chemistries (Kutyavin et al., 2000). Despite their significant success, their use is limited in centralized laboratories due to their high cost, tedious nucleic acid modification, and bulky (Higuchi et al., 1992). In recent times, electrochemical methods are likely to be the best alternative to replace laborious optical methods attributed to their additional advantages of low cost, portable and easier to handle. Therefore, current research in this area has been motivated to develop adaptable electrochemical approaches for the gene detections (Defever et al., 2011; Patterson et al., 2013). The first such effort was demonstrated in 2006 by Hsing et al.; within 8 years, numerous approaches have already been developed which were shown comparable performances to optical methods. The electrochemical monitoring of DNA amplification is based on electrochemical changes revealed by the redox reporters associated with amplification process (Yeung et al., 2006). Patterson et al. (2013) classified the electrochemical PCR monitoring methods into four classes; (1) solid-phase PCR amplification with electrode-bound redox reporters (Yeung et al., 2006), (2) solution-phase PCR using intercalating redox reporters (3) solution-phase PCR with redox labeled probes (Luo et al., 2011; Defever et al., 2009) and (4) isothermal amplification method (Nagatani et al., 2011). Among them, the solution-phase PCR coupled with DNA intercalating reporter has great possibility for sensitive DNA analysis due to its principle similarity with DNA-intercalating fluorescent dyes of optical qPCR. Defever et al. (2011) developed osmium based intercalating redox probes to detect human cytomegalovirus DNA; however, the use of Os metal imposed toxicity concern. Won et al., developed methylene blue based redox probe to detect *Chlamydia*

* Corresponding author at: Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, Taiwan, ROC.
E-mail address: ws75624@ntut.edu.tw (S.-T. Huang).

http://dx.doi.org/10.1016/j.bios.2015.12.042
0956-5663/



Scheme 1. The schematic representation for syntheses of anthraquinone–pyrrole redox intercalating probes and their application in real-time electrochemical monitoring of DNA amplification.

trachomatis genomic DNA; (Won et al., 2011) however, methylene blue encounters thermal instability in miniaturized PCR chips. Although numerous intercalating redox probes are available for DNA binding (Seio et al., 2005), so far only two intercalating redox probes are exploited in solution-phase PCR.

Anthraquinones are well-known feasible redox system and critically acclaimed for their DNA binding abilities (Wei et al., 2012). Besides, polyamide anchors comprising pyrrole moiety are efficient DNA binding motifs for the recognition of DNA minor groove (Schmidt and Heckel, 2009). Considering the excellent DNA binding attributes of anthraquinone and pyrrole, herein, we have designed anthraquinone–pyrrole (AP1 and AP2) based DNA intercalating redox probes to electrochemically monitor DNA quantification in PCR (Scheme 1 and Scheme S1). Structurally, the probes AP1 and AP2 are equipped with anthraquinone as redox reporter and pyrrole as binding motif through amide linkage. The main objective of this work is to develop anthraquinones–pyrrole based intercalating redox probes for rapid and low-cost gene detection. The probes are thermally stable under PCR conditions without causing PCR inhibition. A real-time like electrochemical monitoring of DNA amplification in PCR was demonstrated which detects $\sim 10^3$ copies of *tpc* plasmid DNA.

2. Experimental

2.1. Reagents and instrumentation

Calf thymus DNA was purchased from Sigma and dissolved in double-distilled water. The concentration of DNA was determined by spectrophotometry at wavelength of 260 nm with molar extinction coefficient of 6600 cm^{-1}. The stock solutions were prepared using double-distilled water and stored at 4 °C. DNA ladder (100–10000 bp), primer TPC-F (CAGGCGCGGATCTCCAG) and primer TPC-R1 (GTCGTCCAGCGCCGTGA) were purchased from Genomics. Plasmid miniPREP bought from GeneDireX was employed for the purification of *tpc* plasmid. Taq. DNA polymerase kits bought from NovelGene was used for the PCR amplification analyses. All the other chemicals were purchased from Sigma-Aldrich, Alfa Aesar and Wako.

N,N-dimethyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxamide (AQ-amide) was prepared by known procedure (Degrand and Miller, 1981) and characterized by ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The reactions which require anhydrous conditions were performed in oven-dried glassware under an Ar or N_2 atmosphere. Chemicals and solvents were either puriss p.a. or purified by standard techniques. Analytical thin-layer chromatography (TLC) was performed on a glass plate-mounted silica gel $60F_{254}$ (Merck) (0.2 mm thickness). Flash column chromatography was performed using Silicycle silica gel 60. The synthesized compounds were characterized using ^{1}H NMR (Bruker Advance 300 MHz) and ^{13}C NMR (Bruker Advance 75 MHz). Mass spectra were recorded using Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source. The UV–vis spectroscopy and Fourier-transform infrared spectroscopy analyses were carried out using JASCO V-630, and HORIBA 720, respectively. The Biometra TPersonal Thermocycler was employed for thermal cycling process. Circular dichroism (CD) spectroscopy was performed using Jasco J-810 Spectropolarimeter. Electrochemical measurements were carried out using CHI 612D electrochemical work station. Electrochemical studies were performed in conventional three electrode cell using gold electrode as a working electrode (2 mm diameter), Ag|AgCl (saturated KCl) as a reference electrode and Pt wire as a counter electrode. Prior to use, the working electrode was polished with 0.05 μm alumina powder and thoroughly washed with double distilled water. Prior to each electrochemical experiment, the electrolyte solutions were deoxygenated with pre-purified nitrogen for 5 min unless otherwise specified. All the electrochemical experiments were carried out at room temperature (20 °C). Differential pulse voltammetry (DPV) parameters: amplitude 0.05 V, pulse width 0.05 s and pulse period 0.5 s. The PCR amplification *tpc* (220 bp) sequence conditions were optimized using gel electrophoresis. The PCR products were electrophoresed using agarose gel and visualized by ethidium bromide staining.

2.2. Synthesis of AP1 and AP2

2.2.1. Synthesis of tert-butyl methyl(2-(N-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxamido)ethyl)carbamate (**3**)

Anthraquinone-2-carbonyl chloride (**1**, 0.91 g, 3.4 mmol) was dissolved in dry dichloromethane (20 mL), subsequently [2-