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tert-Butyl Hydroquinone: an Effective Electrochemical Probe for Sensing Polymerase Chain Reaction and Loop-Mediated Isothermal Amplicons

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The quantitative monitoring of nucleic acid amplifications via polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) are current approaches for high sensitive detection of DNA from pathogenic (micro) organisms. This work is continuous effort of our research group to find inexpensive and readily available DNA-redox intercalating probe for monitoring of amplicons. In this regard, we found *tert*-butyl hydroquinone (TBHQ), a readily available and low-cost redox molecule popularly used in food additive has good DNA binding ability and it is successful in the quantitative monitoring of DNA amplicons in PCR and LAMP. Our studies revealed that TBHQ has excellent DNA binding abilities and electrochemical behaviors in both PCR and LAMP buffer, and its binding parameters, binding constant, binding site size and diffusion coefficient are suitable for end point detection. The electrochemical end-point detections for monitoring DNA amplifications are established using target genomic DNA as models including λ phage, calf thymus and *E. coli* and the method is verified and authenticated by traditional methods, such as fluorescence and turbidimetric methods. Besides, we found good recoveries in the determination of target tpc gene spiked in *λ* phage, calf thymus and *E. coli* DNA.

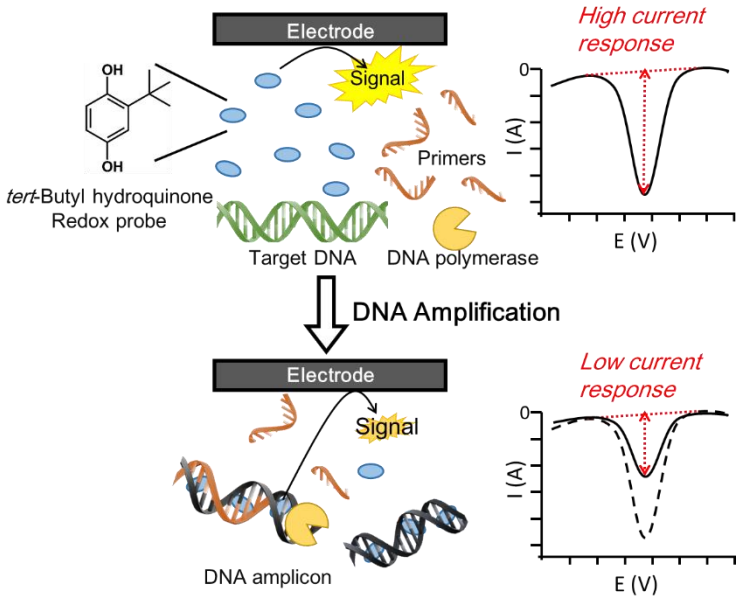
Keywords: Polymerase chain reaction, LAMP, DNA, Biosensor, Molecular design, redox reporter

1. INTRODUCTION

The sensitive and selective gene detection is highly important in clinical and food analysis [1-3]. The nucleic acid amplification is the widely used method due to its accurate quantification of initial copy numbers of target DNA which greatly improves sensitivity [4]. Numerous amplification methods are available such as polymerase chain reaction (PCR) [5-8], nucleic acid sequence based amplification (NASBA) [9], strand displacement amplification (SDA) [10], helicase-dependent amplification (HDA) [11], loop-mediated isothermal amplification (LAMP) [12-15], rolling circle amplification (RCA) [16-18] and self-sustained sequence replication [19]. Among them, PCR is widely established, while recently LAMP has been proved as promising method for DNA amplification [20]. PCR amplification strategy is the current successful method for DNA amplification; however it requires different temperature cycling and involve post–PCR analysis [21-24]. On the other hand, LAMP method amplifies the target gene under isothermal conditions (~ 65°C) which eliminates the need of thermal cycler. Moreover, the gene amplification and detection can be accomplished in single step at short time period (~ 1 h). Besides, LAMP uses four different specifically designed primers to identify six distinct regions of the target gene which offers high level of specificity and hence LAMP is emerging as a powerful amplification method in recent times [13, 25].

Generally, the signal read-outs in PCR and LAMP instruments are optical (colorimetric/fluorescence) methods [21, 26, 27]. Importantly, optical methods require transparent samples and rely on additional expensive optical tools to convert underlying chemical signal (analyte-probe interaction) into electronic signal. In addition, optical methods encounter certain limitations such as low quantum yield, pre-sampling steps and auto-absorption/auto-fluorescence [28, 29]. Electrochemical read-outs are suitable alternative to optical methods due to their additional advantages of low cost, portable and easier to handle [30]. In addition, the electrochemical instruments do not require transparent solutions and they can directly convert the chemical reaction into electronic signal. Indeed, by replacing optical read-outs with electrochemical read-outs, we can set up good platform in order to achieve simple and cost-effective gene detection. Generally, our research group has keen interest to find inexpensive and readily available DNA–redox intercalating probes for monitoring of amplicons considering the importance of gene detections. Recently, we have reported anthraquinone-pyrrole based DNA intercalating redox probes to monitor amplicons in PCR and successfully detected ~10^3 copies of *tpc* plasmid DNA [31]. From our continuous research in this area, we understood that the intercalating probe should (1) possess good DNA intercalating property, (2) low-cost, and (3) readily available as commercial compound in order to be used in amplicons monitoring. In this aspect, *tert*-butyl hydroquinone (TBHQ), a popularly used food preservative satisfies all the aforementioned conditions [32]. TBHQ is electrochemically active and stable redox molecule ascribed to the inherent hydroquinone-quinone reversible redox process. Herein, we have investigated the possibility of using TBHQ as DNA intercalating probe for monitoring DNA amplification process in PCR and LAMP and we found that TBHQ is highly suitable for rapid and low-cost gene detection. To the best of our knowledge, this is the first report employing TBHQ as a redox probe to monitor nucleic acid amplicons in PCR and LAMP. The end point detection of DNA amplification in PCR and LAMP are

demonstrated in λ phage, calf thymus and *E. coli* and our studies proved that TBHQ is a good redox probe for end point detection in PCR and LAMP.



Scheme 1 The schematic representation for electrochemical monitoring of DNA amplification using a DNA intercalating redox probe TBHQ.

2. EXPERIMENTAL

2.1 Reagents and instrumentation

Calf thymus DNA was purchased from Sigma. The concentration of DNA was estimated via spectrophotometry [33]. 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 was bought from GeneDireX which was employed in the purification of *tpc* plasmid. Taq. DNA polymerase kits were bought from NovelGene.

Electrochemical measurements were performed in Atolab PGSTAT128N electrochemical work station. Electrochemical studies were carried out in conventional three electrode cell using glassy carbon electrode (GCE) (BASi) as a working electrode (3 mm diameter), Ag|AgCl (saturated KCl) as a reference electrode and Pt wire as a counter electrode. All the electrochemical experiments were carried out at ambient conditions. The optimized Differential pulse voltammetry (DPV) parameters: pulse width 0.02 s, amplitude 0.09 V, and pulse period 0.5 s.

2.2 Experimental conditions for PCR

1X PCR buffer was used for all experiments which were prepared by following reagents: 15 mM Tris-HCl (pH = 8.75), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100 and 1% DMSO. The

electrochemical PCR analyses of *tpc* DNA (220 bp) were carried out in 1X PCR buffer containing 0.25 mM dNTP, 0.25 µM of each forward (Primer-R1) and reverse (Primer-F) primers, 1.25 units Taq. DNA polymerase, *tpc* plasmid DNA and DMSO. The PCR analyses were carried out in accordance with the following thermal cycling conditions (total of 40 cycles): preheating period of 5 min at 95°C, followed by denaturation for 30 sec at 94°C, annealing at 55.2°C for 20 sec and elongation period of 1 min at 72°C.

2.3 Experimental conditions for LAMP

1X LAMP buffer was used for all experiments which were prepared by following reagents: 20 mM Tris-HCl (pH = 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 1% DMSO. The electrochemical PCR analyses of *tpc* DNA (220 bp) were carried out in 1X LAMP buffer containing 1.4 mM dNTP, 1.6 µM of each inner primers (FIP and BIP) and 0.2 µM outer primers (F3 and B3), 320 unit/mL *Bst* DNA polymerase, *tpc* plasmid DNA and DMSO. The LAMP analyses were carried out by incubating 50 µM TBHQ in presence of respective DNA for heating a period of 70 min at 65°C.

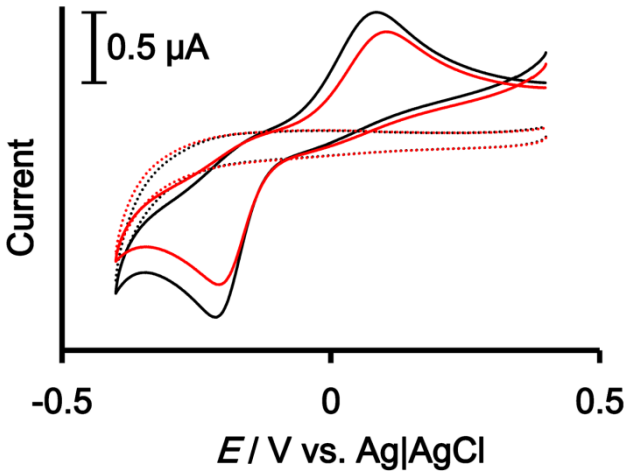


Figure 1. CVs obtained in absence (dotted lines) and presence of 50 µM TBHQ under PCR (black) and LAMP conditions (red). The potential range was applied between 0.4 V and – 0.4 V and the scan rate was held at 50 mV s^{-1}.

3. RESULTS AND DISCUSSION

3.1 Electrochemical properties of TBHQ

Although TBHQ is known to intercalate with DNA, its intercalation characteristics in PCR and LAMP buffer conditions has never been studied. Therefore, first we characterized the DNA binding properties of TBHQ under PCR and LAMP buffer conditions. The electrochemical behaviour of TBHQ was investigated by cyclic voltammetry in both PCR and LAMP buffer conditions (Fig. 1). The potential range was 0.4 V and – 0.4 V. The scan rate was 50 mV s^{-1}. TBHQ exhibits characteristic

reversible redox couple corresponding to the redox reaction of quinone-hydroquinone and the reaction involves two electrons and two protons. The important electrochemical parameters such as anodic (E_{pa}) and cathodic peak potential (E_{pc}), anodic (I_{pa}) and cathodic peak current (I_{pc}), half-wave potential ($E_{1/2}$), peak potential (E_p), and I_{pa}/I_{pc} ratio were evaluated (Table 1). As shown in the table, the redox potentials and $E_{1/2}$ of TBHQ are significantly away from the usual oxygen reduction potentials. Therefore, dissolved oxygen present in the buffer doesn't produce any disturbance with the redox reaction of TBHQ and hence the electrolyte does not require deaeration. TBHQ in LAMP buffer showed comparatively higher redox peak currents (I_{pa} and I_{pc}), presumably due to the higher salt concentration in LAMP buffer condition.

Table 1. Electrochemical behavior of TBHQ presented in PCR and LAMP conditions.

Condition	E_{pc}/V	E_{pa}/V	E_p/mV	$E_{1/2}$/V	I_{pc}/μA	I_{pa}/μA	I_{pa}/I_{pc}
PCR	-0.213	0.085	298	-0.064	0.804	0725	0.902
LAMP	-0.220	0.121	341	-0.050	1.062	0.745	0.702

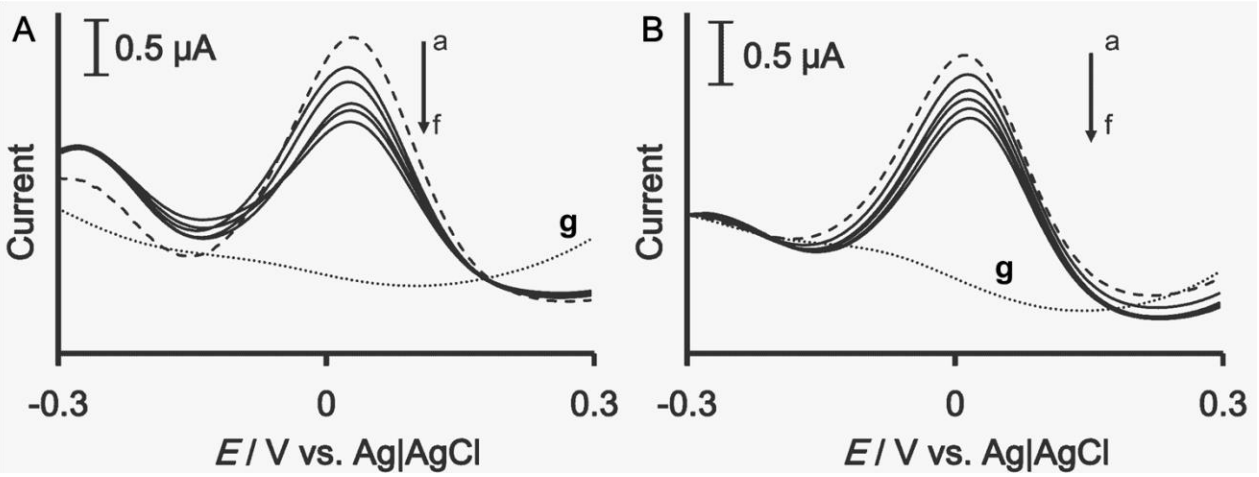


Figure 2. DPVs obtained for 50 μM TBHQ in the absence of DNA (a) and presence of 25 μM (b), 50 μM (c), 100 μM (d), 200 μM (e), 400 μM (f) calf thymus DNA in PCR (A) and LAMP buffer (B) conditions. Curve g represents the DPV obtained for only buffer conditions.

3.2 DNA intercalating ability of TBHQ

Next, we have studied the DNA binding ability of TBHQ in PCR and LAMP conditions. Fig. 2 displays the DPVs of TBHQ under PCR (A) and LAMP (B) buffer solutions containing different concentrations of calf thymus DNA. The peak currents of TBHQ are considerably dropped upon DNA addition which is due to the binding of DNA with TBHQ. The intercalation of DNA with TBHQ makes the TBHQ molecules less accessible and which causes the drop in redox peak currents. As the

concentration of DNA increases, the redox peak currents of TBHQ decrease in both PCR and LAMP conditions. The planar structure of TBHQ efficiently intercalated by DNA double helix through hydrogen bonding, van der Waals, and π-π stacking interactions which reasoned for the good DNA interacting ability of TBHQ. Therefore, TBHQ can be used as potential DNA intercalating molecule in PCR and LAMP. Notably, TBHQ has shown higher DNA binding ability (large decrease in peak currents) in PCR buffer compared to LAMP buffer (small decrease in peak currents). Both PCR and LAMP buffer have cations such as Mg^{2+}, K^+ and NH_4^+ and these cations form ionic bonds with negatively charged DNA which occupies the binding sites of DNA. As a result of this effect, the DNA binding efficiency of TBHQ is decreases. This type of effect is more prominent in LAMP buffer conditions because LAMP buffer has higher cation concentrations. Comparatively, PCR buffer have less cations and hence TBHQ possess comparatively higher DNA binding ability in PCR buffer over LAMP buffer.

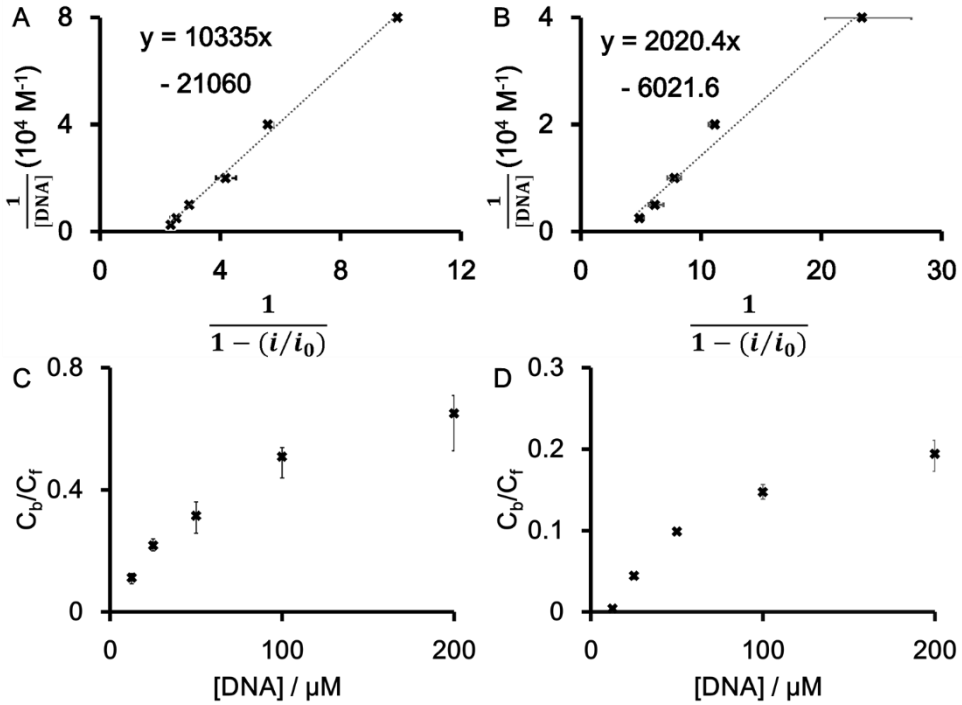


Figure 3. Binding constant of TBHQ for PCR (A) and LAMP (B): Plots of 1/[DNA] vs. 1/(1-i/i_0). Binding sites sizes of TBHQ for PCR (C) and LAMP (D): Plots of C_b/C_f vs. [DNA].

Next, binding constants (K_b) for TBHQ–DNA in PCR (Fig. 3A) and LAMP (Fig. 3B) buffer conditions are calculated from the plots of 1/[DNA] and 1/(1–i/i_0). To make the plots, the values of peak currents were taken from fig. 2 and the equation (1) was used to calculate K_b,[34]

$$\frac{1}{[DNA]} = \frac{K_b\,(1-A)}{1-(i/i_0)} - K_b \qquad (1)$$

Where, K_b stands for binding constant, A is proportionality constant and terms i and i_0 represents peak currents of TBHQ in presence and absence of DNA, respectively. The K_b values of TBHQ obtained for PCR and LAMP are 2.106×10^4 M^{-1} and 6.022×10^3 M^{-1}, respectively (Table 2). The K_b value obtained for PCR conditions is comparatively smaller than for LAMP

conditions. As mentioned earlier, the DNA intercalation to TBHQ is comparatively stronger in PCR buffer than LAMP buffer. However, we observed that the K_b value presented in our system are much lower than the previous report of TBHQ demonstrated in 0.01 M Tris-HCl buffer solution (K_b = 1.07×10^5 M^{-1}) [32]. The lower K_b value is referred to the high amounts of cations present in the solution and the cations will contend the binding sites of DNA with TBHQ.

Table 2. DNA intercalating parameters estimated for TBHQ. Here, K_b is binding constant, *s* is binding site size, D_f is diffusion coefficient of free probes (absence of calf thymus DNA) and D_b is diffusion coefficient of bound probes.

Condition	K_b (M^{-1})	*s* (bp)	D_f (cm^2 s^{-1})	D_b (cm^2 s^{-1})
PCR	2.106×10^4	4	1.660×10^{-13}	1.042×10^{-13}
LAMP	6.022×10^3	5	5.670×10^{-13}	4.775×10^{-13}

3.3 Binding site size, diffusion co-efficient and thermal stability of TBHQ

The binding site size (*s*) was calculated form plot between C_b/C_f and [DNA] for PCR (Fig. 3C) and LAMP (Fig. 3D) buffer conditions. The equation (2) was adopted to calculate binding site size [34].

$$\frac{C_b}{C_f} = \frac{i-i_0}{i} = \frac{K_b}{2s}[DNA] - K_b C_b \qquad (2)$$

Here, *s* is binding site size per base pair (bp), C_b is the concentration of bound TBHQ and C_f is the concentration of free TBHQ. The parameters *i* and i_0 stands for the peak currents obtained for TBHQ in presence and absence of DNA. The obtained *s* values of TBHQ for PCR and LAMP are 4 and 5, respectively (Table 1), which are in good agreement with previously reported DNA intercalating probes [35]. Because the LAMP buffer contains more cations which will compete with DNA binding domain of TBHQ, and therefore the *s* value is little higher in LAMP buffer than PCR buffer.

The diffusion co-efficient values of TBHQ for PCR and LAMP in absence and presence of DNA were estimated using following equation (3), [36]

$$i = 2.69\times10^5\, n^{3/2}\, A\, C_0^*\, D^{1/2}\, v^{1/2} \qquad (3)$$

Here, n_a is number of electrons participated in rate determination step, *D* (cm^2 s^{-1}) is diffusion coefficient, *v* is scan rate, C_o* is concentration, *i* is the response current and *A* is the electrode area. The plots between currents and square root of scan rate for PCR and LAMP buffer conditions are given as Fig. 4A and Fig. 4B, respectively. The experiments were performed in 50 µM TBHQ in absence and presence of DNA (solid line) at the scan rates ranging from 0.01 to 0.1 Vs^{-1} under experimental condition. The diffusion coefficient values in absence and presence of calf thymus DNA were calculated and given in Table 2. The diffusion co-efficient values of TBHQ have been decreased significantly after its intercalation with DNA in both PCR and LAMP buffer conditions.

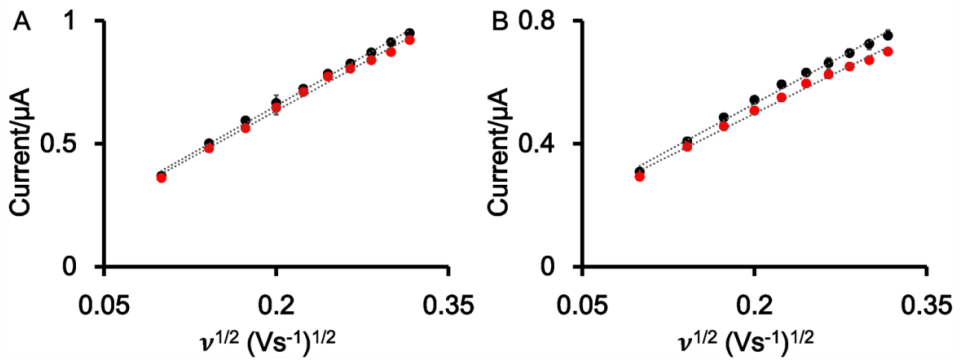


Figure 4 Peak currents versus $v^{1/2}$ for 50 µM TBHQ in absence of DNA (black line) and presence of 200 µM calf thymus DNA (red line) at scan rates ranging from 0.01 to 0.1 Vs^{-1} under PCR (A) and LAMP (B) buffer conditions.

The thermal and chemical stability of TBHQ during different heating process of PCR thermal cycle and also under isothermal (T= 65°C) conditions are investigated. The electrochemical signal of TBHQ has been reduced over time due to its degradation under thermal conditions of PCR and LAMP and hence TBHQ is not thermostable (Figure not shown). Perhaps, TBHQ molecule has to be designed in a different way in order to make it thermally stable under PCR and LAMP conditions which will be our future work. Nevertheless, our studies indicating that TBHQ is an excellent DNA intercalating probe and it can be used for end-point detection of nucleic acid amplifications in PCR and LAMP.

3.4 Detection of PCR amplicons

The TBHQ is added into PCR amplicons of different cycles; 0, 4, 8, 12, 16, 20, 24, 28, 32 and 36 cycles. Then, DPVs were carried out for each cycle and the normalized responses were used to plot against their corresponding cycle numbers (Fig. 5A). The normalized currents are decreased from 4 to 36 PCR amplifications. However, PCR sample with the absence of template have shown no obvious changes in peak currents (negative control). As the PCR cycle reaction progress, TBHQ electrochemical signal exhibits downward trend with the initial copy numbers. Furthermore, the numbers of DNA amplicons produced on each 4 cycles were tested and verified by fluorescent quantification and capillary electrophoresis methods. The electrochemical results are consistent with fluorescent (Fig. 5B) results which validated the working principle and accuracy of our method.

Next, we have evaluated the developed TBHQ based quantitative end-point detection results with standard fluorescent qPCR. A plot was made between threshold electrochemical signal values in terms of cycle number (C_t) versus logarithm of initial copy number of target DNA for both electrochemical detection based on TBHQ and standard fluorescent qPCR (Fig. 5D). As shown in the plot, the end-point detection calibration line is in good agreement with fluorescent qPCR. This method can detect upto ~10^3 copies of target DNA which is competitive to the previously reported electrochemical redox probes such as $Os(bpy)_2DPPZ^{2+}$ [8] and methylene blue [7] and our previously reported anthraquinone-pyrrole probe.[31] It is considerable to believe that a lower detection limit can be achieved by optimizing the concentration of TBHQ and the PCR condition.

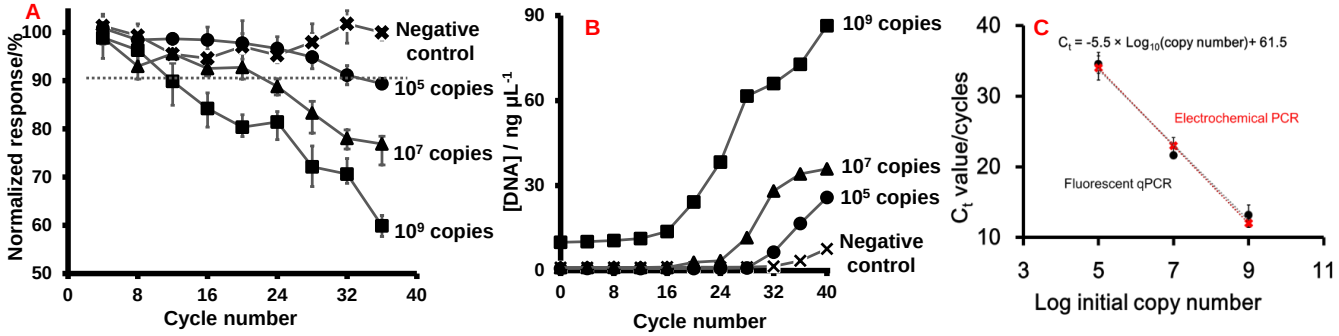


Figure 5 (A) Amplification curve obtained from the electrochemical end-point PCR detection with serially diluted samples containing 10^9 (■), 10^7 (▲), 10^3 (●), and 0 (NC, ×) copies of template. NC = negative control. The horizontal dashed line indicates the threshold level used to establish the C_t values. (B) Quantity of DNA amplicons quantification through PCR cycles by fluorescent quantification. (C) Standard calibration plots of C_t vs the logarithmic initial HTP copies for electrochemical end-point PCR detection (red) and fluorescent qPCR (black).

3.5 Detection of LAMP amplicons

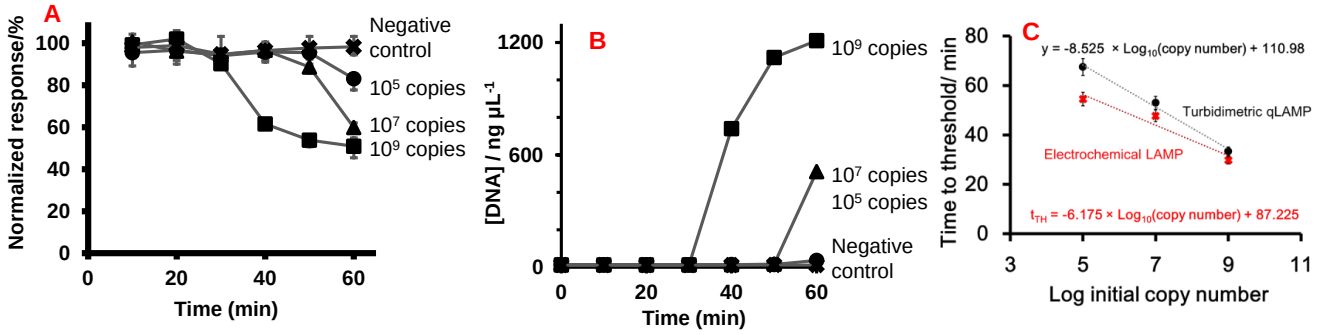


Figure 6. (A) Amplification curve obtained from the electrochemical end-point LAMP tracing detection with serially diluted samples containing 10^9 (■), 10^7 (▲), 10^3 (●), and 0 (NC, ×) copies of template. The horizontal dashed line indicates the threshold. (B) Quantity of DNA amplicons detected through LAMP reaction using fluorescent quantification. (C) Standard calibration plots of time to threshold vs the logarithmic initial HTP copies for electrochemical end-point LAMP detection (red) and turbidimetric quantitative LAMP (black).

The endpoint detection of DNA amplification through LAMP is performed for every 10 min after adding TBHQ followed by mixing. The electrochemical signals were measured using DPV and the normalized current responses were plotted against time (Fig. 6A). The result revealed that as the LAMP progress with time, the electrochemical signal of TBHQ started to decrease and followed a downward trend as the initial copy number of target DNA. At high concentration, we can able to detect 10^9 copies of the initial copy numbers, and the signal begins to decay from 30 min because dNTP were depleted and hence no new DNA fragments were available. As a result, the electrochemical signal slowed down to about 50%. We have observed current decreasing of end-point electrochemical detection in LAMP reaction after 50 min (10^5 copies), and it was faster than PCR reaction (36 cycles =

~140 min). In contrary, the negative control doesn't have any changes for continuous 70 min. In addition, the numbers of amplicons quantified by TBHQ have been verified by fluorescent quantification (B) and the results are in good agreement with electrochemical methods.

Next, we have evaluated the developed TBHQ based quantitative end-point detection results by comparing it with standard turbidimetric qLAMP (Fig. 6D). As shown in the plot, our TBHQ based endpoint detection calibration line is in good agreement with turbidimetric qLAMP which validates the ability of TBHQ to accurately detect DNA amplification. The traditional turbidity approach is based on the quantitative detection of pyrophosphate turbid in Mg^{2+} buffer as a white solid and normally the efficiency of precipitation formation is poor. On the other hand, electrochemical method directly converts the information into electronic digital signal. Besides, it can also work well in non-transparent solutions. Therefore, the developed electrochemical gene detection platform has great significance over traditional turbid formation methods. However, the detection limit of TBHQ platform is higher than the known MEQ-LAMP method which used methylene blue as the DNA intercalating redox probe [13]. Nevertheless, TBHQ provides a cheaper and different signal potential redox for electrochemical detection of DNA and future work will be directed to improve the detection limit.

3.6 End-point detection

From the experimental results, at a high concentration of copy number (10^9 copies), the PCR using an electrochemical method requires 14 cycles of the electrical signal which is about 47 min. However, for the same copy number, LAMP requires only 30 min to observe significant changes in the signal which is about 1.57 times faster than PCR method. At low concentrations (10^5 copies), PCR method requires further 34 cycles before the electrochemical signal is observed which is about 107 min, while the LAMP method able to reveal the signal in just 55 min which is about 1.95 times faster. Thus, LAMP method possesses significantly better detection efficiency over PCR method in terms of sensitivity and reaction time.

Using our probe incorporated LAMP method, we set up a complex nucleic acid samples assay on a selected target tpc gene on HTP plasmid incorporated in various DNA sample respectively, such as λ phage DNA, calf thymus DNA and *E. coli* genomic DNA, and LAMP for 60 min prior to the addition of TBHQ and then the electrochemical end (end-point) is detected before and after the electrochemical signals. Fig. 7 compares the DPVs of genomic DNA incorporated HTP plasmid (λ phage (A), calf thymus (B) and *e. coli* (C); solid line) and unincorporated HTP plasmid (dotted line). The current signal is considerably reduced after the incorporation of DNA samples. The signal decline has been converted into percentage and given in Fig. 8. The quantities of HTP plasmid were measured under three circumstances and recoveries of 98%, 101% and 90%, respectively, were observed which revealing that indeed a variety of DNA samples can be accurately detected in the mixture to the selected target gene and accurately distinguish the difference between the positive control and the negative control.

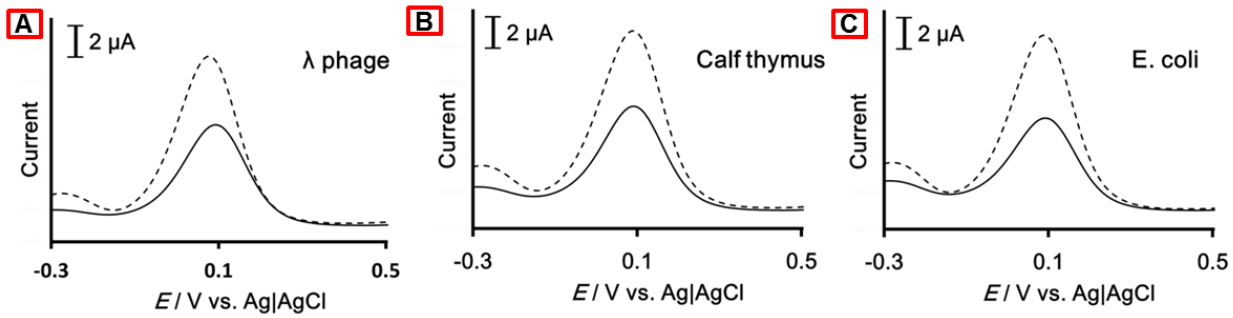


Figure 7. The electrochemical monitoring of amplification of target genomic DNA, λ phage (A), calf thymus (B) and *E. coli* (C) using TBHQ-LAMP system.

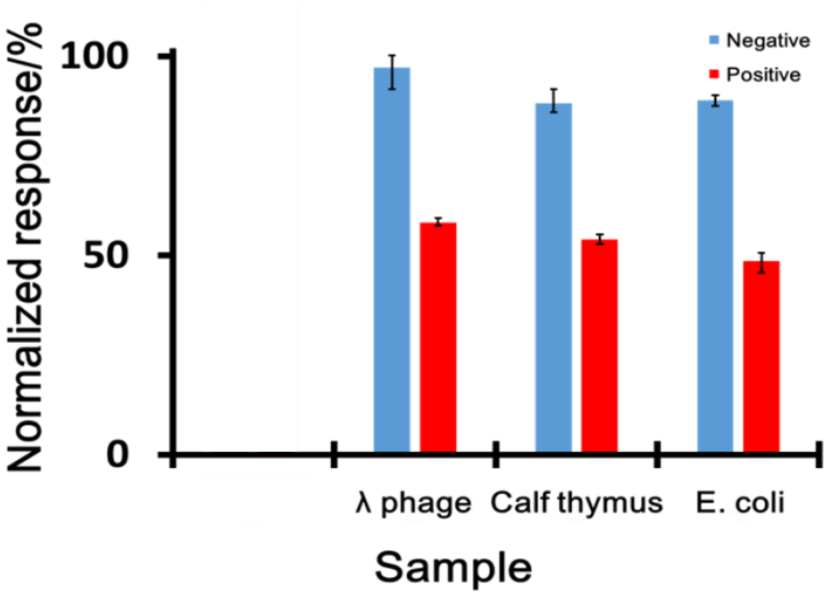


Figure 8. Normalized current responses vs. real samples

4. CONCLUSIONS

In summary, TBHQ had been verified as successful DNA intercalating redox probe for the quantification of DNA in λ phage, calf thymus and *E. coli*. The probe TBHQ has shown good DNA intercalating ability and its binding ability were estimated. Although, TBHQ was not thermostable, it has shown good recoveries in the end point detection of *tpc* gene on HTP plasmid. The results of our method are verified and authenticated by traditional methods, such as fluorescence and turbidimetric methods. TBHQ has added advantages such as cheap and metal-free. The future work will be directed to redesign TBHQ in order to make it thermally stable in PCR and LAMP conditions.

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