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Optimizing electrochemical DNA biosensors for the detection of avian infectious bronchitis virus

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ABSTRACT

Aims: The aim of the study is to develop the optimised parameters of electrochemical DNA biosensors for the specific detection of the Infectious Bronchitis Virus (IBV) in chickens. The goal is to further create a highly sensitive and specific biosensor that can be used for on-site monitoring of IBV on poultry farms.
Methodology and results: In this study, an electrochemical DNA biosensor was developed for detecting a specific sequence in the IBV genome. The process involved attaching a NH2-ssDNA probe to a gold electrode, followed by hybridization with the target DNA. Various parameters like buffer, pH, scan rate, incubation time, redox indicators and temperature were optimised using cyclic voltammetry. The probe DNA was designed to enhance hybridization efficiency, which was assessed by measuring current signals. The biosensor, under optimal conditions, demonstrated high sensitivity and specificity when tested with different sequences, including complementary, non-complementary and mismatched ones. Cross-reactivity studies against non-IBV viruses showed distinguishable current signals. These findings have implications for developing a portable on-site IBV monitoring device for use on farms.
Conclusion, significance and impact of study: The optimised parameters and specificity of the electrochemical DNA biosensor suggest its potential for the development of a portable device for on-site monitoring of IBV on poultry farms. This device could prove to be a valuable tool for the early detection of IBV, helping to prevent further spread of the disease. However, it's essential to conduct further research to ensure the practicality and accuracy of the biosensor in real-world farm settings.

Keywords: Avian infectious bronchitis virus, cyclic voltammetry, electrochemical biosensor, hybridization, immobilisation

INTRODUCTION

Infectious bronchitis virus (IBV) is an avian coronavirus of the Coronaviridae family that has the greatest impact on broiler, breeder and layer production worldwide. Based on genome sequencing, IBV belongs to the gamma-coronavirus and only some numbers are in Delta-CoV (de Wit and Cook, 2020) genus, which is essential for understanding its genetic relationship to other coronaviruses (Wickramasinghe *et al.*, 2011; Sharma *et al.*, 2020). However, beta- and gamma-coronaviruses (CoV) carry an open reading frame (ORF) that encodes the structural proteins in the 3'-terminal and the non-structural proteins in the 5'-terminal regions of the genome, which bind to the host cell as distinct receptors for pathogenesis.

IBV has led to economic losses in the poultry industry as the number of local IBV variants has increased due to frequent genetic mutations, replications and gene deletions. When infected with IBV, the chicken becomes immunosuppressed and quickly becomes infected with secondary bacterial infections. This therefore poses a major challenge for the prevention and control of IBV (Jackwood, 2012; Bhuiyan *et al.*, 2021). IBV variants or strains can be mutually protective and response to vaccines ranges from very low to moderate, which can lead to an outbreak of IBV in vaccinated flocks (Xu *et al.*, 2007; Xu *et al.*, 2015). Between 1990 and 2020, studies on IBVs were conducted across various states in Malaysia, including Sabah where the predominant IBV strain was identified as the IBV QX-like variant (47%) followed by the Malaysia Variants (13%) and vaccine

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strains (11%) (Zulperi *et al.*, 2009; Leow *et al.*, 2018). The prevalence of the QX-like strain, especially once combined with the IBV Malaysian variant, has been associated with issues like kidney disease and false layer syndrome in infected chickens, resulting in mortality rates ranging from 20% to 30%. However, in a few cases, due to the severity and coinfection with IBV disease, mortality during outbreaks can reach up to 80% of chicken flocks (He *et a*l., 2012; Bande *et al*., 2016; Ismail *et al.*, 2020; Besar *et al.*, 2023).

The diagnosis of Infectious Bronchitis Virus (IBV) in poultry involves various laboratory methods. Conventional assays such as cell culture or various steps of isolation techniques have several drawbacks as they are time-consuming and take up to 2–14 days; yet they are unsuitable for rapid detection (Oberste *et al.*, 2003; Rajapaksha *et al.*, 2019). Alternatively, the most common techniques used to identify IBV are reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), which are faster and more sensitive (Belák, 2007), however, they are all lab-based and very difficult to use in small-scale chicken farmers. Alternatively, these molecular assays are exclusive and require a high-tech instrument and complete laboratory equipment. The pre-amplification of genomic DNA is required by the PCR method prior to analysis, which requires a high concentration of target DNA compared to unrelated sequences. Moreover, the target DNA is being influenced by impurities during PCR from environmental DNA throughout the amplification process which is highly sensitive to contamination and sometimes not linear with cycle number (Peccoud and Jacob, 1996; Smith and Osborn, 2009).

Faster diagnosis of IBV in poultry farms is crucial for early intervention, effective disease management, minimising economic losses, preserving biosecurity, maintaining consumer confidence and preventing further disease spread. A biosensor is a simple device for detecting a specific target analyte by combining the response of biological recognition components with a transducer that converts the bio-recognition event into an appropriate electrical signal (Lim and Ahmed *et al*., 2016; Saylan et al., 2019; Saylan and Denizli, 2020). The electrochemical DNA biosensor based on nucleotide assays is routinely used as it is more specific to identify the viral/bacterial genotypes. To minimise economic losses in the poultry industry, it would be very important to develop a rapid test to overcome the difficulties of current techniques. Similarly, DNA biosensors can meet the following criteria since it is very simple to construct, inexpensive, portable and can be transported from farm to farm. Since then, the electrochemical DNA biosensor has performed amazingly well in this field of research and has been used for the detection of various viruses (Turner, 2013). The DNA biosensor has been more efficient in DNA hybridization than conventional hybridization assays because array-based hybridization assays still depend on the diffusion of target DNA to surface-bound probes, resulting in hybridization times of several hours or more (Peterson *et al.*, 2001; Jobs *et al.*, 2002).

In this work, we developed a simple and effective method to optimise the universal sequence of the 5'-UTR of infectious bronchitis virus (IBV) on a bare gold electrode by a simple chemisorption method to produce an electrochemical DNA biosensor. The chemisorption method was applied to the covalent binding of DNA probes between amine-modified DNA probes and the AuE surface, resulting in a monolayer of DNA probes on the gold electrode surface due to the strong affinity interaction (Benvidi *et al*., 2015). Using the current reaction, we demonstrated that the electrochemical measurement is strongly influenced by various optimization parameters such as pH, scan rate, accumulation time, immobilisation and hybridization time, and temperature, which gives the optimal conditions for the selection of the best current response prior to modification assay of AuE. The selectivity of the proposed electrochemical biosensor was evaluated using different DNA sequences. As a result, PCR-free DNA biochips are a new point-of-care diagnostic and on-site monitoring device that can be used for IBV early diagnosis. The optimization parameters from this study will be useful for future modifications of input devices used as simple smart biosensors.

MATERIALS AND METHODS

Materials

Potassium hydrogen phosphate (K_2HPO_4) potassium dihydrogen phosphate (KH_2PO_4), sodium citrate dihydrate ($C_6H_9Na_3O_9$), aluminium sulphate ($Al_2(SO_4)_3$) and sodium chloride (NaCl) were purchased from Systerm Chemical Sdn Bhd manufactured from Malaysia, anhydrous citric acid ($C_6H_8O_7$) was obtained from Nacalai Tesque manufactured from Japan, ammonium acetate ($C_2H_7NO_2$) was from Ajax Chemical manufactured from Malaysia and Tris-HCl ($NH_2C(CH_2OH)_3{\cdot}HCl$ was from First Base Company manufactured in United States for buffer preparation constituents. Redox indicator chemicals such as potassium hexacyanoferrate (III) and potassium ferrocyanide (II) trihydrate were products of Nacalai Tesque, whereas methylene blue and iron (III) chloride were purchased from Systerm Chemicals Sdn Bhd and Sigma-Aldrich manufactured from United States, respectively. The PCR-amplified real IBV samples were collected from the Veterinary Department of Sabah, Malaysia. The tested oligonucleotides were synthesised by First BASE Laboratories Sdn. Bhd. in Selangor, Malaysia. The sequences are listed sequentially in Table 1. The other solution was a 50 mM Tris-(hydroxymethyl) aminomethane-HCl (Tris-HCl) (Sigma, USA) buffer solution containing 20 mM NaCl (Sigma, USA) (pH 7.0) as a supporting electrolyte buffer and washing buffer for CV measurements. Every chemical used was analytical reagent grade, and deionized water was obtained from a Millipore purification system that made it.

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Table 1: The base sequences of avian infectious bronchitis virus (*Gammacoronavirus*).

Primers	Sequences	References
Universal probe sequences	5'-NH_2-CACCACCAGAACCTGTCACCTC-3'	Callison *et al*. (2006)
Target DNA	5'-GAGGTGACAGGTTCTGGTGGTG-3'	N/A
One-base mismatch	5'-GAGGTGACACGTTCTGGTGGTG-3'	N/A
Two-base mismatch	5'-GAGGCGACAGGTTATGGTGGTG-3'	N/A
Three-base mismatch	5'-GAGGTCACAGATTCTGGCGGTG-3'	N/A
Non-complementary	5'-GCCATGTTGTCACTGTCTATT-3'	N/A
Target DNA of ND	5'-GTGCAGGCACCCCRAGTGCT-3'	Nidzworski *et al*. (2013)
Target DNA of MG	5'-CGCAATTTGGTCCTAATCCCCAACA-3'	Hantow *et al.* (1998)
Target DNA of ILT	5'-CTAACCCGTTCGCCGCACTCG-3'	Zhao *et al*. (2013)
Target DNA of AIV	5'-TCAGGCCCCCTCAAAGCCGA-3'	Nidzworski *et al*. (2013)

NP: IBV - Infectious Bronchitis Virus; NDV - Newcastle disease virus; ILT - Infectious laryngotracheitis; MG - *Mycoplasma gallisepticum*; AI - avian influenza (AI).

Methods

All electrochemical experiments were conducted with a µAutolab system potentiostat–galvanostat (Model PGSTAT) packaged with NOVA Autolab 1.8 software. The CV potentiostat indicated the existence of target ions through current responses, shown in a cyclic voltammogram graph. Every experiment was carried out at room temperature (20 ± 2 °C). The instrument was associated with the three electrodes, which involved the working electrode (AuE), counter electrode (platinum wire, Pt) and reference electrode (silver-silver chloride, Ag|AgCl, KCl/3M). A Metrohm pH-meter was used to measure the pH of every experiment (Model 691, Switzerland).

Pre-treatment of bare gold electrode (AuE)

According to the procedure previously reported by Siddiquee *et al*. (2010), the bare AuE was pre-treated. AuE was briefly polished for two min with a 0.3–0.5 µm alumina slurry. After that, AuE was ultrasonically sonicated for 2 min in sterile distilled water. The electrode was gently cleaned with distilled water. The electrode was then dried with nitrogen gas and placed in an electrochemical cell with three other electrodes and a buffer solution of Tris-HCl (50 mM in Tris-HCl) to monitoring the current flows.

Preparation of DNA oligonucleotides

DNA oligonucleotides stock (100 µM) solutions were prepared in a TE buffer solution containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) and kept frozen. Additional dilute solutions of the oligomers were prepared in a 50 mM Tris-HCl and 20 mM NaCl buffer solution (pH 7.0). An appropriate dilution was made prior to use and the working solution was maintained at 4 °C. The additional solution was employed in deionized water with a 50 mM Tris-HCl buffer solution containing 20 mM NaCl (pH 7.0) as a supporting electrolyte buffer and washing buffer.

Optimization of immobilisation and hybridization

The bare AuE was covalently bound with a single-stranded IBV DNA probe (1 mL) and dried for at least 8 h in the dark at room temperature. To remove unbound ssDNA probe, wash with the wash buffer solution (50 mM Tris-HCl + 20 mM NaCl, pH 7.0) for 30 sec. (Siddiquee *et al*., 2001). This electrode captured with the probe is referred to as ssDNA. The probe was then hybridised with the target DNA on the AuE surface for 2 h at room temperature. The electrode surface was then washed with the wash buffer for 30 sec to remove unbound hybridised target DNA. Finally, this hybridised electrode was designated as dsDNA. The volume study of immobilisation was conducted over a wide range of 5–30 µL (0.25 µM) and a hybridization range of 5–25 µL (0.2 µM). Hybridization efficiency was optimised by the two main effects, which were time (15 to 30 min) and temperature (20 to 40 °C). The terms "single-stranded probe DNA" (ssDNA probe) and "double-stranded DNA" (dsDNA), which has already undergone hybridization.

Redox indicators binding to the DNA-gold electrode

The significant hybridization efficiency of the target DNA was tested with a series of redox indicators, including the redox pair $[Fe(CN)_6]^{3-}$/ $[Fe(CN)_6]^{4-}$ (Hassan *et al*., 2019), methylene blue (MB) (Nordin *et al*., 2017) and Prussian blue (PB) (Wen *et al*., 2018). The reason for using redox was to study to increase the current signals of bare AuE between ssDNA and dsDNA. A schematic diagram shows the sequence of immobilisation and hybridization reactions between the probe ssDNA and the target dsDNA on the surface of the electrode (Figure 1). To allow attachment to the AuE surface, these redox indicators (5 mM) were soaked on the electrode surface for 2 min and then rinsed in the same concentration of 50 mM Tris-HCl buffer (pH 7.0) at room temperature. Then, the current signal was measured by cyclic voltammetry (CV) to detect the IBV target. The same protocol was used for non-complementary DNA, mismatched DNA (single, double and triple) and all non-IBV of target DNA.

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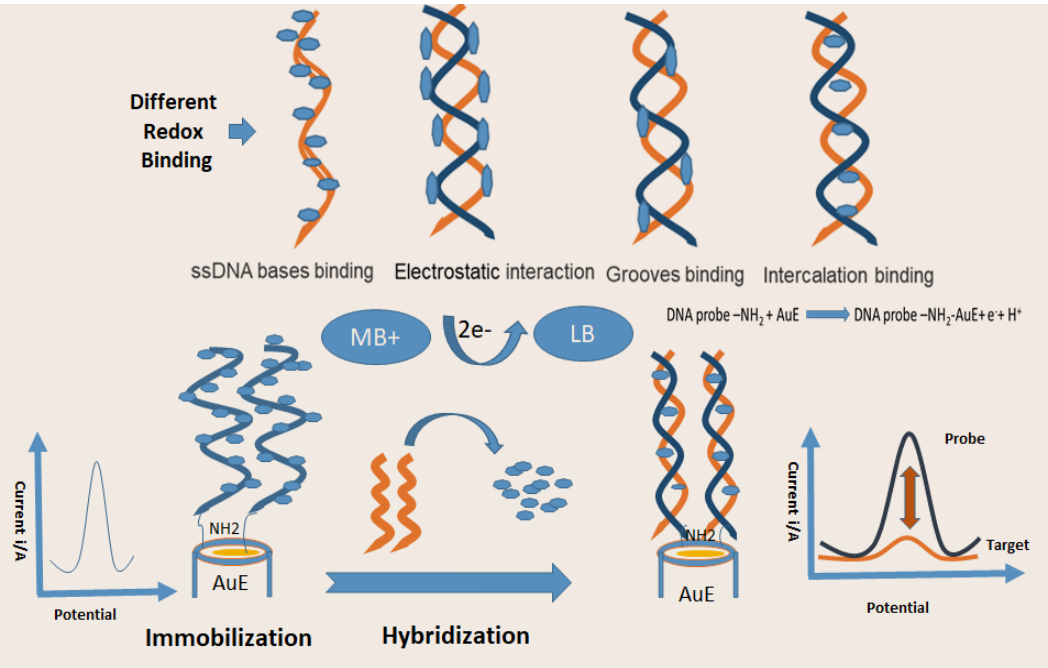

Figure 1: A schematic diagram showing MB interactions between ssDNA (Blue) and dsDNA (pink) with the covalently bonding of NH_2-DNA onto the bare AuE.

Table 2: Optimization parameters of electrochemical DNA-biosensor for detecting avian infectious bronchitis virus (*Gammacoronavirus*).

Parameter	Variation
Potential applied	0.0 V to 0.7 V
Buffer solution	Phosphate; Tris-HCl; Ammonium; Citrate
Redox indicator	MB, PB and $K_3[Fe(CN)_6]$
Scan rate/mVs^{-1}	50; 100; 150; 200; 250; 300
pH	6.0; 6.5; 7.0; 7.5; 8.0
Accumulation time/s	5; 10; 15; 20, 25
Probe volume/µL	5; 10; 15; 20; 25; 30
Target volume/µL	5; 10; 15; 20; 25
Hybridization temperature/°C	20; 25; 30; 35; 40
Hybridization time/min	15; 20; 25; 30
Cross reactivity study	Target DNA of IBV, NDV, MG, ILT, AIV

Voltammetric analysis of IB virus

The optimizations of all parameters were performed using the CV method and the data analysis was constructed using a Potentiostat/Galvanostat device. The initial electrochemical measurement of the CV method ranged from 0.0 V to 1.7 V for potential applied, 0.8 V stop/start potential: 100 mV/s scan rate and 5 sec reaction time in the analytical buffer (50 mM Tris-HCl, pH 7.0). All experiments were conducted at room temperature unless otherwise stated.

Cross reactivity study

The target DNA was tested with different types of respiratory viruses in chickens by using RT-PCR (reverse transcription PCR) and conventional PCR. The developed DNA biosensor was analysed for the selectivity test on different chicken viruses such as the Newcastle disease virus (NDV) (Nidzworski *et al*., 2013), Infectious laryngotracheitis (ILT) (Zhao *et al*., 2013), *Mycoplasma gallisepticum* (MG) (Hantow *et al*., 1998) and avian influenza (AI) (Nidzworski *et al*., 2013).

Optimization of the parameters

The optimization parameters were applied to the detection of IBV by using an electrochemical DNA biosensor. The below proposed parameters were applied for the detection of a specific IBV sequence using methylene blue as a redox indicator for monitoring the hybridization as well as the immobilisation efficiency

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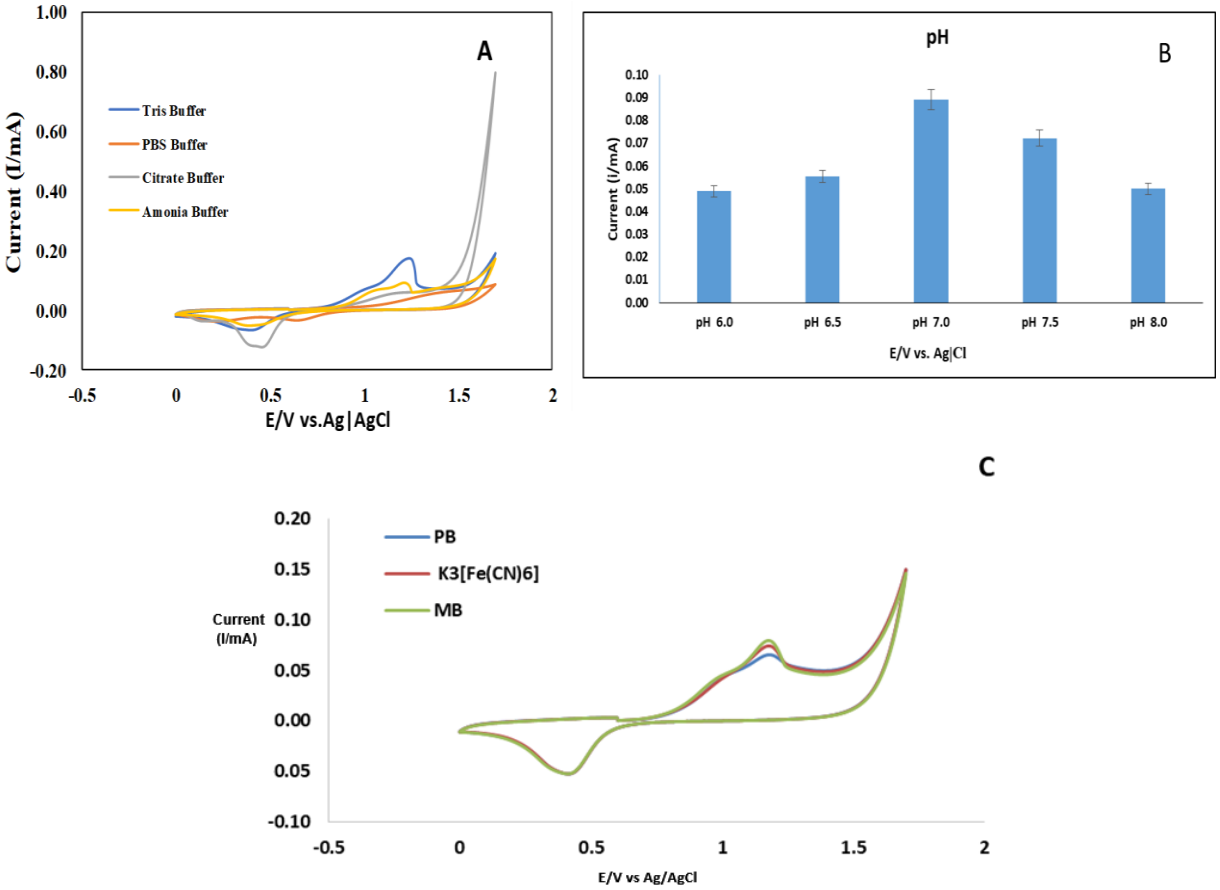

Figure 2: A) Cyclic voltammetry recorded on bare AuE + buffer + dsDNA in various kinds of buffer selection (50 mM, pH 7) ; B) Various ranges of pH values from pH 6–8 supported by Tris-HCl buffer (50mM); C) Different redox indicator (5 mM) supported by Tris-HCl buffer (50mM, pH 7) at a scan rate of 0.10 mV/s vs Ag|AgCl by the presence of 5 mM redox (I — current, E — potential) (n=3).

(Table 2). Under these optimum conditions, the selectivity assay was performed using a non-specific of the IBV DNA.

RESULTS AND DISCUSSION

The optimization parameters are an important requirement for the detection of a specific IBV sequence. The probe ssDNA was hybridised with target, mismatch and non-complement DNA to observe the strong hybridization stability.

Influence of the buffer, pH and redox indicator

Based on the current response from CV measurement for optimization, four types of buffers (50 mM, pH 7.0) were tested as supporting electrolytes in the presence of target DNA (2 µM) as shown in Figure 2A. The development of hybridised label-free DNA biosensors requires optimization of the measurement buffer solution, as it reduces solution resistance. The effects of the measurement buffer solution shows that the Tris buffer (50 mM) had the lowest resistance value, resulting in a larger current signal compared to other buffer solutions. In an electrolyte solution, anions and cations are normally in equilibrium because the ions move in two directions. While the negatively charged ion moves along different paths, the positive charge (cation) attracts the negative charge (anion). Although electrolytic reactions also produce reverse charged ions, charged ions are still attracted to the anode or cathode medium when an external force is applied. The presence of charged ions in the chambers enables the transport of electricity and the display of current signals, which are represented as voltammograms. The lowest resistance of the buffer solution will improve electron transport and thus increase the activation of the DNA biosensor (Andrade *et al*., 2011; Radhakrishnan *et al*., 2014). Several crucial factors, including the type of buffer and the concentration and dissolved salts in the buffer solution, most affect the electrochemical measurement. The three different types of redox indicators measured in CV are shown in Figure 2C along with the current responses as a function of pH (7.0) and buffer concentration (50 mM), respectively.

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Methylene blue (5 mM) was selected as the redox indicator that provided the largest peak current compared to the other redox indicators. According to Silva *et al*. (2009), the optimised 5 mM MB was chosen because the dsDNA-MB complex has a stronger ability to bind DNA compared to ssDNA. The cationic charge of the MB redox molecules can interact with the negatively charged phosphate backbone of DNA in a variety of ways, including electrostatic interactions, intercalation binding within G-C base pairs in dsDNA, groove binding, and MB interactions with an exposed guanine base in ssDNA (Nasef *et al*., 2010; Nordin *et al*., 2018). The fourth number of the principle used in this study includes in the list of MB-DNA interaction mechanisms that can be uniquely identified as IBV target sequence. After immobilisation, there was an increase in attraction between MB and the ssDNA probe. Alternatively, the bound MB molecules were replaced by the ssDNA probe with its target sequence, or only a few MB molecules were bound during the hybridization of the ssDNA probe with its target sequence causing lowering the signal (Farjami *et al*., 2010). The Tris-HCl buffer provides a complete circuit and serves as the electrolytic solution for detection in all electrochemical biosensor experiments (Ringgit *et al*., 2020). The oxidation signal was nearly twice as large as the reduction signal, indicating that both positive and negative radical ions diffused at the top of the active surface. The oxidation peak was found at 1.237 V (1.98 mA), while the reduction peak was recorded at 0.358 V (–56.10 mA). The current signals with different buffers were not at the same location as the potency region.

The current DNA biosensor response is increased in direct proportion to the availability of alkalinity pH. According to Ning *et al.* (2014), various buffer solutions have monitored the peak current with MB sensitivity at varied pH ranges. The effects of pH and ionic strength in the upper or lower pH range were compared with pH 7.0, which affected the different current signals of the DNA biosensor (Hassan *et al*., 2019). The solubility and stability of DNA molecules were supported by the hybridization buffer and pH in the neutral condition. In this study, the optimal buffer pH ranged from 6.5 to 8.0 for detection of the IBV target. From the results shown in Figure 2B, the calculation of the highest peak signals, a pH of 7.0 was selected as the optimal hybridization buffer because the oxidation and reduction peak currents had the highest value and were used for all analytical assays in DNA biosensor experiments. The results also show that the oxidation and reduction peak are shifted from pH 7.5 to 7.8, as the hydrogen bonds between the nitrogen-containing base pairs are broken down and the helical dsDNA is denatured. However, the MB intercalation reaction with dsDNA cannot be followed due to DNA fragmentation (Zhai *et al.*, 1997; Ariffin *et al*., 2018). Several researchers have performed similar experiments (Rahman *et al.*, 2017; Ariffin *et al*., 2020) and found that CV peak current signals are increased under neutral (pH 7) conditions and decreased under acidic and basic electrolytes. A 50 mM Tris-HCl + 20 mM NaCl buffer, pH 7.0, was used to immobilise the target DNA. As shown in Figure C, the selected optimised Tris-HCl buffer (50 mM Tris-HCl) is shown with the different redox indicators for the electrochemical detection of the IBV target at CV. The results showed that the HCl buffer containing 5 mM methylene blue (MB) produced the highest peak current responses compared to the other redox indicators. Therefore, MB was chosen as the electrolytic solution for IBV target sequence detection.

Effects of accumulation time and scan rate

Optimisation of accumulation time was performed for the current flow that generates oxidation and reduction peaks in the electrochemical cell. The accumulation time was measured from 5 sec to 30 sec with the target DNA bound to the bare AuE (see Figure 3A). The results show that the peak current decreases rapidly with increasing accumulation time, having reached its largest signal at 5 sec. Therefore, it is suggested that the saturation adsorptions on AuE create a rough surface that may eventually reduce the biological activity of DNA biosensors (Niu *et al*., 2012; Zhang *et al*., 2012). For various applications of DNA biosensors, researchers have used different accumulation times, including 5 sec (Zhang *et al*., 2010), 10 sec (Fayazfar *et al*., 2014), 50 sec (Zhang *et al*., 2011), 60 sec (Sani *et al*., 2019), 75 sec (Siddiquee *et al*., 2011) and 120 sec (Ziyatdinova *et al*., 2011). Therefore, an accumulation time of 5 sec was determined as the optimal reaction time for further studies.

In addition, the effects of scan rate were studied in the range of 50 mV/s to 350 mV/s. The current response is determined based on the current kinetics, which is measured by the diffusion control factor. The signal response transitions to a linear line when the scan rate is above 100 mV/s of the oxidation peak. It is hypothesised that the experiment's stability is compromised by the current response being out of balance as a result of excessive oxidation (Yuan *et al.*, 2019). As can be seen in Figure 3B, a value of 100 mV/s was chosen for the following experiments because the correlation coefficient (R^2) for the peak oxidation current versus the square root of the scan rate was 0.980. The scan rate found was supported by several researchers and it was mentioned that the active electrode surface improves the electron transfer system and increases the sensitivity of the biosensor (Nordin *et al*., 2017; Nordin *et al*., 2018).

Influence of the probe and target volume

To optimise the volume study for the immobilisation, the adsorption-associated ssDNA probe was applied on bare AuE at a potential of 0.7 V in a 50 mM Tris-HCl buffer solution (pH 7.0). The probe DNA solution was run using a volume range spanning from 5 µL to 30 µL (equivalent to 0.25 µM). The results in Figure 4A showed an increase in volume up to 25 µL of the ssDNA probe, where the maximum current signal was found, followed by a decrease in signal. As a result, the optimal immobilisation

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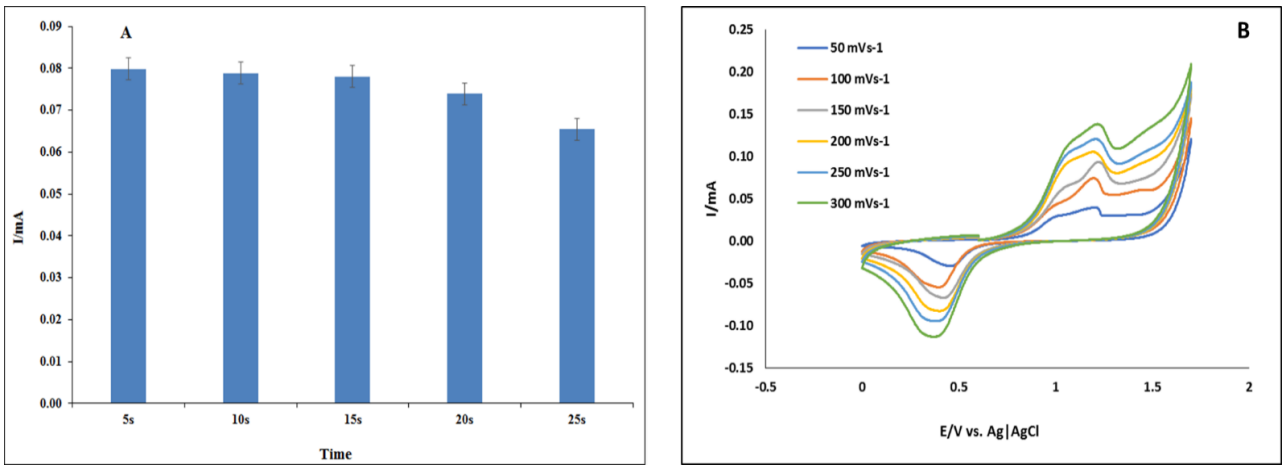

Figure 3: A) Cyclic voltammograms (CV) obtained on the bare AuE + buffer + dsDNA (0.2 µM target DNA) at various stages of accumulation time; B) Scan rates from 50–300 mV/s in buffer + dsDNA by the presence of MB (5 mM). In addition, the extracted peak currents are linearly proportional to the square roots of the scan rate of 100 mV/s vs Ag|AgCl (I — current, E — potential) (n=3).

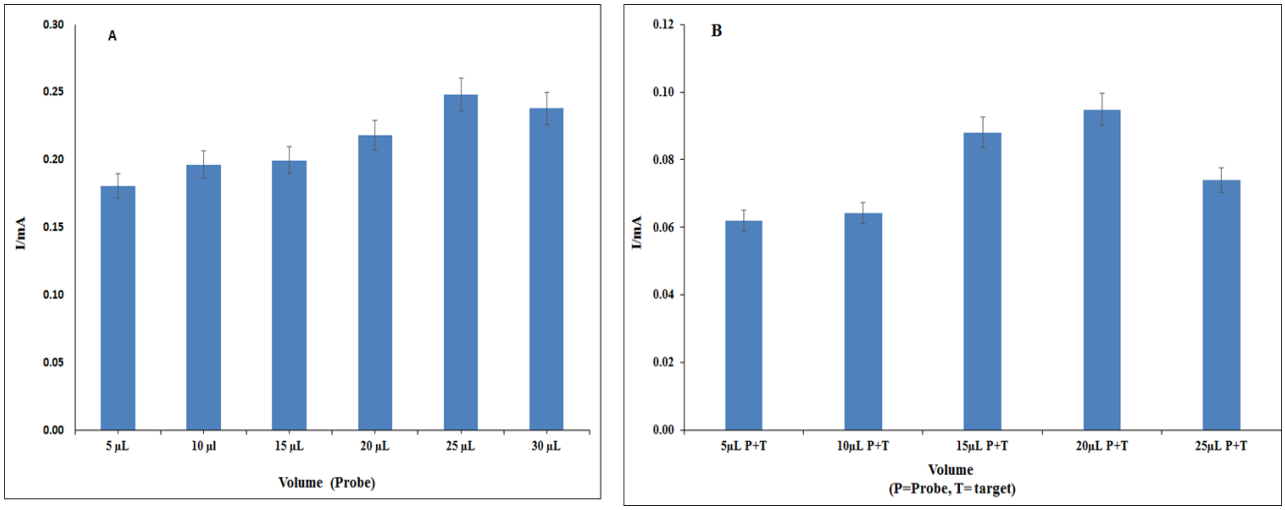

Figure 4: A) Optimisation study of probe volume (0.25 µM) on AuE + buffer + ssDNA; B) Probe volume (0.25 µM) on AuE + buffer + dsDNA supported by MB (5 mM) using CV at a scan rate of 100 mV/s vs Ag|AgCl (I — current, E — potential) (n=3).

volume was set at 25 µL for this study. Volume experiments were conducted to reduce the stability or affinity of DNA during hybridization with low functionalization period and solvent compatibility (Movilli *et al*., 2018). Similar volumes of concentrated DNA have been studied by several researchers where the density of ssDNA applied to sensor surfaces to organise the precise surface orientation and uniformity of ssDNA (Wipawakarn *et al*., 2012; Gao *et al*., 2017). According to previous research, depositing too many ssDNA probes on the electrode surface can reduce the efficacy of hybridization because the ssDNA has a substantial electrostatic repulsion effect on the surface electrode (Idili *et al*., 2014). The same protocol was performed for hybridization experiments, and it was discovered that the peak currents signal increased with an increasing amount of target ssDNA in the range of 5 µL to 25 µL and then signals were subsequently decreased. Thus, the optimum conditions were found to be 20 µL of target DNA (0.2 M) and higher hybridization efficiency shown in Figure 4B. The DNA hybridised with the immobilised probe was strengthened by the electrostatic attraction between the positive charge of the AuE surface and the negative hydrophilic sugar phosphate backbone of the DNA charged with the bases facing the buffer solution (Souza *et al*., 2014).

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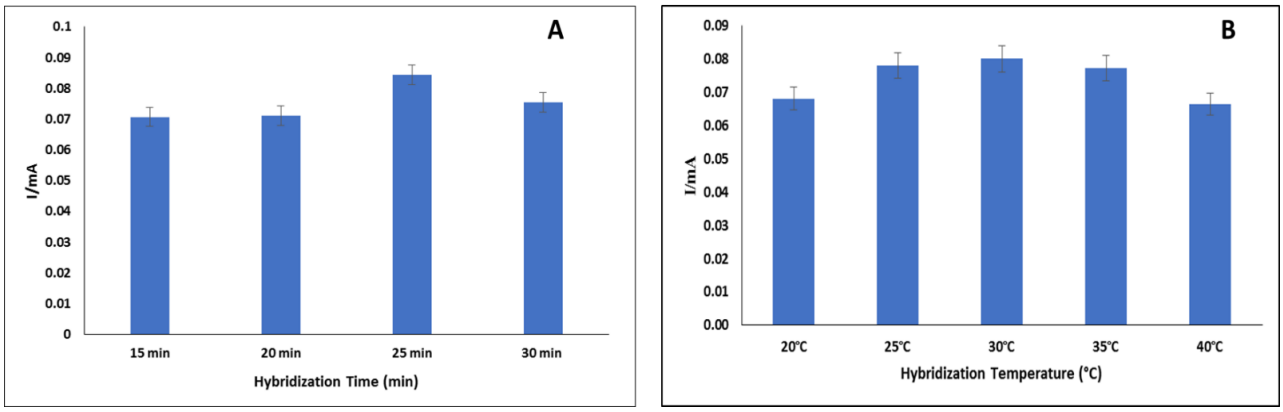

Figure 5: A) Effect of hybridization time; B) Temperature on AuE + buffer + dsDNA (0.2 µM target DNA) supported by MB (5 mM) using Cyclic voltammograms (CV) at a scan rate of 100 mV/s vs Ag|AgCl (I — current, E — potential) (n=3).

Effects of hybridization time and temperature

Bare AuE with the ssDNA probe (0.25 µM) was soaked with 20 µL of the target DNA solution (0.2 µM) for different time periods (15, 20, 25 and 30 min). As shown in Figure 5A, the current signal of the target DNA increased until 25 min and then decreased. Therefore, 25 min was chosen as the ideal time for the hybridization experiment. According to Hwang *et al*. (2017), quick hybridization times always provide a number of benefits, including quick detection, high intensity, and high sensitivity. In the previous studies by Yogeswaran and Chen (2008) and Benvidi *et al.* (2018), it was shown that the DNA hybridization efficiency increases with the hybridization time up to an unchanged condition, indicating that DNA hybridization is not possible with increasing time because the hybridization on the electrode surface is complete. According to earlier studies by Yogeswaran and Chen (2008) and Benvidi *et al.* (2018) reported that DNA hybridization is not possible with increasing time because the hybridization on the electrode surface is complete, it was confirmed that the DNA hybridization efficiency increases with the hybridization time up to an unchanged condition. Additionally, the efficacy of hybridization was evaluated at temperatures ranging from 20 to 40 °C shown in Figure 5(B). The current signal gradually decreased above 30 °C, indicating a slow degradation of DNA binding. This is primarily caused by faster DNA contact and increased mass transfer at higher temperatures. Due to the dsDNA helix structure becoming denatured during the hybridization process, the signal was reduced. Therefore, 30 °C was established to be the ideal hybridization temperature. According to Lin *et al.* (2006), the effects of increased hybridization temperatures may cause DNA molecules to drive faster. However, it is possible that the dsDNA would be denatured as the temperature increases and it near the melting point as described in the related studies by Nimse *et al*. (2014) and Benvidi *et al.* (2015). The efficiency of hybridization is greatly influenced by the temperature of hybridization because it affects how quickly the immobilised probe DNA can interact with the target DNA.

Selectivity test

The selectivity study was performed using the optimal parameters for immobilisation and hybridization. Under optimal parameters, this study was performed using the probe ssDNA (0.25 µM), a similar concentration (0.2 µM) of the target DNA, a single-base mismatched DNA, a three-base mismatched DNA, and non-complementary DNA, respectively. The results of CV showed stronger hybridization of the IBV probe with the target DNA and differentiation with mismatched and non-complementary DNA in the presence of MB as a redox indicator (Figure 6). The current signal of the target DNA had the lowest signal compared to all oligonucleotide sequences, indicating the sensitivity of the oligonucleotide sequences. Based on the potential current response, the hybridization of the target DNA showed the lowest peak current of 56.94 mA, which was gradually increased thereafter by the single-base mismatched DNA (83.28 mA), the three-base mismatched DNA (85.60 mA), the non-complementary DNA (224.91 mA), and the probe DNA (251.86 mA). Percent selectivity was calculated using the technique of Nordin *et al.* (2017). Selectivity percentage (%) = $(A_t/A_0) \times 100$, where A_0 is the mean MB peak current without hybridization (n=3) and A_t is the mean MB peak current (n=3) followed by different hybridization samples like target DNA, one-base mismatched DNA, third-base mismatched DNA, non-complementary DNA used in this study as shown in Table 3.

The percentage (%) of selectivity level was significantly higher, while the oxidation signal of the target DNA (56.94 mA) had the lowest signal, which was about 4.76 times lower than the signal of the probe DNA (251.86 mA). Compared with the target DNA, the oxidation current gradually increased by 31.19% and 32.05% after hybridization with single-base mismatched DNA and three-base mismatched DNA, respectively.

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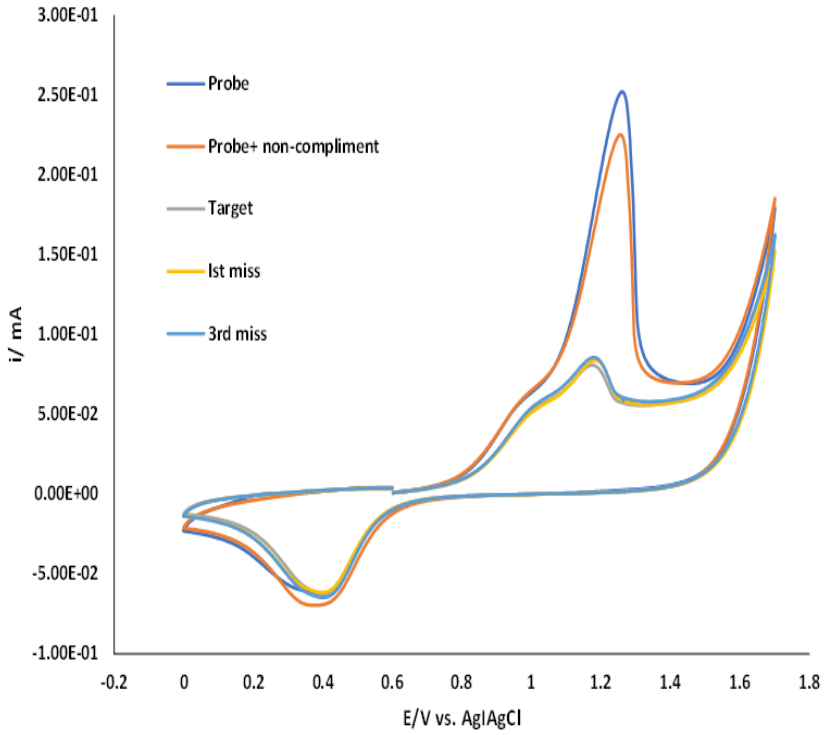

Figure 6: Cyclic voltammogram of the MB reduction peak current using different hybridization effect (selectivity test) supported by MB (5 mM) using CV at a scan rate of 100 mV/s vs Ag|AgCl (I — current, E — potential) (n=3).

Table 3: Selectivity percentages of MB reduction peak current apply with different types of oligomer DNA.

Test	i_{pa} (× 10^{-3} A)	Selectivity %
Bare electrode with MB	267	-
Probe DNA	251.86	-
Non-complementary DNA,	224.91	83
Third-base mismatched DNA	85.60	32.05
one-base mismatched DNA	83.28	31.19
Target/complementary DNA	56.94	20.98

According to the results, weak hybridization occurred as the hybridization activity gradually decreased. In similar studies, it was found that the peak current of MB reduction decreased almost four to five times compared to the probe signal after hybridization. This could be due to the reducible group of MB being sterically hindered by the double helix of the DNA hybrid, or MB being partially intercalated into the DNA helix (Meric *et al.*, 2002; Kusnin et *al.*, 2020). In contrast, the target DNA and non-complementary DNA signals are weakly bound to MB, and the probe DNA generates the highest current signal. The results suggested a lower MB accumulation carried on by uneven hybridization and a higher interaction between non-complementary and probe DNA as a result of weak hybridization. However, uneven hybridization may occur between the probe and non-complementary DNA due to a minor drop in MB signal that is not seen throughout the hybridization process (Souza *et al.*, 2014). The results indicated that hybridization activity gradually decreased, indicating that only weak hybridization occurred.

In addition, inaccessible interactions between MB and guanine bases could be the cause of the lowest peak of target DNA. However, a number of factors, including pH, temperature, DNA concentration, ionic strength and buffer type, have a significant interaction between the DNA and MB. Most studies on DNA biosensors have reported that the mode of MB accumulation was the dominant binding mode with dsDNA in the groove and intercalative processes rather than ssDNA (Siddiquee *et al.*, 2014; Nordin *et al.*, 2017). However, in this study, the redox complex interacted strongly with ssDNA, resulting in greater MB ac accumulation on the surface during immobilisation and producing a higher current signal than with dsDNA. This is mainly because small amounts of MB were deposited on the surface of dsDNA, which was triggered by an inaccessible interaction between guanine bases, and the effect of MB decreased the CV signal of

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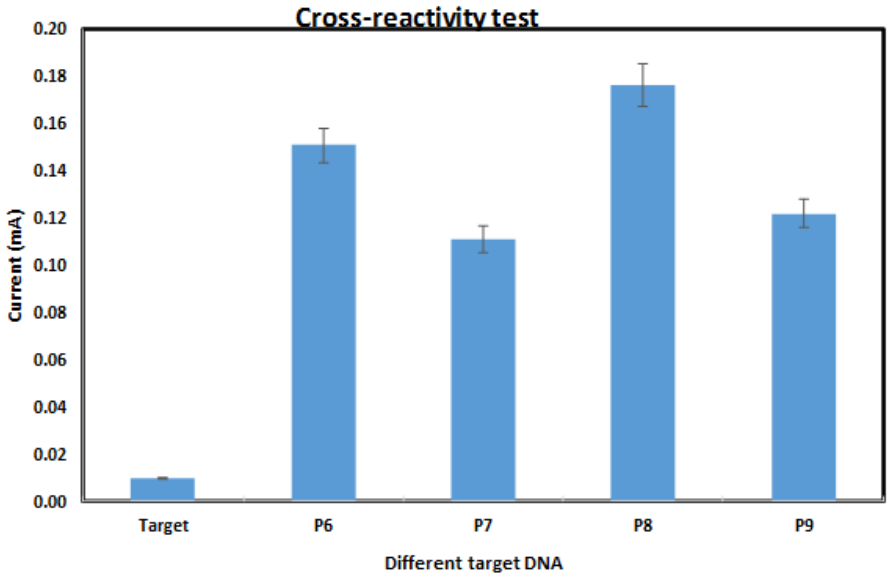

Figure 7: Bar chart of the MB reduction peak current for the cross-reactivity test against non-IBV virus target DNA supported by MB (5 mM) at a scan rate of 100 mV/s vs Ag|AgCl (I — current, E — potential) (n=3). (Here, Target = Target DNA of IBV, P6 = Target DNA of NDV, P7 = Target DNA of MG, P8 = Target DNA of ILT, P9 = Target DNA of AIV).

the immobilised ssDNA probe. In addition, a similar procedure was used to immobilise the probe DNA on single-base mismatched DNA and on three-base mismatched DNA, which constantly increased the peak currents compared with the target DNA, weak hybridization was shown. The hybridization process immobilising the probe ssDNA with the target ssDNA can only form a stable duplex under ideal conditions, which can improve and stabilise the selectivity of the electrochemical DNA biosensor (Abu-Salah *et al*., 2015; Rashid and Yusof, 2017).

Influence of the cross-reactivity study

The optimal parameters showed faster detection of IBV with high cross-reactivity. The parameters were used to differentiate from other non-IBV viruses associated with avian respiratory pathogens. Differentiation of IBV was based on the different values of the current signals and revealed that they were higher than the IBV target DNA due to lower hybridization, i.e., low stringency allows some unpaired bases. As shown in Figure 7, the current signal of IBV target DNA was distinguished from other non-IBV specific DNAs such as ILT, AI, MG, and ND. The finding of cross-reactivity is similar to the results of other DNA biosensors previously published by several researchers using different analytics (Wang *et al*., 2016; Nordin *et al*., 2018).

CONCLUSION

Electrochemical DNA biosensors have recently become a potential diagnostic tool for viral infections. Optimization parameters are performed on bare AuE for detection of IBV strains using a simple chemisorption method to produce an electrochemical DNA biosensor. Our studies showed NH_2-modified IBV ssDNA in an electrochemical DNA biosensor due to a strong affinity interaction between the NH_2 bond and the AuE surface, which increased the efficiency of DNA hybridization in the presence of MB. The study initially focused on the optimal buffers, redox indicators, pH, scan rate and reaction time with temperature to enable the larger binding capacity of target DNA of IBV to improve the hybridization efficiency. The ideal optimization parameters will be helpful in developing a portable device for IBV.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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