Rapid detection of pork DNA in food samples using reusable electrochemical sensor

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Abstract

This study aims to demonstrate the potential use of reusable electrochemical sensor for detecting pork DNA in solution. The approach was based on electrochemical principle in which the electrostatic interaction between DNA and redox species will generate detectable signal upon introduction of electrical charge. In this study, Ruthenium Hexaamine (RuHex) was used as the redox species and result was based on the output current. Coupled with highly specific Polymerase Chain Reaction (PCR) primers designed for pork DNA, this study has successfully demonstrated the reliability of proposed novel detection approach that utilized reusable electrochemical sensor and can potentially be developed into a rapid detection tool for halal and kosher food industry.

Index Terms: Pork DNA, Halal, Kosher, Electrochemical Sensor, PCR, Ruthenium Hexaamine

1. Introduction

The concept of "halal" and "kosher" have been well acknowledged and put into practice by Muslims and Jews respectively. These two terms are frequently mentioned in their respective scriptures and are commonly practiced amongst the Muslims and Jews as part of their food law. When applies in food law, the term "halal" is referring to permissible food consumed by Muslim that does not contain any prohibited component.[1]

PCR, a DNA-based technology, has been successfully performed to detect pig meat and fat. It has also been deemed as one of the most effective and reliable detection methods.[2,3] Conventionally, agarose gel electrophoresis is subsequently performed to detect the PCR amplicons. However, this detection method possesses several drawbacks arisen from its requirement for time consuming post-PCR sample preparation, high voltage, bulky instruments and may yield a rather vague qualitative result. Moreover, the long-term cost of using agarose is relatively expensive when compared to the cost of a reusable sensor and this method has also been proven to display poor separation on samples with low molecular weight.[4-7]

Several alternative methods have been well applied in place of gel electrophoresis to avoid the above mentioned drawbacks, electrochemical method being one of them. Modern electrochemical method often uses a three-electrode electrochemical system that has been re-designed into miniature forms and integrated onto a biochip to increase its compatibility as an electrochemical sensor.[8] The sensor requires minimal current flow in nanoampere (nA) range and small sample volume in microlitre (μL) range for the electrode reaction to occur.

The detection of pork DNA profoundly relied on the capability of redox active compound to interact with DNA to initiate electrochemical reaction. RuHex being electrochemically active and capable of binding to DNA via electrostatic interaction made it an ideal redox species candidate for this study [9-11]. Moreover, this approach requires no modification to the electrode surface. Electrostatic interaction occurs between DNA and redox species when the cationic RuHex

binds to the anionic phosphate group in DNA backbone (***Figure 1***).

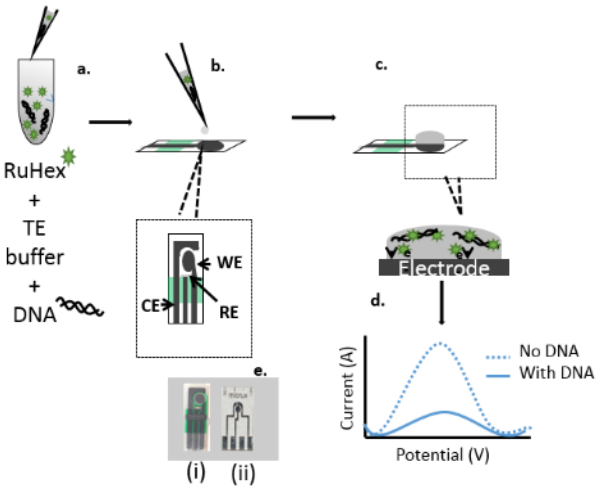

Figure 1. Schematic diagram of DNA detection in solution using electrochemically active RuHex on carbon screen printed electrode (SPE) chip. (a) solution composition mixture comprises of amplicons (dsDNA) : RuHex : buffer in a ratio of 2:1:1 respectively; (b) solution spiked onto the surface, covering the whole area of working electrode; (c) Electrostatic interaction between RuHex and dsDNA; (d) peak current generated where solution of free RuHex gives higher peak current than solution containing dsDNA which gives lower peak; (e) two types of electrodes used; (i) SPE chip made from carbon ink and (ii) thin film microelectrode (ME) made of platinum or gold.

Although electrostatic interaction achieved via the use of RuHex was applied in this study, but the innovativeness was profoundly focused on PCR amplicons, carbon screen-printed electrodes (SPE chip) and thin-film microelectrodes (ME) as the mediator. A novel pork-specific primer set was employed in PCR to amplify a specific region of pork mitochondrial DNA.

This modern electrochemical sensor equipped with different substrate materials was utilized in this analytical study as a detection device. The major aims of this study are namely: a) to develop new PCR primers for pork DNA amplification, b) to detect pork DNA in food samples using two types of reusable electrochemical sensors, the thin film microelectrodes and screen printed chip and c) to make comparison on efficiency, rapidity, sensitivity and cost for the two types of electrochemical sensors employed.

2. Experimental approach

Meat samples

23 food products, including raw meat, processed meat and canned food of different brands and origins were collected from various local supermarkets. These include raw meats of six species that are commonly consumed by the locals, namely pork (*Sus scrofa*), goat (*Capra hircus*), chicken (*Gallus gallus*), ostrich (*Struthio camelus*), beef (*Bos indicus*) and duck (*Anas platyrhynchos*). Out of the 23 samples, 8 samples analysed were pork samples, 6 were beef samples including beef gelatin from marshmallows, 4 chicken samples, 3 goat samples, 1 ostrich and 1 duck sample (***Table 1***).

DNA extraction and quantification

DNA was extracted from meat samples using Biokits DNA extraction kit (Neogen Corporation, Lansing, Michigan, USA). The kit isolation method was based on the use of magnetic beads. The extracted DNA was quantified using Nanophotometer P-Class (Implen, München, Germany). Five measurements were taken to obtain the average DNA concentration to be used as final DNA concentration. Subsequent to quantification, PCR amplification was carried out.

Primer selection and PCR amplification

Primers were designed in our laboratory and synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). The forward primer (5'-CAC ATC AGA CACA AAC AAC -3') and the reverse primer (5'- CCT ACG TGG ATG AAT AGG -3') generated amplicon of 132 base-pair (bp) in length at annealing temperature of 56^{o}C.

PCR amplification was executed in a 25 µL volume reaction mixture, which contained autoclaved water, 10× PCR buffer II, 25 mM $MgCl_2$, 10 mM dNTP mix, 20 µM forward and reverse primer, 0.625 U AmpliTaq DNA Polymerase (Thermo Fisher Scientific, USA) and 10 ng of template DNA. PCR was then run using a Veriti® thermal cycler (Applied Biosystems, Thermo Fisher Scientific, USA). The PCR cycling conditions used include an initial denaturation at

Table 1. Samples of different species from different brand and origin for the development of pork-specific PCR

No	Name	Brand	Origin
1	Negative control	-	-
2	Wild boar	-	-
3	Spiced pork cubes	Narcissus	China
4	Pork mince with beans paste	Narcissus	China
5	Chopped pork and ham	Greatwall	China
6	Pork and bamboo shoot	Gulong	China
7	Pork meat	-	Brunei
8	Sliced ham	Pinoy Fiesta	Philippines
9	Pork short sausages	-	Unknown
10	Corned beef	Banquet	Brazil
11	Beef loaf	CDO	Philippines
12	Curry beef	Amocan	Singapore
13	Canned beef luncheon meat	Mei Ning	China
14	Mallow bakes (beef gelatin)	Betta	Australia
15	Marshmallow (beef gelatin)	Haribo	Turkey
16	Chicken luncheon meat	Mei Ning	China
17	Chicken luncheon meat	Tulip	Denmark
18	Chicken luncheon meat	Hana	United Arab Emirates
19	Chicken luncheon meat	Golden Bridge	Singapore
20	Duck meat	Perak Duck Food	Malaysia
21	Mutton luncheon with chicken	El-Dina	Singapore
22	Corned mutton	Carters	Australia
23	Lamb curry with potatoes	Adabi	Malaysia
24	Corned ostrich	Mulaut Abbatoir	Brunei

95ºC for 2 min, followed by 35 cycles of three steps of denaturation at 95ºC for 20 s, primer annealing at 56ºC for 30 s and extension at 72ºC for 1 min. The final extension was performed at 72ºC for 5 min. The PCR products were held at 4ºC until further analysis. For initial confirmation, PCR amplicons were ran on 1.0% agarose gel submerged in a 10x Tris-Borate EDTA (TBE) electrolyte buffer for 75 minutes at 80 V, 400 mA using Bio-Rad Sub-Cell FT (Bio-Rad Laboratories, USA). The agarose gel was visualized under UV illuminator (UVP, USA). The length of the amplicons was measured against a 50 bp ladder (New England BioLabs Inc., Ipswich, USA).

Specificity, sensitivity and real samples analysis

Specificity was analyzed with 7 genomic DNA of non-specific species, namely sheep (*Ovis aries*), goat (*Capra hircus*), buffalo (*Bubalus bubalis*), horse (*Equus caballus*), duck (*Anas platyrhynchos*), ostrich (*Struthio camelus*) and turkey (*Meleagris*). Wild boar (*Sus scrofa*) was used as positive control for every PCR reaction. These non-specific species and all the 23 samples were further diluted to 10 ng prior to PCR amplification. For sensitivity, 10-fold serial dilutions of wild boar DNA were prepared, ranged from 10 ng to 10^{-5} ng.

RuHex preparation

1 mM of RuHex stock solution was prepared from 2.7315 mg of RuHex dissolved in 10 mL of water in a 15 mL polypropylene tube. The tube was wrapped up with aluminium foil to prevent RuHex from light exposure as ruthenium complexes are found to have resemblance as chlorophyll which is capable of absorbing light.[12] This stock solution was stored at 4ºC until usage.

Electrochemical sensors

Two kinds of electrodes were used in this detection method; Screen-Printed Electrodes chip (SPE chip) and thin-film microelectrodes (ME). SPE chip made from carbon ink with a working

electrode (WE) area of 2.64 mm^2 was obtained from Biodevice Technology, Co. (Ishikawa, Japan). This SPE chip has an external dimension of 12.5 mm by 4 mm, with cell diameter of 2 mm. The chip requires sample volume of 10 - 30 μL. Two MEs were attempted in this study, having noble metal substrate materials such as platinum and gold based.

These microelectrodes were obtained from MicruX Technologies (Asturias, Spain), with area of WE of 0.79 mm^2, external dimension of 10 mm by 6 mm and cell diameter of 1 mm. These microelectrodes only require sample volume of 1 - 10 μL. Prior to electrochemical analysis, the electrochemical sensors were cleaned for each reaction to avoid background interferences. Sulfuric acid (H_2SO_4) was used to clean the platinum and gold microelectrode, whereas phosphate-buffered saline (PBS) buffer was used as cleaning solvent for SPE chip. For PCR amplicons detection, all the electrodes were optimized based on their measuring condition for SWV, pH of Tris-EDTA buffer (TE buffer) and final concentration of RuHex. Salmon DNA was used for optimization purposes prior to amplicons analysis.

Measuring condition for Square Wave Voltammetry (SWV)

The measuring technique opted was square wave voltammetry (SWV). In this electrochemical analysis, potentiostat (Autolab system PGSTAT 101, Metrohm, Netherlands) was connected to a computer. The program used to process the data was NOVA 1.10. Three readings were taken and the average of the peak current (A) was calculated and regarded as the final peak current expressed in μA. The measurement condition for SWV varies for each electrode after optimization. The measurement condition for SPE chip were frequency = 25 Hz; amplitude = 0.0495 V; scan rate = 0.04875 Vs^{-1}; step potential = 0.00195 V. The potential range was slightly shifted from -0.5 to -0.1 V to -0.7 to -0.2 V, whereas the rest of the variables were maintained. The shift was made due to the availability of peak response. This measurement was also used in platinum ME but only differ by their potential range (-0.5 to 0.5 V). For gold ME, the measuring condition was personally optimized based on peak response obtained. The measurement condition for gold ME were frequency = 24 Hz; amplitude = 0.2 V; scan rate = 0.5 Vs^{-1}; step potential = 0.02 V with a potential range of -1 to 0 V.

The detection of PCR amplicons on the electrode was carried out in amplicon-buffer-RuHex mixture with composition ratio of 2:1:1 respectively. The mixture was spiked onto the electrode surface of SPE chip, platinum and gold ME, covering the area of working electrode. These electrodes were inserted into their respective connectors which were connected to the potentiostat and detector computer. The instrument setup for platinum and gold ME used drop-cell connector (MicruX Technologies, Asturias, Spain).

3. Results and Discussion

PCR-based method and pig-specific primers

The newly designed pork-specific primers targeted the mitochondrial pork DNA sequences and yielded amplicons of 132 bp in length. The amplification can also be achieved using several other PCR-based methods such as real time PCR and probes such as TaqMan or SyBr-Green. [13-15] However, real time PCR instrumentation is known to be not economical when compared to conventional PCR.[16] Our approach in this study using PCR-based method is not only cost-effective but also highly specific with the use of novel primers.

Following the optimization, cross-reactivity with non-specific species was performed with seven non-specific species. The primers did not cross-react with any of the mentioned non-specific species and only amplified the DNA sequence from wild boar. After the cross-reactivity analysis, sensitivity analysis was tested with sample concentration of 10 ng - 10 pg. The designated primers set has successfully amplified detectable wild boar DNA of as low as 5 ng and hence 5 ng was determined as the limit of detection (LOD) for this approach.

pH working condition of TE buffer

The optimum pH determined by this study was pH 9.23 for the two electrodes used (platinum ME and carbon SPE chip). At a higher pH range, destabilization of DNA may have occurred. The bonds that hold the two strands of DNA bound together might have instantly ruptured at high pH, causing the double stranded DNA to be unwound and denatured into a single stranded.[17] In addition, nitrogenous bases of DNA molecules; guanine's N (1) and thymine's N (3), could become deprotonated in basic condition and as a result the charge of the whole DNA could become negative.[18] An electrostatic interaction became possible when negatively charged DNA interacted with positively charged RuHex. The interaction of RuHex is commonly reported with double stranded DNA. The ratio of RuHex to DNA in this case was 1:1. As discussed, it is essential to keep the pH of the TE buffer used to store amplicons or DNA constant throughout the analysis. Depurination of DNA tends to occur at acidic and neutral pH leading to lose of purine bases and eventually breaking the DNA chain.[19-20] The chain breaking induces the free distribution of RuHex in solution thus giving contradicting results when detected using SWV. Therefore the detection mode was not executed at a lower pH range.

Optimized RuHex concentration for DNA sensor

When fixed measuring conditions for SWV were applied (scanning range= -0.7 to -0.2 V; step potential=0.00195 V; amplitude=0.04950 V; frequency=25 Hz; scan rate=0.04875 Vs^{-1}) at pH 9.23, the final concentration of RuHex was determined to be 10 μM according to the best signal-to-noise ratio (S/N ratio) when compared with lower RuHex concentration of 5 and 1 μM. S/N ratio was calculated based on the differences of peak current height between the blank and the amplicon. The S/N ratio obtained for 10, 5 and 1 μM RuHex was 2.369, 1.749 and 1.724 respectively.

Carbon SPE chip as the preeminent electrochemical sensor

An extensively used electrode substrate of carbon can be fabricated into many forms due to its soft properties, such as carbon SPE chip (made from carbon paste) glassy carbon electrode (GCE), graphene biochip etc. This carbon material was found to be chemically inert, showing a rich surface chemistry and can generate low background current.[21] These superior features offer a great support in yielding a more reliable and reproducible outcome. In addition, carbon SPE chip is more economical than ME due to fabrication cost. When these noble metals ME were experimentally compared to carbon SPE chip, the carbon SPE chip managed to scan at a more negative potential. Therefore as predicted, the response peak was observed at a more negative potential range. Ultimately, carbon SPE chip was opted as the preeminent electrochemical sensor due to its low-cost and tendency to give a promising conclusion.

During optimization, many problems were encountered when using the metal based microelectrodes. Both of the electrodes, platinum and gold ME, gave contradictory readings at some point and failed to present reproducible outcome. The quality of these particular electrodes was highly doubted and incapable to meet the expectation of the study. The discrepancy may arise from irreversible adsorption of significant amounts of DNA onto a scratched thin metal film surface.[22] This adsorption may generate inconsistent results. Therefore, platinum and gold ME were exempted in this study for further analysis.

Comparisons were established between platinum ME and carbon SPE chip by retaining SWV parameters, using final RuHex concentration of 10 μM and pH of TE buffer of 9.23. It turned out that the SPE chip gave a higher R^2 value of 0.9794 (***Figure 2a***) than R^2 value of Platinum ME 0.7961 (***Figure 2b***). After careful consideration based on the overall quality of performance from the aspect of its reproducibility and sensitivity, SPE chip was chosen as the final electrochemical detection tool for this study.

Cross-reactivity, sensitivity and real samples electrochemical analysis

Carbon SPE chip was used throughout the analysis. After optimization, cross-reactivity analysis, sensitivity analysis and analysis with real

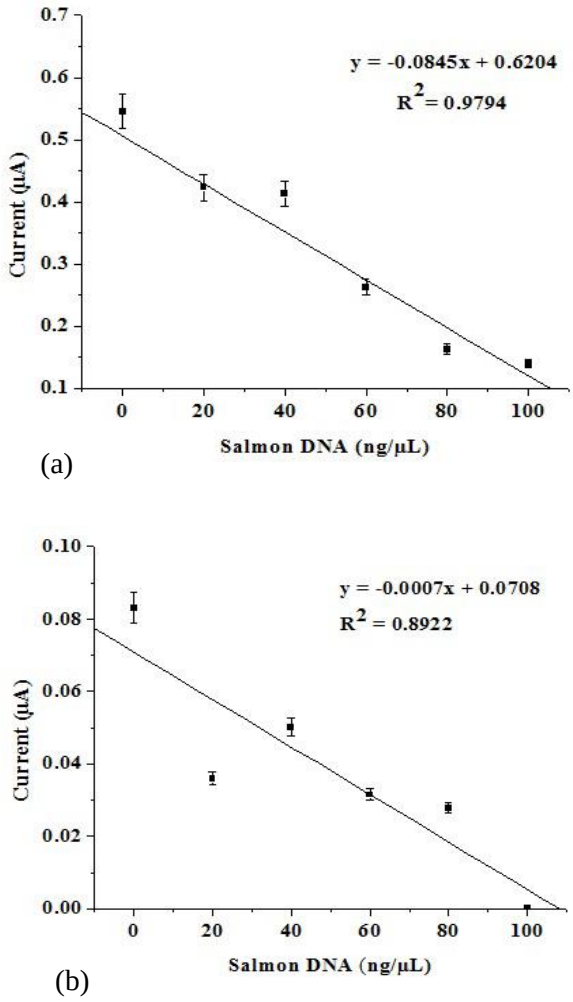

Figure 2. Electrochemical detection of 0, 20, 40, 60, 80 and 100 ng/µL of Salmon DNA using (a) carbon SPE chip and (b) platinum ME at 10 µM RuHex and pH 9.23. Measuring condition for SWV; scanning range=-0.7 to -0.2 V; step potential=0.00195 V; amplitude= 0.04950 V; frequency= 25 Hz; scan rate= 0.04875 Vs^{-1}

samples were conducted using electrochemical detection approach. For blank which comprised of RuHex and TE buffer, the peak current height was expected to be the highest. Positive control and samples that contain pork DNA significantly reduced the current flow and therefore expected to be yielding lower peak current height. Negative control and samples without pork DNA were expected to give higher peak than the one contained pork DNA but slightly lower than blank. The reason for such observation was because even amplicons of the negative control contains primers which consist of DNA fragments. Even without the presence of pork DNA, the primers can also develop electrostatic interaction with RuHex hence insignificantly reduced the current.

In cross reactivity analysis, specific species wild boar gave the lowest peak whereas non-specific species gave higher peaks (***Figure 3a***). For sensitivity analysis, 10 ng/µL of wild boar DNA gave the lowest peak than 5 ng/µL of wild boar (***Figure 3b***).

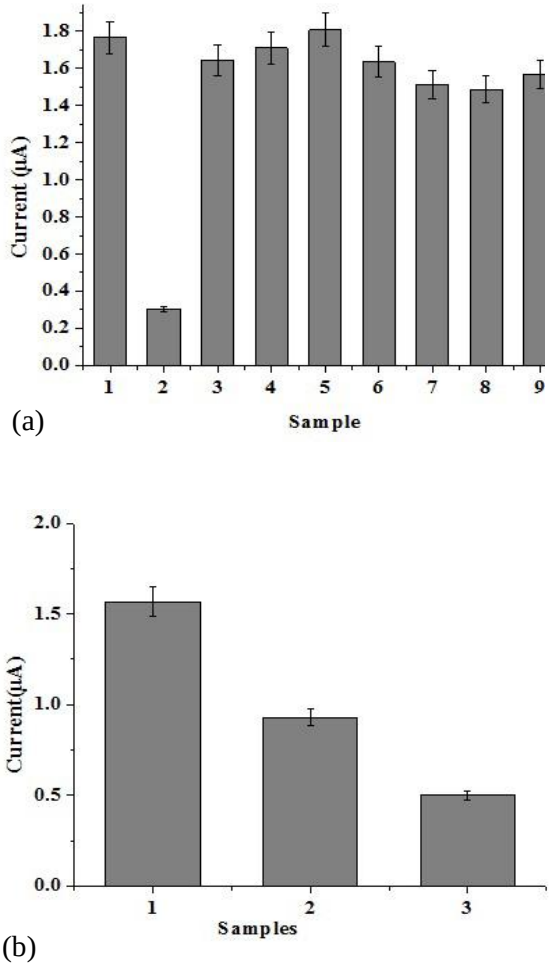

Figure 3. SWV behaviour of 10 µM RuHex with carbon SPE chip using pork primers for (a) specificity. Sample 1: Negative control, 2: Wild boar, 3: Duck, 4: Ostrich, 5: Turkey, 6: Buffalo, 7: Goat, 8: Horse and 9: Sheep; (b) sensitivity. Sample 1: Negative control, 2: 5 ng/µL wild boar and 3: 10 ng/µL wild boar.

For that reason, concentration of DNA and current response can be correlated. The linearity followed

increasing DNA concentration with decreasing peak current and vice versa. For sample analysis, the presence of pork DNA was indicated by lowest peak, whereas the absent of pork DNA gave higher peak. The extracted values from current response of 23 food samples in SWV were translated and illustrated in ***Figure 4***.

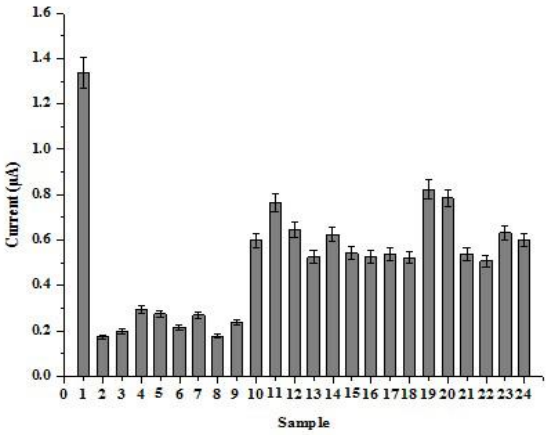

Figure 4. SWV behaviour of 10 µM RuHex with carbon SPE chip for all 23 real sample analysis (concentration of 10 ng/µL) using pork primers as mentioned in table 1. Sample 1: Negative control, 2-9: pork; 10-15: beef; 16-19: chicken; 20: duck; 21-23: goat and 24: ostrich.

Diffusion coefficient of Ruthenium Hexaamine

The current signal was proportional to the diffusion rate of molecules. The diffusion rate or diffusion coefficient can be determined using the Randles-Sevcik equation by entering the extracted data of current peak into the equation $i_p = 2.69 \times 10^5\ n^{3/2}\ A\ D^{1/2}\ v^{1/2}\ C$, where i_p = peak current in ampere A, n = no of electrons, A = electrode area in cm^2, D = diffusion coefficient in $cm^2\ s^{-1}$, v = scan rate in Vs^{-1}, C = concentration in mol cm^{-3}.

To verify that the diffusion activity of RuHex was hindered by the presence of DNA, diffusion coefficient using Randles-Sevcik equation was calculated for blank and positive control of PCR amplicons. Based on the calculation, the diffusion coefficient for blank (only RuHex) was determined to be 0.03762 cm^2s^{-1}. The diffusion coefficient of positive control (RuHex and DNA) was determined to be 0.001835 cm^2s^{-1}. In the solution where DNA was absent, RuHex in the solution rapidly diffuse onto the electrode surface. Being an electrochemically active species, such high diffusion activity increased the tendency to yield an intense current peak. However, when DNA is present, the electrostatic interaction took place between RuHex and DNA which showed slow diffusion of RuHex in the solution.[10] Slow diffusion reduced the current peak and hence reduced the current peak intensity as predicted. As a result, the expected diffusion coefficient of RuHex has been confirmed the presence and absence of DNA in the solution.

4. Conclusion

In conclusion, a PCR-based method to detect pork in food samples was successfully developed using rapid and reusable electrochemical sensor. With the newly designed primers, the primers were able to amplify as low as 5 ng/µL of pork DNA. It was highly recommended to use carbon SPE chip as a detection tool to detect the presence of specific species DNA. The reason for such recommendation was that, the outcome reported was highly reproducible when compared to metal film microelectrodes due to the existing broad characteristics of carbon materials. Moreover, the detection method was found to be rapid, sensitive and cost effective as it can be reused after several rounds of testing. As discussed above, since PCR amplicons were generally used in this study to detect via electrochemical detection, a further improvement using novel and high throughput reusable integrated microfluidic device is imminent for on-site food analysis. Our group is currently working on the development of field deployable and cost-effective system for point-of-care analysis.

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