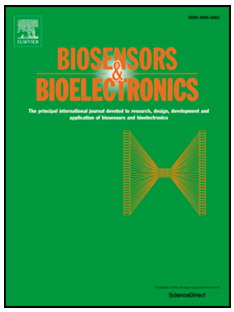

Design a highly specific sequence for electrochemical evaluation of meat adulteration in cooked sausages

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Aboulfazl Barzegari: Methodology, Software, Formal analysis.

Shahram Shoeibi: Writing - Review & Editing.

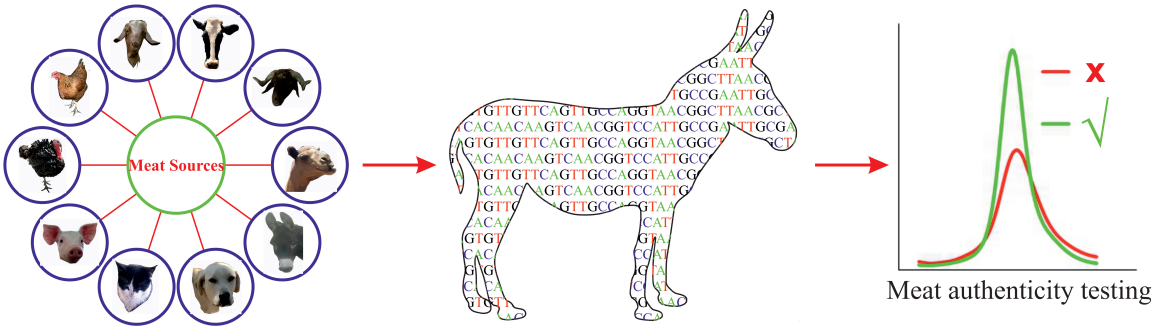
Selim Isildak: Writing - Review & Editing.

Nasrin Bargahi: Methodology.

Siavoush Dastmalchi: Methodology.

Yadollah Omidi: Validation.

Mohammad-Reza Rashidi: Conceptualization, Methodology, Validation, Writing - Review & Editing, Data Curation, Investigation, Supervision.



Design a highly specific sequence for electrochemical evaluation of meat adulteration in cooked sausages

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Abstract

A specific and unique sequence probe was designed for detection of donkey adulteration in cooked sausages and its species specificity was confirmed bioinformatically in the common software and website (ClustalX and NCBI). Subsequently, a novel species-specific electrochemical DNA probe (locked nucleic acid, LNA) was synthesized and implemented in a construction of DNA-based electrochemical genosensor for sensitive, convenient and selective detection of donkey adulteration. The electrochemical behavior of the fabricated genosensor was studied by linear sweep, square wave, differential pulse voltammetry and electrochemical impedance spectroscopy techniques. Due to inherent optimal hybridization conditions, the lower limit of quantification (LLOQ) was obtained as 148 pM with a relative standard deviation of 0.16%. Eventually, as a proof of concept, the designed biosensor was successfully used for detection of donkey genetic element in consumable beef sausages preparations, as a real sample. It is predicted that the proposed biosensor will provide a sensitive, inexpensive, fast, and reliable bioassay for application in food analysis, forensic investigations, genetic screening and biodiagnostics. As a prominent feature of this study, the recorded results were confirmed by quantitative real time–polymerase chain reaction (QRT-PCR) as a standard method in adulteration analysis. Our future perspective is minutralization of the development bioassay for making on-desk device and specially merging the designed system by microfluidic systems for accelerating the analysis time.

Keywords: Electrochemistry, adulteration, sausage, genosensor, LNA, food analysis

1. Introduction

Meat species adulteration is touted as very important global issue (von Bargen et al. 2014; Zhang 2013). In this regard, authenticity testing of the animal species present in food is serious issue from safety, health, economic, legal, import and export aspects. Moreover, food labeling regulations postulate that meat speciation in food products is exactly declared to the consumer (Doosti et al. 2014; Zhang 2013). It is an important task for food control laboratories to identify the species and distinct differentiation of crude materials utilized in food products preparation which may be replaced or mixed with other undeclared animal species (Doosti et al. 2014; Mehdizadeh et al. 2014).

So far, several analytical methods based on protein and DNA extraction analysis have been developed for the species authentication in food products. Protein-based methods include sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Azira et al. 2012), isoelectric focusing (IEF) (Ortea et al. 2010), ELISA (Liu et al. 2006) and HPLC (von Bargen et al. 2014), which are time-consuming, inadequate and/or expensive, not specific enough, need advanced instrumentation and greatly technical proficiency.

DNA-based analyzing methods, especially polymerase chain reaction (PCR)-based techniques are currently common in meat species discrimination and mislabeling detection. These methods include single or multiplex PCR (El-Azeim et al. 2016; Kesmen et al. 2007; Zhang 2013), PCR-restriction fragment length polymorphism (PCR-RFLP) (Haider et al. 2012; Zahran and Hagag 2015), randomly amplified polymorphic DNA (RAPD–PCR) (Calvo et al. 2001; Saez et al. 2004), TaqMan or SYBR GREEN Real-time PCR (Chisholm et al. 2005; Hird et al. 2006; Kesmen et al. 2014; Rojas et al. 2010) and single-stranded conformational polymorphisms PCR (SSCP analysis) (Rehbein et al. 1999). Recent published PCR approaches displayed an excellent

sensitivity in the analysis of raw products. One of the main limitations of PCR methods is the sensitivity of DNA-related assays to food processing. However some authors declared that the PCR robustness in processed foods can be increased by using small mitochondrial DNA templates (Arslan et al. 2006), while others displayed that the DNA templates can be degraded during the procedures with minor heating (Matsunaga et al. 1999). Moreover, mitochondrial DNA can also be prone to degradation (Şakalar et al. 2012; Ulca et al. 2013). This has raised concerns about the reliability and application of PCR analyses to validate species in processed foods wherever DNA degradation is detected.

Sausage among other meat products is a customary meat product which has high consumption volume by most countries (Kesmen et al. 2009). For example, according to the U.S. Census data and Simmons National Consumer Survey (NHCS), 247.93 million americans consumed sausages in 2017 that was expected to increase to 256.78 million in 2020 (www.statista.com). Moreover, that is a high-risk potential meat product, as various meat species which are low quality or not consumed by the public can be used in its manufacturing (Kesmen et al. 2009). Since donkey is not a commercial species for human consumption, its presence in sausage shows adulteration for economic interest and so gives the hint that it has been processed in unsanitary conditions demonstrating possible risks to human health (Zahran and Hagag 2015). Therefore, determining of the meat fraud is an important issue for food control laboratories. To prosecute manufacturers for their adulteration, analytical techniques should be able to quantify all anticipated components in this complex matrix (Eugster et al. 2008). Due to the mentioned facts and the high request for greater transparency in the food industry, it is serious to develop precise, specific and reliable detection methods such as biosensors in order to detection of the meat species origin used in sausages.

In recent years, biosensors have been developed for detection of species-specific sequences for meat products authentication. Nanobiosensors has been designed for detection and quantification of pork-specific sequences in degraded mixed meats (Ali et al. 2011a) and degraded biological samples (Ali et al. 2011b). Also using labeled single-stranded probe, a nanobiosensor for the analysis of pork fraud in processed burger, has been prepared (Ali et al. 2012b). Moreover, a nanoparticle based sensor has been developed for detection of pork adulteration in mixed biological samples (Ali et al. 2011c) and meatball formulation (Ali et al. 2012a).

The function of the mentioned biosensors is based on fluorescence spectroscopy and colorimetric assays. However, those techniques are expensive, not user-friendly, need greatly technical expertise and sophisticated instrumentation.

Electrochemical methods for its top features (Like; quick response time, selectivity and sensitivity) were used for detection of most analytes like; drugs (Saghatforoush et al. 2009), biomolecules (Babaei et al. 2010; Emran et al. 2018d), cancer biomarkers (Nakhjavani et al. 2019; Nakhjavani et al. 2018; Pakchin et al. 2018) and specially for monitoring of cancer and living cells (Emran et al. 2019; Emran et al. 2018a; Emran et al. 2018e).

Due to high sensitivity, simplicity, low cost and portability, electrochemical DNA biosensor has attracted more attention from diagnostics to food safety. The development of an electrochemical DNA probe is the main point for fabricating an electrochemical DNA biosensor. Most conventional short-electrochemical DNA probes indicate poor stability, sensitivity and specificity. Locked nucleic acid (LNA) designated in 1998 (Koshkin et al. 1998a; Koshkin et al. 1998b), as an innovative oligonucleotide derivative, has fascinated by people's widespread and applied to investigation of gene in the earlier few years. The LNA nucleotides due to a methylene bridge between the 4′-carbon and the 2′-oxygen of the ribose moiety, lock efficiently

of the furanose ring in the C3′-endo conformation and by declining the conformational flexibility of the ribose, leading to the local organization of the phosphate backbone (Koshkin et al. 1998a; Koshkin et al. 1998b; Wang et al. 1999).

This process highly raises the affinity of LNA oligomers intended for DNA complementary sequences and with each LNA replacement, the melting temperature (TM) is increased to 3.0–9.6 °C (Bondensgaard et al. 2000; Yang et al. 2007). In addition, the LNAs are more resistant to nuclease digestion (Wahlestedt et al. 2000). Moreover, these oligomers have displayed the particular properties, such as synthesis by standard methods (Koshkin et al. 1998a), enhanced triplex formation (Obika et al. 2000; Obika et al. 2001), low toxicity (Wahlestedt et al. 2000), and accessibility from a commercial supplier.

In this study, for first time, we designed a LNA-probe based electrochemical biosensor to detection of donkey meat adulteration in consumable beef sausage preparations with various percentages of donkey meat. This biosensor presented excellent specificity and selectivity under the optimal hybridization conditions in simple hybridization system, for the differentiation between completely complementary and mismatch sequences. However the fabricated biosensor has the advantages such as using nontoxic compounds, user-friendly, ability of miniaturization, short response time and low detection limit (DL).

2. MATERIALS AND METHODS

All of the materials and methods except, specific probe design, electrode preparation steps and PCR section, have been discussed in detail in supplementary section.

2.1. Designing of Equus-Specific genosensor

A 25-nt fragment (2236–2260 bp) of Equus (*asinus africanus*) cytb gene (GenBank accession no. KX669267.1 in NCBI data base) as a donkey-specific sequence, was selected. This probe is designed by comparing *Equus asinus africanus* cytochrome b (cytb) gene (GenBank: KX669267.1) with *Bos taurus* (beef; GenBank: KF926377.1), *Gallus gallus* (chicken; GenBank: KF939304.1), *Ovis aries* (sheep; GenBank: KX669267.1), *Sus Scrofa* (pig; GenBank: KX094894.1), *Felis Catus* (cat; GenBank: U20753.1), *Canis lupus familiaris* (dog; GenBank: KX379529.1), *Camelus* (camel; GenBank: EU159113.1), *Meleagris Gallopavo*(Turkey; GenBank: EF153719.1) and *Capra hircus breed* (goat; GenBank: KP271023.1) cytb genes by ClustalX multiple sequence alignment tool (http://www.genome.jp/tools/clustalX/). NCBI-BLAST analysis revealed a high degree of interspecies polymorphism and dissimilarities with the others alongside non-redundant nucleotide collection. The oligoprobe was custom synthesized with a hexamethyl-thiol at the 5′-end and an inserted T_{10} spacer as shown in Table 1. The oligoprobe and the synthetic targets (complementary, non-complementary, and single-mismatched) were purchased from Exiqon Company, Denmark.

‘Table.1’’

2.2. Preparation of LNA based biosensor

The gold electrode (3 mm diameter) was polished with 0.3 µm alumina slurry on polishing micro cloth for 5 min followed by washing with Milli-Q water. To remove residual alumina powder from the electrode surface, polished electrode was sequentially sonicated in ethanol and Milli-Q water (each for 5 min). Following this step, the cleanness of the electrode was electrochemically checked in fresh 0.5 M H_2SO_4 solution. Subsequently, the electrode was washed with a copious amount of Milli-Q water and dried immediately with nitrogen stream before LNA immobilization. The LNA modified gold electrode was prepared by casting the clean electrode

with 25 µL of I-buffer solution supplemented 0.74 µM thiolated capture probe (CP) for 10 hours at room temperature (RT) which was pre-activated by Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) as previously indicated elsewhere (Dosi et al. 2012). Then, the electrode (indicated as single-stranded DNA (ssLNA) capture probe/ gold electrode (AuE)) was soaked in PBS buffer (pH = 7.4) to remove any non-adsorbed LNA probe and air-dried. By using 1mM mercaptohexanol (MCH) for 2 hours, the nonspecific binding sites were blocked followed by soaking in PBS and exposure to the nitrogen stream. Finally, the electrode (indicated as MCH/ssLNA capture probe/AuE) was incubated with 12.5 µL H-buffer solution containing 0.74 µM target DNA for 6 hours. In the next step, the electrode (indicated as target DNA/MCH/ssLNA capture probe/AuE) was washed again to remove the non-hybridized target and blow-dried. The electrode preparation steps were represented in Scheme 1.

Scheme 1

2.3. Quantitative Real-Time PCR Analysis

Quantitative Real-time PCR (qRT-PCR) was implemented for analysis of donkey DNA using species-specific primers (Table 1) and a SYBR Master Mix (Real Q Plus) on a Corbett (RG-6000) real-time PCR detection system. qRT-PCR was executed using the following profiles: 95 °C for 5 min and 45 cycles of 95 °C for 30 seconds, 60 °C for 60 seconds, and 72 °C for 30 seconds. Then, melting curves were obtained using step by step increasing of the temperature from 60 to 95°C to confirm the amplification of a unique product in the reaction.

3. Results and discussion

3.1 Characterization of the LNA based biosensor preparing process by linear sweep voltammetry (LSV) (OX) and electrochemical impedance spectroscopy (EIS)

The LSV (OX) and EIS techniques were used to characterize the preparation process of the proposed biosensor, in 10 mM of $[Fe(CN)_6]^{3-/4-}$ solution, and the results are presented in Fig. 1A, B and C respectively. From LSVs (OX) shown in Fig. 1A, it can be clearly seen that the peak current had maximum value on the bare electrode surface, and self-assembly of the thiolated ssLNA capture probe caused that the decrease of the oxidation peak significantly. Based on the results, a negative-charge interface was composed at the surface of the bare electrode, and the electrostatic repulsion between DNA backbones and $[Fe(CN)_6]^{3-/4-}$ largely delayed the electron transfer at the electrode interface. Incubation of the capture probe modified bare electrode in MCH solution managed a decrease in the insulating property due to the short alkyl chain molecule of MCH made a self-assembled monolayer that comprised many pinholes and the redox was adequately small to easily pierce through these pinholes (Porter et al. 1987). Then the capture probe was hybridized with the target sequence and the oxidation peak decreased. The reasonable description is that after hybridization of the capture probe with the target sequence, the density of negative charge at the electrode surface was increased and therefore the strong electrostatic repulsion between DNA backbones and $[Fe(CN)_6]^{3-/4-}$, resulted in a more decline in peak current and growth in peak to peak separation.

Fig. 1B and C displays the EIS plots at different preparation stages of the proposed biosensor. The bare gold electrode illustrated the smallest semicircle in the Nyquist curves. Once immobilization of the thiolated ssLNA capture probe, an upper interfacial electron transfer resistance (R_{et}) was detected from 105.4 to 134.8 Ω. Incubation with MCH for the removal of nonspecific probe adsorption, resulted in decreased R_{et} to 111.7 Ω. Subsequently, hybridization of capture probe with target DNA was performed and the value of R_{et} was significantly increased

to 277.0 Ω. These results confirmed the LSV (OX) results. Finally, the electrode preparation process was stepwise confirmed by the both LSV (OX) and EIS result.

Figure 1

3.2 Optimization of parameters:

3.2.1 Effect of CP immobilization

Deposition concentration of thiol-ssLNA oligonucleotides on gold surface affects the probe density while electrochemical responses influenced by the probe density (Steel et al. 2000). So, the effect of CP deposition concentration on the electrochemical signal of gold electrode was studied with LSV (Red) and EIS techniques by casting of bare electrode with different concentrations of CP. The LSVs (Red) and variations of the reduction peak currents *versus* CP concentration of the proposed biosensor, EIS responses and equivalent circuit model of the EIS results were illustrated in Figs. S1A, S1B, S1C, S1D and S1E, respectively. According to Figs. S1A and S1B, by increasing CP concentration to 0.617 µM, the peak current of bare electrode decreased, followed by an increasing in the peak current. Also, the EIS responses in Fig. S1C exactly confirm the results of the previous technique. Thus, the optimum CP concentration was selected as 0.617 µM. In addition, the fitting circuit values of charge resistance and charge diffusion the various immobilized electrodes were calculated and summarized in table S1 (Emran et al. 2018b; Emran et al. 2018c), all of the interpretations were based on the charge transfer resistance (R2). Furthermore, the immobilization process of CP on the bare electrode was confirmed by field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDS). The surface morphology and coverage was successfully confirmed. The FE-SEM and EDS, images and results were illustrated in Fig. S2 and S3, individually.

Figures S1, S2 and S3
Table S1

3.2.2 CP immobilization temperature effect

The effect of the CP immobilization temperature on the electrochemical response of gold electrode was studied using square wave voltammetry (SWV) technique by coating of CP optimum concentration for 120 min at different incubation temperatures (i.e., 4, 37 °C and RT). Figs. S4A and S4B show that the best temperature was set up to be RT.

Figure S4

3.2.3 CP immobilization time effect

Optimization of CP immobilization time was performed with LSV (RED) technique. For this purpose, The optimum concentration of CP was incubated on Au electrode for different time points at RT for 2, 4, 6, 8 and 10 h. Surface density of thiol-modified oligonucleotide on gold that effects electrochemical responses, as a function of deposition period (Steel et al. 2000). The LSV (RED) and variation of reduction peak currents *versus* CP immobilization time was illustrated in Figs. S5A and S5B, respectively. Based on the results, in the early hours of the deposition, variation of reduction peak currents steeply decreased to 6 h and after that, variations was gradually. On the other hand, after completing the chemical bonding between the thiol groups and gold atoms on the surface of gold electrode and also saturating the surface of the gold electrode with CPs, by increasing the incubation time, the recorded electrical signals were approximately constant (Steel et al. 2000). Hence, for next optimization steps, the optimal time was considered as 6 hours.

Figure S5

3.2.4 Effect of target probe (TP) hybridization temperature

Experiments specified that the hybridization temperature plays a very important role. Melting temperature (T_m) as a physical nature of nucleic acids, can provide information around the

stability of duplexes in an identified environment to approve nucleic acids potential for specific recognition and hybridization with complementary sequences (Wang et al. 2011). So as to confirm reasonably more hybridization productivity and expand the proficiency of LNA/DNA recognition, hybridization temperature of the target probe was optimized using differential pulse voltammetry (DPV) measurements at various incubation temperatures (4, 37 °C and RT) by incubation of LNA modified electrode for 2 h with 0.74 µM concentration of target probe. According to Figs. S6A and S6B the best of hybridization temperature was found to be 37 °C and selected as an optimum hybridization temperature.

Figure S6

3.2.5 Effect of TP hybridization time

The SWV technique was chosen to investigate the hybridization time influence on the performance of the LNA based biosensor. For this task, the finally modified electrode was incubated with 0.74 µM target at 37 °C for different times (2, 4, 6, 10 and 12 h). As shown in the Fig. S7A by increasing the hybridization time, the peak currents of SWVs were decreased. In another word, the after 6 h, the hybridization process between the CP and target probe was completed. Fig. S7B shows the variation of peak currents *versus* TP hybridization time. The peak currents reached a plateau when the hybridization time was longer than 6 h, so this hybridization time period (6 h) was selected as an optimum hybridization time.

Figure S7

3.3 Analytical behavior of prepared biosensor

The electroanalytical behavior of the donkey marker specific biosensor was studied using SWV technique under optimized experimental conditions. As seen in Figs. 2A and C the responses of

SWV decreased by increasing of donkey marker concentrations. The calibration plot was prepared for SWV responses *versus* natural logarithm of proposed marker different concentrations (Fig. 2B), across the range of 148 pM to 0.74µM covering a response expanse of 6 orders of magnitude. Based on the results observed in Fig. 2B the lower limit of quantification (LLOQ) of the prepared biosensor was obtained as 148 pM. Relative standard deviation (RSD, n=10) of the ssLNA capture probe/target DNA/Au electrode was calculated as 0.44%. The recorded RSD confirm that, the results of the developed bioassay system are repeatable.

The selectivity of prepared biosensor for target detection was also investigated using perfectly complementary, single base-mismatched and non-complementary sequences using the DPV technique as presented in Figs. 2D and 2E. As can be seen, the DPV signal resulting from the capture probe hybridization with the single base-mismatched sequence has increased in comparison with the perfectly complementary sequence, which specified that the whole hybridization was not accomplished attributable to the base mismatch. Additionally, the significantly increased DPV signal from hybridization with a non-complementary sequence, indicated that the prepared biosensor was more selective in the presence of single base-mismatched and non-complementary sequences.

Figure 2

The precision and stability as important features of the biosensors were checked. In this regard, we test repeatability and reproducibility in this study. The repeatability was checked for at least five times for 0.074, 0.00037 and 0.00074 µM concentrations of target probe and the desirable RSD were recorded as 0.614%, 0.444% and 0.198%. The reproducibility as between lab test was examined for two different electrodes for 0.000148 µM concentration of target probe and the acceptable RSD was recorded as 5.89%. For checking the stability of the designed genosensor,

the electrochemical results were checked for 10 times (for 0.0074 μM concentration of target probe) and after 10 times recording the electrochemical signal, the acceptable RSD was calculated as 0.409 %. It means that, after 10 times recording the electrochemical signal, the electrochemical signal approximately was unchanged. In other word, the developed biosensor has enough stability to use as commercial tool.

3.4 Application of the prepared biosensor for analysis of donkey adulteration in cooked sausages of donkey-beef binary mixture and various species

After optimization of RFLP (Fig. S8) all samples were digested and used. The SWVs of sausages prepared from donkey-beef binary mixtures containing a number of percentages of spiked donkey also prepared from pure turkey, chicken, beef and mix 25% of each of these four species are illustrated in Figs. 3A and 3B. The donkey specific LNA-based biosensor obviously detected 1% donkey meat containing 10 ng μl^{-1} of related DNA in consumable sausage prepared from donkey-beef dual blends. Therefore, this noticeably reflects the more specificity and sensitivity of the designed biosensor to detection of target DNA in processed food products by vigorous pressure and heat, in which, sometimes led to degrades DNA (Brodmann and Moor 2003; Haider et al. 2012; Hird et al. 2006; Kesmen et al. 2009; Rojas et al. 2010).

Prepared sausages of other species (chicken, turkey and beef) presented SWV responses that are approximately different form the baseline response of the unreacted probes (Figs. 3A and 3B). The nucleotide sequence of cytb genes of above-mentioned meat sources was retrieved and aligned with the origin probe using ClustalW alignment program. Inset of Fig. 3A shows the mismatched nucleotide sequences of each species and their number of mismatches. Only the Equus asinus africanus (donkey) species showed 100% matching, and all other species indicated 9–14 nucleotide mismatching, defined the surprising selectivity of the developed bioprobe and supportive the results.

Haider et al. (Haider et al. 2012) identified species origin in raw meat samples of chicken, cow, sheep, turkey, buffalo, camel, pig and donkey using PCR-restriction fragment length polymorphism (RFLP) of a portion of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene. A 710-bp fragment was yielded in all species. In contrast, Chisholm et al. (Chisholm et al. 2005) detected 1 pg donkey template DNA in model food samples under raw states via a relatively small fragments (119 bp) of different mt genes by real-time PCR. Recently, using the polymerase chain reaction (PCR), was identified donkey and/or pork various adulterants in mixture of fresh minced beef by Abd El-Azeim (El-Azeim et al. 2016) with targeting 439 bp mt-DNA fragment of donkey. Kesmen and coworkers (Kesmen et al. 2009) successfully quantified the 0.0001 ng adulteration in pork, donkey and horse DNAs in raw and cooked meat mixtures by TaqMan based real-time PCR using a 183 bp fragment of ND2 (NADH dehydrogenase subunit 2) gene for donkey and other fragments of different genes for other species. Conversely, with a relatively shorter fragments (<120 bp) of various mt genes, Kesmen et al. (Kesmen et al. 2014) quantified 0.001% adulteration of donkey and pork DNAs in raw and heat processed binary meat mixtures by TaqMan-based duplex real-time PCR. Recently, Zahran et al. (Zahran and Hagag 2015) indicated that 5% of minced meats and Egyptain sausages were contaminated with donkey meat using PCR-RFLP of 359 bp fragment from mitochondrial cytochrome b gene sequence. Also Kesmen et al. (Kesmen et al. 2007) developed a species-specific PCR approach and detected 0.01 ng of DNAs of donkey, horse and pork meat in cooked sausages using a 145 bp fragment of ND2 gene for donkey and other fragments of different genes for other species. Although these studies evidently exhibit improvement in sensitivity through the decrease of the size of capture DNA, excessively decrease of the target DNA size is described to improve the selectivity, creating the PCR test unreliable (Brodmann and Moor 2003; Hird et al. 2006).

Furthermore, the PCR tests as small as 27 bp cannot be probable due to they reflect the similarity of the length of a PCR primer (Ali et al. 2012c; Brodmann and Moor 2003; Catuogno et al. 2011; Chisholm et al. 2005; El-Azeim et al. 2016; Haider et al. 2012; Hird et al. 2006; Kesmen et al. 2009; Kesmen et al. 2014; Kesmen et al. 2007; Rojas et al. 2010; Zahran and Hagag 2015). Therefore, while the DL of this study is more sensitive than the routine qRT-PCR, but the analysis method has main applications for the quantification of small size DNA targets which can persist in the severe situations that expansively breakdown DNA sequence into short fragments lead to amplification fail in PCR (Kesmen et al. 2009; Rojas et al. 2010). Detection in small size (15–30 nucleotides) DNA sequences, such as microRNAs, is progressively vital in the distinction of cancers and other genetically disorders at the initial stages, and the current biosensor can be a proper candidate for this propose (Catuogno et al. 2011). Routine qRT-PCR tests not only have restrictions in detection of small size DNA fragments, but also suffer enormous budget of consumables and instrumentations and comprises sophisticated electrophoresis and usage of dangerous substances like ethidium bromide (Houhoula et al. 2017; Zhang et al. 2007; Zhou et al. 2012). In return, the current methodology just includes the initial cost of Autolab potentiostat and reusable electrodes.

Donkey Mb (myoglobin) sequence, as an important constituent of meat and appropriate molecular marker for identifying meat species in transformed and raw products, determined by a complementary method based on Edman degradation and mass spectrometry (Dosi et al. 2012). This technique provide a very accurate and sensitive platform for determining the protein sequences, however, it requires the special infrastructures.

Identification of specific DNA species helps to detect permissible and non-permissible border limitations of adulteration investigation of food and also to screen the progress of infectious and

inherited diseases in sub-molecular detections (Kesmen et al. 2009). The present evaluation has a substantial potential to be used for mentioned aims, due to its lower cost and simplicity in comparison with other common techniques like real-time PCR.

Figure 3

3.5. Detection of target DNAs with qRT-PCR

qRT-PCR analysis was achieved to confirm the applicability of the designed biosensor for analysis of donkey adulteration in cooked sausages as a meat product. For this purpose, the donkey-specific primers were utilized to amplify an internal fragment (145 bp) of ND2 gene. A primer pair (18SEU-F, 18SEU-R shown in Table S2) as endogenous controls was used to amplify the nuclear 18S rRNA. To evaluate the PCR efficiency for the designed primer pair, serial ten-fold dilutions of the extracted DNA from positive control sausage sample (including 100% donkey meat) starting from 0.1 ng, were prepared. The PCR efficiency was reported as 113% (Fig. S8). Specificity of the primer pair was evaluated by adding the extracted DNA from sausage samples containing 100% of donkey, beef, turkey and chicken (Refer to section 2.3 in supplementary information) as well as quaternary mixture of four meat species to the qRT-PCR reaction as a template. Results obtained from qRT-PCR analysis showed that the primer set was specific enough to distinguish between donkey and other meat species (Fig. S9). In the next step, sausage samples prepared from donkey-beef mixtures containing various percentages of 1, 5, 10, 25, 50 and 100 of donkey were analyzed using qRT-PCR. The analysis results using both SWV and qRT-PCR methods are shown in Fig. 3 and 4, respectively. Based on the results, although the origin and the composition of samples are known in our work, qRT-PCR analysis was not able to accurately quantify donkey meat content in beef at percentages higher than 50%. If the sample origin is already known, calibration standard solutions could be prepared through

elimination of background DNA by applying proteinase K for digestion of unwanted contaminants (Ballin et al. 2009). Thus, this method certifies the best judgment between samples and calibration plots, because it prevents bias from diversities in tissue, DNA extraction, and genome size. Whereas if the sample origin is unknown (which is routinely the case in regulatory control with supervisory organs), for example a 5 percent difference in PCR efficiency between standard curves and unknown samples after 30 PCR cycles, can be result in a systematic error of 113%, if calculation is based on the following equation, Percentage error = $(2^n / E^n - 1) \times 100$. (E = amplification efficiency, n = the number of relative cycle at which the percentage error is determined) (Ballin et al. 2009). So non-linear amplification of target DNA during PCR assay lead to the error in quantification of adulteration in w/w for meat products by qRT-PCR.

In addition, extracted DNA by itself is another issue during w/w quantification in meat products using qRT-PCR procedure. Concentration of extracted DNA is associated with type and the nature of tissue/organ and the extraction procedure as the tissues differs in cell density and more cells would result in more DNA extract (Ballin et al. 2009).

According to the purpose, various types of DNA are utilized in PCR techniques. For species determination in w/w, PCR amplification of mitochondrial DNA is widely applied and its strength by the plentiful mitochondria present in most cell extracts is proved. Moreover, mitochondrial DNA is polymorphic and so greatly proper in differentiation of various species (Brown et al. 1979). Nonetheless, the content of mitochondria and mitochondrial DNA differs in various tissues (Robin and Wong 1988) and therefore, quantitative analysis does not sense unless the tissue is known, which is not routinely the case. The lack of sense in the quantitative detection of mitochondrial DNA was described in a study displaying the various results recorded from muscle and liver tissues. The content of mitochondria in muscle cells is lower than that of

liver cells. The mitochondrial DNA in muscle cell is ~ 3 times less than that of liver cell, which is why the assay results in the lower limit of detection (Rodriguez et al. 2003).

However, the newly developed biosensor could detect the various percentages of spiked donkey meat in donkey-beef binary mixtures. The qRT-PCR technique is relatively time-consuming and requires a well-equipped and sterile laboratory with expert personnel. Therefore, the engineered LNA-based biosensor is more sensitive than the qRT-PCR method in detection of donkey meat adulteration.

Figure 4

4. Conclusion

In this study, a unique and specific LNA based genosensor was designed and successfully applied for quantification of donkey adulterations in cooked sausages. The high sensitive bioassay with LLOQ of 148 pM and desirable precision with RSD of 0.16% for ten repetitive analysis were obtained. As a proof of concept, the prepared genosensor was successfully applied for detection of donkey adulterations with different percentages as a real sample and the obtained results were compared with qRT-PCR as standard method. It is important to point out that, the developed genosensor will provide a sensitive, inexpensive, fast, and reliable approach for application in food analysis, forensic investigations, genetic screening and biodiagnostics. The most restriction factors in this study were the time of analysis and use of un-portable instrument. In our future project we will miniaturized the bioassay system to make an on-desk device. Especially, for reduce the analysis time we will merge the developed genosensor with microfluidic systems.

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Table legend

Table 1: synthetic oligonucleotides sequences used in this study.

Table 1: synthetic oligonucleotides sequences used in this study. (L: 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides LNA) .

Synthetic oligonucleotides	**sequence**
immobilized LNA modified capture probe (CP)	5'-SH-$(CH_2)_6$-TTTTTTTTTTGATTC^{L}AATCA^{L}AAAGT^{L}TTTGT^{L}TGTCC^L-3'
complementary target	5'-GGACAACAAAACTTTTGATTGAATC-3'
non-complementary target	5'-CCTGTTGTTTTGAAAACTAACTTAG-3'
single-base mismatch target	5'-GGACAAAAAAACTTTTGATTGAATC-3'

Figures captions

Scheme 1: The schematic illustration for preparation of DNA biosensor.

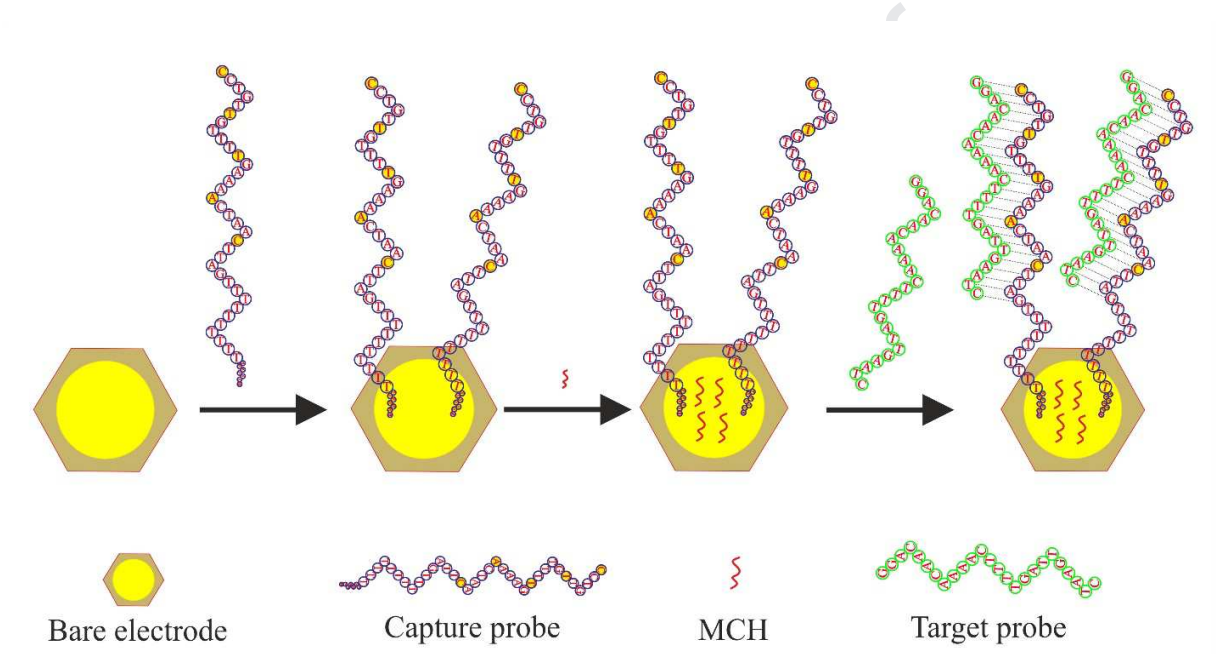
Figure 1: The **A**) LSVs(OX); **B**) EISs; **C)** the enlarged part of EIS in 10 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl recorded at Bare Au, Bare Au + CP, Bare Au + CP + MCH, Bare Au + CP + MCH + TP.

Figure 2: A) SWVs results for ssLNA capture probe/MCH/Au electrode incubated with synthetic target at different concentrations; **B)** Calibration plot of donkey marker; **C)** The bar graphs of the peak currents versus different concentrations of synthetic target. **D)** DPVs related to comparison of hybridization response intensities for capture probe/MCH/Au electrode with complementary, single base-mismatched and non-complementary sequences, respectively. **E)** The bar graphs of the DPV peak currents related to synthetic sequences Referenced in Fig. 2D.

Figure 3: A) SWVs results of donkey detection in consumable donkey-beef dual mixed, pure and mix sausages of various species. The inset is the comparison of nucleotide sequences of various species with donkey oligoprobe. **B)** The bar graphs of the SWV peak currents Referenced in Fig. 3A.

Figure 4: The Ct values are related to the concentration of 10 ng/µl of extracted DNA from pure donkey samples and other percent's from donkey-beef binary mixtures.

Scheme 1



The locked nucleic acids on the capture probe were illustrated by yellow filled nucleotides

Figure 1

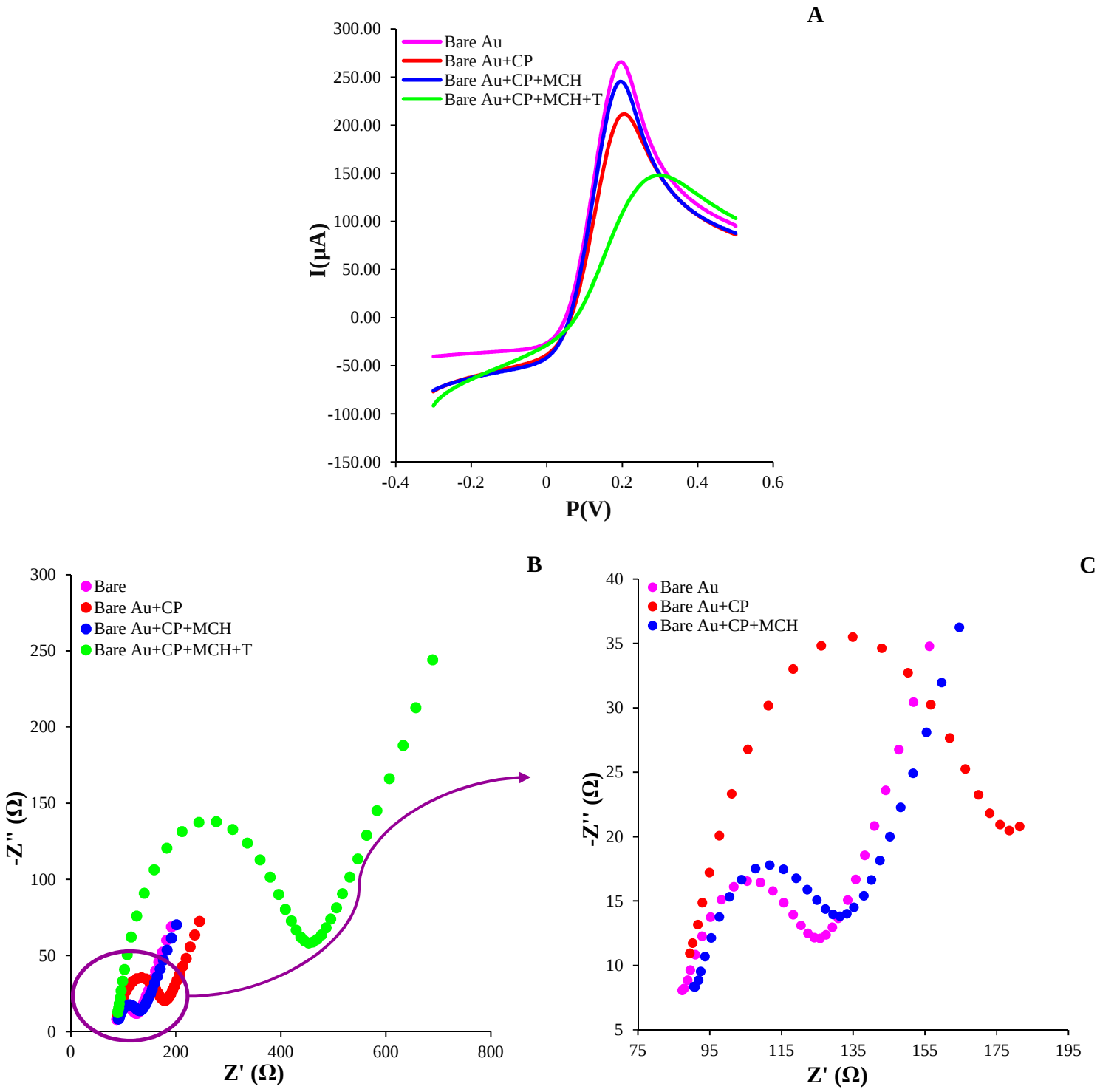
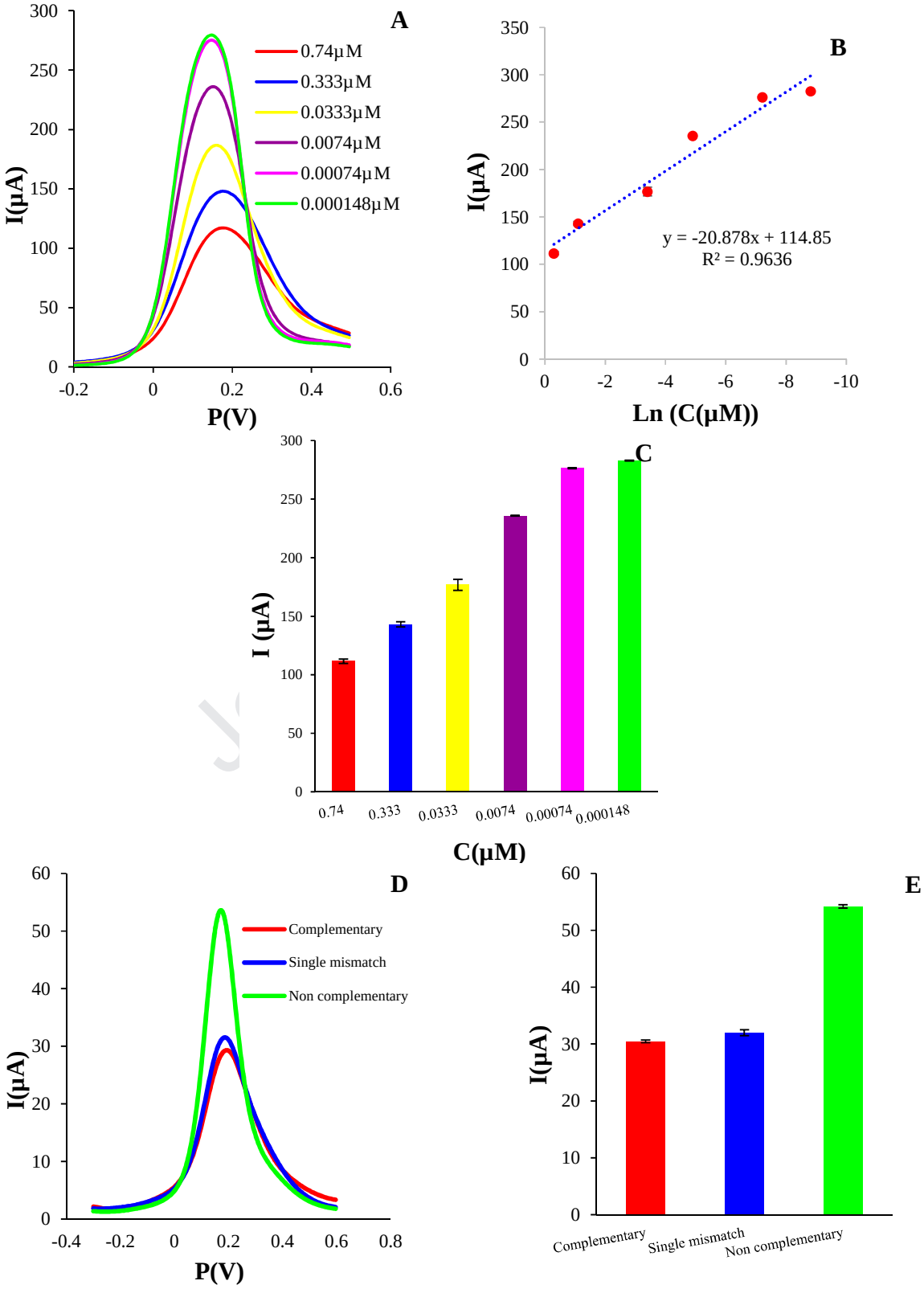
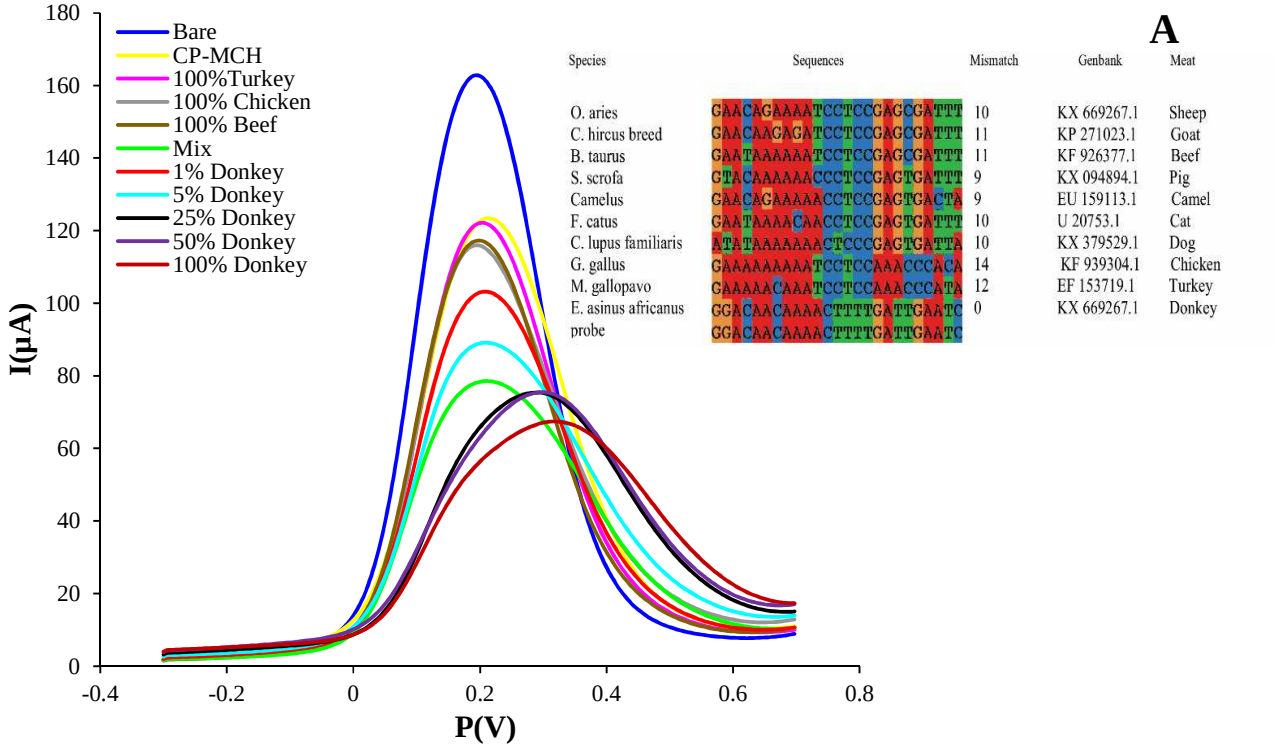

A
Bare Au
Bare Au+CP
Bare Au+CP+MCH
Bare Au+CP+MCH+T
I(μA)
P(V)
B
Bare
Bare Au+CP
Bare Au+CP+MCH
Bare Au+CP+MCH+T
-Z'' (Ω)
Z' (Ω)
C
Bare Au
Bare Au+CP
Bare Au+CP+MCH
-Z'' (Ω)
Z' (Ω)

Figure 2


A
0.74μM
0.333μM
0.0333μM
0.0074μM
0.00074μM
0.000148μM
I(μA)
P(V)
B
y = -20.878x + 114.85
R² = 0.9636
I(μA)
Ln (C(μM))
C
I (μA)
0.74
0.333
0.0333
0.0074
0.00074
0.000148
C(μM)
D
Complementary
Single mismatch
Non complementary
I(μA)
P(V)
E
I(μA)
Complementary
Single mismatch
Non complementary


A
Bare
CP-MCH
100%Turkey
100% Chicken
100% Beef
Mix
1% Donkey
5% Donkey
25% Donkey
50% Donkey
100% Donkey
I(μA)
P(V)
Species
Sequences
Mismatch
Genbank
Meat
O. aries
C. hircus breed
B. taurus
S. scrofa
Camelus
F. catus
C. lupus familiaris
G. gallus
M. gallopavo
E. asinus africanus
probe
KX 669267.1 Sheep
KP 271023.1 Goat
KF 926377.1 Beef
KX 094894.1 Pig
EU 159113.1 Camel
U 20753.1 Cat
KX 379529.1 Dog
KF 939304.1 Chicken
EF 153719.1 Turkey
KX 669267.1 Donkey

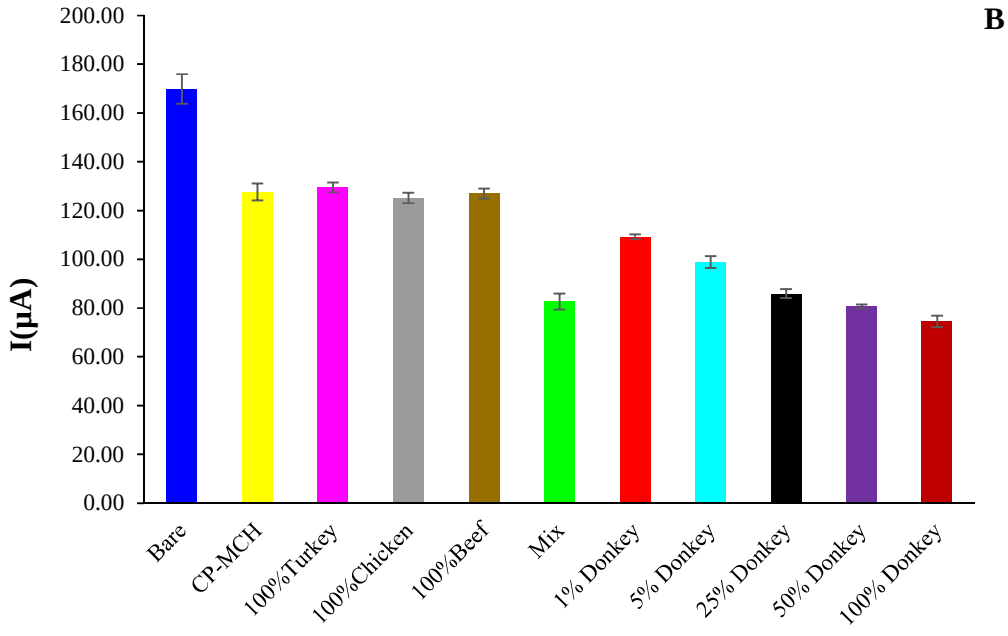

B
I(μA)
Bare
CP-MCH
100%Turkey
100%Chicken
100%Beef
Mix
1% Donkey
5% Donkey
25% Donkey
50% Donkey
100% Donkey

Figure 3

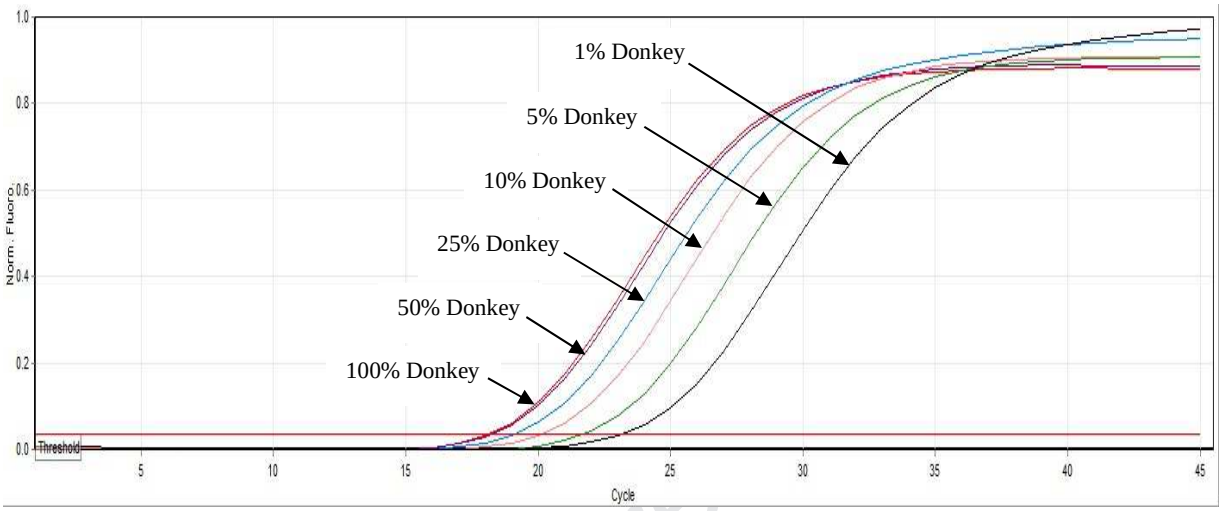


Figure 4

- A species-specific DNA probe for the electrochemical biosensing of food adulteration was developed.
- LNA was used for the improvement of the genosensor performance based on high affinity of the target sequence.
- As a proof of concept the designed LNA-based genosensor was successfully applied for sensitive and selective detection of meat adulteration.

The author contributions list of the manuscript entitled “Design a highly specific sequence for electrochemical evaluation of meat adulteration in cooked sausages” (BIOS-D-19-02916) is as below:

Maryam Mansouri was contributed in all experimental analysis, writing and editing.

Balal Khalilzadeh was participated in idea, development of the method, validation of data and editing.

Aboulfazl Barzegari helped in designing of the specific capture probe.

Shahram Shoeibi assisted in drafting.

Selim Isildak contributed in final edition.

Nasrin Bargahi and Siavoush Dastmalchi participate in PCR analysis.

Yadollah Omidi contributed in data interpretations.

Mohammad-Reza Rashidi was the supervised the study and helped in research design, method development, validation, data analysis, writing and editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: