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Disposable amperometric PCR-free biosensor for direct detection of adulteration with horsemeat in raw lysates targeting mitochondrial DNA

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ABSTRACT: A novel electrochemical disposable nucleic acid biosensor for simple, rapid and specific detection of adulterations with horsemeat is reported in this work. The biosensing platform involves immobilization of a 40-mer RNA probe specific for a characteristic fragment of the mitochondrial DNA D-loop region of horse onto the surface of magnetic microcarriers. In addition, signal amplification was accomplished by using a commercial antibody specific to RNA/DNA duplexes and a bacterial protein conjugated with an HRP homopolymer (ProtA-HRP40). Amperometric detection at -0.20 V vs. Ag pseudo-reference electrode was carried out at disposable screen-printed carbon electrodes. The methodology achieved a LOD of 0.12 pM (3.0 attomoles) for the synthetic target and showed ability to discriminate between raw beef and horsemeat using just 50 ng of total extracted mitochondrial DNA ($\sim 16,660$ bp in length) without previous fragmentation. The biosensor allowed also discrimination between 100% raw beef and beef meat samples spiked with only 0.5% (w/w) horse meat (levels established by the European Commission) using raw mitochondrial lysates without DNA extraction or PCR amplification in just 75 min. These interesting features made the developed methodology an extremely interesting tool for beef meat screening and it can be easily adapted to the determination of other meat adulterations by selection of the appropriate specific fragments of mitochondrial DNA region and capture probes.

1. INTRODUCTION

Authentication and traceability of meat and meat products have become a concern for governments, consumers, and food industries.¹ The alarming regularity with which major food fraud and contamination events occur lately seem to be closely related to globalization and rapid distribution systems. As a consequence, issues related to large-scale food adulteration and contamination have now more international impacts and are beginning to be realized, discussed and analyzed in far more detail, by the food industry, regulators, as well as by consumers.² Within food contamination, adulteration of meat products has become a very serious problem nowadays which limits the development of the industry and connects with the public health, religious factors, wholesomeness and unhealthy competition in meat market. Therefore, it is critically important to accurately and rapidly identify the mammalian species in meat.^{1,3}

Indeed, one of the best-known food fraud and contamination events reported, which demonstrated the vulnerabilities currently inherent within complex international food supply chains, was the horse meat scandal (so-called 'Horsegate' scandal) in 2013 centered in the UK and Europe. This event also focused the attention of governments, industry, researchers and regulatory bodies across the world, and involved the large-scale replacement of processed beef products with horsemeat and other undeclared meat products, such as pork, sometimes up to levels of 100% substitution.²

Therefore, to protect consumer rights and avoid unfair market competition, it is imperative to provide reliable, efficient, rapid and accurate methods facilitating routine control tests to identify the different species of mammals within raw and processed meats.³ Currently many different assays and strate-

gies are available for tracing meat adulteration and differentiating species present in mixed meats such as lipid analysis,⁴ immunological procedures,⁵ sensory evaluation,⁶ spectroscopic methods,^{2,7} and molecular biology techniques.⁸ However, analytical methods based on identification of species-specific proteins by means of electrophoretic and/or immunological assays are sometimes not sensitive enough to differentiate closely related species due to cross-reactivity and not reliable for identifying species in heated or baked products due to denaturation and degradation of the proteins. Therefore, DNA-based assays have also been used for the accurate detection of animal species because of the larger stability of DNA compared to proteins at higher temperature and its conserved structure within all organisms in a species.^{1,3,4} For this purpose, different approaches based on a wide variety of nucleic acid amplification strategies including conventional PCR,^{1,9} real-time PCR,^{8,10,11} primer multiplex-PCR¹² and loop-mediated isothermal amplification^{3,13} have been reported. However, most of these methods involve PCR amplification of target DNAs that are typically more than hundred base-pair long. Interestingly, several studies demonstrated that longer DNA targets are broken down to small fragments during extensive food processing causing failure of PCR-based identification methods.¹⁴

In this sense, an interesting alternative source of candidate markers for developing PCR-free methods for meat species identification even in processed foods is the mitochondrial (Mt) genome. Mt-DNAs are circular and hence more stable than linear nuclear DNAs. Moreover, Mt-genes rarely undergo recombination and are resistant to degradation by the protective shape and size of mitochondrion. Given that Mt-DNAs are present in multiple copies in each mitochondrion and that several copies of mitochondria are present per cell, targeting

Mt-genes will ensure available targets even in extremely degraded samples and without PCR amplification. The Mt-genomes of all bilateral animals contain 13 protein-coding genes (cytochrome oxidase subunits 1, 2, and 3, CO1-CO3, cytochrome b subunit, *cytb*, NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6, ND1-ND6, ND4L, and ATPase subunits 6 and 8, ATP6 and ATP8) 2 ribosomal RNA genes (12S and 16S rRNAs) and 22 transfer RNA (tRNA) genes.¹⁵ The inter-to intra-species gap (barcode gap) of all the genes of Mt origin is larger than 10, which makes them effective markers for species differentiation in foods and feeds.¹⁶ The mitochondrial DNA comprises also a D-loop region which is the main non-coding area of the mitochondrial DNA molecule. Although the function of this D-loop is still unclear, it seems to be a control region in mitochondrial DNA. To allow detection of meat species in processed foods, several sensitive PCR-based approaches targeting small regions of Mt-genes have been developed.^{4,17,18} Moreover, given the large copy number of Mt-genes, attractive PCR-free strategies have been developed for detection of pork adulteration both in raw and heat processed foods using hybrid nanobioprobes composed of gold nanoparticles and fluorophore-labeled oligo-probe of swine-Mt origin (*cytB* gene).^{14,16,19} These methods provided LODs of 58.6 pM for synthetic target and 230 $\mu\text{g L}^{-1}$ for total DNA and showed ability to detect 1% pork in mixed meats cooked under heat and pressure conditions that extensively degraded longer DNA targets causing failure of PCR-based identification methods.¹⁹ However, it is important to mention that to our knowledge no PCR-free biosensing strategy has been reported so far for horsemeat adulteration detection.

Therefore, to fulfil the existing demands for user friendly methodologies that allow the detection of meat adulterations in a sensitive, selective, fast and with no complex nucleic acid amplification strategies, this work describes for the first time the development of a PCR-free electrochemical biosensing platform for the detection of horsemeat by targeting a 40-mer fragment of the mitochondrial DNA D-loop region of horse. The proposed assay involves direct hybridization of the target mitochondrial DNA fragment with a specific RNA capture probe immobilized onto Streptavidin-functionalized magnetic microcarriers (Strep-MBs), recognition of the captured DNA/RNA heteroduplexes with a commercial antibody and labeling with a bacterial protein conjugated with a horseradish peroxidase homopolymer (ProtA-HRP40). The variation in the cathodic current measured using the $\text{H}_2\text{O}_2/\text{HQ}$ system, after magnetic capture of the modified MBs onto SPCE, could be related with the presence of the target DNA in the analyzed sample. The optimized method allowed a LOD of 0.12 pM of the synthetic target DNA as well as unequivocal identification of beef meat samples adulterated with only 0.5% (w/w) of horsemeat using raw mitochondrial lysates without DNA extraction or PCR amplification in just 75 min.

2. EXPERIMENTAL

2.1. Apparatus and electrodes

Amperometric measurements were performed with a CH Instruments (Austin, TX) model 812B potentiostat controlled by software CHI812B. Screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens, Spain), consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode, were used as electrochemical transducers in conjunction with a specific cable

connector (DRP-CAC, DropSens). All measurements were performed at room temperature. A neodymium magnet (AIMAN GZ) embedded in a homemade Teflon casing was used to magnetically capture in a reproducible way the modified-MBs on the surface of SPCEs.

A Raypa steam sterilizer, a biological safety cabinet Telstar Biostar, a thermocycler (SensoQuest LabCycler, Progen Scientific Ltd), an incubator shaker Optic Ivymen® System (Comecta S.A, Sharlab), a Bunsen AGT-9 Vortex for homogenization of the solutions, a magnetic particle concentrator DynaMag™-2 (123.21D, Invitrogen Dynal AS) and the centrifuges Heraeus Multifuge 3 SR plus (Thermo Scientific) and 5424 (Eppendorf) were also employed. The quality and quantity of extracted DNAs were evaluated by using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies) and a horizontal electrophoresis system (Bio-Rad).

2.2. Reagents and solutions

All reagents used were of the highest analytical grade. Streptavidin-modified magnetic beads (Strep-MBs, 2.8 μm Ø, 10 mg mL^{-1} , Dynabeads M-280 Streptavidin, 11206D) were purchased from Dynal Biotech ASA.

NaCl, KCl, NaH_2PO_4 , Na_2HPO_4 and Tris-HCl were purchased from Scharlab. ProtA, HRP conjugate (ProtA-HRP), hydroquinone (HQ) and H_2O_2 (30%, w/v) were purchased from Sigma-Aldrich. Protein A-Poly-HRP40, a ProtA labeled with a Poly-HRP40 (an enhanced enzymatic label comprising covalent HRP homopolymer), was purchased from antibodies-online. RNA-DNA hybrid antibody (clone: D5H6) (anti-DNA-RNA) was purchased from Covalab. Ethylenediaminetetraacetic acid (EDTA) from Merck (Germany) and streptavidin-horseradish peroxidase (Strep-HRP) conjugate from Roche were also used. A commercial blocker casein solution (a ready-to-use, PBS solution of 1% w/v purified casein) was purchased from Thermo Scientific. G-Spin™ Total DNA extraction kit (Intron Biotechnology) and Speedtools plasmid DNA purification kit (Biotools) were also used. All the used oligonucleotides (probes and synthetic target), whose sequences are described in Table S1, were purchased from Sigma-Aldrich. Upon reception, they were reconstituted in nuclease-free water to a final concentration of 100 μM , divided into small aliquots and stored at -20 or -80 °C.

All the probes used (bRNACp-40mer, bRNACp-24mer, EQ2 F, EQ2 R, bDNACp and bDNADp) were designed to be fully complementary to both synthetic target DNA and a specific fragment of the horse mitochondrial DNA D-loop region (see corresponding sequences in Table S1). Synthetic target sequence designed is a copy of a specific region of the 851 bp *Equus caballus* mitochondrial DNA haplotype ID65 D-loop, partial sequence.²² We decided to target the mitochondrial DNA D-loop region of horse (GenBank number: DQ327940.1) because its hypervariable character. The sequences of this region available in databases (NCBI) were studied to select an invariant region of this species being absent in the rest of mammals. In order to get the optimal hybridization yield in sandwich assays, there was no gap between hybridization sites of the designed probes with the target.^{20,21}

All the required buffer solutions were prepared in deionized water from a Millipore Milli-Q purification system (18.2 MΩ cm): phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 0.137 M NaCl and 0.0027 M KCl, pH 7.5; 0.05 M phosphate buffer, pH 6.0;

Binding and Washing buffer (B&W) consisting of 10 mM Tris-HCl solution containing 1 mM EDTA and 2 M NaCl, pH 7.5; Solution I consisting of 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0; MLB solution consisting of 150 mM NaCl, 50 mM Na₂EDTA and 10 mM Tris-HCl, pH 8.0; SDS solution (prepared just before using) consisting of 2% SDS in 0.4 N NaOH (in H₂O sterilized) and Solution III consisting of 29.5% (v/v) of glacial acetic acid, pH 4.8. PBS, B&W, I and MLB solutions were sterilized after their preparation to avoid RNases degradation.

2.3. Samples

Different meat muscle samples (beef, horse, turkey, chicken and pork) were purchased from a local supermarket and stored at -80 °C.

Beef samples containing different percentages of horse meat (0.5, 1.0, 2.5, 5.0, 10, 25 and 50 % (w/w)) were prepared by mixing the appropriate amounts of beef and horse meats completely crushed. A 10% (w/w) horse meat sample was used for the preparation of beef spiked samples in the 0.5-5.0% (w/w) range by mixing with the appropriate amount of 100% beef meat. All samples were ground and homogenized with liquid nitrogen using a mortar and pestle.

2.4. Mitochondrial DNA extraction and preparation of mitochondrial lysates

Three different protocols were evaluated for mitochondrial DNA extraction: G-SpinTM Total DNA extraction kit (A); mitochondrial isolation and G-SpinTM Total DNA extraction kit (B) and mitochondrial isolation and Speedtools plasmid DNA purification kit (C). The manufacturer's instructions were followed in all cases.

To perform the mitochondrial DNA extraction using G-SpinTM Total DNA extraction kit (protocol A), 25 mg of raw sample were employed.

Mitochondrial isolation was accomplished by crushing 3.0 g of sample in 15 mL of Solution I and centrifuging at 1,000 g (5 min, 4 °C) to remove un-dissolved materials. Subsequently, the supernatant was re-centrifuged at 12,000 g (10 min, 4 °C) and the mitochondrial mass from the sample was isolated. Mitochondrial DNA was extracted from the collected pellet by using G-SpinTM Total DNA extraction kit (protocol B) or Speedtools plasmid DNA purification kit (protocol C). Regarding protocol C and prior to DNA extraction, the collected pellet was re-suspended in 800 µL of MLB solution, and 400 µL of SDS solution and 800 µL of Solution III were sequentially added and manually shaken, keeping in ice for 5 min. The resulting solution was centrifuged at 13,000 rpm (5 min, 25 °C) and mitochondrial DNA was finally extracted from the supernatant.

Raw mitochondrial lysates were obtained by re-suspending the mitochondrial mass (obtained as described above by centrifugation) in 200 µL of MLB solution and 100 µL of the SDS solution.

Just before making the determination with the biosensor both mitochondrial DNA extracted and raw mitochondrial lysates were denatured by heating at 97 °C for 5 minutes in the thermocycler and transfer immediately to ice for 10 minutes.

2.5. Hybridization and labeling

A 5-µL aliquot of the Strep-MBs commercial suspension was transferred to a micro-centrifuge tube and washed twice with 50 µL B&W buffer (pH 7.5). After each washing step, MBs were magnetically concentrated for 3 min at the magnetic concentrator and the supernatant was discarded. Strep-MBs were incubated for 60 min (950 rpm at 37 °C) in 25 µL of a 0.1 µM bRNACp-40mer solution prepared in B&W. Then the modified MBs (bRNACp-40mer-MBs) were washed twice with 50 µL of commercial blocker casein solution. The as prepared bRNACp-40mer-MBs could be stored (in filtered B&W buffer pH 6.0 at 4 °C) until they were used. Subsequently, the bRNACp-40mer-MBs were incubated for 30 min (950 rpm at 37 °C) in 25 µL of a solution containing the target DNA (synthetic DNA, mitochondrial DNA extracted or 2-times diluted mitochondrial lysate). After two additional washings with 50 µL of blocker casein solution, the target DNA-bRNACp-40mer-MBs were incubated during 30 min in 25 µL of a mixture solution containing anti-DNA-RNA (2 µg mL⁻¹) and ProtA-HRP40 (1 µg mL⁻¹) (prepared in blocker casein solution and incubated previously 1 h). Finally, after two additional washing steps with blocker casein solution, modified MBs were re-suspended in 45 µL of 0.05 M sodium phosphate buffer solution (pH 6.0) to perform the amperometric detection.

It is worth to mention that all the MBs manipulations carried before the amperometric measurements were performed in the laminar flow cabinet to avoid RNase contamination and prevented RNA capture probe degradation

2.6. Amperometric measurements

The above mentioned 45 µL of the modified MBs suspension were magnetically captured on the surface of the working electrode after placing the SPCE on the magnetic holding block.²³ Amperometric measurements were performed in stirred solutions by immersing the ensemble SPCE/magnet holding block into an electrochemical cell containing 10 mL of 0.05 M sodium phosphate buffer solution (pH 6.0) and 1.0 mM HQ (prepared just before performing the electrochemical measurement) and applying a detection potential of -0.20 V vs. Ag pseudo-reference electrode. Once the baseline was stabilized (~60 s), 50 µL of H₂O₂ solution (0.1 M) were added and the current was recorded until the steady state was achieved (~100 s). The magnitude of the measured cathodic current was directly proportional to the target DNA concentration.

2.7. Amplification protocol of a 144 bp-fragment specific from horse meat by PCR

PCR amplification was carried out in a total volume of 20 µL. This mixture consisted of 10 µL of DNA AmpliTools Fast Master Mix 2×, 5 pmol of each primer (forward and reverse), 2 µL of DNA extract and nuclease free water. PCR was performed using a heated lid thermal cycler applying an initial denaturation at 94 °C for 5 min, 35 subsequent cycles of denaturation at 94 °C for 15 s and annealing at 55 °C for 30 s and a final elongation step at 72 °C for 5 min. PCR products (no further purified) were stored at -20 °C.

3. RESULTS AND DISCUSSION

Strategies involving antibodies as specific and versatile bio-receptors with unique binding properties towards RNA/DNA heterohybrids have demonstrated to be particularly attractive for the determination of nucleic acids.^{24–30} Apart from their selectivity towards a particular type of duplexes, these specific antibodies are versatile recognition elements for the detection of any target nucleic acid. In addition, their small epitope (~6 base pairs in size) makes feasible tailoring the sensitivity of the analytical strategy by varying the length of the DNA/RNA heteroduplexes. Therefore, in order to develop an electrochemical sensor to detect horsemeat adulterations, we have designed an RNA probe for the specific recognition of the target horse mitochondrial DNA and we have monitored the hybridization by using anti-DNA-RNA specific antibodies. A schematic display of the fundamentals involved in the developed methodology is shown in Figure 1. In brief, streptavidin-functionalized magnetic microcarriers (MBs) were modified with a specific biotinylated RNA probe and used to selectively capture by direct hybridization the synthetic target DNA or the target mitochondrial DNA fragment either in the extracted total mitochondrial DNA or directly in the raw mitochondrial lysate. The resulting heteroduplexes immobilized on the MBs were labeled with a specific DNA-RNA antibody previously labeled with a Protein A (ProtA) conjugated with an HRP homopolymer (Poly-HRP40). The MBs bearing the HRP-labeled heterohybrids were magnetically captured on the SPCE working electrode surface previously placed on a custom-fabricated magnetic holding block. The extent of the hybridization reactions was monitored by measuring the reduction current arising from the benzoquinone (BQ) formed in the HRP catalyzed oxidation of HQ upon addition of a H_2O_2 solution³¹ using amperometry in stirred solutions as described in section 2.6.

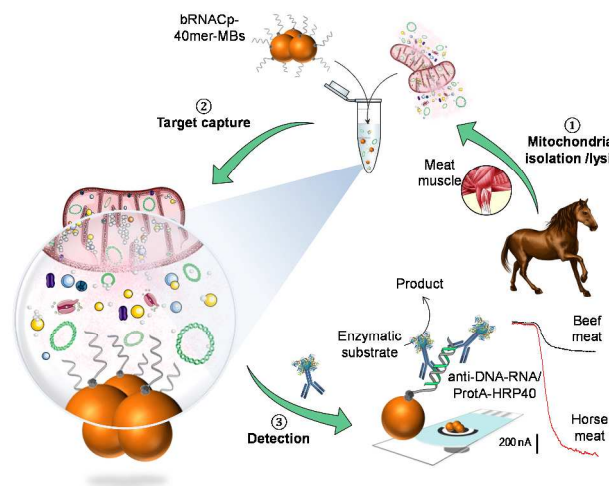


Figure 1. Schematic display of the fundamentals involved in the development of an electrochemical sensor for horse mitochondrial DNA detection.

3.1. Optimization of experimental variables

All the experimental variables affecting the performance of the methodology developed for horse meat detection were optimized. The criterion of selection was the largest ratio between the amperometric responses measured at -0.20 V (vs. the Ag pseudoreference electrode) for 0.0 (blank, B) and 0.1 nM of synthetic target DNA (signal, S) (signal-to-blank, S/B, ratio). Both the detection potential and the volume of the

Strep-MBs suspension used were the same that those previously optimized.^{32,33} All the checked variables, the ranges into which they were tested, and the selected values are summarized in Table S2.

The influence of the RNA capture probe length immobilized onto the Strep-MBs was evaluated. Figure 2a shows as the amperometric responses obtained when the 40-mer RNA sequence was used was 2.2 times larger than those measured using a 24-mer capture probe. These results can be attributed to the larger number of anti-DNA-RNA antibodies that can bind to longer RNA/DNA heteroduplexes, which is also in agreement with the small size of the binding epitope (~6 base pairs) of the DNA-RNA antibodies.^{34,35}

An important variable to be tested because of its practical significance is the number of steps used to perform the hybridization assay. Different protocols were compared:

- 1-step protocol: hybridization and labeling was performed in a single step by incubating during 30 min the bRNACp-40mer-MBs in a mixture solution containing the target DNA, the anti-DNA-RNA and the ProtA-HRP40.

- 2-steps protocol: involving a former hybridization step by incubating the bRNACp-40mer-MBs with the target DNA (30 min) and a further labeling step by incubating the target DNA-bRNACp-40mer-MBs during 30 min with a mixture solution of anti-DNA-RNA and ProtA-HRP40.

- 3-steps protocol: involving firstly the hybridization of the target DNA onto the bRNACp-40mer-MBs (30 min) followed by two independent and successive steps of the target DNA-bRNACp-40mer-MBs with anti-DNA-RNA (30 min) and ProtA-HRP40 (30 min).

The amperometric responses measured for 0.0 and 0.1 nM target DNA as well as the corresponding signal-to-blank (S/B) ratios using each of these protocols are displayed in Figure 2b. As it is clearly seen, although all the employed assay protocols allowed discrimination of the target DNA against the blank, the largest S/B ratio was achieved when the target DNA-bRNACp-40mer-MBs were incubated in a mixture solution containing the anti-DNA-RNA and ProtA-HRP40. These results can be attributed to a better recognition of the anti-DNA-RNA by the ProtA-HRP40 when both reagents are free in solution. Consequently, a 2-step protocol involving the former hybridization of the target DNA onto the bRNACp-40mer-MBs and further labeling of the heterohybrids attached onto MBs by incubation in an anti-DNA-RNA and ProtA-HRP40 mixture solution was selected for further work.

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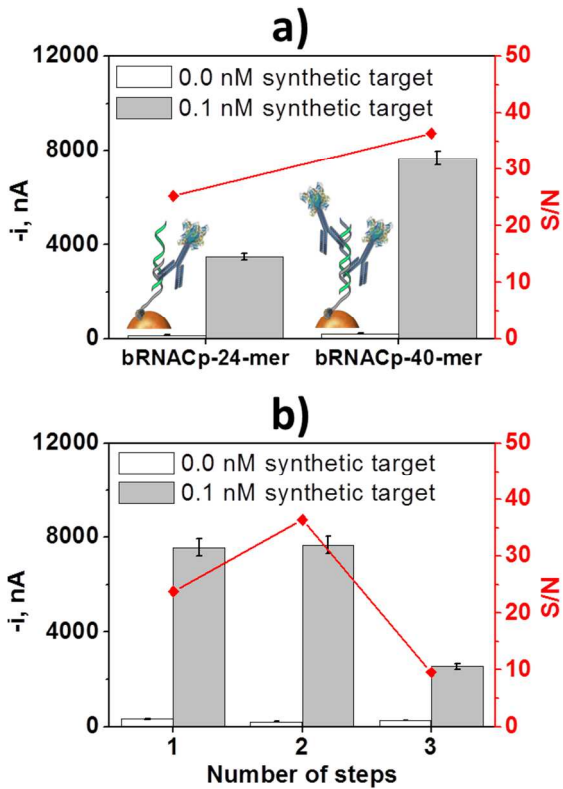


Figure 2. Influence of the length of the RNA capture probe immobilized onto Strep-MBs (a) and the number of steps used to perform the assay (b) on the amperometric responses measured with the developed amperometric sensor for 0.0 (blank, B, white bars) and 0.1 nM of synthetic target DNA (signal, S, grey bars) and the corresponding S/B ratio values (in red circles). Error bars were estimated as triple of the standard deviation (n=3).

3.2. Analytical characteristics for the synthetic target DNA determination

Under the selected experimental conditions, the calibration plot for synthetic target DNA (Figure 3) exhibited a linear dependence ($r=0.9984$) with DNA concentration between 0.39 and 75 pM, with slope and intercept values of $(91,796 \pm 728)$ nA nM⁻¹ and (244 ± 20) nA, respectively. Detection (LOD) and quantification (LQ) limits were calculated according with the $3 \times s_b/m$ and $10 \times s_b/m$ criteria, respectively, where m is the slope of the linear calibration plot and s_b was estimated as the standard deviation of ten amperometric measurements obtained in the absence of target DNA. Values of 0.12 and 0.39 pM (3.0 and 9.75 attomol), respectively, were obtained.

These characteristics were compared with those obtained by preparing DNA sensors using a conventional sandwich assay involving 24mer-biotinylated DNA capture and detector probes and enzymatic labeling with Strep-HRP and DNA/RNA hybridization assays using the above mentioned shorter 24-mer RNA capture probe and enzymatic labeling with ProtA-HRP instead of ProtA-HRP40. The comparison of the corresponding calibration graphs is displayed in Figure 3 and the analytical characteristics summarized in Table 1. As it can be deduced, both the formation of longer DNA-RNA heterohybrids allowing labeling with a larger number of antibodies and the use of the ProtA conjugated with the HRP homopolymer (Poly-HRP40), greatly amplified the signal and resulted in an enhanced sensitivity of the developed DNA

sensor. Table 1 shows that the optimized methodology provided an amplification factor of 771 and a LOD more than 3 orders of magnitude lower in the same assay time than the conventional sandwich hybridization assay. Moreover, the use of a longer RNA capture probe and the ProtA-HRP40 label improved the sensitivity 2.2 and 9.2 times with respect to the use of bRNACp-24mer/ProtA-HRP40 and bRNACp-40mer/ProtA-HRP, respectively. These results highlight the potential of the developed methodology for the detection, without previous nucleic acid amplification, of a specific Mt-DNA fragment associated with the presence of horsemeat.

It is important to mention also that the achieved LOD for the synthetic target is 488 times lower (0.12 vs. 58.6 pM) than that reported for the only PCR-free fluorescent biosensor developed so far for meat adulteration.¹⁹ Moreover, although this LOD value is higher than that reported using nucleic acid-based amplification methods, this methodology could be particularly relevant for the analysis of samples where PCR-based detection cannot be applied due to limited amounts of DNA templates or compromised stability of longer DNA targets during food processing.

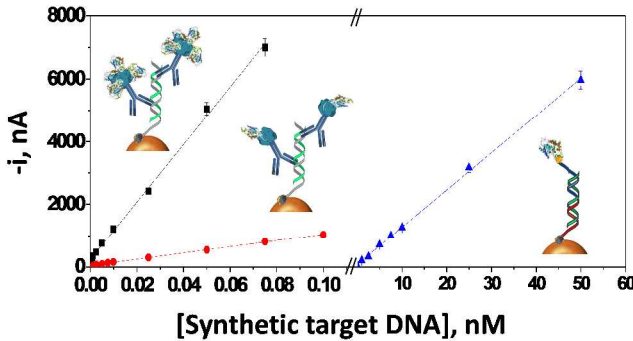


Figure 3. Calibration plots obtained for the synthetic target DNA with the developed sensor (in black), using ProtA-HRP instead of ProtA-HRP40 for enzymatic labeling (in red) and with a sandwich conventional strategy (in blue). Error bars were estimated as triple of the standard deviation (n=3).

Table 1. Comparison of the analytical characteristics achieved with DNA sensors constructed following different strategies.

Parameter	Conventional sandwich hybridization assay/Strep-HRP	bRNACp-24mer/ProtA-HRP40	bRNACp-40mer/ProtA-HRP	bRNACp-40mer/ProtA-HRP40
LR /pM	812-50,000	1.4-250	2.5-100	0.39-75
r^2	0.999	0.999	0.999	0.998
Slope /nA nM ⁻¹	119.0 ± 1.3	$40,629 \pm 295$	$9,986 \pm 80$	$91,796 \pm 728$
Intercept /nA	86 ± 27	206 ± 25	51 ± 4	244 ± 20
Amplification factor vs. conventional	—	341	84	771

LOD/pM	157	0.4	0.8	0.12
Assay time, min	75	75	75	75

The reliability of the whole procedure was evaluated by calculating the relative standard deviation (RSD) value for the amperometric measurements for 5 pM synthetic target DNA provided by 8 different sensors prepared in the same manner. A RSD of 4.0%, demonstrated the great reproducibility of the protocols used for the sensor preparation including modification of MBs, affinity reactions, magnetic capture on the SPCE surface and the amperometric transduction.

In addition, the storage stability of the bRNACp-40mer-MBs conjugates was tested by storing them at 4 °C in Eppendorf tubes containing 50 μ L of sterilized and additionally filtered B&W and measuring the amperometric responses for 0.0 and 5 pM target DNA with different DNA sensors prepared in different working days, according with the protocols described in sections 2.5 and 2.6. As it can be seen in Figure 4, no significant differences in the measured S/B ratio were apparent for a 23 days-period (no longer times were assayed), indicating great stability of the bRNACp-40mer-MBs conjugates which can be prepared and stored in advance until the determination is required.

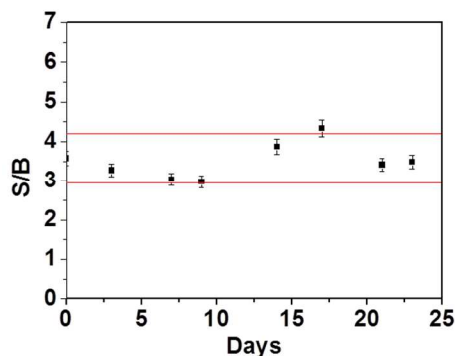


Figure 4. Control chart constructed to check the stability of bRNACp-40mer-MBs conjugates stored in filtered B&W buffer (pH 7.5) at 4 °C after preparation. Values of S/B obtained with the amperometric signals provided by the sensors fabricated with the conjugates on different days in the absence and in the presence of 5 pM synthetic target DNA. Error bars were estimated as triple of the standard deviation ($n=3$).

3.3. Determination of a specific fragment of horse mitochondrial DNA

In order to apply the developed methodology to the determination of a specific fragment of horse mitochondrial DNA, different mitochondrial DNA extraction protocols were assayed. A commercial kit for the extraction of DNA from animal tissue (bars A, Figure 5a), and protocols involving the previous isolation of mitochondrial mass present in the sample and subsequent extraction of DNA using the same commercial kit mentioned above (bars B, Figure 5a) or specific extraction of the mitochondrial DNA using a plasmid DNA extraction kit, due to the similar characteristics of plasmid and mitochondrial DNAs (bars C, Figure 5a). The same protocols were also applied to extract mitochondrial DNA from beef meat. As it can be seen in Figure 5a, a noticeable discrimination between horse and beef meat was only achieved when the DNA extraction protocol involves mitochondrial isolation and specific

extraction of mitochondrial DNA. This finding is in good agreement with the larger concentration of mitochondrial DNA found using the protocol C (Figure 5b). This was also confirmed by amplifying a 144-bp specific fragment of mitochondrial DNA (see Figure S1 and protocol described in section 2.7 for additional details). It is important to note that the absence of amplicon obtained in all cases using DNA extracted from beef meat confirmed the high specificity of the primers used and the fragment selected for amplification. According with the obtained results, the protocol involving mitochondrial isolation and the use of the Speedtools plasmid DNA purification kit was selected for further studies.

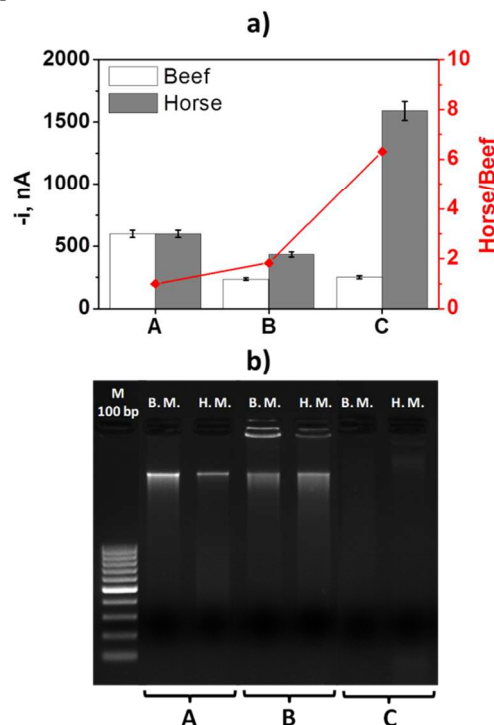


Figure 5. Comparison of the amperometric signals obtained with the developed DNA sensor for beef meat (B.M.) and horse meat (H.M.) and their respective ratios (Horse/Beef ratio) using different mitochondrial DNA extraction protocols: G-Spin™ Total DNA extraction kit (A); mitochondrial isolation and G-Spin™ Total DNA extraction kit (B) and mitochondrial isolation and Speedtools plasmid DNA purification kit (C). Error bars were estimated as triple of the standard deviation ($n=3$) (a). Agarose gel (1.2%) electrophoresis detection of 250 ng of the DNA extracted using the three protocols (b).

The usefulness of the methodology to discriminate beef and horse meats was tested by analyzing different amounts of mitochondrial DNA extracted from horse and beef meat (Figure 6). As it can be seen, it was possible a clear discrimination between both types of meat, with no need for any target DNA amplification protocol, when amounts of mitochondrial DNA equal to or larger than 50 ng were analyzed. The decrease in the amperometric responses observed for DNA amounts larger than 100 ng could be attributed to a hook effect occurring when the concentration of the target DNA exceeds in a large amount the concentration of the capture RNA probe.³⁶

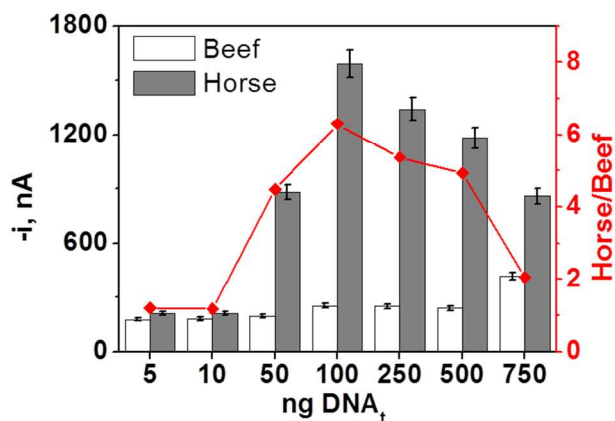


Figure 6. Amperometric responses measured with the electrochemical DNA sensor using different amounts of mitochondrial DNA extracted from beef and horse meat. Error bars were estimated as triple of the standard deviation ($n=3$).

It is worth to mention at this point that a lower hybridization efficiency was observed in the analysis of mitochondrial DNA extracted, as expected considering the secondary and tertiary structures formed by longer targets that hinder efficient hybridization to a surface-bound probe due to steric hindrance.^{37,38} However, we consider these results highly satisfactory since, after the extraction protocol used, most mitochondrial DNA molecules extracted should be intact and their size about 16,660 bp, which is a length 347 times higher than the synthetic one (48-mer).

It should be noted also that the great sensitivity achieved comes from the sum of different factors. Besides the resulting electrochemical response amplification as a consequence of using a relatively long RNA capture probe, the small size of the binding epitope of the anti-DNA-RNA and the ProtA conjugated with multiple HRPs, the nature of the selected target DNA fragment as well as the extraction protocol used are also responsible for the enhanced sensitivity, since mitochondrial DNA is constituted by a smaller number of genes than genomic DNA but with a higher copy number for each mitochondrial gene.^{19,39} In addition, the extraction protocol used allows the concentration of the mitochondrial DNA present in the sample due to the previous isolation of the mitochondrial mass and the subsequent extraction of the DNA.

3.4. Direct determination in mitochondrial lysates

In an attempt to simplify the protocol and shorten the assay time, the determination of the target DNA was carried out directly in the extracts resulting from alkaline lysis of isolated mitochondria without previous DNA extraction. Figure 7 compares the amperometric signals measured with the biosensor in the analysis of extracted mitochondrial DNA using the selected protocol described in the former section with those measured directly in raw mitochondrial lysates prepared as described in section 2.4 and diluted at different ratios with blocker casein solution. As it can be observed, the best discrimination between beef and horse meat was obtained by analyzing directly the crude mitochondrial lysates after a 1:1 dilution (MI(1/2)) with blocker casein solution.

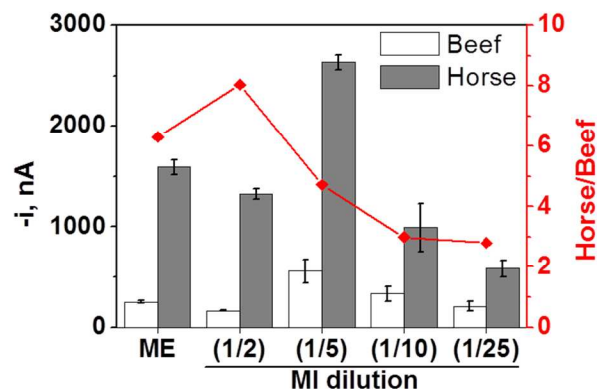


Figure 7. Amperometric responses measured with the developed electrochemical DNA sensor in the analysis of mitochondrial DNA extracted (ME) from 3.0 g of beef and horse meat and directly in the raw mitochondrial lysates (MI) obtained from the same amount of meat diluted different times with blocker casein solution. Error bars were estimated as triple of the standard deviation ($n=3$).

The selectivity of the developed methodology was checked by comparing the amperometric responses measured for 1:1 diluted raw mitochondrial lysates obtained from 3.0 g of beef, turkey, pork, chicken and horse meats. Figure 8 shows that only amperometric responses significantly different to that obtained in the absence of target DNA were apparent for the mitochondrial lysate prepared from horse meat which confirmed the specificity of both the RNA capture probe and the fragment selected for the unequivocal detection of this species meat.

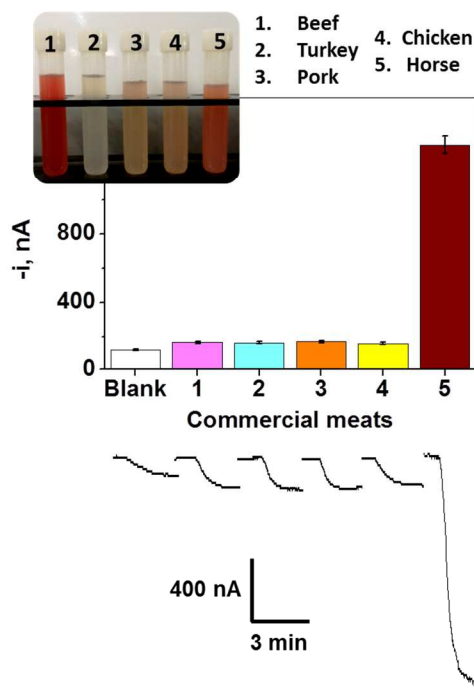


Figure 8. Amperometric responses measured with the developed DNA sensor in the absence of target DNA (blank) and for 1:1 diluted raw mitochondrial lysates obtained from 3.0 g of beef, turkey, pork, chicken and horse meats. Error bars were estimated as triple of the standard deviation ($n=3$). Picture of the corresponding extracts resulting after centrifugation (1,000 g, 5 min, 4 °C) of the sample crushed in MLB/SDS solution.

The real detection limit of horse meat in the presence of beef meat achieved with the developed biosensor, which is the most relevant parameter to ensure real applicability to detect fraudulent adulterations, was established by preparing different mixtures of beef and horse meats (as described in section 2.3). The determination in the 1:1 diluted raw mitochondrial lysates obtained from 3.0 g of each prepared mixture provided the results shown in Figure 9. As it can be seen, it is possible a clear discrimination between 100% beef meat and samples spiked with different percentages of horse meat. Importantly, the detection of the presence of only 0.5% (w/w) of horse meat in beef meat samples, which is the level required by legislation,⁴⁰ is possible. In fact, statistically significant differences (at a confidence level of 95%) were found between the amperometric signals provided by the DNA sensor for 100% beef and 99.5/0.5% beef/horse meat samples ($n = 10$) (see left up inset in Figure 9). Interestingly, a linear dependence ($r = 0.999$) between the measured cathodic current and the percentage of horse meat used to spike beef meat was found over the 1.0–10.0% range (see left down inset in Figure 9), thus showing the usefulness of the methodology not only for adulteration detection but also for quantification of this adulteration. It is worth to remark that the method described in this work is the first approach reported so far for horse meat detection directly from raw mitochondrial cell lysates without DNA extraction or PCR amplification in an assay time as short as 75 min.

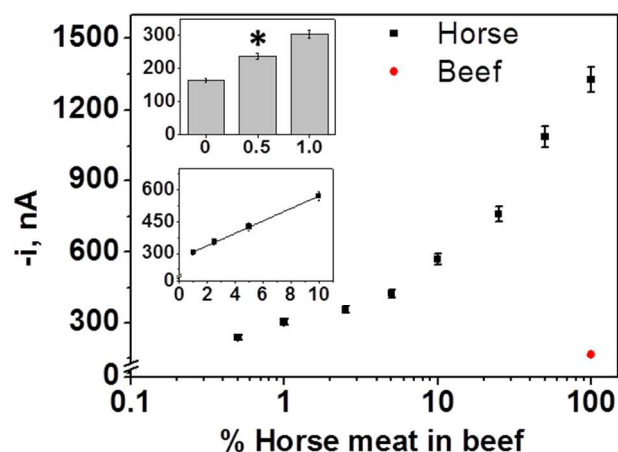


Figure 9. Dependence of the amperometric signals obtained with the electrochemical sensor for 1:1 diluted raw mitochondrial lysates obtained from 3.0 g of mixtures prepared with beef and different percentages of horse meats. Insets: amperometric signals provided by the DNA sensor for mitochondrial lysates obtained from 100% beef, 99.5/0.5% and 99/1% beef/horse meat samples and linear dependence between the measured cathodic current and the percentage of horse meat used to spike beef meat in the 1.0–10.0% range. Error bars were estimated as triple of the standard deviation ($n=3$).

4. CONCLUSIONS

This work reports the first disposable electrochemical sensor combining the use of a selective capture RNA probe with magnetic microbeads technology for the unequivocal identification of horse meat through the detection of a specific fragment of mitochondrial DNA D-loop region. The achievement of a sensitive and selective strategy without nucleic acid amplification relied, apart from a careful optimization of all the

experimental variables involved, on the use of a relatively long RNA capture probe, a specific antibody for RNA/DNA heteroduplexes with a small size binding epitope as detector bioreceptor and ProtA conjugated with an HRP homopolymer for amplification purposes. The analytical performance of the developed method allowed a LOD of 0.12 pM (3.0 attomoles) for the synthetic target DNA. This value is three orders of magnitude lower than that achieved using a conventional sandwich hybridization assay. The feasibility of the method to perform the detection of the target DNA both in extracted intact mitochondrial DNA (~16,660 bp in length) and directly in the raw lysate resulting from alkaline lysis of the mitochondrial mass isolated from meat (without DNA extraction) has been demonstrated. Indeed, results presented confirmed the feasibility to statistically discriminate between 100% beef meat and beef meat samples spiked with only 0.5% horse meat directly in these mitochondrial lysates, thus complying with the current legislation for adulteration with horse meat. It is worth to mention also, that apart from the great advantages of sensitivity, selectivity, simplicity and assay time, the developed methodology paves the way toward the detection of other meats of animal origin, provided a deep study of their specific genes is performed leading to the design of appropriate probes. This smart biosensing platform, with great portability and versatility to be transferred to the determination of other mammalian DNAs or to be multiplexed, can be envisaged as a very promising tool for the identification of species and meat traceability, a hotspot for food research worldwide, to be positioned even in locations where more complex laboratory equipment is not available, such as points of vulnerability along food supply networks. Moreover, this biosensor offers a very attractive on site alternative to PCR-based methodologies for detecting shorter size DNA sequences in degraded samples to address a range of biological problems such as food analysis, biodiagnostics, environmental monitoring, and genetic screening.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information including Tables S1 and S2 and Figure S1 is available free of charge on the ACS Publications website.

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VRM, MLG, RMTR, EP and EV performed research. AJR, RL, FJG, SC and JMP designed the experiments. FJG, SC and JMP wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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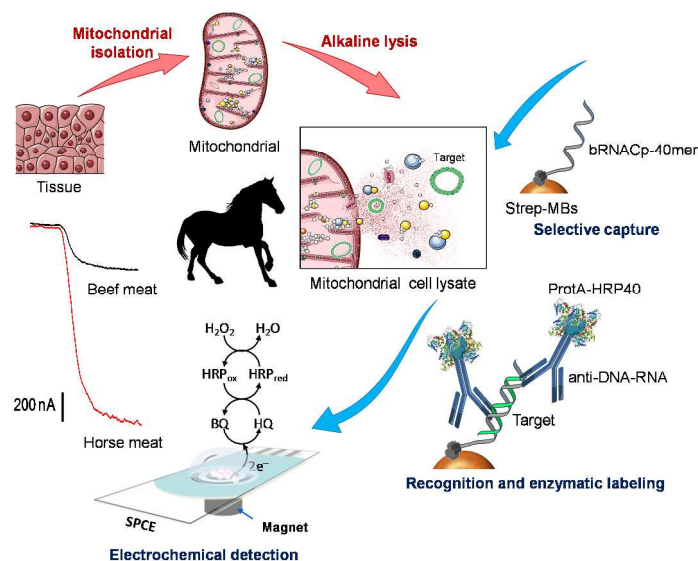
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Disposable amperometric PCR-free biosensor for rapid detection of adulteration with horsemeat in raw lysates targeting mitochondrial DNA

Victor Ruiz-Valdepeñas Montiel, María L. Gutiérrez, Rebeca M. Torrente-Rodríguez, Eloy Povedano, Eva Vargas, Á. Julio Reviejo, Rosario Linacero, Francisco J. Gallego*, Susana Campuzano*, José M. Pingarrón*



Disposable PCR-free electrochemical sensor combining the use of a selective capture RNA probe with magnetic microbeads technology for the unequivocal identification of horse meat through the detection of a specific fragment of mitochondrial DNA D-loop region directly in raw mitochondrial lysates