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Direct PCR-free electrochemical biosensing of plant-food derived nucleic acids in genomic DNA extracts. Application to the determination of the key allergen Sola l 7 in tomato seeds

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ABSTRACT

A novel and disposable electrochemical biosensor for PCR-free and selective detection of Sola l 7, a non-specific lipid transfer protein (nsLTP) found in tomato seeds associated to severe symptoms of tomato-allergic patients, is reported in this work. The methodology involves the formation of DNA/RNA heterohybrids by sandwich hybridization of a specific fragment of the *Sola l 7* allergen coding sequence with appropriate RNA probes designed and described for the first time in this work. Labeling was carried out with commercial antibodies specific to the heteroduplexes and secondary antibodies conjugated with HRP onto the surface of magnetic beads. Amperometric transduction was performed upon magnetic capture of the resulting magnetic bioconjugates on screen-printed electrodes using the system H_2O_2/HQ. A comparison of the sandwich hybridization format with a direct approach as well as between different labeling strategies was performed. The LOD value achieved was 0.2 pM (5 amol in 25 μL). The biosensor was successfully applied to the selective analysis of the targeted *Sola l 7* specific region directly in just 100 ng of non-fragmented denatured genomic DNA extracted from tomato seeds.

1. Introduction

Food allergy is gradually growing worldwide and is one of the major current health concerns (Eissa et al., 2013), (Martín-Pedraza et al., 2016). The presence of hidden allergens in food, by unintentional cross-contamination or non-accurate labelling, is of great importance for both allergic consumers and producers (Zurzolo et al., 2012). Therefore, highly specific and sensitive analytical methods able to detect traces of these allergens in a rapid, robust, reliable, end-user friendly and cost-effective way are highly demanded for ensuring the compliance of food labeling and improving consumer protection (Ruiz-Valdepeñas Montiel et al., 2016).

Vegetal origin allergens are especially relevant because they affect approximately 2–4% of the European adult population (Asero, 2013). Among them, tomato (*Solanum lycopersicum*), one of the most consumed vegetables worldwide both as fresh and processed food (Martín-Pedraza et al., 2016), has been claimed as a prevalent plant-derived food sensitizer (Larramendi et al., 2008), (Bassler et al., 2009) with an allergenic frequency ranging between 1.5 and 20% in different populations of patients with specific IgE (Ballmer-Weber and Hoffmann-Sommergruber, 2011), (Geroldinger-Simic et al., 2011). Particularly, 6.5% of this allergic population is sensitized to tomato fruit in the Spanish Mediterranean region although 80% of these patients still tolerated its consumption. Recent studies have demonstrated that the non-specific lipid transfer protein (nsLTP) Sola l 7 is the most allergenic protein in tomato seeds. This allergenic protein, very stable and found in numerous occasions masked in some commercial foods and sauces, causes severe and unexpected reactions, including anaphylaxis (Martín-Pedraza et al., 2016). Therefore, its presence in food is a major concern for the food industry and food-allergic consumers.

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Currently, the main methods to detect traces of food allergens focus on testing allergic proteins by SDS-PAGE, immunoblotting, ELISA, dipstick assays, or detecting specific fragments of genes encoding the target allergens usually by conventional, real-time, digital PCR, microarray or PCR-ELISA. However, commercial production processes involve heat treatment and/or high-pressure industrial practices which often destroy partially or totally the tertiary structure of food proteins despite the high stability of many of them, and hence cannot be detected using conventional techniques (Arlorio et al., 2007), (Sun et al., 2012), (Iniesto et al., 2013). Therefore, methods based on detecting specific DNA fragments are advantageous because DNA remains intact longer under heat and pressure processing, shows constant concentrations and sequence conserved within all species and allows better extraction yields (even from processed and heat-treated samples) (Santiago-Felipe et al., 2014). However, conventional PCR methods may fail when longer DNA targets are broken down to small fragments during extensive food processing (Ali et al., 2012) and require the use of external standards, these hindering the application of such methods to processed foods or complex food matrices (Holzhauser et al., 2014). In this context, PCR amplification strategies coupled to electrochemical nucleic acid biosensing methodologies have been proposed to determine specific fragments of characteristic allergen coding sequences (Bettazzi et al., 2008), (Ruiz-Valdepeñas Montiel et al., 2017a). These strategies offer a convenient and flexible alternative tool to conventional PCR-based methodologies and combine the high selectivity and sensitivity of nucleic acid amplification strategies with the advantages provided by electrochemical sensors in food analysis (Mishra et al., 2018) such as user friendliness, multiplexing capabilities, quantitative results and suitability to fabricate compact and low cost devices. Moreover, to develop strategies of easy implementation in portable devices to perform routine determinations at decentralized settings, isothermal nucleic acid amplification strategies have been used together with electrochemical sensors (Barreda Garcia et al., 2015), (Li et al., 2015), (Barreda Garcia et al., 2016), (Torrente-Rodríguez et al., 2016), (Barreda Garcia et al., 2017), (Jirakova et al., 2019). Despite the attractive characteristics provided by these strategies, the design of affordable, quick and simple nucleic acid electrochemical biosensors enable achieving the required sensitivity without involving the use of nanomaterials and/or target nucleic acid amplification strategies is still a practical challenge (Ruiz-Valdepeñas Montiel et al., 2018).

Recent results reported by Pingarrón's group have shown the possibility of achieving significant and tunable amplification by exploring novel assay configurations and labeling strategies leading to larger biomolecule or multienzyme loadings on appropriate detector bioreceptors (oligonucleotides or specific antibodies) (Ruiz-Valdepeñas Montiel et al., 2018). Indeed, an attractive electroanalytical platform involving direct hybridization assay onto magnetic microbeads (MBs) and amperometric transduction at screen-printed carbon electrodes (SPCEs) using the system HRP/H_2O_2/HQ, was constructed to detect unequivocally the presence of horsemeat without nucleic acid amplification by targeting a specific region of its D-loop mitochondrial genome directly in raw mitochondrial lysates. The method was based on the efficient capture of the target mitochondrial DNA on streptavidin-functionalized magnetic microcarriers previously modified with a specific biotinylated RNA probe. The resulting heteroduplexes immobilized on the MBs were labeled with a specific DNA-RNA antibody ($Ab_{RNA/DNA}$) previously incubated with Protein A conjugated with a homopolymer containing 40 units of HRP (Ruiz-Valdepeñas Montiel et al., 2017b).

Considering these recent findings, this work reports the first electrochemical nucleic acid biosensor for the sensitive and selective determination of the Sola l 7 allergen by targeting a 60-mer specific fragment of its coding sequence in tomato seeds. The method implies a specific DNA/RNA sandwich hybridization coupled to the use of antibodies specific to the formed heterohybrids further conjugated with HRP-labeled secondary antibodies (anti-IgG-HRP). These affinity reactions were implemented on the surface of MBs while the amperometric transduction was carried out at SPCEs using the system H_2O_2/HQ. The optimized biosensing platform exhibited a great performance for the analysis of non-fragmented genomic DNA (gDNA) extracted from tomato fruits. Importantly, this target DNA has smaller copies and is much longer than that determined in (Ruiz-Valdepeñas Montiel et al., 2017b).

2. Materials and methods

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) used as electrochemical transducers in conjunction with a specific cable connector (DRP-CAC, DropSens), consisted of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode. A homemade Teflon casing with a neodymium magnet (AIMAN GZ) embedded and placed just below the working electrode, once the SPCE was set on the casing, was used to magnetically capture in a reproducible way the modified-MBs on the surface of the SPCE. All measurements were made at room temperature.

An incubator shaker Optic Ivymen® System (Comecta S.A, Sharlab), a biological safety cabinet Telstar Biostar, a Raypa steam sterilizer, a thermocycler (SensoQuest LabCycler, Progen Scientific Ltd.), a Bunsen AGT-9 Vortex for homogenization of the solutions, a magnetic particle concentrator DynaMag™-2 (123.21D, Invitrogen Dynal AS), a stove (CH-100, Boeco), a centrifuge MiniSpin® Eppendorf, a Powerpac™ HV high-voltage power supply (BIO-RAD) and a spectrophotometer NanoDrop ND-1000® were also employed.

2.2. Reagents, solutions and samples

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

Sodium chloride, potassium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, and Tris–hydroxymethyl aminomethane–HCl (Tris–HCl) were purchased from Scharlab. Protein A-HRP conjugate (ProtA-HRP, stock concentration of 1 mg mL^{-1} after solid reconstitution), hydroquinone (HQ) and hydrogen peroxide (30%, w/v) were purchased from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) from Merck (Germany) was also used. RNA-DNA hybrid antibody (clone: D5H6) ($Ab_{RNA/DNA}$) from Covalab and HRP-labeled antimouse IgG Fc (antiIgG-HRP) from Abcam were also used.

A blocker casein solution (a ready to use, PBS solution of 1% w/v purified casein) was purchased from Thermo Scientific. All the oligonucleotides used (probes, synthetic target and mismatched and non-complementary sequences), whose sequences are described in Table S1 in the Supporting Information, 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 °C.

All the buffer solutions were prepared in deionized water obtained from a Millipore Milli-Q purification system (18.2 MΩ cm) and sterilized after their preparation: 0.05 mol L^{-1} phosphate buffer, pH 6.0 and Binding and Washing buffer (B&W) consisting of 10 mM Tris–HCl solution containing 1 mM EDTA and 2 M NaCl, pH 7.5.

Agarose (Laboratorios Conda) and a NucleoSpin® Plant II kit (Ref: 740770.50, MACHEREY-NAGEL), including hexadecyltrimethylammonium bromide (CTAB), SDS, RNase A, guanidine hydrochloride, NucleoSpin columns, 2-propanol, and elution buffer Tris/HCl were used for the gDNA extraction.

Tomato and corn samples were purchased in a local supermarket.

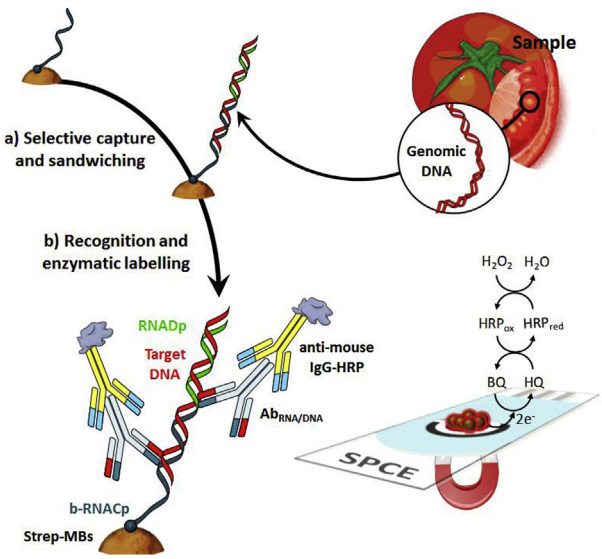

Fig. 1. Schematic display of the MBs-based amperometric biosensing strategy developed at SPCEs for targeting a specific fragment of the *Sola l 7* allergen coding sequence.

Details about all the protocols used (Genomic DNA extraction, Fig. S1, Preparation of the amperometric genosensor and functionalization of MBs and Amperometric measurements) are described in detail in the Supporting information.

3. Results and discussion

A schematic display of the fundamentals involved in the nucleic acid biosensing platform prepared for the determination of a specific fragment of the *Sola l 7* allergen coding sequence is shown in Fig. 1. In brief, magnetic microcarriers modified with a specific b-RNACp were used to selectively capture the synthetic target DNA (or the denatured gDNA extracted) which was further hybridized with a specific RNA detector probe (RNADp). The resulting sandwiched 60-bp-RNA/DNA heterohybrids attached to the MBs were recognized by specific DNA-RNA antibodies (Ab$_{RNA/DNA}$ with a binding epitope of about 6 bp in size (Qavi et al., 2011)) previously labeled with secondary antibodies conjugated with HRP (anti-IgG-HRP). The as prepared MBs with the sandwich RNA/DNA heterohybrids captured and bearing a large number of HRP molecules, were magnetically captured on the SPCE working electrode surface previously placed on a custom-fabricated magnetic holding block. The extent of the affinity reactions was detected by amperometry at −0.20 V (vs the Ag. pseudoreference electrode) in stirred solutions using the system H_2O_2/HQ (both the detection potential and the H_2O_2/HQ concentrations were previously optimized for the HQ/HRP/H_2O_2 system (Eguílaz et al., 2010), (Gamella et al., 2012)). The measured cathodic current is due to the HRP reduction of H_2O_2 mediated by HQ (see scheme reaction in Fig. 1) (Volpe et al., 1998), (Camacho et al., 2007) where the oxidized form of the mediator, benzoquinone (BQ), was reduced at the applied detection potential on the electrode surface providing a cathodic current proportional to the concentration of the target DNA concentration.

3.1. Optimization of experimental variables

Most of the experimental variables involved in the biosensor functioning were optimized taking as the selection criterion the largest ratio between the amperometric signals measured at −0.20 V (*vs.* the Ag pseudoreference electrode) for 0 (blank, B) and 50 pM of synthetic target DNA (signal, S) (signal-to-blank, S/B, ratio) (Fig. 2).

Results obtained using direct hybridization of the 60 nt-selected

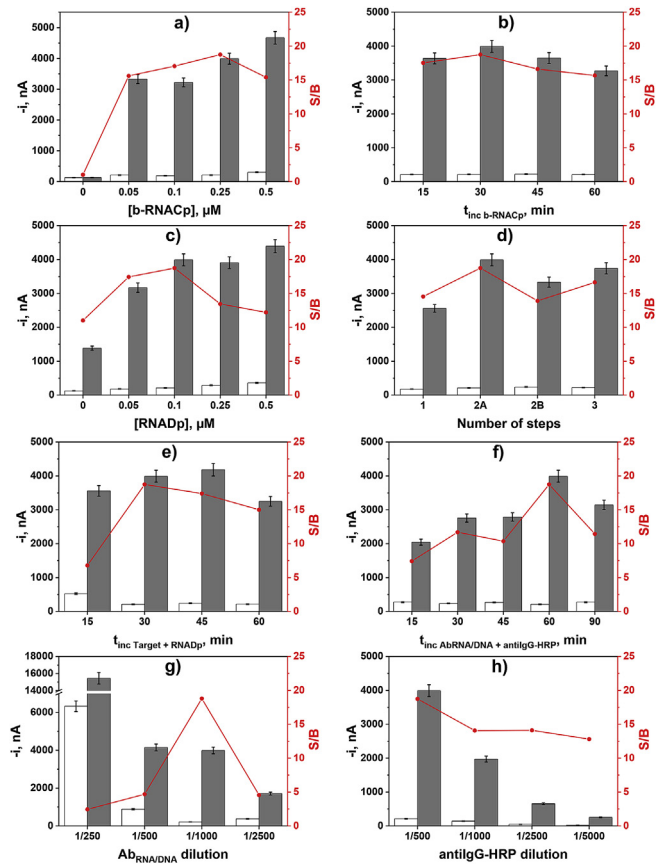

Fig. 2. Optimization of the experimental variables involved in the preparation of *Sola l 7* amperometric biosensors. Dependence of the amperometric responses measured for 0 (blank, B, white bars) and 50 pM of synthetic target DNA (signal, S, grey bars) specific to the *Sola l 7* allergen coding sequence, and the corresponding S/B ratio values (red circles) with the b-RNACp concentration (a), incubation time of the Strep-MBs on the b-RNACp solution (b), RNADp concentration (c), number of steps involved in the protocol (d), incubation times in the mixture solutions containing the target DNA and RNADp (e), and Ab$_{RNA/DNA}$ and anti-IgG-HRP (f), concentration of Ab$_{RNA/DNA}$ (g) and antiIgG-HRP (h). Amperometric detection at −0.20 V (*vs.* Ag pseudoreference electrode) at SPCEs. Error bars were estimated as triple of the standard deviation (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

target with just a capture probe of 30 nt were compared with those provided by a sandwich-type hybridization configuration involving, in addition to the same capture probe, a detection probe of 30 nt. The sandwich-type hybridization format provided a 5.6-times higher sensitivity (calibration curve slope), compared to the direct hybridization strategy (in the absence of detector probe). These experimental results (displayed in Fig. S2 in the Supporting Information) are in agreement with that expected considering that the binding epitope of the Ab$_{RNA/DNA}$ is about 6-bp in size (Qavi et al., 2011). In this particular case, since there were 30 additional nucleotides, ideally there should be 5 additional Ab$_{RNA/DNA}$ molecules attached to each single 60 bp-heterohybrid which is consistent with the achieved amplification factor. This finding opens the possibility of tailoring the sensitivity of the assay by varying the heterohybrid length.

In addition, the analytical performance exhibited by biosensors based on the sandwich hybridization using the secondary antibody (anti-IgG-HRP) or a bacterial immunoglobulin-binding (Protein A) conjugated with HRP (ProtA-HRP dil. 1/1000 (Ruiz-Valdepeñas Montiel et al., 2018)) to label the Ab$_{RNA/DNA}$ was also compared. Both methodologies provided attractive analytical characteristics using simple protocols with no need for the use of nanomaterials or strategies

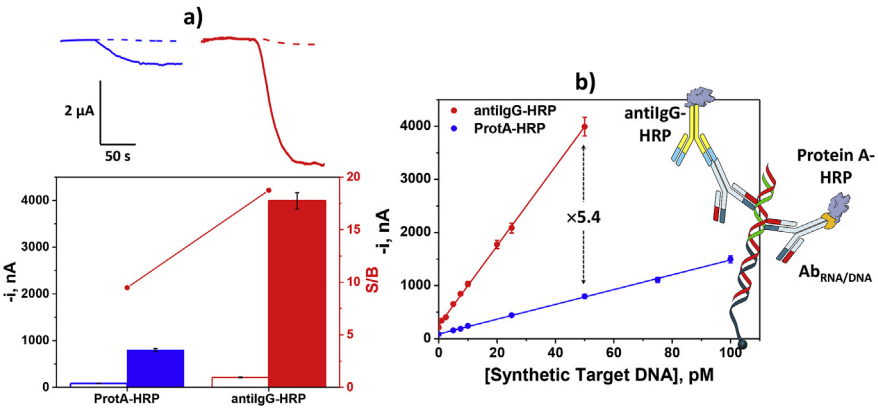

Fig. 3. Comparison of the amperometric responses and amperograms recorded for 0 (dashed lines and empty bars) and 50 pM (solid lines and full bars) target DNA with biosensors prepared by labeling the $Ab_{RNA/DNA}$ with anti-IgG-HRP (in red) or ProtA-HRP (in blue) (a). Calibration plots constructed for the amperometric determination of the *Sola l 7* allergen coding sequence using both labeling strategies (b). Amperometric detection performed at −0.20 V (*vs*. Ag pseudoreference electrode) with SPCEs. Error bars are 3 times the standard deviation of three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to amplify the target DNA. Nevertheless, a 5.4-times higher sensitivity was achieved using the secondary antibody compared to the Protein A (see Fig. 3). These results are in agreement with previous reports (Ruiz-Valdepeñas Montiel et al., 2018) and are attributed to a better affinity of the antiIgG-HRP for the $Ab_{RNA/DNA}$ than the ProtA-HRP.

Accordingly, a sandwich assay format and an $Ab_{RNA/DNA}$ labeling with antiIgG-HRP was selected for further work.

Regarding the other experimental variables involved, Fig. 2a) and c) show that the S/B ratios increased with the concentration of the capture (b-RNACp) and detection (RNADp) probes up to 0.25 and 0.1 μM values, respectively, and decreased with larger concentrations most likely due to the restricted hybridization efficiency when large amounts of probes are used (Liu et al., 2014), (Zouari et al., 2018). Fig. 2b) shows that 30 min were enough to allow the efficient b-RNACp immobilization on the Strep-MBs to ensure an effective hybridization.

A relevant optimization study in order to develop simpler and shorter assay protocols is the one on the number of steps involved in the biosensor preparation. Accordingly, the amperometric responses measured with biosensors prepared using the following protocols (all of them with incubation steps of 30 min and starting from the b-RNACp-MBs) were compared:

- Protocol 1: one incubation step with a mixture solution containing the target DNA, RNADp, $Ab_{RNA/DNA}$ and antiIgG-HRP.
- Protocol 2A: two sequential incubation steps with solutions containing (1) the target DNA and RNADp and (2) $Ab_{RNA/DNA}$ and antiIgG-HRP.
- Protocol 2B: 2 sequential incubation steps with (1) the target DNA and with (2) a mixture solution containing RNADp, $Ab_{RNA/DNA}$ and anti-IgG-HRP.
- Protocol 3: three sequential incubation steps with the (1) target DNA, (2) RNADp and with (3) a mixture solution containing $Ab_{RNA/DNA}$ and anti-IgG-HRP.

Fig. 2d) shows as the protocol 2A provided better S/B ratio and, therefore, was selected for the construction of the biosensors fabrication. These results can be attributed to the improved efficiency of the hybridization reaction of the target DNA with RNADp and the labeling of $Ab_{RNA/DNA}$ with anti-IgG-HRP in homogeneous solutions.

The time required for the two incubation steps (Fig. 2e) and f)) as well as the $Ab_{RNA/DNA}$ and antiIgG-HRP concentrations were also optimized (Fig. 2g) and h)). Table 1 summarizes all the selected experimental variables as well as the ranges in which these variables were optimized.

3.2. Analytical characteristics

The analytical performance of the developed biosensor was tested using the 60 nt-synthetic target DNA. Under the selected experimental conditions, the obtained calibration plot is displayed in Fig. 3 (calibration in red). A linear relationship (r = 0.9988) between the measured cathodic current and the target DNA concentration was found in the 0.8–50 pM range, with slope and intercept values of (74,794 ± 1677) nA nM^{-1} and (254 ± 34) nA, respectively. The detection (LOD) and quantification (LQ) limits were calculated according to the $3 \times s_b/m$ and $10 \times s_b/m$ criteria, respectively, where m is the slope value of the linear calibration plot and s_b was estimated as the standard deviation of ten amperometric measurements made in the absence of target DNA. LOD and LQ values were 0.2 and 0.8 pM (5 and 20 amol), respectively. The achieved LOD is 5.5 times lower than that obtained with the biosensor constructed by labeling the $Ab_{RNA/DNA}$ with ProtA-HRP (1.1 pM). Moreover the LOD achieved is similar to that reported previously with an electrochemical biosensor involving direct hybridization of a target DNA fragment with a specific 40-mer-b-RNACp immobilized onto Strep-MBs, recognition of the captured DNA/RNA heteroduplexes with the same $Ab_{RNA/DNA}$ but labeling it with a ProtA conjugated with an homopolymer bearing 40 HRP molecules (ProtA-poly-HRP40) of 0.12 pM for a 48 nt synthetic target DNA (a specific fragment of the horse mitochondrial DNA D-loop region) (Ruiz-Valdepeñas Montiel et al., 2017b).

The relative standard deviation (RSD) value obtained from the amperometric measurements provided by 8 different biosensors prepared in the same manner for 25 pM of the synthetic target DNA was 4.4%, thus indicating a good reproducibility of the fabrication and amperometric transduction protocols involved.

The storage stability of the b-RNACp-MBs conjugates was tested after storing them at 4 °C in Eppendorf tubes containing 50 μL of filtered B&W buffer. Each working day, amperometric responses were measured for 0 and 10 pM synthetic target DNA. No significant differences in the measured S/B ratio were apparent for a period of 69 days (no longer times were assayed), indicating an excellent stability of the b-RNACp-MBs conjugates.

Table 1
Optimization of the different experimental variables involved in the preparation of biosensors for the amperometric determination of *Sola l 7* coding sequence.

Variable	Tested range	Selected value
Strep–MBs suspension, μL	—	**5**
[b-RNACp], μM	0–0.5	**0.25**
$t_{inc\ b\text{-}RNACp}$, min	0–60	**30**
Number of steps	1–3	**2A**
[RNADp], μM	0–0.5	**0.1**
$t_{inc\ target\ DNA+RNADp}$, min	0–60	**30**
$t_{inc\ AbRNA/DNA\ +\ antiIgG\text{-}HRP}$, min	15–90	**60**
$Ab_{RNA/DNA}$ dilution	1/2500–1/250	**1/1000**
antiIgG-HRP dilution	1/5000–1/500	**1/500**

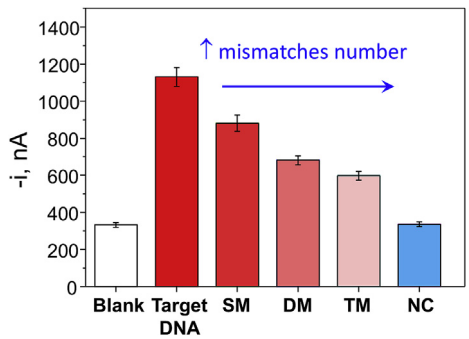

Fig. 4. Amperometric responses obtained with the biosensors constructed to determine the *Sola l 7* allergen coding sequence in the absence and in the presence of 10 pM synthetic target DNA, SM, DM, TM and NC sequences. Amperometric detection made at −0.20 V (*vs.* Ag pseudoreference electrode) with SPCEs. Error bars were estimated as triple of the standard deviation (n = 3).

3.3. Selectivity

The selectivity of this strategy was checked by comparing the amperometric responses obtained in the absence and in the presence of 10 pM of the following synthetic sequences: target DNA, single mismatched (SM), double mismatched (DM), triple mismatched (TM) and non-complementary (NC). Fig. 4 shows as total discrimination was achieved against the NC sequence, while an acceptable selectivity was found against the mismatched sequences. The selectivity improved with the number of mismatched bases, showing a decrease in the amperometric responses obtained for the target DNA of 23, 40 and 50% for the SM, DM and TM sequences, respectively. These results suggest the feasibility of the developed biosensors to discriminate even between the numbers of mismatches in the analyzed sequence.

3.4. Analysis of real samples

The electrochemical biosensor constructed for the determination of the *Sola l 7* allergen coding sequence was applied to the analysis of 100 ng of non-fragmented plant-derived genomic DNA extracts. It is worth to mention that a lower hybridization efficiency is expected considering the secondary and tertiary structures formed by such long targets which hinder an efficient hybridization to a surface-bound probe due to steric hindrance (Liu et al., 2007), (Cederquist and Keating, 2010).

Fig. 5 compares the results obtained with the biosensor for the measurements of the gDNA extracted from tomato (seeds and peel) and corn using two different protocols (CTAB or SDS-based methods). It is important to note that corn was selected for this study because it has an allergenic nsLTP, Zea m 14, which shows one of the highest percentages of identity and similarity in its primary sequence (amino acidic) with Sola l 7. The 46% sequence identity and 64% similarity are higher than for the nsLTPs present in the skin of peach or apple. Both nsLTPs (Zea m 14 and Sola l 7) come from seeds and if the developed biosensor does not recognize non-targeted *Zea m 14* specific region, therefore it can be concluded that it would not recognize other nsLTPs with lesser sequence similarity. It is important to highlight also that corn and tomato can be consumed together in many processed foods (corn pancakes, purees, ketchup or pizza/pasta sauces) and that it has been reported that Zea m 14 and Sola l 7 possess similar thermal stability and maintain their immunogenic capacity, triggering anaphylactic reactions, after severe processing at high temperatures for long periods of time (Pastorello et al., 2000), (Pastorello et al., 2003), (Guillen et al., 2014). As it is clearly seen, better results were achieved using the CTAB extraction protocol. In addition, only amperometric responses significantly different from the blank were obtained for gDNA extracted

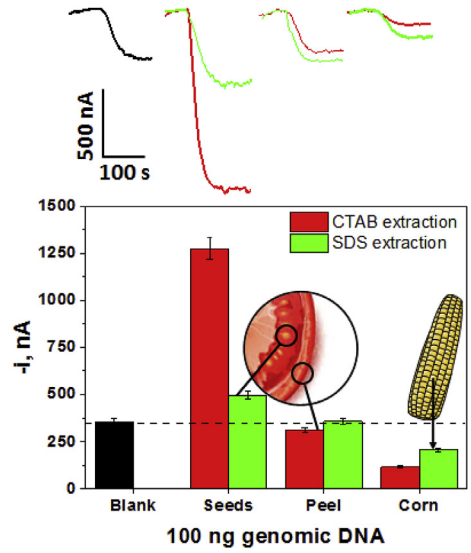

Fig. 5. Amperometric responses and amperograms measured with the biosensor constructed to determine the *Sola l 7* allergen coding sequence in the absence of target DNA (blank) and for 100 ng of denatured gDNA extracted from tomato peel and seeds and corn. Amperometric detection performed at −0.20 V (*vs.* Ag pseudoreference electrode) with SPCEs. Error bars were estimated as triple of the standard deviation (n = 3).

from the tomato seeds which is fully consistent with the specific location of the target nsLTP in this part of the fruit (Martín-Pedraza et al., 2016), thus demonstrating the high selectivity and sensitivity of the biosensor in these challenging samples. These important features can be attributed to the combination in the same methodology of the hybridization processes selectivity, the unique specificity of commercial antibodies for RNA heterohybrids and the use of MBs as solid supports for the affinity reactions to minimize matrix effects in complex samples (Tran et al., 2013), (Campuzano et al., 2017), (Serafín et al., 2017), (Ruiz-Valdepeñas Montiel et al., 2018). These results can be considered as highly relevant taking into account the much bigger length of the intact analyzed gDNA (~792 Mbp) compared with the synthetic target (60-mer). Indeed, this gDNA is actually the longest target DNA detected to date with an electrochemical biosensor. In addition, this is the first electrochemical DNA sensor applied to perform the direct determination in intact genomic DNA extracted from plants (with smaller copies and a length 5×10^4 times longer than the horse mitochondrial DNA detected previously of ~16 kbp (Ruiz-Valdepeñas Montiel et al., 2017a,b)) without previous fragmentation or amplification and after just a denaturing step.

4. Conclusions

This work reports the first disposable electrochemical nucleic acid sensors for the unequivocal detection of tomato seeds through the sensitive and selective PCR-free detection of a specific fragment of the characteristic *Sola l 7* allergen coding sequence. The developed biosensors exhibit a good sensitivity (LOD of 0.2 pM, 5 amol for the synthetic target) and selectivity and the analysis can be made in just 90 min with feasibility to be performed directly in 100 ng of extracted raw gDNA without previous fragmentation and after a denaturing step. The excellent analytical performance of the implemented strategy without target nucleic acid amplification relies on the use of a sandwich hybridization assay leading to the formation of quite long RNA/DNA heteroduplexes and a commercial antibody with a high affinity to bind regions of only 6 bp in the RNA heterohybrids. The constructed biosensing platforms offer the possibility of tailoring the sensitivity by varying the bioassay format, heterohybrid length or labeling strategy. Moreover, these bioplatforms pave the way towards the sensitive

detection of other nucleic acids regardless of their naturally occurring variety (DNA or RNA), origin (animal or plant), copy number (mitochondrial vs genomic) and length (from very long intact DNAs to degraded samples, difficult to be analyzed by conventional PCR-based methodologies), with no need for using nanomaterials or nucleic acid amplification. The simple handling of the biosensors with applicability at different settings and the relatively low cost per detection (~3.5 €) postulate these novel biotools as the basis for the electrochemical biosensing in routine analyses, even in locations where more complex laboratory equipment is not available, and with potential applications not only in food safety control and consumer protection but also in diagnostics, environmental monitoring, and genetic screening.

Declaration of interest statement

This work reports the first disposable electrochemical nucleic acid sensors for the unequivocal detection of tomato seeds through the sensitive and selective PCR-free detection of a specific fragment of the characteristic *Sola l 7* allergen coding sequence. The developed biosensors exhibit a good sensitivity (LOD of 0.2 pM, 5 amol for the synthetic target) and selectivity and the analysis can be made in just 90 min with feasibility to be performed directly in 100 ng of extracted raw gDNA without previous fragmentation and after a denaturing step. The nice analytical performance of the implemented strategy without target nucleic acid amplification relies on the use of a sandwich hybridization assay leading to the formation of quite long RNA/DNA heteroduplexes and a commercial antibody with a high affinity to bind regions of only 6 bp in the RNA heterohybrids. The constructed biosensing platforms offer the possibility of tailoring the sensitivity by varying the bioassay format, heterohybrid length or labeling strategy. Moreover, these bioplatforms pave the way towards the sensitive detection of other nucleic acids regardless of their naturally occurring variety (DNA or RNA), origin (animal or plant), copy number (mitochondrial vs genomic) and length (from very long intact DNAs to degraded samples, difficult to be analyzed by conventional PCR-based methodologies), with no need for using nanomaterials or nucleic acid amplification. The simple handling of the biosensors with applicability at different settings and the relatively low cost per detection postulate these novel biotools as the basis for the electrochemical biosensing in routine analyses, even in locations where more complex laboratory equipment is not available, and with potential applications not only in food safety control and consumer protection but also in diagnostics, environmental monitoring, and genetic screening.

CRediT authorship contribution statement

Magda A. Pereira-Barros: Investigation. **M. Fátima Barroso:** Supervision, Writing - original draft, Funding acquisition. **Laura Martín-Pedraza:** Investigation, Writing - original draft. **Eva Vargas:** Investigation. **Sara Benedé:** Investigation, Writing - original draft. **Mayte Villalba:** Supervision, Funding acquisition. **João M. Rocha:** Supervision, Writing - original draft, Funding acquisition. **Susana Campuzano:** Supervision, Writing - original draft, Funding acquisition. **José M. Pingarrón:** Supervision, Writing - original draft, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.05.011.

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SUPPORTING INFORMATION

Direct PCR-free electrochemical biosensing of plant-food derived nucleic acids in genomic DNA extracts. Application to the determination of the key allergen Sola l 7 in tomato seeds

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Table S1. Oligonucleotides used in the preparation of the electrochemical DNA sensor for a specific region of *Sola l 7* allergen coding sequence.

Role	Short name	Sequence 5´ → 3´
Biotinylated capture RNA probe*	b-RNACp	Biotin-CUACAGCAUGCUGCACCGGGCUCGCCACCC
Detector RNA probe*	RNADp	UGUGUCAGGAACGGGACACAGGGUGCCAAG
Target DNA**	Target	CTTGGCACCCTGTGTCCCGTTCCTGACACA GGGTGGCGAGCCCGGTGCAGCATGCTGTAG
Target DNA single mismatch	SM	CTTGGCACCCTGTGTCCCGTTCCTGACACA GGGTGGCGAGCCCGGCGCAGCATGCTGTAG
Target DNA double mismatch	DM	CTTGGCACCCTGTGGCCCGTTCCTGACACA GGGTGGCGAGCCCGGCGCAGCATGCTGTAG
Target DNA triple mismatch	TM	CTTGGCACCCTGTGGCCCGTTCCTGACACA GGGTGGTGAGCCCGGTGCAGCATACTGTAG
Non-complementary sequence***	NC	GAATGGGAGCGACAGGAGAGACAAGAGA GGGAGAGTGAGCAAGAGCGGGA

**To get the optimal hybridization yield in the sandwich hybridization assays, both b-RNACp and RNADp (of 30 nt each) were designed to hybridize contiguously with the selected 60 nts target, with no gap of non-hybridized nts between their hybridization sites (Ruiz-Valdepeñas Montiel et al., 2017), (Liao et al., 2007JMD).*

***This sequence is a copy of a specific region (30-90 in 5´ ì 3´) of the 482 bp Sola l 7 without the region that encodes the protein signal peptide. The 60 bp target selected for Sola l 7 determination is the fragment that encodes the region with the highest degree of similarity and identity with other highly allergenic nsLTP distributed in numerous species of the plant kingdom.*

**** Specific fragment of the hazelnut Cor a 9 allergen coding sequence (Ruiz-Valdepeñas Montiel et al., 2017).*

Genomic DNA (gDNA) extraction

CTAB (hexadeciletrimethylammonium bromide) or SDS (sodium dodecyl sulphate)-based extraction methods followed by the NucleoSpin® Plant II (MACHEREY-NAGEL) kit, were used to extract gDNA from the samples to be analyzed (Knebelsberger and Stöger, 2012), (Telfer et al., 2013), (Drábková, 2014). The plant material (tomato seeds and peel and corn) was lyophilized and subsequently pulverized with liquid nitrogen maintaining minimum sterility conditions. Both methods have a first step of cell lysis in which the plant material was incubated with each detergent (CTAB or SDS) and RNase A for 10 min at 65 °C. After a filtration process of the cell lysate and centrifugation 2 min at 11000×g, the supernatant is collected and prepared to binding into columns provided with guanidine hydrochloride 24–36 % and ethanol 35–55 %. Subsequently, NucleoSpin columns are incorporated for DNA binding and, finally, after washing and dry the silica membrane with guanidine hydrochloride 36–50 % and 2-propanol 20–35 %, the total gDNA was eluted in 100 µL of elution buffer (5 mM Tris/HCl, pH 8.5).

The quality and concentration of extracted gDNA were evaluated by measuring the absorbance at the appropriate wavelength (A260/A280) with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and bands visualization through agarose gel (0.8%, see Fig. S1). Just before the analysis with the biosensor, the extracted gDNA was denatured by heating at 97 °C for 5 min in the thermocycler and transferred immediately to ice for 10 min.

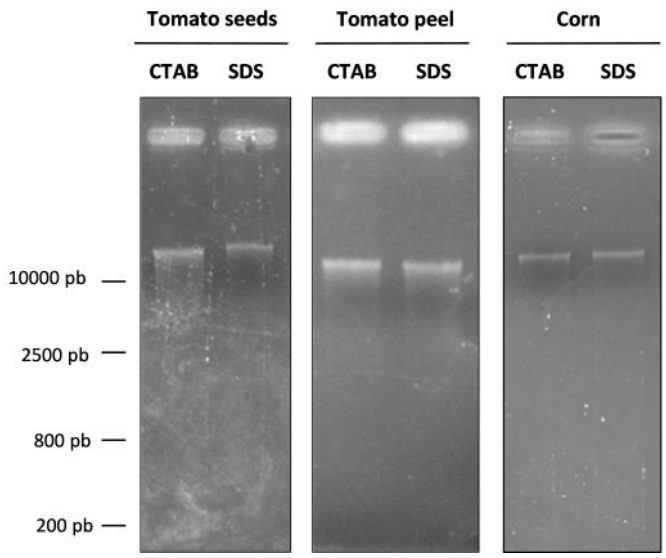

Fig. S1. Agarose gel (0.8%) electrophoresis detection of the gDNA extracted.

Preparation of the amperometric genosensor and functionalization of MBs

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 30 min in an incubation shaker (950 rpm at 37 ºC) in 25 µL of a 0.25 µM b-RNACp solution prepared in B&W. The resulting b-RNACp-Strep-MBs were washed two times with 50 µL of the blocker casein solution. Thereafter, the b-RNACp-Strep-MBs were incubated for 30 min in an incubation shaker (950 rpm at 37 ºC) in 25 µL of a solution prepared in blocker casein solution containing the target DNA (synthetic DNA or denatured extracted gDNA) supplemented with 0.1 µM of RNADp, prepared in blocker casein solution. After two additional washing steps with blocker casein solution, the enzymatic labeling of the RNADp-Target DNA-b-RNACp-Strep-MBs was carried out in a single step by incubation for 60 min in an incubation shaker (37 °C, 950 rpm) in 25 µL of a mixture solution, prepared in blocker casein solution, containing the $Ab_{RNA/DNA}$ (1 µg mL^{-1}) and anti-mouse IgG-HRP (40 µg mL^{-1}). Before performing the amperometric

detection, the modified-MBs were washed twice with 50 µL of blocker casein solution and re-suspended in 45 µL of 0.05 M sodium phosphate buffer solution (pH 6.0). All the MBs manipulations were made in the laminar flow cabinet to avoid RNase contamination and prevent RNA probes degradation.

Amperometric measurements

The 45 µL of the modified MBs suspension were magnetically captured on the surface of the working electrode by previous placing of the SPCE on the magnetic holding block. The ensemble SPCE/magnet holding block was immersed 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 the electrochemical measurement). Amperometric measurements were performed in stirred solutions by applying a detection potential of −0.20 V *vs.* Ag pseudo-reference electrode. Upon addition of 50 µL of a freshly prepared 0.1 M H_2O_2 solution, the steady state current was recorded (~100 s).

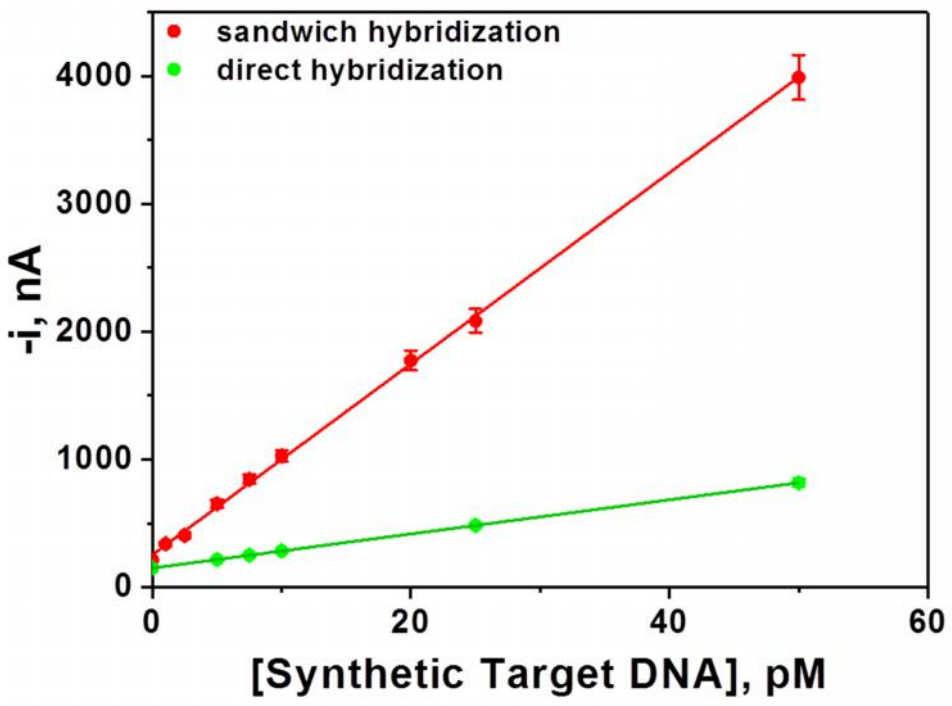

Fig. S2. Calibration plots constructed for the amperometric determination of the *Sola l 7* allergen coding sequence using direct and sandwich hybridization formats. Amperometric detection performed at −0.20 V (vs. Ag pseudoreference electrode) with SPCEs. Error bars are 3 times the standard deviation of three replicates.

Table S2. Comparison of the analytical characteristics obtained for the amperometric determination of the *Sola l 7* allergen coding sequence with biosensors prepared by labeling the $Ab_{RNA/DNA}$ with anti-IgG-HRP or ProtA-HRP.

	Labeling	
Parameter	ProtA-HRP	antiIgG-HRP
r	0.9992	0.9997
Slope, nA nM^{-1}	13,936 ± 149	74,794 ± 711
Intercept, nA	89 ± 7	254 ± 14
LR, pM	3.7 - 100	0.8 - 50
LOD, pM	1.1	0.2

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