Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM) soy

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Dear Editor,
the manuscript was further revised according to the reviewer's comments on the first revision.
We would like to thank the reviewers for their feedback and insightful comments on the paper.
A point-to-point response to the comments raised by the referees is reported as follows*:
*Blue text: Referee's comment; red text: author's answer.

Reviewer #2: I didn't think the authors made convincing and positive responses to the comments of Reviewers. Furthermore, the novelty and application of the proposed method is indeed deficient.
Answer R2.1: We disagree with the Reviewer on this point. As stated by all the other Reviewers, the manuscript has been thoroughly and carefully revised, responding to each question/statement raised by all Referees and improving all sections.
The novelty of our study, which relies on the combination of the peculiar properties of carbon nanotubes in terms of amplification of the electrode processes with the recognition efficiency of PNA probes for the determination of non-amplified genomic DNA extracted from real samples of GM-Soy, was underlined both in the Introduction and in the Conclusions, as required from the Reviewers and the Editor. Similarly, in the revised version the scope of the proposed method were stated more clearly also in the Conclusions.

Reviewer #3: After careful modification, the paper has met the requirements of Journal of Biosensors and Bioelectronics; however the following questions should be explained:

1. The thickness of the bare SPE and modified SPE should be investigated and clarified.
   Answer R3.1: We thank the Reviewer for the suggestion, but we do not believe that further characterization of the electrode surface (for example by AFM), before and after the immobilization of the PNA probes, would provide useful information. The efficiency of the immobilized PNAs in DNA hybridization as well as the evaluation of their optimal loading have been faced and discussed in section 3.1 and in the supporting infos (Figure S12)
2. I wonder why the Authors' response for question" Role of surfactants in buffer solutions" not added to the main manuscript.
   Answer R3.2: We thank the reviewer for this relevant comment. The role of surfactants in the buffer solution was elucidated in section 3 (pages 10-11) of the revised manuscript (R2).
3. References for extinction coefficients of PNAs should be added.
   Answer R3.3: A reference for extinction coefficients of PNAs (Nielsen and Appella, 2014) was already included at page 7 of the revised manuscript (R1), and now another more specific has been added.
4. The benefits of UV measurements for DNA extracted from Soy flours at 260 nm.
   Answer R3.4: As explained in section 3.5 (page 15) of the manuscript, the spectrophotometric analysis of the extracted DNA concentration was aimed to find the

proper dilution ratio in order to obtain current signals within linear range, thus allowing quantitative assays.

5. I asked authors to thoroughly revise their manuscript but some phrases appear nonsense such as page 5, line 16 (Hydroquinone diphosphate (HQDP) was from Metrohm Italiana).
   Answer R3.5: We have to disagree with the Referee. Such ALP-enzyme substrate is a Dropsens patent and can only be purchased by the official Metrohm distributor (https://www.metrohm.com/en/products/electrochemistry/reagents-for-electrochemistry/HQDP). However, the sentence was rewritten for more clarity.

Reviewer #4: Authors did perform the revison according to each comments raised by reviewers.
However the resolution of each figures should be improved.
Then, the revised mansucript can be accepted for a publication.
Answer R4: We thank the reviewer for the suggestion related to the improvement of the resolution of the figures. However, in the "Response to Reviewers" related to the first revision we already pointed out that the original TIFF files of all figures and schemes are provided as high-resolution files, so that the edited manuscript will include images of proper resolution.

Reviewer #6: The authors have addressed all my concerns, and i agree to recommend this journal to accept the manuscript. The present manuscript has been revised carefully, and the novelty in this revision was listed and emphasized by comparing with the report. All my concerns proposed last time has been answered and amended. So I agree to recommend the editor to accept the revision.
Answer R6: We thank the reviewer for his thoughtful and thorough review and believe his input has been invaluable to improve the manuscript.

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DIPARTIMENTO DI SCIENZE
CHIMICHE, DELLA VITA E DELLA
SOSTENIBILITÀ AMBIENTALE

Parma, January 7, 2019

Dear Editor,

please consider the second revision of the manuscript BIOS-D-18-03717R1, entitled "*Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM) soy*", by Simone Fortunati, Andrea Rozzi, Federica Curti, Marco Giannetto, Roberto Corradini and Maria Careri.

The manuscript was further revised according to the reviewer's comments on the first revision.

Further changes compared to R1 manuscript have been marked in green, while previous changes are still marked in red and the deleted text has been marked with double strikethrough.

We would like to thank the reviewers for their feedback and insightful comments. We believe we have addressed all of the major and minor comments that were raised by the reviewers and, in doing so, have crafted a paper that is more rigorous in content and clearer in presentation.

A point-to-point response to the comments raised by the referees on R1 manuscript is attached as a separate file (Response to reviewers R2), highlighted according to the following style:

Blue text: Referee's comment; red text: authors' answer.

We hope that the manuscript could be acceptable for publication in its present form on "Biosensors & Bioelectronics" as full-length article.

Best regards

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***Highlights (for review)**

- First amperometric genosensor based on PNA immobilized on carbon nanotubes
- PNA provided high selectivity while carbon nanotubes exhibited high sensitivity
- Direct determination of genomic DNA from GM Soy, without preamplification
- Validation on soybean flour, matching the EC Regulation (0.9% threshold limit)
- Screening tool valuable for food authenticity assessment.

Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM)~~O~~ soy

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Abstract

A novel amperometric genosensor based on PNA probes covalently bound on the surface of Single Walled Carbon Nanotubes – Screen Printed Electrodes (SWCNT-SPEs) was developed and validated in samples of non-amplified genomic DNA extracted from genetically modified (GM)-Soy. The sandwich assay is based on a first recognition of a 20-mer portion of the target DNA by a complementary PNA Capture Probe (CP) and a second hybridization with a PNA Signalling Probe (SP), with a complementary sequence to a different portion of the target DNA. The SP was labelled with biotin to measure current signal by means of a final incubation of an Alkaline Phosphatase-streptavidin conjugate (ALP-Strp). The electrochemical detection was carried out using hydroquinone diphosphate (HQDP) as enzymatic substrate. The genoassay provided a linear range from 250 pM to ~~1.75~~ 2.5 nM, LOD of ~~71~~ 64 pM and LOQ of ~~256~~ 215 pM. Excellent selectivity towards one base mismatch (1-MM) or scrambled (SCR) sequences was obtained. A simple protocol for extraction and

analysis of non-amplified soybean genomic DNA without sample treatment was developed and validated. Our study provides insight into how the outstanding recognition efficiency of PNAs can be combined with the unique properties of CNTs in terms of signal response enhancement for direct detection of genomic DNA samples at the level of interest without previous amplification.

Keywords

PNA; genosensing; GMO; Soy; Carbon NanoTubes

1. Introduction

During the last decade, much attention has been devoted to the development of genosensing techniques because of high versatility of these systems and wide applicability to many fields, ranging from diagnosis of genetic diseases (D'Agata et al., 2017) to determination of food contaminants (Martín-Fernández et al., 2017; Silva et al., 2018; Neethirajan et al., 2018) and much more. Genosensors are established based on the principle of specific pairing between complementary nucleobases in nucleic acids, either natural, synthetic or mimics, as recognition method. DNA can be found and extracted from most organisms and, given its high stability under processing conditions, it represents an efficient analytical target for food safety and authenticity (Marchelli et al., 2012; Bianchi et al., 2018; Abdalhai et al., 2015; Manzanares-Palenzuela et al., 2015). For this purpose, genosensing techniques can be applied to detect the presence of genetically modified organisms (GMOs) (Marmiroli et al., 2008), taking into account that, according to EU Regulation (1829/2003/EC), all food and feed containing a concentration of GMO higher than 0.9% must be labelled as containing GM products.

Genosensors are designed to provide signal variation according to the mode of signal transduction (i.e. optical, electrochemical or mechanical), upon hybridization of target DNA or RNA with specific oligonucleotide probes. A wide variety of molecules have been synthesized to obtain efficient mimics of nucleic acids, which, besides the ability to hybridize complementary target

sequences, are requested to possess new properties, e.g. ease of modification, resistance to enzymatic processing etc. Among these, Peptide Nucleic Acid (PNA) (Nielsen, 2004, 2010) have proven to be a very promising class of nucleic acids mimics. Their structure is made of a *N*-(2-aminoethyl)glycine repeating units, each linked through a spacer to a nucleobase (Scheme 1). This structure guarantees the correct intra-base distance, which allows for Watson-Crick interactions to occur. Furthermore, due to the absence of negatively charged groups on its backbone, PNA yields hybrids with DNA of higher stability than homologous DNA-DNA hybrids. Another important property is that the PNA:DNA duplex is more sensitive to the presence of mismatches in the complementary DNA strand. Given its synthetic nature, PNA is also easily modifiable to include functional groups that can be used, for example, for covalent immobilization on substrates as Screen-Printed Electrodes (SPEs). Carbon Nanotubes (CNTs) are very promising nanomaterials thanks to their unique properties which can be exploited in electrochemical sensing (Rivas et al., 2017; Shrestha et al., 2016; Da Silva et al., 2016; Giannetto et al., 2017). They can be defined as an allotropic form of carbon with cylindrical shape, with a micrometric length and a width of approximately 2 nm. They can be obtained as concentric cylinders, i.e. multi-walled carbon nanotubes (MWCNTs) or with a single cylindrical unit as in the case of single-walled carbon nanotubes (SWCNTs). When such structures are implemented using screen printing technology for development of disposable CNTs-mediated SPEs, an enhancement of the electrochemical signal is obtained. By incorporating CNTs on all-carbon SPEs it is possible to exploit the native carboxylic moieties present on the edges of the tubes for immobilization of functional molecules and probes.

Numerous approaches to electrochemical detection of ~~In~~ DNA~~-based electrochemical sensors numerous detection approaches~~ have been devised. For example, an electrochemical genosensor was developed for the detection of *salmonella typhi* based on the use of gold nanoparticles-mercaptosilane modified SPEs: a 50 pM limit of detection was achieved with a good ability to discriminate against one-base, two-base and three-base mismatched target sequences (Das et al., 2014). Recently, Manzanares-Palenzuela et al. proposed an electrochemical genosensor based on a

multiplex electrochemical platform for the determination of *Roundup Ready* transgenic Soy using a sandwich format through the immobilization of two different Capture Probes (**CP**) on magnetic beads and subsequent detection with two different signalling probes (Manzanares-Palenzuela et al., 2015); the system was capable to achieve sub-picomolar limits of detection, associated to a response saturation above 250 pM of synthetic DNA. The suitability of the multiplex assay for the quantification of RR in relation to the taxon content was assessed using synthetic GMO mixtures, artificially simulating the GMO content around the labelling threshold value (0.9%). However, it has to be noted that the analysis of genomic DNA extracted from soybean flour was not challenged. ~~could detect a 0,9% content of transgenic material, thus resulting useful for food authenticity assessment.~~

In the present paper we developed and validated a simple and effective amperometric genosensor based on PNA probes covalently bound on the surface of SWCNT-SPEs and electrochemical read-out using Differential Pulse Voltammetry (DPV). At the best of our knowledge, this is the first study dealing with the use of PNA receptors covalently linked to the electrode nanostructured substrate, thus combining the outstanding performance of PNAs in terms of recognition efficiency with the unique properties of CNTs, in terms of amplification of the electrode processes. The effectiveness of this analytical method was tested in the detection of *Roundup Ready* transgenic soybean. Genosensor was successfully validated on genomic DNA extracted from GM-Soybean European Certified Reference Materials (ERM®) and selectivity was assessed comparing the responses obtained using Full Match (FM) sequence DNA with two targets containing different mismatches on the **CP**-complementary portion, i.e. a single mismatch (1-MM) and a totally scrambled (SCR) DNA sequences.

2. Experimental

2.1 Materials

N,*N*-Diisopropylethylamine (DIPEA), piperidine, *N*,*N*,*N*′,*N*′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), acetic anhydride, trifluoroacetic acid (TFA), formic acid (FA), *m*-cresol, biotin, Fmoc-glycine, N^{α}-Fmoc-N^{ε}-Boc-L-lysine , Rink Amide resin, acetonitrile HPLC grade (purity >99,9%), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 4-morpholineethanesulfonic acid monohydrate (MES), sodium bicarbonate ($NaHCO_3$), sodium dodecyl sulfate (SDS), disodium hydrogen phosphate (Na_2HPO_4), Trizma® base, ethylendiaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), sodium chloride (NaCl), albumin from bovine serum (BSA), magnesium chloride anhydrous ($MgCl_2$), hydrochloric acid (HCl, 37 % w/w), sodium hydroxide (NaOH), streptavidin−alkaline phosphatase from *Streptomyces avidinii* (ALP-Strp) and Tween® 20 were purchased from Sigma-Aldrich (Milan, Italy). Solvents for peptide synthesis and purification were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification, except for *N*,*N*-dimethylformamide (DMF) which was dried over 4 Å molecular sieves and purged with nitrogen to avoid the presence of dimethylamine. Hydroquinone diphosphate (HQDP) was purchased from Metrohm Italiana (DropSens DRP-HQDP) (Origgio, Varese, Italy). PNA monomers and spacers AEEA (2-[2-(Fmoc-amino)ethoxy]ethoxyacetic acid) were from LGC LINK (Teddington, UK).

Synthetic DNA probes were purchased from biomers.net GmbH (Ulm, Germany), having the following sequences:

Full-Match (FM) DNA: 5'-ACT TGG GGT TTA TGG AAA TTG GAA TTG GGA TTA AGG GTT TGT ATC-3'

Single Mismatch (1-MM) DNA: 5'-ACT TGG GGT TTA TGG AAA TTG GAA TTG GGA TTA AG**T** GTT TGT ATC-3'

Scramble (SCR) DNA: 5'-ACT TGG GGT TTA TGG AAA TTG GAA T**GG** **TT**A **GAA TTG TG**T **GTA TG**C-3'

Wildtype and GM (Roundup Ready, RR) Soy European certified reference materials were provided by European Commission, Joint Research Centre (JRC) (Geel, Belgium). In particular, soy flours ERM-BF410ak, ERM-BF410dn and ERM-BF410gn, which contain respectively 0% (blank), 1% and 10% Roundup Ready soybean in wildtype soybean, were used.

The DNA extraction kit "ION Force DNA Extractor FAST" used for extraction of genomic DNA from soy flour samples was purchased from Generon (San Prospero, Modena, Italy).

Double-distilled and deionized water purified with a Milli-Q system was used for the preparation of the buffered solutions as well as for mobile phase preparation for UPLC.

Buffer solutions were prepared according to the following compositions:"

"MES buffer": 0.1 M MES (pH adjusted to 5 with NaOH).

Tris buffered saline (TBS): 0.1 M Trizma® base, 0.02 M $MgCl_2$ (pH adjusted to 7.4 with HCl).

Tris buffered saline-Tween (TBS-T): 0.1 M Trizma® base, 0.02 M $MgCl_2$, 0.05% w/v Tween 20® (pH adjusted to 7.4 with HCl).

"Carbonate buffer" (CB): 0.1 M $NaHCO_3$, 0.1% w/v SDS (pH adjusted to 9 with NaOH).

"Hybridization buffer": 0.3 M NaCl, 0.02 M Na_2HPO_4, 0.1 mM EDTA (pH adjusted to 7.4 with HCl).

"Blocking Buffer" (BB): 20 mg mL^{-1} BSA in TBS (pH 7.4).

"Reading buffer" (RB): 0.1 M Trizma® base, 0.02 M $MgCl_2$ (pH adjusted to 9.8 with HCl).

2.2 Equipment

Most of PNA synthesis was performed by an automatic synthesizer Biotage Syro I in 2.5 mL polypropylene reactors.

PNA purity and identity was checked by UPLC-ESI-MS (Waters Acquity Ultra Performance LC equipped with Waters Acquity SQ Detector and electrospray interface) using a Waters Acquity UPLC BEH C18 column, 300 Å (50x2.1 mm, 1.7 µm) (Waters Corporation, Milford, USA). UPLC

conditions: 0.90 min in water 0.2% formic acid (FA), then linear gradient to 50% acetonitrile 0.2% FA in 5.70 min at a flow rate of 0.25 mL min^{-1}.

PNA oligomers were purified by RP-HPLC using a XTerra® Prep RP18 column (7.8x300 mm, 10 µm) (Waters). HPLC conditions: 5.00 min in water 0.1% TFA, then linear gradient from water 0.1% TFA to 50% acetonitrile 0.1 % TFA in 30 min at a flow rate of 4.0 m~~l~~L min^{-1}.

PNA concentrations were determined by UV absorption at 260 nm using a Lambda BIO 20 Perkin Elmer Spectrophotometer (Perkin Elmer, San Antonio, TX, USA) and calculated from the following extinction coefficients of the nucleobases: Adenine 13700 l mol^{-1} cm^{-1}, Cytosine 6600 l mol^{-1} cm^{-1}, Guanine 11700 l mol^{-1} cm^{-1}, Thymine 8600 l mol^{-1} cm^{-1} (Faccini et al. 2008). ~~(Nielsen and Appella, 2014).~~

The concentration of Genomic DNA extracted from soybean flours was determined by a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) according to the procedure established by the manufacturer.

Genosensors were assembled on SWCNT-SPEs purchased by Metrohm Italiana Srl (DropSens DRP-110SWCNT) (Origgio, Varese, Italy).

All electrochemical measurements were performed using a PGSTAT-204 potentiostat/galvanostat produced by Metrohm Italiana Srl, equipped with NOVA 2.1.3 Advanced Electrochemical Software and connected to DropSens DRP-DSC plug.

An ESEM instrument Quanta™ 250 FEG (FEI, Hillsboro, OR) was used for the visualization of working electrode nanostructure by recording secondary electron signal.

2.3 PNA Synthesis

PNAs were synthesized on Chemmatrix Rink Amide resin pre-loaded with Fmoc-Glycine for **CP** PNA and Fmoc-Lysine for signalling probe (**SP)** PNA. The latter has a positively-charged amino-acid capable of enhancing attractive electrostatic forces between **SP** PNA and negatively-charged DNA, thus helping **SP** in promoting strand invasion reaction in the double helix of genomic DNA during annealing procedure.

PNAs were synthesized in 5 μmol scale, automatically from the 1st to the 15th monomer for **CP**, from the 1st to the 20th monomer for **SP**. For automatic synthesis PNA monomers and HBTU were dissolved in dry DMF at concentration of 0.1 M and 0.47 M, respectively; DIPEA was dissolved in dry 0.40 M DMF. After swelling in dichloromethane (DCM) it was followed a cyclic procedure: a) deprotection with 20% piperidine in DMF (2 times, 8 min), b) coupling with PNA monomer (5 equivalents), HBTU (5 equivalents), DIPEA (10 equivalents) in dry DMF (2 subsequent coupling reaction, 30 min as reaction time each), c) capping with acetic anhydride/DIPEA in dry DMF respectively 5:6:95 (2 times, 1 min).

After that, terminal monomers and spacers AEEA for **CP** and biotin for **SP** were added in polypropylene reactors for solid phase synthesis following Fmoc protocol: a) deprotection with 20% piperidine in DMF (2 times, 8 min), b) coupling with PNA monomer, spacer or biotin (10 equivalents at c = 0.05 M), HBTU (10 equivalents at c = 0.05 M), DIPEA (20 equivalents, c = 0.1M) in dry DMF (2 min activation followed by 40 min as reaction time), c) capping with acetic anhydride/DIPEA in dry DMF respectively 5:6:95, (2 times, 1 min), d) washing with DIPEA 20% in DMF to remove traces of acetic anhydride (2 times, 2 min).

Both PNAs were cleaved from resins using a 10% m-cresol solution in TFA and then precipitated in ethyl ether. After removal of ether, PNAs were dissolved in water and purified in reversed phase HPLC. PNAs identity and purity were checked using UPLC-MS (data are reported in Supplementary Information section (SI)). Finally, as already described in the 2.2. section, PNAs were quantified by UV-Vis absorption spectroscopy at 260 nm, using the following extinction

coefficients: **CP** ε = 201900 l mol^{-1} cm^{-1}, **SP** ε= 203900 l mol^{-1} cm^{-1}. **CP-Bio** ε= 129900 l mol^{-1} cm^{-1} (Nielsen and Appella, 2014, Faccini et al. 2008).

CP: H-AEEA-AEEA-GAT ACA AAC CCT TAA TCC CA-Gly-NH_2 yield 5.3%

CP-Bio: H-AEEA-AEEA-CTA CGC CAT CAG CT-Lys(biotin)-NH_2 yield 5.5%

SP: Biotin-AEEA-AEEA-AAT TTC CAT AAA CCC CAA GT-Lys(NH_3^+)-NH_2 yield 5.2%

2.4 Genosensor Setup

2.4.1 Capture Probe immobilization on SWCNT-CSPEs

The carboxylic function of SWCNTs was activated by incubation of 50 µL of 0.2 M EDC and 0.05 M NHS in MES buffer for 1 h at room temperature. After removal of the solution by rinsing with water, 50 µL of 500 nM **CP** in carbonated buffer was incubated for 2 h at room temperature, after which unreacted species were removed by rinsing with water. In order to prevent non-specific interaction of probes with the electrode substrates, a blocking step was performed by depositing 50 µL of 500 nM pyrene in DMSO. The SPEs surface were then washed with DMSO followed by water.

2.4.2 Extraction of genomic DNA from soy flour

The extraction of genomic DNA was carried using the DNA extraction kit "ION Force DNA Extractor FAST", according to the standard procedure. A total amount of soy flour of 0,04 g was used for each sample. The extracted DNA was quantified and directly used for the following phase without amplification. A proper dilution of the extracted solution (see discussion) was performed prior to analysis with the developed genosensor.

2.4.3 Hybridization of Target DNA and Signalling Probe in homogeneous phase

Properly diluted solutions of Target DNA and **SP** in Hybridization Buffer were mixed together in order to reach a final concentration of 20 nM **SP** and the desired Target DNA concentration. The mixture was left under agitation at 1000 rpm for 3 h at room temperature. This solution was subsequently transferred on the electrode surface and incubated for 2h. The SPEs were then rinsed with Tween 0.05% followed by water.

2.4.4 Enzymatic labelling and reading of the electrochemical genoassay

The ALP-Strp conjugate was 100-fold diluted in BB and incubated on the SPEs surface for 15 min at room temperature before washing with TBS-~~t~~T followed by TBS. Electrochemical read-out was performed by DPV using 50 µL of a 1 mg mL^{-1} solution of HQDP dissolved in RB, which was left in contact with the sensor surface for a fixed time of 2 min 30 s immediately prior to measurement. DPV curves were acquired by scanning potential between -0.5 V and +0.3 V (step potential= +0.00495 V, modulation amplitude= +0.04995 V, modulation time= 0.102 s, interval time = 0.4 s) and recording the signal ascribable to the oxidation of hydroquinone (HQ), generated by ALP-promoted enzymatic dephosphorylation of HQDP, to Quinone (Q). The peak current is associated with the amount of produced HQ, which is related to the amount of the **CP**/Target/**SP** sandwich formed. At least three replicate measurements were carried out for all standards of synthetic DNA and samples of genomic DNA.

All the measurements carried out for the assessment of the best experimental conditions, as well as the signal values for construction of the calibration curve were replicated three times. Mean values and standard deviation are shown in all figures.

Method validation was performed by calculating linearity range, Limit of Detection (LOD) and Limit of Quantification (LOQ) according to Eurachem Guide second ed., 2014, (https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf)

3 Results and Discussion

A general scheme of the sensing format used in this work is depicted in Scheme 2: a PNA **CP**, bearing an amino function, was covalently bound to the electrode surface through the carboxylic functions of the SWCNT-SPEs. We have chosen on purpose SWCNTs rather than MWCNTs, which would have resulted both in an increased resistance of the electrode substrate and in a "confinement" of the PNA CPs in between multiple concentric nanotubes, thus resulting in reduced accessibility of the receptor probes towards target DNA hybridization. It has to be noted that the SDS included in the carbonate buffer used for the immobilization of CP on CNTs increases wettability of hydrophobic surfaces by the aqueous PNA solutions.

The sequence of the **CP** was complementary to a 20-mer portion of the Target DNA; a second biotin-tagged PNA **SP**, with sequence complementary to a different contiguous portion of the target DNA, was used to obtain a sandwich hybrid with an Alkaline Phosphatase-streptavidin conjugate (ALP-Strp). The portion of the sequence was chosen on the basis of our previous works using SPR sensor (D'Agata et al 2010) or microstructured optical fibers (Bertucci et al 2015). In this study, longer CP and SP probes (i.e. 20mer instead of 15-mer) were used, since preliminary studies using 15-mer CP and shorter reporter probes were not satisfactory in terms of specific vs non-specific signal (results not shown); these data are in agreement with the signal increase observed using PNA-microarrays with increasing PNA length (Germini et al, 2014). The read-out of the electrochemical genoassay is carried out using hydroquinone diphosphate (HQDP) as enzymatic substrate, which is enzymatically converted to Hydroquinone (HQ), yielding a voltammetric signal by the SWCNT electrode proportional to the amount of PNA-**SP** hybridized on the electrode surface (Preechaworapun et al., 2008; Akanda et al., 2013).

The protocol of the genoassay is based on a first versatile hybridization of the genomic DNA with **SP**, carried out in homogeneous phase in a disposable plastic tube; this first hybrid is subsequently transferred on the **CP**-functionalized SWCNT-SPEs. Tween-20 is added to the washing buffers to remove any non-specifically absorbed material, thus avoiding non-specific binding.

The final step is the incubation of the enzyme-conjugate ALP-Strp, reacting with the biotin tag of **SP**, followed by drop-casting of HQDP on the electrode surface in order to undergo the dephosphorylation leading to the analytical signal acquired by DPV. The incubation time for the ALP-Strp conjugate was set at 15 min since longer times (30 and 45 minutes) did not result in significant improvements ($p>0,05$) in the current response. An analogous criterion was applied also for the assessment of the proper incubation time of HQDP substrate. In this way, signal intensity is proportional to the ALP quantity, since the reaction rate depends on the enzyme density in the proximity of the electrode, which in turn depends on the density of the SP immobilized and hence on the quantity of DNA captured on the surface.

3.1 PNA probe synthesis and loading

The PNAs necessary as **CP** and **SP** in the above-mentioned scheme were synthesized on Chemmatrix Rink Amide resin, as described in the experimental part.

After synthesis of the PNA part, the AEEA spacer for **CP** and biotin for **SP** were added. The sequence of the PNA probes and the positioning with respect to the target DNA sequence is depicted in Scheme 1. The **CP** and **SP** were obtained in moderate yields (5.2 and 5.3% respectively).

It has to be noticed that a crucial aspect for the development of a genoassay is to reach an adequate coverage of the electrode surface by capture probes PNA in order to quantitatively address the target DNA present in solution. To evaluate the loading of **CP** onto the activated carboxylic functions of the CNTs, an analogous of the **CP** PNA bearing not only the amino function necessary for the coupling reaction, but also a biotin label (**CP-Bio**) was used: for this purpose, four levels of **CP-Bio** concentration were explored, namely 50 nM, 100 nM, 500 nM and 1 µM (see Figure S12 of supporting information). Thus, through deposition of the enzymatic conjugate ALP-Strp, an evaluation of loading capability was carried out for **CP**. It was observed that the signal increases up

to 500 nM, whereas there was no statistically significant difference (p>0.05) between 500 nM and 1 µM levels, thus suggesting a saturation of the available active sites on the surface. On the basis of these findings, the **CP** concentration for deposition was set at 500 nM for the following experiments.

3.2 Assessment of signal to background ratio

In order to find the best operating conditions for the detection procedure in the genoassay, we first tested the DNA detection assay using a synthetic oligonucleotide including target sequences for both **CP** and **SP** and corresponding to the RR-soy tract of interest (**DNA-1,** 45-mer portion of the Roundup Ready soy DNA).

For this purpose, first it was necessary to take into account the non-specific interactions occurring between nucleobases and the electrode support, a well-known limitation of these carbon-based systems (Varghese, 2009). In fact, preliminary results showed a very strong blank signal obtained in absence of Target DNA, ascribable to non-specific interactions established between **SP** and the electrode surface. A significant improvement in terms of signal to background (S/B) ratio was obtained by using a solution of pyrene in DMSO as backfilling agent. These findings can be explained on the basis of stacking interactions taking place between the carbon-based substrate and such compound containing multiple benzene rings, preventing the non-specific absorption of the oligos nucleobases.

A concentration of 500 nM in DMSO was found to be sufficient to drastically reduce the non-specific signal, whereas no significant ($p>0.05$) difference was observed upon increase of pyrene concentration to 5µM. Therefore, a blocking step with 500 nM pyrene in DMSO was introduced after the immobilization of the **CP**. The influence of the backfilling agent is shown in Fig. 1, which illustrates comparison of the signals acquired in absence and presence of target with and without the use of pyrene.

The effect of concentration of SP, namely 10, 20 and 50 nM, on the S/B ratio was investigated by comparing the signals obtained in absence of target **DNA-1** (background) with the corresponding signals obtained using **DNA-1** concentrations equimolar to **SP**. As shown in Fig. 2, the background signal obtained using 50 nM SP showed an increase with respect to lower concentrations investigated, leading to a moderately lower S/B ratio, compared to that obtained in the case of the 20 nM level. However, since a higher data dispersion was observed in correspondence to 50 nM **SP**, the **SP** concentration was set at 20 nM for further development of the genosensor.

3.3 Analytical performance of the genosensor

The response signal measured at different Target **DNA-1** concentrations ranging from 250 pM to 20 nM was tested under the previously assessed best experimental conditions. As shown in Fig. 3, the dynamic range of response current is limited to a concentration of 5 nM of target **DNA-1**, where the saturation of the signal was reached. As for the linear dynamic range, it was assessed from 250 pM to ~~1,75~~ 2,5 nM (inset in Fig. 3). The limit of detection (LOD) of the biosensor was ~~71~~ 64 pM and the limit of quantitation (LOQ) ~~256~~ 215 pM.

3.4 Selectivity of the genosensor

The selectivity of the electrochemical genoassay was assessed through mismatched sequences in order to demonstrate that the signal observed was due to the selective binding of DNA to the capture probe. For this purpose, two non-complementary synthetic DNA targets (**DNA-1MM** and **DNA-SCR**) were used, one containing a single mismatch (1-MM) and one totally non-complementary (SCR). A central mismatch was chosen since in early studies the mismatch in the central bases has been shown to affect the recognition to a greater extent (Ratilainen et al, 2000), as recently found also in molecular modelling studies (Verona et al, 2017). The signals obtained were compared with that of complementary target FM **DNA-1** at the same concentration (10 nM) for all

sequences (see Figure S13 of supporting information). Excellent selectivity was obtained, as evidenced by a signal reduction by 28% in the case of 1-MM sequence and 98% for the SCR sequence. These results are in line with the general properties of PNA probes, which are known to possess an increased ability to discriminate non-complementary sequences. Although this property can depend on the length of the probe, in the present case the target application was RR soy detection, so the recognition of single point mutation was not strictly required; accordingly, a long PNA sequence was chosen in order to maximize full-match response. It is reasonable to propose that probe length should be optimized when developing a method for detection of mutated DNA.

3.5 Validation on soybean samples

Using the detection scheme developed, direct sensing without prior PCR or other amplification procedure was tested. Although PCR-based methods are widely diffused, it has to be noted that the possibility of directly measuring the DNA content using a simple extraction-sensing protocol would enormously simplify sensing methodologies, making them suitable for point-of-care and portable assays. Several genosensing methods have been developed, especially using nanoparticles for signal enhancement (Spoto, 2012, D'Agata, 2008, Bertucci, 2015). Comparing with previously devised methods, the goal of our work was to assess if the genosensor assay developed could work avoiding pre-amplification of the target DNA.

The system was thus validated on genomic DNA extracted from European Reference Material soy flours containing different percentages of Roundup Ready GM soy in wildtype soy (ERM-BF410ak, ERM-BF410dn and ERM-BF410gn). In particular, 1% and 10% levels were tested and compared with the wildtype soy.

A crucial aspect to be addressed when working with genomic material extracted from food samples is the need to adopt a protocol that allows the sensor probes to access the target sequence on DNA double strand and to form Watson-Crick base pairs with it. For this purpose, a step of heating at

95°C was introduced prior to addition of the **SP** to sample. The **SP** was added during the subsequent cooling phase in order to allow occurrence of hybridization with **SP** PNA before the self-reannealing of the genomic DNA. The extracts were diluted in HB to a final DNA concentration of 180 ng mL^{-1} and assessed through spectrophotometric assay, considering that an optical density of 1, measured at 260 nm, corresponds to a double strand DNA concentration of 50 ng μL^{-1} (Barbas et al., 2001). The dilution ratio was set to obtain current signals within linear range (Fig. 3), thus allowing quantitative assays. Under these conditions an analysis of variance (ANOVA) carried out on the recorded responses showed a statistically significant difference (p<0.001) for each level of RR Soy (Fig. 4), thus demonstrating suitability of our genosensor as a reliable and time/cost effective tool for labeling purposes and assessment of food authenticity.

4 Conclusions

The main goal of the present study was the development and the validation of a high selective genosensor for the analysis of non-amplified genomic DNA extracted from GM-Soy, high selectivity being provided by the sandwich approach based on a double recognition from capture and signalling probes. The good performance of the genosensor was achieved thanks to a combination of the enhancing properties of carbon nanotubes with the recognition efficiency of PNA probes. Although the detection of DNA derived from food and biological samples in principle is a very potent tool for a large set of tasks, going from food analysis to biological screenings and point-of-care tests, its full exploitation is still limited to the need for complex procedures and expensive instrumentation that can only be used in specialized labs. The present findings show that amperometric sensors can be used in a simple and effective way for direct detection of genomic DNA samples using a simple and inexpensive instrumentation combined with an easy procedure, with performance matching the Regulation (EC) No. 1829/2003. ~~stating that all food and feed containing a concentration of GMO greater than the threshold level, set at 0.9%, must be labelled as~~

~~containing GM products.~~ In addition, apart from the intrinsic limitation due to a relatively narrow linear range, the findings attested that the use of SWCNT-screen printed electrodes in combination with enzyme-mediated voltammetric read-out allows us to reach a sensitivity which is high enough to attain ~~this~~ the goal of discrimination of GM soy at threshold level as from EC Regulation~~;~~.This is further improved by drastic reduction of the non-specific signal obtained applying the treatment with pyrene, which overcomes one of the most demanding problems of this type of analysis, i.e. fouling of the sensor surface by assay or sample components.

Future efforts will be addressed to exploit this device also for developing portable and remote sensors to be used for the *in situ* detection of DNA without amplification procedures.

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Schemes and Figures Captions

- **Scheme 1.** a) Sequences of CP PNA, SP PNA and Target DNA-1, b) PNA:DNA interaction in antiparallel conformation (blue DNA, black PNA).
- **Scheme 2.** Genosensor setup and working principle.
- **Figure 1.** Effect of pyrene backfilling on current responses in presence (signal) and in absence (background) of 50 nM target DNA-1. Inset: DPV curves when using pyrene as blocking agent.
- **Figure 2.** Trends of response current and signal to background ratios (S/B) for different concentrations of signalling probe.
- **Figure 3.** Calibration curve generated by synthetic DNA-1 (unfitted). Inset: Data fitting within the linearity range.
- **Figure 4.** DPV signals from genomic DNA extracted from European Reference Materials (ERM) containing different percentages of GM soy referred to wildtype soy (blank) and GM soybean at different percentages. a) and b) refer to the replicated measurements carried out on independent samples. Inset: ANOVA plot of current responses from ERM; mean values and standard deviation ($n=3$) are reported.

Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM) soy

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Abstract

A novel amperometric genosensor based on PNA probes covalently bound on the surface of Single Walled Carbon Nanotubes – Screen Printed Electrodes (SWCNT-SPEs) was developed and validated in samples of non-amplified genomic DNA extracted from genetically modified (GM)-Soy. The sandwich assay is based on a first recognition of a 20-mer portion of the target DNA by a complementary PNA Capture Probe (CP) and a second hybridization with a PNA Signalling Probe (SP), with a complementary sequence to a different portion of the target DNA. The SP was labelled with biotin to measure current signal by means of a final incubation of an Alkaline Phosphatase-streptavidin conjugate (ALP-Strp). The electrochemical detection was carried out using hydroquinone diphosphate (HQDP) as enzymatic substrate. The genoassay provided a linear range from 250 pM to 2.5 nM, LOD of 64 pM and LOQ of 215 pM. Excellent selectivity towards one base mismatch (1-MM) or scrambled (SCR) sequences was obtained. A simple protocol for extraction and analysis of

non-amplified soybean genomic DNA without sample treatment was developed and validated. Our study provides insight into how the outstanding recognition efficiency of PNAs can be combined with the unique properties of CNTs in terms of signal response enhancement for direct detection of genomic DNA samples at the level of interest without previous amplification.

Keywords

PNA; genosensing; GMO; Soy; Carbon NanoTubes

1. Introduction

During the last decade, much attention has been devoted to the development of genosensing techniques because of high versatility of these systems and wide applicability to many fields, ranging from diagnosis of genetic diseases (D'Agata et al., 2017) to determination of food contaminants (Martín-Fernández et al., 2017; Silva et al., 2018; Neethirajan et al., 2018) and much more. Genosensors are established based on the principle of specific pairing between complementary nucleobases in nucleic acids, either natural, synthetic or mimics, as recognition method. DNA can be found and extracted from most organisms and, given its high stability under processing conditions, it represents an efficient analytical target for food safety and authenticity (Marchelli et al., 2012; Bianchi et al., 2018; Abdalhai et al., 2015; Manzanares-Palenzuela et al., 2015). For this purpose, genosensing techniques can be applied to detect the presence of genetically modified organisms (GMOs) (Marmiroli et al., 2008), taking into account that, according to EU Regulation (1829/2003/EC), all food and feed containing a concentration of GMO higher than 0.9% must be labelled as containing GM products.

Genosensors are designed to provide signal variation according to the mode of signal transduction (i.e. optical, electrochemical or mechanical), upon hybridization of target DNA or RNA with specific oligonucleotide probes. A wide variety of molecules have been synthesized to obtain efficient mimics of nucleic acids, which, besides the ability to hybridize complementary target

sequences, are requested to possess new properties, e.g. ease of modification, resistance to enzymatic processing etc. Among these, Peptide Nucleic Acid (PNA) (Nielsen, 2004, 2010) have proven to be a very promising class of nucleic acids mimics. Their structure is made of a *N*-(2-aminoethyl)glycine repeating units, each linked through a spacer to a nucleobase (Scheme 1). This structure guarantees the correct intra-base distance, which allows for Watson-Crick interactions to occur. Furthermore, due to the absence of negatively charged groups on its backbone, PNA yields hybrids with DNA of higher stability than homologous DNA-DNA hybrids. Another important property is that the PNA:DNA duplex is more sensitive to the presence of mismatches in the complementary DNA strand. Given its synthetic nature, PNA is also easily modifiable to include functional groups that can be used, for example, for covalent immobilization on substrates as Screen-Printed Electrodes (SPEs). Carbon Nanotubes (CNTs) are very promising nanomaterials thanks to their unique properties which can be exploited in electrochemical sensing (Rivas et al., 2017; Shrestha et al., 2016; Da Silva et al., 2016; Giannetto et al., 2017). They can be defined as an allotropic form of carbon with cylindrical shape, with a micrometric length and a width of approximately 2 nm. They can be obtained as concentric cylinders, i.e. multi-walled carbon nanotubes (MWCNTs) or with a single cylindrical unit as in the case of single-walled carbon nanotubes (SWCNTs). When such structures are implemented using screen printing technology for development of disposable CNTs-mediated SPEs, an enhancement of the electrochemical signal is obtained. By incorporating CNTs on all-carbon SPEs it is possible to exploit the native carboxylic moieties present on the edges of the tubes for immobilization of functional molecules and probes.

Numerous approaches to electrochemical detection of DNA have been devised. For example, an electrochemical genosensor was developed for the detection of *salmonella typhi* based on the use of gold nanoparticles-mercaptosilane modified SPEs: a 50 pM limit of detection was achieved with a good ability to discriminate against one-base, two-base and three-base mismatched target sequences (Das et al., 2014). Recently, Manzanares-Palenzuela et al. proposed an electrochemical genosensor based on a multiplex electrochemical platform for the determination of *Roundup Ready* transgenic

Soy using a sandwich format through the immobilization of two different Capture Probes (**CP**) on magnetic beads and subsequent detection with two different signalling probes (Manzanares-Palenzuela et al., 2015); the system was capable to achieve sub-picomolar limits of detection, associated to a response saturation above 250 pM of synthetic DNA. The suitability of the multiplex assay for the quantification of RR in relation to the taxon content was assessed using synthetic GMO mixtures, artificially simulating the GMO content around the labelling threshold value (0.9%). However, it has to be noted that the analysis of genomic DNA extracted from soybean flour was not challenged.

In the present paper we developed and validated a simple and effective amperometric genosensor based on PNA probes covalently bound on the surface of SWCNT-SPEs and electrochemical read-out using Differential Pulse Voltammetry (DPV). At the best of our knowledge, this is the first study dealing with the use of PNA receptors covalently linked to the electrode nanostructured substrate, thus combining the outstanding performance of PNAs in terms of recognition efficiency with the unique properties of CNTs, in terms of amplification of the electrode processes. The effectiveness of this analytical method was tested in the detection of *Roundup Ready* transgenic soybean. Genosensor was successfully validated on genomic DNA extracted from GM-Soybean European Certified Reference Materials (ERM®) and selectivity was assessed comparing the responses obtained using Full Match (FM) sequence DNA with two targets containing different mismatches on the **CP**-complementary portion, i.e. a single mismatch (1-MM) and a totally scrambled (SCR) DNA sequences.

2. Experimental

2.1 Materials

N,N-Diisopropylethylamine (DIPEA), piperidine, *N,N,N′,N′*-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), acetic anhydride, trifluoroacetic acid (TFA), formic acid

(FA), *m*-cresol, biotin, Fmoc-glycine, N^{α}-Fmoc-N^{ε}-Boc-L-lysine , Rink Amide resin, acetonitrile HPLC grade (purity >99,9%), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 4-morpholineethanesulfonic acid monohydrate (MES), sodium bicarbonate ($NaHCO_3$), sodium dodecyl sulfate (SDS), disodium hydrogen phosphate (Na_2HPO_4), Trizma® base, ethylendiaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), sodium chloride (NaCl), albumin from bovine serum (BSA), magnesium chloride anhydrous ($MgCl_2$), hydrochloric acid (HCl, 37 % w/w), sodium hydroxide (NaOH), streptavidin−alkaline phosphatase from *Streptomyces avidinii* (ALP-Strp) and Tween® 20 were purchased from Sigma-Aldrich (Milan, Italy). Solvents for peptide synthesis and purification were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification, except for *N*,*N*-dimethylformamide (DMF) which was dried over 4 Å molecular sieves and purged with nitrogen to avoid the presence of dimethylamine. Hydroquinone diphosphate (HQDP) was purchased from Metrohm Italiana (DropSens DRP-HQDP) (Origgio, Varese, Italy). PNA monomers and spacers AEEA (2-[2-(Fmoc-amino)ethoxy]ethoxyacetic acid) were from LGC LINK (Teddington, UK).

Synthetic DNA probes were purchased from biomers.net GmbH (Ulm, Germany), having the following sequences:

Full-Match (FM) DNA: 5’-ACT TGG GGT TTA TGG AAA TTG GAA TTG GGA TTA AGG GTT TGT ATC-3’

Single Mismatch (1-MM) DNA: 5’-ACT TGG GGT TTA TGG AAA TTG GAA TTG GGA TTA AG**T** GTT TGT ATC-3’

Scramble (SCR) DNA: 5’-ACT TGG GGT TTA TGG AAA TTG GAA T**GG** **TT**A **GAA TTG** **TG**T **GTA** **TG**C-3’

Wildtype and GM (Roundup Ready, RR) Soy European certified reference materials were provided by European Commission, Joint Research Centre (JRC) (Geel, Belgium). In particular, soy flours

ERM-BF410ak, ERM-BF410dn and ERM-BF410gn, which contain respectively 0% (blank), 1% and 10% Roundup Ready soybean in wildtype soybean, were used.

The DNA extraction kit "ION Force DNA Extractor FAST" used for extraction of genomic DNA from soy flour samples was purchased from Generon (San Prospero, Modena, Italy).

Double-distilled and deionized water purified with a Milli-Q system was used for the preparation of the buffered solutions as well as for mobile phase preparation for UPLC.

Buffer solutions were prepared according to the following compositions:"

"MES buffer": 0.1 M MES (pH adjusted to 5 with NaOH).

Tris buffered saline (TBS): 0.1 M Trizma® base, 0.02 M $MgCl_2$ (pH adjusted to 7.4 with HCl).

Tris buffered saline-Tween (TBS-T): 0.1 M Trizma® base, 0.02 M $MgCl_2$, 0.05% w/v Tween 20® (pH adjusted to 7.4 with HCl).

"Carbonate buffer" (CB): 0.1 M $NaHCO_3$, 0.1% w/v SDS (pH adjusted to 9 with NaOH).

"Hybridization buffer": 0.3 M NaCl, 0.02 M Na_2HPO_4, 0.1 mM EDTA (pH adjusted to 7.4 with HCl).

"Blocking Buffer" (BB): 20 mg mL^{-1} BSA in TBS (pH 7.4).

"Reading buffer" (RB): 0.1 M Trizma® base, 0.02 M $MgCl_2$ (pH adjusted to 9.8 with HCl).

2.2 Equipment

Most of PNA synthesis was performed by an automatic synthesizer Biotage Syro I in 2.5 mL polypropylene reactors.

PNA purity and identity was checked by UPLC-ESI-MS (Waters Acquity Ultra Performance LC equipped with Waters Acquity SQ Detector and electrospray interface) using a Waters Acquity UPLC BEH C18 column, 300 Å (50x2.1 mm, 1.7 µm) (Waters Corporation, Milford, USA). UPLC conditions: 0.90 min in water 0.2% formic acid (FA), then linear gradient to 50% acetonitrile 0.2% FA in 5.70 min at a flow rate of 0.25 mL min^{-1}.

PNA oligomers were purified by RP-HPLC using a XTerra® Prep RP18 column (7.8x300 mm, 10 μm) (Waters). HPLC conditions: 5.00 min in water 0.1% TFA, then linear gradient from water 0.1% TFA to 50% acetonitrile 0.1 % TFA in 30 min at a flow rate of 4.0 m~~l~~L min^{-1}.

PNA concentrations were determined by UV absorption at 260 nm using a Lambda BIO 20 Perkin Elmer Spectrophotometer (Perkin Elmer, San Antonio, TX, USA) and calculated from the following extinction coefficients of the nucleobases: Adenine 13700 l mol^{-1} cm^{-1}, Cytosine 6600 l mol^{-1} cm^{-1}, Guanine 11700 l mol^{-1} cm^{-1}, Thymine 8600 l mol^{-1} cm^{-1} (Faccini et al. 2008).

The concentration of Genomic DNA extracted from soybean flours was determined by a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) according to the procedure established by the manufacturer.

Genosensors were assembled on SWCNT-SPEs purchased by Metrohm Italiana Srl (DropSens DRP-110SWCNT) (Origgio, Varese, Italy).

All electrochemical measurements were performed using a PGSTAT-204 potentiostat/galvanostat produced by Metrohm Italiana Srl, equipped with NOVA 2.1.3 Advanced Electrochemical Software and connected to DropSens DRP-DSC plug.

An ESEM instrument Quanta™ 250 FEG (FEI, Hillsboro, OR) was used for the visualization of working electrode nanostructure by recording secondary electron signal.

2.3 PNA Synthesis

PNAs were synthesized on Chemmatrix Rink Amide resin pre-loaded with Fmoc-Glycine for **CP** PNA and Fmoc-Lysine for signalling probe (**SP)** PNA. The latter has a positively-charged amino-acid capable of enhancing attractive electrostatic forces between **SP** PNA and negatively-charged DNA, thus helping **SP** in promoting strand invasion reaction in the double helix of genomic DNA during annealing procedure.

PNAs were synthesized in 5 μmol scale, automatically from the 1st to the 15th monomer for **CP**, from the 1st to the 20th monomer for **SP**. For automatic synthesis PNA monomers and HBTU were dissolved in dry DMF at concentration of 0.1 M and 0.47 M, respectively; DIPEA was dissolved in dry 0.40 M DMF. After swelling in dichloromethane (DCM) it was followed a cyclic procedure: a) deprotection with 20% piperidine in DMF (2 times, 8 min), b) coupling with PNA monomer (5 equivalents), HBTU (5 equivalents), DIPEA (10 equivalents) in dry DMF (2 subsequent coupling reaction, 30 min as reaction time each), c) capping with acetic anhydride/DIPEA in dry DMF respectively 5:6:95 (2 times, 1 min).

After that, terminal monomers and spacers AEEA for **CP** and biotin for **SP** were added in polypropylene reactors for solid phase synthesis following Fmoc protocol: a) deprotection with 20% piperidine in DMF (2 times, 8 min), b) coupling with PNA monomer, spacer or biotin (10 equivalents at c = 0.05 M), HBTU (10 equivalents at c = 0.05 M), DIPEA (20 equivalents, c = 0.1M) in dry DMF (2 min activation followed by 40 min as reaction time), c) capping with acetic anhydride/DIPEA in dry DMF respectively 5:6:95, (2 times, 1 min), d) washing with DIPEA 20% in DMF to remove traces of acetic anhydride (2 times, 2 min).

Both PNAs were cleaved from resins using a 10% m-cresol solution in TFA and then precipitated in ethyl ether. After removal of ether, PNAs were dissolved in water and purified in reversed phase HPLC. PNAs identity and purity were checked using UPLC-MS (data are reported in Supplementary Information section (SI)). Finally, as already described in the 2.2. section, PNAs were quantified by UV-Vis absorption spectroscopy at 260 nm, using the following extinction coefficients: **CP** ε = 201900 l mol^{-1} cm^{-1}, **SP** ε= 203900 l mol^{-1} cm^{-1}. **CP-Bio** ε= 129900 l mol^{-1} cm^{-1} (Nielsen and Appella, 2014, Faccini et al. 2008).

CP: H-AEEA-AEEA-GAT ACA AAC CCT TAA TCC CA-Gly-NH_2 yield 5.3%

CP-Bio: H-AEEA-AEEA-CTA CGC CAT CAG CT-Lys(biotin)-NH_2 yield 5.5%

SP: Biotin-AEEA-AEEA-AAT TTC CAT AAA CCC CAA GT-Lys(NH_3^+)-NH_2 yield 5.2%

2.4 Genosensor Setup

2.4.1 Capture Probe immobilization on SWCNT-CSPEs

The carboxylic function of SWCNTs was activated by incubation of 50 µL of 0.2 M EDC and 0.05 M NHS in MES buffer for 1 h at room temperature. After removal of the solution by rinsing with water, 50 µL of 500 nM **CP** in carbonated buffer was incubated for 2 h at room temperature, after which unreacted species were removed by rinsing with water. In order to prevent non-specific interaction of probes with the electrode substrates, a blocking step was performed by depositing 50 µL of 500 nM pyrene in DMSO. The SPEs surface were then washed with DMSO followed by water.

2.4.2 Extraction of genomic DNA from soy flour

The extraction of genomic DNA was carried using the DNA extraction kit "ION Force DNA Extractor FAST", according to the standard procedure. A total amount of soy flour of 0,04 g was used for each sample. The extracted DNA was quantified and directly used for the following phase without amplification. A proper dilution of the extracted solution (see discussion) was performed prior to analysis with the developed genosensor.

2.4.3 Hybridization of Target DNA and Signalling Probe in homogeneous phase

Properly diluted solutions of Target DNA and **SP** in Hybridization Buffer were mixed together in order to reach a final concentration of 20 nM **SP** and the desired Target DNA concentration. The mixture was left under agitation at 1000 rpm for 3 h at room temperature. This solution was subsequently transferred on the electrode surface and incubated for 2h. The SPEs were then rinsed with Tween 0.05% followed by water.

2.4.4 Enzymatic labelling and reading of the electrochemical genoassay

The ALP-Strp conjugate was 100-fold diluted in BB and incubated on the SPEs surface for 15 min at room temperature before washing with TBS-T followed by TBS. Electrochemical read-out was performed by DPV using 50 µL of a 1 mg mL^{-1} solution of HQDP dissolved in RB, which was left in contact with the sensor surface for a fixed time of 2 min 30 s immediately prior to measurement. DPV curves were acquired by scanning potential between -0.5 V and +0.3 V (step potential= +0.00495 V, modulation amplitude= +0.04995 V, modulation time= 0.102 s, interval time = 0.4 s) and recording the signal ascribable to the oxidation of hydroquinone (HQ), generated by ALP-promoted enzymatic dephosphorylation of HQDP, to Quinone (Q). The peak current is associated with the amount of produced HQ, which is related to the amount of the **CP**/Target/**SP** sandwich formed. At least three replicate measurements were carried out for all standards of synthetic DNA and samples of genomic DNA.

All the measurements carried out for the assessment of the best experimental conditions, as well as the signal values for construction of the calibration curve were replicated three times. Mean values and standard deviation are shown in all figures.

Method validation was performed by calculating linearity range, Limit of Detection (LOD) and Limit of Quantification (LOQ) according to Eurachem Guide second ed., 2014, (https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf)

3 Results and Discussion

A general scheme of the sensing format used in this work is depicted in Scheme 2: a PNA **CP**, bearing an amino function, was covalently bound to the electrode surface through the carboxylic functions of the SWCNT-SPEs. We have chosen on purpose SWCNTs rather than MWCNTs, which would have resulted both in an increased resistance of the electrode substrate and in a "confinement" of the PNA CPs in between multiple concentric nanotubes, thus resulting in reduced accessibility of the receptor probes towards target DNA hybridization. It has to be noted that the

SDS included in the carbonate buffer used for the immobilization of CP on CNTs increases wettability of hydrophobic surfaces by the aqueous PNA solutions.

The sequence of the **CP** was complementary to a 20-mer portion of the Target DNA; a second biotin-tagged PNA **SP**, with sequence complementary to a different contiguous portion of the target DNA, was used to obtain a sandwich hybrid with an Alkaline Phosphatase-streptavidin conjugate (ALP-Strp). The portion of the sequence was chosen on the basis of our previous works using SPR sensor (D'Agata et al 2010) or microstructured optical fibers (Bertucci et al 2015). In this study, longer CP and SP probes (i.e. 20mer instead of 15-mer) were used, since preliminary studies using 15-mer CP and shorter reporter probes were not satisfactory in terms of specific vs non-specific signal (results not shown); these data are in agreement with the signal increase observed using PNA-microarrays with increasing PNA length (Germini et al, 2014). The read-out of the electrochemical genoassay is carried out using hydroquinone diphosphate (HQDP) as enzymatic substrate, which is enzymatically converted to Hydroquinone (HQ), yielding a voltammetric signal by the SWCNT electrode proportional to the amount of PNA-**SP** hybridized on the electrode surface (Preechaworapun et al., 2008; Akanda et al., 2013).

The protocol of the genoassay is based on a first versatile hybridization of the genomic DNA with **SP**, carried out in homogeneous phase in a disposable plastic tube; this first hybrid is subsequently transferred on the **CP**-functionalized SWCNT-SPEs. Tween-20 is added to the washing buffers to remove any non-specifically absorbed material, thus avoiding non-specific binding.

The final step is the incubation of the enzyme-conjugate ALP-Strp, reacting with the biotin tag of **SP**, followed by drop-casting of HQDP on the electrode surface in order to undergo the dephosphorylation leading to the analytical signal acquired by DPV. The incubation time for the ALP-Strp conjugate was set at 15 min since longer times (30 and 45 minutes) did not result in significant improvements ($p>0,05$) in the current response. An analogous criterion was applied also for the assessment of the proper incubation time of HQDP substrate. In this way, signal intensity is proportional to the ALP quantity, since the reaction rate depends on the enzyme density in the

proximity of the electrode, which in turn depends on the density of the SP immobilized and hence on the quantity of DNA captured on the surface.

3.1 PNA probe synthesis and loading

The PNAs necessary as **CP** and **SP** in the above-mentioned scheme were synthesized on Chemmatrix Rink Amide resin, as described in the experimental part.

After synthesis of the PNA part, the AEEA spacer for **CP** and biotin for **SP** were added. The sequence of the PNA probes and the positioning with respect to the target DNA sequence is depicted in Scheme 1. The **CP** and **SP** were obtained in moderate yields (5.2 and 5.3% respectively).

It has to be noticed that a crucial aspect for the development of a genoassay is to reach an adequate coverage of the electrode surface by capture probes PNA in order to quantitatively address the target DNA present in solution. To evaluate the loading of **CP** onto the activated carboxylic functions of the CNTs, an analogous of the **CP** PNA bearing not only the amino function necessary for the coupling reaction, but also a biotin label (**CP-Bio**) was used: for this purpose, four levels of **CP-Bio** concentration were explored, namely 50 nM, 100 nM, 500 nM and 1 µM (see Figure S12 of supporting information). Thus, through deposition of the enzymatic conjugate ALP-Strp, an evaluation of loading capability was carried out for **CP**. It was observed that the signal increases up to 500 nM, whereas there was no statistically significant difference (p>0.05) between 500 nM and 1 µM levels, thus suggesting a saturation of the available active sites on the surface. On the basis of these findings, the **CP** concentration for deposition was set at 500 nM for the following experiments.

3.2 Assessment of signal to background ratio

In order to find the best operating conditions for the detection procedure in the genoassay, we first tested the DNA detection assay using a synthetic oligonucleotide including target sequences for both **CP** and **SP** and corresponding to the RR-soy tract of interest (**DNA-1,** 45-mer portion of the Roundup Ready soy DNA).

For this purpose, first it was necessary to take into account the non-specific interactions occurring between nucleobases and the electrode support, a well-known limitation of these carbon-based systems (Varghese, 2009). In fact, preliminary results showed a very strong blank signal obtained in absence of Target DNA, ascribable to non-specific interactions established between **SP** and the electrode surface. A significant improvement in terms of signal to background (S/B) ratio was obtained by using a solution of pyrene in DMSO as backfilling agent. These findings can be explained on the basis of stacking interactions taking place between the carbon-based substrate and such compound containing multiple benzene rings, preventing the non-specific absorption of the oligos nucleobases.

A concentration of 500 nM in DMSO was found to be sufficient to drastically reduce the non-specific signal, whereas no significant ($p>0.05$) difference was observed upon increase of pyrene concentration to 5µM. Therefore, a blocking step with 500 nM pyrene in DMSO was introduced after the immobilization of the **CP**. The influence of the backfilling agent is shown in Fig. 1, which illustrates comparison of the signals acquired in absence and presence of target with and without the use of pyrene.

The effect of concentration of SP, namely 10, 20 and 50 nM, on the S/B ratio was investigated by comparing the signals obtained in absence of target **DNA-1** (background) with the corresponding signals obtained using **DNA-1** concentrations equimolar to **SP**. As shown in Fig. 2, the background signal obtained using 50 nM SP showed an increase with respect to lower concentrations investigated, leading to a moderately lower S/B ratio, compared to that obtained in the case of the

20 nM level. However, since a higher data dispersion was observed in correspondence to 50 nM **SP**, the **SP** concentration was set at 20 nM for further development of the genosensor.

3.3 Analytical performance of the genosensor

The response signal measured at different Target **DNA-1** concentrations ranging from 250 pM to 20 nM was tested under the previously assessed best experimental conditions. As shown in Fig. 3, the dynamic range of response current is limited to a concentration of 5 nM of target **DNA-1**, where the saturation of the signal was reached. As for the linear dynamic range, it was assessed from 250 pM to 2,5 nM (inset in Fig. 3). The limit of detection (LOD) of the biosensor was 64 pM and the limit of quantitation (LOQ) 215 pM.

3.4 Selectivity of the genosensor

The selectivity of the electrochemical genoassay was assessed through mismatched sequences in order to demonstrate that the signal observed was due to the selective binding of DNA to the capture probe. For this purpose, two non-complementary synthetic DNA targets (**DNA-1MM** and **DNA-SCR**) were used, one containing a single mismatch (1-MM) and one totally non-complementary (SCR). A central mismatch was chosen since in early studies the mismatch in the central bases has been shown to affect the recognition to a greater extent (Ratilainen et al, 2000), as recently found also in molecular modelling studies (Verona et al, 2017). The signals obtained were compared with that of complementary target FM **DNA-1** at the same concentration (10 nM) for all sequences (see Figure S13 of supporting information). Excellent selectivity was obtained, as evidenced by a signal reduction by 28% in the case of 1-MM sequence and 98% for the SCR sequence. These results are in line with the general properties of PNA probes, which are known to possess an increased ability to discriminate non-complementary sequences. Although this property can depend on the length of the probe, in the present case the target application was RR soy

detection, so the recognition of single point mutation was not strictly required; accordingly, a long PNA sequence was chosen in order to maximize full-match response. It is reasonable to propose that probe length should be optimized when developing a method for detection of mutated DNA.

3.5 Validation on soybean samples

Using the detection scheme developed, direct sensing without prior PCR or other amplification procedure was tested. Although PCR-based methods are widely diffused, it has to be noted that the possibility of directly measuring the DNA content using a simple extraction-sensing protocol would enormously simplify sensing methodologies, making them suitable for point-of-care and portable assays. Several genosensing methods have been developed, especially using nanoparticles for signal enhancement (Spoto, 2012, D'Agata, 2008, Bertucci, 2015). Comparing with previously devised methods, the goal of our work was to assess if the genosensor assay developed could work avoiding pre-amplification of the target DNA.

The system was thus validated on genomic DNA extracted from European Reference Material soy flours containing different percentages of Roundup Ready GM soy in wildtype soy (ERM-BF410ak, ERM-BF410dn and ERM-BF410gn). In particular, 1% and 10% levels were tested and compared with the wildtype soy.

A crucial aspect to be addressed when working with genomic material extracted from food samples is the need to adopt a protocol that allows the sensor probes to access the target sequence on DNA double strand and to form Watson-Crick base pairs with it. For this purpose, a step of heating at 95°C was introduced prior to addition of the **SP** to sample. The **SP** was added during the subsequent cooling phase in order to allow occurrence of hybridization with **SP** PNA before the self-reannealing of the genomic DNA. The extracts were diluted in HB to a final DNA

concentration of 180 ng mL^{-1} and assessed through spectrophotometric assay, considering that an optical density of 1, measured at 260 nm, corresponds to a double strand DNA concentration of 50 ng μL^{-1} (Barbas et al., 2001). The dilution ratio was set to obtain current signals within linear range (Fig. 3), thus allowing quantitative assays. Under these conditions an analysis of variance (ANOVA) carried out on the recorded responses showed a statistically significant difference (p<0.001) for each level of RR Soy (Fig. 4), thus demonstrating suitability of our genosensor as a reliable and time/cost effective tool for labeling purposes and assessment of food authenticity.

4 Conclusions

The main goal of the present study was the development and the validation of a high selective genosensor for the analysis of non-amplified genomic DNA extracted from GM-Soy, high selectivity being provided by the sandwich approach based on a double recognition from capture and signalling probes. The good performance of the genosensor was achieved thanks to a combination of the enhancing properties of carbon nanotubes with the recognition efficiency of PNA probes. Although the detection of DNA derived from food and biological samples in principle is a very potent tool for a large set of tasks, going from food analysis to biological screenings and point-of-care tests, its full exploitation is still limited to the need for complex procedures and expensive instrumentation that can only be used in specialized labs. The present findings show that amperometric sensors can be used in a simple and effective way for direct detection of genomic DNA samples using a simple and inexpensive instrumentation combined with an easy procedure, with performance matching the Regulation (EC) No. 1829/2003. In addition, apart from the intrinsic limitation due to a relatively narrow linear range, the findings attested that the use of SWCNT-screen printed electrodes in combination with enzyme-mediated voltammetric read-out allows us to reach a sensitivity which is high enough to attain the goal of discrimination of GM soy at threshold level as from EC Regulation.This is further improved by drastic reduction of the non-specific signal

obtained applying the treatment with pyrene, which overcomes one of the most demanding problems of this type of analysis, i.e. fouling of the sensor surface by assay or sample components.

Future efforts will be addressed to exploit this device also for developing portable and remote sensors to be used for the *in situ* detection of DNA without amplification procedures.

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Schemes and Figures Captions

- **Scheme 1.** a) Sequences of CP PNA, SP PNA and Target DNA-1, b) PNA:DNA interaction in antiparallel conformation (blue DNA, black PNA).
- **Scheme 2.** Genosensor setup and working principle.
- **Figure 1.** Effect of pyrene backfilling on current responses in presence (signal) and in absence (background) of 50 nM target DNA-1. Inset: DPV curves when using pyrene as blocking agent.
- **Figure 2.** Trends of response current and signal to background ratios (S/B) for different concentrations of signalling probe.
- **Figure 3.** Calibration curve generated by synthetic DNA-1 (unfitted). Inset: Data fitting within the linearity range.
- **Figure 4.** DPV signals from genomic DNA extracted from European Reference Materials (ERM) containing different percentages of GM soy referred to wildtype soy (blank) and GM soybean at different percentages. a) and b) refer to the replicated measurements carried out on independent samples. Inset: ANOVA plot of current responses from ERM; mean values and standard deviation ($n=3$) are reported.

Scheme 1

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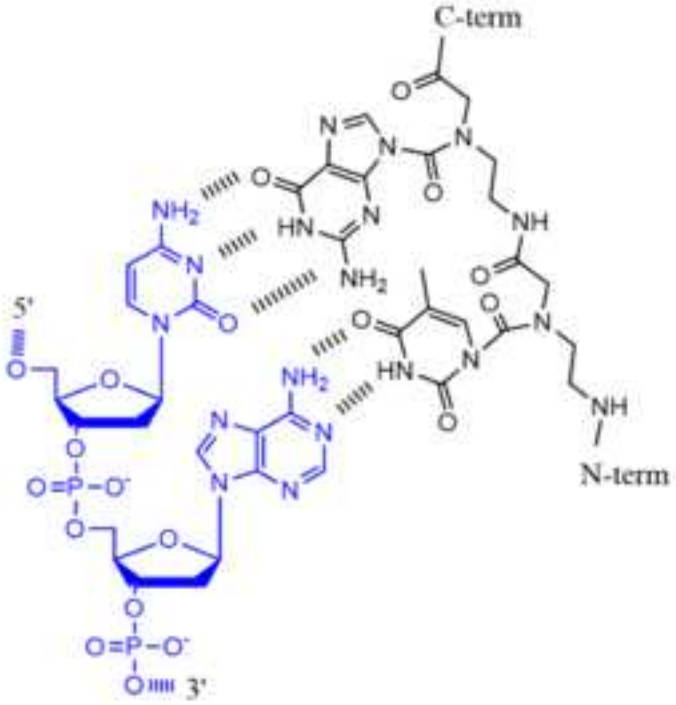
a)

b)



Scheme 2

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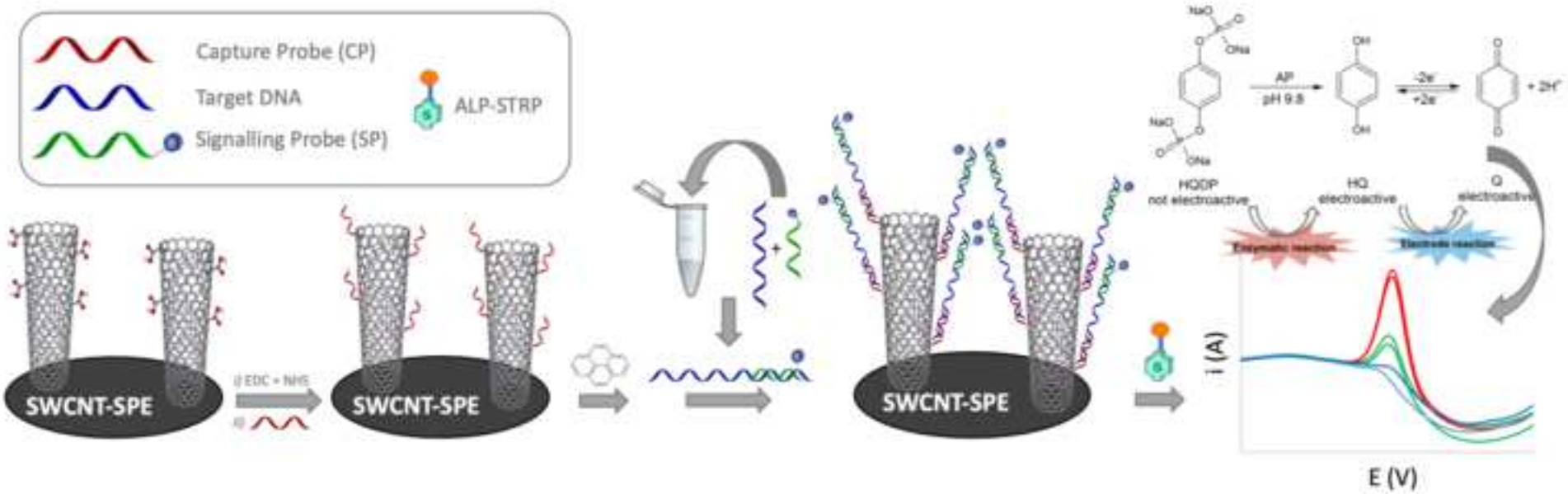

Figure 1

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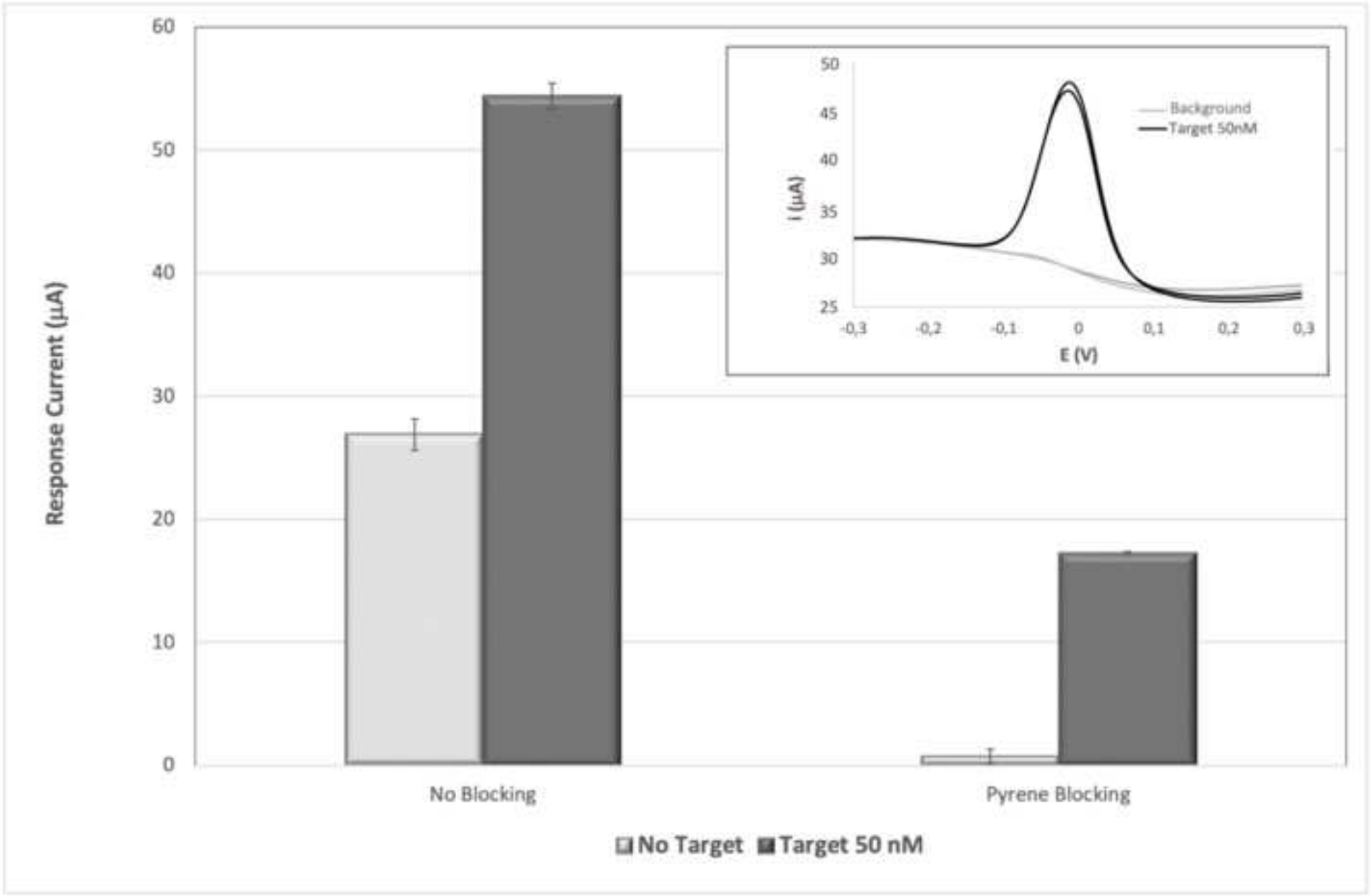

Figure 2

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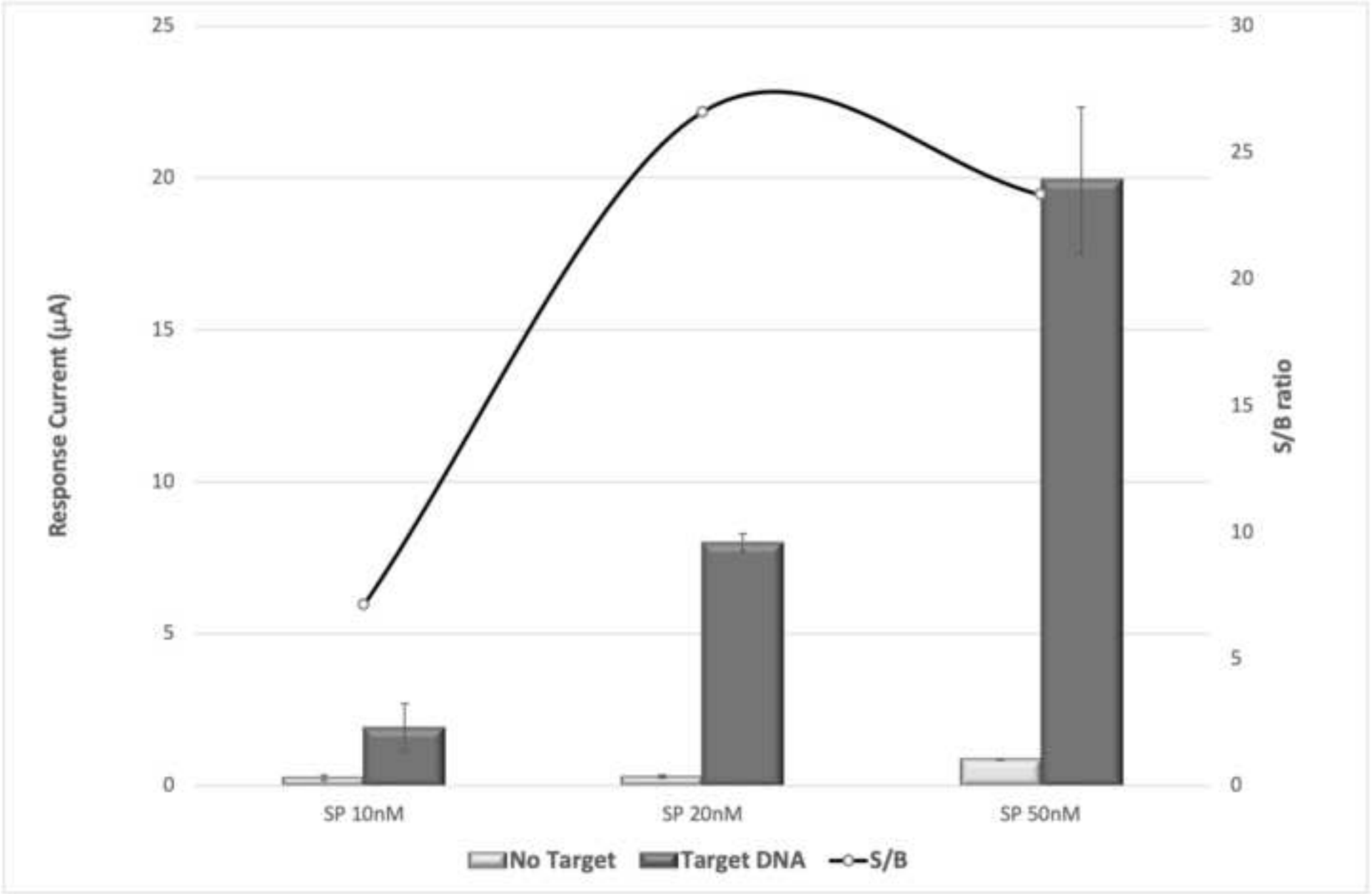

Figure 3

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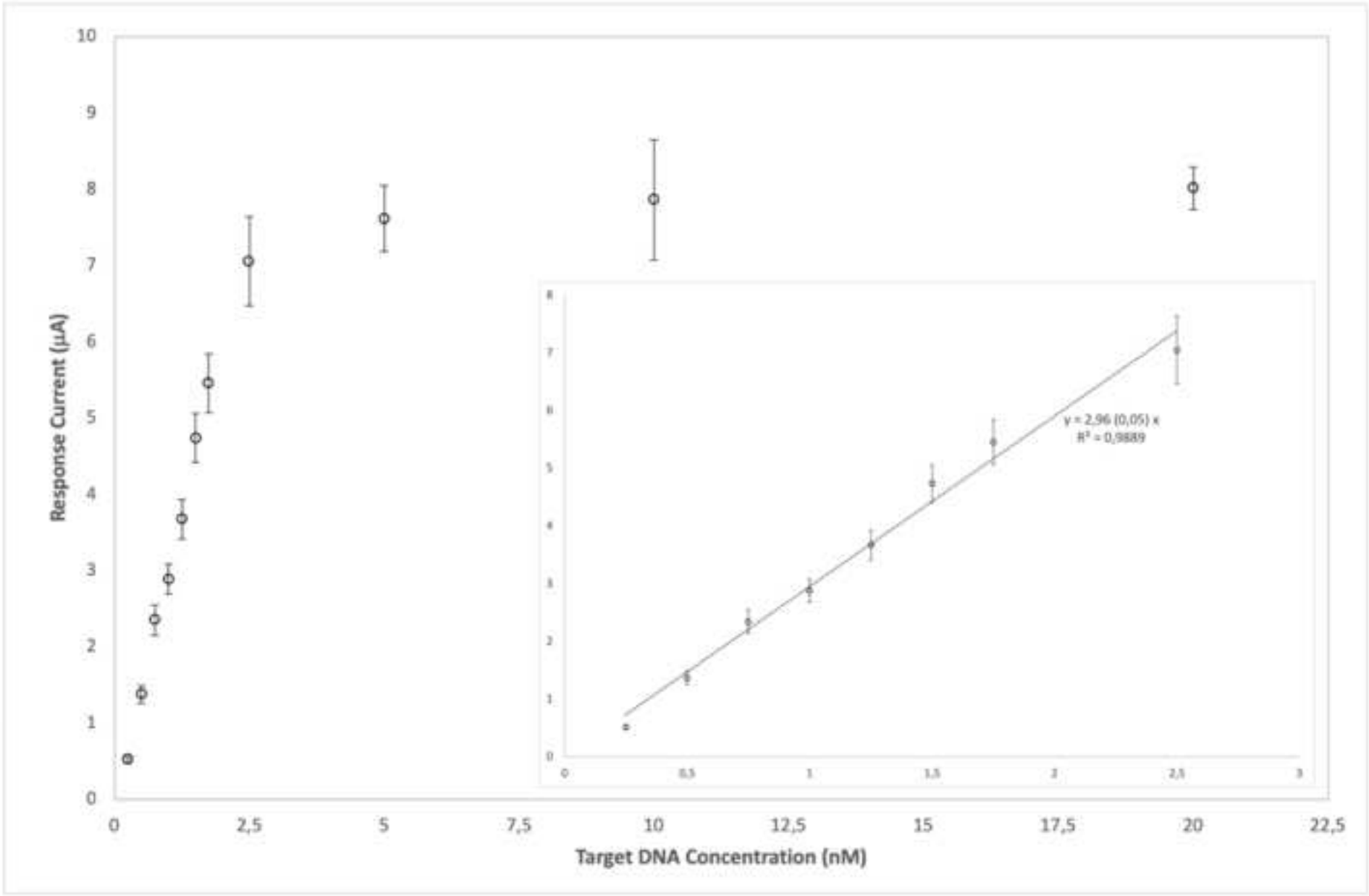

Figure 4

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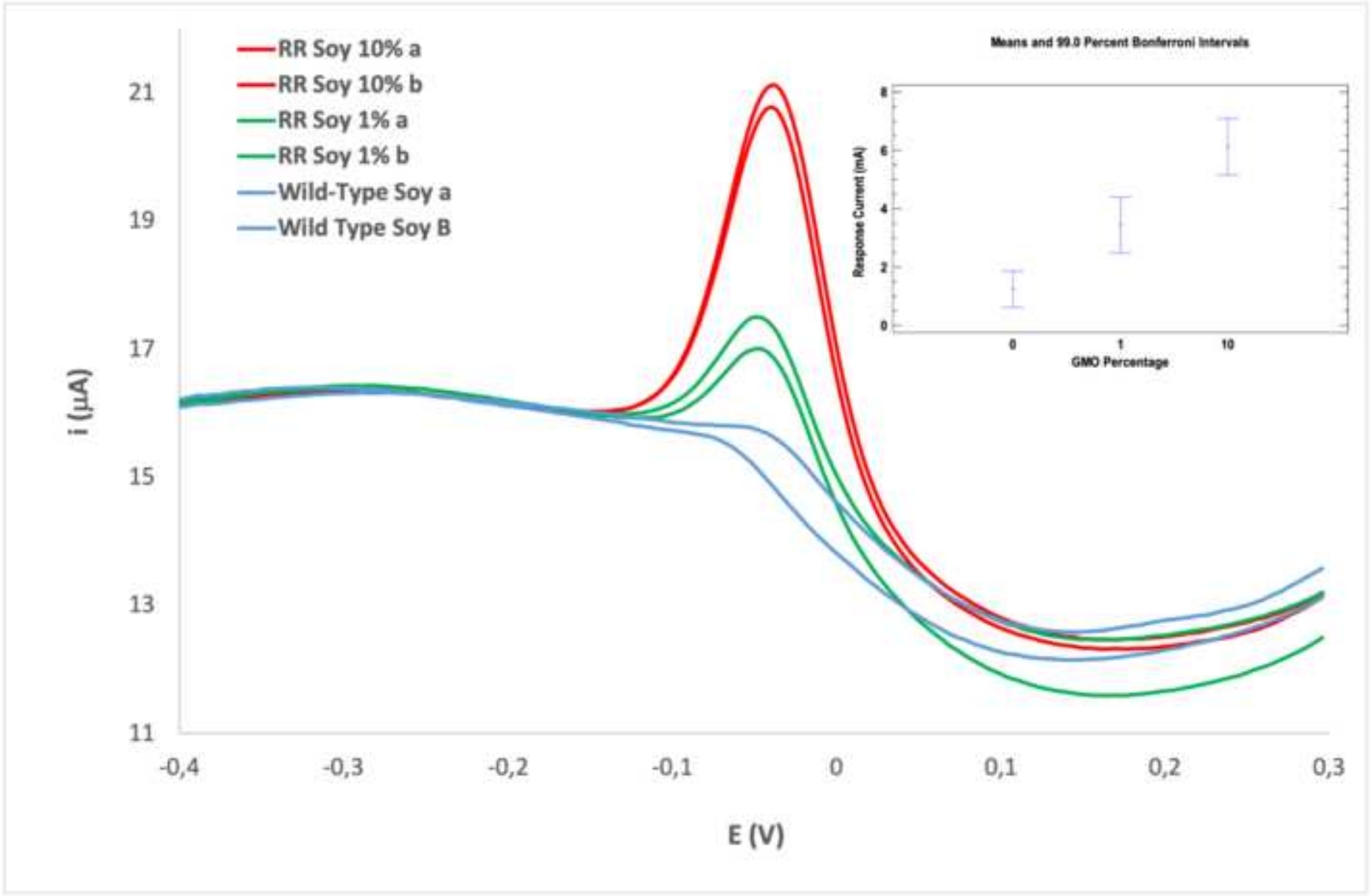

Supplementary Material

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Conflict of interest

There are no conflicts to declare

Novel amperometric genosensor based on peptide nucleic acid (PNA) probes immobilized on carbon nanotubes-screen printed electrodes for the determination of trace levels of non-amplified DNA in genetically modified (GM)soy

Simone Fortunati, Andrea Rozzi, Federica Curti, Marco Giannetto*, Roberto Corradini*, Maria Careri

CRediT author statement

Simone Fortunati: Investigation, Writing- Original Draft, Validation, Visualization

Andrea Rozzi: Investigation

Federica Curti: Investigation

Marco Giannetto: Conceptualization, Methodology, Validation, Resources, Writing- Original Draft, Writing – Review & Editing, Visualization, Supervision

Roberto Corradini: Conceptualization, Methodology, Resources, Writing- Original Draft, Writing – Review & Editing, Supervision, Funding Acquisition

Maria Careri: Resources, Writing- Original Draft, Writing – Review & Editing, Supervision, Funding Acquisition

***Declaration of Interest Statement**

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: