UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Química Analítica

TESIS DOCTORAL

Electrochemical DNA biosensors and sensing platforms for the detection and quantification of genetically modified soybean in food and feed

Biosensores y plataformas sensoras de ADN con transducción electroquímica para la detección y cuantificación de soja genéticamente modificada en alimentos y piensos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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BIOSENSORES Y PLATAFORMAS SENSORAS DE ADN CON TRANSDUCCIÓN ELECTROQUÍMICA PARA LA DETECCIÓN Y CUANTIFICACIÓN DE SOJA GENÉTICAMENTE MODIFICADA EN ALIMENTOS Y PIENSOS

Memoria para optar al grado de Doctor

Presentada por

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ELECTROCHEMICAL DNA BIOSENSORS AND SENSING PLATFORMS FOR THE DETECTION AND QUANTIFICATION OF GENETICALLY MODIFIED SOYBEAN IN FOOD AND FEED

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

By

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SUMMARY

In the nearly two decades since they were first commercialized, genetically engineered crops have gained ground on their conventional counterparts, reaching nearly 180 million hectares worldwide in 2015. The technology has bestowed most of its benefits on enhancing crop productivity with two main traits currently dominating the market: insect-resistant and herbicide-tolerant crops.

Genetically modified organisms (GMOs) are conventionally obtained through the introduction of foreign DNA fragments into the host genome *via* genetically engineering techniques. The modified organism, *i.e.* plant, will then be able to express new protein(s) confering it with the novel, desired trait(s), *e.g.* herbicide tolerance. Plants such as maize and soybean have been modified to withstand weed-killing chemicals or resist insect pests to increase yields and improve profits to farmers.

Despite their rapid and vast adoption by farmers worldwide, GMOs have generated heated debates, especially in European countries, driven mostly by consumers concerned about safety of transgenic foods and about the potential impact of their release into the environment. The European Union (EU) has established the mandatory labeling of GMOs in food and feed above a certain threshold (0.9%, based on the ingredient). In the list of ingredients the term "genetically modified" must appear (next to the ingredient in question). Below such level, labeling is not mandatory provided that the presence of GM material is proven to be accidental or technically unavoidable. The need to monitor GMOs and to verify compliance with EU legislation has driven the development of analytical methods able to detect and quantify GMOs in crops, and in food and feed products.

GMO detection is generally carried out by enzymatic amplification of DNA sequences specific of the transgenic insert by polymerase chain reaction (PCR)–based methods. Quantitative methods are based in the real-time variant of this technique, which relies on the use of fluorescent molecules to generate real-time data during the

different stages of amplification. This allows to collect fluorescence in the exponential phase, where it is possible to achieve quantification of the amplified DNA fragment (amplicon). Despite the fact that PCR is the reference methodology for DNA detection and quantification, there are still some drawbacks that have motivated researchers into developing alternative methods. These are intended to be less expensive and suitable for decentralized applications and for resource-limited settings.

Electrochemical DNA biosensors and sensing platforms have been proposed as low-cost, sensitive and robust alternatives for DNA sequence-specific detection. It is not surprising that electrochemical-based DNA detection represents an active area of research with increasing publications year after year. The interest in this field, demonstrated by research groups worldwide, has been encouraged by the simple and relatively low-priced instrumentation, the high selectivity of the base-pairing biorecognition process (hybridization) and the high sensitivity and versatility of electrochemical detection principles through which DNA hybridization can be monitored (*e.g.*, redox enzyme-amplified signaling, surface impedance measurements, electron transfer mediated by DNA-binders or intercalators, etc.).

This PhD thesis describes the development of electrochemical DNA biosensors and sensing platforms for the detection and quantification of genetically modified soybean. The soybean event GTS 40-3-2 or Roundup Ready® Soybean (RRS) was chosen as model analyte being the most widely adopted GMO, accounting for 75% of the total soy production in the world. Soybean is present at a high percentage in the compound feed used in the EU for breeding animals. Most of it is imported into the EU from countries that cultivate genetically engineered soybean.

This thesis is structured in eight chapters, five of which are based on bibliographical and experimental work that were published during this doctorate. It begins with an introduction (Chapter 1) describing the state-of-the-art and current status of GMO development and commercialized traits with special emphasis in the EU region, as well as the most common detection techniques used for GMO monitoring. Finally, a general description of electrochemical DNA biosensor/sensing methods is presented, followed by an entire chapter devoted to a comprehensive review of the electrochemical genosensors reported for GMO detection (Chapter 2). Sorting through what has been

done allowed us to detect the most relevant gaps in the field and, with that, the motivation to provide pertinent contributions to it.

Chapter 3 describes the aims and scope of this thesis. The main purpose of this work was to achieve accurate RRS quantification through relating the contents of transgenic and taxonomic sequences present in a variety of samples, from flours to highly processed samples, using newly developed electrochemical methods. To reach such goal, on one hand, a labeled-based method was proposed using enzymatic signal amplification and magnetic microparticles as immobilization platform. Sandwich hybridization was performed, granting a high level of specificity to the assay. Two variants of this method were pursued: single assays to separately detect both sequences and a multiplex assay that simultaneously immobilize-hybridize-labels and sequentially detects both analytes.

On the other hand, towards designing simple and easy-to-fabricate analytical devices, a label-free biosensor was proposed as a rapid and low-cost screening tool for transgenic soybean, based on layer-by-layer assemblies of copper phthalocyanine built onto nanostructured electrodes.

Chapter 4 describes the development of the single and multiplex platforms and their pre-validation with synthetic oligonucleotide mixtures containing GMO levels around the labeling threshold set by European authorities. In Chapter 5, the quantitative coupling of the single electrochemical assays with a PCR pre-amplification step is presented and, for the first time, accurate GMO levels were determined in flours and reference material. These minimally processed samples with known GMO percentages were used as proof-of-concept to evaluate the quantitative performance of the electrochemical assays, comparing the data to a real-time PCR method. In Chapter 6, the quantitative approach moves a step forward by detecting PCR-derived amplicons with the multiplex platform, achieving lower limits of detection for both analytes. Accurate quantification of RRS in highly complex supermarket samples was accomplished. Finally, in Chapter 7, a label-free strategy based on electrochemical impedance spectroscopy is described using nanostructured electrodes with layer-by-layer phthalocyanine assemblies. A full characterization of this novel platform is presented and its use to detect synthetic DNA from transgenic soybean without requiring label molecules is discussed.

Overall, this thesis encompasses the development of analytical methods for the complex task of detecting and quantifying genetically engineered material, under two main detection principles: labeled-based and label-free DNA-detection. The accomplishment of such analytical challenges has been demonstrated in practical terms using food matrices with the labeled-based approaches. The impedimetric method, based upon a novel strategy, allowed detecting transgenic soybean in a label-free fashion using synthetic sequences.

RESUMEN

Los cultivos genéticamente modificados han ganado terreno desde que han sido comercializados hace casi dos décadas, habiendo alcanzado en 2015 casi 180 millones de hectáreas en el mundo, lo que sobrepasa el área destinada a cultivos convencionales. La tecnología ha permitido mejorar la productividad de los cultivos, existiendo dos tipos de modificación genética que actualmente dominan el mercado: la resistencia a insectos y la tolerancia a herbicidas.

Los organismos genéticamente modificados (OGMs) se obtienen, normalmente, mediante la inserción de un fragmento de ADN de una especie diferente a la especie receptora, a través de la introducción de fragmentos de ADN al genoma receptor, a través de técnicas de ingeniería genética. El organismo modificado (planta) será capaz de expresar una o más proteínas nuevas que le conferirá la característica deseada (*p. ej.*, tolerancia a herbicidas). Plantas como el maíz o la soja han sido modificadas para tolerar agentes químicos que eliminan malas hierbas o para expresar proteínas insecticidas y resistir enfermedades, siendo el resultado un aumento en el rendimiento de los cultivos y en los beneficios económicos adquiridos por los agricultores.

A pesar de la rápida y creciente adopción de los OGMs por los agricultores a nivel mundial, se ha generado una gran controversia y desconfianza en los consumidores, encontrándose una gran oposición, sobre todo en Europa, debido principalmente a los temores sobre la seguridad de los alimentos y a las consecuencias medioambientales. La Unión Europea (UE) ha considerado necesario establecer reglamentos sobre el etiquetado y la trazabilidad de los OGMs, de modo que es obligatorio indicar la presencia de OGMs en el etiquetado de un alimento o pienso cuando contenga algún OGM autorizado en cantidades superiores al 0,9% del ingrediente. Por debajo de este nivel, no es obligatorio el etiquetado, siempre que se demuestre que la presencia de material genéticamente modificado es accidental o técnicamente inevitable. En la lista de ingredientes debe de aparecer el término “modificado genéticamente” (al lado del

ingrediente pertinente). Es por ello que es necesario disponer de métodos analíticos que permitan detectar y cuantificar OGMs en cultivos, alimentos y piensos.

Generalmente, la detección de OGMs se lleva a cabo amplificando una secuencia de ADN específica de la modificación o inserción transgénica, mediante métodos basados en la técnica de la reacción en cadena de la polimerasa (PCR). Los métodos cuantitativos se basan en la PCR en tiempo real, la cual hace uso de marcadores fluorescentes para generar datos en tiempo real durante el proceso de amplificación. Esto permite registrar la fluorescencia en la fase exponencial, donde es posible llevar a cabo la cuantificación de secuencias específicas de ADN. A pesar de ser el método de referencia para la detección y cuantificación de ADN, ciertas limitaciones vinculadas a la técnica han impulsado el desarrollo de métodos alternativos de menor coste, adecuados para aplicaciones descentralizadas y para laboratorios con recursos limitados.

Los biosensores y plataformas sensoras de ADN con transducción electroquímica han sido propuestos como alternativas económicas, sensibles y robustas para las detección de secuencias específicas de ADN. De modo que no sorprende el hecho de que la detección electroquímica de ADN sea un área activa de investigación con un elevado número de publicaciones, que incrementa año tras año. El interés mostrado por este campo por investigadores a nivel mundial se debe a diferentes factores, entre los cuales se encuentran: el bajo coste y la simplicidad del equipamiento electroquímico, la alta selectividad del proceso de hibridación como evento de biorreconocimiento, y la alta sensibilidad y versatilidad de los principios de detección electroquímicos (p. ej., amplificación enzimática de la señal, medidas de impedancia en la interfaz electrodo-electrolito, medida de la transferencia electrónica mediada por moléculas que se unen o intercalan al ADN, entre otras estrategias).

La presente tesis tiene como objetivo el desarrollo de biosensores y plataformas sensoras de ADN con transducción electroquímica para la detección y cuantificación de soja genéticamente modificada. Se eligió como analito la soja en su variedad transgénica GTS 40-3-2, también conocida como Soja Roundup Ready® (SRR), por ser el OGM más extendido, ocupando actualmente el 75% de las plantaciones mundiales de soja. Los piensos utilizados para alimentar al ganado en la UE contienen un alto porcentaje de soja, en su mayoría importada de países que cultivan soja transgénica.

La tesis se estructura en ocho capítulos, de los cuales cinco se presentan como publicaciones, que comprenden una revisión bibliográfica y cuatro artículos de investigación publicados durante el doctorado. La tesis comienza con una introducción (Capítulo 1) en la que se realiza una revisión del estado de arte del tema, abarcando el estado de desarrollo y comercialización de los OGMs en Europa y las metodologías convencionales de detección de ADN, incluyendo una descripción general de los biosensores y plataformas sensoras electroquímicas. En el segundo capítulo, se presenta una revisión bibliográfica sobre los genosensores electroquímicos reportados para la detección de OMGs. Las conclusiones extraídas de esta revisión permitieron detectar las limitaciones de los trabajos anteriores para así proponer soluciones que contribuyan al avance de este campo. Los objetivos de la tesis expuestos en el Capítulo 3, están encaminados al diseño y desarrollo de métodos analíticos cuantitativos para el análisis de alimentos con soja transgénica, que permitan conocer la relación entre el contenido de dos secuencias, una específica de la inserción transgénica, y la otra específica del taxón o de la especie, presentes en muestras de distinta complejidad, desde harinas hasta alimentos altamente procesados. Para alcanzar este objetivo, se desarrolló un método basado en marcadores enzimáticos como sistemas de amplificación de la señal electroquímica y micropartículas magnéticas como plataforma de inmovilización. La hibridación de realizó mediante un formato tipo sándwich, que permite alcanzar un alto nivel de especificidad. Se propusieron dos variantes de este método: dos ensayos sencillos para detectar ambas secuencias individualmente, y un ensayo múltiplex para inmovilizar-hibridar-marcar simultáneamente y detectar secuencialmente ambos analitos.

Por otro lado y, con el fin de conseguir un dispositivo analítico sencillo y de fácil fabricación, se diseñó un biosensor libre de marcadores como una herramienta rápida y de bajo coste para la detección de soja transgénica, basado en el ensamblado molecular mediante la técnica de capa-por-capa de ftalocianina de cobre sobre electrodos nanoestructurados.

En el cuarto capítulo, se presenta el diseño y desarrollo de distintas plataformas sensoras para la detección de ambos analitos y su pre-validación utilizando mezclas sintéticas con un contenido de material transgénico similar al que establece el reglamento Europeo como límite para etiquetar el producto. En el capítulo 5, se describe

cómo se llevó a cabo el acoplamiento de las plataformas electroquímicas sensoras con el paso previo de amplificación por PCR. Se logró cuantificar con exactitud la cantidad de soja transgénica en material de referencia y muestras de harina con porcentajes conocidos de SRR. Nunca antes se había realizado con los genosensores electroquímicos propuestos para la detección de OMGs. Los resultados así obtenidos se correlacionaron con los registrados mediante PCR a tiempo real. En el capítulo 6, se avanzó un paso más consiguiendo la detección de fragmentos amplificados por PCR con la plataforma múltiplex. Se alcanzaron límites de detección inferiores y se consiguió cuantificar SRR en muestras con ADN degradado y de elevada complejidad, adquiridas en supermercados locales. Finalmente, en el capítulo 7 se presenta el diseño de una nueva estrategia basada en la espectroscopia de impedancia electroquímica con electrodos nanoestructurados modificados con capas moleculares de ftalocianina. Se presenta una caracterización completa de esta plataforma novedosa, así como su uso en la detección de ADN sintético de soja transgénica, sin requerir el uso de molécular marcadoras.

En definitiva, esta tesis engloba el desarrollo de métodos analíticos para la compleja tarea de detectar y cuantificar material genéticamente modificado bajo dos principios de detección: por un lado, la amplificación de la señal electroquímica utilizando marcadores y, por otro lado, la detección de la hibridación sin marcadores. Se alcanzaron los objetivos propuestos en la tesis y los retos analíticos inherentes a ellos, habiéndose demostrado la aplicabilidad real de los métodos desarrollados, basados en marcadores enzimáticos, utilizando matrices de alimentos. El método impedimétrico, por otro lado, libre de marcadores, permitió la detección de soja transgénica utilizando secuencias sintéticas.

LIST OF PUBLICATIONS

1. C. L. Manzanares-Palenzuela, B. Martín-Fernández, M. Sánchez-Paniagua López, B. López-Ruiz. **Electrochemical genosensors as innovative tools for the detection of genetically modified organisms.** *TrAC Trends Anal Chem.* 66 (2015) 19–31.

2. C. L. Manzanares-Palenzuela, N. de-los-Santos-Álvarez, M. J. Lobo-Castañón, B. López-Ruiz. **Multiplex electrochemical DNA platform for femtomolar-level quantification of genetically modified soybean.** *Biosens and Bioelect* 68 (2015) 259–265.

3. C. L. Manzanares-Palenzuela, I. Mafra, J. Costa. M. F. Barroso, N. de-los-Santos-Álvarez, C. Delerue-Matos, M. B. P.P. Oliveira, M. J. Lobo-Castañón, B. López-Ruiz. **Electrochemical magnetoassay coupled to PCR as a quantitative approach to detect the soybean transgenic event GTS40-3-2 in foods.** *Sensor Actuat B* 222 (2016), 1050-1057.

4. C. L. Manzanares-Palenzuela, E.G.R. Fernandes, M.J. Lobo-Castañón, B. López-Ruiz, V. Zucolotto. **Impedance sensing of DNA hybridization onto nanostructured phthalocyanine film-modified electrodes.** *Electrochimica Acta* 221 (2016) 86–95.

5. C. L. Manzanares-Palenzuela, J.P. Martin Clemente, M. J. Lobo-Castañón, B. López-Ruiz. **Electrochemical detection of magnetically-entrapped DNA sequences from complex samples by multiplexed enzymatic labelling: Application to a transgenic food/feed quantitative survey.** *Talanta* 164 (2017) 261-267.

LIST OF FIGURES

List of Abbreviations

DNA: deoxyribonucleic acid
dsDNA: double-stranded DNA
ssDNA: single-stranded DNA
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CRISPR-Cas: CRISPR-Associated Proteins/nucleases
CaMV: Cauliflower Mosaic Virus
PEP: phosphoenolpyruvate
P-FMV: Figworth Mosaic Virus 35S
nptII: Neomycine Phosphotransferase II
ISAAA: International Service for the Acquisition of Agri-Biotech Applications
EU: European Union
GM: genetically modified
EC: European Commission
EU-RL GMFF: European Union Reference Laboratory for GMO Food and Feed
ENGL: European Network of GMO Laboratories
JRC: Joint Research Centre
EIP-AGRI: Agricultural European Innovation Partnership
FEFAC: European Feed Manufacturers' Federation
RRS: Roundup Ready® soybean
EPSPS: Enzyme 5-Enolpyruvylshikimate-3-Phosphate Synthase
ELISA: Enzyme-Linked Immunosorbent Assay
Bt: (from) *Bacillus thuringiensis*
dNTPs: deoxynucleotide triphosphates
GC content: Guanine-Cytosine content
SYBR Green I®: (commercial name of) N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
FRET: Fluorescence Resonance Energy Transfer
DST: Double-Surface Technique
ITO: Indium Tin Oxide
FTO: Fluorine-Doped Tin Oxide
SAMs: Self-Assembled Monolayers
EIS: Electrochemical Impedance Spectroscopy
AC: Alternate current
DPV: Differential Pulse Voltammetry
SWV: Square Wave Voltammetry
CV: Cyclic Voltammetry
SWASV: Square Wave Anodic Stripping Voltammetry
DPASV: Differential Pulse Anodic Stripping Voltammetry

| CHAPTER 1 |

INTRODUCTION

1

INTRODUCTION

1.1. Genetically Modified Organisms (GMOs)

The advent and application of GMOs have undoubtedly revolutionized agronomic practices over the past 20 years. GMOs are defined as "organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination"[1]. The result is the expression of new, specific protein(s) conferring desirable feature(s) to the –genetically– modified crops, *e.g.* insect and herbicide resistance. Some benefits of genetic engineering in agriculture include increased crop yields, reduced costs for food production, reduced need for pesticides, enhanced nutrient composition and food quality, resistance to pests and disease, among others. Progress has also been made in developing crops that mature faster and tolerate environmental stressors, allowing plants to grow in conditions where they might not otherwise flourish[2]. All of which is aimed at facing the critical challenge of producing sufficient food for a growing human population living in a changing and unstable climate[3].

Advances in the field of genetic engineering have allowed for precise control over the genetic changes introduced into an organism. In a broad sense, this is achieved by selecting and extracting genes of interest normally from other organisms, such as bacteria, and inserting the desired DNA fragment into the plant genome[2]. This plant-breeding process is called *transgenesis* and it refers to the incorporation of foreign/new genes from one species into a completely unrelated species. Other types of GMOs

involving genetic material from closely related species or from the same species are discussed ahead. Genetic transfer is commonly achieved using *Agrobacterium tumefaciens* (biological vector) or biolistic (particle-bombardment) technologies[4]. Although both methods have been practiced for more than three decades now, recent contributions in genome editing techniques, *e.g.* the CRISPR–Cas9 tool, have dramatically enhanced plant genome research and transformation in recent years[3, 5].

The use of a biological vector is the most frequent transformation method, which involves the infection of the host plant by *Agrobacterium* strains leading to genetic transfer from the bacterium and integration into the plant nuclear genome. Fig. 1 shows a simplified illustration of this process. The transferred DNA (T-DNA) naturally resides on the Ti-(tumor inducing) (*1*) or Ri-(root inducing) plasmid, but in the laboratory, T-DNA can be "launched" from binary vectors (*2*) or from the bacterial chromosome (*3*)[3, 6]. *Agrobacterium*-mediated transformation consists of a complex process comprising a series of biochemically-triggered routes that allow for T-DNA transfer to occur: (*a*) after bacterial attachment to the plant cell, induction of virulence (*vir*) genes takes place forming a site-specific nuclease that nicks the T-DNA region at border sequences; (*b,c*) by covalently linking to single-stranded T-DNA, a *vir* protein complex leads T-DNA into the plant by a secretion system; (*d*) T-DNA/protein complexes target the nucleus of the plant; (*e*) once inside, proteins are stripped from T-DNA; (*f*) integration takes place into the plant chromosomes, resulting in stably transformed cells[3].

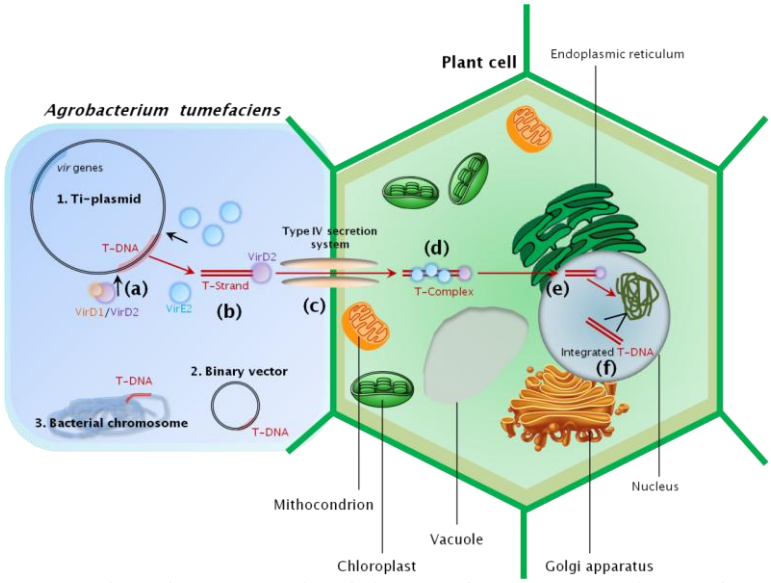

Fig. 1. Simplified illustration of *Agrobacterium*-mediated plant transformation initiated *via* Ti-plasmid (*1*) (binary vector (*2*) or bacterial chromosome (*3*) are other examples) containing the transgenic construct (T-DNA): (*a*) *VirD1/VirD2* endonucleases nick T-DNA at border sequences, releasing single-stranded T-DNA. (*b*) A T-stand/*VirD2* complex is formed

and (*c*) introduced to the plant cell by a Type IV secretion system. (*d*) The T-complex formed also by *VirE2* proteins enters the plant cell. Finally, (*e*) T-DNA penetrates into the nucleus and (*f*) it is integrated in the plant chromosome. Adapted from ref. 3.

Biolistic –the result of combining biological and ballistic- delivery, also known as "particle bombardment" or "gene gun technique", consists on the acceleration of DNA-coated high density carrier particles into tissues (or cells), by a high-voltage electric spark or a helium discharge. The particles are usually heavy metal microparticles (usually gold beads) of approximately 1–1.5 μm in diameter, which are smaller than a plant cell, functionalized with genetic constructs containing the trait gene(s) desirable for crop modification. 'Naked' DNA is then gradually released within the cell post-bombardment, resulting in the integration of DNA into the host genome and ultimately in gene expression (Fig. 2). There are commercially available hand-held gene guns to perform this transformation method. Protoplasts, organized tissues like meristems (a group of non-differentiated cells with active mitosis), cells, embryos or callus (vegetable tissue with disorganized growing) can be used as target. Gene delivery using biolistics is a useful mechanism to transfect DNA into cells that cannot readily be transferred by other methods. However, *Agrobacterium*-mediated –indirect- transformation offers more advantages in terms of transformation efficiency, transgene copy number, expression, inheritance, etc[7-8].

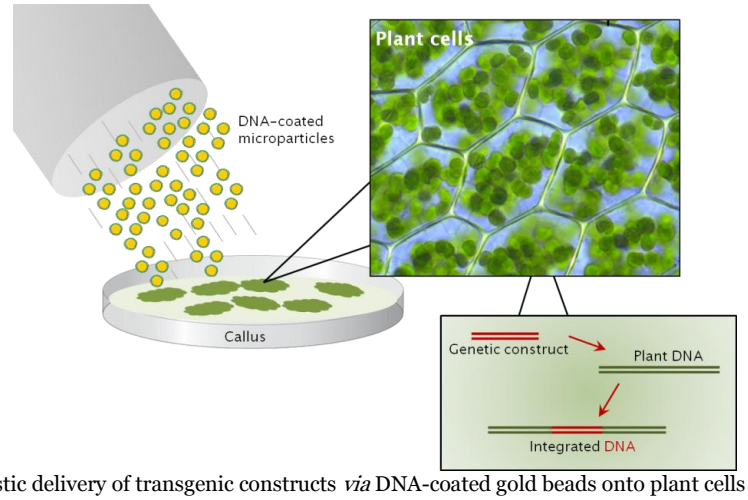

Fig. 2. Biolistic delivery of transgenic constructs *via* DNA-coated gold beads onto plant cells from callus.

Vector constructs for plant transformation contain several genetic elements required for insertion into the plant genome. In addition to the trait gene(s), *i.e.*

sequences that are intended to be inserted into the target organism to confer the desirable trait, a vector construct includes promoter and terminator sequences that enable the plant to express the gene of interest. Promoters are regions of the DNA upstream of a gene's coding region that contain specific sequences recognized by proteins involved in the initiation of transcription[9]. One source of such promoters is CaMV, which is a double-stranded DNA virus affecting plants in the Cruciferae, Resedaceae and Solanaceae. The 35S promoter of CaMV is a functional, well-characterized, and constitutively expressed promoter that enables high levels of gene expression in the host organism. Hence it has been incorporated into numerous constructs and used to produce many of the genetically engineered crops commercially used today, such as maize, soy, canola, and papaya. Other promoters, such as PEP carboxylase promoter, which encodes a photosynthetic enzyme, and P-FMV, are used less frequently in GMOs[9-10]. The NOS sequence from the *Agrobacterium tumefaciens* nopaline synthase gene serves as a polyadenylation site (terminator sequence indicating the end of transcription) in many constructs[11].

According to Holst-Jensen *et al.*[12-13], GMOs can be classified into four generations based upon the origin of the inserted genetic elements (Fig. 3):

a. **First generation**, obtained by insertion of fully transgenic constructs, *i.e.* those involving genetic elements (promoters, genes and terminators) from species other than the recipient taxon. This generation represents the most of the present commercial GMOs and will be the focus of this thesis. The cloning vectors usually also contain marker genes meant to confer an easily detectable characteristic to the successfully transformed cells, such as the ability to survive against specific antibiotics. This allows the selection and propagation of those cells in which the vector had been transfected[12], *e.g.* neomycin-kanamycin resistant gene called nptII gene.

b. **Second generation**, represented by the so-called stacked GMOs, which consists of hybrid crosses between two or more events (*e.g.* Bt11 × GA21 maize) (not shown in Fig. 3).

c. **Third generation**, or the so-called near-intragenics GMOs, in which the major part of the insert is derived from a closely related, sexually compatible species and

the recombinant part of the insert is very restricted (*e.g.* limited to short segments derived from the cloning vector). The high-amylopectin potato line named 'Modena' (AV43-6-G7) is an example of this type of GMO[14].

d. **Fourth generation**, which includes true intragenics and in particular cisgenics. In the latter, the inserted elements are derived from the recipient species itself. These technologies have been successfully exploited to obtain the cisgenic Arctic™ "Golden Delicious" and "Granny Smith" apples (Okanagan Specialty Fruits Inc., Summerland, BC, Canada), a cisgenic alfalfa with altered lignin production (Monsanto) and the intragenic potatoes of the Innate™ line (J.R. Simplot Co., Boise, ID, USA) that are currently cultivated for commercial purposes[5].

Currently, intragenic/cisgenic plants are regulated as transgenic plants worldwide[14], although the regulation of these crops is presently under evaluation in the EU.

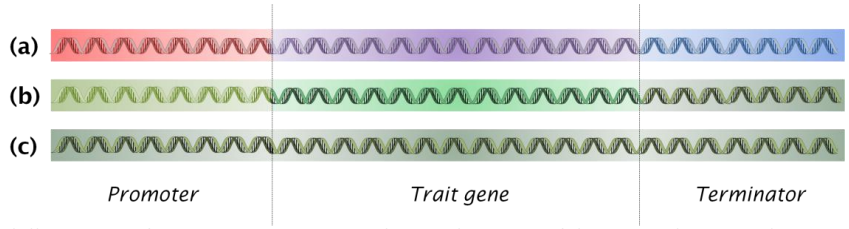

Fig. 3. Simplified illustration of GMO generations according to the origin of the inserted genetic elements (adapted from ref. 13). Genetic sequences distantly related to the taxon recipient are shown as red, purple and blue shaded sequences for virus, fungus and bacterium origin, respectively. DNA from crossable species is shown in various tones of green. (a) Fully transgenic construct where promoter, trait gene and terminator elements are distantly related; (b) Intragenic or nearly-intragenic, where each element is from a closely related, sexually compatible species; and (c) cisgenic construct made through genome editing within the same modified species.

According to ISAAA[15], 179.7 million hectares of biotech crops (mainly transgenic - 1st and 2nd generation-) have been cultivated in the world until 2015, a year that marked the 20th anniversary of the commercialization of biotech crops. An unprecedented cumulative hectarage were cultivated globally, from 1.7 million hectares in 1996 to a 100-fold increase last year, which makes it the fastest adopted crop technology in recent times. The United States (US) is the lead country with 70.9 million hectares (39% of global) with over 90% adoption for the principal crops of maize (92% adoption), soybean (94%) and cotton (94%). Brazil is the second largest grower globally with 44.2 million hectares (25%), followed by Argentina, India and Canada. Fig. 4

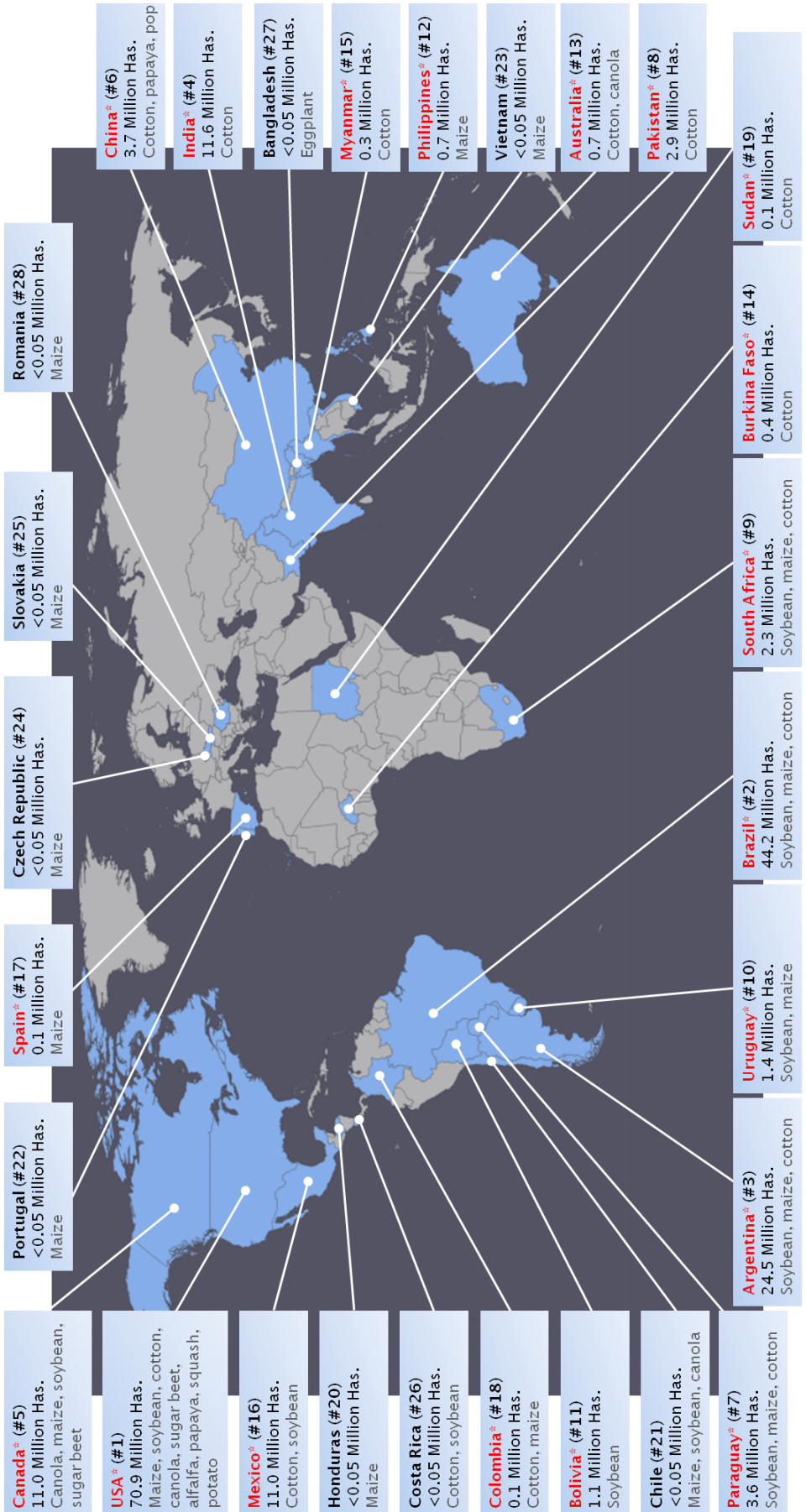

Fig. 4. Global distribution of biotech crops and country ranking by cultivated areas in 2015. *Mega-countries (red) growing ≥50 thousand hectares of biotech crops. Adapted from ref. 15.

shows the global distribution of planted biotech crops.

Stacked traits occupy 33% of the biotech crops cultivated worldwide and are thought to be favored by farmers in all countries given their increasing adoption in the last years. Herbicide resistance is the most planted trait (53%) and insect resistance occupied 14% of the global cultivated area. Soybean is the most planted GMO (51%), followed by maize (30%), cotton (13%), canola (5%) and others (1%)[15].

1.1.1. GMOs in Europe: Public opposition and stringent legislation

The rapid adoption of transgenic crops in the US, Argentina, and Canada stands in strong contrast to the situation in the EU where there is a high level of consumer rejection and strict legislation concerning official approval[16]. The arrival of the first shipments of GM soy in Europe from the US in 1996 was met by intense protests from environmental nongovernmental organizations framing GMOs as a threat to biodiversity, farmer autonomy and food safety[17]. Opposition to GMOs has been based on concerns about the potential impact of releasing transgenic crops into the environment ranging from gene flow, to the development of insect resistance, to impacts on nontarget organisms. Health-related concerns include possible transfer of antibiotic resistant genes to bacteria in the gastrointestinal tract, toxicity (presence of anti-nutrients) and allergenicity of foods derived from GMOs[16, 18].

Safety studies regarding toxicity of GM food and feed for consumption are usually subchronic (90 days) trials. A recently published review of the latest toxicity studies conducted with some GM plants (soybeans, rice, maize and wheat) concluded that these should be as safe as their non-GMO counterparts when used in feed or human food[19]. However, the author highlighted the fact that in long-term studies[20] the results have been highly controversial. There is clearly a lack of consensus on GMO-related risks between authorities, manufacturers and some independent researchers. However, despite of this lack of agreement, there are other important factors influencing the skeptical attitude of consumers towards GMOs: when transgenic products first went on

sale in Europe, one of the main determinants of consumer hostility was the lack of a clear labeling policy[16].

As a result of all of the above, the EU has established one of the strictest legal frameworks for regulation of biotech crops aimed at: establishing safety assessment before any GMO is placed on the market, drawing harmonized procedures for risk assessment and authorization of transgenic events, setting labeling thresholds for GMOs placed on the market in order to provide freedom of choice to consumers as well as professionals *(e.g.* farmers, and food feed chain operators), and establishing procedures to ensure the traceability of GMOs on the market.

The building blocks of the European GMO legislation are:

- **Directive 2001/18/EC**: procedure for granting consent for the deliberate release of GMOs into the environment. Such consent is limited to a period of 10 years (renewable) and introduces compulsory monitoring after GMOs have been placed on the market.
- **Regulation (EC) 1829/2003**: rules on how GMOs are authorized and supervised (safety assessments) and on mandatory labeling.
- **Directive (EU) 2015/412** (amending Directive 2001/18/EC): refers to the possibility for the Member States to restrict or prohibit the cultivation of GMOs in their territory. The following countries have placed bans on the cultivation and sale of GMOs so far: France, Germany, Austria, Hungary, Greece, and Luxembourg.
- **Regulation (EC) 1830/2003**: concerns the traceability and labeling of GMOs and the traceability of food and feed products produced from GMOs at all stages of the supply chain.
- **Directive 2009/41/EC:** refers to the contained use of genetically modified microorganisms.
- **Regulation (EC) 1946/2003:** concerns transboundary movements of GMOs, *.i.e.* GMO exports to non-EU countries.

According to Regulation (EC) 1829/2003, all products containing GM-based materials must be labeled when the content of any authorized GM ingredient exceeds

0.9% of the food/feed ingredients when considered individually. Below this threshold, labeling is not mandatory provided that the presence of GM material is proven to be accidental or technically unavoidable. For non-authorized GM ingredients, the threshold is set at 0.5%, given that the source of the GMO has been pre-evaluated and an appropriate detection method for its presence is available. For GMOs in feed for which an authorization procedure is pending or the authorization of which has expired, recent EU regulation is setting the non-compliance limit to 0.1 %.

Traceability is defined as the ability to track GMOs and products produced from GMOs at all stages of the production and distribution chain. In this sense, sellers have to inform trade buyers in writing that a product contains GMOs with their unique identifiers (event names), specifying each GM-derived ingredient. Final consumer packaging or pre-packaged products containing GMOs should be labeled: '*This product contains genetically modified organisms [or the names of the organisms]*. EU countries must carry out inspections, sample checks and tests, to ensure the rules on GMO labeling are complied and it is also mandatory that each country imposes effective penalties for infringements. The EU-RL GMFF is in charge of the scientific assessment and validation of detection methods for GM food and feed as part of the EU authorization procedure and the coordination of the national reference laboratories for GMO in the member states. The EU-RL GMFF is supported by ENGL and hosted by JRC.

Currently there are 55 GM events registered in the EU, most of them authorized for their use in foods/feed and food/feed ingredients containing, consisting of, or produced from transgenic crops. In Fig. 5, a schematic representation of authorized events in the EU is shown. The majority of these authorized events are stacked traits combining herbicide tolerance with insecticide resistance. In addition, there are currently 9 products (6 maize and 3 swede rape events) subject to the decisions made by the EC on withdrawal from the market. There are also 28 pending authorizations, many of which are stacked traits.

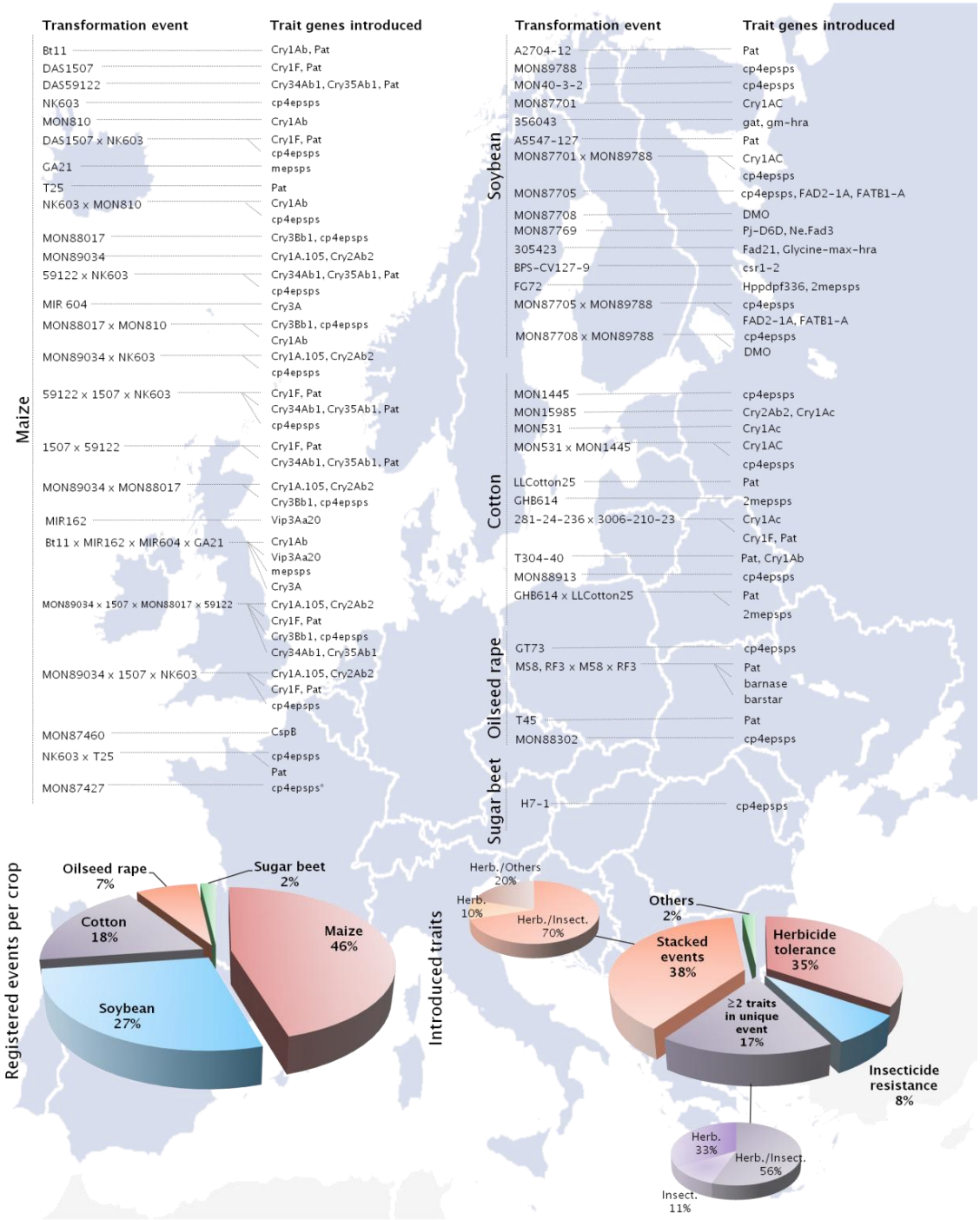

Fig. 5. Schematic representation of the transgenic events registered in the EU: listing of events per crop and their introduced genes (above). Pie charts (below) showing distribution of events (%) per crop (left) and of the type of traits (%) (right). A comprehensive thesaurus on trait genes can be found in https://isaaa.org/gmapprovaldatabase/geneslist/default.asp

Due to the highly restrictive regulatory environment and a growing support for organic farming and local food production, just a single GM plant, the insect resistant maize MON810, is authorized for cultivation in the EU. Spain is the only European country with significant plantings of this GM crop (Fig. 4) occupying more than 30% of

the total Spanish maize area. Four other EU countries (Portugal, Czech Republic, Romania, and Slovakia) grow a limited amount of GM maize[17].

1.1.2. Genetically engineered soybean: event GTS 40-3-2 or Roundup Ready® Soybean

Soybean (*Glycine max)* is a legume widely used as feed ingredient for animal breeding given its high protein content (≈ 40%) with high ileal lysine digestibility, as well as its relative low cost. In addition, it is part of the 3% of the daily protein intake of European consumers[21]. According to EIP-AGRI[21], a high percentage of soybean is present in the compound feed used in the EU, especially for monogastric animals, *i.e.* 37% for broilers, 29% for pigs, 22% for layers, 10% for dairy cattle and 14% for beef cattle. In total, around 60 % of the protein source in animal feed comes from soybean meal.

Around 478 million tons of feedstuffs are consumed by EU livestock on a year basis, according to FEFAC[22]. Of this amount, 233 Mt are roughages being produced on-farm and 245 Mt are compound feed. The latter is manufactured from a mixture of raw materials designed to achieve pre-determined performance objectives among animals. While some raw materials are obtained from the co-products of the food industry, other important ingredients which cannot be grown in sufficient quantity in the EU are imported from third countries. Such is the case for soybean.

The EU is almost 70% dependent on imports of feed ingredients, and for soybean meal this figure is over 97%[21]. The lack of wide adaptation to northern latitudes leading to low and unsteady yields explains why this crop is mainly bred outside Europe[23]. Most soy imports come from Argentina, USA and Brazil and are genetically modified varieties, resulting in the need for traceability within the EU legal framework.

Among GM soybean varieties, line GTS 40-3-2, commercially known RRS, dominates the market contributing 94% of the entire soybean production in the US. Globally, RRS crops account for 75% of the total soy production[24-25]. This biotech crop was the first-generation glyphosate-tolerant GM-soy produced and patented by Monsanto Company, which began to be commercially grown in 1996, quickly becoming

a predominant trait. It was genetically modified to tolerate exposure to glyphosate-based herbicides during the entire growth season.

Glyphosate, the active ingredient of Roundup®, is an herbicide used worldwide as a non-selective weed control agent. Glyphosate acts as a competitive inhibitor of the enzyme EPSPS, an essential enzyme of the shikimate biochemical pathway involved in the production of the aromatic aminoacids phenylalanine, tyrosine and tryptophan. The inhibition of EPSPS results in growth suppression and plant death. The development of glyphosate-tolerant soy has allowed the use of glyphosate as an alternative weed control system in soybean production. As a result, the farmer may eradicate all kinds of plant weeds by spraying with glyphosate, and not harm the GM crop plants[24].

The development of GTS 40-3-2 was based on recombinant DNA technology through the introduction of a gene encoding for EPSPS, isolated from *Agrobacterium tumefaciens* strain CP4, into the commercial soybean variety "A5403" (Asgrow Seed Company)[26]. The A5403 variety was transformed by means of gold particle bombardment using the PV-GMGT04 plasmid vector harvested from *Escherichia coli*. This plasmid contained the CP4 EPSPS gene, the gus gene for production of ß-glucuronidase as a selectable marker, the nptII gene for antibiotic resistance (kanamycin) and other common regulating sequences (Fig. 6 –above-).

The original selected transformed cells showed two sites of integration, one with the gus selectable marker and the other with the glyphosate tolerance gene. These two sites subsequently segregated independently in the following sexual generation. Upon analysis, line GTS 40-3-2 was found to contain just one insertion site, in which only the glyphosate tolerance gene is integrated[26-27]. In the genetic construct, the EPSPS gene is under the regulation of CaMV 35S and terminates with NOS (Fig. 6 –below-). A plant-derived DNA sequence coding for a chloroplast transit peptide (CTP4 from *Petunia hibrida*) was cloned at the 5' of the glyphosate tolerance gene in order to facilitate the import of the newly translated enzyme into the chloroplasts, where both the shikimate pathway and glyphosate sites of action are located[26].

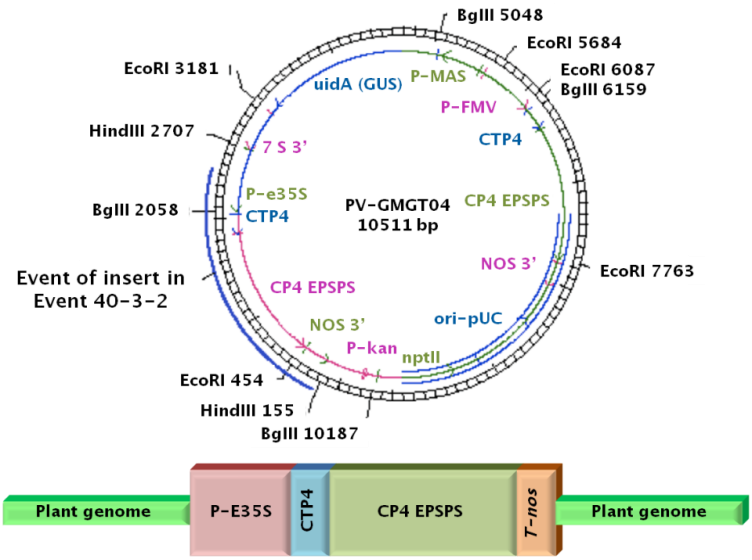

Fig. 6. Plasmid PV-GMGT04 introduced in A5403 cells by particle bombardment (above) and the resulting cassette inserted in the plant genome (below). Adapted from ref. 26.

Often, cells transformed *via* particle bombardment contain rearranged and truncated transgene fragments besides the intact transgene copies. Windels *et al.*[27] characterized the genetic arrangement of line GTS-40-3-2 and found that no major rearrangements occurred at the 35S border during integration of the insert DNA and that plant DNA is present immediately adjacent to the 35S promoter end-point. In contrast to the junction structure at the NOS border site, in which a 254 bp portion of truncated CP4 EPSPS coding sequence is present. This 254 bp DNA segment is followed by an unknown DNA segment of 534 bp, followed by adjacent plant DNA. Characterizing the resulting inserts of the transformed lines aids the further design of target sequences for GMO detection, as will be discussed in the next section.

1.1.3. GMO detection and quantification

Threshold labeling levels set in different countries vary from 0 to 5%. They are either mandatory (Australia, Brazil, Chile, China, EU, India, Indonesia, Israel, Japan, Philippines, Russia, Saudi Arabia, South Korea, Taiwan and Thailand) or voluntary (Argentina, Canada and USA)[28]. This fact has driven the need to develop analytical methods able to detect and quantify GMOs in different types of samples, from raw material (agricultural crops) to food and feed commercial samples. The most common detection approach relies on the knowledge that part of the genetic information in GM

plants differs from that of the wild type line. Thus, the genetic modification is by definition detectable at the DNA level. But also, another approach involves the detection of the protein(s) encoded by the inserted trait gene *via* immunoassays such as ELISA. However, given their –generally- lesser stability, proteins are often not considered suitable for GMO detection in a wide range of products (*e.g.* processed food/feed)[12].

According to the DNA targets present in GMOs, DNA-based methods can be categorized into different levels of specificity (Fig. 7)[12]:

a. **Screening methods**, which are the least specific methods because the targets include common DNA elements in GMOs, such as promoters and terminators that are present in many different events. Sometimes, marker genes are also used as screening targets, such as the nptII gene.
b. **Gene-specific methods**, which detect a part of the trait gene associated with the specific genetic modification. Examples are the Bt or the CP4 EPSPS genes. If a positive signal is obtained, the presence of GM-related sequences is highly probable, but it is not possible to identify the specific GM crop because the trait gene can be used in different transformation events. Both screening and gene-specific methods are based on DNA sequences present in nature and that significantly increases the risk of obtaining false positives.
~~c.~~ **Construct-specific methods**, which target the junction between two DNA elements, such as the promoter-trait gene or trait gene-terminator. These methods target DNA sequences that are not present in nature. However, different GMOs may share the same constructs. Such is the case for two distinct GMO maize, MON809 and MON810, which have the same promoter-trait gene junction.
d. **Event-specific methods,** which provide the highest level of specificity because the target is the unique junction, characteristic of each event, found at the integration locus between the inserted DNA construct and the recipient genome. Although, stacked events cannot be distinguished with these methods.

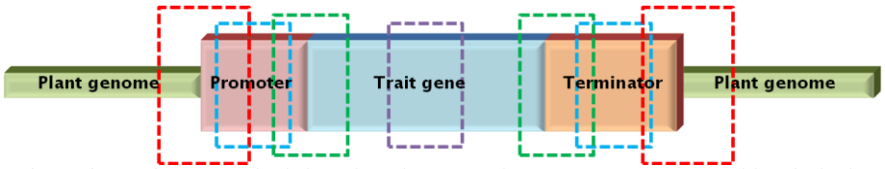

Fig. 7. Levels of specificity of GMO methods based on the targeted DNA region: screening (blue dashed square), gene-specific (purple square), construct-specific (green) and event-specific (red). Adapted from ref. 12.

Qualitative detection methods can be used as an initial screening of food products, to investigate whether GMO specific fragments are present. Qualitative analysis could thus be performed on packaged products sampled from the shelves of supermarkets, from stocks at the supply chain or from raw material. If the qualitative analysis provides an indication of the presence of GMOs, a subsequent quantitative test might give a decisive answer concerning the labeling requirement[29].

In the EU, legislation on GMO labeling drove analysts to harness the initially complex analytical challenge of quantifying GMOs. When implemented, the legal tolerance level did not explicitly specified which measurement units were to be used to calculate the final GMO content in a sample[30]. In 2004, the EU Recommendation 2004/787/EC proposed that this should be done in terms of DNA copy number, *i.e.* results should be expressed as the ratio of event-specific DNA copy numbers in relation to the target taxon-specific DNA copy numbers, calculated in terms of haploid genomes[31]. This is because the labeling threshold was established for each individual ingredient, so that quantification is based on each GM ingredient in proportion to the global amount of the same ingredient, *e.g.* GM soybean in proportion to the total amount of soybean. This has to be carried out with event-specific methods.

The GMO analytical procedure can be approached as a modular process starting with sample collection and including all steps performed to determine the presence, identify and quantify (when necessary) GMOs until finally a measurement result is provided (GMO %). Accordingly, sample preparation, DNA extraction and detection of individual target sequences can be treated as separate modules that together form a method. A module can therefore be defined as a distinct and limited operation, each of which involves its own input and output material/data. In GMO monitoring, the following modules are usually performed (Fig. 8): 1) a sample preparation module where the input material is processed to its homogenized form, *e.g.* grains to flour; 2) a

DNA extraction and purification module where the input material is the homogenized sample and the output material is purified DNA in aqueous solution; 3) a detection module where the input material is purified DNA in aqueous solution and the output material is measurement data, *e.g.* collection of fluorescence data and translation into a number of target sequence copies; and 4) a data evaluation module, *e.g.* the number of copies of the taxon-specific and event-specific targets are processed into a final quantitative result[12].

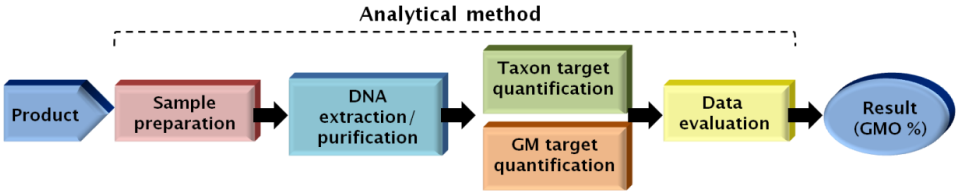

Fig. 8. GMO analytical procedure. Adapted from ref. 12.

The most commonly accepted and used analytical methods for identification and quantification of GMOs are based on the Polymerase Chain Reaction (PCR). PCR is a powerful technique in which a specific DNA region is delineated and amplified into billions of copies (amplicons) mimicking the basic mechanism of DNA replication, *i.e.* making use of the ability of DNA polymerase to synthesize new strands of complementary DNA from a template strand. The technique enables large amounts of DNA to be produced from very small amounts of starting material. Not only can DNA be amplified to levels detected by conventional methods (*e.g.* gel electrophoresis and imaging) but it also allows the selection of specific segments occurring at low frequency in a complex mixture of other DNA sequences, by incorporating a minimum of two oligonucleotides primers designed to flank the region of interest.

The amplification process comprises a series of temperature-dependent steps, for which specific instrumentation (thermal cycler) is required. These steps are illustrated in Fig. 9. Amplification takes place in repeated cycles made up of three defined stages, namely denaturation, annealing and extension. In the first stage the template DNA is heated usually at 90-98 ºC to separate the double stranded DNA in order to generate two single strands. This is followed by annealing of the primer sequences, which takes place typically at 45-65 ºC. Primers are designed to hybridize to the opposite strands flanking the sequence of interest. After primer hybridization, a fixed temperature of

generally 72 ºC is programmed for DNA polymerase-mediated polymerization or extension of the sequence located between the primer pair, using free nucleotides as building blocks. This enzyme is able to withstand the high denaturation temperature. It was initially isolated from *Thermus aquaticus* found in hot springs, giving it the name of Taq DNA polymerase.

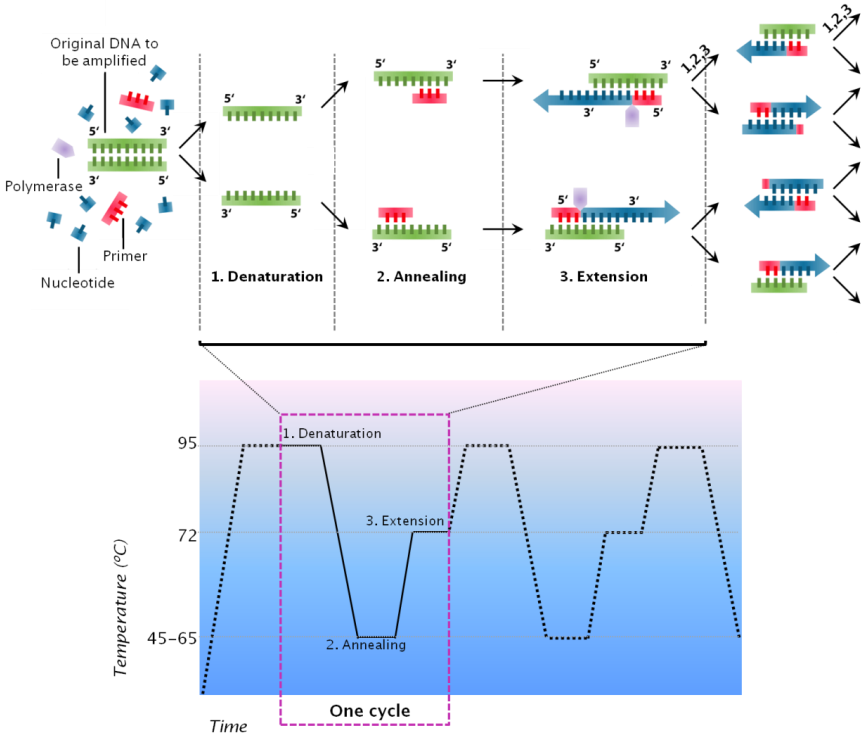

Fig. 9. Temperature-dependent stages of the PCR reaction.

After each cycle, the newly synthesized DNA strands can serve as templates in the next cycle. In the first round of amplification, the products are heterogeneously sized DNA molecules with lengths that may exceed the actual size of the target sequence. In the second round, these molecules start to generate DNA strands of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Thus, amplification is conventionally expressed by the following equation: $(2^n-2n)x$, where n is the number of cycles, $2n$ is the first product obtained after the first cycle and second products obtained after the second cycle with undefined length and x is the number of copies of the original template[32].

A PCR of only 20 cycles amplifies the initial template DNA over a million-fold (assuming 100% reaction efficiency). With this remarkable gain there is also potential for considerable errors, *e.g.* a 95% efficient PCR will only amplify the original DNA over

600,000-fold[33]. The necessary number of amplification cycles depends on the starting concentration of the target DNA: in order to amplify 50 target molecules, 40 - 45 cycles are recommended, whereas 25 - 30 cycles are enough to amplify $3x10^5$ molecules to the same level[34]. The non-proportionality behind PCR kinetics is owed to the so-called plateau effect, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when the product reaches 0.3-1.0 nM and, theoretically, all of the samples will reach the same total amount of amplified DNA[32-33]. The plateau phase is caused by degradation of reactants (dNTPs, enzyme), reactant depletion (primers, dNTPs), end-product inhibition (pyrophosphate formation), competition for reactants by non-specific products, competition for primer binding by re-annealing of the concentrated (10 nM) product[34]. This phase is usually reached after a high number of cycles (usually >35-40 cycles), yet it depends on the sample and on the amount of template DNA. This has important implications for quantification using end-point PCR, *i.e.* detecting amplicons after the reaction is stopped usually after an elevated number of cycles. The different phases of PCR are shown in Fig. 10.

The amplification products are generally visualized through agarose gel electrophoresis after staining with an intercalating dye that fluoresces upon binding to dsDNA. Imaging software is usually required for band intensity analysis (*i.e.* densitometry)[33]. Gel electrophoresis is normally used for qualitative detection of PCR-amplified DNA based on size determination, but it used to be the gold standard for quantification as well. One of the first developed PCR-based quantitative method for GMOs involved gel electrophoresis with a competitive quantification strategy[35]. This method was based on the co-amplification of target DNA template and defined amounts of an internal DNA standard (competitor) carrying the same primer binding sites. Since the initial amount of the competitor is known, and given that the amplification efficiencies of the target and competitor DNA are the same, the ratio of the amounts of the two PCR products determined by *e.g.* gel electrophoresis, is representative of the ratio of target DNA and competitor present in the reaction mix pre-amplification[36]. However, the problems with gel-based quantification after end-point PCR rely on several aspects: on one hand, densitometry has limited dynamic range and lacks sensitivity and reproducibility[33]; on the other hand, by quantifying PCR products at the end of the reaction after a high number of cycles, most likely the

resulting correlation between the final product concentration and the number of initial target molecules is inaccurate[33, 36] given the saturating nature of the PCR reaction. Other forms of post-PCR detection may overcome some of these limitations provided that the method is sensitive enough to be able to stop the amplification reaction at a lower cycle number so that an appropriate correlation between starting DNA amount and measured amplicon signal can be established.

At present, real-time PCR (herein referred as 'qPCR', from quantitative PCR) has become the reference method for DNA quantification and, as such, it is the most commonly used technology for quantification of GMOs. This technique relies on fluorescence-based detection of amplicon DNA as it forms during PCR and allows the amplification kinetics to be monitored in real time (Fig. 10), making it possible to reliably quantify DNA in the exponential phase of amplification.

Fluorescence is measured after each temperature cycle and is proportional to the amount of synthesized amplicon. The exponential growth of the amplicon concentration in the reaction mixture at cycle n, X_n, can be described as an exponential function of the template starting concentration, X_o; the efficiency of the qPCR, E; and the number of qPCR cycles, n: $X_n = X_o\ (1 + E)^n$. Two parameters are essential for quantification: the threshold cycle, C_T, and the qPCR E. The C_T is the number of cycles necessary to reach a certain fluorescence threshold (cutting threshold in Fig. 10). In one experimental setup, the cutting threshold is the same for all samples. Since fluorescence is a relative measure of the DNA content, all samples contain the same number of amplicons when passing the C_T. The quantitative parameter is the C_T value as this will increase with decreasing amounts of template DNA[37].

E is a measure of amplification quality and depends on factors such as the primer GC content, primer mismatches and the presence of PCR inhibitors. If E equals 2, the number of amplicons doubles per cycle, *i.e.*, the efficiency is 100%[37]. Two distinct methods can be used to estimate E: E_{fi} is the efficiency estimated from the fluorescence increase using linear[38-39] or nonlinear regression models[40] and E_{ds} is the efficiency estimated from the slope of a dilution series. The latter is the most common approach[37] (see inlets in Fig. 10).

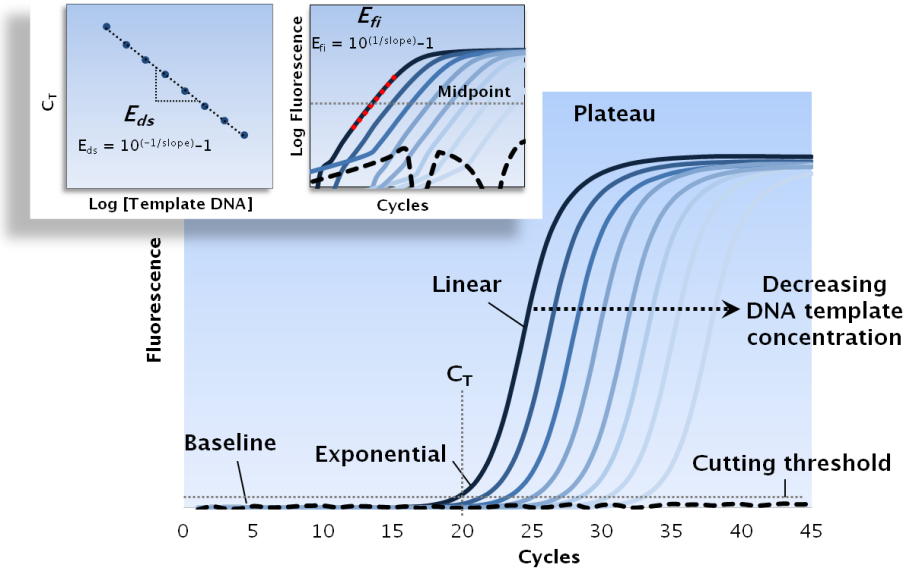

Fig. 10. Real-time PCR amplification curve with its different phases (inlet: efficiency determination *via* two methods: from the slope of a dilution series in the calibration plot (E_{ds}) and from the fluorescence increase (slope) in the linear phase (E_{fi}) of the amplification plot.

The simplest and cheapest principle for fluorescence real-time data acquisition is based on the binding of fluorescent dyes (*e.g.* intercalation/binding) to dsDNA (Fig. 11A). There is a large family of commercially available cyanine dyes frequently used in qPCR, *e.g.* SYBR Green I®. Dye-based qPCR can be easily applied to already established PCR assays. However, specific and nonspecific PCR products are both detected with this approach, thus melting curve analysis is required to differentiate specific fragments from by-products[41-42]. This type of assays has been widely described for GMO detection[43-44].

Amplicon-related fluorescence can also be monitored with more specific strategies: either *via* hybridization of one (molecular beacon) or two (hybridization probes (*e.g.* FRET probes) to the amplicon or involving probe cleavage (hydrolysis probes, *e.g.* Taqman® probes)[45]. FRET probes hybridize with the central region of the amplicon in the annealing phase. FRET system takes place after hybridization by the quenching of the donor and the sensitization of the acceptor fluorescence (Fig. 11B). Hydrolysis probes -dually labeled with a quencher molecule in one end and a fluorophore in the other end- hybridize with the central region of the amplicon; during extension, DNA polymerase hydrolyzes the probe and fluorescence emission takes place (Fig. 11C). Fig. 11 illustrates some of the most common real-time PCR chemistries.

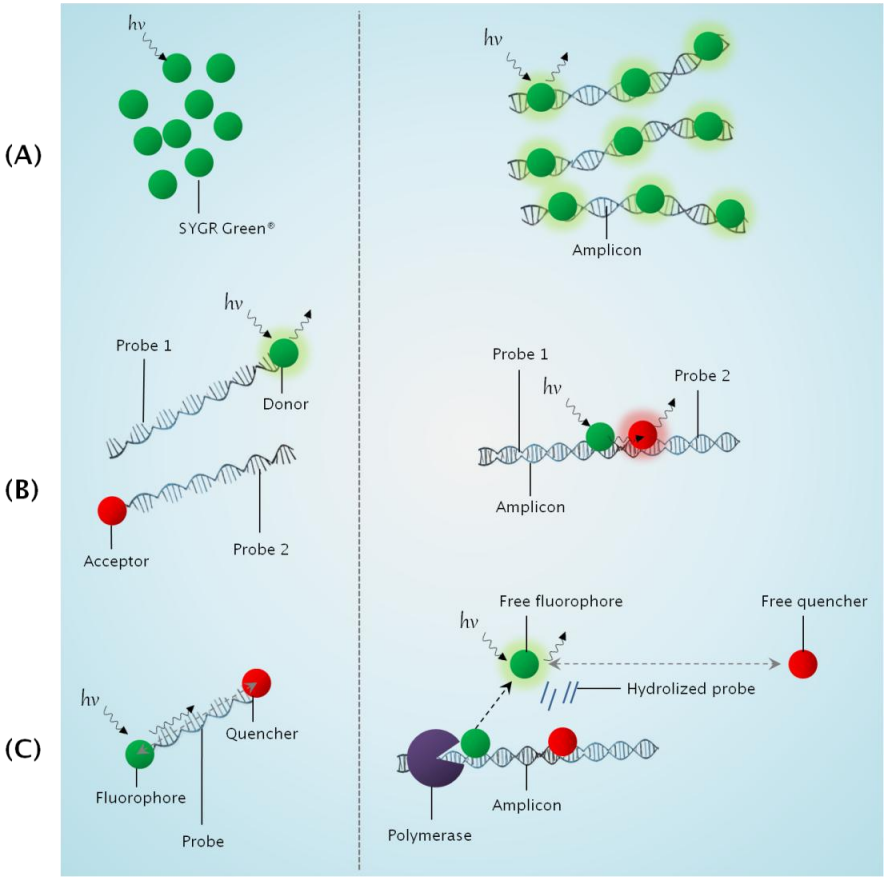

Fig. 11. Common real-time PCR chemistries: (A) Intercalating/binding dyes, (B) hybridization FRET probes, and (C) hydrolysis probes.

Introduction of additional probes increases the specificity of the quantified PCR product and allows the development of multiplex reactions. Beside these four main principles, other technologies have been described, *e.g.* hairpin primer-probes[41]. Most of the qPCR methods that have been validated by the EU-RL GMFF are based on hydrolysis probes.

In spite of the fact that qPCR technology is so far the method of choice for GMO detection and quantification, its application in the simultaneous detection of several targets is somewhat limited. Moreover, qPCR-based systems are often too expensive for resource-limited environments. Alternative PCR-based strategies as well as combinations of conventional PCR with hybridization or capillary electrophoresis have been explored and have resulted in promising alternatives capable of overcoming the drawbacks linked to qPCR technology[26]. Hybridization-based approaches, *e.g.* microarrays and biosensors, have been widely developed for GMO monitoring given their high level of specificity as these methods rely on hybridization of specific probes

with the selected targets, usually after amplification by conventional PCR or isothermal systems. Detection is often based on optical, piezoelectric and electrochemical techniques[46-48].

All of these techniques, including PCR-based methods, are based upon the knowledge of sequence composition of the transgenic constructs and integration sites. Other types of methods are available for 'unknown' genetic regions (*e.g.* unauthorized events), such as next generation sequencing[13]. This type of methods falls out of the scope of this thesis.

1.2. Electrochemical DNA detection by biosensors and sensing systems

By revisiting the basic definition of a 'chemical sensor' from nearly 40 years ago - 'a device that transforms chemical information into an analytically useful signal'-, biosensors can then be defined as chemical sensors in which the recognition system utilizes a biochemical mechanism. In general, biosensors contain usually two basic components connected in series: a biochemical (biomolecular) recognition system (receptor, probe) and a physicochemical transducer (electrode, in electrochemical biosensors) (Fig. 12)[49]. When this recognition system involves nucleic acids as receptors and the hybridization reaction as recognition event, the term 'DNA biosensor' or 'genosensor' is used[50]. The biorecognition event (hybridization reaction) takes place *via* Watson-Crick base-pairing fundamentally between two complementary sequences, *i.e.* the support-immobilized synthetic probe and the target sequence.

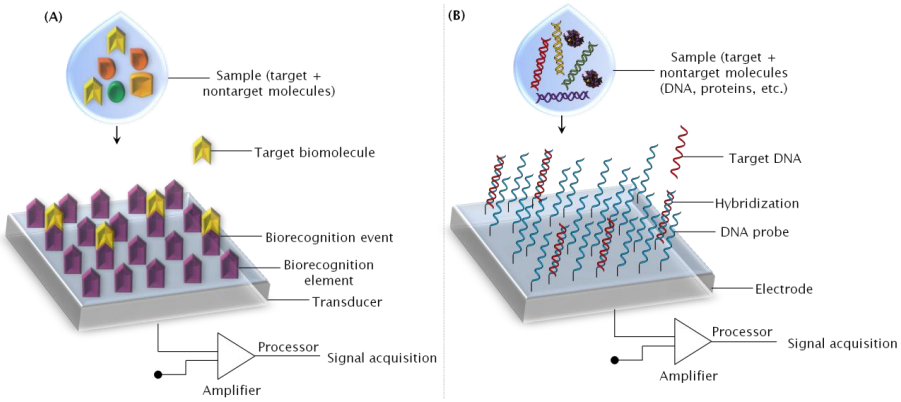

Fig. 12. Classical representation of (A) a general biosensor and (B) an electrochemical genosensor.

According to the IUPAC technical report by Labuda *et al.*[50], the terms 'nucleic acid biosensors' and 'nucleic acid sensing' should be strictly distinguished from one another: in electrochemical DNA biosensors, the DNA has to be in intimate contact with the electrode surface prior to and during the interaction between the recognition element (probe) and the analyte (target sequence). Whereas, DNA electrochemical sensing –also called assay- has a broader meaning: the product of an interaction of any nucleic acid with an analyte (generated either in solution or at another surface) or the DNA itself can be detected electrochemically, usually after accumulation onto the electrode surface.

A specific class of approaches, which has expanded the classical concept of electrochemical DNA sensors during the last decade, employs magnetic beads as the surface on which DNA hybridization occurs. The electrochemical detection of target DNA, signaling probe or other indicator molecules is then done at the electrode surface. Due to the two different surfaces involved, such techniques are called 'double-surface techniques' (DSTs)[51]. In this thesis, the term 'magnetoassay' is employed as well. Fig. 13 shows the difference between a DNA biosensor and a DNA sensing strategy based on DST.

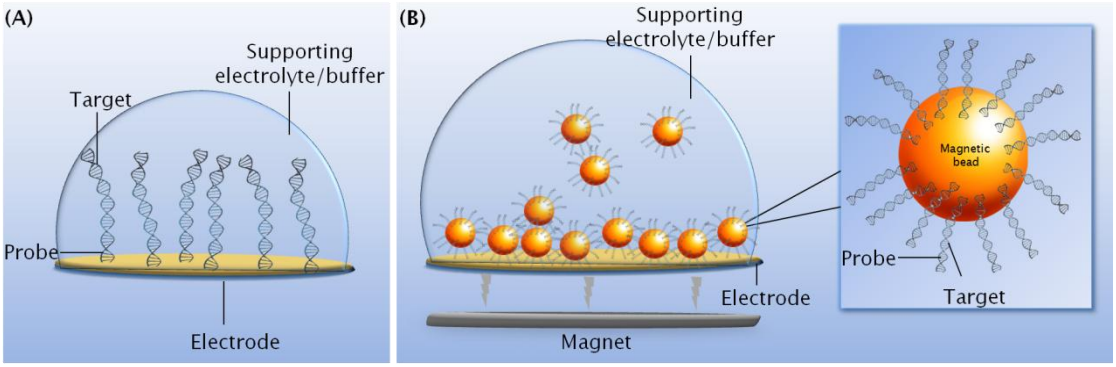

Fig. 13. DNA biosensor *versus* DNA sensing (DST): (A) the biosensor involves probe immobilization, target hybridization and electrochemical measurement, all onto the surface of an electrode; (B) DNA sensing *via* DST involves probe immobilization and target hybridization onto the surface of magnetic beads, while the electrochemical readout is carried out onto the electrode surface, *e.g.* after magnetic accumulation of the beads onto the surface of an electrode.

1.2.1. Probe immobilization: surfaces and strategies

Control of the surface chemistry and coverage is crucial for the analytical performance of DNA biosensors and sensing schemes. The key features of DNA-modified surfaces are DNA density and hybridization accessibility[52]. Moreover, the

immobilization chemistry should be sufficiently specific for probe binding and offer efficient surface blockage in order to avoid unspecific adsorption by other molecular species, *e.g.* proteins, short oligonucleotides, genomic DNA, etc.

The immobilization strategy ultimately depends on the electrode material used for transduction or, in the case of DSTs, the surface chemistries of the nano/micromaterial. Carbon and gold electrodes are the most common substrates for probe immobilization[53], although other substrates such as transparent conducting oxides (ITO, FTO)[54-55] and nanostructured surfaces[56-57] have also been reported for DNA biosensors. Magnetic beads are the most common probe immobilization support in DST-based assays[51]. The most frequently reported probe-immobilization schemes include SAMs onto gold, biotin-streptavidin non-covalent interaction onto practically any modifiable surface, and, finally, electrostatic-based probe immobilization[53, 58].

Given the plethora of surface chemistries and immobilization systems reported for the design of DNA biosensors, only two specific strategies will be discussed at detail in this thesis: streptavidin/biotin interaction onto magnetic beads and electrostatic adsorption onto charged surfaces.

Streptavidin-biotin bioaffinity interaction is one of the strongest non-covalent bindings in nature (K_A = 10^{15} mol·L^{-1}). It can be a highly efficient and remarkably fast way to capture probes onto solid surfaces, *e.g.* 15-30 min in DSTs. Streptavidin, a 52.8 kDa tetrameric protein, in its surface-bound form has at least two free sites for binding biotinylated oligonucleotides. A streptavidin monolayer can thus anchor a high number of biotynilated probes. Additionally, streptavidin-coated surfaces depict little unspecific adsorption[52]. The fact that this tetramer acts as a bridge between the solid surface and the oligonucleotides providing an appropriate intermolecular probe spacing, diminishes steric-hindrance effects and renders the probes more accessible for hybridization as compared to direct immobilization of oligonucleotides onto electrode surfaces[59]. Accordingly, this approach does not require spacer molecules, although functionalization of electrode surfaces with streptavidin monolayers can be time-consuming and laborious. In the case of DST-based assays, streptavidin-modified magnetic beads (Fig. 14) are commercially available[51, 60] with very low size dispersion

and high sedimentation times, making them a practical strategy for fast probe immobilization without the need of lingered surface functionalization protocols.

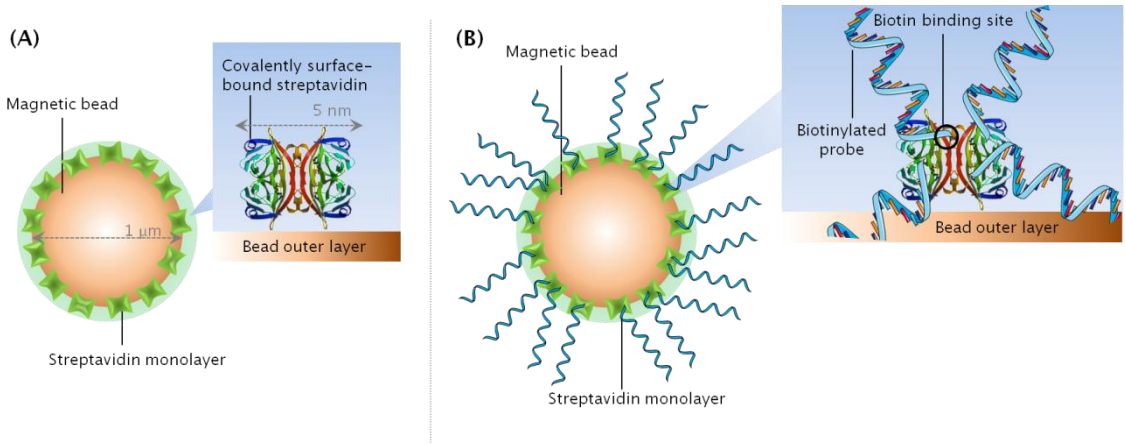

Fig. 14. Streptavidin-coated magnetic beads as immobilization system: (A) streptavidin-coated magnetic microparticles and (B) biotinylated probes immobilized onto the surface of streptavidin-coated magnetic microparticles. There are four biotin binding sites in free streptavidin, which are represented in this figure for illustrative purposes, although there could be fewer sites in surface-bound streptavidin.

Probe immobilization onto magnetic beads enables between-steps washes using magnetic separation. This is a highly efficient way to isolate the desired target biomolecule from complex biological media and from other molecules involved in the assay (enzymes, secondary probes, etc.). The separation relies on the concentration of superparamagnetic particles –usually micrometer sized (0.5-10 µm)- under a strong magnetic field, which do not retain residual magnetism in the absence of magnetic field[51, 60-61].

On the other hand, electrostatic adsorption onto electrode surfaces offers a much less costly platform because it does not require functionalized probes or expensive biological reagents for surface modification. This strategy relies of the negatively charged nature of the DNA phosphate backbone and its interaction with positively charged surfaces, *e.g.* polyelectrolyte-modified surfaces[62]. As a result, DNA probes are 'lying down' on the electrode surface, which can have some advantages in the label-free electronic detection of hybridization[62-63]. However, it is important to point out that electrostatically-bound DNA can leak off the surface when using stringent washes or detergent-based buffers[53]. In addition, these highly charged surfaces can lead to unspecific adsorption by nontarget molecules[58]. Despite of these disadvantages, the simplicity of the approach makes it one of the most commonly used in electrochemical

DNA sensors, especially suitable for simple qualitative monitoring, *i.e.* yes-or-no systems.

In recent years, material-modified nanostructured electrodes have been widely reported for the fabrication of ultrasensitive DNA biosensors. The use of this type of surfaces as immobilization platforms are at the state-of-the-art of the biosensor field. Nanocavities or nanopores can bear a considerably high amount of probes as compared to nearly flat –smooth- surfaces[64-66]. Furthermore, the nanostructure may play an important role in the orientation and assembly density control of probe DNA, making it 'more accessible' for hybridization[64, 66-67]. Increase in conductivity is another important feature exhibited by some nanometric structures[66]. All of these characteristics readily translate into enhanced sensitivity, *i.e.* attomolar to femtomolar-level limits of detection.

Fig. 15 shows a representation of a nanostructured surface, with cavities and grains below 100 nm. The use of nanostructured electrodes has been widely reported in recent years for DNA detection using different materials, *e.g.* graphene[68], gold nanostructures[66], conducting polymers[69], etc.

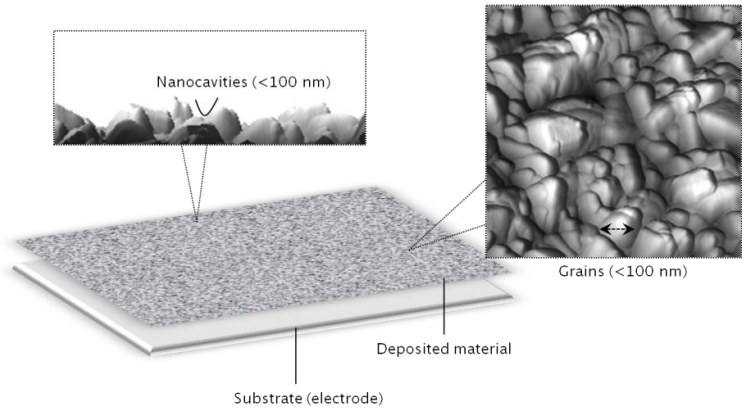

Fig. 15. Example of a nanostructured surface with cavities and grains in the nanometric scale.

1.2.2. Electrochemical transduction of the hybridization event

In order to translate the hybridization event into a measurable signal, both label-free and label-based approaches have been reported in DNA biosensor/sensing schemes.

Label-free strategies are highly pursued given their advantages as simple, fast and low cost platforms. While in earlier reports label-free DNA detection was achieved *via* inherent electroactivity of the nitrogenous bases in DNA[70], most recent reports convey the use of EIS to detect unlabeled DNA by monitoring changes in surface impedance when the target sequence hybridizes with an immobilized probe[71] (Fig. 16). EIS is a powerful technique that measures changes in 'charge transfer resistance' (R_{ct}) at the surface level in the presence of a redox probe (before and after hybridization), across a wide range of frequencies under AC mode. After duplex formation, an increase of negative charges at the surface owed to the phosphate backbone of dsDNA usually translates in the increment of impedance. Typically, the data is represented in a Nyquist plot, which usually shows a semicircle with a linear region at low frequencies (Warburg diffusion). This type of behavior is often fitted with an equivalent electrical circuit called 'Randles circuit'. The diameter of the semicircle is considered the R_{ct} value, and it usually increases proportionally to target concentration, *i.e.* when more target molecules are hybridized with the surface-immobilized probes, more negative charges are present at the interface. EIS-based DNA detection systems that deviate from this typical behavior have been attributed to more complex phenomena involving DNA-material interactions, desorption post-hybridization, DNA-mediated charge transfer, changes in ionic transport, structural effects, etc[68, 72-74].

EIS-based genosensors have been widely reported for DNA detection in the last decades and, more specifically, for GMO detection (Chapter 2). Most of these reports rely on the combination of material-modified electrodes to increase conductivity and surface area, and EIS as detection system[75]. These two features, when combined, often result in low detection limits (pico-, femto-, attomolar range). When EIS alone is used to detect changes between ssDNA and dsDNA, the sensitivity usually falls within the nanomolar range.

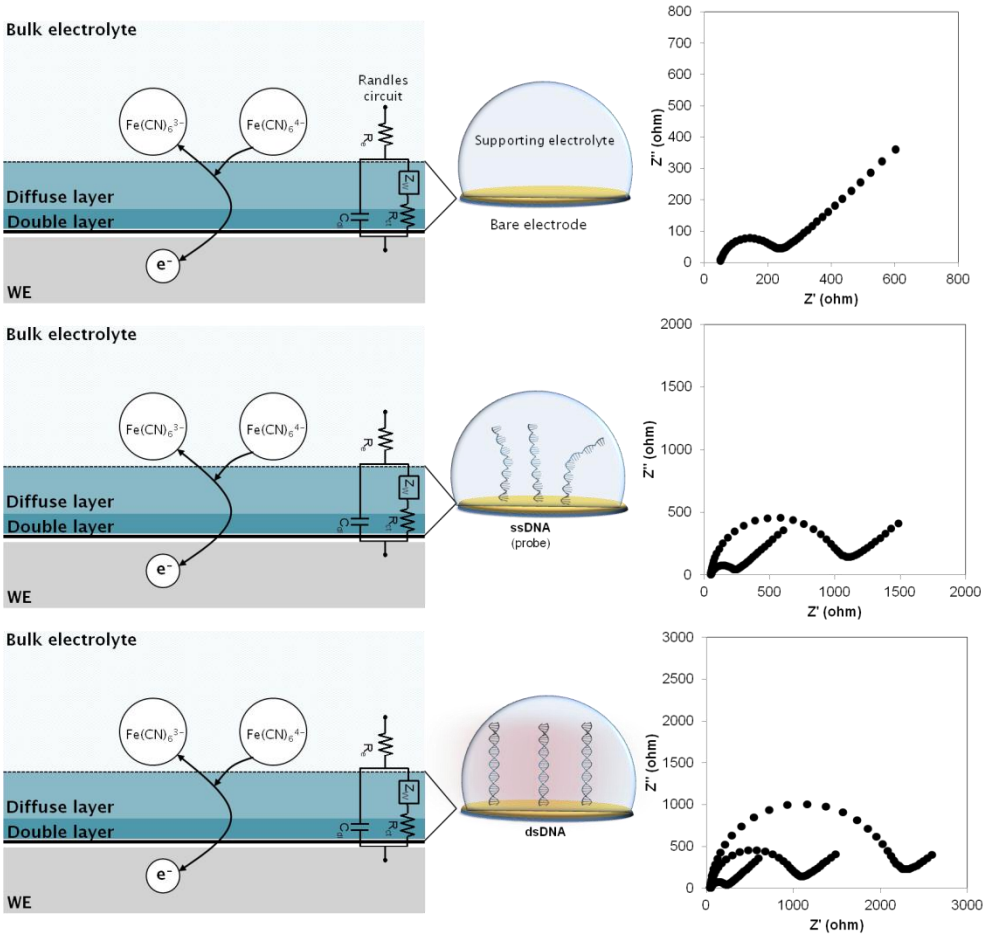

Fig. 16. EIS-based DNA detection. The diameter (red circle) of the semicircle in the Nyquist plots is usually measured as R_{ct}. In the typical Nyquist representation, imaginary impedance is plotted against real impedance across a wide range of frequencies.

Daniels and Purmand[71] reviewed the topic of impedance biosensors stating that "the most promising applications of electrical biosensors are situations where low cost, small instrument size, and speed of analysis are crucial, but cutting-edge accuracy and detection limits are not". It is probably for these reasons that label-free impedance DNA detection has been most successful at qualitative analysis.

With the aim of improving method performance, signal amplification is usually carried out *via* label-based strategies. Labels are usually based on electroactive molecules (*e.g.* ferrocene[76], methylene blue[76-77], anthraquinone[78-79]), nanoparticles (*e.g.* quantum dots[80], gold[81] and silver nanoparticles[82]) or enzymes (*e.g.* horseradish peroxidase[83], alkaline phosphatase[84]). Each type of label involves different transduction schemes. Here are some examples of labeling/transduction strategies (Fig. 17):

- Electroactive molecules can be incorporated in molecular beacons in signal-off methods[85] (Fig. 17A), *i.e.* hybridization is detected by means of a decrease in the electrochemical signal of the electroactive probe (usually by DPV or SWV). The mechanism is the following: the redox molecule remains near the electrode surface in the absence of the target sequence, and moves away from the surface after hybridization due to breakage of the hairpin structure of the probe.
- Redox intercalators (*e.g.* methylene blue[86], osmium complexes[87]) are also used for transduction of the hybridization event (Fig. 17B). CV, DPV or SWV are usually the electrochemical techniques used to detect intercalator/groove binders. These assays are usually signal on, *i.e.* signal increases upon intercalation/binding of the redox molecule to the dsDNA structure due to DNA-mediated electron transfer. Their use is especially useful for discrimination of mismatches that disrupt current flow through the duplex structure[86, 88].
- Nanoparticles have been used in several formats. Heavy-metal quantum dots are usually integrated as end-labels in a second probe, namely signaling probe, in the sandwich-hybridization format[89]. After hybridization occurs between the target and the signaling probe carrying the label, usually an acidic solution is added to dissolve the quantum dots releasing free metals into solution, which are then electrochemically detected usually by means of SWASV or DPASV (Fig. 17C). This strategy has been widely reported for multiplex analysis. Another common strategy involves the use of gold nanoparticles, which can also be detected in a similar fashion[90] or by silver-enhancement strategies. The latter are based on the precipitation of silver on gold nanoparticle tags and the subsequent electrochemical stripping detection of silver[91-92].
- Enzyme-amplified transduction consists of end-labeling usually the signaling probe with an enzyme that turns a specific substrate into an electroactive product. This is then measured by amperometric or voltammetric techniques. EIS has also been used for transduction of an enzyme-labeled assay consisting of the enzymatic conversion of a soluble substrate into an insoluble, electrode-passivating product at the electrode surface upon DNA hybridization[93] (Fig. 17).

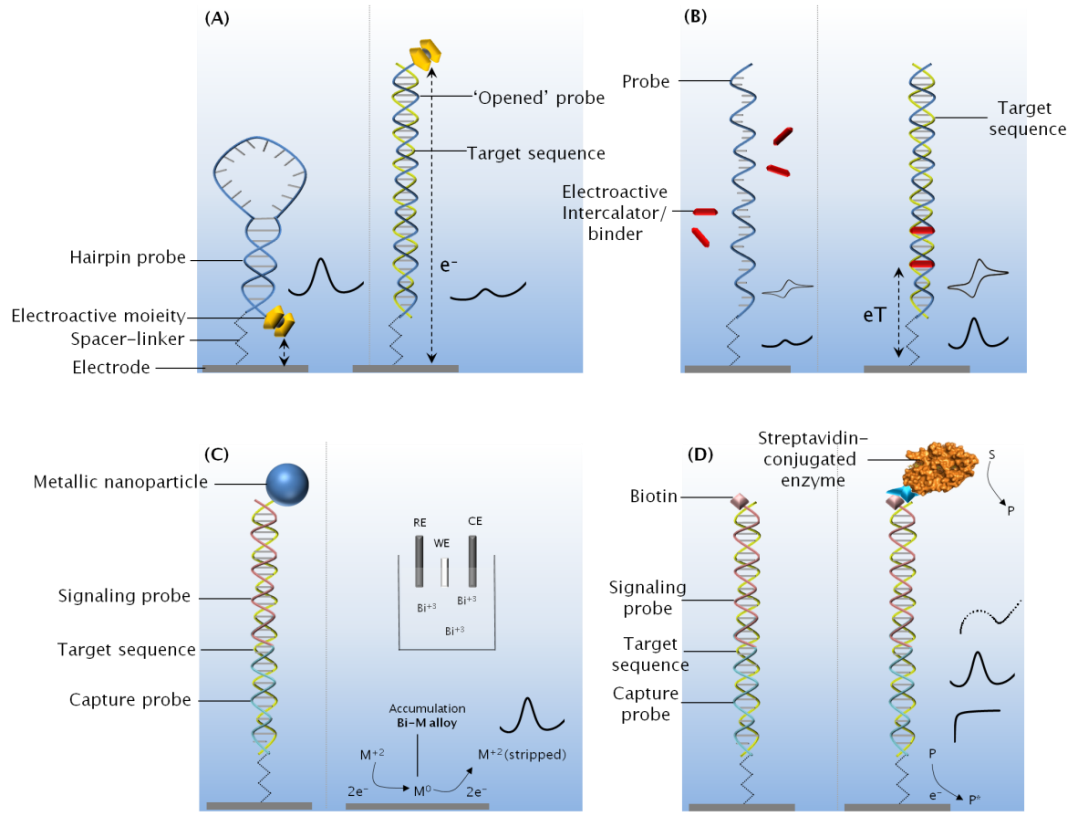

Fig. 17. Most common labeling strategies: (A) electroactive molecule (proximity assay, 'signal-off'); (B) electroactive double-helix intercalator ('signal-on'); (C) Nanoparticle label and ASV technique for metal detection using bismuth as alloying metal; (D) Enzymatic signal amplification.

1.2.3. From sample to measurement: Analytical overview of DNA detection using biosensor/sensing technologies

While in enzymatic biosensors the sample-to-measurement process is usually short and ideally no pre-treatment is required to isolate the analyte from the sample, this is not the case for DNA biosensors. The whole concept of a biosensor is attractive because it should not require extensive sample pre-treatment and the analyte would generate a signal on the basis of the high selectivity of the device even in the presence of non-target molecules. Yet, DNA brings an entirely different scenario: samples *must* be processed to isolate genomic DNA from cell components and other matrix-derived interfering species (*e.g.* carbohydrates and phenols, usually present in food samples). Fig. 18 shows a schematic diagram of this process.

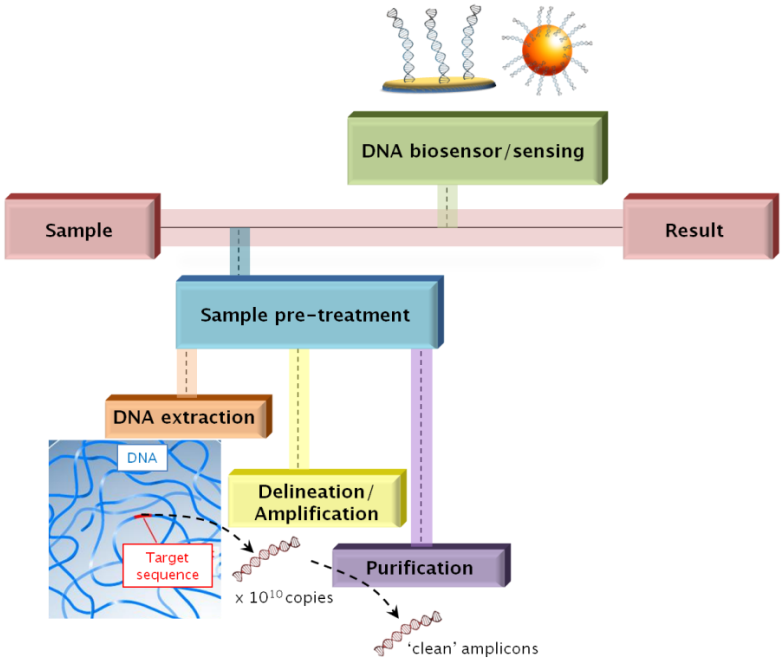

Fig. 18. Analytical overview of DNA detection with electrochemical biosensors/sensing platforms from the sample to the final result.

The genetic material is extracted from a sample in the form of supercoiled genomic DNA of great size. The target sequence represents a small fragment of this complex genomic structure. Hybridization directly using genomic DNA is unlikely to be efficient on the electrode/bead surface, mainly due to steric effects. Moreover, in the case of processed food samples, DNA is extracted in ultralow quantities. Hence, target size restriction and amplification are necessary to reach detectable levels of the analyte, both of which can be accomplished *via* PCR. In some cases, if the biosensor surface is not specific enough against physical adsorption or other types of non-specific interactions that could lead to high background currents, then amplified DNA must be purified post-PCR. However, to this day surface chemistries have been extensively optimized to surpass this problem. Finally, electrochemical detection of the amplicons takes place. The whole process can take approximately from one to three days, depending on the protocol for sensor fabrication, which can involve overnight procedures and/or lingered nanoparticle synthesis/bioconjugations in the case of nanoparticle-labeled strategies.

Given the complexity of this workflow, little reports convey the analysis of samples from 'real-life' situations, especially in the field of food control. An excellent

revision of this topic was carried out in 2010 by Tosar *et al.*[94] regarding biological samples. Food samples can be considered analytically more challenging due to their diverse content in chemical and biological ingredients[95].

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| CHAPTER 2 |

ELECTROCHEMICAL GENOSENSORS AS INNOVATIVE TOOLS FOR THE DETECTION OF GENETICALLY MODIFIED ORGANISMS

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ELECTROCHEMICAL GENOSENSORS AS INNOVATIVE TOOLS FOR THE DETECTION OF GENETICALLY MODIFIED ORGANISMS

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In this work a thorough review of electrochemical DNA biosensors and assays reported until 2014 for GMO detection was carried out. The analytical challenges surrounding GMO detection and quantification with electrochemical methods involving DNA as target are highlighted in this bibliographical work. The main conclusions drawn from this review include:

- From a technological perspective, the fact that ultralow limits of detection were achieved by methods that combined nanostructured or material-modified surfaces with EIS-based transduction is worth of mention. Yet, while these platforms represent convenient strategies for GMO screening, their fabrication can be time-consuming and laborious owed to multiple-step synthesis procedures and lingered electrode modification. It would advantageous to develop simpler and easy-to-execute protocols in label-free mode.
- From an analytical standpoint, scarce reports on 'real-sample' applications and lack of quantitative analysis regarding taxon-specific-to-event-specific ratios stand out as two of the most important gaps of this technology towards this specific application. In addition, most reported GMO sensors were for screening purposes, while very few attained event-specific detection.

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Electrochemical genosensors as innovative tools for detection of genetically modified organisms

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ABSTRACT

A genetically modified organism (GMO) is defined as a living organism whose genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics, such as enhancement of the nutritional properties, herbicide resistance or insect protection. The need to monitor and to verify the presence and the amount of GMOs in agricultural crops and in food products has generated interest in analytical methods for accurate, sensitive, rapid, cheap detection of these products. A novel DNA-detection technology was developed: genosensors. This article reviews electrochemical DNA biosensors reported for the qualitative and quantitative determination of transgenic traits. We discuss critical aspects of genosensor design with particular emphasis on analytical characteristics and analysis of real samples.

Contents

Abbreviations: ASV, Anodic stripping voltammetry; AuNP, Gold nanoparticle; CNT, Carbon nanotube; CPE, Carbon-paste electrode; CV, Cyclic voltammetry; DNA, Deoxyribonucleic acid; DPASV, Differential pulse anodic solid voltammetry; DPV, Differential pulse voltammetry; dsDNA, Double-stranded DNA; EDC, 1-ethyl-3(3′-dimethylaminopropyl)-carbodiimide; EIS, Electrochemical impedance spectroscopy; ELISA, Enzyme-linked immunosorbent assay; EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; FITC, Fluorescein isothiocyanate; GAT, gene encoding glyphosate *N*-acetyltransferase; GCE, Glassy carbon electrode; GDL, Gluconolactone; Glu, Glucose; GMO, Genetically modified organism; GOx, Glucose oxidase; GS, Sugar beet taxon-specific target; HMGa, Rapeseed taxon-specific target; HRP, Horseradish peroxidase; MB, Methylene blue; MCH, Mercaptohexanol; MWCNT, Multi-walled carbon nanotube; NOS, Nopaline synthase; PANI, Polyaniline; PAT, Phosphinothricin acetyltransferase gene; PATP, *p*-aminothiophenol; PCR, Polymerase chain reaction; PDC, 2,6-pyridinedicarboxylic acid; PDDA, Poly(diallyldimethyl ammonium chloride); PEP, Phosphoenolpyruvate carboxylase promoter; PPy, Polypyrrole; RT-PCR, Real-time polymerase chain reaction; SAM, Self-assembled monolayer; SPCE, Screen-printed carbon electrode; SPE, Screen-printed electrode; SPEAu, Screen-printed gold electrode; ssDNA, Single-stranded DNA; SSIIb, Taxon-specific gene from maize; SWCNT, Single-walled CNT; SWV, Square wave voltammetry; TMB, Tetramethylbenzidine.

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1. Introduction

The advent and the application of genetically modified organisms (GMOs) have undoubtedly revolutionized agronomic practices over the past 10 years [1]. GMOs are defined as "organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" [2]. Since the beginning of recombinant DNA technology, GMOs have offered many advantages, such as considerable improvement in the yield and the quality of crops and enhancement of the nutritional properties of plants. These genetic modifications are possible by selecting and extracting genes of interest from other organisms, such as bacteria, and inserting the desired DNA fragment into the plant genome. Herbicide-resistant soybean serves as an example of one of the most important genetic modifications in crops, in which a specific gene is inserted in order to produce herbicide resistance, providing great benefits for agriculture and the food industry.

However, public attention has been growing in the past few years regarding the use of genetic engineering in the production of food. Concerns about the possible impact of transgenic food on public health and the environment have driven some governments to introduce food-labeling regulations [3,4]. Labeling helps customers to identify the product of interest and its contents, allowing them to exercise choice [1].

According to European legislation, the presence of genetically modified (GM) material in food and feed is governed by Regulation EC 1829/2003 [5] of the European Parliament and the European Council, which insists on a labeling procedure for all products containing GM-based materials. The Regulation is supplemented by Regulation EC 1830/2003 [6], which ensures traceability and labeling of GMOs placed on the market. Labeling is required when the content of any authorized GM ingredient exceeds 0.9% of the food or feed product; in this case, the term "genetically modified" must appear in the list of ingredients immediately following the relevant ingredients. Below this threshold, the presence of GM material is considered to be accidental or technically unavoidable, and the products can be sold without labeling. For non-authorized GM ingredients, the threshold is set at 0.5%, provided the source of the GMO has been pre-evaluated, and an appropriate detection method for its presence is available [5]. Unlike in the European legislation, the labeling of transgenic products is voluntary in the USA, an issue currently under discussion among consumers and environmentalists.

The need to monitor and to verify the presence and the amount of GMOs in agricultural crops, food and feed has generated interest in analytical methods for the accurate, sensitive, rapid, cheap detection of these products. Two classical approaches, based on the detection of two kinds of macromolecules, proteins and DNA, have been used in order to reveal the presence of GMOs [7–10]. Protein-based methods detect the new protein(s) encoded by the integrated gene(s). DNA-based methods detect the DNA sequence related to the genetic modification.

The protein-based methods rely on the binding between the protein and the specific antibody; most of these methods are based on enzyme-linked immunosorbent assay (ELISA). However, technological processes that affect the integrity of the protein must be considered. Furthermore, these approaches are often unable to discriminate between various GMOs that express the same or similar proteins [11].

Official DNA-based methods for GMO detection are based on the real-time polymerase chain reaction (RT-PCR) because of the high sensitivity of this method [12,13]. However, the technique requires relatively expensive equipment and qualified personnel. Hence, several other analytical technologies that can provide alternatives in the detection of GMOs are emerging [14]. Methods for the detection of DNA hybridization have attracted much attention in the past decade. Biosensors have emerged as a new DNA-detection technology for GMO analysis due to their simplicity, automation, low cost and good analytical properties.

This article reviews the state of the art in electrochemical DNA biosensors reported for the detection of GMOs. We review the basic elements present in a GM plant, and give a brief description of the plant-transformation methods, followed by classification of the analytical methods for GMOs in terms of specificity. We assess the design and the construction of electrochemical genosensors, describing the contributions so far in the GMO field and the analytical challenges involved in analyzing real samples.

2. Overview of plant transformation

A genetic transformation is defined as stable incorporation and expression of foreign genes into a host organism. This process is a complex multi-stage procedure involving three phases:

(a) selection and application of a gene delivery system for transferring the DNA of interest into a viable host cell;
(b) integration of the DNA into plant cells resulting in cells that successfully express the gene of interest; and,
(c) recovery of a viable transgenic plant [15].

Numerous methods have been developed to introduce and to integrate the "foreign" DNA into plant cells. These methodologies are based on biological vectors and physical methods. The most frequent transformation method involves the use of a modified plasmid of *Agrobacterium sp.* (biological vector). Plasmids are extrachromosomal short molecules of dsDNA, usually circular, which occur naturally in bacteria. These circular DNA molecules are used as vectors because they contain all the necessary genetic elements for replication, expression of specific proteins, and additional elements of interest, such as markers. These markers are usually antibiotic-resistant genes used to identify cells that contain the plasmid, allowing selection and recovery of the transformed cells [15,16].

A GMO insert consists of three genetic elements: promoter, trait gene and terminator (Fig. 1). The promoter is needed to achieve high levels of gene expression in plants. 35S from Cauliflower Mosaic Virus (CaMV) is the most common promoter in GMO inserts. Other promoters, such as phosphoenolpyruvate (PEP) carboxylase promoter, which encodes a photosynthetic enzyme, and Figworth Mosaic Virus 35S (P-FMV), are used less frequently in GMOs. The terminator gene is a DNA sequence that serves as the "stop signal" for gene transcription. The Nopaline Synthase (NOS) from *Agrobacterium tumefaciens* is the most frequent terminator element [18].

Both promoter and terminator flank the gene of interest (trait gene), which confers the plant with the desirable characteristic. Although many trait genes, herbicide and insect resistant, are currently authorized for use in GMOs, the following are the most employed in the design of GMO genosensors:

(a) CP4 EPSPS gene that encodes the enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, enzyme resistant to the herbicide glyphosate, known commercially as Round-Up;
(b) bar and PAT genes, both encoding a phosphinothricin acetyltransferase that induces tolerance to the herbicide agent phosphinothricin (glufosinate); and,
(c) Bt, Cry1Ab and Cry1Ac genes, both encoding an insecticidal protein derived from *Bacillus thuringiensis*, acting as an endotoxin for susceptible insect species, such as the European corn borer, a major insect pest of maize in agriculture.

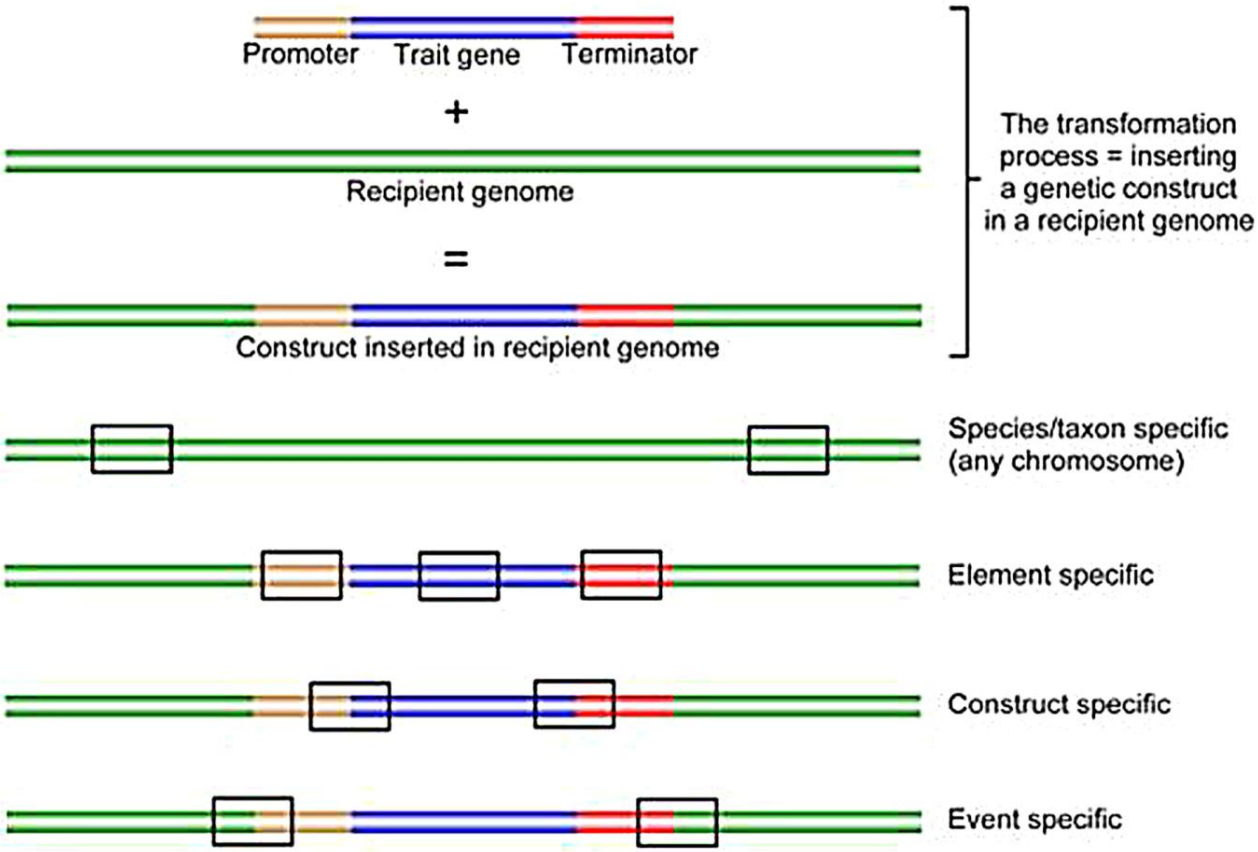

Fig. 1. Transformation process and the four levels of specificity of analytical modules targeting DNA sequences. In some cases, there are additional partial or complete constructs inserted. These can be located at the same loci or other loci (BCH, 2011; CERA, 2011; GMO Compass, 2011). The typical species or taxon-specific screening target is a single copy seed specific for the housekeeping gene. {Reproduced from [17] with permission from Elsevier}.

Soybean and maize are the main crops that have been modified with at least one of these genes. Although there are GMs aiming at nutritional improvement, there are no genosensors to detect these GMOs.

According to the European legislation, the amount of transgenic DNA has to be expressed as the ratio GMO DNA copies/taxon-specific DNA copies (Commission Recommendation, 2004/787/CE), so the analysis of samples containing GMOs requires quantification of the taxon-specific target present in the wild-type organism (Fig. 1), to provide an estimation of the total amount of DNA from the plant species; hence, results have to be expressed as relative GMO proportion [19]. These sequences are also useful as reference targets to identify the species. These reference genes do not exhibit allelic variation, and, ideally, have a constant number of copies per haploid genome across different cultivars of the target species, in order to ensure reliable quantitative data when analyzing samples from unknown sources. Examples of these targets are: lectin, high mobility group protein gene and UDP-glucose pyrophosphorylase from soybean, maize and potato, respectively.

3. Specificity of the methods for GMO detection

According to the DNA targets present in GMOs, DNA-based GMO tests can be categorized into different levels of specificity. The least specific methods are frequently called "screening methods" and include common DNA elements in GMOs, such as promoters and terminators that are present in many different GMOs [3,4,20,21]. Sometimes, marker genes are also used as screening targets, such as the neomycin-kanamycin resistant gene called neomycine phosphotransferase II (*nptII*) gene [22,23].

The second level includes "gene-specific methods", which detect a part of the trait gene associated with the specific genetic modification. Examples are the Bt or the CP4 EPSPS genes. If a positive signal is obtained, the presence of GM-related sequences is highly probable, but it is impossible to identify the specific GMO because the trait gene can be used in transformation events of different organisms [20]. Both screening and gene-specific methods are based on the detection of naturally, or very close to naturally, occurring DNA sequences – and that significantly increases the risk of obtaining false positive analytical results in tests. In Fig. 1, the first and second levels are located in the "element-specific" category.

The third level covers "construct-specific methods", which target the junction between two DNA elements, such as the promoter-trait gene and trait gene-terminator. These methods target DNA sequences that are not present in nature. However, different GMOs may share the same DNA elements, as is the case for two distinct GMO maizes, MON809 and MON810, that have the same promoter and trait genes. Genosensors for the detection of construct-specific sequences have not been reported.

The highest specificity is reached when the target is the unique junction, characteristic of each event, found at the integration locus between the inserted DNA and the recipient genome. These

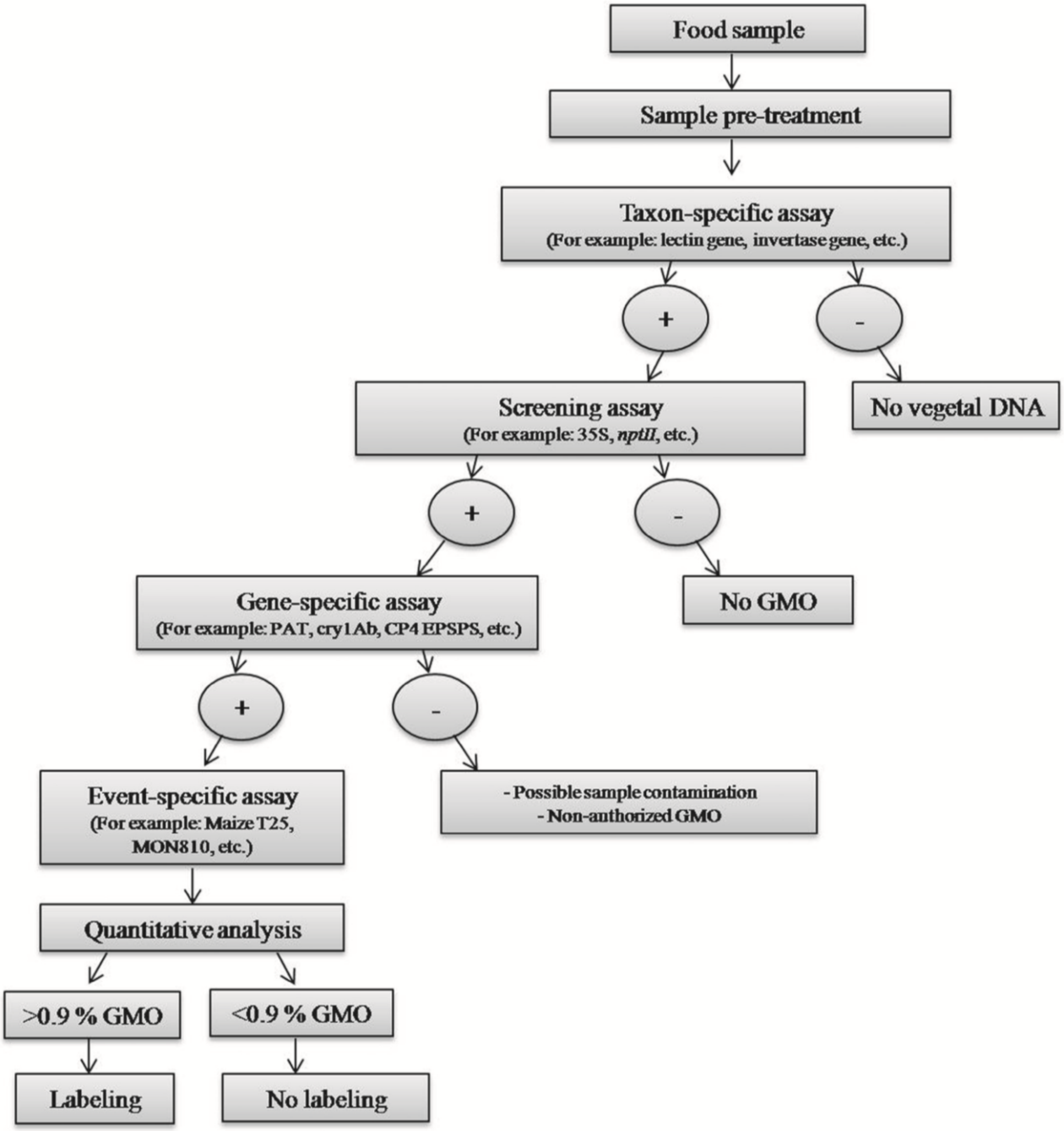

Fig. 2. General scheme for testing genetically modified organisms.

are called "event-specific methods". Unfortunately, even these methods have their limitations. Crossbreeding between two GMO lines may lead to so-called stacked genes. For example, a herbicide-tolerant GMO can be combined with an insecticide-tolerant GMO. Both sets of trait genes are present in the resulting crossbreed. Quantitative methods cannot distinguish between the gene-stacked GMO and a mixture of its two parental GMOs. In the USA, this type of hybrid GMO is not regulated if both parent GMOs are authorized. However, in the European Union, gene-stacked cross-breeds are considered new single GMOs and require their own authorization [20,24].

In order to reveal the presence of GMOs in food and feed samples, the protocol begins with a taxon-specific assay, after an appropriate pre-treatment step. A positive result will be obtained in the case of vegetal DNA. The following step seeks to verify the presence of any GMO by a screening assay. It is important to discriminate the specific event and/or the inserted gene, followed by a quantitative determination of the GMO content in the sample (Fig. 2).

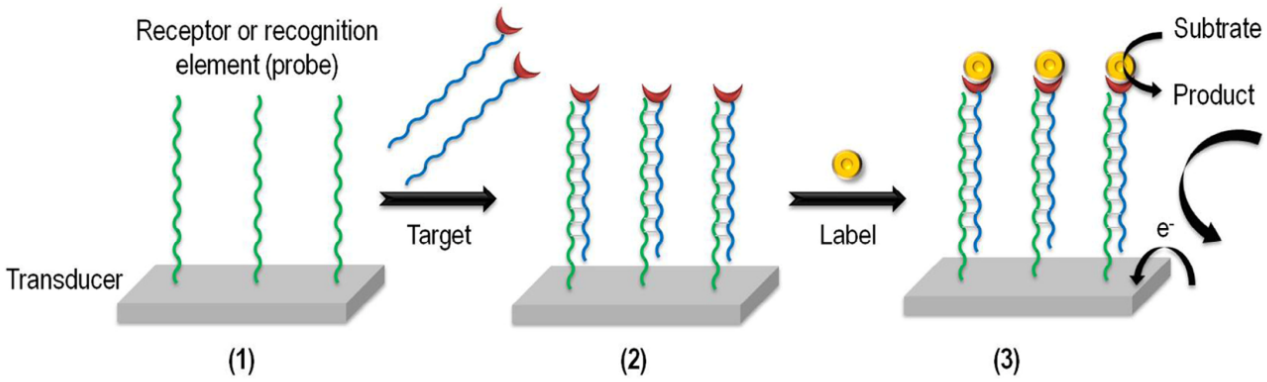

Fig. 3. Steps in the design of a genosensor with a labeled target.

4. Genosensors, electrochemical platform for GMO detection

Biosensors are analytical devices that translate a biological event into a measurable signal. Genosensors are DNA biosensors in which the recognized event consists of the hybridization reaction. The biological recognition elements in genosensors are DNA sequences called probes, complementary to the DNA sequence of interest (target). DNA is especially suited to get selective devices because of the high specificity of the base-pairing interaction between complementary sequences, even in the presence of mismatches [25].

Although several transducers have been used in biosensors and genosensors, as far as we know, only DNA biosensors with optical (surface-plasmon resonance), piezoelectric (quartz-crystal microbalance) and electrochemical transduction have been successfully developed for the detection of GMO sequences, as was recently reviewed by Arugula et al. [26]. However, electroanalytical techniques provide important advantages, such as relative simplicity, low cost of equipment and automatic on-line and portable options.

The construction of an electrochemical genosensor usually involves the following stages (Fig. 3): (1) immobilization of the DNA probe onto the electrode surface; (2) hybridization with the target sequence; (3) labeling and electrochemical readout [27]. Optimization of each step is required to improve the overall performance of the device. The probe immobilization step plays a major role in determining the performance of an electrochemical DNA biosensor. Many methods have been employed for the immobilization of DNA, including adsorption, covalent binding, self-assembled monolayers (SAMs), and electropolymerization.

5. Design and performance of GMO genosensors

The steps involved in the design of an electrochemical genosensor are:

(a) transducer selection;
(b) target-sequence selection;
(c) selection of a convenient probe-immobilization strategy; and,
(d) evaluation of labeling marks and detection methods.

Table 1 summarizes the electrochemical genosensors reported for GMOs.

5.1. Electrochemical transducers

Among electrochemical transducers, gold and carbon electrodes have been widely employed in the development of DNA sensors. Glassy-carbon electrodes (GCEs) and carbon-paste electrodes (CPEs) are popular electrodes used in the design of genosensors because of their wide potential window and chemical inertness. In contrast to the relatively complicated modifications of solid substrates, carbon pastes can be easily modified to obtain sensors with the desired, often predefined, properties. Glassy carbon combines glassy and ceramic properties with those of graphite, conferring the electrodes with resistance to high temperatures and chemicals. However, gold electrodes provide a suitable surface for the highly organized immobilization of thiolated compounds giving SAMs as a result. Almost all papers describing electrochemical genosensors are based on the use of conventional gold and carbon electrodes obtained by sealing gold bars or carbon pastes into polymeric supports [43,44,49,57,58,60]. The availability of disposable printed, photolithographed and screen-printed electrodes [28,29,35,36,38] has greatly facilitated genosensing protocols, eliminating the regeneration of a new bare electrode surface through tedious and time-consuming mechanical and chemical procedures.

Taking into account the huge quantity of GMO events, one of the most important challenges in this field is the development of multiplex analysis. In this sense, electrode arrays provide a great platform for a complete, fast analysis, as recently reported by Liao et al. [59]. In their work, electrochemical measurements were performed on a 16-sensor gold-electrode-array chip; each sensor featured a central gold working electrode (2.5 mm in diameter) surrounded by a pseudo-reference electrode and an auxiliary electrode.

5.2. Selection of the target sequence

In GMO detection, the selection of the target sequence is relatively easy. It depends on the aim of the assay (i.e., screening, qualitative or quantitative). The nucleic-acid sequences of authorized or pre-evaluated transformed plants are well characterized and described in open-access databases (http://www.gmo-compass.org/eng/gmo/db/, http://gmo-crl.jrc.ec.europa.eu/gmomethods/, and http://gmdd.shgmo.org/). The taxon-specific or reference genes have also been described in official PCR methods, which are necessary for the relative quantification of GMOs and for species identification.

Half the reported DNA sensors are screening methods, which are least specific, and most of them targeted 35S (Fig. 4). Gene-specific methods can be anchored to screening assays with the goal of reducing false positive results. Many authors have reported the simultaneous determination of NOS and PAT by genosensors [50–54]. As shown in Fig. 4, most genosensors based on gene-specific detection use the PAT gene as the target.

Table 1
Electrochemical genosensors for detection of genetically modified organisms (GMOs)

Target	Electrode type	DNA immobilization strategy	Detection method	Sample type	Linear range (*M*)	Detection limit (M)	RSD	Ref.
35S	SPEAu	SAMs	DPV with enzymatic amplification	Synthetic DNA and bacterial plasmid with PCR amplification	0–$2.46{\cdot}10^{-8}$ (synthetic) 0–$1.20{\cdot}10^{-7}$ (amplicons)	$2.5{\cdot}10^{-10}$ (synthetic) $1{\cdot}10^{-9}$ (amplicons)	12%	[28]
	SPEAu	SAMs	EIS with enzymatic amplification	Synthetic DNA and reference material with PCR amplification	$1.2{\cdot}10^{-11}$–$1.2{\cdot}10^{-8}$ (synthetic) Not linear for amplicons	$1.2{\cdot}10^{-12}$ (synthetic)	10%	[29]
	GCE	Covalent attachment with ethylenediamine	DPV with MB as indicator	Synthetic DNA	$5{\cdot}10^{-9}$–$1.2{\cdot}10^{-7}$	Not reported	4.9%	[30]
	GCE	Adsorption	DPV with $[Co(NH_3)_6]^{+6}$ as indicator	Synthetic DNA and reference material with asymmetric PCR	Not quantitative	Not reported	Not reported	[31]
	GCE	Adsorption on Pt-nanoparticles	SWV with $[Co(phen)_3]^{+3}$ as indicator	Synthetic DNA and reference material with enzymatic digestion	$2.14{\cdot}10^{-9}$–$2.14{\cdot}10^{-7}$ (synthetic)	$1{\cdot}10^{-9}$ (synthetic)	5.89%	[32]
	GCE	SAMs onto Au electrodes	DPASV using PbS-nanoparticles onto GCE	Synthetic DNA	$1.2{\cdot}10^{-11}$–$4.8{\cdot}10^{-8}$	$4.38{\cdot}10^{-12}$	Not reported	[33]
	CPE	Adsorption of the DNA in the PbSe/Chitosan composite	DPV with Methylene Violet as indicator	Synthetic DNA	$5{\cdot}10^{-11}$–$5{\cdot}10^{-6}$	$1.6{\cdot}10^{-11}$	4.97%	[34]
	Au interdigitated microelectrodes	Electrocopolymerization with PPy/MWCNTs	EIS (label-free)	Synthetic DNA	$2.5{\cdot}10^{-11}$–$8{\cdot}10^{-11}$	Not reported	Not reported	[35]
	SPCE	Covalent attachment to succinimide-functionalized acrylic microspheres onto the AuNPs/SPCE	DPV with anthraquinone-2-sulfonic acid monohydrate sodium salt as indicator	Synthetic DNA and reference material	$2{\cdot}10^{-15}$–$2{\cdot}10^{-9}$	$7.79{\cdot}10^{-16}$	2.7–4.7%	[36]
35S and NOS	Au	SAMs	SWV with MB as indicator	Real sample (DNA extracted with kit)	Not quantitative	Not reported	Not reported	[37]
NOS	SPCE	Adsorption by controlled potential	SWV with MB as indicator	Synthetic DNA and reference material with PCR amplification	Not reported	$2.4{\cdot}10^{-6}$	2.4%	[38]
	GCE	SAMs onto Au electrodes	DPASV using CdS-nanoparticles onto GCE	Synthetic DNA	$8{\cdot}10^{-12}$–$4{\cdot}10^{-9}$	$2.75{\cdot}10^{-12}$	Not reported	[39]
	Au	SAMs	CV with MB as indicator	Synthetic DNA and reference material with PCR amplification	$5{\cdot}10^{-8}$–$1{\cdot}10^{-4}$ (synthetic)	$3.6{\cdot}10^{-8}$ (synthetic)	3.89%	[40]
nptII	CPE	Adsorption by controlled potential	SWV with MB as indicator	Synthetic DNA	Not quantitative	Not reported	Not reported	[41]
	Au	SAMs	SWV with enzymatic amplification (aniline polymerization)	Synthetic DNA and plant samples with PCR amplification	$1{\cdot}10^{-10}$–$1{\cdot}10^{-9}$ (synthetic) $2{\cdot}10^{-10}$–$1{\cdot}10^{-9}$ (amplicons)	$1{\cdot}10^{-10}$ (synthetic) $2{\cdot}10^{-10}$ (amplicons)	Not reported	[23]
Bar	CPE	Covalent attachment with ethylenediamine	SWV with $(Co(bpy)_3$ as indicator	Synthetic DNA	Not quantitative	Not reported	Not reported	[22]
Bar and CP4 EPSPS amplicons	CPE	Adsorption onto aluminum films	DPV with MB as indicator	Synthetic DNA (bar) and real samples with PCR amplification (CP4 EPSPS)	$1{\cdot}10^{-7}$–$1{\cdot}10^{-4}$ (bar) Not quantitative for amplicons	$2.25{\cdot}10^{-8}$ (bar) Not reported for amplicons	Not reported	[42]
Bt	Au	SAMs	Solid state voltammetry using Ag nanoparticles	Synthetic DNA	$1{\cdot}10^{-12}$–$1{\cdot}10^{-6}$	$1{\cdot}10^{-14}$	7.38%	[43]
	SPE	Covalent attachment with -COOH	EIS with Ag signal amplification	Synthetic DNA	10^{-14} -$2{\cdot}10^{-12}$	$72{\cdot}10^{-15}$	12.8%	[44]
PAT	GCE	Adsorption onto nanogold/nanoPANI-chitosan	EIS (label-free)	Synthetic DNA	$1{\cdot}10^{-12}$–$1{\cdot}10^{-6}$	$3.1{\cdot}10^{-13}$	4.05%	[45]
	Au	Potential-controlled adsorption onto a SiO_2-PATP	EIS (label-free)	Synthetic DNA	$1{\cdot}10^{-11}$–$1{\cdot}10^{-6}$	$1.5{\cdot}10^{-12}$	5.8%	[46]
	GCE	Adsorption onto nanogold/PDC	EIS (label-free)	Synthetic DNA	$1{\cdot}10^{-10}$–$1{\cdot}10^{-5}$	$2.4{\cdot}10^{-11}$	Not reported	[47]
	GCE	Potential-controlled adsorption on ZrO_2/nanogold	DPV with MB as indicator	Synthetic DNA	$1{\cdot}10^{-10}$–$1{\cdot}10^{-6}$	$3.1{\cdot}10^{-11}$	4.32%	[48]
	GCE	Adsorption on nanoPANI-ZrO_2/Tyrosine	EIS (label free)	Synthetic DNA	$1{\cdot}10^{-13}$–$1{\cdot}10^{-6}$	$2.68{\cdot}10^{-14}$	3.56%	[49]

(*continued on next page*)

Table 1 *(continued)*

Target	Electrode type	DNA immobilization strategy	Detection method	Sample type	Linear range (*M*)	Detection limit (M)	RSD	Ref.
PAT and NOS amplicons	GCE	Adsorption on ZrO_2/SWNTs/PDC	EIS (label-free)	Synthetic DNA (PAT) and real samples with PCR amplification (NOS)	$1{\cdot}10^{-11}$–$1{\cdot}10^{-6}$ (PAT)	$1.38{\cdot}10^{-12}$ (PAT) Not reported for amplicons	2.46%	[50]
	GCE	PDDA/PDC-SWNTs films	DPV with MB as indicator	Synthetic DNA (PAT) and real samples with PCR amplification (NOS)	$1{\cdot}10^{-11}$–$1{\cdot}10^{-6}$ (PAT) Not quantitative for amplicons	$2.6{\cdot}10^{-12}$ (PAT) Not reported for amplicons	5.16%	[51]
	CPE	Potential-controlled adsorption on Poly-lysine/SWNTs	EIS (label-free)	Synthetic DNA (PAT) and real samples with PCR amplification (NOS)	$1{\cdot}10^{-12}$–$1{\cdot}10^{-7}$ (PAT) Not quantitative for amplicons	$3.1{\cdot}10^{-13}$ (PAT) Not reported for amplicons	3.16%	[52]
	CPE	Adsorption on PANI-MWNTs/chitosan	EIS (label-free)	Synthetic DNA (PAT) and real samples with PCR amplification (NOS)	$1{\cdot}10^{-13}$–$1{\cdot}10^{-7}$ Not quantitative for amplicons	$2.7{\cdot}10^{-14}$ (PAT) Not reported for amplicons	Not reported	[53]
	CPE	Potential-controlled adsorption on nanogold-CNT/nanoPANI	EIS (label-free)	Synthetic DNA (PAT) and real samples with PCR amplification (NOS)	$1{\cdot}10^{-12}$–$1.0{\cdot}10^{-6}$ (PAT)	$5.6{\cdot}10^{-13}$ (PAT) Not reported for amplicons	3.6%	[54]
PEP gene	GCE	Adsorption on nano(Au-Pt)-polytyramine	EIS (label-free)	Synthetic DNA	$1{\cdot}10^{-12}$–$1{\cdot}10^{-7}$	$3.6{\cdot}10^{-13}$	5.16%	[55]
SSIIb ivrp	Au	SAMs	SWV with $[OsO_4(bipy)]$ as indicator	Synthetic DNA	$2.5{\cdot}10^{-8}$–$2{\cdot}10^{-7}$ (ivrp and MON810)	Not reported	Not reported	[56]
cryIa/b event MON810	Au	SAMs	SWV with $[OsO_4(bipy)]$ as indicator	Real samples with asymmetric PCR amplification	Not reported	0.6%	Not reported	[57]
MON810	CPE	Adsorption on CILE/ p-ERG film	DPV with MB as indicator	Synthetic DNA and GM maize sample	10^{-11} -10^{-6} Not quantitative for amplicons	$4.52{\cdot}10^{-12}$	3.6%	[58]
35S G1b1 CP4-EPSPS PAT cordapaA lectin SSIIb	Au (array)	SAMs	Chronoamperometry with enzymatic amplification	Synthetic DNA and soybean powder with known GM levels	Up to $2{\cdot}10^{-5}$ total (genomic) DNA 0–5% GM content	$2.25{\cdot}10^{-10}$	Not reported	[59]
A2704-12 soybean	CPE	Ionic liquid modified and partially reduced graphene. SAMs	DPV with MB as indicator	Synthetic DNA and real samples with PCR amplification	$1{\cdot}10^{-12}$–$2{\cdot}10^{-6}$ Not quantitative for amplicons	$2.9{\cdot}10^{-13}$	3.2%	[60]

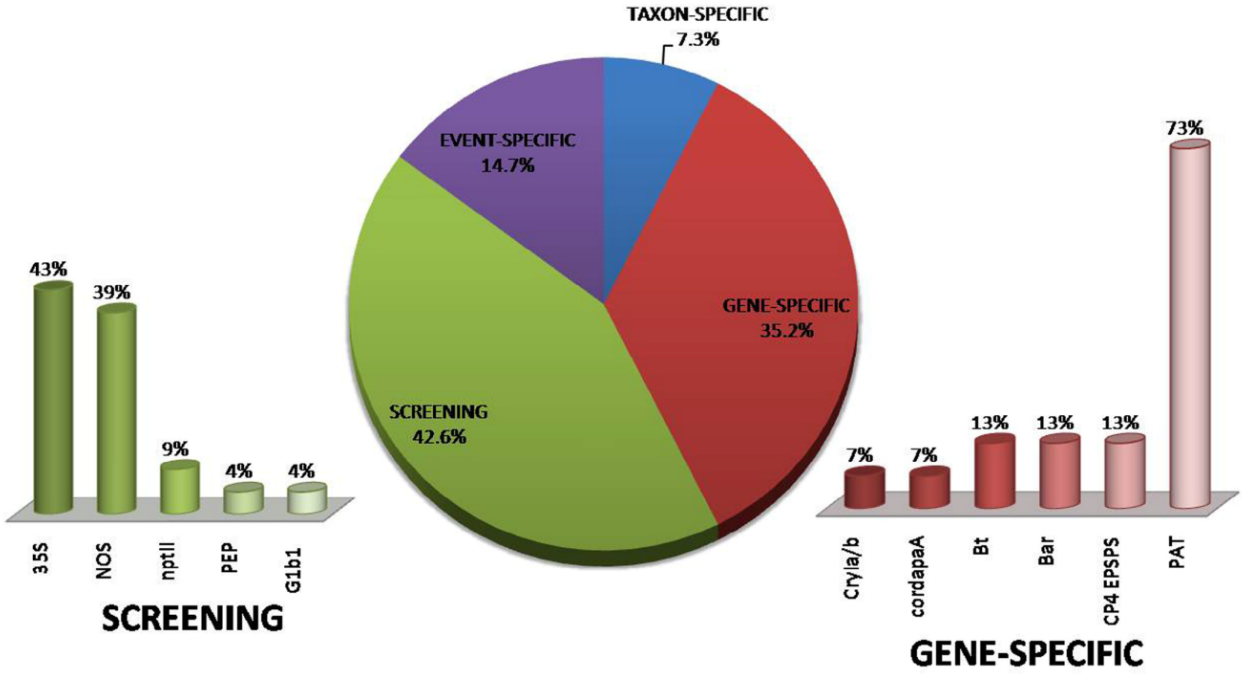

Fig. 4. Frequency of reported genosensors based on the target sequences and specificity level.

Only a few works reported event-specific genosensors. Duwensee et al. [56], Sun et al. [58] and Mix et al. [57] described the detection of MON810 maize, Liao et al. [59] tested soybean 40-3-2 and A5547-127, maize NK603, T25 and LY038, and Sun et al. [60] targeted soybean A270412.

5.3. Immobilization strategy

Once the target has been selected, the next step involves the choice of a convenient probe-immobilization strategy. In GMO genosensors, direct adsorption of DNA probes, governed by time or potential, has been reported using carbon substrates [31,38]. Although it is easy to immobilize DNA probes using this approach, the immobilized oligonucleotides could be attached on multiple sites, resulting in a lower reactivity for hybridization and a decrease in the specificity of the recognition layer. In addition, some of the probes could detach the support during the assay due to the weak immobilization system. Covalent immobilization exhibits some advantages because DNA is easily attached by its end to the electrode surface, ensuring structural flexibility and the increasing hybridization efficiency. Xu [30] and Ligaj [22] used ethylenediamine as a connector for one-site covalent immobilization for the detection of 35S and Bar, respectively.

Gold surfaces are advantageous due to the spontaneous formation of SAMs, providing well-defined and organized surfaces, as well as a convenient, flexible, simple system to immobilize thiolated DNA [27,61]. In order to control the density and availability of the capture probe, post treatment of the probe-modified surface with a short secondary thiol, such as mercaptohexanol, should be carried out [62–64]. This spacer molecule displaces the non-specifically adsorbed DNA molecules [65], and enhances the hybridization efficiency due to the better disposition of the oligonucleotide molecules. Mixed SAMs have permitted very low limits of detection (LODs), such as picomolar levels for the detection of 35S [29] or even femtomolar levels for a gene-specific target [43].

Carbon nanotubes (CNTs), single-walled (SWCNT) or multi-walled (MWCNT), are also used due to their high electrical conductivity, long-term stability and good mechanical strength. The excellent properties of these nanostructures can be enhanced if they are homogeneously embedded into light-weight matrices, such as those offered by polymers [35,50–54]. However, hybrid materials consisting of nanoparticles (NPs) and polymers are used with the aim of increasing the immobilization surface area [45–48,50,55]. All these complex matrixes provide remarkable increases in reproducibility and sensitivity of the sensor. For example, the synergistic effect of polyaniline nanofibers, MWCNTs and chitosan resulted in a very sensitive, stable genosensor that could be reused several times [53]. Another example of the synergy between materials was recently reported by Ulianas et al. [36], who obtained a linear range of six orders of magnitude with a sub-femtomolar LOD, attributed to robust covalent binding of the DNA probe onto acrylic microspheres/gold NPs.

5.4. Detection methods

Initially, the intrinsic electrochemistry of nucleic acids was utilized to detect the hybridization event. Later, electroactive labels were introduced into DNA to increase the sensitivity of the analysis. Some of these electroactive molecules, such as methylene blue, methylene violet, anthraquinone compounds, osmium and cobalt complexes, including $[OsO_4(bipy)]$, $[Co(NH_3)_6]^{+6}$, $[Co(phen)_3]^{+3}$ and $Co(bpy)_3$, bind specifically to guanine bases in the DNA molecule and have been employed in GMO genosensors. However, enzymatic labels are convenient tools in GMO biosensing due to its inherent catalytic signal amplification: one enzyme molecule can undergo many catalytic turns, thus producing many indicator molecules per hybridization event [66]. A bond is required to attach the enzyme to the DNA sequences. Avidin-biotin, streptavidin-biotin and fluorescein-anti-Fab-fluorescein conjugations have been used for this purpose. The most commonly used enzymes are alkaline phospha-

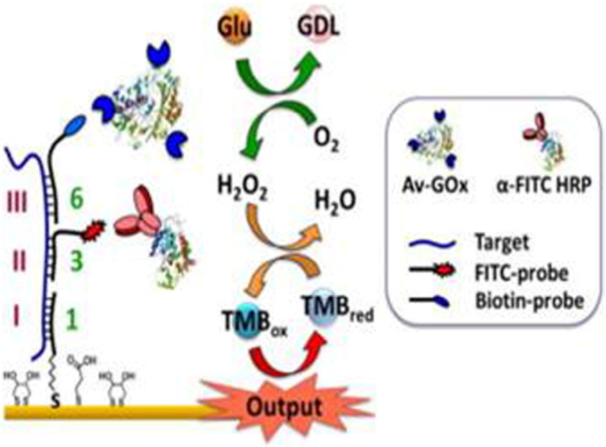

Fig. 5. Envisioned configuration of biomolecular detection of genetically modified organisms (GMOs) on the chip, wherein the computing relies on three hybridization reactions, two affinity recognition events, and biocatalytic cascade reactions with the resulting output displayed in terms of the electrochemical transformation of oxidized TMB (TMB_{ox}) to reduced TMB (TMB_{red}). Right: various substances involved in the gate. {Reproduced from [59] with permission from Elsevier}.

tase [28,29], catalase [23], glucose oxidase and horseradish peroxidase [59]. An interesting bienzymatic approach, recently described by Liao et al. [59], involves an enzymatic cascade reaction with two different enzymes. The aim is to obtain an electrochemical output only when the three targets are present in a sample, promoter, gene-specific and taxon sequences (Fig. 5).

The use of an NP-based signaling system has also been reported, in which AgNPs [43], CdS-NPs [39] and PbS-NPs [33] were proposed. As shown in Fig. 6, the NPs are bound to functionalized probes that will hybridize with the target, previously bound to the electrode surface via SAM. In the end, the NPs are dissolved in an acid solution and measured by voltamperometric techniques.

Sandwich hybridization is occasionally used to avoid labeling the target sequence [23,29,43]. Two probes are involved in this kind of assay:

- a capture probe partly complementary to the target and attached to the electrode; and.
- a labeled signaling probe that hybridizes with the rest of the target (Fig. 7).

Electroactive labels are measured by different electrochemical techniques, including cyclic voltammetry (CV), differential pulse voltammetry (DPV) (Fig. 8), anodic stripping voltammetry (ASV), chronoamperometry and square wave voltammetry (SWV). Electrochemical transduction of the hybridization process can also be performed by electrochemical impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$, which allows the study of the surface properties of a modified electrode measuring changes in values of electron-transfer resistance R_{et}. An increase in the R_{et} value is obtained after hybridization due to the higher resistance to electron transfer generated by the presence of dsDNA. Impedance-based devices have outstanding sensitivity compared to other techniques [44,53].

6. Challenges in applying GMO genosensors to real samples

In the early stages of GMO-sensor development, they were applied only to pure solutions of synthetic oligonucleotides. These types of experiment are required to know relevant parameters, such as sensitivity and selectivity of the analytical assay under research. From these results, the immobilization and detection method could be demonstrated, and the capability to discriminate between complementary targets and unspecific DNA sequences. These results have great scientific value and these contributions are really encouraging.

However, the performance of genosensors with a real, complex sample may be entirely different {e.g., when the target sequence is present within a relatively large excess of non-complementary nucleic acids, proteins, organic molecules, salts and other undefined components [67]}. This is the great challenge these analytical devices face.

Most GMO genosensors were applied to the analysis of powdered plant tissues [23,29,31,32,36–38,40,42,50–54,57–60], but commercial food and feed samples have not so far been assessed with electrochemical genosensors.

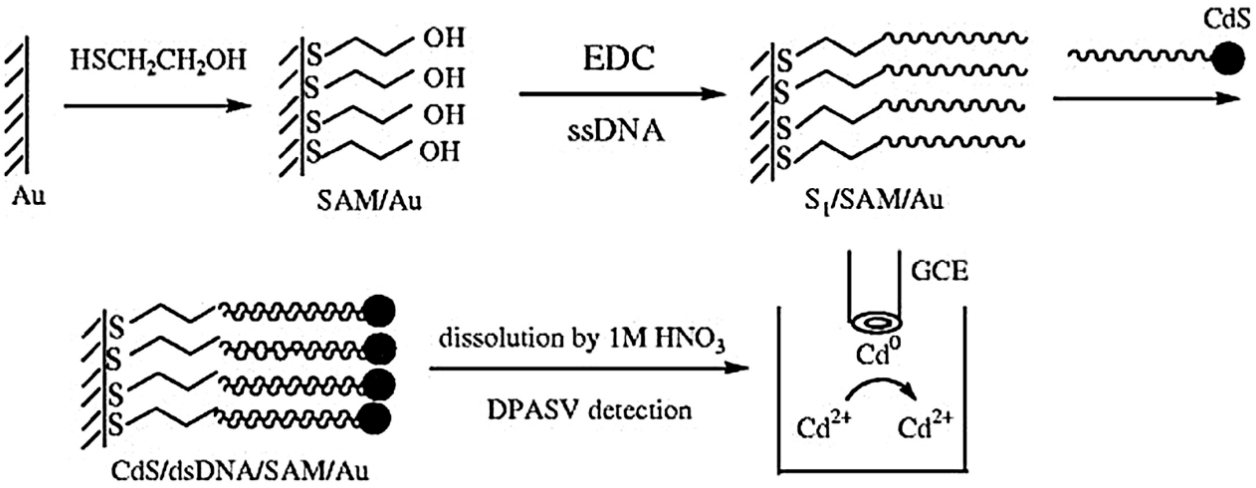

Fig. 6. Electrochemical DNA detection with CdS nanoparticle labels. {Reproduced from [39] with permission from Springer}.

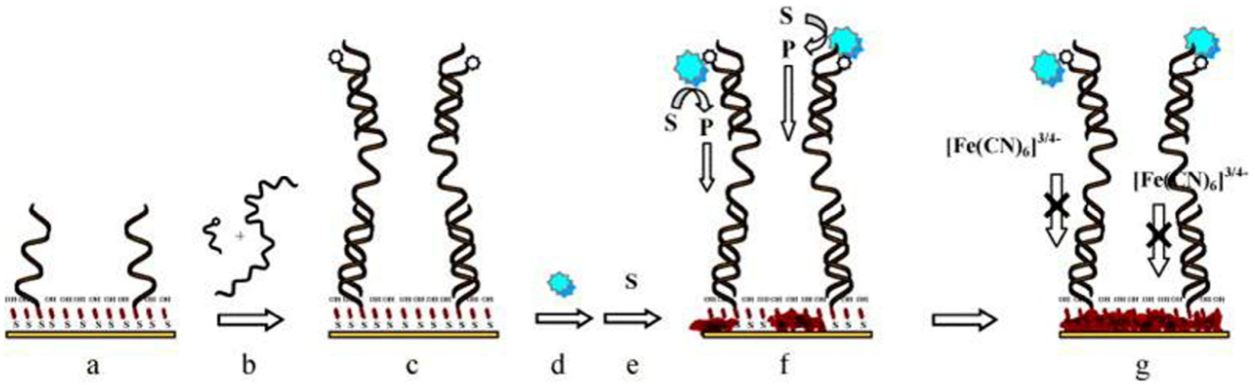

Fig. 7. The impedimetric genosensor (sandwich hybridization assay). Unmodified PCR products (b) were captured at the sensor interface (a) via sandwich hybridization with the surface-tethered probe and a biotinylated signaling probe. The biotinylated hybrid (c) was then coupled with a streptavidin–alkaline phosphatase conjugate (d) and finally exposed to the substrate solution (e). The bio-catalyzed precipitation of an insulating product (f) blocked electrical communication between the gold surface and the $[Fe(CN)_6]^{3/4-}$ redox probe (g); Faradic impedance spectroscopy was finally used to detect the enhanced electron-transfer resistance. {Reproduced from [29] with permission from Elsevier}.

The complexity of the matrix in real samples makes the detection of GMO a challenge, primarily due to the minute portion of the specific oligonucleotide sequence of the organism selected as target *versus* the complete genome, but also due to the double-helix and coiled conformation of DNA, which makes the hybridization reaction in the genosensor extraordinarily difficult [68,69]. A sample pretreatment step is therefore generally required. This step involves DNA extraction and amplification of the short target sequence.

In GMO analysis, even ultrasensitive methods are unable to avoid amplification of the target sequence and need a pre-concentration step. The genosensor is designed to detect a specific fragment in a genomic DNA extract, in which the ratio between the target and interfering species will often be very low [67]. Although some genes are present in high copy numbers in the genome of some species, in GM plants, analysts generally deal with single-copy genes, making amplification necessary. So far, PCR has been the most frequent amplification process coupled to the genosensing protocol. However, other amplification strategies, such as isothermal amplification, could be coupled to the biosensors [70–72], but this type of amplification has not yet been reported coupled to electrochemical GMO genosensors. The amplification of DNA by PCR involves multiple components, including templates, primers, ions, nucleotides, and variables, such as enzyme activity and reaction temperature [73]. Except for the reaction temperature, which is well controlled, the rest of the parameters will change during the course of the reaction, without the possibility of control. These changes affect the amplification efficiency. During PCR, the amplification occurs exponentially until saturation is reached [74], and that could explain the difficulty of getting quantitative results with conventional PCR [69,74,75]. Lucarelli [29] considered that the amplicons could be properly quantified only in the exponential phase of their amplification.

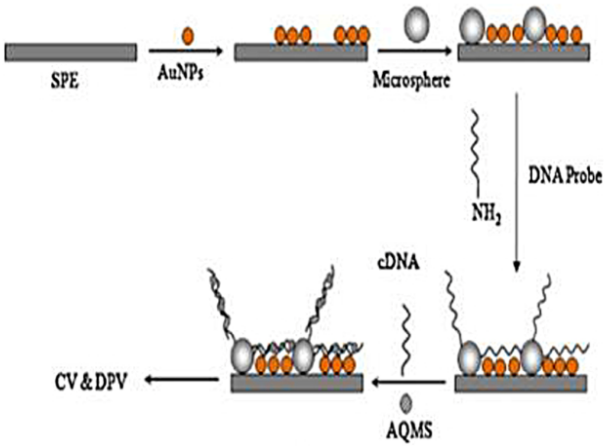

Fig. 8. Design mechanism of electrochemical genetically modified DNA biosensor based on acrylic microsphere-modified gold-nanoparticle screen-printed electrode (2). {Reproduced from [36] with permission from Elsevier}.

The re-annealing of the amplicons is another important factor to be considered. Conventional PCR leads to a double-stranded DNA, but the two strands have to be separated for successful hybridization in the genosensor. For this purpose, a thermal denaturation step is usually performed, followed by an ice-cold bath to maintain the strands separated. In the sensor, the hybridization reaction is favored against re-annealing because of the excessive number of probes. Another strategy is the use of asymmetric PCR, in which an overproduction of single-stranded target is obtained [31,57].

The success of DNA amplification depends on the presence of a large quantity of high-quality DNA. The DNA present in highly processed food is usually fragmented into small sequences (60–100 bp) [76,77]. This problem can usually be overcome by designing specific primers targeting short sequences. Food products are complex matrices that might contain a number of PCR inhibitors and have frequently been subjected to one or more steps of processing (mechanical, thermal, chemical or enzymatic treatments) affecting the integrity of DNA. Consequently, the isolation and the amplification of DNA from foods comprise the most difficult step in food analysis [19,76,78–80]. Generally, the effects of the inhibitors may be reduced by evaluating several extraction methods according to the matrix, by choice of a more robust DNA polymerase or use of specific PCR additives. To determine the inhibitory effect, an internal control assay (e.g., the taxon-specific reference system) should be performed [81]. This is useful to exclude false-negative results, which may result from complete inhibition of the PCR, even in the presence of the target sequence [82].

Restriction enzymes generate shorter sequences by cutting the genomic DNA at specific sites, making them more accessible for hy-

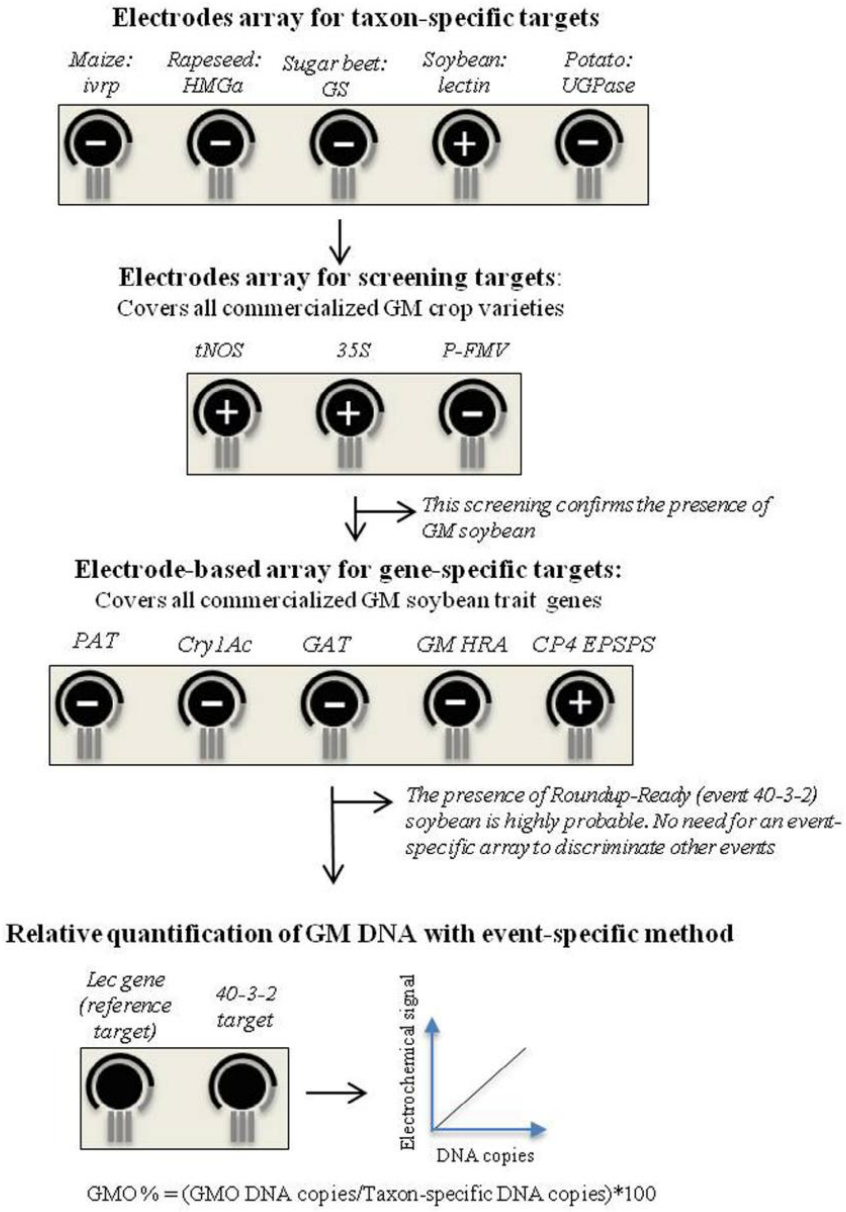

Fig. 9. Example of Roundup Ready Soybean determination using an electrochemical-array sensor.

bridization [32]. However, its application is limited by sensor sensitivity.

Moreover, if quantification of GMOs with genosensors is intended to be in line with legislation, the ratio of event-specific target to taxon-specific target expressed in copy numbers must be reported. However, none of the reviewed works reported this ratio.

7. Future of GMO genosensors

Many challenges related to GMO determination should be addressed, such as the detection of unauthorized GMOs and multitarget analysis. Each year, new GMOs are entering the world market. Their number and diversity are growing rapidly, and they challenge the established system of traceability and detection schemes. One of the possibilities for improving the efficiency of GMO diagnostics is to analyze several targets simultaneously. The ability to multiplex greatly expands the power of electrochemical-genosensor analysis. Electrode arrays provide an excellent electrochemical platform for GMO testing. As shown in an example in Fig. 9, discrimination between different plant species is the first step, followed by identification of common transgenic elements, such as promoter 35S. Negative samples for screening targets would be considered GMO free, while those that are positive would be submitted to a more specific analysis of construction or event-specific sequences. Thus, the GM event would be recognized and could be quantified by co-analyzing the reference gene. Standard curves for both transgenic and reference targets would allow calculation of the GMO percentage in unknown samples.

8. Final remarks

Detection and traceability of GMOs are attracting interest worldwide due to their growing presence and the related socio-economic implications. Electrochemical DNA-hybridization sensors have very interesting, promising applications in relation to multi-target analysis and their implementation in miniaturized and automated devices suitable for in-field analysis, because of their simplicity, low cost and portability.

In order to achieve feasible application of these electrochemical devices in GMO detection, more efforts should focus on optimizing these systems, especially when working with real samples. Currently, the sensitivity of genosensing systems is insufficient to eliminate the need for PCR coupling. Most DNA sensors for GMOs have achieved quantification only when synthetic oligonucleotides were analyzed. However, only qualitative responses have generally been reported on powdered plant materials. There have been no genosensors for highly processed foods (e.g., sauces and syrups).

Although the determination of the relative GMO content is recommended using a taxon-specific gene, it was not reported for the electrochemical genosensors proposed so far. More efforts should therefore be devoted to achieve the quantification required by the current European labeling legislation.

Finally, with all this in mind, we can conclude that electrochemical genosensors are a powerful option in DNA detection, and offer a suitable strategy for GMO monitoring.

Acknowledgments

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| CHAPTER 3 |

AIMS AND SCOPE

3

AIMS AND SCOPE

European policies regarding GMOs are highly strict, which is why they could benefit from the availability of low-cost and readily-applicable analytical methods for GMO monitoring. Compliance with labeling thresholds must be guaranteed throughout the production chain, for which GMO quantification is ultimately required. This is a highly demanding analytical task that is, at present, solely fulfilled by qPCR methods, which are not utterly available in resource-limited environments and are often inadequate for decentralized and on-field analysis.

While electrochemical DNA biosensor/sensing technologies represent viable alternatives for this end, there are still fundamental limitations in the field. The inability to accurately quantify specific DNA sequences derived from 'real' samples is a critical drawback. Moreover, qualitative biosensors aimed at screening purposes should be based on simpler and faster platforms with superior sensitivity than the already available gel-based technologies. These aspects, which were highlighted in the previous chapter, are clearly holding back the widespread use of electrochemical devices for food control.

This thesis is aimed at the design, development and application of electrochemical DNA biosensors and sensing platforms for the detection and quantification of genetically modified soybean (RRS) in food and feed samples. To achieve this main objective, the following specific aims are proposed:

1. Selecting taxon-specific (*Lec*) and event-specific (*RR*) targets for the relative quantification of RRS. Designing complementary, biotin and hapten-tagged capture and signaling probes, respectively, for sandwich hybridization with the target sequences.

2. Developing electrochemical DNA sensing platforms based on magnetic beads and enzymatic labeling for the separate chronoamperometric detection of *Lec* and *RR*.

3. Designing and developing a multiplex electrochemical DNA sensing platform based on magnetic beads and bi-enzymatic labeling for the subsequent chronoamperometric and voltammetric detection of *Lec* and *RR*, respectively.

4. Developing and applying an analytical method for relative GMO quantification in flour samples comprising DNA extraction, amplification of the target sequences by end-point PCR and hybridization/detection of the analytes with the separate chronoamperometric sensing platforms. Assessing quantitative results with qPCR.

5. Developing and applying an analytical method for relative GMO quantification in processed, commercial samples of food and feed, comprising DNA extraction, amplification of the target sequences by end-point PCR and simultaneous hybridization/subsequent detection of the analytes with the multiplex sensing platform. Assessing quantitative results with qPCR. Surveying the prevalence of RRS in the Spanish market by analyzing these samples with conventional PCR, qPCR and with the multiplex electrochemical sensing platform.

6. Designing and developing an impedimetric DNA biosensor for the label-free, fast and simple detection of *RR* sequences based on LbL phthalocyanine assemblies onto nanostructured electrodes.

| CHAPTER 4 |

MULTIPLEX ELECTROCHEMICAL DNA PLATFORM FOR FEMTOMOLAR-LEVEL QUANTIFICATION OF GENETICALLY MODIFIED SOYBEAN

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MULTIPLEX ELECTROCHEMICAL DNA PLATFORM FOR FEMTOMOLAR-LEVEL QUANTIFICATION OF GENETICALLY MODIFIED SOYBEAN

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This first work is aimed at the design and development of a DST-based sensing method directed towards GMO quantification, using as model analyte the Roundup Ready® soybean line. For this end, event-specific and taxon-specific methods were developed. Magnetic beads with streptavidin-biotin and hapten-antibody-enzyme conjugates as immobilization and labeling chemistries, respectively, were used to obtain the best possible analytical features, especially required for quantitative DNA-based methods.

In this work, on one hand, two separate electrochemical DNA sensing platforms were developed and optimized for the detection of *RR* and *Lec* sequences. On the other hand a multiplex platform is proposed as novelty in the field, in which the two sequences necessary for relative RRS quantification are simultaneously entrapped onto the surface of magnetic beads and detected subsequently *via* bi-enzymatic labeling. The assays were pre-validated using synthetic mixtures with highly dissimilar content of both sequences to check whether the multiplex platform had any competition-related problems between the different strands co-existing in solution.

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Multiplex electrochemical DNA platform for femtomolar-level quantification of genetically modified soybean

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ABSTRACT

Current EU regulations on the mandatory labeling of genetically modified organisms (GMOs) with a minimum content of 0.9% would benefit from the availability of reliable and rapid methods to detect and quantify DNA sequences specific for GMOs. Different genosensors have been developed to this aim, mainly intended for GMO screening. A remaining challenge, however, is the development of genosensing platforms for GMO quantification, which should be expressed as the number of event-specific DNA sequences per taxon-specific sequences. Here we report a simple and sensitive multiplexed electrochemical approach for the quantification of Roundup-Ready Soybean (RRS). Two DNA sequences, taxon (lectin) and event-specific (RR), are targeted via hybridization onto magnetic beads. Both sequences are simultaneously detected by performing the immobilization, hybridization and labeling steps in a single tube and parallel electrochemical readout. Hybridization is performed in a sandwich format using signaling probes labeled with fluorescein isothiocyanate (FITC) or digoxigenin (Dig), followed by dual enzymatic labeling using Fab fragments of anti-Dig and anti-FITC conjugated to peroxidase or alkaline phosphatase, respectively. Electrochemical measurement of the enzyme activity is finally performed on screen-printed carbon electrodes. The assay gave a linear range of 2–250 pM for both targets, with LOD values of 650 fM (160 amol) and 190 fM (50 amol) for the event-specific and the taxon-specific targets, respectively. Results indicate that the method could be applied for GMO quantification below the European labeling threshold level (0.9%), offering a general approach for the rapid quantification of specific GMO events in foods.

1. Introduction

Genetically modified plants have brought great benefits in agriculture, such as increased crop yields, reduced need for pesticides, enhanced nutrient composition and food quality (Phillips, 2008). However, biotech crops are also the cause of great controversies among consumers, who question the safety of these modified organisms for human consumption and for the environment. These arguments have resulted in public demand for labeling transgenic-derived foods (Frewer et al., 2004). In the European Union, there are specific regulations in this matter that involve the control and monitoring of genetically engineered traits throughout the production chain of food and feed. Product labeling is mandatory, except when the GMO content is below 0.9% of the food or feed product, provided that this presence is adventitious or technically unavoidable (European Commission, 2003). Consequently, reliable quantification of genetically modified organisms (GMOs) is required to fulfill these regulations. The need to verify the presence and the amount of GMOs requires accurate, sensitive, rapid and cheap analytical methods. Approaches based on the direct analysis of the genetic modification (s) are preferred over targeting the expressed protein(s) (Garcia-Canas et al., 2004; Mafra et al., 2007). In this regard, Real-time Polymerase Chain Reaction (RTi-PCR) has proven to be the most successful technique so far for GMO quantification. However, a rising interest on alternative methods is driven by the high-priced instrumentation required by the RTi-PCR technique (Holst-Jensen, 2009). Efforts are focused on low-cost methods that generate simple and fast measurements and data easy to interpret. Analytical methods with possibility for automation and microfabrication based on simple and portable detection systems, such as visual-based devices or electrochemical sensors, represent a valuable tool for GMO analysis (Kamle and Ali, 2013; Randhawa et al., 2013).

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In the field of electrochemical DNA sensors, several devices have been reported to detect GMOs (Arugula et al., 2014; Kamle and Ali, 2013). Most of these methods are element-specific or gene-specific, serving as screening tools. Although qualitative assays are useful for preliminary analysis, event-specific methods provide the highest level of specificity, serving also for quantification purposes. These methods target the junction between the inserted DNA and the recipient genome, which is characteristic of each event (Miraglia et al., 2004). A key element for the authorization of GMOs within the European Union is the provision of an event-specific quantitative method (European Commission, 2003). For the relative quantification of GMOs according to the European legislation, two reactions must be performed on the same template DNA: one targeting an endogenous gene that is specific to the plant taxon, and the other targeting an event-specific sequence. The relative percentage of GMO is often calculated as the percentage of transgene target copies relative to the total number of DNA (taxon) copies (Chaouachi et al., 2013) and taking into account the transgenic plant zygosity.

Most GMO genosensors claim that they are suitable for quantitation by measuring DNA from a certified material of a certain percentage. Although the feasibility of detecting such samples is not under question, they fail to demonstrate their ability to quantify the GMO percentage from an unknown sample as it is mandatory to verify the European labeling compliance. The reason behind this is the need for developing the corresponding genosensor for the taxon and establishing the relationship between both measurements to give the result as GMO percentage. To the best of our knowledge, this has not been accomplished yet.

Multiplex DNA detection is an attractive strategy for quantification purposes given the possibility of targeting the taxon and the transgenic sequences simultaneously in a single-tube assay (Foti et al., 2006). Few electrochemical genosensors have been proposed as multiplexed platforms for GMO detection (Duwensee et al., 2009; Liao et al., 2013; Mix et al., 2012), and all these sensors are intended as screening methods or to identify a specific event, while currently, quantification remains almost exclusively achievable by the RTi-PCR technology.

In this work, we propose a quantitative approach based on multiplex eletrochemical transduction to serve as a sensitive analytical tool in GMO monitoring. As a model GMO we selected Round-Up Ready Soybean (RRS), a genetically engineered herbicide-resistant form of soybean that represents one of the most successful achievements of crop biotechnology, occupying more than 75% of worldwide soybean plantations in 2013 (James, 2013). Two sequence-specific DNA probes, targeting an event-specific sequence from RRS and a fragment of the endogenous lectin gene specific for soybean (Mazzara et al., 2007), are simultaneously entrapped onto the surface of magnetic beads. These probes are interrogated together with the sample containing two signaling probes, each modified with a distinct tag. The magnetic platform offers a highly efficient approach to achieve the isolation of the target DNA from complex matrices with simple washing steps (Palecek and Fojta, 2007), avoiding the adsorption of non-specific species directly at the electrode surface, common in conventional sensors (Palecek et al., 2002). After a sandwich hybridization assay, each tag is labeled with a different enzymatic reporter, allowing the parallel electrochemical readout of both genes. The quantification of the percentage of a specific transgenic event is thus achieved for the first time by genoassay technology instead of RTi-PCR. The practicability and low-cost features of this platform make it highly suitable for routine analysis.

2. Experimental

2.1. Chemicals and materials

Streptavidin-coated magnetic beads Dynabeads MyOne streptavidin C1 (1 μm diameter) were from Life Technologies (Spain). The modification of the beads and the hybridization and labeling assays were performed in a 12-tube mixing wheel (Dynal MX1) and magnetic separations were carried out with a magnet DynaMag2, both from Life Technologies.

Tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H_2O_2), diethanolamine (DEA) and Tween 20 were purchased from Sigma-Aldrich (Spain). Saline sodium phosphate-EDTA (20 × SSPE) pH 7.4 and 1-naphthyl phosphate were also obtained from Sigma-Aldrich. Ethanol was purchased from Panreac (Spain). 1% casein buffer in 1 × PBS was obtained from Fischer Scientic (Germany). Anti-fluorescein-peroxidase (antiFITC-POD), anti-fluorescein-alkaline phosphatase (antiFITC-ALP) and anti-digoxigenin-peroxidase (antiDig-POD) Fab fragments were received from Roche Diagnostics GmbH (Mannheim, Germany). MilliQ water was used throughout this work.

The oligonucleotide sequences used and the specific Gibbs energy for their most stable secondary structures are shown in Table S1 in the Supplementary material. Oligomers were synthetized by Sigma-Genosys. All stock solutions were prepared in MilliQ water and stored at −20 °C.

The names and composition of the buffers used were:

1. Washing buffer: SSPE-T (2 × SSPE, 0.005% of Tween-20)
2. Hybridization and immobilization buffer: SSPE-N (2 × SSPE, 0.9 M NaCl, pH 7.4)
3. POD buffer: PBS-C (Phosphate buffered saline 0.1 M phosphate, 1.54 M NaCl, 1% casein, pH 7.4)
4. ALP buffer: DEA-M (DEA 1 M, 0.5 mM $MgCl_2$, pH 9.8)

2.2. Apparatus

Electrochemical measurements were carried out with disposable screen-printed carbon electrodes (SPCEs, DropSens-110, Spain), using a computer-controlled μ-AutoLab type II potentiostat with GPES 4.9 software and PGSTAT101 potentiostat with NOVA 1.9 software (EcoChemie, The Netherlands).

Before carrying out the measurement step, the SPCEs were washed with ethanol and deionized water, and dried with nitrogen. No electrochemical pretreatment was required. All experiments were carried out at room temperature, and each measurement took place in a new disposable electrode. pH measurements were performed with a Crison micropH 2001 pH-meter(Spain).

2.3. Biomodification of streptavidin-coated magnetic beads

The experimental procedures were based on the described by González-Álvarez et al. (2013) with slight modifications. 5 or 10 μL of the commercial preparation of magnetic beads (10 mg/mL) were transferred into an eppendorf tube and mixed with 245 or 490 μL of SSPE-T buffer, for the single and multiplex assays, respectively. Once magnetically separated they were washed twice with 250 or 500 μL of the same buffer. Then, they were resuspended in 250 μL of a solution containing 0.25 nmol of biotinylated capture probe prepared in salt-adjusted buffer SSPE-N, according to manufacturer's instructions. In the multiplex assay, the particles were resuspended in 500 μL of an equimolar mixture (0.25 nmol) of both capture probes. The resulting suspension was incubated for 30 min at room temperature and then washed twice with 250 or 500 μL of SSPE-T.

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2.4. Hybridization

Biomodified microparticles were used in a sandwich-type hybridization assay. First, the desired concentration of target DNA and 10 nM signaling probe were mixed in SSPE-N to a final volume of 250 or 500 μL and subjected to a thermal cycle, 5 min at 98 °C and 5 min in an ice bath, to facilitate homogeneous hybridization. The mixtures were left 25 min at room temperature. Subsequently, heterogeneous hybridization between the partial duplex and the magnetic beads previously modified with capture probe took place. With this aim, 250 or 500 μL of the homogenous hybridization mixture was used to resuspend the washed microparticles. The heterogeneous hybridization reaction proceeded for 1 h at room temperature under rotation. The hybrid-conjugated beads were then washed twice with SSPE-T buffer and subsequently washed with the PBS-C buffer.

2.5. Enzymatic labeling and electrochemical detection

Enzymatic labeling was performed by resuspending the beads in 250 μL of antiFITC-POD conjugate and antiDig-POD conjugate 0.25 U/mL, both prepared in PBS-C buffer. For the multiplex assay, the beads were resuspended in a mixture (500 μL) containing 6.25×10^{-2} units of antiFITC-ALP and antiDig-POD also prepared in PBS-C. After 30 min of incubation time, the beads were washed twice with PBS-C and finally resuspended in 100 μL of 2 × SSPE for the single assays and 200 μL of DEA 1 M pH 8.5 for the multi-labeled system.

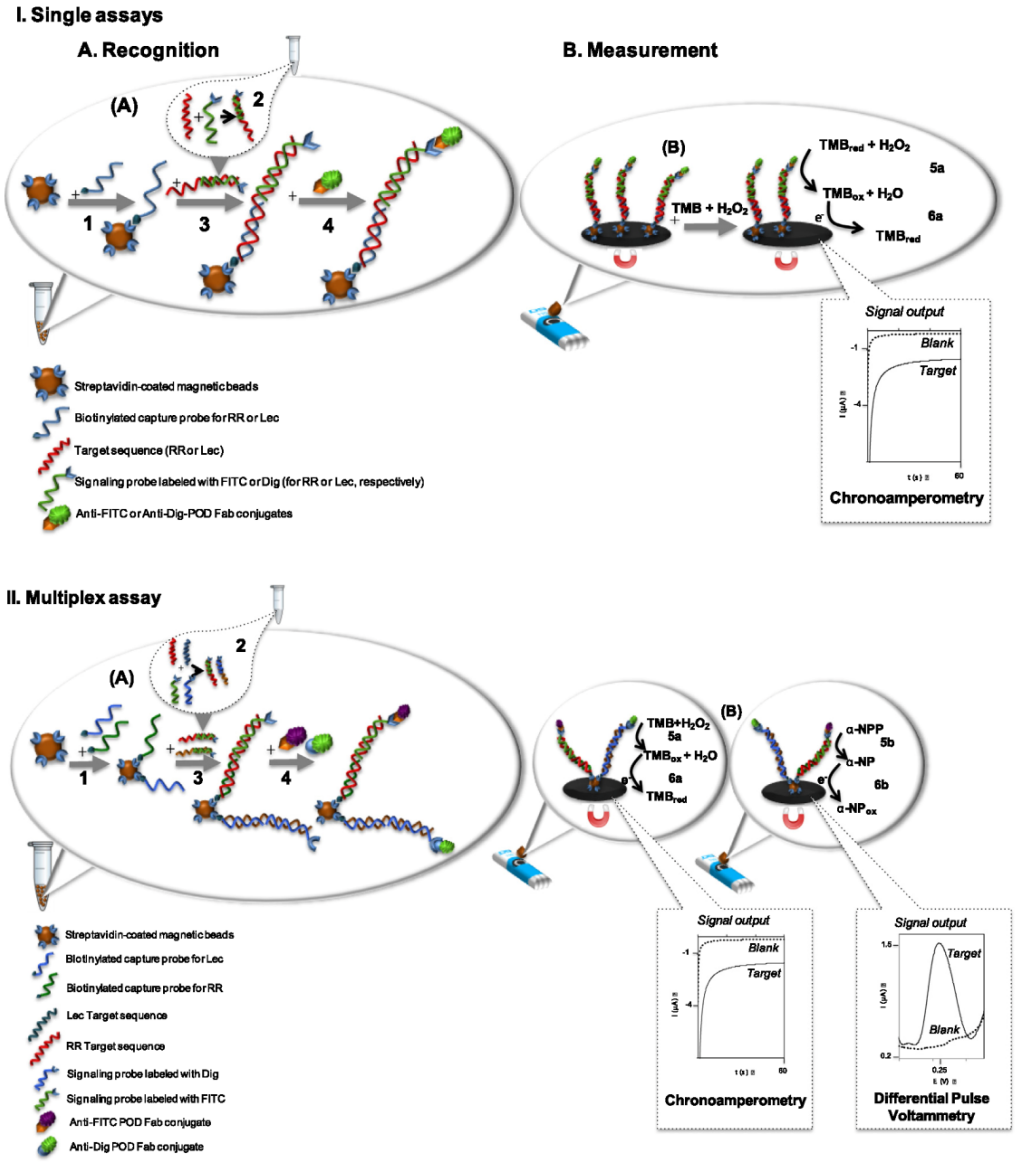

Fig. 1. Scheme of the (I) single assays (valid for either RR or Lec detection) and (II) multiplex assay (simultaneous detection of RR and Lec). The assays are divided into two steps, recognition (A) and measurement (B): (1) attachment of capture probe(s) to the surface of magnetic beads; (2) homogeneous hybridization between a labeled-probe and target sequence; (3) heterogeneous hybridization with capture probe bound to the beads; (4) addition of the Fab-enzyme conjugate; (5a–6b) enzymatic reactions occurring after adding the enzymatic substrate (TMB/α-NPP); (6a) chronoamperometric measurement of TMB_{ox} reduction at the electrode surface; (6b) voltammetric measurement of naphthol oxidation current at the electrode surface.

The electrochemical detection of the enzymatic products was performed onto the surface of a disposable carbon electrode, by means of differential pulse voltammetry (DPV) for ALP or chronoamperometry for POD. 15 μL of the fully modified beads were magnetically captured onto the working electrode with a magnet placed under it. After 2 min, the liquid was carefully removed with a pipette. Finally, 45 μL of the enzymatic substrate (4 mM α-NPP in DEA-M for ALP and commercial TMB substrate for POD) was placed on the cell, covering the three electrodes, and after a fixed time the product was detected. α-Naphthol, the product of the ALP reaction, was measured after 10 min of enzymatic reaction by DPV from 0 to 0.55 V with a modulation amplitude of 0.05 V, a step potential of 0.0015 V, an interval time of 0.5 s, and a modulation time of 0.05 s. When POD-conjugates were used, chronoamperometric measurements were performed after 60 s of enzymatic reaction using a potential pulse to 0 V, and sampling the current during 60 s.

3. Results and discussion

Initially, we designed single assays to separately detect both the event-specific and taxon-specific targets with the aim of optimizing the working variables. Once the operating conditions were selected we developed the multiplex platform. Fig. 1 shows the schemes of both single and multiplex methods. The absolute quantification of GMO requires the construction of standard curves for the genetically modified event and total plant DNA, which in a multiplex format involves the use of two distinguishable reporter molecules. For electrochemical detection two different enzymatic labels were selected, namely peroxidase (POD) and alkaline phosphatase (ALP), both incorporated to the double-stranded DNA formed onto the surface of the magnetic beads after hybridization reaction with the sample through the affinity interaction with a Fab fragment recognizing the specific tag in the signaling probe (FITC or digoxigenin). We first evaluated the performance of the single assays for both sequences using POD as label due to its high turnover rate. Optimization was carried out with the event-specific target, and the resulting experimental conditions were extrapolated to the lectin assay. In parallel, we comparatively evaluated the analytical performance of the RR single assays using the two enzymes as label, selecting for the multiplex assay the enzyme that provides the lowest limit of detection for the event-specific target, to be found at lower levels in the sample.

3.1. Single assay optimization

We evaluate several variables involved in the assay that we identify to be highly relevant for a good analytical performance. The amount of magnetic beads deposited on the electrode surface proved to be the most important variable in the assay, causing important shifts in the signal-to-blank ratios. In order to evaluate this parameter, 50, 100, 300 and 1000 μL of 2 × SSPE were added to the washed beads prior to the measurement step, with the aim of depositing from 0.75 to 15 μg of beads onto the working electrode. Other conditions were: Tween-20 0.005% in the washing buffer, 0.5 μM signaling probe, 0.5 U/mL enzymatic conjugate and 2 × SSPE buffer for hybridization. Incubation times were the same as described in Section 2. Results are shown in Fig. 2A. The current response measured for 10 nM of transgenic target increased when the amount of beads rose to 7.5 μg. By contrast, the blank response did not change significantly, leading to consistently higher *S*/*B* ratios when 7.5 μg of particles were deposited on the electrode. With twice the amount of beads, the *S*/*B* value decreased, possibly indicating steric hindrance of the biomodified particles on the electrode surface, which limit the diffusion of the product of the enzymatic reaction to the sensor surface. Thus, 7.5 μg was the amount of bead selected for subsequent studies.

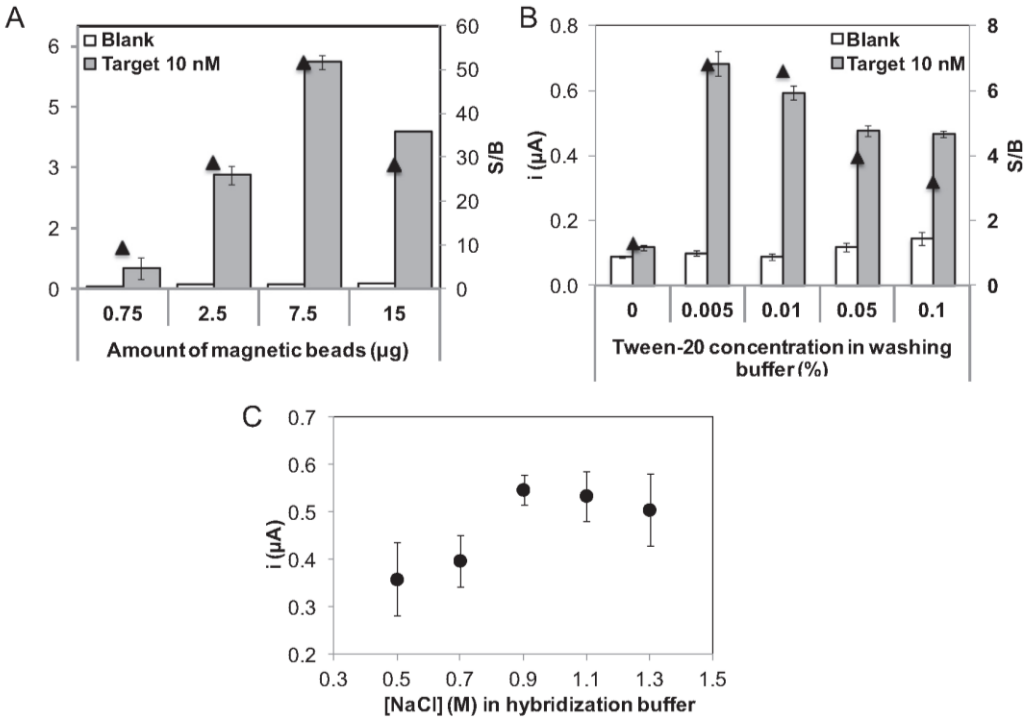

Fig. 2. Effect of (A) magnetic beads mass deposited on the electrode; (B) Tween-20 concentration in the washing buffer and (C) NaCl concentration in the hybridization buffer on the current measured for the event-specific target. *S*/*B* ratio is indicated with triangles in the secondary axes. For experimental conditions see text.

The influence of the signaling probe and enzymatic conjugate concentrations on the *S/B* ratio was also examined. Four different concentrations of the FITC-probe were mixed with 10 pM of transgenic target. The blank responses increased with increasing probe concentration, suggesting that non-specific adsorption is favored with higher concentration of signaling probe. Consequently, the lowest concentration evaluated (10 nM) yielded the highest *S/B* value, 6.3 (Fig. S1). In contrast, the concentration of the enzymatic conjugate anti-FITC POD hardly influenced the target and blank signals (data not shown) in the studied range (0.25–1 U/mL). Thus, 10 nM and 0.25 U/mL of signaling probe and enzymatic conjugate, respectively, were chosen for the assays.

The buffer composition in the different steps is another critical parameter. Surfactants are used to stabilize magnetic particles by both steric and electrostatic effects. Therefore, Tween-20 concentration in the washing buffer was thought to have an important effect on the dispersion and magnetization of the microparticles, and also on the removal of non-specific adsorbed nucleic acids and proteins on the surface of the beads (Shen et al., 2011). In order to evaluate this effect, we assayed different Tween concentrations in the range of 0.005–0.1%. The highest *S/B* value for the event-specific target 10 pM was reached with 0.005%, followed by 0.01% (Fig. 2B). Above this value the blank signal began to increase while the target response decreased, possibly because high concentrations of Tween yield the formation of surface structures that may disrupt the biomodified surface of the beads, giving higher background currents and lower target responses. On the other hand, a very low *S/B* ratio was obtained when no Tween was added to the buffer, indicating the need of incorporating this detergent for magnetic bioseparations, even at low concentrations.

The hybridization buffer was also assessed considering that ionic strength is one of the most important factors in DNA hybridization (Levicky and Horgan, 2005) and immobilization efficiency (Sánchez-Paniagua López et al., 2014). The amount of NaCl has a pronounced influence on these steps due to the effect of the ionic density around the DNA strands. In order to study this variable, hybridization was performed in 2 × SSPE solution (pH 7.4) with different concentration of NaCl in the range of 0.5–1.3 M and a transgenic target concentration of 10 pM. The signal increased up to 0.9 M of NaCl (Fig. 2C), confirming the expected effect of salt ions on the enhancement of hybridization efficiency. Thus, NaCl 0.9 M was chosen for subsequent experiments (buffer SSPE-N).

The use of optimum ionic strength resulted in a considerable improvement of the linear range of the assay. Comparing calibrations performed using as hybridization medium 2 × SSPE (Fig. S2-A) and SSPE-N (Fig. S2-B), we observed that the linear range was extended from 10–100 pM to 5–350 pM. This fact was attributed to the effect of saline ions on the stabilization of the DNA duplex because ionic strength governs the magnitude of electrostatic interactions (Gong and Levicky, 2008). Sodium cations are attracted to the negative charged phosphate backbone of the DNA strands, which results in a decrease of the electrostatic repulsion between strands and as a result, the hybridization is favored. When using 2 × SSPE, hybridization occurred linearly with target concentration up to 100 pM. On the contrary, when working under optimal ionic strength conditions (SSPE-N buffer), hybridization took place efficiently up to a target concentration of 350 pM.

3.2. Assessment of the dual enzymatic label for the multi-target scheme

The design of the multi-target scheme required the incorporation of a different enzyme for each target in order to obtain two distinct signals. Toward this goal, we explored the use of antiFITC-ALP conjugate for RR detection. ALP activity depends on the buffer composition and the presence of magnesium ions. The activity decreases in phosphate buffers due to inhibition of ALP by inorganic phosphates (Fernley and Walker, 1967). Therefore, the phosphate-based buffer used in the single assays had to be replaced for another pH-regulating system compatible with ALP, such as Tris–HCl and DEA buffers. These buffers support high ALP activity due to transphosphorylation, wherein the net transfer of phosphate from substrate to the hydroxyl group of the buffer is a more rapid reaction than transfer to water (hydrolysis) (McComb and Bowers, 1972). Given this, we performed preliminary tests with two different buffers i.e. Tris–HCl 0.5 MpH 9.8 with $MgCl_2$ 0.5 mM and KCl 0.1 M and DEA-M (Walter and Schütt, 1974). These buffers were used to dissolve the enzymatic substrate, α-NPP. The *S/B* ratio obtained for an event-specific target concentration 5 pM in DEA buffer was 11, more than two times than that obtained when the enzyme activity is measured in Tris–HCl (Fig. S3). This behavior was attributed to the amino group and the two hydroxyl moieties in DEA that play an important role in transphosphorylation, hence this buffer was selected for the incorporation of ALP in the multiplex assay.

Subsequently, a comparison between ALP and POD and the respective electrochemical techniques, DPV and chronoamperometry, was carried out using the RR target and the FITC-probe tag. Results indicated that the ALP-DPV combination exhibited higher sensitivity (slope=39 ± 3 nA pM^{-1}; *S/B* for 5 pM=5.6) and lower blank current (30 ± 2 nA) than the POD-chronoamperometry system (slope=27.5 ± 0.9 nA pM^{-1}; *S/B* for 5 pM=3.2; blank=100 ± 8 nA). A linear fit was obtained up to 200 pM for ALP, while the POD-based combination permitted to detect up to 350 pM (Fig. 3). However, since one of the requirements of these assays is to achieve detection of low levels of RR, saturation with high target concentrations is not an issue for the GMO-related sequence. While for Lec, it is expected to obtain responses in the upper region of the calibration curve, due to the high content of Lec predicted in a soybean sample with respect to GMO content. Hence, we chose the ALP-label for RR and the POD-label for Lec in the multiplex protocol.

3.3. Effect of probes co-immobilization: multiplex assay

Once the labeling system was revised for the multiplex assay, it seemed imperative to evaluate if the simultaneous immobilization of the two capture probes, both with the same length, had any competition-related problem that could compromise the analytical response of one of the targets. This was assessed by immobilizing both probes on the magnetic beads separately and subsequently joining the two suspensions in a single tube to

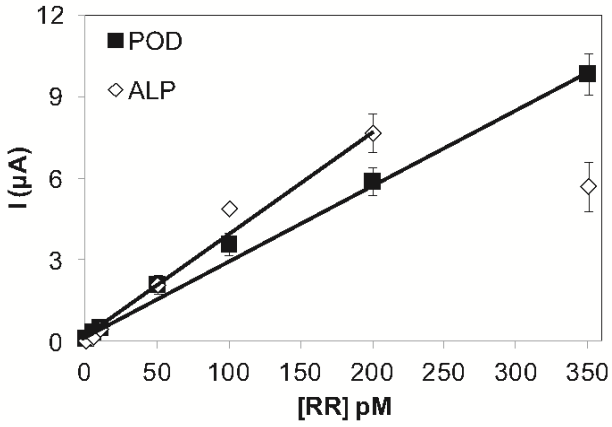

Fig. 3. Comparison of the response obtained with ALP and POD as labels for RR.

perform simultaneous hybridization and labeling. At the same time, the complete multiplexing of both targets, i.e. simultaneous immobilization, hybridization and labeling, was carried out for comparison (Fig. 4).

The Lec system resulted in similar S/B ratios when the capture probes were simultaneously ($S/B=6$) and separately immobilized ($S/B=5$). However, results indicated that when RR probes were co-immobilized, the target current dropped compared to the separate procedure, while the S/B ratios remained similar. Competition probably takes place between both capture probes and, as pointed by these results, RR is less favoured than Lec. This disparity may be due to differences in the formation of secondary structures. Both capture probes have similar free energy values (Table S1), but different loop formation (Fig. S4). The RR capture probe forms a larger harpin-like structure than the Lec probe. This 16-nucleotide-loop, starting at the first guanine base at the 5′ end, is thought to induce steric hindrance with the biotin molecule attached at the same site. In spite of these results, co-immobilization exhibits the best characteristics in terms of less time consumption and practicability in the multiplex method, therefore it is preferred over the separate procedure.

3.4. Analytical performance

The analytical characteristics of the separate methods are shown in Table S2. A linear range from 5 to 350 pM was achieved for both targets (Fig. 5A), with a limit of detection (LOD) of 900 fM (226 amol) for the transgenic target (anti-FITC labeling system), calculated as three times the standard deviation of the blank response/slope ratio. For the Lec target (anti-Dig label), the LOD value was 300 fM (80 amol). Adequate precisions were obtained (RSD = 10% and 7% for the transgenic and reference targets 10 pM, respectively). Selectivity was assessed against a non-complementary sequence and also with both transgenic and taxon-specific targets in the same tube, more likely to be present in a real sample. Both experiments resulted in low non-specific signals, near to the blank response. This confirmed the specificity of the assays.

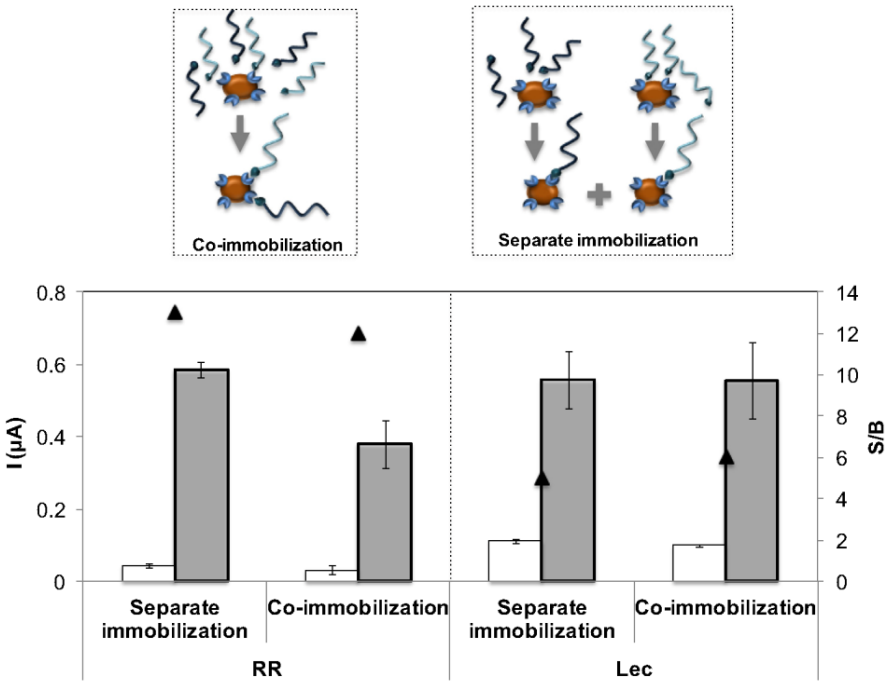

Fig. 4. Comparison of the response obtained after co-immobilization and separate immobilization of the capture probes.

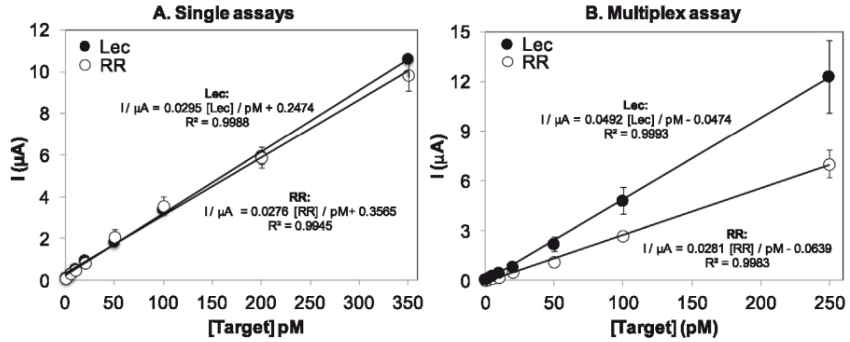

Fig. 5. Calibration plots for the event-specific and reference targets: (A) single assays and (B) multiplex assay.

Table 1
Accuracy of the multiplex method with synthetic GMO mixtures.

Theoretical value			Experimental value			Relative error (%)
Lec (ng)	RR (ng)	GMO (%)	Lec (ng)	RR (ng)	GMO (%)	
1.4	0.0112	**0.8**	1.4 ± 0.1	0.012 ± 0.004	**0.85**	6.3
0.56	0.0113	**2.0**	0.558 ± 0.001	0.0117 ± 0.0005	**2.10**	5

The multiplex method was slightly more sensitive than the single approach (Table S2). Calibration with Lec resulted in a higher slope value than the one obtained with RR, confirming our previous observation. As stated before, this finding may be due to the different loop structures of the capture probes affecting the binding to the streptavidin-modified beads.

Furthermore, system saturation is observed above 250 pM for both targets in the multiplex assay (Fig. 5B), while for the single assays this occurs above 350 pM. An overload of the beads in the multiplex assay may explain this behavior. The LOD values were 160 amol and 100 amol for the event-specific and reference targets, respectively.

The design of a multi-target platform involves a double challenge: on one hand, the simultaneous detection of two target sequences may be hampered by unspecific interactions between the DNA strands and, on the other hand, detecting a transgene event and a taxon-related sequence implies that the content of both is extremely different, i.e., in a sample containing 1% of GMO, the taxon sequence is present at a 100-fold excess respect to the transgenic sequence.

The suitability of the multiplex assay to quantify RR in relation to the taxon content, as recommended by the Commission Recommendation 2004/787/EC (European Commission, 2004), was assessed with mixtures of both sequences containing 0.8% and 2% of RR. This was intended to simulate artificially the GMO content around the labeling threshold value (0.9%). The current values obtained were interpolated in the calibration plots of both targets and the GMO percentage was calculated as the ratio RR/Lec nanograms. The experimental values were very close to the theoretical ones in both cases (Table 1), suggesting that the multiplex method is highly suitable for quantifying both sequences simultaneously as expected in a real sample at GMO proportions below the labeling threshold.

4. Conclusions

We have shown that GMO quantification may be achievable by other approaches and is not limited to RTi-PCR. Most DNA-hybridization methods that focus on the detection of the specific-event do not address the concomitant taxon-specific detection that allows real quantification. To fill this gap, we have developed a multiplex electrochemical platform for sensitive GMO quantification in compliance with European guidelines. The immobilization of two different DNA sequences into the same sensing platform allows simultaneous discrimination of two sequences with high dissimilar content, i.e. event and taxon-specific at a 0.8:100 ratio. Thus, it is possible to apply the genoassay to achieve GMO quantification below and above 0.9% in synthetic mixtures. This multiplex platform represents a simplification of the analysis procedure when compared with the two single analyte assays needed for absolute quantification of GMOs. Our approach is simple, sensitive (subnanomolar) and suitable to perform double-target detection on the same sample or DNA template for the quantitative detection of GMOs. Overall, the electrochemical method presented in this work represents a low-cost alternative for DNA detection with remarkable analytical performance.

Acknowledgements

C.L.M.P. thanks to Universidad Complutense de Madrid (Grant no. BE43/11) for a predoctoral grant. This work has been developed in the frame of the GMOsensor project of International Research Staff Exchange Scheme (FP7) PEOPLE-2013-IRSES (Marie Curie actions), and supported by the Ministerio de Educación y Ciencia (Spain) Project CTQ2012-31157, and the European Regional Development Fund.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.01.007.

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Supplementary Material
Multiplex Electrochemical DNA Platform for Femtomolar-level Quantification of Genetically Modified Soybean

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Table S1

Probes and target sequences and Gibbs energy of their most stable secondary structure

Description	Sequences of oligonucleotides 5' → 3'	ΔG (kcal/mol)*
Transgenic target (RR)	TTCATTCAAAATAAGATCATACATACAGGTTAAAATAAACATAG GGAACCCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCC	-5.6
RR signaling probe-FITC	TTCCATTTGGGTTCCCTATGTTTATTTTAACCTGTATGTATGATC TTATTTTGAATGAA-FITC	-3.2
RR capture probe	Biotin- GGCATTTGTAGGAGCCACCTTCCTT	-2.6
Taxon-specific target (lec)	CCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCCTGACA CAAAAAGGCTTGCAGATGGGCTTGCCTTC	-7.7
Lec signaling probe-Dig	Dig-GAAGGCAAGCCCATCTGCAAGCCTTTTTGTGTCAGGGGCATAG AAGGTG	-7.0
Lec capture probe	AAGTTGAAGGAAGCGGCGAAGCTGG-Biotin	-3.6

*ΔG values were obtained with Mfold Web Server (http ://mfold.rna.albany.edu/)

Table S2

Analytical characteristics of the methods

	Single assays	
	Event-specific target (RR)	**Taxon-specific target (Lec)**
LOD	900 fM (226 amol)	300 fM (80 amol)
Linearity	5-350 pM I/nA = (28±1) C/pM + (356±125) R^2 = 0.9944	5-350 pM I/nA = (29.5±0.4) C/pM + (247±62) R^2 = 0.9988
RSD (10 pM)	10 %	7 %
Label	Anti-FITC-POD	Anti-Dig-POD
Technique	Chronoamperometry	
	Multiplex assay	
LOD	650 fM (160 amol)	200 fM (100 amol)
Linearity	2-250 pM I/nA = (28.0±0.4) C/pM - (64±46) R^2 = 0.9983	2-250 pM I/nA = (49.1±0.5) C/pM - (47±53) R^2 = 0.9992
RSD (10 pM)	6 %	11 %
Label	Anti-FITC-AP	Anti-Dig-POD
Technique	DPV	Chronoamperometry

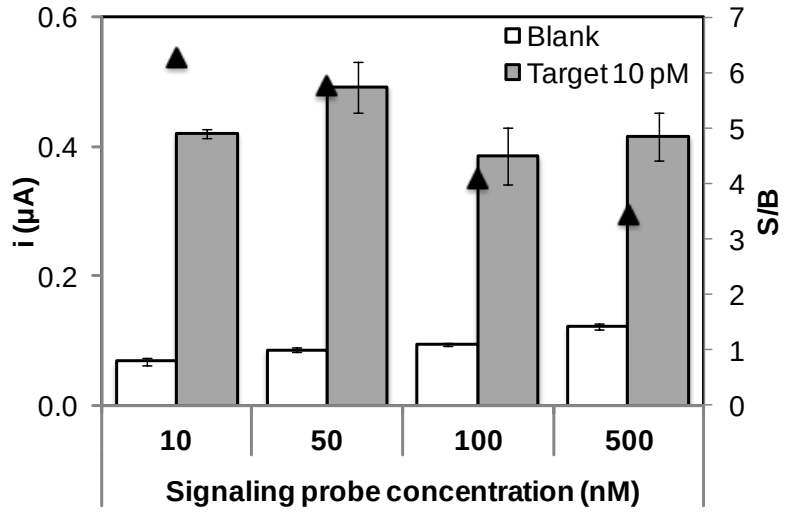

Fig. S1. Effect of signaling probe concentration on the analytical response.

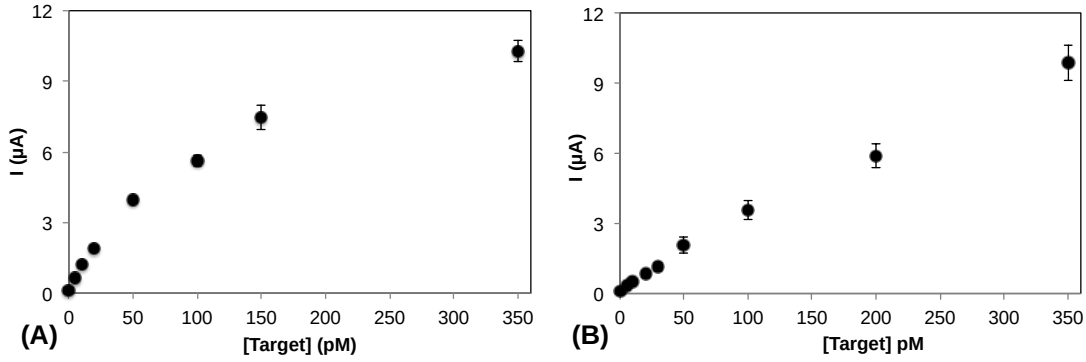

Fig. S2. Effect of ionic strength in linear range: (A) Calibration plot in buffer 2xSSPE and (B) in optimized hybridization buffer (SSPE-N).

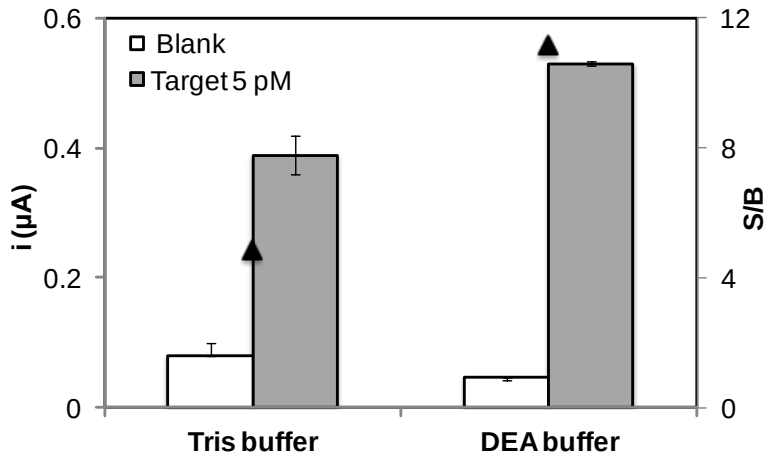

Fig. S3. Evaluation of buffer composition for antiFITC-ALP activity with the event-specific assay: Tris-HCl 0.5 M pH 9.8 $MgCl_2$ 0.5 mM and KCl 0.1 M and DEA 1 M pH 9.8 $MgCl_2$ 0.5 mM for substrate solution.

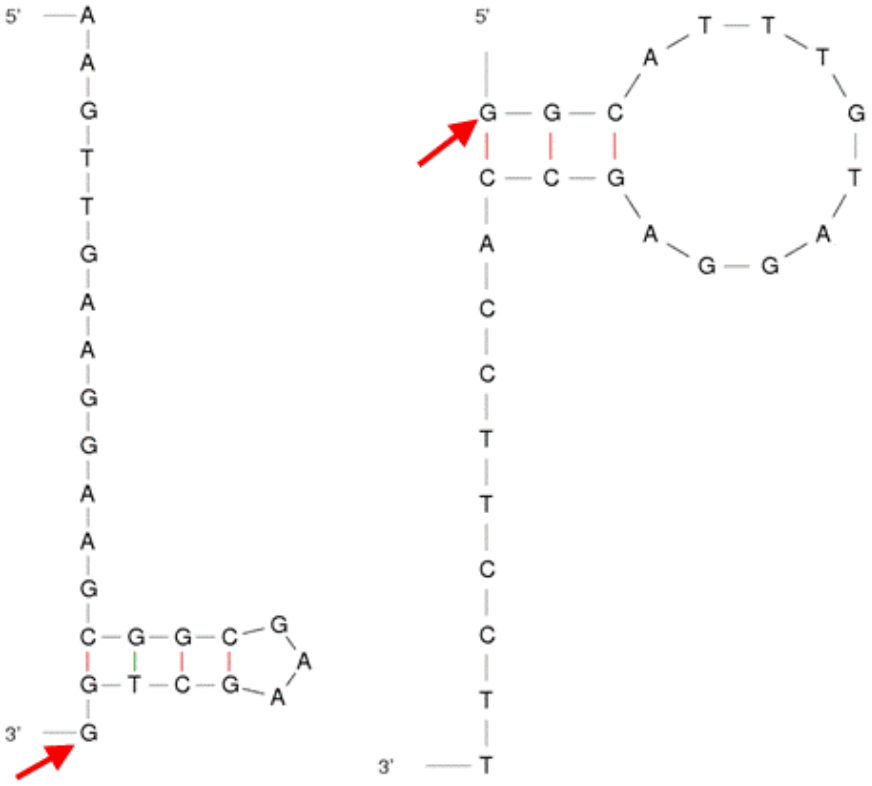

Fig. S4. Secondary structures of (A) Lec and (B) RR capture probes. Red arrow indicates the location of the biotin molecule.

| CHAPTER 5 |

ELECTROCHEMICAL MAGNETOASSAY COUPLED TO PCR AS A QUANTITATIVE APPROACH TO DETECT THE SOYBEAN EVENT GTS 40-3-2 IN FOODS

Sens & Actuat. 222 (2016) 1050-1057

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ELECTROCHEMICAL MAGNETOASSAY COUPLED TO PCR AS A QUANTITATIVE APPROACH TO DETECT THE SOYBEAN EVENT GTS 40-3-2 IN FOODS

Sens & Actuat. 222 (2016) 1050-1057

In this following experimental work, the aim was to demonstrate the quantitative potential of the previously developed platforms after PCR-coupling. Given that end-point PCR is known to lose its ability to quantify DNA after a given number of cycles, the optimization of this specific parameter was carried out with each target sequence. For the first time, accurate quantification was accomplished after PCR coupling to an electrochemical method. The literature reveals how GMO quantification using genosensors had remained an unfulfilled task due to the difficulty in performing quantitative coupling of the sensors to amplification procedures. The number of PCR cycles had not been previously optimized to achieve a quantitative relationship between the electrochemical signal and pre-PCR template amounts. Taxon-specific sequences had not been co-quantified together with the event-specific sequences in order to relate the contents of both. Due to these reasons, accurate quantitative data had been unreached with genosensors, which is why this work represents a distinctive contribution to the field. The analytical method developed in this chapter, comprising DNA extraction with a partially modified kit, PCR amplification and electrochemical sensing, was applied in flour samples as proof-of-concept.

Sensors and Actuators B 222 (2016) 1050–1057

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Electrochemical magnetoassay coupled to PCR as a quantitative approach to detect the soybean transgenic event GTS40-3-2 in foods

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ABSTRACT

Simple, cost-effective and reliable tools for the quantification of genetically modified organisms (GMO) in food and feed are highly demanded to enforce labelling legislation in the EU. Herein, we report a novel method for quantitative analysis of genetically modified soybean with the event GTS-40-3-2, also known as Roundup Ready (RR) soybean, using magnetoassays with electrochemical detection, coupled to DNA amplification by end-point polymerase chain reaction (PCR). For the proposed work, two DNA sequences were targeted via hybridisation onto magnetic beads, one specific for the transgenic event and the other for the taxon or species-specific *lectin* gene. Enzymatic labelling was performed to obtain an electrochemically active product measured by chronoamperometry. By optimising the number of PCR cycles, among other parameters, two magnetoassays coupled to PCR were successfully accomplished and linearity was obtained in the ranges of 53–4425 and 1093–88,496 DNA copies for the event-specific and *lectin* sequences, respectively. The proposed method provides accurate and precise RR soybean quantitative results, being effectively compared to those obtained by real-time PCR, as the reference method. These findings confirm the suitability of the method as an alternative tool for GMO quantification.

1. Introduction

Recently, molecular biology techniques have been regarded as excellent tools for the quality and safety assessment of foods. Within the sphere of DNA-based methods, the polymerase chain reaction (PCR) has become a standard technique for food authenticity and food control, being routinely applied to the detection/identification of allergens, microorganisms, pathogens and genetically modified organisms (GMO) [1]. In the last decades, biotech crops have gained public attention, being the fastest adopted crop technology in the history of modern agriculture [2]. Despite all the pros and cons associated with the production and use of GM crops for food and feed, in 2014 the area of biotech crops continued to increase, totalising 181.5 million hectares. Soybean is the most important GM crop, corresponding to 50% of the global biotech area in 2014 [3]. Among the GM events cultivated worldwide, the soybean GTS 40-3-2 event is one of the most representative, being commercially known as Roundup Ready™ (RR) soybean since it was especially developed to be tolerant to the herbicide with the same name.

In response to public demands, especial measures have been engaged to implement labelling regulations for GMO in many countries, despite the lack of consensual evidences on potential health risks [4,5]. Accordingly, the European Union (EU) issued the Regulation No. 1829/2003 that establishes the mandatory labelling of all products consisting of, produced or containing authorised GMO above 0.9%. Below this limit, GMO labelling is not required since its presence is considered adventitious or technically unavoidable [6].

Currently, PCR-based methods are generally used for GMO screening and identification, being real-time PCR the technique of choice for its quantification. Methods based on the application of real-time PCR are specifically designed to determine the relative content of GMO, commonly, by means of two parallel reactions, in

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which one targets a GM-specific sequence (e.g. soybean Roundup Ready) and the other targets the correspondent species-specific endogenous reference gene (*lectin* for soybean). The quantification is further performed, either by comparing the cycle threshold of the two amplified sequences (the ΔCt method), or via a standard curve [7]. In spite of the extensive application of real-time PCR assays for the detection and quantification of GMO in raw and processed foods, both as single or multi-target approaches, new strategies using different analytical methodologies (e.g. biosensor platforms) have also been widely exploited for DNA analysis.

Various types of hybridisation-based biosensors, including surface plasmon resonance (SPR), quartz crystal microbalance or piezoelectric, thin-film optical, dry-reagent dipstick-type and electrochemical have been introduced in GMO screening and/or detection [8–10]. Electroanalytical techniques provide important advantages such as the relative simplicity, low-cost of equipment and automatic on-line and portable options [10,11]. Currently, most of these devices require previous DNA amplification by means of PCR in order to be applied to sample analysis [10,12]. Despite their remarkable sensitivity [13–16], the reported PCR-coupled devices have only been used as confirmatory tools, whereas sensor-based quantitation of GMO has not yet been reported. This has been attributed, in part, to the semi-quantitative kinetics of PCR, compromising reliable quantification of DNA [12,17,18]. Additionally, the proposed sensors do not address the need for developing the detection of the respective taxon-specific gene, which is required for establishing the relationship between both measurements and enable a quantitative GMO analysis. To the best of our knowledge, only screening/identification of GMO has been reported in food samples using DNA sensors.

The aim of this work concerned the development of an alternative GMO quantitative strategy, merging the advantages of both end-point PCR and electrochemical approaches in terms of simplicity and low-cost to propose an accurate and robust new method. Our strategy uses a versatile electromagnetic platform that couples the hybridisation recognition reaction in a sandwich format on magnetic microparticles with enzymatic labelling for electrochemical detection [19]. In this way, two sequence-specific DNA probes, one targeting an event-specific sequence of RR soybean and other the endogenous lectin gene, were both entrapped onto the surface of magnetic beads. Sandwich hybridisation was performed for each PCR fragment in order to indirectly tag the sequences with hapten molecules (fluorescein-isothiocyanate—FITC, or digoxigenin—Dig). The enzyme horseradish peroxidase (HPR) was used as reporter macromolecule, by affinity binding via anti-FITC or anti-Dig Fab conjugation. Although FITC is frequently used as fluorescent dye in biosassays, in this work it was used as intermediate tag to bind the DNA probe to an enzymatic label. This type of conjugation offers strong and fast affinity binding with minimum unspecific adsorption onto streptavidin-coated magnetic beads. FITC/Dig-antiFITC/Dig-HRP conjugates have also been exploited in other biosensing platforms [20–22]. Electrochemical readout was performed on the surface of disposable carbon electrodes. By careful optimisation of the amplification-related parameters, we demonstrate the utility of this approach for reliable quantification of GMO in foods. The method was further validated by quantitative real-time PCR, using RR soybean as the GMO target.

2. Experimental

2.1. Samples

Standards consisting of commercial certified reference material (CRM) developed by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) were used. The CRM consisted of dried soybean flour (IRMM 410) containing 5, 2, 1, 0.5, 0.1 and 0% of Roundup Ready soybean expressed in mass percentage. One ground soybean sample containing 1% of RR soybean, among other events, obtained from the USDA/GIPSA (Grain Inspection, Packers, and Stockyards Administration) Proficiency Program was also included in this study.

2.2. DNA extraction

DNA was extracted from 100 mg of soybean flour using the Wizard method as described by Mafra et al. [23]. All extractions included a blank for the control of reagents and potential contaminations during the extraction procedure.

Yield and purity of extracts were assessed by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA (1 absorbance unit corresponds to 50 ng μL^{-1} of dsDNA) in the Gen5 data analysis software version 2.01 (BioTek Instruments Inc., Vermont, USA).

The quality of extracted DNA was analysed by electrophoresis in a 1.0% agarose gel carried out in 1× of SGTB buffer (Grisp, Porto, Portugal) for 30 min at 200 V, previously stained with 1× of GelRed (Biotium Inc., Hayward, CA, USA). The agarose gel was visualised with a UV light tray Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and the digital image was obtained with Image Lab software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

2.3. Target gene selection and oligonucleotides

In this work, two sequences of each selected DNA region, namely a taxon-specific gene (*lectin*) and RR soybean-specific (event or construct) were targeted. In both cases, primers were selected from validated real-time PCR methods [24,25]. Primers Lec-F/Lec-R and RRS-Fm/RRS-Rm were used for amplification by end-point PCR to produce fragments to be detected by the magnetoassay (Table 1). The primers RRS-Fm/RRS-Rm, targeting the recombination region between the transgenic insert and the plant genome, were used as event-specific, being slightly modified in order to increase the melting temperature (Table 1). Other primers and probes were used in quantitative real-time PCR to validate the method (Table 1). The primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4. End-point PCR

The PCR amplifications were carried out in 25 μL of total reaction volume containing 2 μL of template DNA, 1× buffer (67 mM of Tris–HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20), 200 μM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 2.0 mM of $MgCl_2$ and 480 nM or 280 nM of each set of primers, Lec-F/Lec-R and RRS-Fm/RRS-Rm, respectively (Table 1). The PCR amplifications were performed in a MJ Mini thermal cycler (BioRad Laboratories, Hercules, CA, USA) using the following programme of temperatures: 95 °C for 5 min; 30 or 35 cycles of 95 °C for 30 s, 66 or 60 °C for 30 s for primers Lec-F/Lec-R or RRS-Fm/RRS-Rm, respectively, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel stained with GelRed and carried out in 1× SGTB for 30 min at 200 V. The agarose gel was visualised with a UV light tray Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and the digital image was obtained with Image Lab software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

Table 1
Sequence of primers and probes used to target the endogenous reference gene (*lectin*) and RR soybean by end-point PCR and real-time PCR amplifications, and oligonucleotides used in the magnetoassay.

Target region	Name/description	Sequence (5′ → 3′)	Amplicon (bp)	Reference
End-Point PCR				
Lectin gene	Lec-F	CCA GCT TCG CCG CTT CCT TC	74	[24]
	Lec-R	GAA GGC AAG CCC ATC TGC AAG CC		
35S/plant junction	RRS-Fm	TTC ATT CAA AAT AAG ATC ATA CAT ACA GG	84	[24]
	RRS-Rm	GGC ATT TGT AGG AGC CAC CTT C		
Real-time PCR				
Lectin gene	Lectin-F	TCC ACC CCC ATC CAC ATT T	81	[25]
	Lectin-R	GGC ATA GAA GGT GAA GTT GAA GGA		
	Lectin-TMP	FAM-AAC CGG TAG CGT TGC CAG CTT CG-BHQ1		
CTP/35S junction	RRS-F	GCC ATG TTG TTA ATT TGT GCC AT	83	[25]
	RRS-R	GAA GTT CAT TTC ATT TGG AGA GGA C		
	RRS-TMP	FAM-CTT GAA AGA TCT GCT AGA GTC AGC TTG TCA GCG-BHQ1		
Magnetoassay				
Lectin gene	Taxon-specific target	CCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGCCCC TGACACAAAAAGGCTTGCAGATGGGCTTGCCTTC	74	[19]
	Signalling probe-Dig	Dig-GAAGGCAAGCCCATCTGCAAGCCTTTTT GTGTCAGGGGCATAGAAGGTG		
	Capture probe	AAGTTGAAGGAAGCGGCGAAGCTGG-Biotin		
35S/plant junction	Transgenic target	TTCATTCAAAATAAGATCATACATACAGGTTAAAATAAACATA GGGAACCCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCC	84	[19]
	Signalling probe-FITC	TTCCATTTGGGTTCCCTATGTTTATTTTAACC TGTATGTATGATCTTATTTTGAATGAA-FITC		
	Capture probe	Biotin-GGCATTTGTAGGAGCCACCTTCCTT		

2.5. Real-time PCR

The amplifications by real-time PCR were performed in 20 μL total reaction volume containing 2 μL of DNA template, 1× iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 600 nM or 900 nM of each primer Lectin-F/Lectin-R or RRS-F/RRS-R, and 200 nM or 100 nM of each probe Lectin-TMP or RRS-TMP (Table 1), respectively, for lectin gene and event-specific region. Parallel reactions were prepared for each target sequence. The assays were performed on a fluorometric thermal cycler CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following temperature conditions: 95 °C for 5 min, 45 cycles at 95 °C for 30 s and 60 °C for 1 min, with collection of fluorescence signal at the end of each cycle. Data were collected and processed using the BioRad CFX Manager software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR trials were repeated in two independent assays using three replicates for each unknown or blind sample and two replicates for each standard.

2.6. Electrochemical magnetoassays

The experimental procedures were based on single assays described in our previous work [19]. In Fig. 1, a general overview of the magnetoassays coupled to PCR is illustrated and the oligonucleotide probes used are presented in Table 1. Oligonucleotides were obtained as lyophilised desalted salts from Sigma-Life Sciences.

2.6.1. Biomodification of the magnetic beads

Streptavidin-coated magnetic beads (MB) Dynabeads MyOne streptavidin C1 (1-μm diameter—10 mg mL^{-1}) were acquired from Life Technologies (Spain). The modification of the MB, the hybridisation and labelling assays were performed in a 12-tube mixing wheel (Dynal MX1) and magnetic separations were carried out with a magnet DynaMag2, both from Life Technologies (Spain). Following a procedure described in previous work [19], the commercial bead suspension was mixed with a Tween-containing phosphate buffer. After washing steps, 1 μM of biotinylated capture probe was added to the beads and incubated for 30 min at room temperature in the mixing wheel. According to manufacturer recommendations, there is a known relationship between the amount of biotinylated ssDNA and the amount of magnetic beads (approximately 500 pmol ssDNA mg^{-1} MB). An excess of capture probe was used to ensure all beads were biomodified.

2.6.2. Sandwich hybridisation with PCR amplicons

A sandwich-type hybridisation assay was carried out with 25 μL of PCR product, undiluted for RR soybean or diluted for *lectin* (50-fold or 100-fold), and 10 nM of the corresponding signalling probe (SP), fluorescein isothiocyanate (FITC-SP) for RR soybean or digoxigenin (Dig-SP) for *lectin*. The mixture was homogenised and subsequently subjected to a thermal cycle, 5 min at 98 °C and 5 min in ice, to facilitate the denaturation of the double-stranded amplicon and to allow homogeneous hybridisation. After resting for 25 min at room temperature, the mixtures were added to the microparticles in order to proceed with heterogeneous hybridisation between the partial duplex and the capture probes. This step took place for 1 h at room temperature under rotation. The hybrid-conjugated beads were then washed three times with phosphate buffer and casein buffer.

2.6.3. Enzymatic labelling

An anti-fluorescein-peroxidase (anti-FITC-POD) or anti-digoxigenin-peroxidase (anti-Dig-POD) conjugate (Roche Diagnostics GmbH, Mannheim, Germany) were added at a final concentration of 0.25 U mL^{-1} to the beads and incubated for 30 min under rotation. After two washing steps, the beads were finally re-suspended in phosphate buffer.

2.6.4. Electrochemical detection

The electrochemical detection of the enzymatic products was performed onto the surface of a disposable carbon electrode, by means of chronoamperometry. Fifteen microliters of the fully modified beads were magnetically captured onto the working electrode with a magnet placed under it. After 2 min, the liquid was carefully removed. Finally, 45 μL of the enzymatic substrate, tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H_2O_2, obtained from

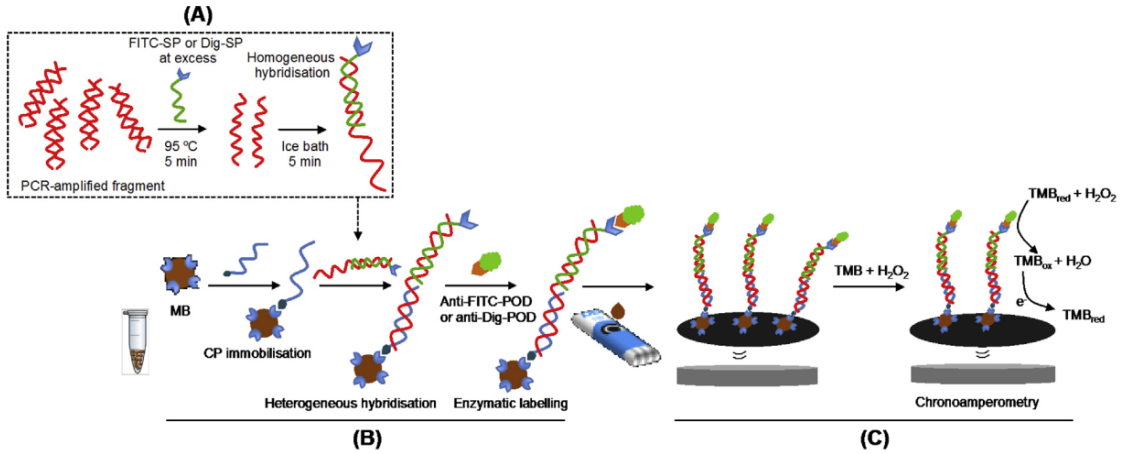

Fig. 1. Scheme of the electrochemical magnetoassays coupled to PCR: (A) after DNA extraction, PCR is carried out and respective fragments are submitted to a thermal-ice cycle in order to denature the dsDNA and perform the hybridisation with the signalling probe (SP); (B) in a separate tube, the attachment of capture probe(s) (CP) to the surface of magnetic beads (MB) takes place, after which the SP-target complex is added to the MB and heterogeneous hybridisation with the capture probe occurs; being followed by the addition of the Fab-enzyme conjugate to allow enzymatic labelling; (C) biomodified MB are entrapped onto the working area of a screen printed carbon electrode via a small magnet placed under it, followed by the addition of the enzymatic substrate (TMB), which undergoes enzymatic oxidation. Subsequently, TMB reduction is recorded at the electrode surface by chronoamperometry.

Sigma-Aldrich, Spain), was placed on the disposable screen-printed carbon electrodes (SPCE, DropSens-110, Spain), covering the three electrodes. Chronoamperometric measurements were performed after 60 s of enzymatic reaction using a potential pulse to 0 V, and sampling the current during 60 s. Electrochemical measurements were performed using a computer-controlled μAutoLab type II potentiostat with GPES 4.9 (EcoChemie, The Netherlands). Before carrying out the measurement step, the SPCE were washed with ethanol and deionised water, and dried with nitrogen.

3. Results and discussion

3.1. Optimisation of end-point PCR

For the effective application of PCR, a critical step to overcome in the case of complex and processed food matrices is the DNA extraction and purification. DNA extracts presented, generally, high concentration (355 ± 71 ng μL^{-1}) and purity (2.0 ± 0.1), as determined by UV spectrophotometry. The evaluation of extracts by agarose gel electrophoresis evidenced high molecular weight genomic DNA (~10 kb), with only little sheared DNA (data not shown). The tested sample and CRM amplified positively by end-point PCR with soybean taxon-specific primers, confirming that all DNA extracts presented the adequate quality, integrity and purity for PCR amplification. These results were in good accordance with other reports for certified reference materials and flours, as those are basically submitted to mechanical processing [26].

Despite using primers from a validated method, it was necessary to optimise the end-point PCR conditions, for both *lectin* gene and soybean RR specific region. Besides, the PCR conditions were particularly optimised to obtain high sensitivity, but also relatively low band saturation to improve linearity and range of application with the proposed magnetoassay. After testing several parameters (e.g. number of amplification cycles, temperatures of annealing, magnesium concentration), the final conditions for *lectin* and RR soybean amplification were set: annealing temperature of 66 °C or 60 °C with 30 or 35 cycles for lectin or RR soybean, respectively, using 2.0 mM of magnesium chloride. For both targets, the PCR assays allowed positive amplifications down to 1.23 ng of template DNA (data not shown).

3.2. PCR coupled to the magnetoassay: GMO calibration strategies

For GMO quantification, the method using two standard curves, one that targets the GM specific-region and other the correspondent taxon-specific gene, is normally elected as the most adequate [26]. Based on this approach, in the present work, it was intended the development of a double calibration curve method by means of two PCR coupled to the magnetoassays for each target sequence of *lectin* gene and RR soybean.

A preliminary test of the PCR coupled to the magnetoassay consisted on the measurement of the electrochemical current related to the RR soybean target amplified from all CRM levels (0, 0.1, 0.5, 1, 2, 5% of RR soybean). This test was carried out aiming at evaluating if the electrochemical response was directly proportional to the amount of RR soybean present in the sample. Additionally, it was also intended to verify whether the 0% RR soybean CRM together with the remaining PCR reagents (enzyme, dNTP, primers) could interfere in the blank response. Therefore, 200 ng of total DNA from the 6 different CRM levels were submitted to PCR amplification using 38 cycles targeting the RR soybean specific region. After amplification, products were hybridised with signalling and capture probes and the responses were registered as described in Section 2. From the results in Fig. 2A, two main issues could be observed. First, the lowest tested level (0.1% RR soybean) could not be accurately detected, and second, the system reached a saturation point at 5% of RR soybean. Moreover, using the referred amplification conditions (38 cycles), the level 0.1% could not be differentiated from the level 0.5% of RR soybean. The logarithm of electrochemical responses versus the logarithm of GMO content confirms the non-proportional correlation for the 5% CRM (Fig. 2B). When PCR reaches later cycles, the reaction enters in its plateau phase, making inaccurate the quantification of end-point products [27]. With 38 cycles, amplicons are most likely to be 'levelled-off', thus disabling their correct quantification. Accordingly, new PCR conditions had to be tested, lowering the number of amplification cycles until reaching linearity. It is also important to point out that the 0% RR soybean gave a similar response to the one from the blank of synthetic oligonucleotides, suggesting that the background genomic DNA and the PCR components do not interfere with the proposed magnetoassay.

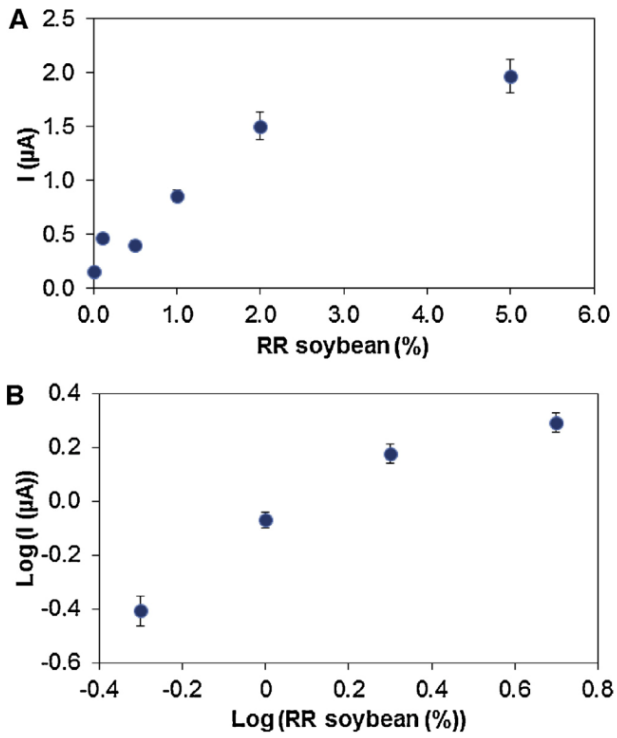

Fig. 2. Electrochemical responses obtained with the magnetoassay applied to CRM materials (0, 0.1, 0.5, 1, 2 and 5% of RR soybean) after 38 cycles of PCR amplification targeting RR soybean. (A) Electrochemical responses vs. RR soybean relative content; (B) Logarithm of electrochemical responses vs. logarithm of RR soybean relative content.

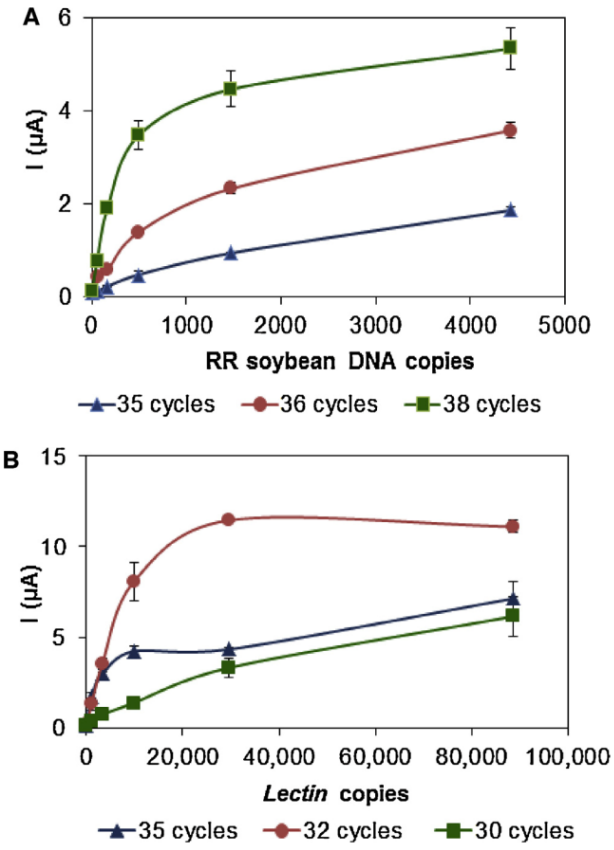

Fig. 3. Electrochemical responses obtained with the magnetoassays coupled to PCR fragments using different amplification cycles from a 3-fold serially diluted DNA extract (5% RR soybean CRM). (A) RR soybean amplified products (5.0 ng of RR soybean DNA (4425 copies) until 61.7 pg (55 copies)); (B) *Lectin* gene amplified products (100 ng of total genomic DNA (88,496 copies) until 1.2 ng (1093 copies)), but to decrease signal saturation, further dilution of amplicons was required prior to hybridisation: 100-fold for the assay with 35 cycles and 50-fold for the other assays.

3.3. Calibration models for absolute determination of lectin and RR soybean DNA: Cycle number optimisation

The evaluation of the electrochemical response of the proposed magnetoassay was performed for PCR products of five serial dilution (3-fold) levels of 5% RR soybean CRM, starting from 100 ng of total template DNA (88,496 copies) until 1.2 ng (1093 copies), which corresponded to 5.0 ng of RR soybean DNA (4425 copies) until 61.7 pg (55 copies), respectively. DNA copies were calculated according to the prime estimate value of the available soybean genome size (1.13 pg) from the Plant DNA C-value database [28] and assuming that the targeted sequences are single copy genes. The electrochemical responses of RR soybean or *lectin* PCR products were assayed using 35, 36 and 38, or 30, 32 and 35 cycles of amplification, respectively. In each assay, the total volume of PCR products (25 μL) was added to the hybridisation mix containing 10 nM of signalling probe, as described in Section 2. In the case of *lectin*, the PCR product was 50-fold diluted before hybridisation. After enzymatic labelling of the target-signalling probe hybrids, the electrochemical readout took place. Chronoamperometry results were plotted against the initial DNA copy numbers of *lectin* or RR soybean sequences. The negative control, which was always included in the assay, presented a very similar response to the ordinary blank obtained with the magnetoassay (all components except the target, submitted to the same treatment of the complete method).

In Fig. 3, it can be noted that as expected the electrochemical signals decreased considerably by lowering the number of PCR cycles. With 36 and 38 cycles, the electrochemical responses showed a slight plateau above ~500 DNA copies of RR soybean, whereas at 35 cycles the responses presented a close linear profile (Fig. 3A). To support these findings, real-time PCR assay targeting the RR construct-specific region was performed, using 3-fold serial dilutions of 5% RR soybean DNA (10 ng down to 41.1 pg). In Fig. 4, the red arrows signal the stage of amplification of each DNA concentration when reaching 35 cycles. The highest amount (10 ng—8850 DNA copies of RR soybean) is located just before the beginning of the plateau phase, whereas the following DNA dilutions are all within the exponential phase of PCR. Therefore, the results from real-time PCR suggested that 35 cycles of end-point PCR amplification ensure linearity in the 3-fold diluted range of concentrations (from 5.0 ng down to 61.7 pg of RR soybean DNA). The electrochemical measurements performed at end-point PCR using such range of concentrations and 35 cycles result in the curve plot shown in Fig. 5A. By fitting the plot to a logarithmic model, linearity was obtained between 53 and 4425 DNA copies of RR soybean (Fig. 5B), with a corresponding regression equation of $\mathrm{Log}(I) = 0.645(\pm 0.006)\,\mathrm{Log}(\mathrm{DNA\ copies}) - 2.077(\pm 0.018)$ and a correlation coefficient of 0.9997. A similar behaviour was previously verified using the band intensities to develop quantitative models based on end-point PCR, in which the number of amplification cycles had to be optimise to reach linear log–log models [29,30]. The absolute LOD of 53 copies of RR soybean DNA, which corresponds to an estimated relative LOD of 0.06%, shows that the proposed method reaches a sensitivity comparable with the one reported in the real-time PCR assay using the same primers (0.045%) [24].

In the case of *lectin* gene amplification, a close linear profile of electrochemical responses was verified with 30 cycles, while with

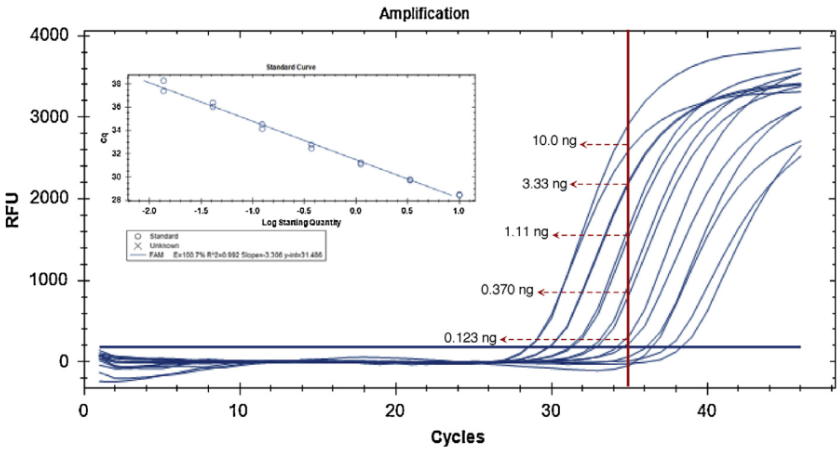

Fig. 4. Real-time PCR amplification curves and respective calibration curve targeting a RR soybean sequence from a serially diluted soybean DNA containing 10 ng to 0.0137 ng of RR soybean DNA (5% RR soybean CRM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

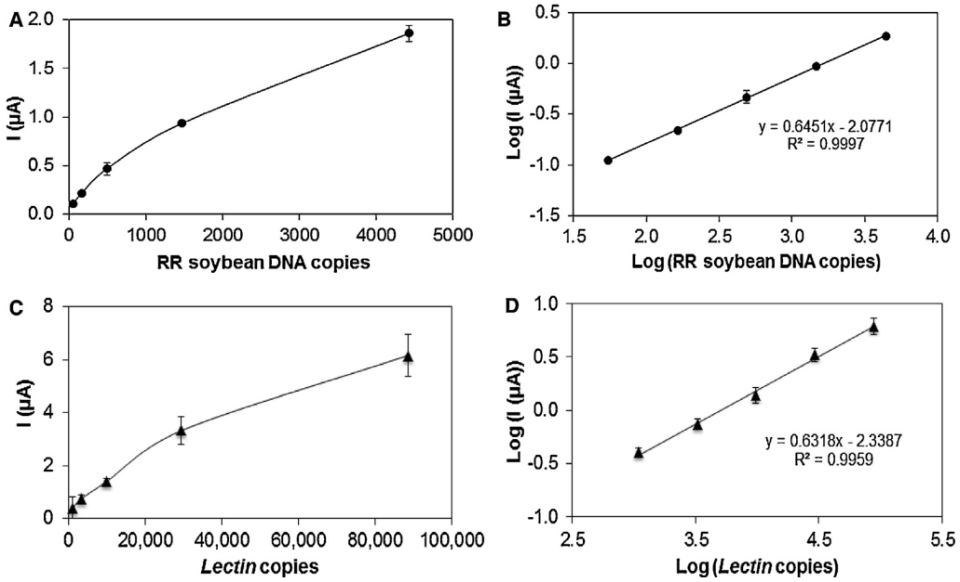

Fig. 5. Calibration plots (A and C) and the corresponding linear log–log models (B and D) of electrochemical responses obtained with the magnetoassays coupled to PCR fragments for RR soybean with 35 cycles (A and B) and *lectin* with 30 cycles (C and D).

32 and 35 cycles a plateau was reached above ~10,000 DNA copies (Fig. 3B). Earlier saturation is observed for *lectin* gene, which can be explained by the high quantity in which is present in the amplified material, compared to RR (5:100 ratio). The electrochemical response presented the best results using 30 cycles (Fig. 5C), but it was necessary to perform a 50-fold dilution of PCR products in order to obtain a linear log–log adjusted fit with the magnetoassay (Fig. 5D). With these conditions, the magnetoassay allowed linearity between 1093 and 88,496 copies of soybean *lectin* gene, with a corresponding regression equation of $\text{Log}(I) = 0.631(\pm 0.023)\ \text{Log}(\text{DNA copies}) - 2.338(\pm 0.094)$ and a correlation coefficient of 0.9959.

In both systems, adequate linearity parameters were obtained using 30 and 35 cycles of amplification for *lectin* and RR soybean products, respectively, as evidenced by their adequate correlation coefficients and response factors (Fig. 5B, 5D). The respective limits of quantification corresponded to the first points of the calibration curves (53 copies for RR and 1093 copies for *lectin*). For the same conditions of PCR, the magnetoassay enabled achieving sensitivities 3- or 9-times higher for RR soybean (61.7 pg) and lectin (1.2 ng), respectively, than the conventional electrophoretic methods (185 pg for RR soybean and 11.1 ng of lectin). These findings emphasise the high sensitivity of electrochemical detection over traditional electrophoretic techniques, with special focus on its use for quantitative determinations.

3.4. Validation of magnetoassays with quantitative real-time PCR

Once the magnetoassays were developed and optimised for lectin and RR soybean quantitative detection, validation tests were carried out using 1% CRM of RR soybean and soybean flour (1%) obtained from the USDA/GIPSA Proficiency Program as blind samples. Parallel quantitative assays were performed by real-time PCR and by the proposed PCR coupled to magnetoassays in order to assess the analytical performance of the latter. The performance parameters of real-time PCR were in good agreement with the acceptance criteria defined for this technique [31], namely correlation coefficient >0.98, slope between −3.5 and −3.1 that

Table 2
Accuracy assessment of RR soybean by the electrochemical magnetosassays in comparison to real-time PCR results.

Assay	Actual RR soybean (%)		Lectin[a]		RR soybean[a]		Estimated RR soybean (%)	Bias (%)
			Signal (Ct or μA)	DNA copies	Signal (Ct or μA)	DNA copies		
Real-time PCR	CRM[b]	1.0	22.17 ± 0.23	29,7375 ± 53,385	29.44 ± 0.06	2824 ± 129	0.95	−5.0
Electrochemical magnetoassay		1.0	4.59 ± 0.14	56,236 ± 2784	0.468 ± 0.018	511.1 ± 29.9	0.91	−9.0
Real-time PCR	GIPSA[c]	0.86[d]	22.19 ± 0.14	29,1711 ± 28,707	29.60 ± 0.10	2439 ± 177	0.84	−1.7
Electrochemical magnetoassay		0.86[d]	7.00 ± 0.98	110,204 ± 24,361	0.653 ± 0.003	856.5 ± 5.8	0.78	−6.9

[a] Values are mean ± standard deviation.
[b] Certified reference material (flour) containing 1.0% of RR soybean.
[c] Ground soybean sample from the USDA/GIPSA Proficiency Program with 1.0% RR soybean fortification.
[d] Value is the average result from the USDA/GIPSA Proficiency Program.

corresponded to PCR efficiency between 90 and 110%. For real-time PCR targeting RR soybean, PCR efficiency was 97.6%, slope −3.380 and a correlation coefficient of 0.982. The real-time PCR performance parameters targeting *lectin* gene were 94.5%, −3.461 and 0.992, respectively, for PCR efficiency, slope and correlation coefficient. With respect to the proposed PCR coupled to the magnetoassays, the correlation coefficients were >0.99, with response factors (slopes) >0.63 in both amplified targets. Although the response factor should be the closest possible to unity to enable accurate determinations, an adequate compromise among linearity, response factor and sensitivity was accomplished [31].

The application of the two proposed magnetoassay models was successful for the estimation of both DNA targets by interpolation, which enabled to determine the relative content of RR soybean of two soybean materials (Table 2). The results showed that both values were estimated with high accuracy since bias was lower than 10% in both samples. It is important to note that the GIPSA material is not a CRM, so we consider as reference value for comparison the average result of 0.86% obtained from the Proficiency Program. The high accurate estimates obtained by real-time PCR confirm and validate the magnetoassay results, particularly in the GIPSA sample. Besides the high accuracy of the proposed quantitative magnetoassay, a high precision was also obtained as evidenced by relative standard deviations lower than 20%. It should be stressed that the reproducibility of the assays is owed mostly to the high level of monodispersity of the magnetic microparticles (coefficient variation of less than 1%) and their stability throughout the different stages of biorecognition events and multiple washing steps.

4. Conclusions

To the best of our knowledge, this is the first effective attempt of developing a biosensing approach for GMO quantification in real food samples since methods described in the literature rely on synthetic oligonucleotides, lacking true applicability. In this work, we have arduously focused on applying the method for GMO quantification by successfully establishing a correlation between the electrochemical response and the initial amount of template DNA. The proposed method relies on a two target approach, in which *lectin* and RR event-specific sequences are both targeted. Reliable quantification of DNA with end-point PCR has been established by decreasing the number of amplification cycles owing to the increased magnetoassay sensitivity, it is possible to obtain linear calibration approaches able to quantify the GMO proportion in flour samples. Further studies are required to evaluate the applicability of the PCR-magnetoassay approach on more complex/processed food matrices. Furthermore, concomitant studies will be carried out with streptavidin-coated magnetic nanoparticles, in order to improve the sensitivity of our current system for analysis of more complex samples.

Considering that simplicity and economy are two of the most desirable features in GMO analysis, this work represents an attractive and reliable alternative for the quantification of RR soybean in commercial foods without requiring real-time PCR instrumentation.

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Dr. Noemí de-los-Santos-Álvarez received her Ph.D. degree in Chemistry in 2002 from Universidad de Oviedo (Spain). She moved to Cornell University for two years under Prof. Abruña supervision working on characterisation of solid and nanostructured intermetallic materials for their use as anodes in fuel cell. She moved back to Spain, where she is currently a Lecturer. Her research interest includes the development of electrochemical (bio)sensors for the detection of allergens and pathogens in food, clinical and environmental samples. She is focused on aptasensors and genosensors as well as SPR characterisation of biorecognition events.

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María Jesús Lobo-Castañón is head of the Electroanalysis research group at the University of Oviedo. She obtained her Ph.D. in Chemistry in 1996, working in NAD-dependent dehydrogenase enzymes-based electrochemical biosensors. Between 1996 and 2001 she held an Assistant Professor position in Analytical Chemistry at Universidad de Oviedo and since 2001 she is Associate Professor in Analytical Chemistry at this University. Her research interests focus on the development of electrochemical and SPR sensors for clinical diagnosis and food analysis, using different molecular recognition elements, such as DNA, aptamers and molecularly imprinted polymers.

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| Chapter 6 |

Electrochemical detection of magnetically-entrapped DNA sequences from complex samples by multiplexed enzymatic labeling: Application to a transgenic food/feed quantitative survey

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6

ELECTROCHEMICAL DETECTION OF MAGNETICALLY-ENTRAPPED DNA SEQUENCES FROM COMPLEX SAMPLES BY MULTIPLEXED ENZYMATIC LABELING: APPLICATION TO A TRANSGENIC FOOD/FEED QUANTITATIVE SURVEY

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This third contribution was aimed at implementing the newly-optimized multiplex approach in the analysis of 33 commercial samples from the Spanish market. The applicability of this sensing strategy was tested and confirmed with highly processed samples with an elevated level of DNA degradation, extracted with a commercial kit. Using the previous PCR-coupling principle, quantitative results were achieved and compared to those derived from a qPCR method. This study also conveys a small evaluation of the Spanish market in terms of GMO-labeling compliance.

The novelty of this work was based upon achieving unprecedented accuracy in DNA quantification in highly processed samples where the ratio *RR*/*Lec* was expected to be very low, using an electrochemical method coupled to end-point PCR.

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Electrochemical detection of magnetically-entrapped DNA sequences from complex samples by multiplexed enzymatic labelling: Application to a transgenic food/feed quantitative survey

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ABSTRACT

Monitoring of genetically modified organisms in food and feed demands molecular techniques that deliver accurate quantitative results. Electrochemical DNA detection has been widely described in this field, yet most reports convey qualitative data and application in processed food and feed samples is limited. Herein, the applicability of an electrochemical multiplex assay for DNA quantification in complex samples is assessed. The method consists of the simultaneous magnetic entrapment *via* sandwich hybridisation of two DNA sequences (event-specific and taxon-specific) onto the surface of magnetic microparticles, followed by bienzymatic labelling. As proof-of-concept, we report its application in a transgenic food/feed survey where relative quantification (two-target approach) of Roundup Ready Soybean® (RRS) was performed in food and feed. Quantitative coupling to end-point PCR was performed and calibration was achieved from 22 and 243 DNA copies spanning two orders of magnitude for the event and taxon-specific sequences, respectively. We collected a total of 33 soybean-containing samples acquired in local supermarkets, four out of which were found to contain undeclared presence of genetically modified soybean. A real-time PCR method was used to verify these findings. High correlation was found between results, indicating the suitability of the proposed multiplex method for food and feed monitoring.

1. Introduction

From verification of foodstuff origin to monitoring of genetically engineered crops, DNA-based technologies have been widely implemented in the field of food control in recent years [1–6]. In cases where remarkable levels of specificity and sensitivity are necessary and sample complexity/processing is elevated, DNA detection is favoured over protein-based approaches [4–7].

In molecular food analysis, quantitative data is often required to assess whether foods in trade are in compliance with particular specifications legally established [8]. Such is the case for genetically modified organisms (GMOs) in food and feed products. In the European Union, labelling is mandatory except when GMO content is in a proportion no higher than 0.9 per cent of the food ingredients considered individually or food consisting of a single ingredient, provided that this presence is adventitious or technically unavoidable [9]. In such strict cases, usually two genetic fragments are targeted in order to perform the relative quantification of the target sequence normalised against a taxon-specific housekeeping gene [10,11].

GMO quantification is routinely achieved by fluorescent real-time or quantitative polymerase chain reaction (qPCR), which represents today nearly the only universally accepted strategy for sequence-specific DNA quantification [12–14]. Intercalating dyes are commonly used to monitor amplicon formation by means of real-time fluorescence measurements [15]. Despite its popularity as a relatively low-cost approach, most GMO detection methods validated by the Joint Research Commission (JRC) are based upon hydrolysis probes, *e.g.* Taqman® probes. By hybridising with the central region of the targeted fragment, these probes deliver superior specificity to the method but fairly increase the overall price [16]. Alternative hybridisation-based strategies based on electrochemical detection techniques can also offer high specificity as well as cost efficiency. This field has a remarkable trajectory as one of the most active areas of research in analytical chemistry [17,18].

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Electrochemical DNA detection represents a simple, portable and robust alternative with an important track of publications in the last decades. However, while important technological progress has been made in terms of sensitivity, there are still plenty of challenges to overcome regarding real-life applications. Such is the case for DNA biosensors reported for GMO screening [19]. Analysis of commercial samples, especially of quantitative nature, has not been thoroughly addressed so far [4,19,20]. This is most likely owed to the high level of complexity of processed food and feed samples, in which genomic DNA is present in extremely low quantities within a mixture of different species and in supercoiled structures that hinder accessibility of the -frequently short- targeted sequences. While coupling PCR amplification to the electrochemical assay can solve these technical problems, guarantying a quantitative relationship between starting DNA amounts and electrochemical responses can be a challenge. However, we have shown in a previous report [21] that the combination of end-point PCR and electrochemical genosensing allows GMO quantification provided that the number of thermal cycles for each specific system is adjusted. As a result, Roundup-Ready® Soybean (RRS) was quantified in flour samples by determining the ratio between transgenic and taxon-specific targets using two separate electrochemical assays.

Having developed a more rapid method targeting both sequences at once [10], namely electrochemical multiplex assay (EMA), and focusing on surpassing current boundaries in sample analysis, this study seeks to assess the applicability of the EMA method in quantifying transgenic material in food and feed samples. The EMA approach relies on the simultaneous entrapment of transgenic and taxon-specific sequences from RRS with magnetic beads and subsequent multiplex enzymatic labelling for sequential detection of both targets. The theoretical suitability of this method was addressed with synthetic mixtures of the two sequences in our previous report [10]. The double –sandwich-type– hybridisation involved in the assay grants it with a high level of specificity. Application of this double-target-detection scheme in highly processed samples not only is meant as proof-of-concept of non-fluorescent, non-real-time DNA relative quantification, but also as a survey to check compliance of the food and feed market with current European regulations.

2. Experimental section

2.1. Chemicals and materials

Streptavidin-coated magnetic beads Dynabeads MyOne Streptavidin C1 (1 µm diameter) were acquired from Life Technologies (Spain). The modification of the beads and the hybridisation and labelling assays were performed in a 12-tube mixing wheel (Dynal MX1) and magnetic separations were carried out with a magnet DynaMag2, both from Life Technologies.

Tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H_2O_2), diethanolamine (DEA) and Tween 20 were purchased from Sigma-Aldrich (Spain). Saline sodium phosphate-EDTA (20×SSPE) pH 7.4 and 1-naphthyl phosphate were also obtained from Sigma-Aldrich. Ethanol was purchased from Panreac (Spain). 1% Casein buffer in 1× PBS was obtained from Fischer Scientific (Germany). Anti-fluorescein-alkaline phosphatase (antiFITC-ALP) and anti-digoxigenin-peroxidase (antiDig-POD) Fab fragments, were received from Roche Diagnostics GmbH (Mannheim, Germany). MilliQ water was used throughout this work. Lyophilised oligonucleotides were obtained from Sigma-Genosys (UK). The specific sequences of the biotin-, Dig- and FITC-labelled probes are shown in our previous work [10].

The names and composition of the buffers used were: Tween buffer (2×SSPE, 0.005% of Tween-20), hybridisation buffer (2×SSPE, 0.9 M NaCl, pH 7.4), casein buffer (phosphate buffered saline 0.1 M phosphate, 1.54 M NaCl, 1% casein, pH 7.4) and DEA buffer (DEA 1 M, 0.5 mM $MgCl_2$, pH 9.8).

2.2. Samples

Calibration standards were prepared from Certified Reference Material (CRM) with 1% of RRS, provided by the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium). A total of 33 food and feed samples were acquired in local supermarkets, out of which 24 were food samples and 9 were feed samples, namely SOYFO and SOYFE, respectively. Solid and semisolid samples were grinded to powder or paste, respectively.

2.3. DNA extraction

DNA was extracted from 200 to 500 mg of previously homogenised solid and semisolid SOYFO and SOYFE samples. Liquid samples such as SOYFO-02, 18 and 24 were left overnight in a 37 °C oven. The resulting viscous liquid (500 µL) was transferred to an eppendorf tube to begin extraction. All samples were extracted using Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with some modifications: lysis buffer and proteinase K volumes were upscale to 1000–1250 and 20–30 µL per sample, respectively; incubation time for cell lysis was set at 4 h; RNAse 5 mg/mL (pre-boiled at 95 °C for 10 min) was added (2 µL) and let it to cleave RNA for 1 min at room temperature (RT), after which two sequential centrifugation steps at 10,000×*g* were carried out; in the final elution step, two volumes of 75 µL were sequentially added to the column in order to improve the yield of extracted DNA.

Quality and quantity of extracted DNA were determined by UV spectroscopy measurements (UV260 Shimadzu, Japan). 10 ng/µL of total genomic DNA were prepared as working solutions using PCR-grade water.

The quality of extracted DNA was analysed by electrophoresis in a 1.2% agarose gel carried out in SGTB 1× buffer (Grisp, Porto, Portugal) for 30 min at 150 V, previously stained with GelRed 1× (Biotium Inc., CA, USA). Digital images of the gels were captured using Kodak Digital Science™ equipment (Rochester, NY, USA).

2.4. End-point polymerase chain reaction (PCR)

Two DNA sequences were targeted for amplification: event- and taxon-specific, namely RR and Lec, respectively. Primer sequences were called RRS and Lec, accordingly.

PCR amplifications were carried out in 25 µL of total reaction volume containing 10 µL of template DNA, 1× buffer (67 mM of Tris-HCl pH 8.8, 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween-20), 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 2 mM of $MgCl_2$ and 400 nM or 280 nM of each set of primers, Lec-F/Lec-R and RRS-F/RRS-R [21], respectively. Amplifications were performed in a PTC-100 thermal cycler (MJ Research Inc, Quebec, Canada) using the following program of temperatures: 95 °C for 10 min; 30 (for Lec fragment) or 37 (for RRS fragment) cycles of 95 °C for 30 s, 66 °C (for Lec-F/Lec-R primers) or 61 °C (for RRS-F/RRS-R primers) for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 5 min. 35 cycles were used for Lec-sequence amplification in the qualitative gel-based screening. The amplified fragments (84 and 74 bp for RR and Lec amplicons, respectively) were analysed in 1.5% agarose gel, as described in the previous section.

2.5. Real-Time PCR using intercalating dye SYBR® (qPCR/SYBR)

Amplifications by qPCR were performed in 25 µL of total reaction volume containing 10 µL of template DNA, 1× of Mastermix SensiFAST™ SYBR® No-ROX Kit (Bioline, Singapore) and 400 nM or 280 nM of each set of primers, Lec-F/Lec-R and RRS-F/RRS-R, respectively. The amplification reactions were performed in a fluorimetric thermal cycler MyGo Pro (IT-IS Life Science, Cork, Ireland)

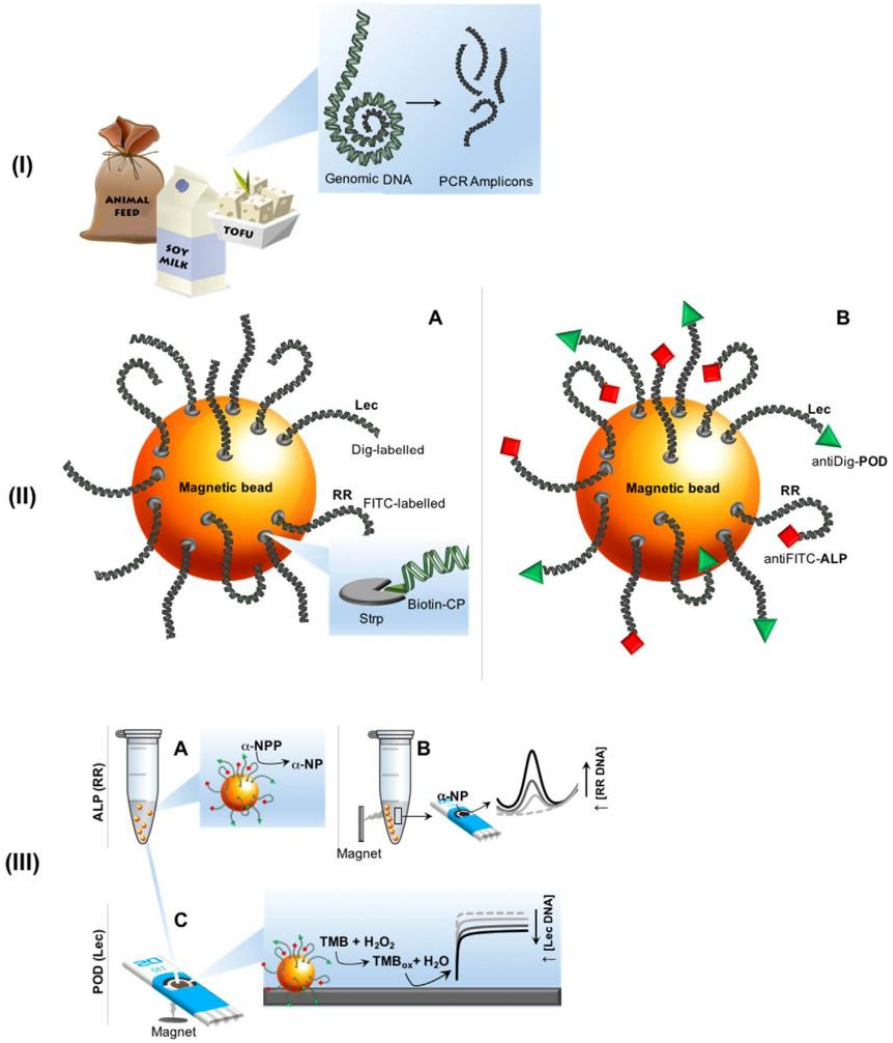

Fig. 1. Schematic representation of the qPCR/EMA method: (I) Sample pre-treatment involving DNA extraction from food and feed samples followed by PCR amplification of two sequences, Lec and RR. (II-A) Magnetic entrapment of the two targets by hybridisation with their specific –biotinylated– capture probes (previously anchored to streptavidin-coated magnetic beads) and with FITC- (RR) and Dig-labelled (Lec) signalling probes (II-B) Bienzymatic labelling using antiFITC-ALP (RR) and antiDig-POD (Lec) conjugated enzymes. (III) Electrochemical measurements consisting of (A) ALP-mediated dephosphorylation of substrate α-NPP in suspension and (B) subsequent transference of the supernatant to a screen-printed carbon electrode for voltammetric measurement of α-NP oxidation. (C) Magnetisation of the beads onto the surface of a screen-printed carbon electrode where POD-mediated oxidation of commercial substrate TMB (+H_2O_2) takes place, followed by amperometric reduction.

using the following temperature conditions: 95 °C for 3 min, 45 cycles at 95 °C for 10 s, 66 °C for 10 s and 72 °C for 15 s, with collection of fluorescence signal at the end of each cycle. Melting analysis was performed from 60 °C to 97 °C at 0.1 °C/s. Data were collected and processed using the MyGo Pro PCR Software v3.0 (IT-IS Life Science, Cork, Ireland). Real-time PCR amplifications were performed in triplicate for each unknown sample and each standard.

2.6. PCR-coupled electrochemical multiplex assay (PCR/EMA)

The experimental procedures were based on a multiplex platform described in our previous work [10], having optimised the measurement step. Fig. 1 shows a schematic representation of the PCR/EMA method consisting of DNA extraction from samples and amplification of RR and Lec sequences (Fig. 1-I), magnetic entrapment of both sequences *via* sandwich hybridisation with bead-anchored biotinylated probe and hapten-labelled probe (Dig and FITC for Lec and RR, respectively) (Fig. 1-IIA), multiplexed enzymatic labelling (antiDig-POD and antiFITC-ALP for Lec and RR, respectively) (Fig. 1-IIB) and sequential detection of electroactive enzymatic products (TMB_{ox} and α-naphthol for Lec and RR, respectively) (Fig. 1-III).

2.6.1. Biomodification of the magnetic beads

10 µL of streptavidin-modified magnetic beads (10 mg/mL) were transferred into an eppendorf tube and mixed with 490 µL of tween buffer. Two washing steps were performed with 500 µL of the same buffer, after which the beads were resuspended in 500 µL of a solution containing 1 µM of biotinylated Lec and RR capture probes and incubated for 30 min at RT. After probe immobilisation, biomodified beads were washed twice with 500 µL of tween buffer.

2.6.2. Hybridisation

The sandwich-type hybridisation assay was performed simultaneously with both PCR products (25 µL each), undiluted for RRS and 50-fold diluted for Lec, and 10 nM of the corresponding signalling probe, Dig- and FITC-labelled for Lec and RR, respectively. The

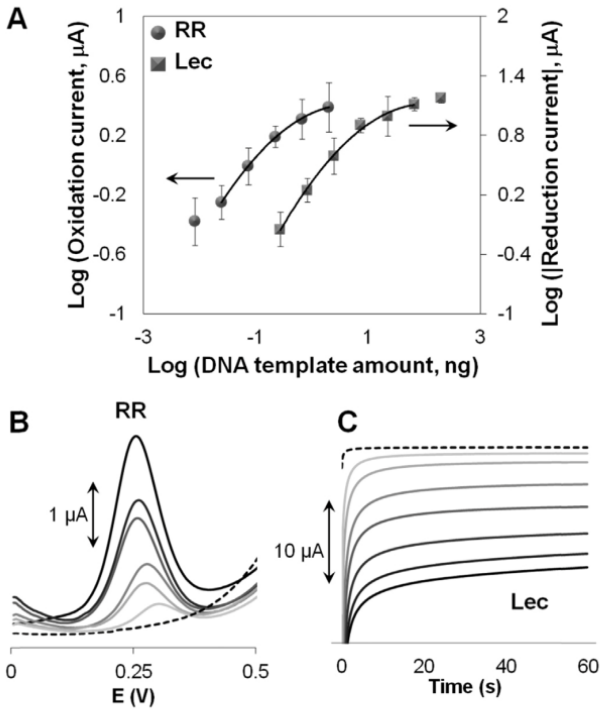

Fig. 2. Analytical response of the PCR/EMA method: (A) calibration plots for Lec and RR, (B) square wave voltammograms, (C) chronoamperometry responses. Increasing DNA amounts are represented by light-to-dark lines. See text for experimental conditions.

mixture was subjected to 95 °C for 5 min following an ice-bath for 5 min. After 25 min at RT, the hybridised structures were added to the multiprobe-modified beads in order to magnetically capture the target hybrids. 1-h incubation was performed under rotation at RT, after which the beads were washed three times with tween and casein buffers.

2.6.3. Enzymatic labelling and electrochemical detection

Enzymatic labelling was performed by resuspending the beads in a mixture (500 µL) containing antiFITC-ALP and antiDig-POD 0.5 U/mL each, prepared in casein buffer. After 30 min of incubation time, the beads were washed twice and finally resuspended in 200 µL of 2×SSPE.

The electrochemical detection of both enzymatic products was performed onto the surface of a disposable carbon electrode, by means of square-wave voltammetry (SWV) for ALP or chronoamperometry (CA) for POD. For the ALP system, 50 µL of the fully modified beads were transferred to a separate eppendorf tube. The buffer was removed after magnetisation and 50 µL 4 mM α-NPP solution in DEA buffer was added to the beads. After ALP reacts with the substrate for 10 min, the beads are magnetised for 1 min and the 50-µL supernatant containing the electroactive product α-naphthol (α-NP) is added to the surface of a screen-printed carbon electrode for electrochemical readout. α-NP oxidation was measured by means of SWV from 0 to 0.5 V with a step potential of 5 mV, 20 mV amplitude and frequency of 25 Hz.

For the POD system, 15 µL of the fully modified beads were magnetically captured onto the working electrode with a magnet placed under it. After 1 min, the liquid was carefully removed with a pipette and 45 µL of the enzymatic substrate (commercial TMB+H_2O_2) was placed on the screen-printed surface. POD-catalised oxidation of TMB takes place after a fixed time (1 min) forming a radical cation in equilibrium with a charge-transfer complex. The reduction of this product is subsequently monitored *via* CA at a potential of 0 V and sampling the current during 60 s.

3. Results and discussion

3.1. Optimisation of the electrochemical multiplex assay

Few changes were made in the measurement step of our previously described multiplex platform [10]: (a) SWV is performed instead of differential pulse voltammetry, as the former provides faster measurements (less than a minute) and well-resolved peaks; and (b) enzymatic dephosphorylation of α-NPP occurs in suspension instead of on the electrode surface. Different signal-to-blank (S/B) ratios were obtained with both suspended and electrode-bound configurations, as shown in Fig. S1-IA and S1-IIA. The beads are spatially well-distributed in the suspended form (Fig. S1-IB) whereas surface-bound beads are in a packed configuration in the electrode surface due to the magnetic force pushing them in close proximity to both the surface and with each other (Fig. S1-IIB).

A higher amount of beads per microliter was used for the suspended configuration (0.5 µg/µL) in order to obtain similar current responses in the presence of target sequence (100 pM) for both electrode-bound and suspended formats. In contrast, the current response in the absence of target sequence (blank) was approximately 37-fold higher in the case of the surface-bound format. It can be argued that the blank signal arises from unspecifically adsorbed enzymes in the surface of the beads, although why this is more noticeable in the packed, electrode-bound configuration is difficult to explain. Given that S/B ratios greatly favour the suspended form, this was the chosen setup for subsequent experiments.

The same variation was tried with the POD system and no improvement was obtained. Thus, a double-detection scheme is proposed in which the ALP reaction occurs in the suspended form and the POD reaction is performed directly on the electrode surface.

3.2. Analytical performance of PCR/EMA

The PCR/EMA method was evaluated against serial dilutions (3-fold) of extracted DNA from CRM 1% submitted to PCR for both RR and Lec systems, starting from 200 ng of total template DNA (~176991 copies) to 0.27 ng (~243 copies). These amounts corresponded to 2 ng (~1770 copies) – 2.7 pg (~2 copies) of RR soybean DNA. DNA copies were calculated taking into account the soybean genome size (1.13 pg) obtained from the Plant DNA C-value database [22], assuming that the targeted sequences are single copy genes [23].

Chronoamperometry and votammetry results were plotted against the initial DNA amount of Lec and RR sequences, respectively (Fig. 2A). Calibration was accomplished from 0.27 ng to 66.67 ng for Lec and from 25 pg to 2 ng for RR. The no template control (NTC) for Lec presented a very similar response to the ordinary blank obtained in our previous work using synthetic oligonucleotides (0.14 ± 0.04 µA). In the case of the RR system, NTC signal was not detectable one out of three times. When detectable, signal was as low as 10 nA. Fig. 2B,C shows the voltammograms and chronoamperograms of the plotted values and of the blanks.

Some adjustments were necessary to perform PCR-coupling under in-house laboratory conditions because these differed from our previous PCR-coupling work [21], *i.e.* different thermal cycler, primer manufacturer, DNA extraction method and reference material. Log-Log quadratic fits were obtained instead of linear, even though the cycle number, primer concentration and annealing temperature were the same for the Lec system. RR amplification changed as to our previous work due to the use of a CRM with lower GMO level. Consequently, DNA copy number was dramatically decreased and the system had to be adjusted to more amplification cycles (37 instead of 35). Additionally, the melting temperature was increased to 61 °C and $MgCl_2$ to 2.5 mM in order to find an appropriate balance between

sensitivity and specificity. All of this indicates the importance of carefully optimizing PCR-related parameters for each individual situation.

The Lec and RR systems were adjusted to the following quadratic fits, respectively:

Log *(Current, μA)*=−0.203(±0.048) (Log (*DNA amount, ng*))^2 +0.775(±0.046) (Log (*DNA amount, ng*) +0.337(±0.026); R^2 =0.996

Log (Current, μA)=−0.126(±0.008) (Log (DNA amount, ng))^2 +0.1703(±0.012) (Log (DNA amount, ng) +0.3494(±0.004); R^2 =0.999

The values of limits of detection (LOD) and quantification (LOQ) were calculated by measuring responses from PCR-submitted extraction blanks (*n*=6). LOD values were calculated as the amount of DNA that gives a current equal to the average of blank currents plus three times the standard deviation, whereas LOQ was determined taking into consideration the average of blank currents plus ten times the standard deviation. Resulting LOD and LOQ values for Lec were 0.11 ng (~97 copies) and 0.21 ng (~186 copies), respectively. For the RR system, although the calculated LOD/LOQ values were extremely low (down to 1 and 3 copies), more 'realistically detectable' values were set at 8.2 pg (~7 copies) for LOD (S/B ~3) and 24.69 pg (~22 copies) for LOQ. The latter corresponds to the first calibration point.

Interday repeatability was evaluated by performing calibration in three separate days. Relative Standard Deviation (RSD) values were in the range of 5–20%.

3.3. Real samples: DNA extraction and qualitative survey

Table SI shows a description of the 33 samples acquired in local supermarkets and their DNA extraction data (yields and purity ratios). The majority of the samples depicted A_{260}/A_{280} ratios near 1.6–1.8, which points to the high purity of the DNA extracts. Yield varied across the different levels of sample complexity. In cases such as SOYFO-02, DNA quantity was as low as 10 ng/μL. Fig. 3-I shows agarose gels of resolved genomic DNA from extracts. Consistent with the data shown in Table SI, samples of low yield are barely seen in the gel, *e.g.* SOYFO-02 and 14 and SOYFE-03. Smeared bands were evidenced in most samples, indicating moderate-to-high levels of DNA degradation, expected for highly processed samples, *e.g.* milk cream, snack bars, fried crackers, dog snacks, *etc.*

Amplification reactions (Fig. 3-II) were performed using primers targeting small amplicons (<100 bp), as recommended for highly processed samples [24]. Lec amplification was positive in all cases (Fig. 3-IIA), indicating that the extracted DNA was amplifiable even in those samples with extremely low concentration and high level of degradation. The CRM 1% lane was included as positive control.

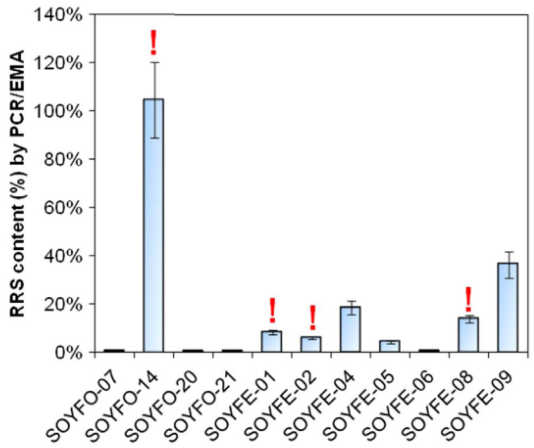

Fig. 4. Bar diagram showing quantitative results expressed as GMO percentages determined by PCR/EMA in 11 samples. Red exclamation points are used to highlight samples with undeclared GMO presence.

The agarose gel shows that RR amplification was significant in SOYFO-07, 14 and 20 and SOYFE-01, 04, 05 and 09 (Fig. 3-IIB). In the case of SOYFO-14, a faint band can be seen in both Lec and RR gels, which indicates a high RR/Lec proportion. SOYFO-07 and 20 were also positive for RR, although given the highly intense Lec bands and, compared to CRM 1%, it is plausible that their GMO content is below 1%. SOYFE-04, 05 and 09 were expected to be GMO-positive because the term 'genetically modified soybean' is included in the ingredients list.

3.4. Quantitative sample analysis

Relative DNA quantification expressed as GMO percentages were calculated with RR/Lec ratios (in nanograms), as established by Recommendation 2004/787/EC in the context of Regulation 1830/2003 by the European Commission [9].

Fig. 4 shows the results from 11 samples with low-to-moderate GMO content. RR was not quantifiable in the rest of the samples (values below LOQ). Surprisingly, 4 samples that did not declare GMO presence in their product labels were found to contain RRS in elevated proportions. As mentioned previously, a proportion higher than 0.9% should be reflected in the ingredients list as 'genetically modified

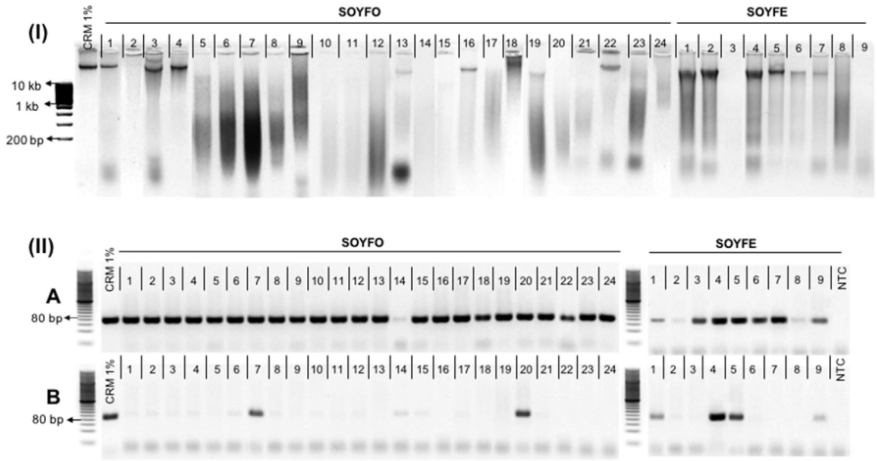

Fig. 3. Agarose gel electrophoresis of (I) DNA extracts from samples and (II) PCR amplified (A) Lec and (B) RR fragments after 35 and 37 cycles of amplification, respectively.

soybean'. In order to verify these findings and check compliance with legislation, a SYBR-based qPCR method was used.

The qPCR method was pre-validated in-house in terms of accuracy, precision, and amplification efficiencies. Three accuracy controls were used consisting on low, medium and high DNA levels, *i.e.* 5, 25 and 100 ng of total DNA corresponding to 0.05, 0.25 and 1 ng of RR DNA. For the Lec system, relative error values were 3.9%, 0.2% and 4.8% for the three levels, respectively, whereas for RR results were 18%, 21% and 24%, respectively. The latter being a system of very low copy number is prone to higher error. Accuracy was also confirmed using 100 ng of DNA from reference material 1% as internal quantitative control. A value of 0.85% was withdrawn. Efficiencies of 91.5% and 100.1% were found for Lec and RR amplifications, respectively. Cycle threshold (C_q) values were plotted against the logarithm of DNA amounts (Fig. S2-A). Calibration was achieved in the ranges of 91 pg–200 ng and 25 pg–2 ng for Lec and RR, respectively. Real-time curves are shown in Fig. S2-B,C.

Despite using primers from the EU GMO method database (JRC), specificity of both primer pairs was evaluated with melting analysis. A well-defined peak was present in each system consistently, resulting in the following melting temperatures: 75.6 °C and 83.5 °C for the RR and Lec amplicons, respectively (Fig. S2-D,E).

RRS (%) values were compared in terms of relative errors (Table 1). In spite of the adequacy of validation parameters, GMO percentages found in samples SOYFE-02 and 06 were much higher with the qPCR method. After running melting analysis, non-specific peaks in RR were observed for these two samples (Fig. S3-A). The post-qPCR products were resolved in an agarose gel and we found several fragments between ~200 bp and ~400 bp (Fig. S3-B). These findings imply lack of specificity of the SYBR assay in these cases, compared to other feed samples, *e.g.* SOYFE-04 and 05, where only specific RR melting peaks were found.

The specificity problem found with qPCR could be linked to several factors. On one hand, the possibility of unspecific primer binding is unlikely given that only specific fragments were observed in the qualitative survey (PCR in previous section) and that specificity was previously confirmed using BLAST. On the other hand, we must consider that a pre-mixed preparation containing all PCR reagents, *i.e.* dNTPs, $MgCl_2$ and hot-start polymerase, was used in qPCR while each individual component was previously optimised for the PCR assay. The ultra-low target amount in the RR reaction together with suboptimal reaction conditions might trigger co-amplification of by-products due to primers hybridising in non-specific regions or to limited fidelity of the DNA polymerase [25]. Another plausible explanation could be related to SYBR binding preferentially to long, G-C rich dsDNA structures [14] that could be circulating in highly degraded extracts. However, this was not observed in other degraded samples.

The result is overestimation of DNA concentration and consequently of GMO levels in these samples, *e.g.* SOYFE-02 was found to contain 22.5% of RRS with qPCR/SYBR while 5.9% was estimated with the PCR/EMA method. The latter has an added degree of selectivity because of the double hybridisation process.

The rest of the samples revealed errors below 25% as shown in Table 1, which is in compliance with validation guidelines [26]. The dispersion of GMO values was higher for the PCR/EMA method, which could be owed to the multi-step nature of the assay. The correlation between the values calculated from both methods was strong (r=0.99) (Fig. S4).

Overall, the survey reflected the undeclared presence of GMO in 4 samples (SOYFO-14, SOYFE-01, 02 and 08) out of the 33 acquired in local supermarkets, which roughly represents 12% of the studied samples. Despite strict labelling regulations in Europe, this is not the first report on inadvertent GMO presence in the European food/feed market [27–29].

3.5. Advantages and disadvantages of the electrochemical method

When drawing the advantages of the electrochemical method, specificity and cost efficiency stand out as important features. Whereas the qPCR/SYBR approach can be rather low-cost in terms of reagents compared to more specific chemistries (*e.g.* Taqman® probes), non-specific amplification can be a problem as it has been revealed in this work. This is because intercalating dyes can bind to any double-stranded structure, which can be a problem because it can lead to overestimation of DNA concentration/gene expression and false positives. While hydrolysis probes can overcome this problem, their design must comply with certain specifications given by the PCR reaction and this is sometimes challenging or even impossible due to the complexity of the target sequence [30,31].

The PCR/EMA approach can be used to circumvent some of these problems as it offers high specificity given the double hybridisation implied. Probes hybridise with the target sequence post-PCR, which can be advantageous since it reduces PCR-related complications. However, one important disadvantage of this strategy is the time-consuming and relatively arduous bench-work compared to the simple qPCR setup. Yet the electrochemical platform presented here has the potential to be further improved, *e.g.* the time and number of steps involved in the protocol can be reduced by using microfluidic integrated devices. Overall costs can be diminished as well by implementing printed electronics in electrode fabrication [18].

4. Conclusions

GMO quantification in real-world samples where genomic DNA is degraded requires highly specific approaches that can deliver accurate

Table 1
Quantitative results from sample analysis with PCR/EMA and qPCR/SYBR methods.

	RRS/Lectin ratio (± standard deviation) (%) (*n=3*)		
Sample	**PCR/EMA**	**qPCR/SYBR**	**Relative error (%)**
SOYFO-07	0.47 ± (0.07)	0.43(± 0.04)	**−10.12**
SOYFO-14	105 ± (16)	87(± 3)	**−20.77**
SOYFO-20	0.17 ± (0.02)	0.210(± 0.001)	**19.05**
SOYFO-21	0.009 ± (0.001)	0.008(± 0.001)	**−12.50**
SOYFE-01	8 ± (1)	9.6(± 0.4)	**14.32**
SOYFE-02	5.9(± 0.6)	22.5(± 0.9)	**73.72**
SOYFE-05	18.3 ± (2.8)	19.3(± 0.1)	**5.14**
SOYFE-06	4.2 ± (0.6)	4.2(± 0.4)	**0.24**
SOYFE-08	0.49(± 0.02)	0.820(± 0.008)	**40.24**
SOYFE-10	13.7(± 1.3)	12.5(± 0.8)	**−9.54**
SOYFE-13	36.3(± 5.4)	44.2(± 1.1)	**17.84**
CRM 1%	0.73(± 0.09)	0.85(± 0.02)	**14.12**

results. While significant progress has been made in the field of electrochemical DNA detection for food analysis, there is still limited demonstration of real-life applicability. We have assessed the suitability of a PCR-coupled electrochemical method in the quantification of two DNA sequences from processed samples. We verified results from our method with a qPCR assay and determined GMO percentages in 11 samples. Bias was below 25% in 9 of them, from low (< 0.01%) to high levels (~ 100%). Two samples depicted non-specific peaks in melting analysis indicating that the qPCR assay based on intercalating dye SYBR® is not suitable for all samples.

The reported method application also involved a transgenic food/feed survey where undeclared GMO presence was revealed in 4 out of 33 samples.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.11.040.

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Supplementary material

Electrochemical detection of magnetically-entrapped DNA sequences from complex samples by multiplexed enzymatic labelling: Application to a transgenic food/feed quantitative survey

C. L. Manzanares-Palenzuela[1,2], J. P. Martin-Clemente[2], M. J. Lobo-Castañón[3], B. López-Ruiz[1*]

Table SI. Samples and DNA extraction data.

Sample code	Description	DNA concentration (ng/µL)	$A_{260/280}$
SOYFO-01	Yellow grains	**75**	1.85
SOYFO-02	Vegetarian milk cream	**10**	1.54
SOYFO-03	Beans with shell	**88.5**	1.99
SOYFO-04	Frozen beans	**47**	1.84
SOYFO-05	Fried soybean	**71**	1.82
SOYFO-06	Fine texturised soybean	**92**	1.79
SOYFO-07	Chopped soybean	**172.5**	1.86
SOYFO-08	Flour extract	**53.3**	1.90
SOYFO-09	Soybean powder drink	**123**	2.02
SOYFO-10	Yellow soybean powder	**49.3**	1.55
SOYFO-11	Youzao powder	**29**	1.53
SOYFO-12	Soybean knot	**180.5**	1.80
SOYFO-13	Soft tofu GMO-free	**154.3**	2.00
SOYFO-14	Fried soybean crackers	**42**	1.74
SOYFO-15	Snack bars	**25.8**	1.47
SOYFO-16	Tofu spaguetti	**24**	1.85
SOYFO-17	Tofu lasagna	**35**	1.89
SOYFO-18	Soy milk	**36.5**	1.90
SOYFO-19	Soy cracker	**85**	1.76
SOYFO-20	Fried tofu	**37**	1.83
SOYFO-21	Firm tofu	**60**	1.56
SOYFO-22	Miso soup	**65**	1.90
SOYFO-23	Roasted edamame	**136.3**	1.83
SOYFO-24	Soy milk	**25**	1.67
SOYFE-01	Rabbit feed	**449.6**	1.80
SOYFE-02	Rodent feed	**274.8**	1.87
SOYFE-03	Soybean snacks for dogs	**21.3**	1.37
SOYFE-05	Feed for broiler chicken*	**233.3**	1.62
SOYFE-06	Feed for laying hens*	**91.8**	1.88
SOYFE-08	Feed for small birds	**35.8**	1.86
SOYFE-09	Granules for cockatiels	**102.5**	1.89
SOYFE-10	Feed for decorating fish	**234.5**	1.72
SOYFE-13	Dog snacks*	**69**	1.80

*GMO-labelled

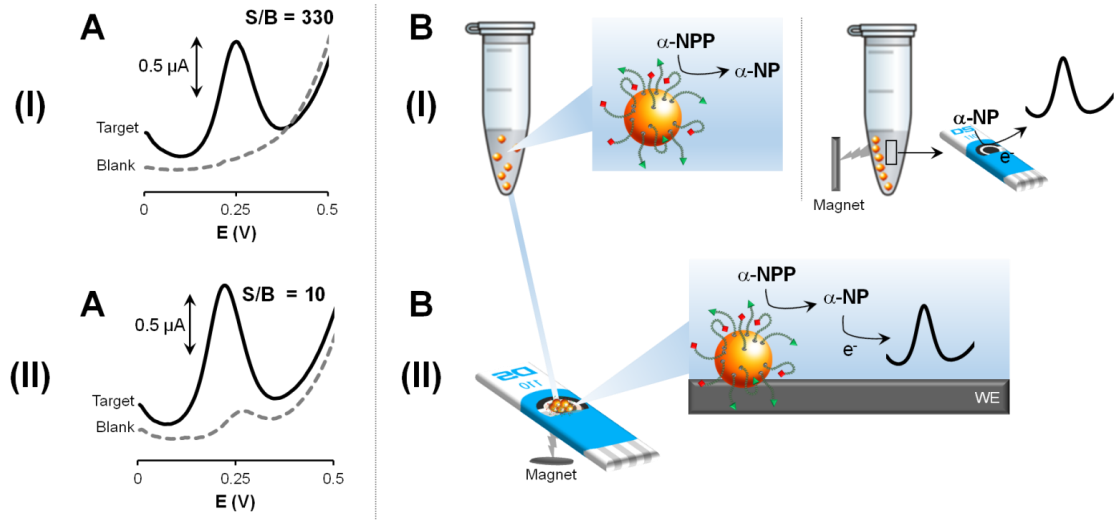

Fig. S1. Electrochemical responses of the ALP-mediated reaction in the (I) suspended-beads form and in the (II) electrode surface-bound form; (A) Voltammetric responses of blank and target 100 pM and (B) measurement setup.

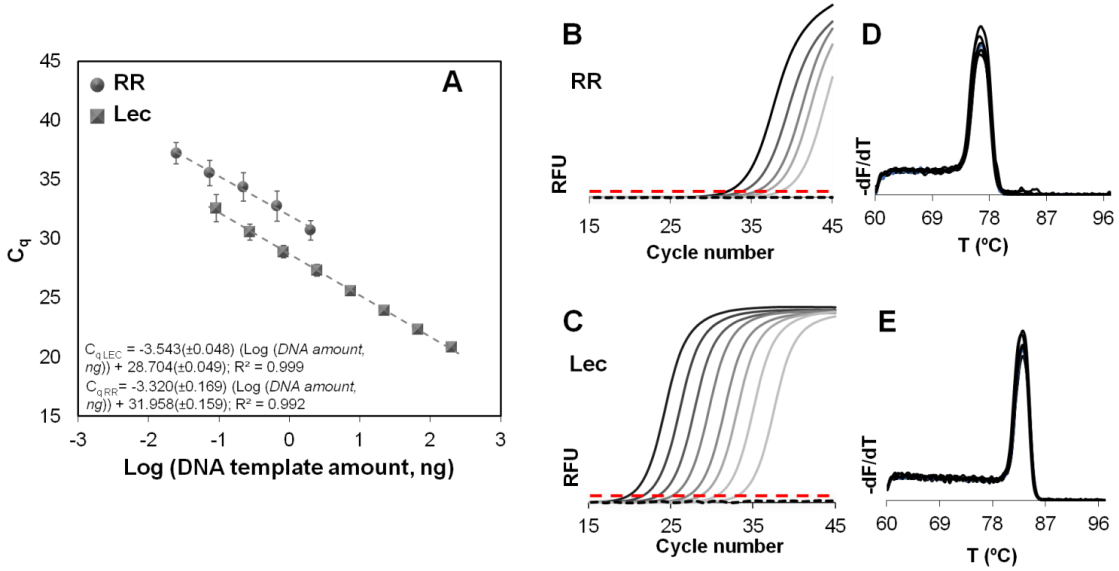

Fig. S2. Analytical response of the qPCR/SYBR method: (A) calibration plots for Lec and RR, (B) real-time curves of RR, (C) real-time curves of Lec, and melting analysis of (D) RR and (E) Lec. Increasing DNA amounts are represented by light-to-dark lines (see text for specific values). Cycle thresholds are represented by red dashed lines.

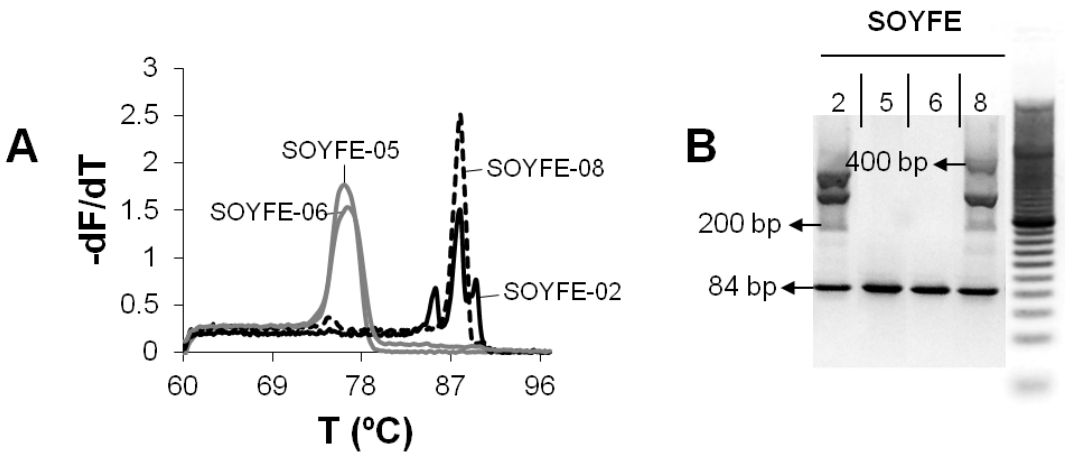

Fig. S3. Analysis of specific and non-specific fragments in SOYFE samples obtained with qPCR/SYBR: (A) Non-specific (black dashed and continuous lines) and specific patterns (gray lines) in melting analysis from SOYFE-05 and 06 and SOYFE-02 and 08, respectively; and (B) agarose gel of post-PCR (45 cycles) samples.

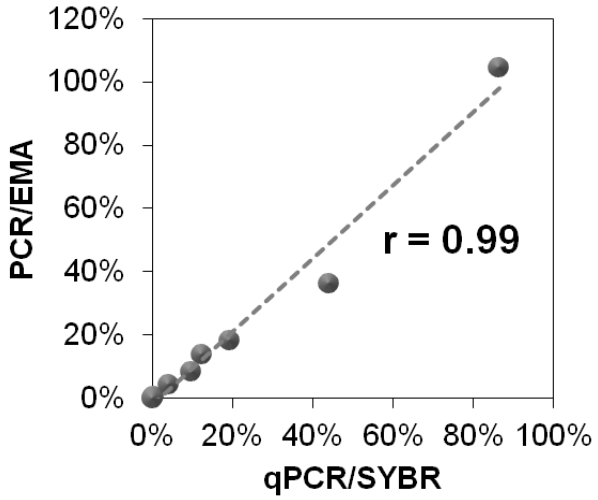

Fig. S4. Correlation between GMO percentages calculated with PCR/EMA and qPCR/SYBR.

| CHAPTER 7 |

IMPEDANCE SENSING OF DNA HYBRIDIZATION ONTO NANOSTRUCTURED PHTHALOCYANINE FILM-MODIFIED ELECTRODES

Electrochimica Acta 221 (2016) 86–95

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IMPEDANCE SENSING OF DNA HYBRIDIZATION ONTO NANOSTRUCTURED PHTHALOCYANINE FILM-MODIFIED ELECTRODES

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In this report, a new DNA analytical method is presented based on a nanostructured, label-free biosensor. The novelty of this work relies on the use of phthalocyanine-modified electrodes for DNA detection. On the other hand, the study also includes a thorough characterization of this specific surface, at the morphological and electrochemical level. Contrary to expectations, DNA hybridization induced a drop in the impedance of the system. This odd phenomenon is discussed and several hypotheses are presented. Having used the RR target sequence and an unlabeled capture probe for the hybridization reaction, this work represents a novel strategy for RRS monitoring.

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Impedance sensing of DNA hybridization onto nanostructured phthalocyanine-modified electrodes

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ABSTRACT

DNA detection is still undergoing major innovations in pursuit of low-cost and simple approaches for decentralized applications. Label-free sensing of DNA hybridization *via* impedance measurements is a popular strategy to fulfil the goals of cost-efficiency and simplicity. Several materials are often reported for electrode modification to improve the sensitivity of impedance-based sensors. Herein we evaluate the electronic properties of copper phthalocyanine tetrasulfonate (CuPcTs) in Layer-by-Layer (LbL) films for impedimetric sensing of DNA hybridization using silanized Fluorine-doped Tin Oxide (FTO) electrodes. 1 to 5 bilayers were prepared by alternate immersion of the substrate in CuPcTs and poly(allylamine hydrochloride) (PAH). DNA probe immobilization was carried out electrostatically onto the last PAH layer, followed by hybridization with the target sequence leading to the formation of a partial double stranded (*pds*) structure onto the films. Impedance decreased after hybridization proportionally with the concentration of the target sequence at picomolar levels. Not only are these findings useful as a potential biosensing strategy, but also leave an open question about the electronic and synergistic properties of DNA interacting with different materials and surfaces.

1. Introduction

Sequence-specific DNA detection is unarguably the core basis of many research fields and routine applications. Several detection technologies already exist for this purpose (e.g., DNA microarrays, pyrosequencing and polymerase chain reaction –PCR–) yet efforts are driven incessantly towards simple and cost-efficient technologies. Interest in electrochemistry for DNA detection became popular decades ago with the introduction of DNA biosensors and more recently with the development of electrochemical real-time PCR [1].

Despite the load of research invested in DNA biosensors their commercial viability and widespread application are still challenged by some limitations [2]. Ideally, one would envisage an easy-to-fabricate biosensor that performs quick and simple electrochemical readout. One earthly limitation hindering this goal is the strenuous assembly of the biosensor itself: time-consuming protocols are often required to construct robust DNA sensing devices. These often involve extended chemical/biochemical modifications of the electrodes, lingered probe immobilization and target labelling using electroactive molecules, enzymes or nanomaterials [3]. These steps could be simplified and shortened by the use of label-free techniques and easy electrode-modification procedures.

Electrochemical Impedance Spectroscopy (EIS) has been widely described for label-free and sequence-specific detection of DNA [4]. Possibly the major motivation for choosing EIS as the detection technique is driven by the need of less costly, more rapid and easier DNA sensing protocols. However, one limitation of EIS-based DNA detection is the sensitivity [2], which can be suboptimal for a number of applications. There have been relevant contributions for improving sensitivity and lowering the limit of detection (LOD) [5]. Most of them focus on one seemingly consensual strategy: modifying the electrode surface to produce highly conductive, catalytic and/or area-enhanced platforms [6–9]. A general example of this material-based strategy can be represented by a graphene-based DNA sensor reported for the detection of specific DNA sequences down to 10^{-12} M level [10].

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Different kinds of materials and methods have been explored in the field of electrode modification. In this sense, the emergence of the layer-by-layer (LbL) film deposition technique has greatly benefited the field by allowing easy and low-cost fabrication of an unprecedented range of functional surfaces with controllable properties [11–14]. The basic principle of this approach involves the self-assembly film fabrication by alternate deposition of oppositely charged species onto a solid substrate. First described a few decades ago [15], this strategy has received growing attention as an effective, simple and environmentally benign way to develop advanced patterned and micro-nanostructured surfaces.

A wide spectrum of building blocks can be assembled with the LbL technique, e.g. polymers, nanomaterials, dyes, biomolecules, etc. [16]. Phthalocyanine derivatives, which share similarities with the biologically-related porphyrin family, have been implemented in important functional materials in many fields for their biomimetic properties and electron transfer abilities [17,18]. There is considerable interest in the semiconductive properties of phthalocyanine films for applications in nanometer-scale electronic devices such as data storage, memory devices and sensors [19–26]. This wide range of applications arises from their unique electronic and electrocatalytic properties, well-defined electrochemical activity and high thermal stability [27].

Driven by the potential of phthalocyanines to engage in electron-exchange processes and by their ability to form highly organized structures, this study seeks to evaluate phthalocyanine films as potential biosensing platforms. We assessed the electrochemical behaviour of silanized Fluorine-doped Tin Oxide (FTO) electrodes modified with phthalocyanine LbL films and their performance towards DNA label-free and sequence-specific detection. Copper phthalocyanine tetrasulfonate (CuPcTs) was selected as the anionic species for LbL assembly based on its chemical stability, water solubility and non-toxicity. Whereas poly (allylamine hydrochloride) (PAH) was used as the −electrochemically inert- polycationic species. The motivation behind this study was to assess whether these well-known films would experience, upon DNA hybridization, changes in terms of electrical/electrochemical phenomena that would arise specifically from the formation of a duplex DNA structure at the electrolyte/electrode interface. EIS was used to monitor such changes in label-free conditions. We propose this DNA-film interaction principle as a potential biosensing strategy.

2. Experimental

2.1. Chemicals and materials

Fluorine-doped tin oxide (FTO) coated glass with sheet resistivity 7 Ω/sq was purchased from Sigma-Aldrich. Copper phthalocyanine-3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CuPcTs), (3-aminopropyl)triethoxysilane (APTES), poly(allylamine hydrochloride) (PAH; average MW ~ 15,000), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]$), Phosphate Buffer Saline (PBS) and Trizma® base were also purchased from Sigma-Aldrich. All chemicals were of analytical grade and used without any further purification. Oligonucleotide sequences were synthesized by Invitrogen (Life Technologies, Brazil) and are listed in Table S1 in the supporting information. All experiments were performed using ultrapure water (Milli-Q® system, resistivity of 18.2 MΩ cm).

2.2. Procedure: film preparation, characterization and DNA sensing

FTO substrates (25 mm × 10 mm × 2 mm) were cleaned ultrasonically with acetone, isopropyl alcohol and ethanol subsequently, followed by a RCA-inspired treatment (H_2O_2:NH_4OH:H_2O 1:1:5 v/v at 70 °C for 15 min). The clean hydrophilic substrates were rinsed with water and dried under nitrogen flow, followed by silanization with APTES 2% in ethanol 95% for 1 h at room temperature (RT). Silanized substrates (FTO_{sil}) were rinsed with ethanol and water subsequently and dried out with N_2.

Aqueous solutions of PAH and CuPcTs (1 g L^{-1}) were prepared at pH 6.5 (±0.2) in an aqueous solution containing NaCl 10 mM. The CuPcTs/PAH film deposition was carried out on FTO_{sil} substrates. The multilayers were assembled by the alternating immersion of the substrate in the CuPcTs and PAH solutions for 10 min each at RT. After each deposition, the substrates were immersed in an aqueous washing solution (NaCl 10 mM) and subsequently dried under a gentle nitrogen flow. Film growth (1–5 bilayers) was monitored with UV–Vis spectroscopy (HitachiU-2001 Spectrophotometer, USA) by directly taking the spectrum at each deposition step. Atomic Force Microscopy (AFM) images (512 × 512 pixels) were recorded on bare, silanized and film-modified substrates with an SPM Multimode–Nanoscope III (Digital Instruments) at RT under tapping mode. Gwyddion® software was used for image analysis and to withdraw roughness values (Root Mean Square, RMS) and grain-size distribution. Film thickness was estimated after removal of the film using a razor blade tip and measuring height differences using the cross-sectional analysis tool [28,29]. The difference between the scratched and non-scratched regions in terms of average height of the profiles was taken as the approximate film thickness.

Gold-sputtered 5-bilayer films were characterized with Scanning Electron Microscopy (SEM) (Shimadzu SS-550 with Energy-Dispersive X-ray Spectroscopy, EDS, Oxford). Fourier-Transform Infrared (FTIR) spectroscopy (Thermo Nicolet 6700 Spectrophotometer with a resolution of 4 cm^{-1} in the transmittance mode) was also employed for film characterization.

Electrochemical characterization of the films was carried out using a three-electrode electrochemical cell with a 1 cm^2 platinum foil and Ag/AgCl electrode as counter and reference electrodes, respectively. Film-modified FTOs (1 cm^2) were used as working electrodes (WE). PBS 1x pH 7.4 was used as supporting electrolyte. Cyclic Voltammetry (CV) measurements were performed at a scan rate of 100 mV s^{-1} to assess inherent film electroactivity and to monitor film growth. EIS measurements were done within a frequency range of 10 KHz to 0.1 Hz, under 5 mV excitation at open-circuit potential using redox probe $Fe(CN)_6^{3-/4-}$ 1 mM.

A PGSTAT40 Autolab electrochemical system (Eco Chemie, Utrecht, Netherlands) equipped with PGSTAT-12 and GPES/FRA 4.9 software (Eco Chemie, Utrecht, Netherlands) was used for electrochemical experiments. Software NOVA 1.9 was also used for electrochemical data analysis and circuit fitting.

The films, namely FTO_{sil}(CuPcTs-PAH)$_{1-5}$, were prepared in batches and left at RT protected from light overnight for oxygen saturation [30], before DNA immobilization and hybridization. Electrostatic immobilization of capture probes (*ss*DNA) 1 μM (prepared in Tris-HCl 10 mM and NaCl 10 mM pH 7.4 ± 0.2) was carried out onto the last PAH layer by adding 40 μL on the WE area for 1 h at RT (protected from light). The DNA-modified electrodes were immersed in an aqueous washing solution (NaCl 10 mM) and let to air-dry. The resulting films were identified as FTO_{sil}(CuPcTs-PAH)$_{1-5}$-*ss*DNA. The same procedure applied for hybridization with the target sequences at a fixed concentration of 1 nM. For calibration purposes, different concentration levels at the pico-molar range were prepared and analyzed using the 5-bilayer films. Given the length difference between the probe and the target sequences, a partial double-stranded structure (*pds*DNA) was formed onto the films after hybridization, namely FTO_{sil}(CuPcTs-PAH)$_{1-5}$-*pds*DNA. EIS measurements were performed before and after hybridization and ΔR_{ct} values were withdrawn from the fitted equivalent circuits.

3. Results and discussion

In a preliminary study, different LbL assemblies for DNA label-free detection were compared using different anionic species of molecular/nanostructured nature, including CuPcTs (data not shown). All LbL assemblies were fabricated onto silanized FTO electrodes using PAH as polycationic species, as described in the Experimental section (nanomaterial synthesis/characterization not included). Absorbance data were collected to monitor film growth and non-linear behaviours were observed for all films excluding CuPcTs. CuPcTs, as will be discussed in the following sections, showed linear and highly reproducible film growth. Impedance data were also collected after film fabrication (for each bilayer) and after DNA immobilization/hybridization. CuPcTs-based films were the only ones showing a clear trend exhibiting significant impedance change after DNA hybridization. Thus, these preliminary findings led to the selection of CuPcTs as anionic species for electrode modification.

3.1. Substrate characterization

FTO electrodes were the primary choice for this study on the basis of their lower cost compared to other electrode materials (indium, gold), their relatively wide electrochemical window, together with their stability in different electrolytes, and finally, the fact that these surfaces are transparent readily enables direct optical characterization during surface modification.

Fig. 1 shows the LbL assembly prepared onto FTO substrates for DNA sensing. The film-modified electrodes portray nanometric structures that will be discussed in the following paragraphs.

The CuPcTs-PAH films were grown on amine-terminated silanized surfaces. Primary amine groups from APTES are protonated ($pK_a = 9.6$) in the pH conditions used for LbL assemblies [31]. The choice of an APTES-covered surface for film fabrication came from early experiments carried out in our group. We compared the electron transfer undergone by redox

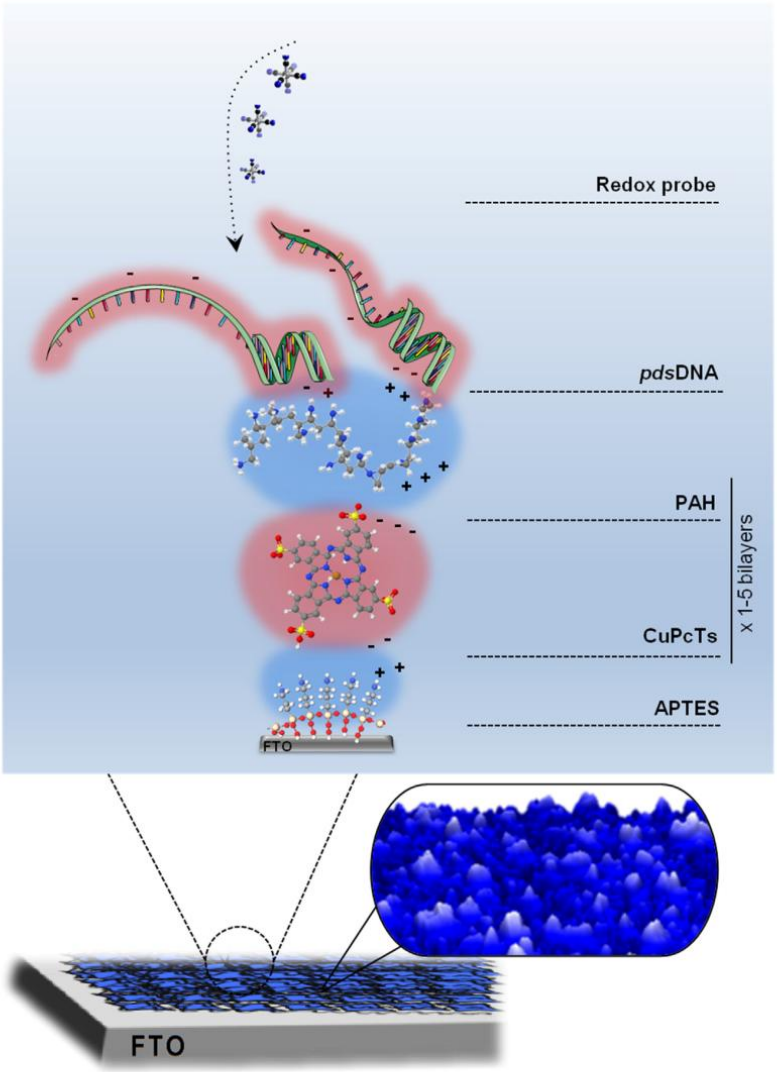

Fig. 1. Representation of the nanostructured LbL-assembly for DNA sensing. Structures are not to scale. Charge-screening effects by counter ions are not shown. For a better interpretation of this image, the reader is referred to the electronic version of this article.

probe $Fe(CN)_6^{3-/4-}$ onto bare FTOs, hydrophilic FTOs (after RCA-like treatment) and silanized FTOs (FTO_{sil}). Fig. S1-A shows a schematic representation of the three surfaces evaluated. AFM imaging of all substrates showed highly similar topographies/roughness. An AFM 3-D surface topography image of FTO_{sil} is shown in Fig. S1-B.

FTO_{sil} depicted less resistance to charge transfer, *i.e.* increased currents corresponding to the redox process of $Fe(CN)_6^{3-/4-}$ (Fig. S1C and D). Impedance spectra also showed that APTES-covered surfaces were seemingly more reproducible whereas the other two surfaces depicted less inter-substrate precision. SEM-EDS analysis showed a homogeneous distribution of Si on the FTO surface after silanization (Fig. S2), which could explain the high reproducibility obtained in impedance studies. The diffusion of $Fe(CN)_6^{3-/4-}$ was possibly favoured by the positively-charged nature of the surface as well [14].

Given the high roughness of all FTO-based surfaces tested here, a constant-phase element (CPE) had to be included in the equivalent circuits used to fit impedance data. CPE is known to account for non-ideal electrical contributions arising from surface heterogeneity [32].

3.2. Film growth and morphology

Roughness of all FTO substrates was found to be around ~28–30 nm, comparable to that found by Lamberti et al. (2013) [33]. Film growth was monitored via absorbance measurements, AFM and electrochemical techniques (to be discussed in the following section). Absorbance values of CuPcTs at Q-band 605 nm increased proportionally with the number of bilayers from 1 to 5, whereas roughness decreased with film growth (Fig. 2A and B). This type of films is known to grow in a linear fashion rather than an exponential one, suggesting that the same amount of material was adsorbed in each deposition cycle [34]. Linearly grown films are more stable than those that experience exponential growth [14] as they are better-packed films. Relative standard deviation (RSD) of absorbance measurements was 3% (n = 10), indicating the repeatability of the manual film-deposition process.

The fact that roughness decreased as the number of bilayers increased was expected. The first two bilayers tend to follow up the highly rough surface of the substrate, whereas from the third bilayer on, the film is expected to present a more packed, less rough configuration. Surface morphology studies using both AFM and

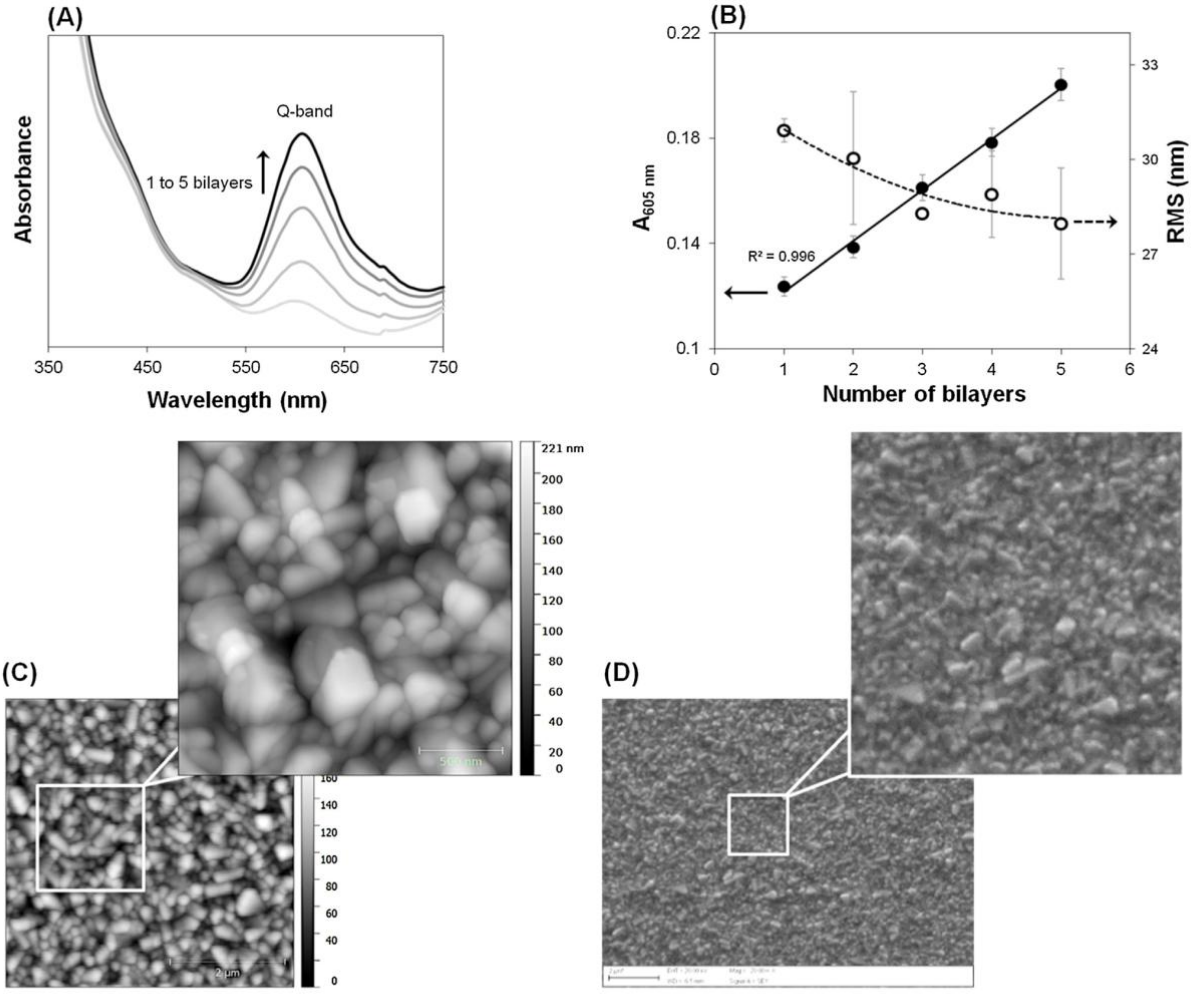

Fig. 2. Film growth: (A) absorbance spectra (B) absorbance values at maximum absorption wavelength 605 nm – phthalocyanine Q-Band (filled circles) and roughness values (empty circles) *versus* number of bilayers. Morphology of 5-bilayer films onto silanized FTO: (C) AFM images of 5 μm and 2 μm dimensions and (D) SEM images (15 × 11.3 μm and 2 × 2 μm).

SEM showed a grain-like structure in the film-modified electrodes (Fig. 2C and D). To see the concomitant evolution of grain size and roughness, grain-size distribution was analyzed from AFM images. The majority of the grains were within 30 and 50 nm in size (equivalent disc radius) (Fig. S3). Silanized FTO had a larger amount of these grains per image and, after film formation, the number of grains decreased to almost half in 5-bilayer films, correlating with the roughness decreased previously mentioned.

Although, for the most part, the electrodes comprise nanostructured grains, there are also scarce micro-populations of 100–300 nm in size. The grain-boundary regions showed 50 to 90 nm in-depth voids, indicating there is porosity in the system. Pores are not caused by faulty film deposition but rather originate from the substrate itself, as it was evidenced by AFM imaging (Fig. S1B). Consequently, the films followed the original roughness arrangement of the substrate, in a way that the film-modified electrodes portray an overall nanostructured arrangement with porosity and heterogeneous grain-size distribution. It is important to consider both the heterogeneity and porosity of the system for later comprehension of its electrical/electrochemical behavior.

Film thickness was estimated after scratching through the 5-bilayer film and comparing the average heights obtained with AFM between the bare and film-coated regions (Fig. S4). Results indicated that the 5-bilayer films were ~13–15 nm thick. Given the thin nature of the films and the highly rough profile of both regions, the estimated thickness here is an approximate value. Similar multilayer films have been reported to have a thickness of ~1.1 nm per bilayer, *i.e.* ~5.5 nm for 5-bilayer films [19]. Our higher values could be due to the use of low-to-moderate ionic strength in film fabrication instead of deionized water [35]. However, Fernandes et al. [36] reported an estimated thickness of *ca.* 3 nm per bilayer for a nickel tetrasulfonated phthalocyanine/poly (propylene imine) dendrimer LbL film (with the same linear growth), which is highly similar to our findings.

3.3. Electrochemical behaviour of film-modified electrodes

CV scans were recorded from −1 to +1 V for each film bilayer with the aim of subtracting mainly qualitative observations of film-inherent redox processes and of film growth. Irreversible reduction and oxidation peaks are evidenced at ~-0.6 V and ~0.5 V, respectively. The latter is assigned to the $PcTs^{-6}/PcTs^{-5}$ unit [37]. It is worth noting that internal metal redox processes affect strongly the ring-redox potentials, although central copper is known to be electroinactive. This means that Cu^{2+}-containing metalloporphyrins and phthalocyanines undergo only reactions involving the π-ring system [38]. The process observed at ~-0.6 V might be arising from Sn present in the electrode material (tin oxide), being reduced to a lower valence (possibly from Sn^{4+} to Sn^{2+}). This reduction seems to be facilitated by phthalocyanine films as the current increases in modified-FTOs. Sn reduction in FTO electrodes has been reported before at this potential as a reversible reaction in bare FTOs [39]. On the other hand, irreversibility has been evidenced with silanized FTOs in another report [40], sharing similarities with our findings. It is possible that silane groups are playing an important role in the irreversibility of this process.

Inherent electroactivity of the films was anticipated given the well-known biomimetic redox properties of phthalocyanines. Fig. 3A shows the electrochemical behaviour of silanized FTO and of the film-modified FTOs in the supporting electrolyte. Three processes stand out as film-specific and are indicated as ***a-a'***, ***b*** and ***c*** in the voltammograms. At approximately −0.85 V (***a***, ***a'***) and +0.85 V (***c***), the redox behaviour of phthalocyanine macrocycle is observed. These two processes are separated by the typical 2 V gap of phthalocyanines and porphyrins [38]. The first process exhibited quasi-reversible behaviour ($I_{pa} \neq I_{pc}$) with $\Delta E \sim 59$ mV for 1 and 2 bilayers, whereas from the third bilayer on, ΔE increases up to ~80 mV. Peak currents (***a-a'***) increased linearly with the number of bilayers, which correlates with the linear growth evidenced by absorbance measurements. Optical band gap energy of the LbL film was determined to be 1.82 ± 0.02 eV. This value is nearly equal to the HOMO-LUMO gap withdrawn from CV data. The results confirm a contribution to the electrical transport in the LbL film due to the overlap of π-orbitals of adjacent organic molecules [41].

Fig. 3B shows the voltammograms of the substrate and the film-modified electrodes in the presence of redox probe. From the third bilayer on, it becomes evident that the films are becoming less permeable to $Fe(CN)_6^{3-/4-}$ and finally after deposition of 5 bilayers, surface becomes almost totally impermeable to ferrocyanide ions. It has been reported that $Fe(CN)_6^{3-/4-}$ ions penetrate in exponentially grown films even when there are negative charges on the surface and this is because of the known permeability of these films [42,43]. Little or no permeability at all is expected for linearly grown films as they form highly packed structures, even

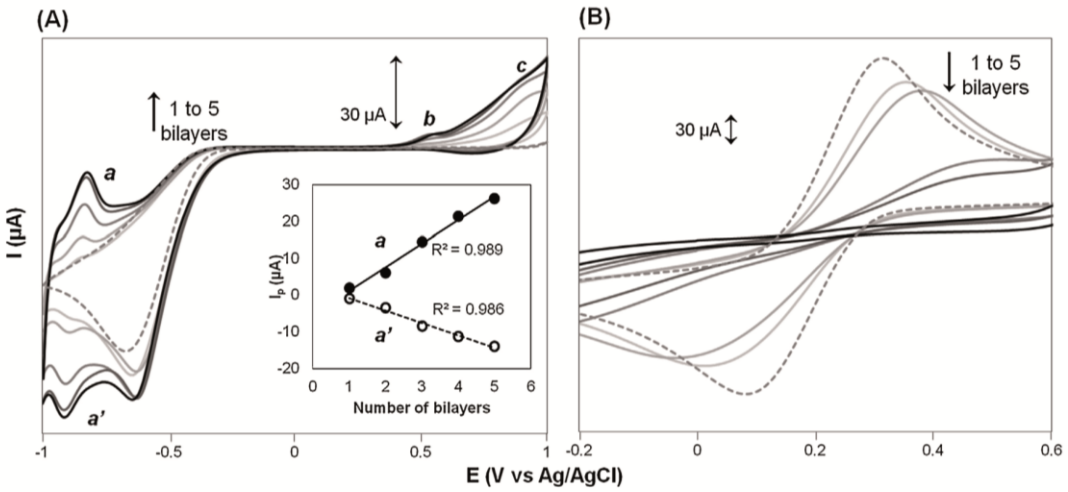

Fig. 3. Cyclic voltammograms of silanized FTO (dashed line) and film-modified electrodes (continuous lines) recorded at 100 mV s^{-1} (A) without redox probe $Fe(CN)_6^{3-/4-}$ (inlet: peak current of the redox process ***a-a'*** as a function of the number of bilayers) and (B) in the presence of redox probe $Fe(CN)_6^{3-/4-}$ (inlet: anodic peak current *versus* number of bilayers).

though CuPcTs/PAH films contain positive charges on the surface. This appears to be occurring after depositing 5 bilayers, given the significant current decrease of $Fe(CN)_6^{3-/4-}$ in these films. However, there is still current flow, although of considerable less magnitude, indicating that the redox probe might still be penetrating the films probably through pinholes [44].

Impedance measurements were done at OCP (0.19–0.24 V), in which none of the previously described film-related processes occur. Instead, processes associated with redox probe (diffusion and charge transfer) are comprised. Fig. 4 shows the different impedance responses of the film-modified electrodes at each bilayer number. Nyquist plots are shown in Fig. 4A, where a clear increase in charge transfer resistance (R_{ct}) is seen with film growth, as it is represented in Fig. 4B using fitted R_{ct} values. The Bode plot (Fig. 4C) shows an increase in angle maxima relative to the capacitive contribution of the films, ranging from −38.2 to −67.6° for 1 and 5 bilayers, respectively. Because each bilayer depicts unique electrical features, arising from differences in charge redistribution, dielectric constants of each coating and thickness, there was also an important shift in the characteristic frequencies of the RC component: ~38.8, ~31.6, ~7.9, 5, 3.2 Hz for 1-, 2-, 3-, 4- and 5-bilayer films, respectively. This again points to the 3rd bilayer as being the turning point in terms of electrical/electrochemical behaviour. Given these differences, we divided the data into three blocks (equivalent circuits are shown in Fig. 4D):

(1) **1 and 2 bilayers.** A Randles circuit was used to model the data of these films ($\chi^2 = 0.01$) (Fig. 4A-a). The contribution of diffusion impedance is evidenced in the Nyquist and Bode plots in the low-frequency region. Warburg impedance was chosen on the basis of bare electrodic regions exposed by incomplete film coverage, in which the redox probe could undergo semi-infinite diffusion.

(2) **3 and 4 bilayers.** These films deviate from the classical Randles behaviour. Redox probe diffusion can still be observed at the low-frequency region, but the behaviour diverges from that of the Warburg impedance. We believe the diffusion impedance might be the result of semi-infinite and finite-length coupled diffusions. The complexity of the system at this point is a result of the distinct electrical contributions arising from grain, grain-boundary, film-coated and bare regions, assuming surface coverage is below ideal. A modified-Randles circuit was used ($\chi^2 = 0.01$) in which an extra R-CPE in-parallel element was added to account for the contribution of the film-coated regions.

(3) **5 bilayers.** From previous CV experiments, we concluded that these films could be separated from the rest in terms of electrochemical behaviour: a well-packed configuration with minimum bare regions is assumed at this point and surface charge excess is thought to contribute to the hindered diffusion of the redox probe. The grainy feature of the films, together with porosity, drove us to fit the system with a transmission line model ($\chi^2 = 0.009$) [45], which takes into account the flux of ions within a pore. Thus, the contributions inside of the pores and in the outer-pore surface were modelled with a series of R-CPE elements in parallel, each corresponding to: impedance of the electrolyte within the pore (X_1), impedance

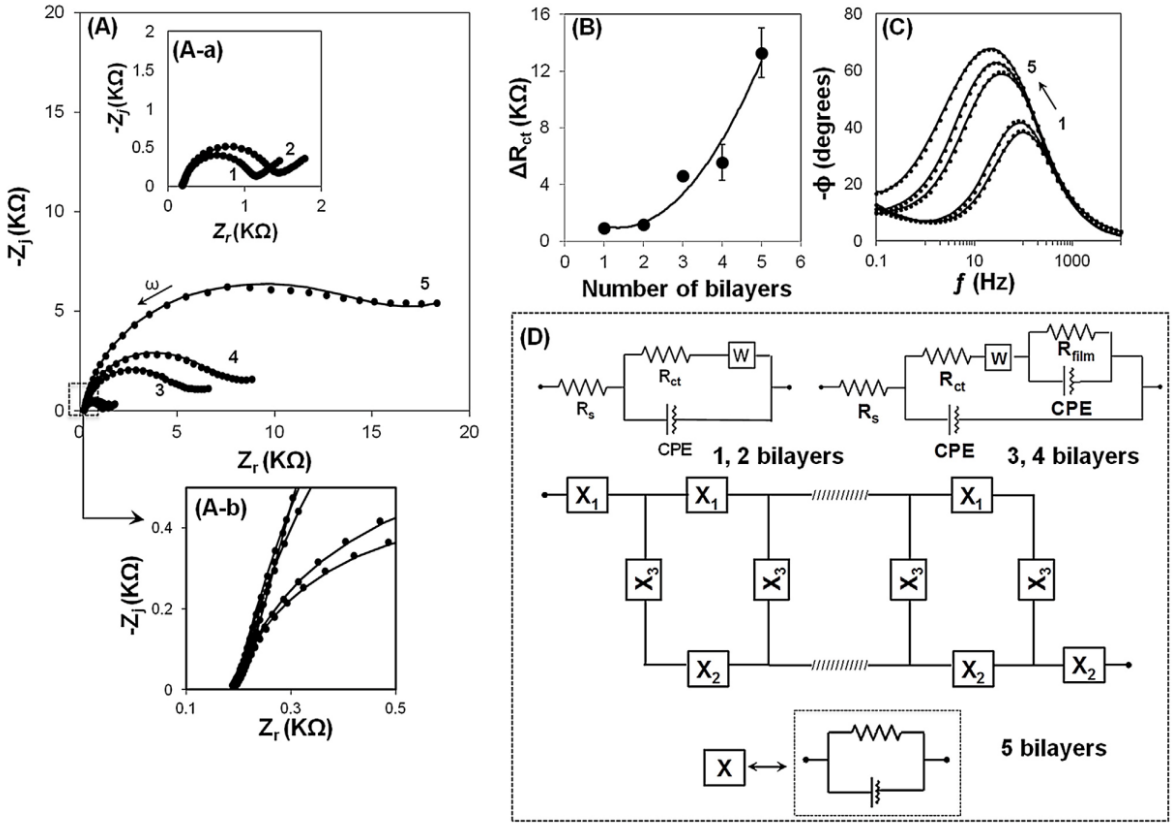

Fig. 4. Impedance spectra: (A) Nyquist plots showing impedance responses of the films from 1 to 5 bilayers (A-a: Nyquist plots of 1 and 2 films; A-b: amplification of the high-frequency region); (B) R_{ct} values versus number of bilayers; (C) Bode plots; (D) Equivalent circuits used for data fitting.

of the electrodic solid phase (X_2) and the impedance of the active surface (X_3) (see schematic representation in Fig. 4D). X_1 differs from bulk electrolyte resistance, thus an additional resistance element was added in series with the transmission line element (not shown in scheme). X_3 was taken as R_{ct}.

As we mentioned previously in the morphology section, a CPE element was used at all times because of the clear non-ideal behaviour of these electrodes. In Fig. 4A-b, the high-frequency region is amplified to show the depressed semi-circle, typically used as diagnostics of non-planar and non-ideal surfaces.

3.4. DNA sensing onto film-modified electrodes

As it would be expected considering the negatively charged nature of DNA, R_{ct} values increased after *ss*DNA immobilization (Fig. 5A–C). In silanized FTOs, ΔR_{ct} ($R_{ct(beforeimmobilization)} - R_{ct(afterimmobilization)}$) was near 0.6 KΩ whereas in film-modified electrodes the changes were 1.2, 2,4, 13.6, 26.3 and 38.2 KΩ (Fig. 5C), from 1 to 5 bilayers, respectively. DNA probes are expected to lay down flat in the surface given the strong interaction predicted between the phosphate backbone of DNA and protonated amines from PAH. This strategy has been used to immobilize DNA in a recent report [46] in which it was necessary to capture the DNA in a laying-down configuration to remain within the Debye length.

A growing distance between the electrode and the electrolyte-facing interface is expected with film growth, which together with immobilized DNA probes at the film surface, explains why the diffusion of the redox probe is hindered proportionally with the number of bilayers after immobilization. Interestingly, the opposite behaviour was obtained after hybridization: ΔR_{ct} values

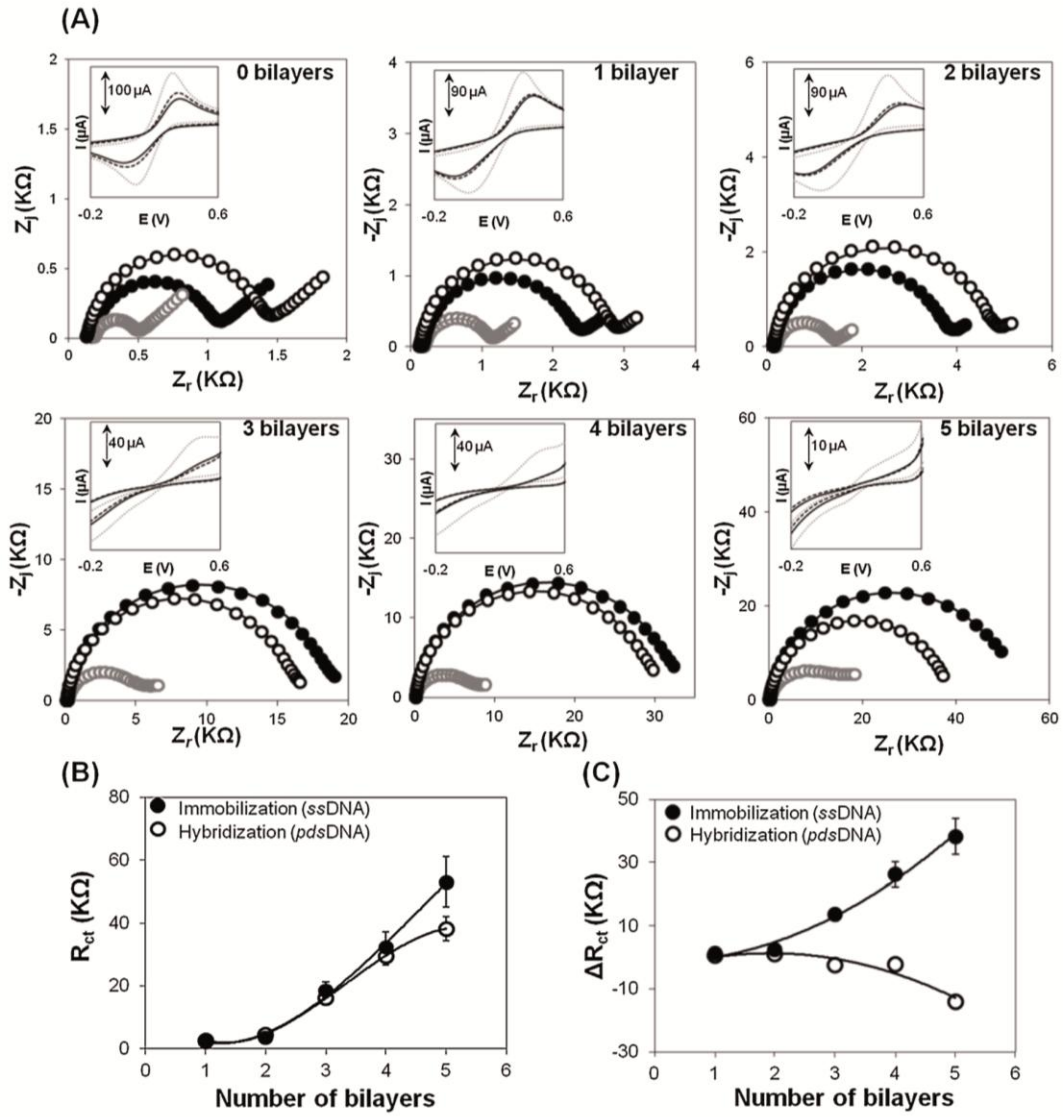

Fig. 5. EIS responses of DNA-film interaction: (A) Nyquist plots of the electrode surface (gray circles), after DNA immobilization (black-filled circles) and after DNA hybridization (white-filled circles). Randles-circuit was used to fit the data (lines). Inlets: Cyclic voltamograms of the surfaces (gray dashed line), after DNA immobilization (black dashed line) and after hybridization (continuous black line). (B) R_{ct} and (C) ΔR_{ct} values versus number of bilayers after DNA immobilization (filled circles) and hybridization (empty circles).

became negative from the 3rd bilayer on, *i.e.* impedance decreased after *pds*DNA formation. Average ΔR_{ct} values were 0.5, 1, −2.4, −2.2 and −14 KΩ for 1, 2, 3, 4 and 5 bilayers, respectively (Fig. 5C). We first considered the possibility of DNA being detached from the surface of the films during post-hybridization washes, possibly due to a charge excess at the film surface in 3-5 bilayers. FTIR (Fig. S5) spectroscopy was employed to assess the presence of DNA-related moieties on top of 5-bilayer films after hybridization. The amide I band (1600–1720 cm^{-1}) (red circle) was observed in the CuPcTs-PAH-DNA spectra, confirming the presence of DNA [47] onto these films. Confocal Microscopy also showed the presence of the duplex structures stained with a fluorescent intercalator (not shown). A partial detachment of DNA strands off the surface is not discarded and could explain the impedance drop, as it will be discussed later on.

Fig. 5 shows the impedance responses and cyclic voltammograms before and after DNA hybridization. CV scans (before and after hybridization) show hindered diffusion of the redox probe in 1- and 2-bilayer films with *pds*DNA. In 3-bilayer films, an increase in the current suggests more penetration of the redox probe through the films after hybridization. Yet in 4- and 5-bilayer films, the voltammograms barely showed any differences, while impedance decreased largely. An early diffusion-based hypothesis was ruled out, by which we believed that *pds*DNA formed a tunnel-like structure that facilitated the diffusion of the redox probe. Given that the data does not systematically point to this theory, other mechanisms have to be considered to explain this $-\Delta R_{ct}$ behaviour.

There are similar reports in the literature concerning EIS-based DNA sensors using material-modified electrodes. Reisberg et al. (2005) [48] was possibly the first to have reported the current-increase behaviour after DNA hybridization, namely "signal-on" response. In this study, they employed a conducting polymer with cation-exchange properties as electrode-modifying sensing interface. The signal-on behaviour was attributed to the possibility of DNA participating in ion-exchange mechanisms. In addition, it was thought that conformational changes (from single-stranded to double-stranded forms) during hybridization could induce a modification of the polymer/solution interface resulting in an increase of polymer electroactivity. On a separate report, Lien et al. (2010) [49], who worked with a multi-wall carbon nanotubes (MWCNTs)-doped polypyrrole sensing platform, ascribed the $-\Delta R_{ct}$ behaviour to an increase in the switching rate of the electronically conducting polymer when DNA hybridization took place at the vicinity of the polymer/solution interface. The mechanism behind this was similarly thought to be owed to DNA-mediated ionic transport to and across the polymer/solution interface.

More recently, Hai Le et al. (2015) [50] observed the same DNA-mediated impedance-decrease phenomenon using nanoporous SnO_2 films. They explained this decrease after hybridization by the hydrophilic character and conformational changes linked to double-stranded forms. The hydrophilic dsDNA could partially facilitate some ionic molecules of electrolyte to reach the electrode surface following their infiltration into the nanoporous structure. It was also argued that the electrode surface could be more "liberated" after hybridization due a more rigid/coiled dsDNA conformation. This unusual behaviour has also been explained elsewhere in terms of DNA intrinsic conductivity [51].

To further investigate these findings, Bonanni and Pumera in 2011 [10], who reported a graphene-based DNA sensor, performed chronocoulometry and fluorescence experiments in addition to impedance measurements. Chronocoulometry revealed that the total DNA density after hybridization with the complementary target was lower than that registered after DNA probe immobilization onto the electrode surface. This confirmed that some DNA probes were released from the electrode surface during hybridization. The same experiment performed after hybridization with the noncomplementary target led to a slight increase in DNA density on the electrode surface, indicating that not only were the DNA probes not released but also that some nonspecific interactions occurred, leading to a partial nonspecific adsorption. The authors also backed this hypothesis using dye-tagged DNA probes onto the electrode surface in a fluorescence study.

We believe the latter mechanism is more accurate to explain our findings. Although, this unusual behaviour of DNA onto phthalocyanine films requires further understanding. It is evident that additional research is needed to assess the nature of the processes behind this phenomenon and whether there are any semiconducting-related mechanisms associated.

Nevertheless, a semilogarithmic relationship (inversely proportional) was obtained between ΔR_{ct} values and target concentration in a narrow picomolar range (Fig. 6), which corresponds to femtomol-level considering the small volumes required. 5-bilayer films were chosen to perform calibration. A noncomplementary (NC) sequence was evaluated to assess the selectivity of the system. An impedance increase was obtained instead with NC, which might be due to unspecific adsorption of these strands onto the PAH layer [10].

Relative Standard Deviation (RSD) (n = 6) was 27% for probe immobilization. This elevated RSD value did not affect the final analytical performance (RSD of calibration points ≤ 15%) because every film-modified electrode was 'normalized' using its own $R_{ct\,(ssDNA)}$ value. However, because phthalocyanine films are very sensitive to ambient conditions, interday repeatability was occasionally compromised, which could also be normalized from the impedance response of the films alone on each day.

4. Conclusions

For the first time, phthalocyanine LbL films have been proposed as DNA sensing platforms in a label-free EIS-based method. This easy-to-fabricate approach, considering there is no need to pre-synthesize conductive nanomaterials or polymers in order to modify the electrode surface, represents a sensitive, fast and noncomplicated way to detect specific DNA sequences at femtomol-level. We used a nanostructured substrate to assemble

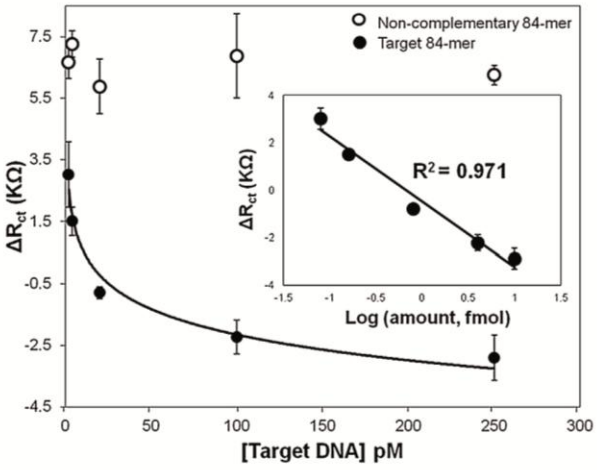

Fig. 6. Calibration curve (ΔR_{ct} versus target concentration) with semilogarithmic fit depicted in inlet figure (each point is the average value for 3 different measurements). EIS spectra were recorded within a frequency range of 10 KHz to 0.1 Hz, under 5 mV excitation at open-circuit potential using redox probe $Fe(CN)_6^{3-/4-}$ 1 mM in PBS 1x pH 7.4 as supporting electrolyte.

the films and characterized their electrochemical responses. A Transmission Line model was used to fit the data from 5-bilayer films, taking into account the porosity of the system. The impedance decrease after DNA hybridization allowed unequivocal sequence-specific detection, although the mechanisms implied require further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.electacta.2016.10.140.

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Supplementary material
Impedance sensing of DNA hybridization onto nanostructured phthalocyanine film-modified electrodes

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Table S1. Oligonucleotide sequences*.

Name/length	5' → 3'
Target sequence/84 bases	TTCATTCAAAATAAGATCATACATACAGGTTAAAATAAACATAGGGAA CCCAAATGGAAAAGGAAGGTGGCTCCTACAAATGCC
Capture probe/25 bases	GGCATTTGTAGGAGCCACCTTCCTT

*C.L. Manzanares-Palenzuela, *et al.* Biosens. Bioelectron. 68 (2015) 259-265.

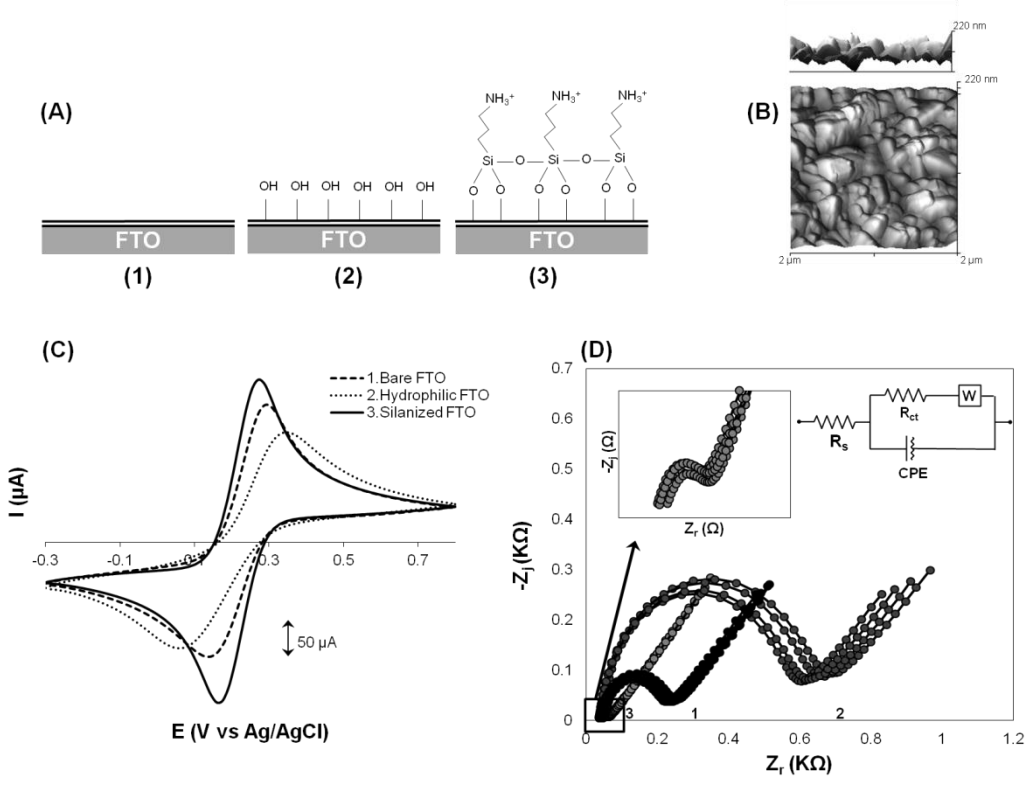

Fig. S1. Substrate characterization: (A) Schematic representation of the different surfaces; (B) AFM 3D image of silanized FTO showing roughness; (C) Cyclic voltammetries of the different FTO-based surfaces in the presence of redox probe; (D) Impedance spectra of different FTO-based surfaces (inlet: amplified spectra of silanized FTO) with their equivalent circuit.

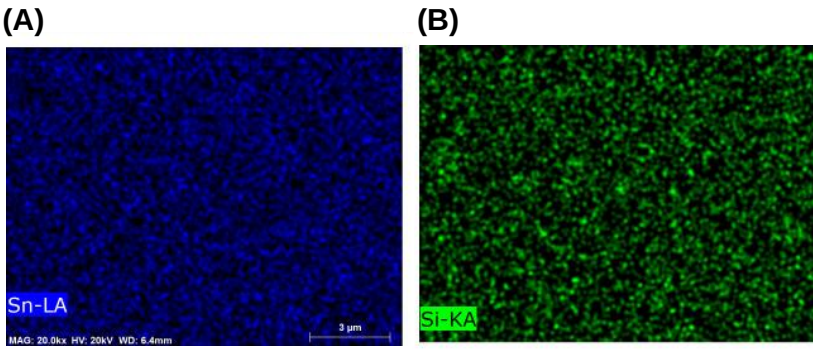

Fig. S2. SEM-EDS images showing distributions of (A) Sn and (B) Si onto the surface after silanization. For a better interpretation of this image, the reader is referred to the electronic version of this article

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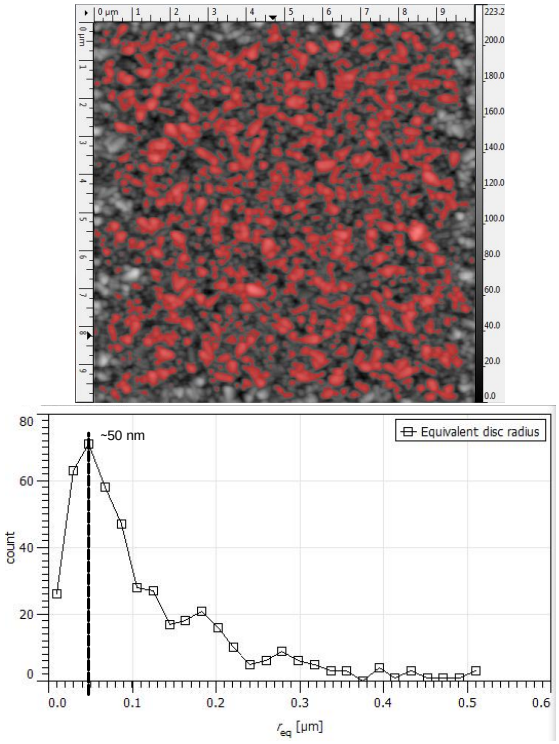

Fig. S3. Grain-size distribution of film-modified electrodes (5 bilayer) analyzed with Gwyddion software. For a better interpretation of this image, the reader is referred to the electronic version of this article.

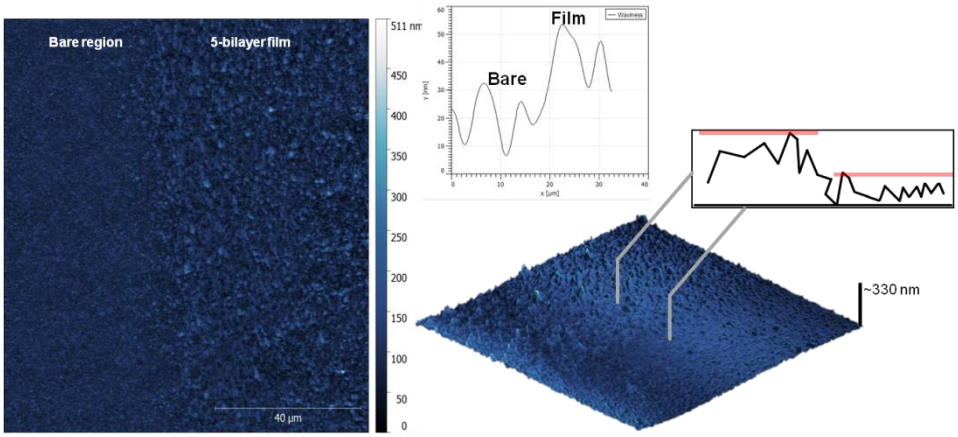

Fig. S4. Film thickness estimation based on AFM-derived average heights after cutting through the 5-bilayer films (image size: 100 µm). For a better interpretation of this image, the reader is referred to the electronic version of this article.

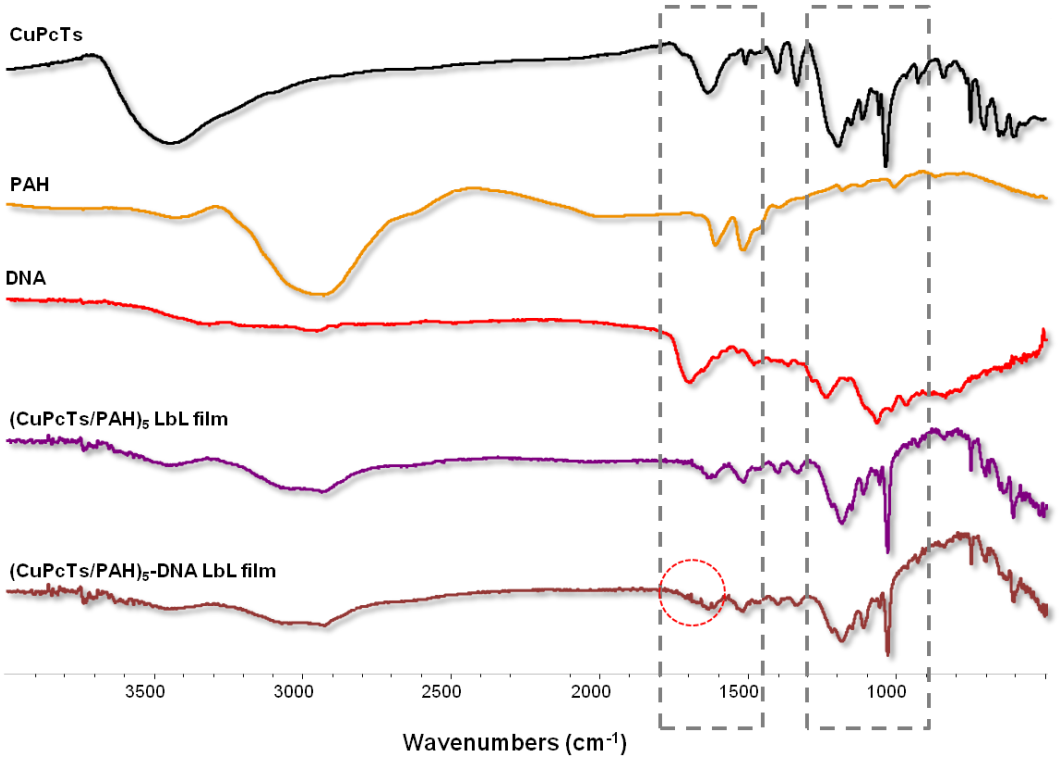

Fig. S5. FTIR spectra in the transmission mode of the different film constituents: CuPcTs, PAH and DNA casting on Si (111), for LbL films ((CuPsTs/PAH)$_5$ LbL film) and for LbL film after DNA hybridization ((CuPsTs/PAH)$_5$-DNA LbL film), fabricated onto silica wafer (111). The amount of DNA in the LbL film is very low, so that the difference in the film spectra is difficult to perceive.

| CHAPTER 8 |

CONCLUSIONS AND FUTURE PERSPECTIVES

8

CONCLUSIONS AND FUTURE PERSPECTIVES

After reviewing the electrochemical contributions made for DNA-based GMO detection (Chapter 2), it became clear that the main gap in the field was related to GMO quantification and method applicability. Another remaining challenge detected from the literature was the lack of truly simple and low-cost approaches for qualitative monitoring at the event-specific level.

Accordingly, the goals attained in this thesis involved (Chapter 3): quantitative determination of RRS; sample analysis -integration of the sensing platforms with DNA extraction and PCR amplification, maintaining the quantitative capacity of the method-; qualitative RRS determination with an easy-to-prepare, low-cost device.

In order to develop a new quantitative method for RRS, the following aspects were first considered (Chapter 4): targets must be short sequences in order to analyze processed samples where a high level of DNA degradation is expected; method sensitivity must be high given that the transgenic DNA level in a food/feed sample is expected to be low; the method should be highly specific for the target sequences even in the presence of similar genetic fragments. Magnetic beads were the eligible choice to achieve these analytical features because of their high superficial area, *i.e.* the amount of probes immobilized onto their surface is elevated, thus providing a large amount of recognition elements for efficient hybridization to occur with the analyte. This feature, together with the low background current generated with this type of assays –efficient magnetic separations-, highly contributes to the sensitivity required. The method was intended to be highly specific due to the double hybridization involved in the performed sandwich format.

Quantitative PCR coupling to an electrochemical platform had been a difficult task in the past due to the saturating nature of the amplification reaction. A pre-requisite for this is that the electrochemical method is sensitive enough (fM-pM in the case of GMO monitoring) to be able to tune the number of PCR cycles in the exponential-linear phase of amplification instead of stopping the reaction in the plateau phase, where quantification is more challenging or even impossible. In Chapter 5 quantitative PCR coupling was investigated and successfully achieved for the first time with the sensitive chronoamperometric platforms previously developed. As proof-of-concept, DNA derived from minimally processed samples –flours- was used to assess the quantitative approach. In Chapter 6, PCR was coupled with the multiplex platform. This time, DNA extracted from complex samples was used as template for electrochemical-based quantification, which had not been reported previously. A portion of the products containing RRS were found to not comply with the EU labeling regulation.

Finally, in Chapter 7, a simple, low-cost biosensor was developed for the label-free detection of *RR* sequences, complementing the portfolio of existing methods, with an important gain in simplicity and ease-of-fabrication.

8.1. Conclusions

The main conclusions obtained from this thesis were:

1. Short target sequences for the taxon-specific (*Lec*) and event-specific (*RR*) systems were selected from the ENGL database of GMO methods. The method was chosen based on the shortest possible amplicons given the expected level of DNA degradation in complex samples. Specificity of such amplicons was confirmed with the bioinformatic tool BLAST. Complementary probes were designed and checked for secondary structures in order to favor hybridization over self-annealing.

2. Two electrochemical DNA sensing platforms based on magnetic beads and enzymatic labeling (peroxidase) were developed for the separate chronoamperometric detection of *Lec* and *RR*. Several variables were optimized,

among which the amount of magnetic beads on the electrode surface was found to have the highest impact on sensitivity. Femtomolar-level detection was achieved for each target and calibration was accomplished spanning two orders of magnitude at the picomolar range.

3. A multiplex platform was designed and developed based on the simultaneous entrapment of the two target sequences onto magnetic beads, followed by bi-enzymatic labeling for the subsequent chronoamperometric and voltammetric detection of *Lec* and *RR*, respectively. A linear range covering two orders of magnitude at picomolar level was achieved. Relative RRS quantification, *i.e. RR*/*Lec* ratio, was addressed with synthetic mixtures of both targets in quantities around the threshold-labeling levels established in the EU.

4. An integral analytical method for relative RRS quantification in flour samples was developed comprising DNA extraction, amplification of the target sequences by end-point PCR at an optimized number of cycles, followed by hybridization/detection of the analytes with the separate chronoamperometric sensing platforms. Results were assessed against a qPCR based on Taqman® probes. Relative errors were found to comply with validation guidelines set for DNA-based methods.

5. An analytical method for relative RRS quantification in processed, commercial samples of food and feed was developed comprising DNA extraction, amplification of the target sequences by end-point PCR at an optimized number of cycles and simultaneous hybridization/subsequent detection of the analytes with the multiplex sensing platform. Quantitative results were assessed against a qPCR method based on SYBR Green® chemistry. The electrochemical method depicted superior specificity compared to qPCR and permitted to detect the unlabeled presence of RRS in 4 out of 33 samples, revealing that some products failed to comply with EU labeling regulations.

6. A qualitative biosensor based on impedance measurements was designed and developed for the label-free detection of *RR* sequences. An easy-to-prepare and low-cost phthalocyanine-modified nanostructured silanized FTO platform was carried out *via* the LbL technique. The films were characterized using AFM,

FTIR, EIS, CV, SEM and EDX techniques. Electrical modeling of such films was presented for the first time. Femtomol-level DNA detection was achieved on the principle of decreased impedance after hybridization.

8.2. Future perspectives

Despite the vast numbers of papers published, the field of biosensors applied in food control is still undergoing active research in pursuit of easy-to-use, portable devices for use by non-specialists for decentralized, in situ or on-field analysis.

The development of accurate devices for sequence-specific quantification remains one of the most demanding challenges of the field. When it comes to food safety and quality assessment, there is a significant number of situations where quantitative data are required over simple 'yes-or-no' results. The integration of the quantitative methods developed in this thesis into microfluidic platforms with isothermal amplification would constitute a major advance in the field with potential use at the industry-level or by official organisms of control. Electrochemical real-time amplicon monitoring has also been a promising technology for quantitative purposes in recent years and it could represent a valuable tool for GMO monitoring.

Nanotechnology brings wide-ranging possibilities for multi-target platforms. These are especially attractive for GMO monitoring given the increasing number of events being authorized worldwide each year.

Finally, the development of advanced methods for the detection of stacked events, unauthorized crops and cisgenic GMOs is one of the most important analytical challenges at the moment. These tasks are currently being addressed mostly with next generation sequencing technologies. Electrochemical DNA-based methods are expected to keep improving towards facing these new analytical challenges in upcoming years.