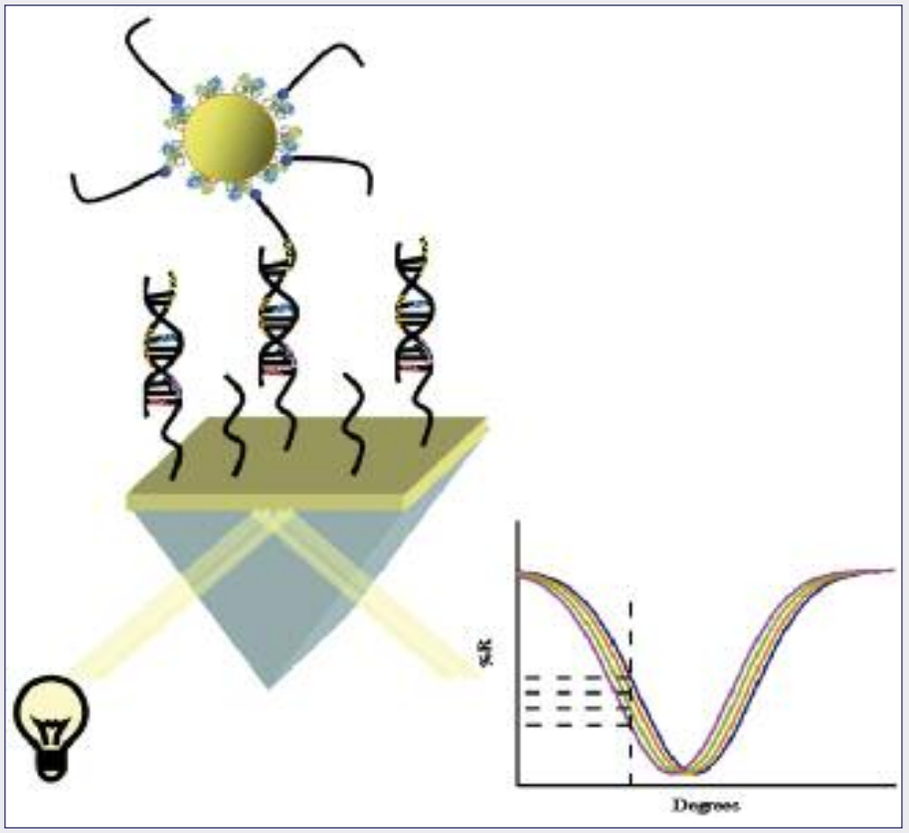


Roberta D'Agata[a], Roberto Corradini[b], Cristina Ferretti[c], Laura Maria Zanoli[d], Marcello Gatti[c], Rosangela Marchelli[b], Giuseppe Spoto[a,e]
[a]Dipartimento di Scienze Chimiche Università di Catania
[b]Dipartimento di Chimica Organica ed Industriale Università di Parma
[c]Neotron SpA S. Maria di Mugnano (MO)
[d]Scuola Superiore di Catania c/o Dipartimento di Scienze Chimiche Università di Catania
[e]Istituto Biostrutture e Bioimmagini-CNR Catania
gspoto@unict.it

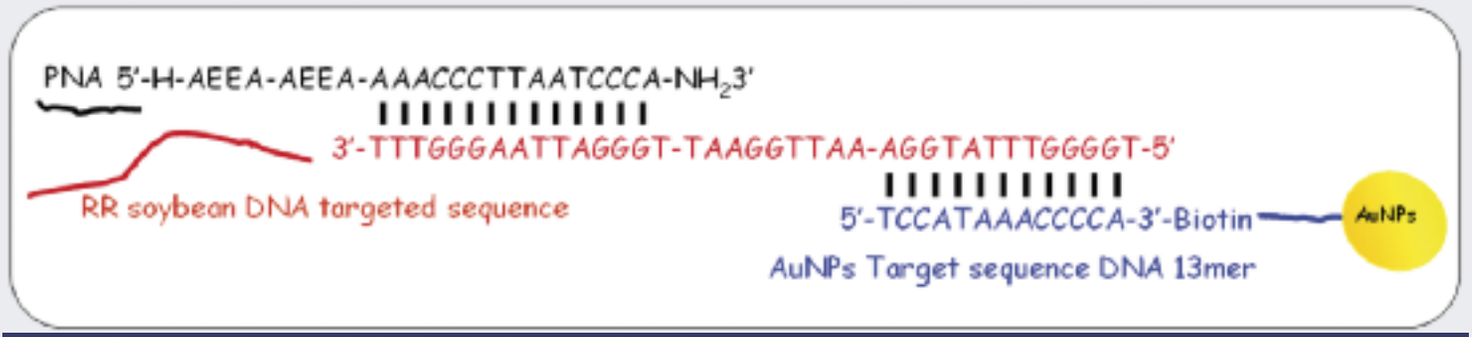
DETECTION OF NON AMPLIFIED GENOMIC DNA BY ADVANCED SPRI METHODS

Current methods for DNA detection are based on the combined use of labeled probes hybridized to target sequences which are amplified by polymerase chain reaction (PCR). New methods that eliminate the requirement for both PCR and labeling steps could afford faster, cheaper and simpler devices for the analysis of small amounts of unamplified DNA. The results we obtained in the ultrasensitive detection of non-amplified genomic DNA are here described.

The fascinating and complex field of DNA detection is a topic of current interest since rapid and highly sensitive DNA detection methods have a wide range of uses from gene expression profiling and DNA diagnostics to identification and traceability of food components. However, the development of new approaches for the detection of specific DNA sequences requires several issues to be addressed. In particular, since small amount of DNA is typically available for the analysis, a detection scheme capable of directly transducing the hybridization events with proper sensitivity is required. The sequence to be targeted is often present in a complex environment such as the whole genomic DNA. For this reason, very specific and highly efficient probes are needed in order to built highly selective detection tools.

The most widely adopted analytical techniques for rapid and multiplexed detection of DNA rely on real-time PCR (polymerase chain reaction) [1] and DNA microarray [2]. Both techniques require the PCR amplification of the DNA to be detected as well as the labeling of the target with a flu-

Questo articolo è ricavato dalla relazione che Roberta D'Agata ha tenuto all'Incontro di Spettroscopia Analitica (ISA 2010), che si è svolto a Rimini lo scorso giugno. La relatrice è stata invitata dal Comitato Scientifico del Convegno come vincitrice del Premio "Ambrogio Mazzucotelli", destinato a un giovane studioso la cui attività di ricerca nel campo della spettroscopia analitica appaia meritevole sia per l'originalità e qualità delle metodiche sia per la rilevanza dei risultati *(Marco Taddia, per il Comitato Scientifico di ISA 2010).*



Scheme 1 - Sequences and acronyms for the oligonucleotides used in this work

orophore, thus introducing steps in the detection procedure which are technically demanding, time-consuming, expensive and vulnerable to contamination and errors.

Innovative ultra-sensitive and label-free devices may avoid the PCR amplification step and can directly target the genomic DNA without any modification.

The need for a label-free detection scheme can be accomplished by using the surface plasmon resonance (SPR) biosensing. SPR is an optical technique used to monitor interactions between receptors immobilized on a metal surface and analytes which are in a solution in contact with the sensor surface [3].

SPR Imaging (SPRI) [4] has emerged as a versatile method for detecting the interaction of biomolecules in a microarray format [5-7]. It uses optical detectors to spatially monitor localized differences in the reflectivity of the incident light (Δ%R), which can be seen as brighter or darker regions in the SPR image, from an array of biomolecules linked to chemically modified gold surfaces. Label-free and real time analyses can be carried out with high-throughput and low sample consumption by coupling microfluidic devices with the SPRI apparatus [8-10].

However, the use of SPRI for genomic assays is limited by the reduced sensitivity in the detection of hybridized DNA samples. Thus, a number of different strategies aimed at amplifying the SPRI response to DNA and RNA hybridization have been recently investigated [11, 12], in particular using colloidal gold nanoparticles (AuNPs) [13-15].

Another aspect which deserves attention is the selectivity in the detection process of a specific sequence. High selectivity is the key issue for practical applications in clinical and food safety analysis of samples in complex and heterogeneous media, especially those involving the detection of mutations and single nucleotide polymorphisms (SNPs) [16-17].

Peptide nucleic acids [18] (PNAs) probes have been shown to be superior to oligonucleotides in targeting complementary DNA and RNA sequences, in terms of both selectivity and sensitivity. PNAs are DNA mimics in which the negatively phosphate deoxyribose backbone is replaced by a neutral *N*-(2-aminoethyl)glycine linkage. Due to their neutral backbone PNA probes offer hybridization characteristics and mismatch discrimination capabilities which make them superior to DNA probes [19]. PNA probes have been shown to contribute to the ultrasensitive nanoparticle-enhanced SPRI detection of oligonucleotide sequences down to 1 fM, maintaining a very high selectivity in the recognition of single nucleotide mismatch [20, 21].

A limited number of label-free applications able to detect non-amplified genomic DNA samples with pM-fM sensitivity have been so far described [22]. A multiplexed and label-free detection of non-amplified genomic DNA with zM sensitivity represents a fundamental step toward the identification of innovative genomic applications.

In this perspective, a new strategy for the detection of target sequences in non amplified genomic DNA samples extracted from genetically modified Roundup Ready Soybean is here described. The described approach was found to be able to detect the transgenic sequences as a minor component of DNA samples with different genetically modified (GM) mass fractions (from <0.03% to 5.00%). The method is able to detect non-amplified genomic DNA down to 70 zM (7.0×10^{-20} M) in concentration, even in the presence of a large excess of non-complementary DNA.

Functionalization of a modified gold surface with a PNA probe

Here we report on the possibility of using a 15-mer PNA sequence (PNA, Scheme 1) specifically designed to identify Roundup Ready genetically modified soybean sequence within of genomic samples [23].

Gold SPRI chips, previously functionalized with the dithiobis-(N-succinimidylpropionate) (DTPS) reagent, were used to immobilize the PNA

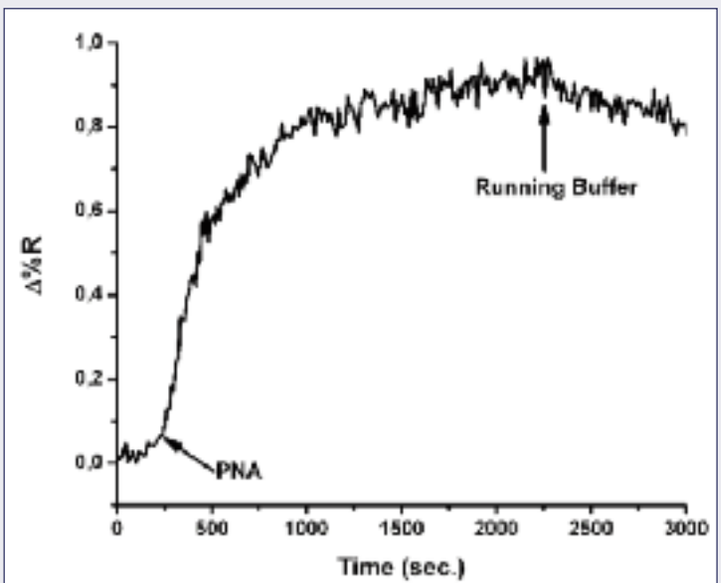


Fig. 1 - The changes in percent reflectivity over time obtained after the immobilization of the PNA probe (0.1 μM)

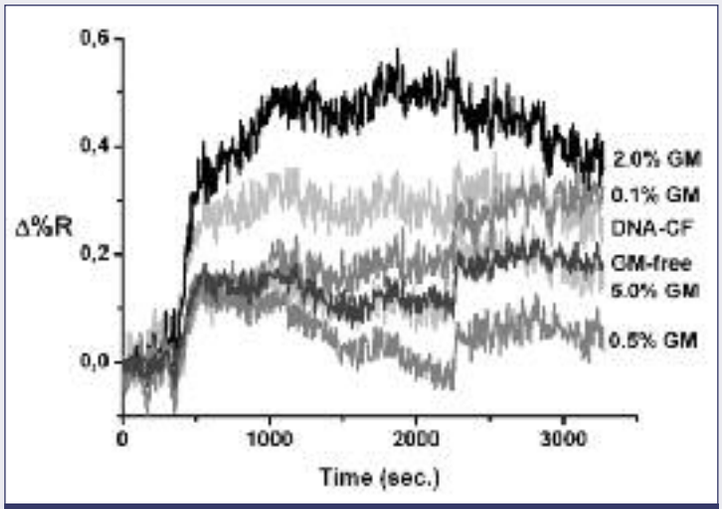


Fig. 2 - Change in percent reflectivity (Δ%R) over time obtained for the interaction between the surface immobilized PNA probe and the DNA targets (total DNA concentration 10 pg μL^{-1}). The SPRI response for the DNA-CF (calf thymus DNA, 10 pg μL^{-1}) adsorption is also shown

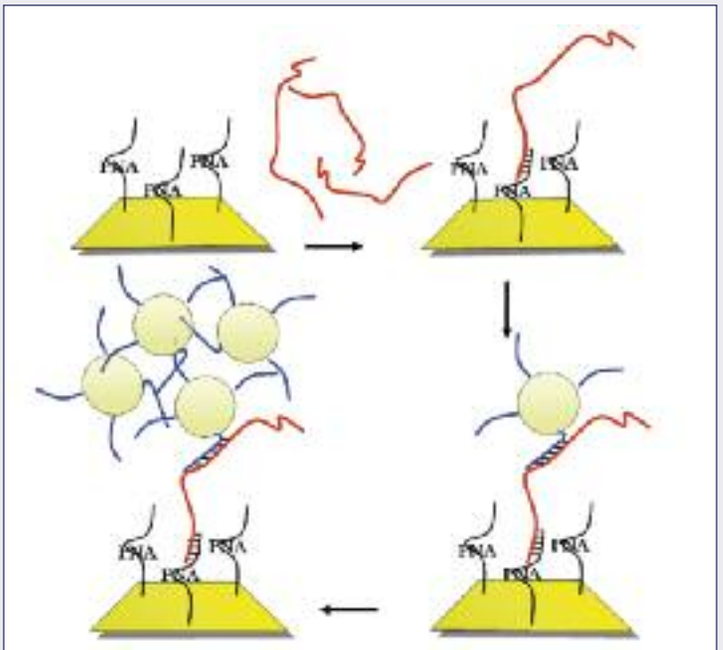
probe through a typical amine-coupling reaction with the N-terminal group of the spacer present at the 5'-position of the PNA. The probe immobilization was obtained by injecting the PNA solution (0.1 μM in PBS, flow rate 5μL min.$^{-1}$) (Fig. 1) into microchannels in contact with DTSP-modified gold surface. Details about the PDMS microfluidic devices fabrication through the well established replica molding technique are reported elsewhere [9].

The two 2-(2-aminoethoxy)ethoxyacetic acid (AEEA) spacers (Scheme 1) were introduced in order to maintain an appropriate distance between the PNA probe and the surface. The surface density of the probe molecules is an important parameter which affects the hybridization efficiency and specificity. Steric effects between adjacent probes and repulsions between the incoming targets prevail when high surface density values are obtained. A PNA surface coverage of $3x10^{12}$ molecules cm^{-2} was estimated in our case [24]. Similar values are reported to minimize both steric effects and electrostatic interactions between target molecules [25, 26].

Genomic DNA sample treatments

The genomic DNAs to be analyzed by SPRI were extracted from Roundup Ready™ soybean certified reference materials (ERM-BF410, Fluka) by using the IonForce kit (Generon, Italy) and quantified with a standard fluorescence-based method (Quant-iT™ PicoGreen®, Invitrogen). In details, the samples were dried powder from soybean containing respectively <0.3 g kg^{-1}, (<0.03%, "GM-free"), 1.0±0.5 g kg^{-1} (0.1±0.05%), 5.0±1.0 g kg^{-1} (0.50±0.10%), 20.0±2.6 g kg^{-1} (2.0±0.26%) and 50.0±5.3 g kg^{-1} (5.0±0.53%) of dried powder from genetically modified Roundup Ready™ soybean.

The mean concentrations of the 10-fold diluted extracted genomic DNA solutions in terms of total (GM and GM-free) genomic DNA were 1.161 (SD = 0.018) μg mL^{-1} (sample



Scheme 2 - Pictorial description of the strategy used for the ultrasensitive nanoparticle-enhanced SPRI detection of the genetically modified DNA sequence

<0.03%), 0.924 (SD = 0.013) μg mL^{-1} (sample 0.1%), 0.812 (SD = 0.145) μg mL^{-1} (sample 0.5%), 0.884 (SD = 0.123) μg mL^{-1} (sample 2%), and 1.027 (SD = 0.171) μg mL^{-1} (sample 5%) respectively.

Solutions for the SPRI detection were prepared by diluting the DNA samples with PBS buffer to a final concentration of 10 pg μL^{-1}. A proper genomic DNA fragmentation was required in order to facilitate the detection. Therefore, before SPRI analysis genomic DNA was fragmented by sonication (2 min.) and by vortexing (1 min.). Gel electrophoresis analyses were carried out in order to confirm the fragmentation of the genom-

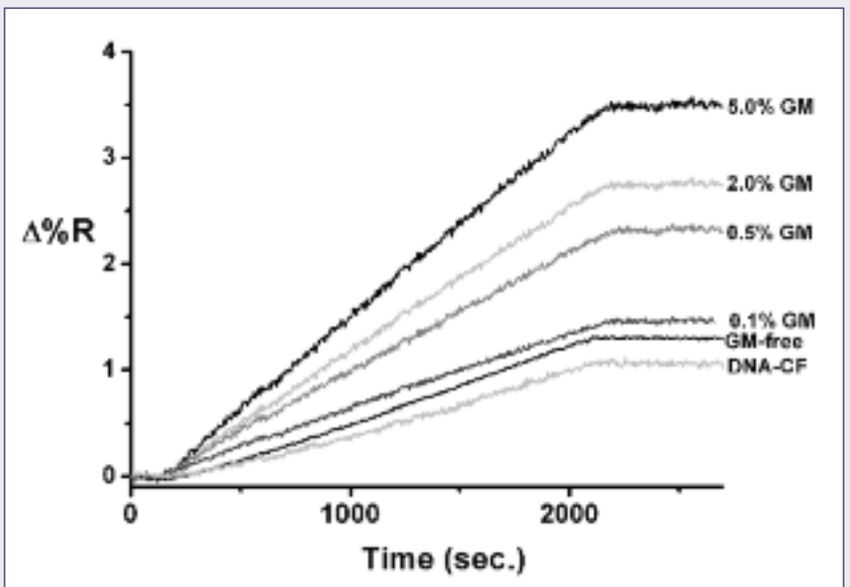


Fig. 3 - Time-dependent SPRI curves obtained after the adsorption of 13-mer-AuNPs on GM DNAs hybridized to the surface immobilized PNA probe and GM-free and DNA-CF adsorbed on surface immobilized PNA, using 10 pg μL^{-1} solutions of genomic DNA

ic DNA (fragments contained from hundreds to thousands of base-pairs). In addition, since the fragmented genomic DNA consisted of double stranded DNA sequences, its denaturation was obtained by heating at 95 °C for 5 min. before each SPRI analysis. The re-annealing was prevented by cooling on ice the samples (1 min.) before their introduction into the SPRI microfluidics apparatus.

The SPRI experiments were carried out by using two different negative control: the 10 pg μL^{-1} solution of the soybean genomic DNA carrying no GM sequence (<0.03%, "GM-free") and a solution of genomic DNA from calf thymus (DNA-CF, 10 pg μL^{-1}).

Direct SPRI detection of genomic DNA

The ultrasensitive SPRI detection was obtained by adopting the protocol depicted in Scheme 2.

The direct adsorption of the four GM DNA solutions (0.1±0.05%, 0.50±0.10%, 2.0±0.26%, 5.0±0.53%), the GM-free DNA solution (<0.03%) and the DNA-CF solution (450 μL of each solution, flow rate 15 μL $min.^{-1}$) on the PNA functionalized surface did not generate SPRI responses useful for samples discrimination. Fig. 2 shows a representative change in percent reflectivity (Δ%R) over time obtained for the absorption of the above mentioned 10 pg μL^{-1} solutions on the PNA functionalized surface. The DNA absorption generated SPRI signals which cannot be attributed to any specific or non-specific interaction involving the genomic DNA. In fact, the detected SPRI signals were closed to the instrumental noise and affected by temperature fluctuations. Because of the extremely high complexity of genomic DNA, it is expected likely that also a low number of non-target sequences were able non-specifically adsorb on the SPRI chip functionalized surface. The following washing step eliminated most of the excess DNA present on the surface preferentially leaving the captured DNA under the form of a single strand.

Indirect SPRI detection of genomic DNA by modified gold nanoparticles

The ultrasensitive detection of the non amplified genomic DNA and the discrimination between the genomic DNA samples with different GM content was achieved by using AuNPs previously conjugated to a 13-mer oligonucleotide (the sequences of the oligonucleotides and their acronyms used in this work are shown in Scheme 1) complementary to a tract of the target DNA not involved in the hybridization with the PNA probe.

The 13-mer modified gold nanoparticles (13-mer-AuNPs) were obtained according to protocols previously described.[27] Fig. 3 shows some SPRI representative responses (change in percent reflectivity (Δ%R) over time) obtained after the adsorption of 13-mer-AuNPs on SPRI chip regions previously treated with 10 pg μL^{-1} solutions of the non-amplified GM DNAs (0.10%, 0.50%, 2.00% and 5.00% GM soy content), GM-free and DNA-CF. The described approach produced significant increases of the SPRI signals for the GM-positive samples with the 0.50%, 2.00% and 5.00% GM soybean content. In particular, a response increasing as a function of the GM percentage was obtained. The 0.1% GM DNA, the GM-free DNA and the genomic DNA-CF, generated SPRI responses which might be attributed to the non specific adsorption of DNA on the functionalized gold surface.

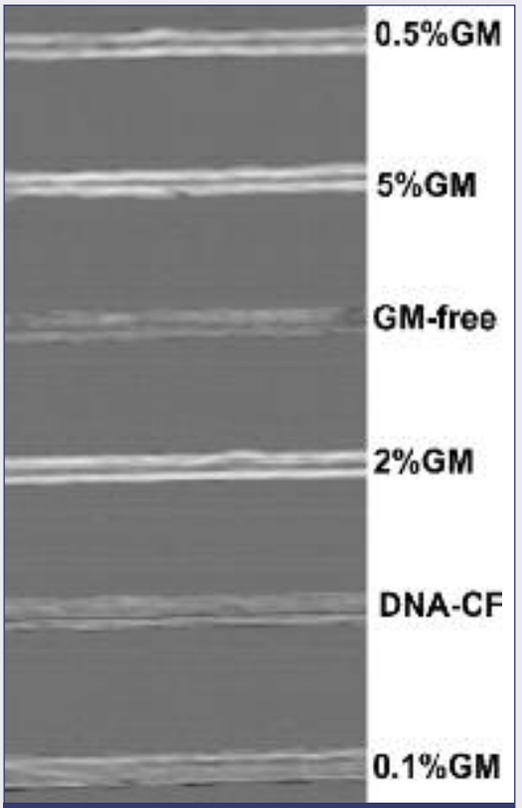


Fig. 4 - Representative SPR difference image showing the parallel detection of the SPRI responses described above

Replicated experiments (Tab. 1) showed that the Δ%R caused by the nanoparticle-enhanced SPRI detection of the 5.00% GM DNA, 2.00% GM DNA and 0.50% GM DNA samples were different from one another (t-test, level 95%, p-value<0.05) and different from the Δ%R caused by the 0.10% GM DNA. The last Δ%R value, instead, was not significantly different from that caused by the GM-free DNA.

Fig. 4 shows a representative SPR difference image obtained after the nanoparticle-enhanced SPRI detection of the non-amplified DNAs.

It is useful to highlight that a molecular weight of about $7.3x10^{11}$ can be estimated for the soybean genome (genome size about 1.1 Gb) [28-30] and that only one copy of the transgene sequence is present in the Roundup Ready™ soybean genome [31].

On account of this, we conclude that the described method can selectively identify the GM target sequence down to a 70 zM concentration (calculated for the 0.50% GM DNA) in solutions containing a total of GM and of GM-free genomic DNA at a 14 aM concentration (10 pg μL^{-1}).

Several factors contribute to the observed SPRI signal enhancement: i)

Tab. 1 - Total DNA concentration 10 pg μL^{-1}

Sample	Mean Δ%R(x)	SD[a]	CI[b](95%)	n[c]
DNA-CF	0,78	0,43	x±0,40	7
GM-free	0,79	0,45	x±0,47	6
%	1,06	0,46	x±0,57	5
0,5%	1,93	0,22	x±0,36	4
2,0%	2,48	0,37	x±0,38	6
5,0%	3,39	0,34	x±0,42	5

a) SD=standard deviation; b) CI=Confidence interval, c) n=number of samples

the specific properties of the PNA probes, ii) the functionalized gold nanoparticles and iii) the specific architecture of the SPRI interface obtained after the immobilization of the large genomic DNA fragments.
The selection of an appropriate conjugated AuNPs concentration is critical for the success of the nanoparticle-enhanced SPRI experiments. The described experiments were carried out by using conjugated AuNPs solutions that were more diluted than those used to detect oligonucleotides instead of genomic DNA. Such evidence allow us to hypothesize the following processes to generate the signal enhancements: i) the PNA probes contribute to selectively and efficiently capture a few single-stranded DNA fragments carrying the target sequences. The captured GM DNAs create a negatively charged layer highly rich in nucleobase residues and polar groups, able to establish weak interactions with the conjugated AuNPs; ii) the alteration of the charge and force balancing the stabilized conjugated AuNPs suspensions, lead to the surface aggregation of the AuNPs. The second hybridization, accomplished by the complementary oligonucleotide on nanoparticle modified, is the event triggering a specific adsorption event, followed by an AuNPs deposition process necessary to produce an increased positive signal. The initial non-cross linking aggregation of AuNPs constitutes a nucleation point around which other conjugated AuNPs aggregates. The different number of starting nucleation points around which aggregation occurs, provides the discrimination power to distinguish targets from non-targets and in the process allows the detection of different GMO contents.

Real-time PCR quantification of genetically modified soybean DNA

In order to compare the real time PCR and nanoparticle-enhanced SPRI sensitivity in detecting genetically modified genomic DNA, real-time PCR quantification of the GMDNA solutions was carried out. The real-time PCR analyses were carried out by using the same solutions of the extracted DNA samples analysed by SPRI. The experiments were carried out by following a validated protocol elsewhere described in detail [32]. Triplicate experiments were carried out from each analyzed sample. Negative control experiments were obtained by using the above described solutions with no template DNA. The average C_T values obtained are shown in Tab. 2. The experiments showed that no amplification of the 0.1% GM DNA sample was obtained from the Roundup Ready soybean solutions with a total DNA concentration of 0.1 ng μL^{-1}.
The correlation existing between the real-time PCR data obtained by analyzing 1 ng μL^{-1} GM DNA
solutions and the data obtained by analyzing 10 pg μL^{-1} GM DNA solutions by using the described nanoparticle-enhanced SPRI method is shown in Fig. 5. In both cases the response allows to discriminate the different levels of GM DNA. However, it should be considered that the concentration of the DNA sample used in the real-time measurement are 100-fold higher than those used for the SPRI analysis. Since both techniques give rise to a non-linear response as a function of analyte concentration, which is logarithmic in the case of real-time PCR, and saturative (Langmuir-type) in the case of SPRI, an empirical linearity is observed in the plot in Fig. 5 (R^2 = 0.9371). Thus, with higher control on the experimental conditions, which could lead to smaller standard deviations, the SPRI method could outperform the real-time PCR, providing parallel results with less material and much simpler sample treatment.

Tab. 2 - Mean CT values obtained from replicated real-time PCR experiments (n=3)

Total genomic DNA concentration				
GM%	10 ng μL^{-1}	1 ng μL^{-1}	0,5 ng μL^{-1}	0,1 ng μL^{-1}
5,0	28,5	31,7	32,3	35,2
2	29,6	32,9	34,0	36,3
0,5	31,9	34,9	36	38,6
0,1	34	384	39,3	40,1[b]
0,01	38	39	38,8[b]	

[a]Threshold ΔRn=0.2
[b]Value obtained from a single experiment. Replicated experiments failed in amplifying the DNA

Conclusions

We have shown that an ultrasensitive detection of non-amplified genomic DNA solutions containing a target sequence as a minor component is obtained by using PNA probes and a nanoparticle-enhanced SPRI detection. The described method can selectively identify the GM target sequence down to a zM concentrations in solutions containing a total of GM and GM-free genomic DNA aM in concentration (10 pg μL^{-1}).
The results obtained with certified samples and the very low limit of detection (0.10% of GM content) parallel some of the performances of the real-time PCR method which is presently the golden standard for

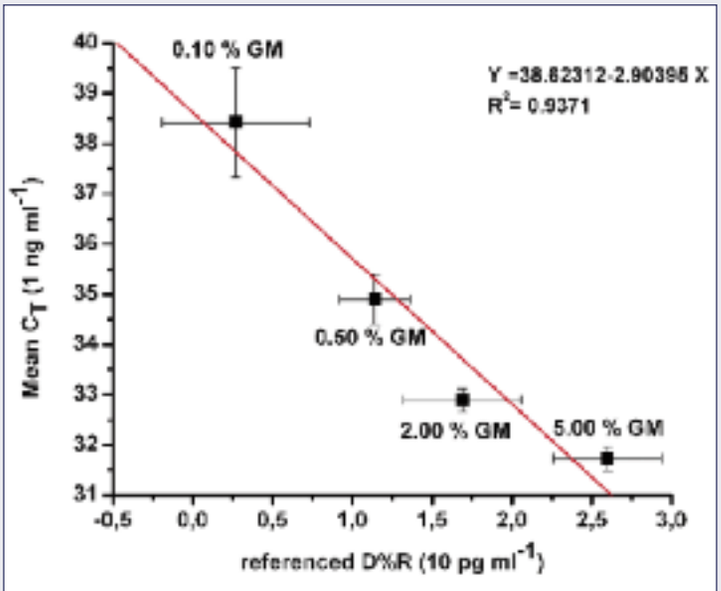


Fig. 5 - The plot shows the correlation existing between the mean C_T values obtained after the real-time PCR analysis of 1 ng μL^{-1} GM DNA samples and the referenced Δ%R values obtained with the nanoparticle-enhanced SPRI method from 10 pg μL^{-1} solutions. The referenced Δ%R value is the difference between the mean Δ%R value and the negative control value

DNA quantification. If one considers that the legal limit for labeling adventitious contamination of food and feed with GM soybean in the European Union is 0.9%, the present method allows to reach high and sound sensitivity levels with no need of PCR amplification.

From a more general point of view, the present results allow to propose that the nanoparticle-enhanced SPRI method in combination with microfluidics can provide an efficient tool for the PCR-free and multiplexed detection of DNA when a very low sample size is available, by using a very simple protocol, thus avoiding enzymatic treatment or complex thermal cycling. On of the most striking features of the system herein described, which is due to the use of very specific PNA probes, is the possibility to trace very low amount of the target modified DNA (0.5%) in the presence of an overwhelming excess of wild-type DNA (99.5%), thus suggesting the possibility to trace small amounts of rare sequences in a large population, a property which can be very useful in biomedical diagnostics.

Finally, the possibility to perform direct read out of the presence of target DNA without amplification could be very useful in the development of diagnostic methods for limited-resource settings, in which the availability of biochemical components (such as DNA polymerases) and their storage are very important limitations [33-34].

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RIASSUNTO

Rivelazione di DNA genomico non amplificato mediante metodi avanzati di SPRI

I metodi attualmente disponibili per la rivelazione del DNA sono basati sull'uso combinato di sonde marcate e ibridate a sequenze target amplificate mediante la reazione a catena della polimerasi (PCR). Metodi innovativi che non richiedono l'esigenza di effettuare sia la PCR che la marcatura potrebbero fornire sistemi più veloci, semplici ed economici per l'analisi di piccole quantità di DNA non amplificato. In questo lavoro sono descritti i risultati ottenuti nella rivelazione ultrasensibile di DNA genomico non amplificato. È stato analizzato del materiale certificato di riferimento contenente quantità differenti di DNA geneticamente modificato utilizzando un protocollo di rivelazione che accoppia la tecnica Surface Plasmon Resonance Imaging (SPRI) all'uso di sonde di acidi peptido nucleici (PNA) e di nanoparticelle di oro opportunamente funzionalizzate. Le sonde di PNA aumentano la selettività e la specificità nel riconoscimento di sequenze di DNA complementari, mentre le nanoparticelle di oro funzionalizzate amplificano il segnale SPRI. Il metodo permette di ottenere una sensibilità di 70 zM nella rivelazione di DNA genomico anche in presenza di un largo eccesso di DNA non complementare.