Full Research Paper

Piezoelectric Sensor for Determination of Genetically Modified Soybean Roundup Ready® in Samples not Amplified by PCR

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Abstract: The chemically modified piezoelectrodes were utilized to develop relatively cheap and easy to use biosensor for determination of genetically modified Roundup Ready soybean (RR soybean). The biosensor relies on the immobilization onto gold piezoelectrodes of the 21-mer single stranded oligonucleotide (probes) related to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, which is an active component of an insert integrated into RR soybean genome. The hybridization reaction between the probe and the target complementary sequence in solution was monitored. The system was optimized using synthetic oligonucleotides, which were applied for *EPSPS* gene detection in DNA samples extracted from animal feed containing 30% RR soybean amplified by the PCR and nonamplified by PCR. The detection limit for genomic DNA was in the range of $4.7 \cdot 10^5$ numbers of genom copies contained *EPSPS* gene in the QCM cell. The properties such as sensitivity and selectivity of piezoelectric senor presented here indicated that it

could be applied for the direct determination of genetically modified RR soybean in the samples non-amplified by PCR.

Keywords: Piezoelectrodes, Roundup Ready soybean, Genomic DNA.

1. Introduction

Nowadays several species of genetically modified plants are used in the production of food and feed (two main species are soybean and maize). EU and Polish legislation (GMO Law from 21 June 2001) imposes a duty to control them by qualitative and quantitative assays. Presently, in EU, labeling is mandatory for foodstuff containing transgenic material in percentage higher than 0.9 %. This implies the need for the availability of analytical methods for rapid semi-quantitative/quantitative GMO detection.

Presently the main assay used for GMO detection is the Polymerase Chain Reaction (PCR) [1-6], and for its quantification is Real-Time PCR [7-9]. Both these methods are costly, require very expensive equipment, reagents and highly experienced personnel. Therefore the duty to monitor the presence of GMOs on the market becomes a serious economical problem.

Relatively cheap and easy to use biosensors based on DNA hybridization might become a very good alternative [10]. Recently, the increasing effort is devoted to develop the electrochemical DNA sensors. Some of these sensors exploit intrinsic electroactivity of the nucleic acids [11-14]. In others, the intercalation of electroactive components into double stranded DNA was used [15-20]. The fact that bonded high molecular weight DNA electrostatically repels or attracts, negatively or positively charged redox markers, was used in DNA electrochemical sensors by Umezawa group [21-25]. The electrochemical impedance spectroscopy also belongs to useful techniques applied for the DNA detection [26, 27].

Some trials have already been undertaken to construct biosensors specific to GMO plant material present on the market, e.g. surface plasmon resonance for quantification of maize BT-176 [28] or disposable electrochemical genosensor to detect NOS (nopaline synthase) terminator [19]. In spite of successful outcome of these trials none of the proposed methods avoided PCR amplification of sequences under investigation, which was necessary due to insufficient sensitivity of the method.

Here we propose application of another class of sensors based on quartz crystal microbalance (QCM) electrodes. The basis of this technique is the relationship between resonance oscillation frequency of piezoelectric crystal and interfacial mass change, described by Sauerbrey equation [29]:

$$\Delta f = -a \, \Delta m$$

where: Δf – frequency change, Δm – mass change , a – proportionality factor

In the QCM technique electrodes consisting of piezoelectric crystal covered by gold are used. Golden surface may be modified by thiols and thiol-related compounds. The layer of these molecules creates the environment in which the bio-molecules such as oligonucleotides, proteins or hormones can be immobilized.

The QCM electrodes are able to operate not only in gas phase, but in fluids as well. This feature offers the possibility to characterize biomolecular systems in their natural aqueous environment [30].

Several successful applications of this method have been already reported for the detection of: herbicide [31], genetic diseases or gene mutations [32, 33], conformational changes of proteins - for example bovine serum albumin [34]. The mass piezoelectric sensors have also been applied for DNA detection [35-37]. Such sensors allow to detect the DNA without the use of labeled probes such as radioisotope like ^{32}P or fluorescent tags.

Among variety of protein immobilization methods, amine coupling is frequently used for covalent attachment via carboxyl-group-containing surfaces [38]. The primary amino groups of lysine residues localized on the surface of avidin are involved in the amide bond formation with carboxyl groups immobilized on the surface of the electrode activated with 1-ethyl-3(3-dimethyl aminopropyl)carbodi-imide hydrochloride / N-hydroxysuccinimide (EDC/NHS) reagents. The role of these reagents is formation of O-acylurea intermediates and NHS esters that promote the formation of amide bonds with the proteins amino groups [39]. This chemistry is sensitive to the temperature and time of exposure. Thus, in the presented method for covalent immobilization of avidin 3,3'-dithiodipropionic acid di (N-succinimidyl ester) (DSP) was used. This made it possible to avoid activating carboxyl groups immobilized on the electrode surface with EDC/NHS reagents. The DSP was also used for immobilization of laccase and dopamine [40].

Glycosylated, positively charged molecule of avidin binds up to four molecules of D-biotin, in a non-covalent interaction [41]. Avidin consists of four identical subunits assembled in a quaternary structure. The biotin binding sites are deep, pear shaped pockets [41]. Thus, for biosensor design, immobilization of avidin molecules on the surface of electrodes retaining their correct orientation and density is crucial.

Therefore, in the present work, avidin-biotin system was used for immobilization of the 21-mer single-stranded deoxyoligonucleotide probes designed for the detection of the Roundup Ready soybean. The probes were related to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene which renders plants' resistance against herbicide glyphosate (Roundup Ready). Glyphosate is toxic for plants because it prevents the production of aromatic aminoacids (tryptophan, tyrosine, phenyloalanine).

The optimization of modification of QCM electrodes (concentration of the modification solution and modification time), conditions of hybridization to immobilized probes and sensor regeneration are presented.

2. Materials and Methods

2.1. Reagents

Avidin, ethanolamine, N-[2-hydroxyethyl]piperazine – N'-[2-ethanesulfonic acid] (HEPES), 6-mercapto-1-hexanol and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Poznań, Poland). Sodium chloride, sodium hydroxide, magnesium chloride hexahydrate, hydrogen peroxide, ethyl alcohol, sulfuric acid, chloroform were from POCh (Gliwice, Poland). 3,3'-dithiodipropionic acid di(N-succinimidyl ester) was from Fluka-Sigma-Aldrich (Poznań, Poland). All chemicals used for investigations were of analytical grade purity. Aqueous solutions were prepared with freshly deionized water (18.2 MΩcm resistivity) obtained with a Simplicity® 185 Water System (Millipore, Molsheim, France).

Synthetic biotinylated oligodeoxynucleotides, used as probes, and unbiotinylated oligodeoxynucleotides complementary or noncomplementary to probes were synthesized in the Laboratory of DNA Sequencing and Oligonucleotides Synthesis, IBB PAS. Samples of fragments of DNA amplified by PCR and genomic DNA were prepared in the Genetic Modifications Analysis Laboratory, IBB PAS.

Stock solutions of oligonucleotides were prepared in deionized water and stored in a freezer. Before experiments the DNA probes were diluted in the appropriate buffer.

All experiments were performed at room temperature (24°C ± 2 °C).

2.2. Characterization of Probes and Target DNA

The probes:
- Biotinylated probe **1**:
5' biotin-TGG GGT TTA TGG AAA TTG GAA-3'   (m.w. 6987)
- Biotinylated probe **2**:
 5' biotin- ATC CCA CTA TCC TTC GCA AGA-3' (m.w. 6717)

The target DNA:
- Complementary oligonucleotide **1**:
5' TTC CAA TTT CCA TAA ACC CCA 3'   (m.w. 6249)

- Complementary oligonucleotide **2**:
5' TCT TGC GAA GGA TAG TGG GAAT 3'   (m.w. 6521)
- Non-complementary oligonucleotide:
 5' GGA TGG GGG TGG AGT AGA GGG C 3'   (m.w. 6980)
- The amplified fragment of the *EPSPS* gene, 169-base pairs long PCR product synthesized on the genomic DNA extracted from RR soybean
- The noncomplementary PCR product, 138-base pairs fragment, amplified by PCR on maize alcohol dehydrogenase gene template (*Adh*)
- The genomic DNA, non-amplified by PCR, extracted from animal feed containing 30 % RR soybean

- The genomic DNA, non-amplified by PCR, extracted from unmodified soybean

2.3. Composition of Buffers

- Immobilization buffer: 40 mM HEPES, 60 mM NaCl, pH = 7.5
- Hybridization buffer: 27 mM HEPES, 55mM NaCl, 2.5 mM $MgCl_2$, pH = 7.5
(hybridization of 21-mer oligonucleotides 1 was carried out in the buffer without $MgCl_2$)
- Denaturation buffer: 27 mM HEPES, 55 mM NaCl, 0.1 mM EDTA, pH = 8.0

2.4. Purification of PCR products

PCR products were purified by electrophoresis in 2% agarose gel. The slice of the gel containing PCR product was cut into small pieces and suspended in extraction mixture (0.5 M ammonium acetate, 0.1% SDS, 1 mM EDTA and 10 mM magnesium acetate), incubated for 10 minutes at 60 ℃, overnight at room temperature and again for 10 minutes at 60 ℃. The volume of DNA extract was measured and 1/10 volume of 3 M sodium acetate was added. After mixing, 4 volumes of cold (-20 ℃) 96% ethanol was added and solution was incubated at -20 ℃ overnight. The sample was then centrifuged for 15 min at 16000 x g, supernatant was discarded and the pelled washed with 150 μl of 85% cold (-20 ℃) ethanol. After final centrifugation as above the pellet was dried and dissolved in 50 μl of sterile water. DNA concentration was assayed spectrophotometrically.

After thermal denaturation of the double helical DNA structures (10 min in 95 °C), the samples were cooled on ice (2 min) and immediately used for the hybridization, which was monitored with QCM method.

2.5. Extraction of Genomic DNA

The genomic DNA from feed contained 30 % Roundup Ready soybean (relative to total soybean content in feed) and unmodified soybean were extracted according to procedure already reported [42] with modifications. 150-250 mg of milled plant material was suspended in 1.5 ml of CTAB extraction buffer (2% CTAB-cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris pH 8.0, 20 mM EDTA) preheated to 65 ℃, 10 μl of proteinase K solution (20 mg/ml) was added, the sample was thoroughly mixed and incubated at 65 ℃ for 30-90 minutes with occasional stirring. After incubation sample was centrifuged (10 min at 14000 x g) and supernatant was transferred to the new 2 ml eppendorf tube. 10 μl of RNAse A solution (10 mg/ml) was added, mixed and incubated at 65 ℃ for 10 min. Then 0.7-1 volumes of chloroform was added, the sample was vigorously vortexed for at least 15 sec to create an emulsion and centrifuged for 10 min at 14000 x g to separate phases. The upper, aqueous phase was transferred into the new 2 ml tube containing 500 μl of chloroform and was extracted as above. The upper, aqueous phase was transferred into the new 2 ml tube, to which 2 volumes of CTAB precipitation buffer (0.5% CTAB, 40 mM NaCl) was added. The content was mixed by gentle inversions of the tube and incubated for at least 60 min at room temperature. The precipitated DNA was subsequently centrifuged 10 min at 14000 x g, supernatant discarded and the pellet dissolved in 350 μl of 1.2 M NaCl. This DNA solution was further purified by a single extraction with 350 μl chloroform as above, centrifuged and upper, aqueous phase was transferred into new

1.5 ml eppendorf tube. Then 0.6 volume of isopropanol was added, mixed by gentle inversions of the tube and incubated for at least 20 min at room temperature. Precipitated DNA was then centrifuged for 10 min at 14000 x g, supernatant was discarded and the pellet thoroughly washed with 500 μl of 70% cold

(-20 ºC) ethanol. After final centrifugation, as above, the pellet was dried and dissolved in 50 μl of sterile water. DNA concentration was assayed spectrophotometrically.

2.6. *Quartz Crystal Microbalance*

The Time-Resolved Electrochemical Quartz Crystal Microbalance (CHI 410, CH Instruments, USA) and 7.995MHz AT-cut quartz crystal with gold coating both sides as working electrode were used in this study.

The parameters of quartz crystal were as follows: shear modulus of the quartz 2.947×10^{11} g cm^{-1} s^{2}, density of the quartz 2.648 g cm^{-3} and area of the gold electrode 0.196 cm^{2}.

The decrease in frequency of 1 Hz corresponds to mass increase of 1.34 ng.

2.7. *Modification of Gold Surface of QCM Electrodes*

The Au electrode surface was cleaned with piranha solution consisting of H_2O_2 (30%) and concentrated H_2SO_4 (98%) in a 7:3 ratio. The crystal was dipped in this solution for 5 min, then thoroughly washed with deionized water and dried under nitrogen. This process was repeated twice. After washing, the gold surfaces of QCM electrodes were immediately immersed for 2 hours into 5 mM solution of 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) in chloroform. Subsequently, the free surface of gold was blocked by immersion for 1 h into 1 mM solution of 6-mercapto-1-hexanol in ethanol. Then, the electrodes were modified by avidin (aqueous solution, 0.2 mg/ml) for 1 h. Next, they were exposed for 1 h to a 1 mM aqueous solution of 2-aminoethanol (pH 8.00, adjusted with HCl). Piezoelectrodes modified according to the above procedure were dept in immobilization buffer in the QCM cell. After frequency stabilization (± 1 Hz), the solution of biotinylated DNA probe was injected. The concentration of the DNA probe in the QCM cell was 300 nM (Figure 1).

The immobilization of biotinylated DNA probe was monitored by measuring the frequency *vs* time. Usually, the probe immobilization was completed within 15 min. After immobilization of biotinylated probe the electrodes were heated for 5 min in 45 ºC to improve the order of the modification of the electrode surface.
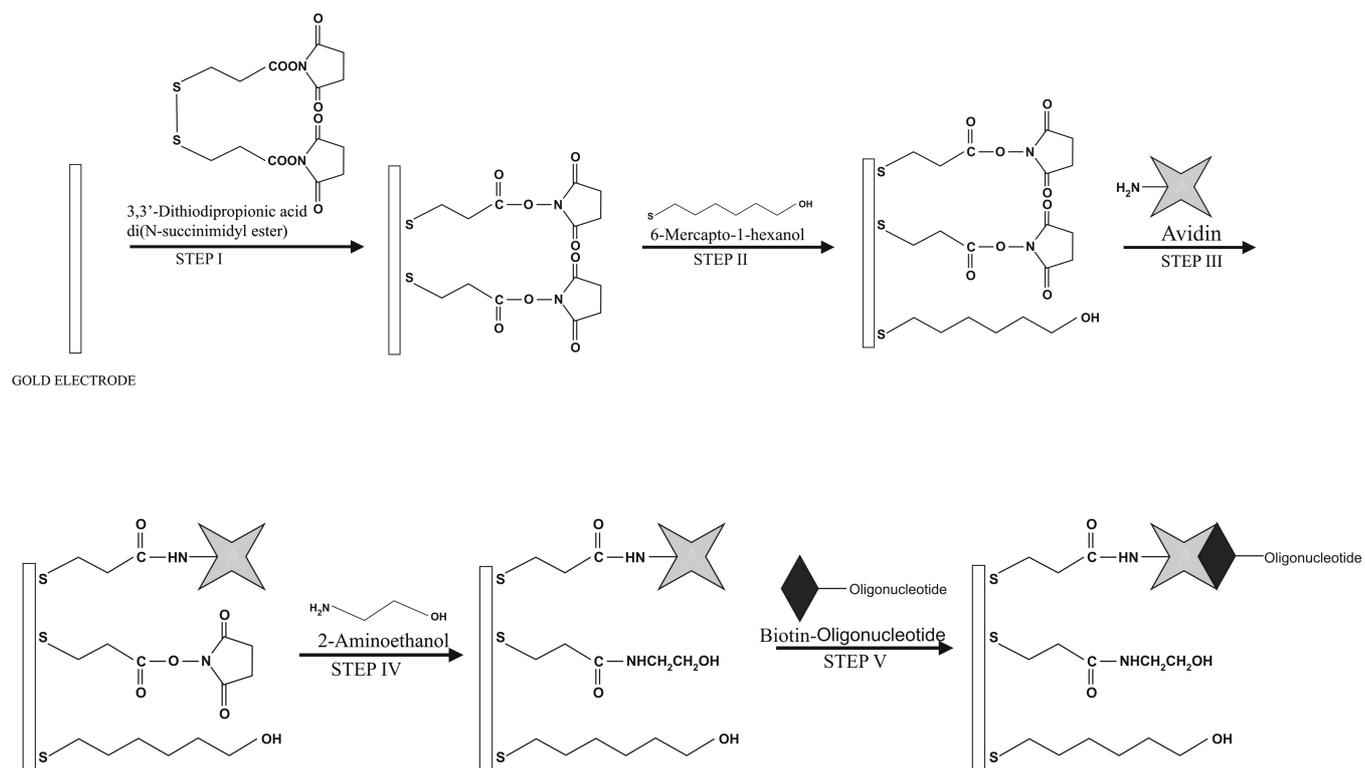
Figure 1. Steps of modification of the gold piezoelectrode.

2.8. Hybridization Process Monitored by QCM Method

The piezoelectrodes, modified as described above, were washed with hybridization buffer and used for the monitoring of the hybridization process with the complementary and non-complementary oligonucleotides.

The amplified products of PCR and genomic DNA were diluted in denaturation buffer and their double helical structures were thermally denatured (10 min in 95 $^{\circ}$C). The samples were cooled on ice for 2 min and immediately added (100 µl) to 100 µl of hybridization buffer placed in QCM cell.

After each run of the hybridization, the QCM electrodes were regenerated by incubation in denaturation buffer pH 8.00 for 10 min at 95 °C. Subsequently, QCM electrodes were kept in denaturation buffer pH 8.00 on the ice for 2 min. Sensors were also regenerated after each hybridization experiment by rising thrice in 10 mM NaOH for 2 min at room temperature.

3. Results and Discussion

3.1. Modification of Gold Surface of QCM Electrodes

Figure 1 illustrates the multistep process of the modification of piezoelectrodes. The presence of 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) on the electrode surface was confirmed by performing the reductive desorption process. It is known that the potential cycled from -0.4V to -1.2V in alkaline solution (0.5 M KOH) disrupts Au-S covalent bonds [43, 44]. The amount of adsorbed ester

may be estimated from the charge required for reductive desorption. The density of 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) on the electrode surface after 2h modification was calculated from the area of the reduction peak (Figure 2), and equaled - 18.8×10^{10} molecules·mm^{-2}.
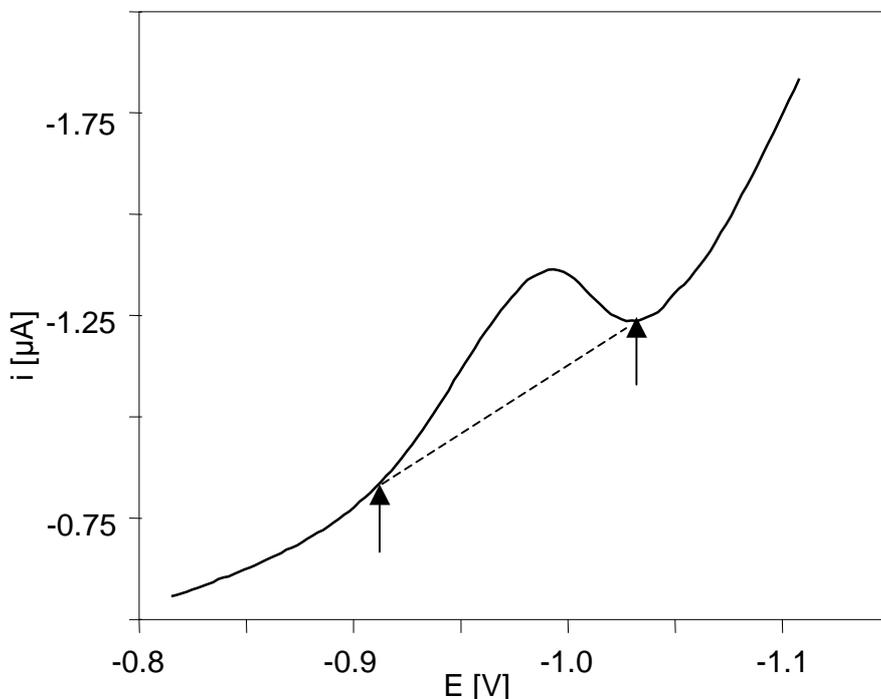

Figure 2. Cyclic voltammetry reductive desorption of 3,3'-dithiodipropionic acid di(N-succinimidyl ester) from gold piezoelectrode after two hours of modification in 5 mM of chloroform solution. Measurement conditions: 0.5 M KOH, potential scan rate 100 mV/s.
This was sufficient for avidin immobilization. The presence of 0.2 mg·ml-1 avidin in the QCM cell resulted in approximately 100 Hz decrease of piezoelectrode frequency. The immobilization of avidin on the electrode surface via creation of amide bonds with 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) was completed after 30 min.(Figure 3).

Two biotinylated oligodeoxynucleotides used in this study (probe **1** and **2**), had similar affinity towards avidin (Table 1). The number and density of DNA molecules immobilized on the QCM electrode surface were calculated from piezoelectrodes frequency changes. The results collected in Table 1 indicated that each step of modification was significantly reproducible. The molar ratios between avidin and both (**1** and **2**) biotynylated probes were 1.8**,** which indicated that their immobilization was very efficient.
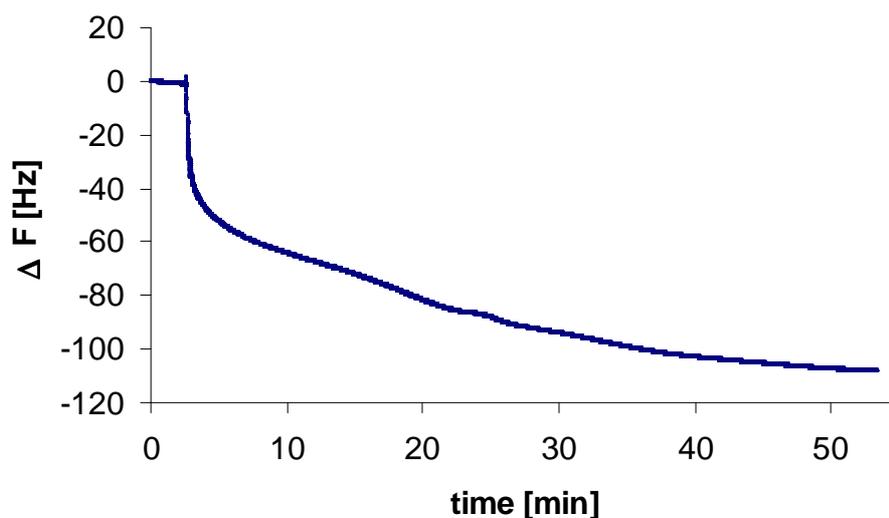
Figure 3. Frequency response of QCM electrode modified with 3,3'-dithiodipropionic acid di(N-succinimidyl ester) in the presence of avidin (0.2 mg/ml).

3.2. Hybridization Process Monitored by QCM Method

The system was optimized using short synthetic oligonucleotides. At the presence of 80 nM of 21-base pairs complementary oligonucleotides in the solution placed in QCM cell, almost all biotinylated
probes (both **1** and **2**) immobilized on the electrode surface were involved in the hybridization process (Table1). The decrease of the frequency of QCM electrodes modified with probe **1** and probe **2** was similar. Representative results are shown on Figure 4, curve a. The hybridization of short oligonucletides (21-22-mer) was completed usually within 15-20 min.

To check the specificity of electrodes bearing immobilized probes **1** or **2** we performed control hybridization experiments with non-complementary oligonucleotides. Observed frequency changes were negligible (Figure 4, curve b), which confirmed that only DNA sequences complementary to probes are detected by this system.

As a next step the sensor was used for the detection of PCR amplified 169 base pairs fragment of EPSPS gene. The electrode modified with probe **1** was more sensitive and in the presence of 2.5 nM EPSPS-derived oligonucleotide a -88.2 ± 7.5 Hz frequency change was observed (Figure 5, curve a).

Table 1. Changes of: frequency, mass, number and density of molecules for the consecutive steps of modification of QCM electrodes (n = 5 - 10).

Molecules immobilized on the QCM electrode	ΔF [Hz]	Δm [ng]	Number of molecules x 10^{12} on the gold electrode	Density of the molecules on the gold electrode x 10^{10} [molecules/mm^2]
avidin	- 104.8 ± 7.3	140.5 ± 9.7	1.3 ± 0.1	6.8 ± 0.4
biotinylated oligonucleotide (probe **1**)	- 19.7 ± 4.8	26.3 ± 6.5	2.3 ± 0.5	11.7 ± 2.8
21-base pairs oligonucleotide complementary to probe **1**	- 17.2 ± 5.9	23.1 ± 7.9	2.2 ± 0.8	11.4 ± 3.9
biotinylated oligonucleotide (probe **2**)	- 19.8 ± 5.1	26.5 ± 6.9	2.4 ± 0.6	12.2 ± 3.1
21-base pairs oligonucleotide complementary to probe **2**	- 19.0 ± 8.4	25.5 ± 11.3	2.4 ± 1.0	12.3 ± 5.6
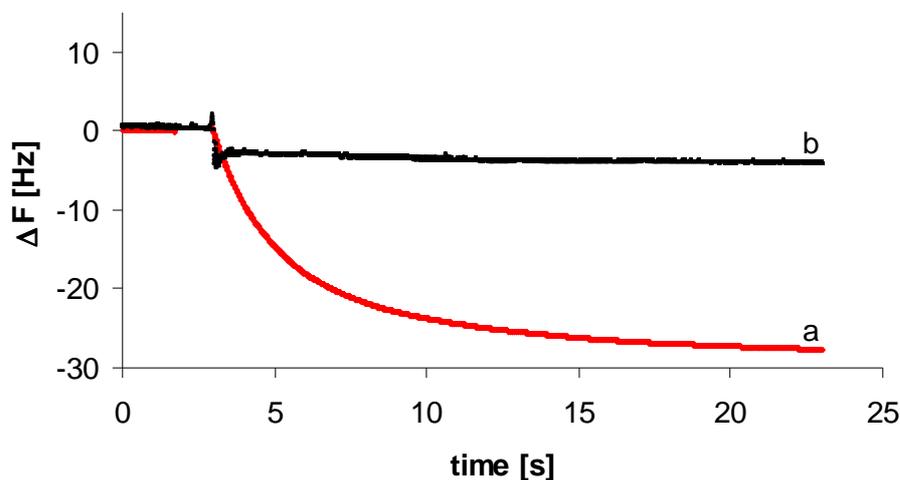
Figure 4. Frequency change of QCM electrode modified with biotinylated probe **1** at the presence of:
(a) complementary oligonucleotide (21-mer) (80 nM)
(b) non-complementary oligonucleotide (22-mer) (72 nM)
The solution composition: 27 mM HEPES, 55 mM NaCl, pH 7.5; total volume in the QCM cell: 200 µl.

In the same experimental conditions, QCM electrode modified with probe **2**, showed the frequency change of only -25.4 ± 5.7 Hz. Different affinity of target DNA fragments towards probes **1** and **2** might be caused by different sequences of probes' 5' biotinylated ends, which are in the vicinity to electrode surface. The following bases are located at the 5' biotinylated end of the probe **1**: one molecule of thymine and four molecules of guanine. In the probe **2**, the sequence at the 5' biotinylated end is as follows: adenine, thymine and two molecules of cytosine. So, at the 5' biotinylated end of the probe **1** there are more bases which form three hydrogen bonds than in probe **2**.

It was recently reported that biophysical parameters e.g. local thermodynamics of DNA baseparing, as well as its kinetics, depend on nucleotide sequences [45]. Therefore it is possible that the different nucleotide sequences make the hybridization process more efficient for probe **1** than for probe **2**.

That is why for subsequent experiments only probe **1** was used. The representative hybridization process with 169-base pairs fragment of EPSPS gene is illustrated on Figure 5. This process demands longer time (50 min) in comparison to hybridization of 21-mer complementary oligonucleotides (Figure 4). Similar tendency was observed by Minunni et al. [37].

The investigated sensor was very selective. The PCR amplified fragment of Adh gene (138bp) used as the negative control caused only negligible frequency shift (Figure 5, curve b).
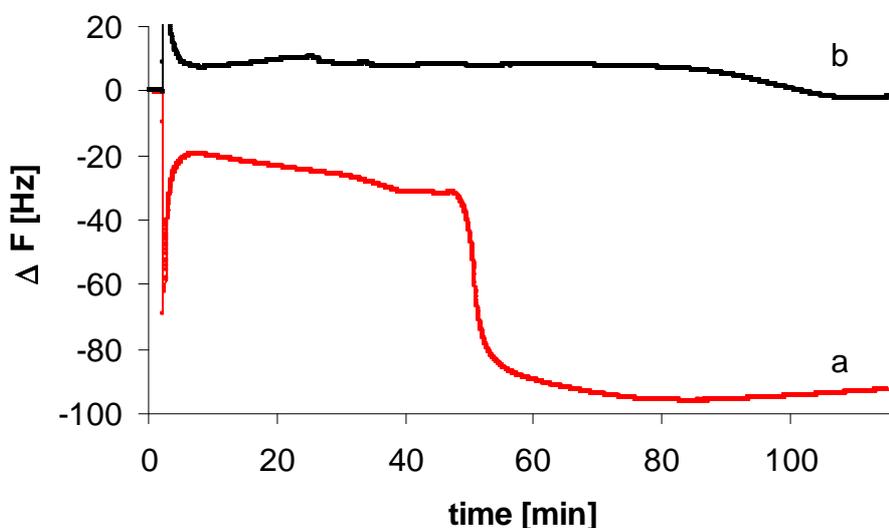
Figure 5. Frequency response of QCM electrode modified with probe **1** via avidin-biotin system
in the presence of:
(a) complementary PCR product - fragment of *EPSPS* gene (2.5 nM)
(b) non-complementary PCR product - fragment of *Adh* gene (2.5 nM)
The solution composition: 27 mM HEPES, 55 mM NaCl, 0.05 mM EDTA, 2.5 mM MgCl$_2$, pH 7.7;
total volume in the QCM cell: 200 μl.

The selectivity of piezoelectric sensor was estimated by competitive studies in which electrode signal generated by PCR product complementary to the probe (*EPSPS)* was measured in the presence of non-complementary PCR product (*Adh)*. In each solution, the total amount of *EPSPS* and *Adh* fragments were kept constant (0.5 pmoles). Results presented in Table 2 allow to conclude that, the responses in the frequency shift were comparable for the same concentration of *EPSPS* gene in samples containing only complementary to probe product of PCR (Table 2A) and with mixture of complementary *(EPSPS)* and non-complementary *(Adh)* products of PCR (Table 2B). The presence of non-complementary *Adh* gene in the solution slightly decreased the signal generated by *EPSPS* sequence hybridization, but did not abolish it. The non-complementary PCR product of *Adh* at the highest concentration studied (2.5 nM) caused negligible changes in frequency shift (-4.8 $\pm$ 1.0 Hz). The reproducibility of the hybridization process expressed as average of coefficient of variation (CV %) calculated for all concentrations of *EPSPS* gene was 28 % and for the mixture of complementary and non-complementary sequences was 19 %. Thus, it might be concluded that piezoelectric sensor presented here displayed low detection limit, in nM range and a good selectivity.

The main reason for low reproducibility is connected with the procedure of gold electrode modification. But still, our results are within the range of reproducibility of such type of sensors reported by others [35, 46].

Table 2. The selectivity of the piezoelectrode modified with probe **1** after hybridization process with complementary PCR product (fragment of *EPSPS* gene) in absence (A) and presence of noncomplementary PCR product (fragment of *Adh* gene) (B).

A

N_o of solution	Concentration of EPSPS [nM]	Changes of frequency ΔF [Hz]
1.	2.5	- 88.2 $\pm$ 7.5
2.	2.0	- 62.1 $\pm$ 19.7
3.	1.5	- 25.2 $\pm$ 8.0
4.	1.0	- 21.4 $\pm$ 7.9

B

N$_o$ of solution	Concentration of EPSPS [nM]	Concentration of Adh [nM]	Changes of frequency ΔF [Hz]
1.	2.5	0.0	- 88.2 ± 7.5
2.	2.0	0.5	- 60.4 ± 1.0
3.	1.0	1.5	- 15.9 ± 6.8
4.	0.0	2.5	- 4.8 ± 1.0

The experimental conditions: see Fig. 5; (n=3-4); in each solution, total number of moles of *EPSPS* and *Adh* (0.5 pmoles) were kept constant.

3.3. Detection of Genetic Modification in Genomic DNA Non-amplified by PCR

On the food control market there is an urgent need to design sensors which will be able to detect the genetic modification in the genomic DNA samples without PCR amplification. In the literature few examples of such sensors were already reported [35, 37, 46]. These sensors were applied only for qualitative or semi - quantitative analysis.

The sensor proposed in this study was tested for the detection of *EPSPS* sequence in PCR non-amplified DNA samples extracted from animal feed containing 30% of the genetically modified soybean Roundup Ready. The sensor was able to distinguish transgene sequence between modified and unmodified soybean DNA at the following level: 3.6, 4.6 and 5.4 µg of genomic DNA in 200 µl of QCM cell. Results of this experiment are shown in Table 3. The negative control, genomic DNA extracted from unmodified soybean, generated small frequency shift, which might be attributed to the direct adsorption of DNA on gold electrode surface without hybridization to the probe or weak, non-specific interactions between the probe and partially complementary sequences present in very long genomic DNA. The DNA amount in 2C nuclei of soybean has been already determined: 2.31 pg/2C [47]. This allows to calculate the number of genom copies with *EPSPS* gene which could be determined by the proposed sensor (Table 3).

Table 3. Determination of the *EPSPS* gene in non-amplified by PCR genomic DNA samples extracted from animal feed contained RR soybean and unmodified soybean (n=3-7).

Amount of genomic DNA extracted from animal feeder contained modified RR soybean in the QCM cell [μg]	Number of genom copies containing *EPSPS* gene in the QCM cell	ΔF [Hz]
3.6	$4.7 \cdot 10^5$	19.6 ± 2.7
4.6	$6.0 \cdot 10^5$	33.1 ± 9.4
5.4	$7.0 \cdot 10^5$	51.2 ± 9.9
Amount of genomic DNA extracted from unmodified soybean in the QCM cell [μg]		
3.6	-	3.4 ± 2.5
4.6	-	7.5 ± 0.2
5.4	-	14.5 ± 7.8

Total volume of the sample solution in the QCM cell: 200 μl. The regeneration of the electrode was done at 95°C in the solution: 27 mM HEPES, 55 mM NaCl, 0.05 mM EDTA, 2.5 mM MgCl$_2$, pH 7.5.

In this experiment two methods of electrode regeneration were applied. In the first one, the electrode was immersed into denaturation buffer containing 27 mM HEPES, 55 mM NaCl and 0.1 mM EDTA, pH 8.00 for 10 min. at 95°C and then cooled on ice (Table 3). In the second one the electrode was regenerated by washing thrice with 10 mM NaOH for 2 min. Only when the first method of regeneration was applied, we observed linear relationship between concentration of genomic DNA and changes of frequency, however the reproducibility of the transgene hybridization with the probe expressed as average CV % has been of the order of 20 % for both methods of the electrode regeneration.

The properties such as sensitivity and selectivity of piezoelectric sensor presented here indicated that it could be applied for the direct detection of genetically modified RR soybean in the samples non-amplified by PCR.

3.4. Conclusions

The proposed piezoelectric sensor was able to detect genetically modified soybean Roundup Ready[®] in genomic DNA samples non-amplified by PCR. The detection limit was in the range of $4.7 \cdot 10^5$ numbers of genom copies with *EPSPS* gene in the QCM cell with 200 μl of investigated samples. The sensor was very specific. The samples of genomic DNA extracted from soybeans free

from genetic modification generated negligible signal. Reproducibility of the hybridization process was 20 %.

The selectivity of the sensor was confirmed by hybridization to the probe immobilized on piezoelectrode of complementary (*EPSPS*) or non-complementary (*Adh*) DNA fragments amplified by PCR. The presence of non-complementary product of PCR (*Adh*) slightly decreased the signal generated by *EPSPS* gene, but did not abolish it.

The advantages of the piezoelectric sensing methods in comparison to classical methods applied for the determination of genetically modified organisms are: relatively low cost, simplicity of use, and avoidance of costly and laborious labelling of DNA samples and their amplification by PCR.

Acknowledgements

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