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Innovative Electrochemical Approach for an Early Detection of microRNAs

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The recent findings of circulating cell-free tissue specific microRNAs in the systemic circulation and the potential of their use as specific markers of disease highlight the need to make microRNAs testing a routine part of medical care. At the present time, microRNAs are detected by long and laborious techniques such as Northen blot, RT-PCR, and microarrays. The originality of our work consists in performing microRNAs detection through an electrochemical genosensor using a label-free method. We were able to directly detect microRNAs without the need of PCR and a labeling reaction. The test is simple, very fast and ultrasensitive, with a detection limit of 0.1 pmol. Particularly feasible for a routine microRNAs detection in serum and other biological samples, our technical approach would be of great scientific value and become a common method for simple miRNAs routine detection in both clinical and research settings.

According to David Baltimore, decades of research went into understanding biological processes without awareness that consideration of a key element, microRNA, was lacking.[1] The mature miRNAs control gene expression interacting with a specific mRNA, either inducing its degradation or blocking the translation process.[2] It is now predicted that as much as 40−50% of mammalian mRNA could be regulated at the translation level by miRNAs; therefore, they have a great impact in biological processes.[3] The discovery of miRNAs as regulators of developmental events in model organisms suggested to many investigators that miRNA might be involved in the regulation of a wide range of biological functions. Specific miRNAs assume a special interest in oncology, immune cell differentiation, hematopoiesis, brain development and function and their role has been also investigated in other human diseases.[4] Mitchell and co-workers have recently showed that tumor cells shed tumor derived microRNAs into the blood stream and that microRNAs are present in the blood and serum in remarkably stable forms.[5,6] Serum circulating microRNAs are promising novel biomarkes for early cancer detection and for improved cancer screening. Therefore there is an urgent need for reliable and ultrasensitive tests for microRNAs. At the present time, microRNAs are detected with techniques such as Northen blot, RT-PCR, and microarrays using many commercial kits. Nevertheless, the kits provided are expensive and the laboratory-procedures require a long time for the assay and the use of equipped laboratory with specialized and well-trained biologists and are not feasible for routine serum-based microRNAs determination. We propose a simple, fast, and very sensitive test for miRNAs detection that can be suitable for research as well as clinic routine. An electrochemical detection on genosensors, based on guanine oxidation consequent to the hybrid formation between the microRNA and its inosine substitute capture probe, has been exploited. The oxidation of guanine during the hybrid formation (RNA/DNA) on the electrode surface generates an electrical signal that is evaluated by a differential pulse voltammetry technique (Scheme 1). This label-free detection method, already described for nucleic acids detection,[7−9] had never been used for microRNAs. In this setting, we used a synthetic miRNA and its substituted inosine capture probe. As a model case, we used the miR-122, a specific liver marker expressed in the 70% of liver cells

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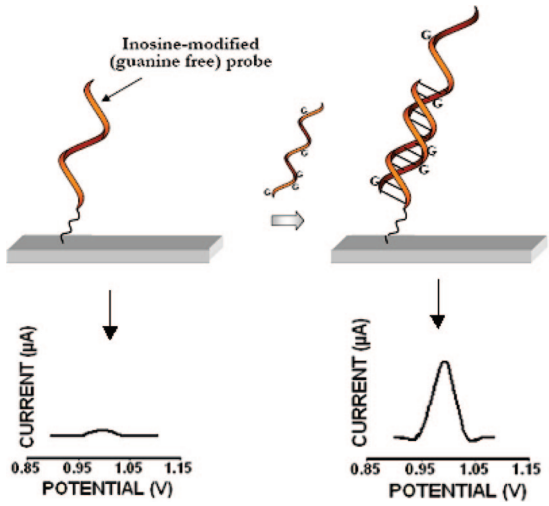
Scheme 1. Electrochemical Detection of the Hybridization between the Inosine-Modified Probe Capture and the Target (miR 122)[a]



[a] The appearance of the oxidation signal of guanine reflects the hybrid formation (yes/no system).

which has been linked with lipid metabolism, liver homeostasis, and hepatitis C virus replication.[10−13]

MATERIALS AND METHODS

An Autolab type II electrochemical analysis system and the GPES software package (Eco Chemie, The Netherlands) were used to obtain the differential pulse voltammograms. The screen-printed graphite electrodes (SPEs) were purchased from BVT Technologies (Czech Republic). Each amperometric sensor, on a corundum ceramic base, consisted of a graphite working electrode (diameter 0.6 mm), the Ag/AgCl reference electrode, and silver auxiliary electrode. At the end of the sensor, there was a contacting field connected with the active part by the silver conducting paths that are covered by a dielectric protection layer. Before the immobilization of the inosine-substituted capture probe, the sensors were pretreated by applying a potential of +1.4 V for 60 s in blank acetate buffer solution (0.5 M, pH 4.8).

Methods. The electrochemical detection procedure consisted of two different immobilization approaches of the inosine-substituted capture probe.

Method 1. Passive Adsorption of the Probe onto SPE Surface. Before the immobilization of the DNA probe, the sensors were pretreated by applying a potential of +1.4 V for 60 s in blank acetate buffer solution (0.5 M, pH 4.8). A probe solution (1 mM in 50 mM potassium phosphate buffer) was immobilized onto the pretreated sensors for 1 h at 37 °C using a passive adsorption method. The screen-printed electrodes were then rinsed with phosphate buffer (pH 7.4) for a short time 5 s.

Method 2. Probe Electrodeposition Onto SPE Surface. After pretreatment, the electrodeposition of a probe solution (1 μM in 50 mM potassium phosphate buffer) was performed by applying a potential of +0.5 V for 180 s. The screen-printed electrodes were then rinsed with phosphate buffer (pH 7.4) for a short time 5 s.

Hybridization with Target. The probe immobilized onto SPEs was dipped into the 20 μL aliquot of target solution. Different concentrations (from 0.005 to 1 μM in phosphate buffer) were used. The hybridization was allowed to proceed for 1 h at room temperature and also at 50 °C. Successive washing steps were applied onto the electrode surface in order to minimize the nonspecific adsorption: 1× SSC, 0.1% SDS once for 5 min, 0.1× SSC, 0.1% SDS twice for 10 min, and 0.1× SSC, twice for 5 min.

Voltammetric Transduction for the Detection of Immobilized DNA. The oxidation signal of guanine was measured by using DPV in blank PBS by scanning from +0.75 to +1.40 with an amplitude of 50 mV at 16 mV/s scan rate. The raw voltammograms were treated by using the Savitzky and Golay filter (level 2) included in the General Purpose Electrochemical software (GPES) of Echo Chemie with moving average baseline correction using a "peak width" of 0.01 V. All the measurements, in triplicate, were carried out by renewing the surface and repeating the above assay format by using DNA-modified SPEs.

Chemicals and Solutions. The 22-mer synthetic oligonucleotides of microRNA 122 and the cDNA inosine (I)-substituted capture probe were purchased as lyophilized powder. To test the specificity of the assay, a similar 22-mer synthetic oligonucleotide of microRNA 29 was used as control. The base sequences of the oligonucleotides were as follows:

miR 122 5'-UGGAGUGUGACAAUGGUGUUUG-3'

miR 29 5'-UAGCACCAUCUGAAAUCGGUUA-3'

probe 5'-CAAACACCATTITCACACTCCA-3'

RESULTS

We compared the two methods to evaluate which one would be more effective for micro-RNAs determination. In the passive adsorption protocol, when a high concentration of the target (1 μM) was used in the hybridization reaction, the signal obtained was not different from the background (probe signal). With scalar dilutions of the target (0.1 μM and 0.01 μM), the electrical signal progressively increased, although the enhancement was not so significant (Figure 1). The highest electric value was observed when 0.01 μM of the target was used, with a detection limit of 0.2 pmol (20 μL of 0.01 μM). However, at 0.005 μM concentration of the target, the amplitude of the electrical signal started to decrease, showing a gradual loss of sensitivity. Therefore, in order to see how much we could improve the sensitivity, we optimized the test by performing the electrical deposition of the probe onto the genosensors. Modifying the procedure in this way, the signal increased proportionally to every scalar dilution of the target and the highest value was observed when 0.005 μM of the target was used, with a detection limit of 0.1 pmol (20 μL of 0.005 μM). (Figures 1 and 2). In order to exclude false positive results, the noncomplementary miR 29 was used as a negative control for the hybridization. In both the immobilization procedures, the electrical signal obtained after the hybridization with 0.005 μM of miR 29 was comparable to the background (probe signal) (Figure 3). The clear difference between the full match and the negative control indicated that the hybridization was not accomplished with a

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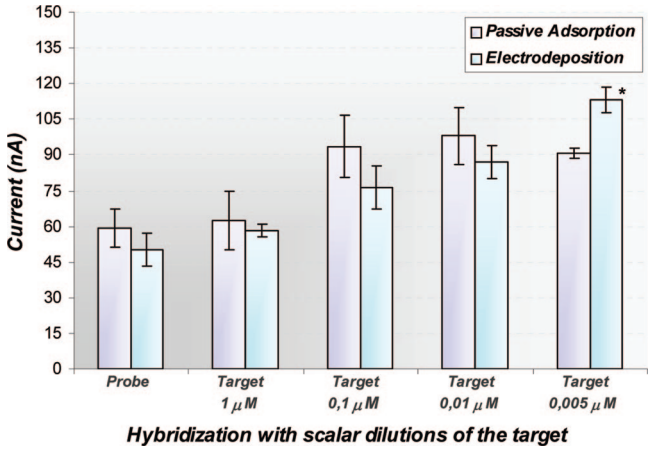

Figure 1. Electrochemical signals of the probe−target hybrid formation in the passive adsorption and electrodeposition method. All the hybridization experiments were performed in triplicate at room temperature. Each point, in the figure, represents the mean current value and the error bars correspond to the standard deviation (*$P <$ 0.05).

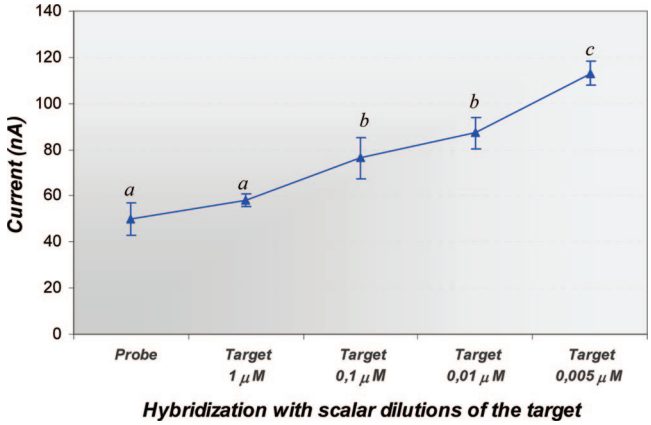

Figure 2. Electrochemical signals of the probe−target hybrid formation in the electrodeposition method. Error bars represent standard deviation of the mean. Points labeled with dissimilar letters (a−c) are significantly different ($P < 0.05$).

noncomplementary sequence. Therefore our test is able to distinguish similar sequences with high specificity. The different temperature conditions used for the hybridization (RT and 50 °C) did not produce significant variations in the amplitude of the electrical signals. (Tables 1 and 2). On the basis of these results, some technical considerations can be made. The low efficiency of the hybridization in the presence of a high concentration of the target is most probably the consequence of steric interaction and electrostatic repulsion between the two complementary strands that strongly reduce their probability of hybridization. The dilutions, minimizing this repulsion, make the detection more sensitive. The electrodeposition method probably leads to a better probe orientation and to a better accessibility of the probe than the passive immobilization, pushing the detection limit to 0.1 pmol. Therefore in order to avoid any potential matrix effect due to the presence of other cellular RNAs in the real biological samples, a selective extraction and enrichment for microRNAs should be recommended.

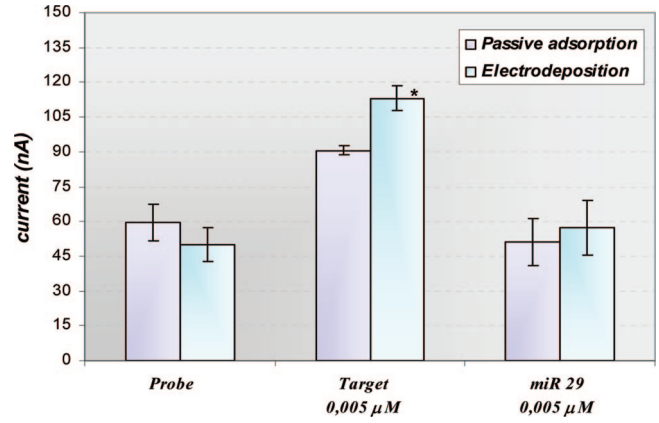

Figure 3. Electrochemical signals obtained during the hybridization with 0.005 μM of the target and 0.005 μM of the noncomplementary miR 29 (negative control) in the passive adsorption and electrodeposition methods. The electrical signal obtained after the hybridization with 0.005 μM of miR 29 was comparable to the background (probe signal). All the experiments were performed in triplicate at room temperature. Each point, in the figure, represents the mean current value, and the error bars correspond to the standard deviation (*$P <$ 0.05).

Table 1. Effect of the Temperature on Hybridization in the Passive Adsorption Method[a]

	hybridization temperature (25 °C)	hybridization temperature (50 °C)
passive adsorption method	mean current (nA) ± σ	mean current (nA) ± σ
probe	59.5 ± 8.0	61.0 ± 5.7
probe−target (1 μM)	62.5 ± 12.3	71.8 ± 15.9
probe−target (0.1 μM)	93.2 ± 13.2	93.5 ± 18.4
probe−target (0.01 μM)	98.0 ± 12.2	96.8 ± 28.4
probe−target (0.005 μM)	90.7 ± 2.1[b]	89.5 ± 3.5[b]

[a] The two different temperature conditions did not produce significant variations in the amplitude of the electrical signals. [b] $P < 0.05$.

Table 2. Effect of the Temperature on Hybridization in the Electrodeposition Method[a]

	hybridization temperature (25 °C)	hybridization temperature (50 °C)
electrodeposition method	mean current (nA) ± σ	mean current (nA) ± σ
probe	50.1 ± 7.1	50.1 ± 7.1
probe−target (1 μM)	58.2 ± 2.8	52.4 ± 10.6
probe−target (0.1 μM)	76.3 ± 9.0	77.3 ± 9.9
probe−target (0.01 μM)	87.1 ± 7.0	90.1 ± 14.1
probe−target (0.005 μM)	113.1 ± 5.3[b]	116.2 ± 8.5[b]

[a] The two different temperature conditions did not influence the electrical signals of the probe−target hybrid formation. [b] $P < 0.05$.

DISCUSSION AND CONCLUSION

The use of electrochemical biosensors for the detection of nucleic acids is already well described.[7−9] Otherwise, here we report, for the first time, a microRNAs detection through an electrochemical genosensor using a label-free method based on guanine oxidation. For the set up of the test, we used a specific

liver microRNA (miR 122) expressed in 70% of the liver cells. This microRNA has been involved in the regulation of liver steatosis, fatty acid metabolism, and in the replication of HCV virus.[10−13] The assay, here described, could be a potential tool for routine detection of miRNA 122 in serum and biopsies of hepatological patients. This electrochemical determination is rapid, portable, ultrasensitive, nonradioactive, and able to directly detect miRNAs without involving ligation, PCR, or microarrays. Moreover it is label free and does not require a direct miRNA labeling with toxic substances to enhance the sensitivity. Our data demonstrate that, by using inosine substituted capture probe, specific miRNAs can be amperometrically detected at subpicomolar levels with high specificity and with a detection limit of 0.1 pmol. Furthermore, this study opens the way to create miniaturized multi-SPE-miRNAs arrays able to detect tissue specific micro-RNAs in human diseases. Considering the surprising and excited discovery that serum and plasma contain a large amount of stable miRNAs derived from various tissues and organs,[5,6] this technical approach would be of great scientific value leading to a simple miRNAs routine detection.

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