

Amperometric Biosensing of miRNA-21 in Serum and Cancer Cells at Nanostructured Platforms Using Anti-DNA–RNA Hybrid Antibodies

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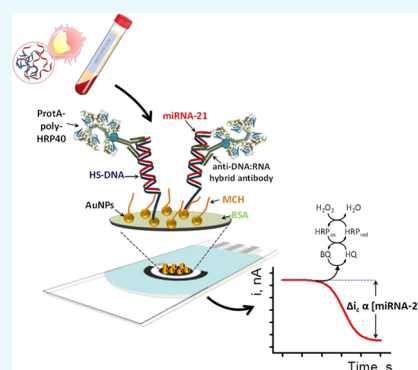
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ABSTRACT: This paper describes a disposable amperometric biosensor for simple and sensitive determination of miRNA-21. The bioplatfrom consists of gold nanoparticles-nanostructured electrode surfaces on which a direct hybridization assay involving the immobilization of a specific thiolated DNA capture probe and recognition of the formed DNA–miRNA-21 heteroduplex by a specific antibody is implemented. The antibody is further recognized through its Fc region by a commercial bacterial protein containing 40 units of horseradish peroxidase (HRP) (ProtA-polyHRP40). The amperometric detection of the hybridization process using the H₂O₂/hydroquinone system allows quantification of the target miRNA in the 0.096–25 pM linear range with a detection limit of 29 fM (0.29 amol in 10 μ L of sample). The bioplatfrom offers excellent selectivity against noncomplementary sequences and very acceptable against sequences with an unpaired base (only 30% of the response obtained for the target miRNA). In addition, the bioplatfrom was shown to be useful for the determination of the endogenous content of the target oncomiR directly in blood serum from breast cancer patients and in breast cancer cells using only 10 ng of total extracted RNA.



1. INTRODUCTION

Nanostructured electrodes with gold nanoparticles (AuNPs) have demonstrated unique properties in the development of nucleic acid electrochemical biosensors with improved analytical performances. The catalytic activity of the AuNPs together with their large surface area and excellent biocompatibility make these nanostructured surfaces more conductive and allow the immobilization of large probe loadings with optimal orientation and spacing for efficient hybridization, keeping their biological activity and storage stability.^{1–4} The resulting bioplatfroms exhibit also interesting advantages in terms of simplicity, cost, assay time, and applicability in different environments. These characteristics make AuNPs-involved bioplatfroms especially attractive compared to conventional methodologies for their implementation in routine determinations.

These properties have been successfully harnessed to develop electrochemical platforms for the determination of biomarkers of high clinical relevance, such as miRNAs,^{5,6} a class of endogenous and small noncoding RNAs that play a relevant role in many diseases, particularly in human cancers.^{7,8} Alterations in miRNA expression levels are involved in the initiation and progression of human cancers, and minimal signature profiles have been identified for various types of human cancers, where miRNAs can be regulated both upward and downward. Therefore, the determination of regulatory patterns is of great importance since they can provide relevant

information about tumor initiation, invasion, and metastasis processes.

However, the determination of miRNAs is highly complex due to their short sequence, low concentration level, and high sequence similarity between members of the same family. Conventional available methods for miRNAs determination, such as northern blotting,^{9,10} in situ hybridization, reverse transcription polymerase chain reaction,^{11–13} and miRNA microarrays,^{14–17} suffer from important limitations (use in centralized laboratories, expensive, laborious, require specialized personnel, and poorly portable instrumentation), which make the development of more simple and rapid alternative strategies highly desirable.

In recent years, several electrochemical platforms have been reported for the single or simultaneous determination of miRNAs either using integrated formats^{4,18–22} or involving magnetic beads (MBs).^{23–27} However, many of these biosensors require amplification strategies, which demand complex and/or long protocols to achieve the required sensitivity. This is an important restriction for their wide use in clinical practice by unskilled personnel. In this context, particularly attractive and relatively simple electrochemical strategies have been reported recently with acceptable

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Scheme 1. Schematic Display of the Biosensor Developed with AuNPs/Screen-Printed Carbon Based Electrodes (SPCEs) for the Amperometric Determination of miRNA-21 Involving a Specific HS-DNA Probe, Direct DNA/miRNA Hybridization, And the Use of Anti-DNA–RNA Antibody As Detection Bioreceptor Further Labeled with ProtA-PolyHRP40

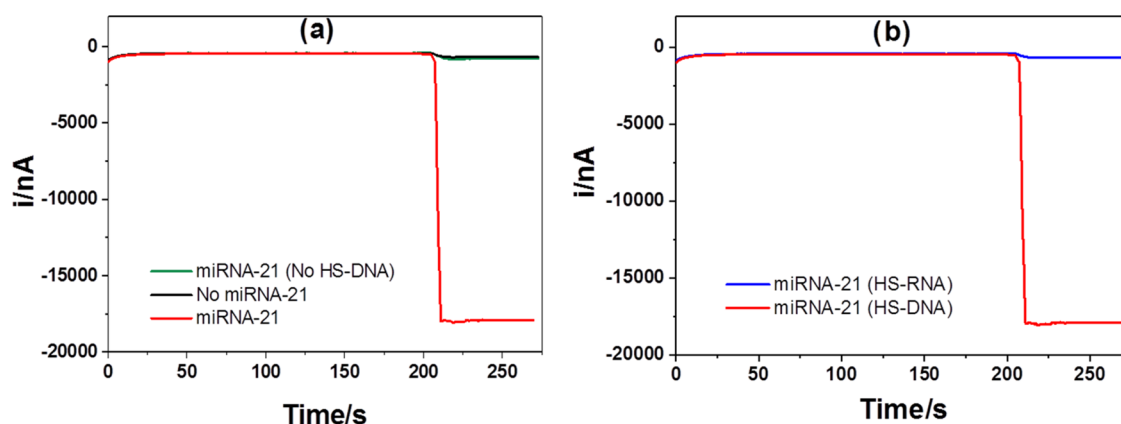
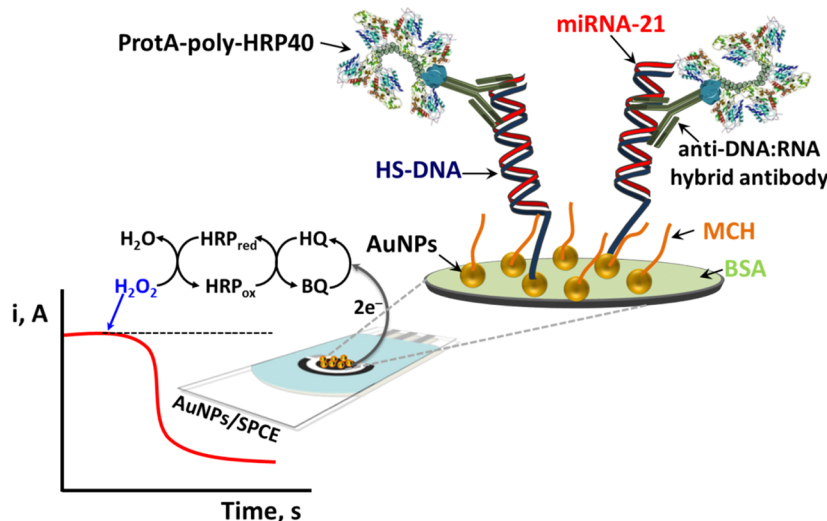


Figure 1. Amperometric responses obtained in the absence and presence of 20 pM synthetic miRNA-21 at AuNPs/SPCEs with and without immobilized HS-DNA probe (a) and in the presence of 20 pM miRNA-21 at AuNPs/SPCEs modified with HS-DNA or HS-RNA probes complementary to miRNA-21 (b).

sensitivity using commercial capture and detector bioreceptors with high affinity toward RNA homohybrids (viral protein p19^{4,28–31}) and heterohybrids (anti-DNA–RNA hybrid antibodies^{19–21,23,25,26}).

This paper describes a novel electrochemical bioplatfor for miRNAs determination, which combines the simplicity of a direct DNA–RNA hybridization format with the advantages of disposable AuNPs-nanostructured platforms and the high specificity and affinity of anti-DNA–RNA hybrid antibodies. To achieve high sensitivity, an easily implementable amplification strategy using a multienzyme bacterial protein able to recognize the Fc region of the anti-DNA–RNA hybrid antibody is used. The hybridization reaction is monitored amperometrically with the H₂O₂/hydroquinone (HQ) system. The resulting biosensor exhibits interesting performance in terms of sensitivity, selectivity, and reduced fabrication and assay times. It was successfully applied for the reliable and accurate determination of the selected target miRNA-21 (a relevant onco-miRNA³²) directly in a five-times diluted human blood serum from breast cancer patients as well as in breast

cancer cells using the lowest amount of total extracted RNA reported to date for an electrochemical biosensor.

2. RESULTS AND DISCUSSION

The developed strategy is displayed in Scheme 1. It involves direct RNA–DNA hybridization implemented on disposable carbon surfaces nanostructured with AuNPs. The target miRNA was selectively captured on the nanostructured surfaces by efficient hybridization with a complementary thiolated DNA probe. The resulting heteroduplex was recognized with an antibody with high affinity for these heteroduplexes, which was labeled with a bacterial binding antibody protein (ProtA) conjugated with a homopolymer containing 40 horseradish peroxidase (HRP) units. The extent of the hybridization reaction and thus of the miRNA-21 concentration was monitored by amperometry in the presence of the H₂O₂/HQ system. Interestingly, both the nanostructured disposable surfaces and all necessary reagents involved in this strategy are commercially available.

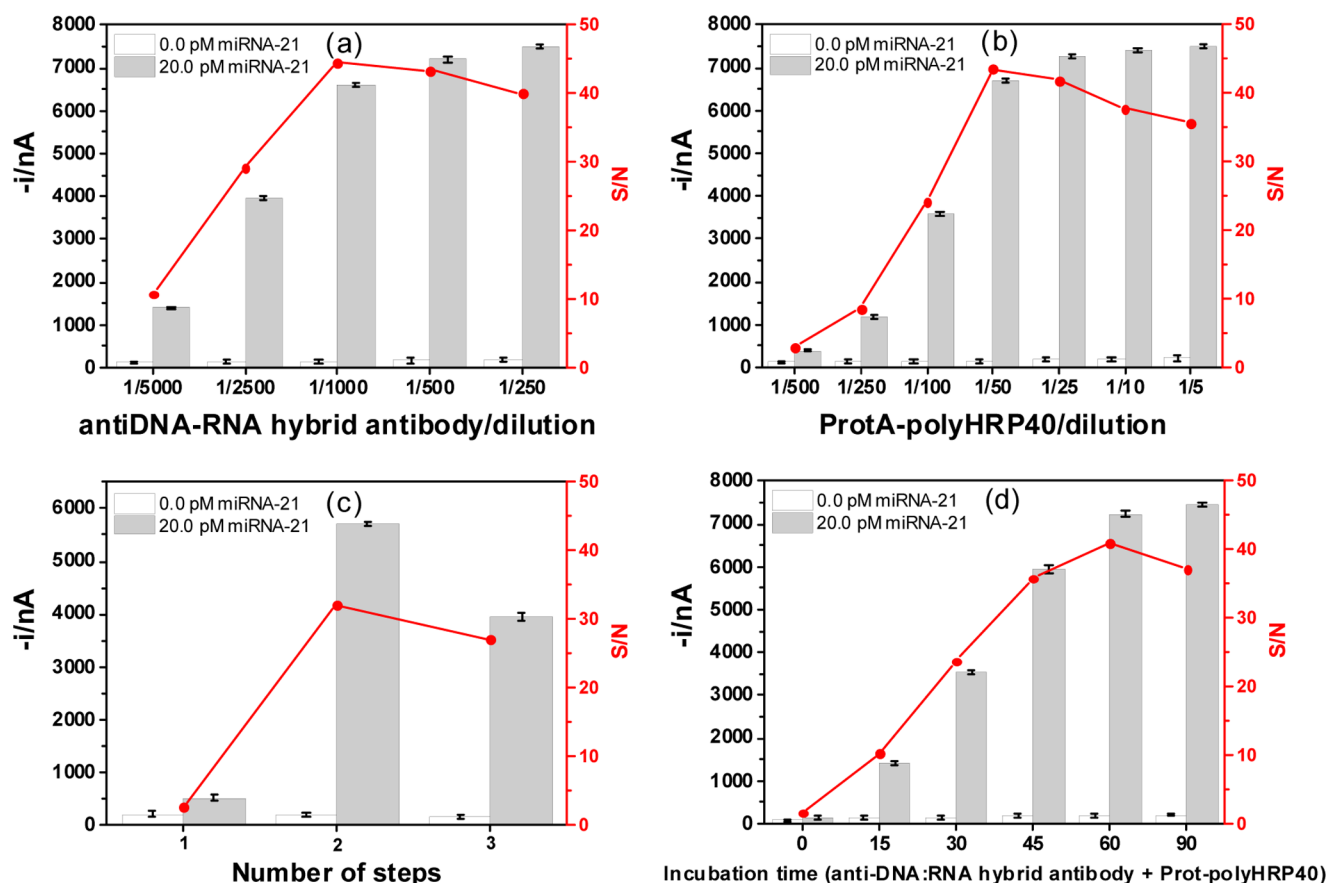


Figure 2. Dependence of the amperometric signals measured at HS-DNA/AuNPs/SPCEs in the absence (N) and presence of 20 pM miRNA-21 (S) on the corresponding S/N ratio with the anti-DNA–RNA antibody (a) and the ProtA-polyHRP40 (b) concentration on the steps number used for the preparation of the biosensor (c) and the incubation time with the mixture solution containing the DNA–RNA hybrid antibody and ProtA-polyHRP40 (d). The error bars are estimated as triple of the standard deviation (SD) of three replicates.

Figure 1 shows the comparison of the amperometric traces recorded in the absence and presence of the target miRNA at unmodified AuNPs/SPCEs and modified with HS-DNA and HS-RNA capture probes. Negligible and similar amperometric responses were observed in the absence of miRNA-21 on HS-DNA/AuNPs/SPCEs and in the presence of 20 pM miRNA-21 on AuNPs/SPCEs with no immobilized HS-DNA capture probe immobilized and on complementary HS-RNA/AuNPs/SPCEs (Figure 1b). These results confirmed that the amperometric responses were due to the efficient hybridization of the HS-DNA capture probe with the target miRNA, the negligible nonspecific adsorption of anti-DNA–RNA hybrid antibody and ProtA-polyHRP40 on the AuNPs/SPCEs surface, and the specificity of the antibody for the selective detection of DNA–RNA heterohybrids.^{33–35}

2.1. Optimization of the Experimental Variables. The most relevant experimental variables affecting the behavior of the designed bioelectrode were optimized. The current measured in the presence of 20 pM (signal, S) and in the absence (noise, N) of the target miRNA-21 as well as the corresponding ratio (S/N ratio) were used as selection criteria. The results obtained are shown in Figure 2 and summarized in Table 1. Apart from these, other variables used involved in the modification of AuNPs/SPCE, such as the HS-DNA probe concentration, the hybridization time with the target miRNA,^{4,22} the pH value, the detection potential, and the concentrations of H_2O_2 and HQ were taken from previous works.^{36–38}

Table 1. Evaluation of Different Experimental Variables Involved in the Preparation and Functioning of the Developed Biosensor

variable	evaluated range	optimal value selected
anti-DNA–RNA hybrid antibody (dilution)	1/5000–1/250	1/1000
ProtA-polyHRP40 (dilution)	1/500–1/5	1/50
incubation time with mixture DNA–RNA hybrid antibody + ProtA-polyHRP40 (min)	0–90	60
number of steps ^a	1–3	2

^aStarting from the preparation of HS-DNA/AuNPs/SPCEs.

Figure 2a,b shows the effect of the concentration of bioreagents used to carry out the heterohybrid recognition and the enzymatic labeling on the biosensor response. The 1/1000 and 1/50 dilutions of the anti-DNA–RNA hybrid antibody and ProtA-polyHRP40 conjugate provided larger S/N ratios, and therefore, they were selected for further work.

In an attempt to simplify the whole working protocol and reduce the assay time, the influence of the number of steps involved in the preparation of the biosensor on its analytical behavior was checked; 30 min incubation steps were assayed in all cases starting from the preparation of HS-DNA/AuNPs/SPCEs. Figure 2c shows a comparison between the results obtained with the following working protocols:

- One single incubation step with a mixture solution consisting of the target miRNA, anti-DNA–RNA antibody, and ProtA-polyHRP40.
- Two successive incubation steps: first with the target miRNA and thereafter with a mixture solution containing anti-DNA–RNA antibody and ProtA-polyHRP40.
- Three successive incubation steps with the target miRNA, anti-DNA–RNA antibody, and ProtA-polyHRP40.

Figure 2c shows clearly that the protocol involving two successive incubation steps provided a larger S/N current ratio. These results suggest that the mixing of all of the bioreagents worsened the efficiency of the hybridization process, whereas the anti-DNA–RNA antibody was better recognized by ProtA-polyHRP40 when both reagents were incubated in homogeneous solution. Once the optimal working protocol was selected, the influence of the incubation time with the mixture of anti-DNA–RNA antibody and ProtA-polyHRP40 to label the DNA/miRNA heteroduplex captured on the electrode was evaluated. As it is shown in Figure 2d, there were no significant differences for the nonspecific responses, but the specific current increased notably with the incubation time up to 60 min. Accordingly, this incubation time ensured an efficient labeling of the immobilized heterohybrids and was selected for further studies.

2.2. Analytical Characteristics. Figure 3 shows the amperometric responses obtained for different concentrations

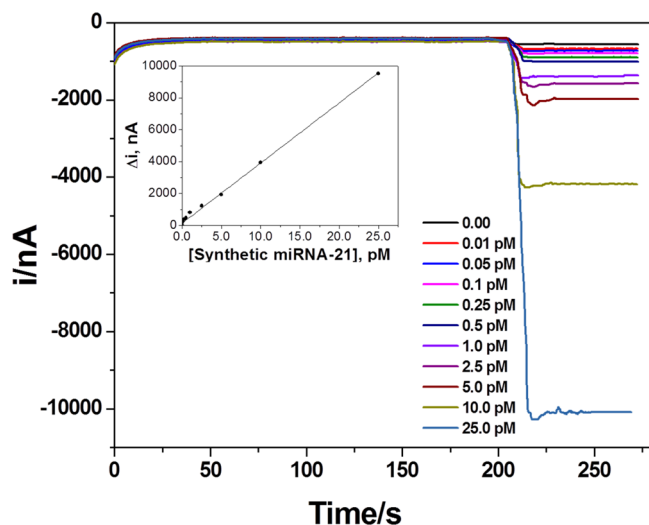


Figure 3. Amperometric traces obtained with the resulting HS-DNA/AuNPs/SPCE biosensors for different concentrations of the synthetic target miRNA-21. Inset: calibration plot. The error bars are estimated as triple of the standard deviation of three replicates.

of the synthetic target miRNA as well as the corresponding calibration plot. As expected for a direct hybridization assay, the Δi values (measured as described in Section 4.5) increased linearly (correlation factor r : 0.998) with the miRNA-21 concentration from 0.096 to 25 pM, with a slope value of $378 \pm 7 \text{ nA pM}^{-1}$. The limit of detection (LOD), defined as the concentration of the analyte that gives a signal that is significantly different from the blank, was estimated from the signal of the blank plus 3 times the standard deviation of the signal values for 10 independent amperometric measurements

obtained without target miRNA. The resulting LOD was 29 fM (0.29 amol in 10 μL sample).

A comparison of the analytical performance of the developed biosensor with that of other electrochemical biosensors reported for the determination of miRNAs using also DNA–RNA heterohybrid-specific antibodies is shown in Table 2. The LOD value provided by the developed biosensor is notably better than that achieved with biosensors involving the use of magnetic microbeads^{23,26} and similar to that claimed using electrodes nanostructured with carbon nanomaterials.^{19,20} Importantly, no application is reported for this latter approach. The achieved LOD is also higher than that given for the sensor using AuNP-modified glassy carbon electrode (GCE) and applied for the determination in RNA_t extracted from rice seedlings.²¹ However, both the fabrication and assay time of the developed sensor are remarkably shorter than those required using these more sensitive strategies (Table 2).

Interestingly, the designed bioscaffold provides about 7-times enhanced sensitivity (55.3 vs 378 nA pM) and a 34-times lower LOD value (0.29 vs 10 amol) than the performance exhibited by a magnetic microbeads-based biosensor involving the same bioreagents.²⁶ The improved performance can be attributed to the inherent conductivity and catalytic activity of AuNPs and the enhanced immobilization of nucleic acid probe with an adequate orientation and spacing to improve the target accessibility at the nanostructured surfaces.^{1–4} Indeed, the LOD achieved with this biosensor is rather similar to the values claimed for other biosensors reported recently for the amperometric determination of miRNAs at AuNPs/SPCEs using competitive and direct RNA/RNA hybridization assays using Strep-HRP and the viral protein p19, respectively.^{4–22} Importantly, as shown below, the sensitivity achieved is sufficient to allow quantification using a significantly small amount of extracted RNA_t. Moreover, the higher stability of DNA probes compared to RNA probes can be considered as another relevant practical advantage of the developed methodology.³⁹

The reproducibility of the measurements carried out with the developed bioplatfrom was checked by comparing the currents obtained for 1 pM miRNA-21 with nine different biosensors prepared in the same manner. A relative standard deviation value of 2.9% was calculated to show an excellent reproducibility in both the biosensor preparation and amperometric measurement procedures.

Regarding the storage stability of the bioplatfroms, the currents measured with HS-DNA/AuNPs/SPCEs, which were stored at 4 °C in a humidified chamber (after incubation with 1% (w/v) bovine serum albumin (BSA)), for 0 and 1.0 pM miRNA-21 showed that they remained within the set control limits over a 2 month period (no longer times were tested) (Figure 4). This attractive storage stability, in good agreement with the behavior observed for bioelectrodes involving thiolated RNA probes at disposable AuNP-nanostructured scaffolds,^{4,22} is attributed to the great biocompatibility and major role played by AuNPs in keeping the activity of the biomolecules immobilized onto their surface.

2.3. Selectivity. Due to the high sequence homology between miRNA family members, a very important feature of miRNAs biosensing platforms is the selectivity toward other nontarget and mismatched miRNA sequences. Figure 5 displays amperometric traces provided by the HS-DNA/AuNPs/SPCEs in the absence and presence of the synthetic target miRNA, a single-central base mismatched (1-m) and a

Table 2. Analytical Characteristics of Electrochemical Biosensors Reported so far for miRNAs Determination Using DNA–RNA Hybrid Antibodies

electrode	role of DNA–RNA hybrid antibody	electrochemical technique	target miRNA	I.L.	LOD	selectivity against 1-m sequences (%) ^a	biosensor or MBs fabrication/assay time ^a	sample (amount)	ref
carbon paper/reduced graphene oxide (RGO)/GCE	detector	SWV	miRNA-29b-1 and miRNA-141	1–1 × 10 ⁶ fM	8 fM		12 h/2 h 30 min		19
oxidized multiwalled carbon nanotubes/RGO/GSPes	detector	SWV	miRNA-29b-1 and miRNA-141	10–1 × 10 ⁶ fM	10 fM		12 h/2 h 30 min		20
SPCE	capture bioreceptor immobilized onto ProtG-MBs	amperometry (H ₂ O ₂ /HQ)	miRNA-21 and miRNA-205	(8.2–250) × 10 ³ fM	2.4 × 10 ³ fM (60 amol)	51	45 min/2 h	RNA _t extracted from cancer cells (500 ng) and human tumor tissues (1000 ng)	23
SPCE	detector bioreceptor for DNA–RNA heterohybrid immobilized onto Strep-MBs	amperometry (H ₂ O ₂ /HQ)	miRNA-21	(1.0–100) × 10 ³ fM	0.4 × 10 ³ fM (10 amol)	48	30 min/1.5 h	RNA _t extracted from cancer cells (250 ng) and human tumor tissues (250 ng)	26
AuNPs/GCE	detector	DPV	miRNA-319a	0.5–500 fM	0.40 fM	14	13 h/5 h 10 min	RNA _t extracted from rice seedlings	21
AuNPs/SPCE	detector bioreceptor	amperometry (H ₂ O ₂ /HQ)	miRNA-21	(0.096–25) × 10 ³ fM	29 fM (0.29 amol)	30	9 h/1 h 30 min	RNA _t extracted from cancer cells (50 ng)	this work

^aOnce modified the electrode or the MBs with the capture bioreceptor. ^b% of the response obtained in comparison to that of the target miRNA.

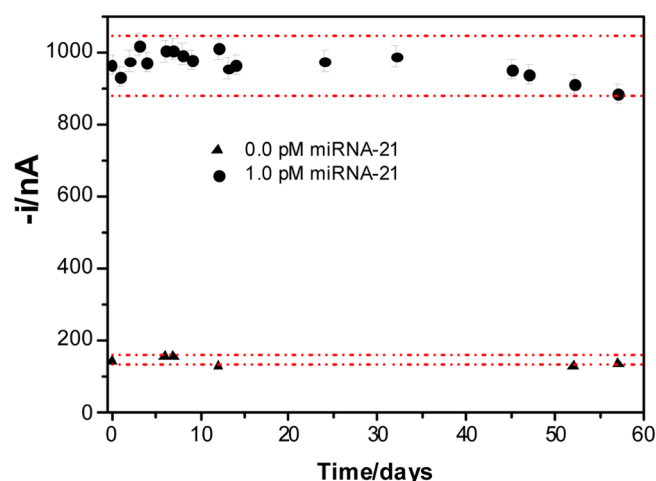


Figure 4. Storage stability of HS-DNA/AuNPs/SPCEs at 4 °C in a humidified chamber. Amperometric signals provided by biosensors prepared with the bioplatfroms stored at different times in the absence and presence of 1 pM synthetic miRNA-21. Control limits were set at $\pm 3 \times$ SD of the mean current values measured with five different sensors prepared on the first day of the study.

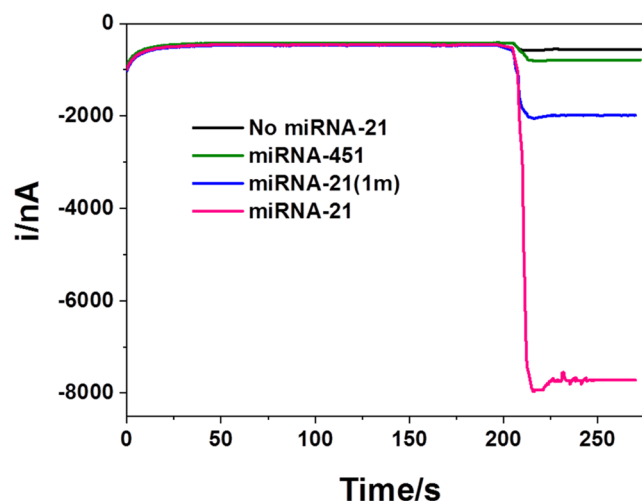


Figure 5. Amperometric traces recorded with HS-DNA/AuNPs/SPCEs in the absence of the target miRNA and in the presence of 20 pM miRNA-21, 1-m and noncomplementary (miRNA-451) sequences.

fully noncomplementary miRNA (miRNA-451). A very small response similar to that measured without target miRNA was obtained for the noncomplementary sequence. The 1-m sequence provided a 30% response of that obtained for the target miRNA. These findings are of particular interest for the applicability of the method in challenging samples, such as raw cellular RNA_t, where the target miRNA is present with many other nontarget miRNAs. The selectivity achieved toward 1-m sequences (30% hybridization efficiency assigning 100% for the target miRNA) is better than that demonstrated for other electrochemical biosensors using anti-RNA–DNA hybrid antibodies (48²⁶ and 51%²³) requiring similar assay times, while it is slightly worse (30 vs 14%) than that claimed for the strategy requiring an assay time 3 times longer.²¹

2.4. Determination of the Endogenous Content of miRNA-21 in Serum from Breast Cancer Patients and RNA_t Extracted from Breast Cancer Cells. The practical

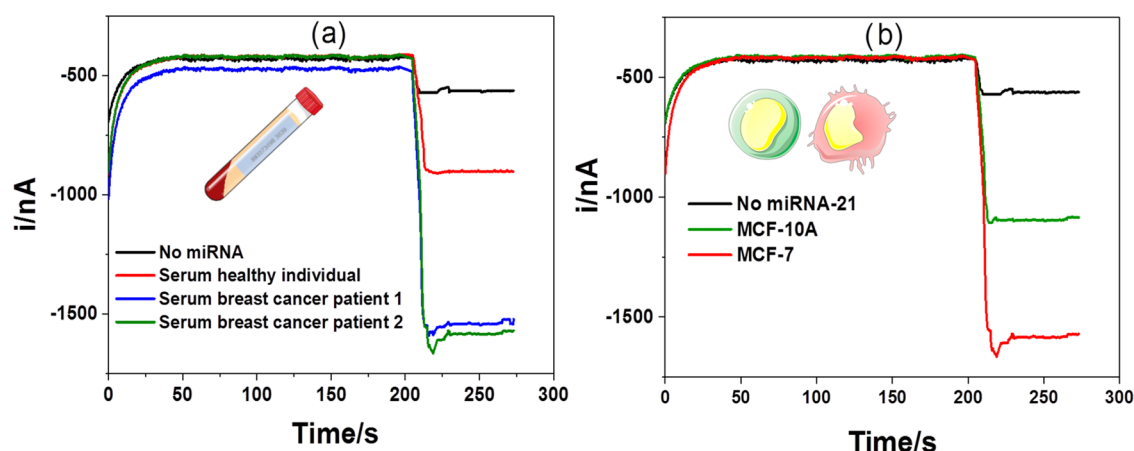


Figure 6. Amperometric responses measured with HS-DNA/AuNPs/SPCEs in the absence of the target miRNA and for five-times diluted and heated serum samples (a) or 10 ng raw RNA_t extracted from breast cells (b).

suitability of the developed methodology was tested by determining the endogenous content of miRNA-21 directly in blood serum from breast cancer patients and in raw RNA_t extracted from breast cancer cells.

Figure 6a shows the amperometric traces recorded with the developed biosensors for the three serum samples analyzed (one from a healthy individual and two from patients diagnosed with breast cancer). The samples were five-times diluted and heated, as described in Section 4.6. These results are in agreement with those reported by other authors⁴⁰ showing that miRNA-21 expression is about 3–4 times larger in breast cancer patients than in healthy individuals using an indirect highly liquid chromatography–mass spectrometry (MS)/MS method.⁴¹ No significant differences were found for the slope values of the calibration plot constructed with miRNA-21 standards (Figure 3, $378 \pm 7 \text{ nA pM}^{-1}$) and of the calibration graphs constructed for five-times diluted and heated sera from the healthy individual ($374 \pm 8 \text{ nA pM}^{-1}$) and the breast cancer patients $353 \pm 8 \text{ nA pM}^{-1}$ spiked with growing amounts of synthetic miRNA-21 up to 20 pM. This made the determination of the endogenous concentration of the target miRNA in serum feasible by simple interpolation of the Δi values measured for the diluted samples into the miRNA standards calibration plot. The concentrations of miRNA-21 obtained were 2.0 ± 0.2 , 8.0 ± 0.3 , and $7.3 \pm 0.4 \text{ pM}$ in the serum samples from the healthy individual and breast cancer patients 1 and 2, respectively.

Regarding the determination of miRNA-21 in cellular extracted RNA_t, Figure 6b shows the amperometric traces provided by the HS-DNA/AuNPs/SPCEs showing the expected miRNA-21 3–4 times higher expression in the tumorigenic (MCF-7) compared to the nontumorigenic (MCF-10A) cells according to the oncogenic role of this miRNA.^{41,42} Similar to that observed in diluted and heated serum samples, no statistically significant differences were found between the slope values of the corresponding calibration plots constructed with the synthetic target miRNA-21 both in buffered solutions and in the presence of 10 ng of RNA_t extracted from MCF-10A cells $354 \pm 9 \text{ nA pM}^{-1}$, thus confirming the absence of apparent matrix effect in the cellular RNA_t analysis. Interpolation in the miRNA-21 standard calibration plot provided concentrations of 0.82 ± 1.05 and $2.97 \pm 3.22 \text{ amol ng}^{-1} \text{ RNA}_t$ in MCF-10A and MCF-7 cells, respectively. These results are in complete agreement

with those reported previously with concentrations ranging from 0.8 to 1.0 amol ng⁻¹ RNA_t in MCF-10A and from 2.3 to 3.3 amol ng⁻¹ RNA_t in MCF-7 cells.^{4,23,43,26} These results demonstrate the bioplatfrom usefulness for the accurate and selective determination of the target miRNA in the presence of many other nontarget miRNAs. Importantly, this determination can be accomplished using the lowest amount of raw RNA_t reported so far with electrochemical biosensors,^{4,24,26,27,29,30,44} which required between 50 and 1000 ng of RNA_t extracted from cells to perform the target miRNA determination.

3. CONCLUSIONS

This work reports a novel amperometric bioplatfrom to determine miRNA-21. Combining the inherent advantages of disposable surfaces nanostructured with gold nanoparticles with the use of selective antibodies to DNA–RNA heteroduplexes and bacterial immunoglobulin-binding protein conjugated to multiple HRP units made this determination possible with an LOD as low as 29 fM and good discrimination toward sequences with a single unpaired base. Interestingly, this bioplatfrom is able to accurately determine the endogenous content of miRNA-21 directly in human blood serum from breast cancer patients and in breast cancer cells using very small amount of RNA_t. It should be emphasized that no target miRNA amplification steps are required with this strategy. In addition, all of the reagents and disposable electrodes used are commercially available, making this bioscaffold particularly attractive to be handled by non-specialized personnel and even on an outpatient basis for routine determinations.

4. EXPERIMENTAL SECTION

4.1. Apparatus and Electrodes. Amperometric measurements were performed with a Metrohm Autolab PGSTAT M204 electrochemical workstation using Nova v1.11 software. The transducers, purchased from Dropsens S.L. (Oviedo, Spain), were AuNP-modified screen-printed carbon electrodes (ref DRP-110GNP) consisting of a 4 mm diameter working electrode, a carbon counter electrode, and a Ag pseudoreference electrode and the specific cable connector with the potentiostat (ref DRP-CAC). Scanning electron microscopy (SEM) characterization studies carried out both by us and by the electrode supplier company confirm the presence of

Table 3. DNA and RNA Used Synthetic Oligonucleotides

oligonucleotide	sequence (5' → 3')
HS-capture DNA probe (HS-DNA)	ACATCAGTCTGATAAGCTA-[thiol-C6]
HS-capture RNA probe (HS-RNA)	ACAUCAGUCUGAUAAGCUA-[tThiol-C6]
target miRNA-21	UAGCUUAUCAGACUGAUGU
1-mismatched miRNA-21 (1-m)	UAGCUUAU <u>A</u> AGACUGAUGU
miRNA-451 (NC)	UUGAGUCAUUACCAUUGCAA

AuNPs homogeneously distributed over the whole surface with a mean diameter of 30 nm.

A Bunsen AGT-9 Vortex, a Raypa steam sterilizer, a Telstar Biostar biological safety cabinet, an Optic Ivymen System incubator shaker (Comecta S.A., Sharlab), and a NanoDrop ND-1000 spectrophotometer were employed.

4.2. Reagents and Solutions. Highest available grade reagents were used. NaH_2PO_4 , Na_2HPO_4 , NaCl, and KCl were purchased from Scharlab. Tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), mercaptohexanol (MCH), hydroquinone, H_2O_2 (30%, w/v), ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Germany).

ProtA-polyHRP40, a native ProtA labeled with a homopolymer containing 40 HRP molecules (antibodies-online), and an anti-DNA–RNA hybrid antibody (AbS9.6) from Kerafast (Boston, MA) were commercially available. A commercial blocker solution, consisting of a phosphate-buffered saline (PBS) containing 1% w/v purified casein, from Thermo Scientific (Waltham, MA) was also used.

The buffer solutions were prepared with Milli-Q water (18 M Ω cm at 25 °C) and sterilized after their preparation to avoid RNase degradation. Phosphate-buffered saline (PBS) consisted of 0.01 M phosphate-buffered solution containing 137 mM NaCl and 2.7 mM KCl (pH 7.5), Tris–EDTA buffer (pH 8.0) formed by mixing 10 mM Tris–HCl, 1 mM EDTA, and 0.3 M NaCl (pH 8.0), and phosphate buffer (0.05 M, pH 6.0) were used.

The synthetic oligonucleotides used (sequences shown in Table 3) were purchased from Sigma-Aldrich (Germany). Once received, they were reconstituted in nuclease-free water to give a final concentration of 100 μM and stored at -80 °C divided into small aliquots.

4.3. Preparation of HS-DNA/AuNPs/SPCEs. Immobilization of HS-DNA onto AuNPs/SPCEs was carried out following the protocol described in our previous works.^{4,22} Briefly, a 10 μL aliquot of a 0.05 μM DNA capture probe solution was incubated over the working electrode in a humidified chamber (8 h, 4 °C). After washing thoroughly with water and drying with nitrogen, the HS-DNA/AuNPs/SPCEs was treated with 10 μL of 0.1 mM MCH and 1% (w/v) BSA solutions (both prepared in Tris–EDTA, pH 8.0) for 5 min and 1 h, respectively.

4.4. Hybridization with the Target miRNA and Labeling of the DNA/RNA Heteroduplex with Anti-RNA–DNA Antibodies and ProtA-PolyHRP40. A 10 μL aliquot of the synthetic target miRNA or the sample (heated and diluted serum or RNA_t extracted from cells, all in sterilized PBS, pH 7.5) solution was incubated onto the HS-DNA/AuNPs/SPCEs at room temperature for 30 min to hybridize the target miRNA with the immobilized DNA probe. Thereafter, electrodes rinsed with sterilized water and dried with nitrogen were 60 min incubated with 10 μL of a mixture solution containing anti-RNA–DNA hybrid antibody and

ProtA-polyHRP40, 1/500 and 1/25 diluted, respectively. This mixture solution, prepared in blocker casein solution, was preincubated for 30 min in an incubator shaker (30 °C, 950 rpm). The modified bioelectrodes were rinsed with sterilized water and dried with nitrogen. To prevent miRNA degradation by RNases, all manipulations, except the amperometric measurements, were made in a laminar flow cabinet.

4.5. Amperometric Detection. Amperometric detection at an applied potential of -0.20 V versus Ag pseudoreference electrode was carried out in stirred solutions by immersing the prepared bioelectrodes into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer (pH 6.0) and freshly prepared 1.0 mM H_2O_2 . Once the background current was stabilized, 50 μL of a 0.1 M H_2O_2 solution was added and the current was recorded for about 100 s until reaching the steady-state current. The Δi values given corresponded to the difference between the currents measured in the presence and absence of the target miRNA and are the average of three replicates ($\alpha = 0.05$).

4.6. Application for the Analysis of Serum and Breast Cells-Extracted RNA_t . The developed bioplatfrom was used for the determination of the endogenous miRNA-21 content directly in human serum samples (without previous RNA_t extraction) and in RNA_t extracted from epithelial non-tumorigenic and tumorigenic breast cells. Blood serum samples were provided by Prof. Besma Loueslati (Department of Biology, University of Tunis El Manar) and collected from informed consenting patients according to ethical guidelines. Serum samples corresponded to healthy individuals and breast cancer patients. A serum aliquot was five-times diluted with the sterilized PBS (pH 7.5) solution and heated for 15 min at 95 °C (in a Stuart block heater) to release the miRNAs from microparticles and exosomes.¹⁸ Subsequently, a 10 μL aliquot of the diluted and heated sample was cast onto HS-DNA/AuNPs/SPCEs and the protocol described in Section 4.4 for the synthetic target miRNA was followed.

Cell culture and RNA_t extraction were performed following protocols described previously.^{29,30} The quality and concentration of the extracted RNA_t were confirmed by measuring the absorbance at appropriate wavelengths.

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Notes

The authors declare no competing financial interest.

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