



A novel zinc finger protein–based amperometric biosensor for miRNA determination

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Abstract

This paper reports a simple electrochemical strategy for the determination of microRNAs (miRNAs) using a commercial His-Tag-Zinc finger protein (His-Tag-ZFP) that binds preferably (but non-sequence specifically) RNA hybrids over ssRNAs, ssDNAs, and dsDNAs. The strategy involves the use of magnetic beads (His-Tag-Isolation-MBs) as solid support to capture the conjugate formed in homogenous solution between His-Tag-ZFP and the dsRNA homohybrid formed between the target miRNA (miR-21 selected as a model) and a biotinylated synthetic complementary RNA detector probe (b-RNA-Dp) further conjugated with a streptavidin–horseradish peroxidase (Strep–HRP) conjugate. The electrochemical detection is carried out by amperometry at disposable screen-printed carbon electrodes (SPCEs) (− 0.20 V vs Ag pseudo-reference electrode) upon magnetic capture of the resultant magnetic bioconjugates and H₂O₂ addition in the presence of hydroquinone (HQ). The as-prepared biosensor exhibits a dynamic concentration range from 3.0 to 100 nM and a detection limit (LOD) of 0.91 nM for miR-21 in just ~ 2 h. An acceptable discrimination was achieved between the target miRNA and other non-target nucleic acids (ssDNA, dsDNA, ssRNA, DNA–RNA, miR-122, miR-205, and single central- or terminal-base mismatched sequences). The biosensor was applied to the analysis of miR-21 from total RNA (RNA_t) extracted from epithelial non-tumorigenic and adenocarcinoma breast cells without target amplification, pre-concentration, or reverse transcription steps. The versatility of the methodology due to the ZFP's non-sequence-specific binding behavior makes it easily extendable to determine any target RNA only by modifying the biotinylated detector probe.

Keywords Zinc finger protein · Screen-printed electrodes · miR-21

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Introduction

The importance of detecting microRNAs (miRNAs or miRs) lies in their involvement in many biological processes implied in normal development, physiology, and diseases [1]. This large group of small non-coding RNAs, with critical regulatory post-transcriptional gene expression functions, has potential applications in diagnosis, prognosis, and therapy control of prevalent diseases including cardiovascular and neurological conditions and cancer [2]. Dysregulation of miRNAs has been shown to affect the hallmarks of cancer [3], covering evading growth suppressors, resisting cell death, activating invasion and metastasis, and inducing angiogenesis [4]. In fact, several studies have shown that altered miRNA expression is a common feature of all human tumors [5, 6].

However, despite the high clinical relevance of miRNAs, several inherent and technical challenges remain regarding

their detection. Current methods include classical nucleic acid detection methods such as microarrays [7, 8], northern blotting [9], nucleic acid amplification techniques (reverse transcriptase polymerase chain reaction, RT-PCR) [10, 11], and next-generation sequencing [12], which are not suitable for resource-limited settings [13, 14]. In this context, biosensors with electrochemical transduction have gained attention due to their high sensitivity, fast response, and simplicity providing quantitative information with affordable and easily miniaturizable instrumentation able to be used at decentralized settings [15].

Despite the noticeable number of electrochemical biosensors reported for the determination of miRNAs [16, 17], it is important to note that many of them require multiple reagents and involve complex and time-consuming working protocols, also including amplification strategies. Some major challenges need to be addressed to achieve the full functionality of miRNA diagnostics due to miRNA intrinsic characteristics such as length (19–23 nucleotides), low abundance (typically femtomolar–picomolar level in clinical samples [14]), and sequence similarity within miRNA families [18]. The use of sandwich-type hybridization configurations has shown to be an interesting approach to achieve sensitive and specific detection with no need for target nucleic acid amplification [19, 20]. However, due to miRNA short length, it is difficult to get efficient hybridization with two different short DNAs or RNA chains (capture and detector probes) [21]. Several strategies have been reported to overcome this problem. They include target hybridization with the short DNA capture and detection probes modified with a high-affinity DNA analogue such as a locked nucleic acid [22], the use of high-affinity commercial bioreceptors toward RNA hybrids (viral protein p19 [23], anti-DNA–RNA hybrid antibodies [24, 25], and zinc finger proteins, ZFPs [26, 27]), and hybridization along with labeling/modification [28]. The biosensing strategies involving commercial bioreceptors and homogeneous hybridization procedures are particularly attractive because they can be readily translated to the interrogation of any target miRNA/RNA simply by adding the complementary probe to the target RNA to form the corresponding RNA hybrid. Thus, these strategies merge the selectivity of target RNA/complementary probe hybridization with the unique and high affinity of these bioreceptors for the particular RNA duplex allowing highly selective and sensitive methodologies [29].

A zinc finger is a DNA-binding domain which has a small peptide with a special secondary structure stabilized by zinc ion-binding two cysteines and two histidines [30]. Commonly, a ZFP is a specific double-stranded (ds)DNA-binding protein [31] that recognizes dsDNA in a sequence-independent manner with high affinity through all its finger domains. ZFP binding mode is simple and natural, and engineered ZFPs can be fused to various functional domains enabling diverse applications such as gene regulation [30] and DNA diagnostics [32]. Improved sensitivity of ZFP-based

methods can be achieved by conjugating ZFP to different signaling labels such as luciferase [33, 34], glucose oxidase [27], glucose dehydrogenase [35, 36], peroxidase [37], or alkaline phosphatase [26, 38].

Although most ZFPs bind dsDNA, some of them also bind RNA such as the so-called Just Another Zinc finger protein (JAZ, ZNF346) and Specificity Protein 1 (SP1) that can recognize RNA–RNA hybrids [39]. JAZs bind non-sequence specifically and preferably A-form dsRNAs over B-form dsRNAs and dsDNAs, and do not bind ssDNAs and ssRNAs [39]. Indeed, because of the similarity of DNA–RNA hybrids to A-form dsRNAs, JAZs also bind these heterohybrids with high affinity [26, 39]. To the best of our knowledge, there is only one electrochemical biosensor reported so far that used this type of ZFP as a detector bioreceptor for miRNAs profiting binding of JAZ to the DNA–RNA hybrid formed between a synthetic DNA capture probe and the target miRNA. This biosensing strategy involved a polymeric self-assembled monolayer–modified and casein-treated indium tin oxide (ITO) electrode and enzymatic amplification using alkaline phosphatase coupled with electrochemical–chemical–chemical (ECC) redox cycling [26].

Herein, we report for the first time a simple strategy for the amperometric determination of the model miRNA, miR-21. The complex produced in homogenous solution between a His-Tag commercial ZFP and the RNA homohybrid formed between miR-21 and a biotinylated synthetic complementary RNA detector probe (b-RNA-Dp) was captured on the surface of commercial functionalized magnetic microbeads (His-Tag-Isolation-MBs). Further conjugation with Strep–HRP allowed amperometric detection involving the H_2O_2 /HQ system upon capturing the magnetic bioconjugates at SPCEs. Compared with the only electrochemical biosensor reported previously for the determination of miRNAs [26], the method reported in this work uses bare SPCEs and, for the first time, a commercial ZFP as a capture bioreceptor without further modification which shortens significantly the whole assay time and reduces the number of utilized reagents. It is important to note that this methodology is clearly different from those reported previously by our group [25, 40, 41]. The use of a ZFP bioreceptor for capturing the RNA hybrid formed between the target miRNA and a complementary synthetic probe and the type of MBs (His-Tag-Isolation-MBs vs Strep-MBs) employed as solid support to perform the bioassay highlight the novelty of this approach with respect to the previous ones.

Materials and methods

Apparatus and electrodes

The apparatus, SPCEs, and cable connector between the potentiostat and the SPCEs used for the amperometric

measurements were as described in previous papers [25, 41], as well as the Teflon casing with an inserted magnet to capture the modified MBs on the surface of the working electrodes. A magnetic stirrer (MS01, ELMI) to homogenize the solutions, a Raypa steam sterilizer, and an incubator shaker (Optic Ivymen® System, Comecta S. A, Scharlab) were also used, together with a biological safety cabinet, a MBs concentrator, and vortex, also used in previous works [25, 41]. The procedure followed to obtain the amperometric measurements was described previously by Vargas et al. [25].

Reagents and solutions

All reagents were of the highest available analytical grade. His-Tag-Isolation-MBs (1.0 μm Ø, 40 mg mL^{-1} , Dynabeads His-Tag Isolation & Pulldown, ref. 10103D, Invitrogen) were purchased from Thermo Fisher Scientific. Sodium chloride (NaCl), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Scharlab. Hydroquinone (HQ) and hydrogen peroxide (H_2O_2 , 30% w/v) were purchased from Sigma–Aldrich. Zinc chloride (ZnCl_2) was from Probus. Recombinant Histidine-Tag-zinc finger protein 346 (ZFP, 0.3 mg mL^{-1} , Cusabio Technology LLC, CSB-EP026691 MO), and streptavidin–HRP conjugate (Strep–HRP, 500 U, Roche) were employed. Commercial blocker casein solution (ready-to-use, PBS solution containing 1% w/v purified casein, BB solution) was purchased from Thermo Scientific. The following buffer solutions, prepared in Milli-Q water (18 $\text{M}\Omega$ cm at 25 °C) and sterilized after their preparation, were used: PBS– ZnCl_2 buffer consisting of 10 mM PBS solution containing 137 mM NaCl, 2.7 mM KCl, 100 μM ZnCl_2 , pH 7.5, and 0.05 M phosphate buffer, pH 6.0. All synthetic oligonucleotides (Table 1) were purchased from Sigma–Aldrich and used after their reconstitution in nuclease-free water to a 100 μM final concentration and stored at -80 °C into small aliquots (5 μL). All manipulations before the amperometric measurements were performed in a laminar flow cabinet to avoid RNase contamination and prevent miRNA and b-RNA-Dp degradation.

MBs modification

First, the hybridization process was carried out in homogeneous solution by mixing the synthetic b-RNA-Dp (0.1 μM) and the target miR-21 (synthetic or RNA_t) in PBS– ZnCl_2 (pH 7.5) in an Eppendorf tube. The mixture was placed in a thermal shaker at 25 °C and 950-rpm mixing speed for 15 min. A control test with no target was included in each hybridization run to evaluate the blank signal. The hybridization mixture was then supplemented with 10 $\mu\text{g mL}^{-1}$ of His-Tag-ZFP (ZFP) and incubated for 45 min at 25 °C under continuous stirring (950 rpm) to form the b-dsRNA/ZFP complex.

Meanwhile, 1.5 μL of the commercial suspension of His-Tag-Isolation-MBs was placed in a 1.5-mL Eppendorf tube and washed twice with 50 μL of PBS– ZnCl_2 (pH 7.5) buffer to remove the NaN_3 used as the preserving agent, which may interfere in the affinity reactions. The washed MBs were re-suspended in PBS– ZnCl_2 , pH 7.5, and incubated for 45 min (25 °C, 950 rpm) with 25 μL of the previously prepared b-dsRNA/ZFP solution to capture the b-dsRNA/ZFP onto the MBs through the ZFP tag. The resulting b-dsRNA/ZFP-MBs were washed three times with 50 μL of commercial BB solution and incubated with 25 μL of Strep–HRP conjugate (1/5,000) for 30 min (25 °C, 950 rpm) to label the attached b-dsRNA. After two final washings with 50 μL of BB solution, the HRP–Strep–b-dsRNA/ZFP-MBs were re-suspended in 50 μL of 0.05 M PB solution, pH 6.0, to carry out the amperometric detection.

Cell culture and RNA_t extraction were undertaken following the protocols described in previous papers by our research group [25, 40, 41]. The final pellet was dried out in a heating plate for 10 min at 80 °C, dissolved in RNase-free water, and stored at -80 °C [23, 42].

Results and discussion

The designed electrochemical strategy combines the benefits of using ZFP for the detection of miR-21, magnetic microbeads as solid supports, and disposable screen-printed carbon electrodes (SPCEs) (Fig. 1). The methodology involves the selective capture of the biotinylated RNA homohybrid formed by hybridization between the target miRNA and a biotinylated complementary RNA detector probe (b-RNA-Dp) (step 1 in Fig. 1) by the commercial His-Tag-ZFP in homogeneous solution (step 2). His-Tag-Isolation-MBs were used as solid supports for further capturing the ZFP/b-dsRNA complex through the ZFP-specific tag (step 3). Enzymatic labeling of the attached b-dsRNA with a Strep–HRP conjugate was further accomplished (step 4). Upon magnetic capture of the resulting modified MBs on the SPCE working electrode, amperometric detection was performed using hydroquinone (HQ) as the electron transfer mediator and H_2O_2 as the enzyme substrate (step 5). Each SPCE was only used to perform a single measurement and discarded afterwards.

Optimization of the experimental variables

The influence of all the experimental variables involved in the preparation of the biosensor was checked. The optimal values were selected considering the largest signal to blank (S/B) current ratio measured in the absence (B) and in the presence of 50 nM (S) synthetic miR-21 solutions at a detection potential of -0.20 V vs the Ag pseudo-reference electrode using the

Table 1 Oligonucleotides used in this work

Oligonucleotide	Name	Sequence (5' → 3')
Biotinylated RNA detector probe	b-RNA-Dp	UCAACAUCAGUCUGAUAGCUA-biotin
Target miR-21	miR-21	UAGCUUAUCAGACUGAUGUUGA
Central single mismatched target miR-21	miR-21 (1-mc)	UAGCUUAUCA <u>AA</u> CUGAUGUUGA
Terminal single mismatched target miR-21	miR-21 (1-mt)	UAGCUUAUCAGACUGAUGUUG <u>G</u>
Non-complementary target	miR-205 (NC)	UCCUUCAUCCACCGGAGUCU
Non-complementary target	miR-122 (NC)	UGGAGUGUGACAAUGGUGUUUG
Biotinylated DNA detector probe	b-DNA-Dp	TCAACATCAGTCTGATAAGCTA-biotin
cDNA of miR-21	cDNA of miR-21	TAGCTTATCAGACTGATGTTGA

H₂O₂/HQ system. This detection potential and the H₂O₂/HQ concentrations were previously optimized [43–45]. All the tested variables and the values selected for further work are summarized in Table S1 of the [Electronic Supplementary Material](#) (ESM).

Figures 2 a and b show the effect of the ZFP and b-RNA-Dp concentration, respectively. The S/B ratio increased with the concentration up to 10 and 0.1 μ M, respectively, and showed a noticeable decrease for larger concentrations due to the considerable enhancement of the non-specific signals measured in the absence of miR-21. In addition, in agreement with the principles of the bioassay, no discrimination between specific and background current was possible in the absence of ZFP and b-RNA-Dp(bars 0 in Figs. 2 a and b).

The influence of the sequence of the steps involved in the preparation of the biosensor was checked. Therefore, different working protocols were tested: (1) immobilization of His-Tag-

ZFP onto the His-Tag-Isolation-MBs surface followed by the capture of the miR-21/biotinylated complementary RNA detector probe (b-RNA-Dp) complex previously formed in homogeneous solution, and further labeling with Strep–HRP conjugate; (2A) hybridization of the target miR-21 and b-RNA-Dp in homogenous solution followed by the selective capture of the resultant b-dsRNA hybrids by the His-Tag-ZFP, capture of the ZFP/b-dsRNA complex onto the His-Tag-Isolation-MBs (all these steps in PBS–ZnCl₂, pH 7.5), and labeling of the b-dsRNA with the Strep–HRP conjugate (in BB solution); (2B) the same protocol as that in 2A but using PBS–ZnCl₂, pH 7.5, during the Strep–HRP labeling step. Figure 2c shows the working protocol 2A provided larger S/B current ratio. The poor results obtained using protocol 1 can be attributed to the poor efficiency to recognize b-dsDNA by the ZFP immobilized on the MBs due to steric hindrance. Importantly, no discrimination between the absence and the

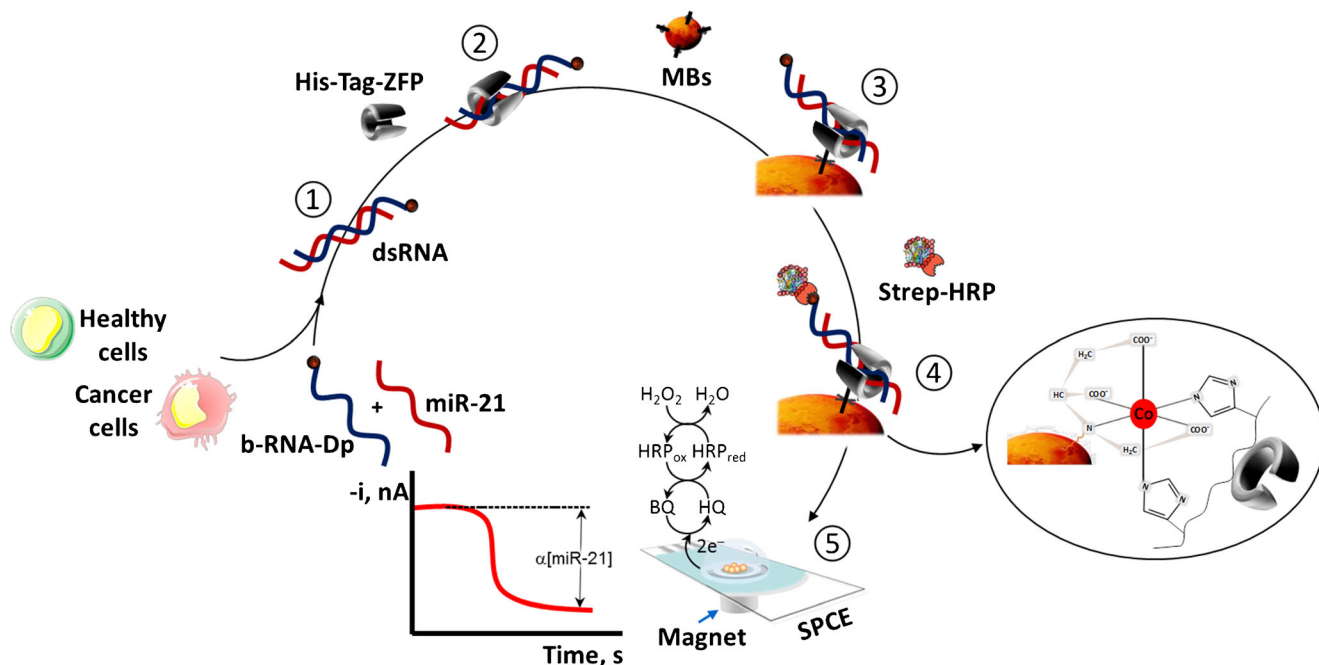


Fig. 1 Preparation of amperometric biosensors for miRNA determination involving ZFP as a biorecognition element, MBs as solid support, and detection at SPCEs

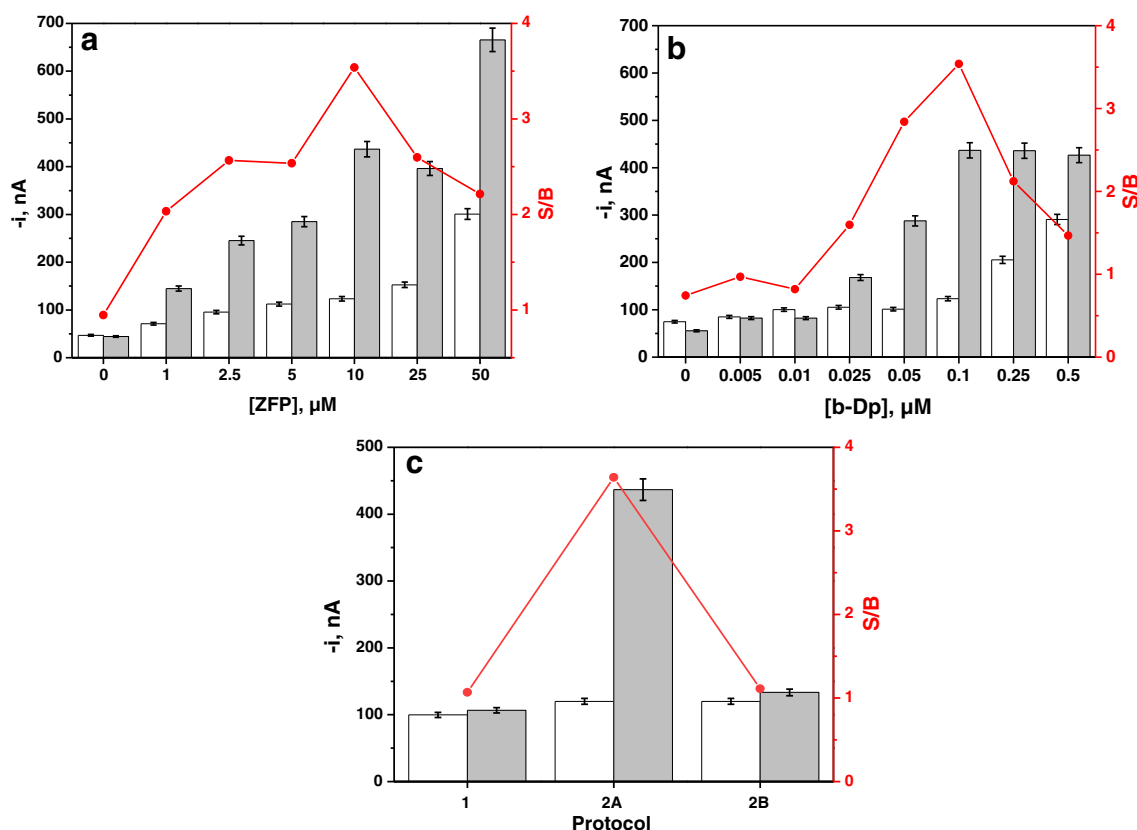


Fig. 2 Dependence of the amperometric responses measured in the absence (white bars) or in the presence (gray bars) of 50 nM miR-21 and the resulting signal to blank (S/B, red lines) current ratios as a

function of the ZFP (a) and b-RNA-Dp (b) concentrations and the different protocols used to perform the assay (c). Error bars estimated as triple of the standard deviation ($n = 3$)

presence of 50.0 nM miR-21 was achieved when the buffer not containing Zn^{2+} was used in the recognition of dsRNA with ZFP, which is in agreement with the reported requirement of this cation to keep the ZFP binding capability [26, 27]. However, protocol 2B showed this cation should be removed from the buffer in which Strep–HRP is incubated because Zn^{2+} inhibits HRP activity [46]. Consequently, protocol 2A was selected to implement the electrochemical biosensing strategy for miR-21 detection.

Results obtained in the evaluation of other experimental variables such as the MBs suspension volume; temperature at which hybridization and ZFP–dsRNA incubation steps were performed; incubation times of b-RNA-Dp+miR-21, b-dsRNA+ZFP, b-dsRNA/ZFP+MBs, and b-dsRNA/ZFP+MBs–Strep–HRP; shaking speed of b-dsRNA/ZFP with MBs; and Strep–HRP dilution are listed in Table S1 and displayed in Fig. S1 of the ESM.

Analytical characteristics

Under optimal experimental conditions, the calibration curve for the amperometric determination of miR-21 exhibited a linear relationship ($R^2 = 0.999$) over the 3.0- to 100-nM concentration range (Fig. 3). The parameters of the calibration

equation were a slope value of $6.4 \pm 0.2 \text{ nA mM}^{-1}$, and an intercept of $123 \pm 8 \text{ nA}$. The limits of detection (LOD) and quantification (LOQ) were estimated according to the $3 \times s_b/m$ and $10 \times s_b/m$ criteria, respectively, where s_b is the standard deviation ($n = 10$) for measurements performed in the absence

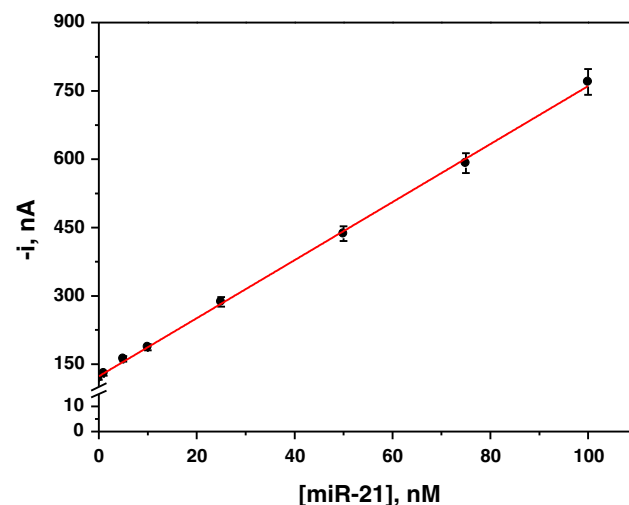


Fig. 3 Calibration plot constructed for the amperometric determination of miR-21 with the ZFP-based biosensor. Error bars estimated as triple of the standard deviation ($n = 3$)

of miR-21, and m is the slope value of the calibration plot. The obtained values were LOD = 0.91 nM and LOQ = 3.0 nM. Considering the required sample volume (25 μ L), the estimated LOD corresponds to detecting 22.7 fmoles of miR-21 with no need for any target miRNA amplification step. Moreover, the amperometric measurements for 50 nM miR-21 made with eight different sensors provided a relative standard deviation (RSD) value of 3.7%, thus demonstrating the robustness of the protocols involved in the whole method.

The achieved LOD is significantly higher than the one claimed for the only electrochemical biosensor reported so far for miRNA determination using ZFP (2 fM) [26]. However, the method described here exhibits relevant comparative advantages over that involving the biosensor reported previously since this demands the use of multiple reagents and time-consuming protocols for the modification of the electrode surface (7 h 20 min) and the enzymatic labeling of the commercial ZFP (~6 h) vs the just 2 h 15 min needed for the MB modification protocol used in this work. These features greatly reduce the cost per determination and simplify the potential implementation of the proposed technology in portable and automatic devices. It is also important to mention that the achieved sensitivity is enough for relevant applications as it is shown in the “Determination of mature miR-21 in RNA_t extracted from cancer cells” section.

Selectivity

Considering the high sequence homology among miRNA family members, an important characteristic of the biosensors developed for the determination of miRNAs is the ability to differentiate the target miRNA from other miRNAs even in complex mixtures. The capability of the developed biosensor to do that was evaluated by comparing the amperometric responses measured in the absence and in the presence of 50 nM

miR-21 with those obtained for two single-base mismatched sequences in the central (1-mc) or terminal (1-mt) position and two fully non-complementary (NC) sequences (miR-205 and miR-122).

Figure 4 shows the amperometric responses measured for the NC sequences were similar to those for the blank solution, thus demonstrating a full selectivity against other non-complementary miRNAs. In addition, the 1-m(c) and 1-m(t) sequences gave only 44% and 65% of the response obtained for the target miR-21, respectively. Apart from the different position of the mismatch in the sequence, these results agree well with the reported stability order of the mismatched base pair: A·G $\geq$ C·A in 1-m(c) and 1-m(t), respectively [26]. Therefore, an acceptable discrimination between homologous miRNA family members was attained, in the unlikely event that the target miRNA coexists with a single mismatched sequence at the same concentration [47].

Moreover, the selectivity of the developed biosensor towards 50 nM ssDNA, ssRNA, dsDNA homohybrids, and DNA–RNA heterohybrids was also checked. As shown in Fig. 4b, the biosensor had higher response for the DNA–RNA heterohybrid, although it was still 14.4% lower than that obtained for miR-21 (Fig. 4a), and provided responses similar to the background current in the presence of ssDNA, ssRNA, and dsDNA, in good agreement with that previously reported by Fang et al. [26]. These results are attributed to the similar structure of the DNA–RNA hybrids and dsRNAs allowing high-affinity binding of ZFP to both types of RNA hybrids [39]. This fact can be profited by the proposed versatile methodology to be used in the analysis of any target miRNA/RNA just by using a DNA/RNA complementary biotinylated detector probe. The affinity of the ZFP used in this work for RNA homo- and heterohybrids makes it advantageous compared with other affinity proteins such as viral p19 protein with affinity only towards small dsRNAs (19–23 bp) [48]. This is

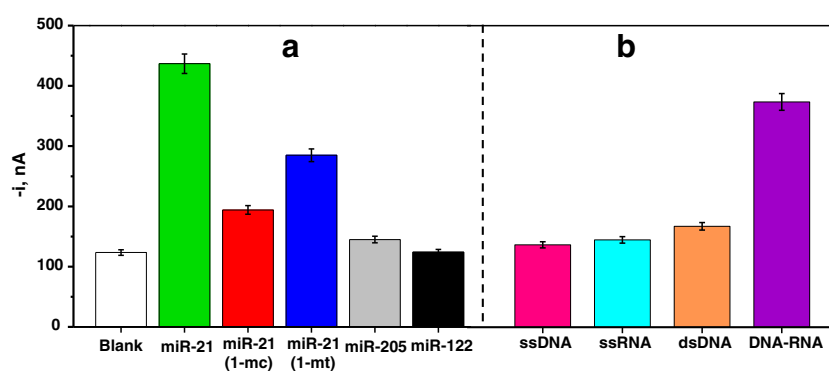


Fig. 4 Selectivity of the developed ZFP-based amperometric biosensor towards miR-21. Amperometric responses measured in the absence or in the presence of 50 nM miR-21 and synthetic sequences with a single-base mismatched at central (1-mc) or terminal (1-mt) position and non-complementary (NC) sequences (miR-122 and miR-205) (a).

Amperometric responses for cDNA to miR-21, cRNA to miR-21, and miR-21 and DNA hybrids (dsDNA: cDNA to miR-21 + b-DNA-Cp) and heterohybrids (DNA–RNA: miR-21 + b-DNA-Cp) (b). Error bars estimated as triple of the standard deviation ($n = 3$)

mainly appealing for synthetic RNA probe stability issues, since miRNAs are reported to be quite stable to pH, temperature changes, and degradation by RNases [49].

Determination of mature miR-21 in RNA_t extracted from cancer cells

The endogenous content of mature miR-21 was determined in RNA_t extracted from cancer (MCF-7) and non-tumorigenic epithelial (MCF-10A) breast cells. The amperometric responses obtained for 1.0 µg RNA_t are depicted in Fig. 5. As can be clearly seen, significantly larger current values were measured in the case of MCF-7 cells, in agreement with the reported overexpression of miR-21 in cancer cells compared with control epithelial breast cells [23, 24, 50], due to the oncogenic function of miR-21 in breast cancer [51]. These results demonstrate that the developed methodology is sensitive enough to detect elevated levels of oncogenic miR-21 directly in real samples without any amplification step.

Conclusions

In this study, we have developed a novel electrochemical biosensor for the facile determination of miR-21, making use of a ZFP as a capture bioreceptor for the first time, MBs as solid supports, and bare SPCEs as electrochemical transducers. This new approach operates at room temperature and it is of outstanding simplicity and with short assay time as compared

with other related methodologies (~2 vs ~7 h). The method is able to perform the selective and sensitive direct determination of miR-21 in RNA_t extracted from breast cancer cells without any amplification, preconcentration, or purification step. Conversely, the only ZFP-based electrochemical biosensor reported so far for the determination of miRNAs requires a more than 3 times longer assay time and involves multireagents/multistep protocols for the modification of both the electrode surface and the affinity protein used as detector bioreceptor, as well as multiple signal amplification (ECC redox cycling involving Ru(NH₃)₆³⁺/Ru(NH₃)₆²⁺, benzoquinone (BQ)/HQ, and tris(2-carboxyethyl)phosphine). Importantly, because ZFP binds non-sequence specifically to any RNA hybrid, the developed method can be readily extended to the single or multiplexed determination of any miRNA/RNA/DNA sequence by using the biotinylated complementary synthetic DNA or RNA strand.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Vidigal JA, Ventura A. The biological functions of miRNAs: lessons from in vivo studies. *Trends Cell Biol.* 2015;25:137–47.
2. Allegra A, Alonci A, Campo S, Penna G, Petrunaro A, Gerace D, et al. Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer. *Int J Oncol.* 2012;41:1897–912.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000;100:57–70.
4. Peng Y, Croce CM. The role of microRNAs in human cancer. Signal transduction and targeted therapy. 2016;1:15004. <https://doi.org/10.1038/sigtrans.2015.4>.
5. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA.* 2002;99:15524–9.
6. Masud MK, Umer M, Hossain SA, Yamauchi Y, Nguyen NT, Shiddiky MJA. Nanoarchitecture frameworks for electrochemical miRNA detection. *Trends in Biomedical Sciences.* 2019;44:433–52.

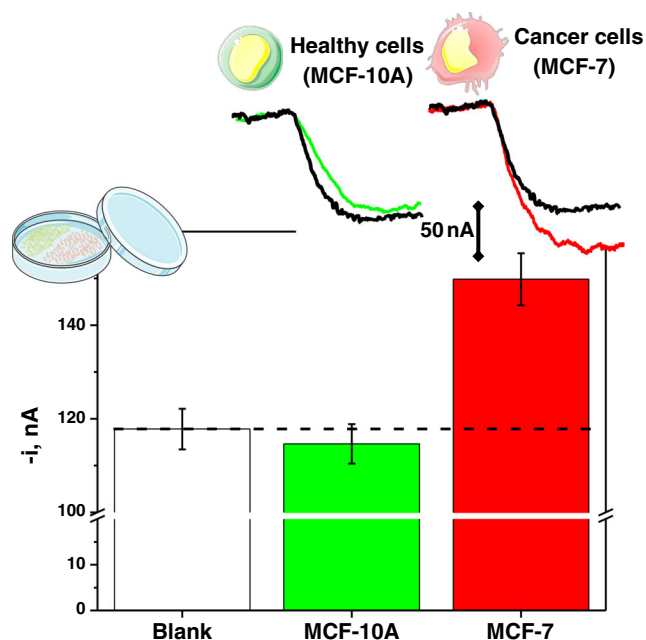


Fig. 5 Amperometric responses measured with the ZFP-based amperometric biosensor for miR-21 in the absence and in the presence of 1.0 µg cellular RNA_t. Error bars estimated as triple of the standard deviation ($n = 3$)

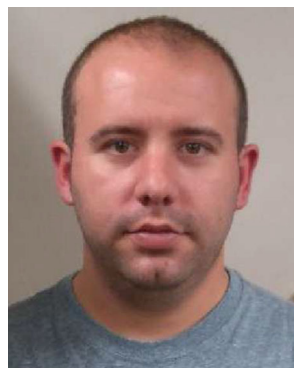
7. Nelson PT, Baldwin DA, Searce LM, Oberholtzer JC, Tobias JW, Mourelatos JZ. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods*. 2004;1:155–61.
8. Castoldi M, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW, et al. A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA*. 2006;12:913–20.
9. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*. 2001;294:862–4.
10. Li J, Yao B, Huang H, Wang Z, Sun C, Fan Y, et al. Real-time polymerase chain reaction microRNA detection based on enzymatic stem-loop probes ligation. *Anal Chem*. 2009;81:5446–51.
11. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods*. 2010;50:298–301.
12. Hunt EA, Broyles D, Head T, Deo SK. MicroRNA detection: current technology and research strategies. *Annu Rev Anal Chem*. 2015;8:217–37.
13. Chen YX, Huang KJ, Niu KX. Recent advances in signal amplification strategy based on oligonucleotide and nanomaterials for microRNA detection—a review. *Biosens Bioelectron*. 2018;99:612–24.
14. Islam MN, Masud MK, Nguyen NT, Gopalan V, Alamri HR, Alothman ZA, et al. Gold-loaded nanoporous ferric oxide nanocubes for electrocatalytic detection of microRNA at attomolar level. *Biosens Bioelectron*. 2018;101:275–81.
15. Voccia D, Palchetti I. Electrochemical biosensors for miRNA detection. In: Erdmann V, Jurga S, Barciszewski J, editors. *RNA and DNA diagnostics*. Cham.: RNA technologies. Springer; 2015. p. 1–19.
16. Campuzano S, Pedrero M, Pingarrón JM. Viral protein-based bioanalytical tools for small RNA biosensing. *Trends Anal Chem*. 2016;79:335–43.
17. Planell-Saguer MD, Rodicio MC. Analytical aspects of microRNA in diagnostics: a review. *Anal Chim Acta*. 2011;699:134–52.
18. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X. MicroRNA: function, detection, and bioanalysis. *Chem Rev*. 2013;113:6207–33.
19. Wu J, Campuzano S, Halford C, Haake DA, Wang J. Ternary surface monolayers for ultrasensitive (zeptomole) amperometric detection of nucleic acid hybridization without signal amplification. *Anal Chem*. 2010;82:8830–7.
20. Campuzano S, Kuralay F, Lobo-Castañón MJ, Bartošik M, Vyavahare K, Paleček E, et al. Ternary monolayers as DNA recognition interfaces for direct and sensitive electrochemical detection in untreated clinical samples. *Biosens Bioelectron*. 2011;26:3577–83.
21. Wark W, Lee HJ, Corn RM. Multiplexed detection methods for profiling microRNA expression in biological samples. *Angew Chem Int Ed*. 2008;47:644–52.
22. Lee JM, Jung Y. Two-temperature hybridization for microarray detection of label-free microRNAs with attomole detection and superior specificity. *Angew Chem Int Ed*. 2011;50:12487–90.
23. Campuzano S, Torrente-Rodríguez RM, López-Hernández E, Conzuelo F, Granados R, Sanchez-Puelles JM, et al. Magnetobiosensors based on viral protein p19 for microRNA determination in cancer cells and tissues. *Angew Chem Int Ed*. 2014;53:6168–71.
24. Torrente-Rodríguez RM, Ruiz-Valdepeñas Montiel V, Campuzano S, Farchado-Dinia M, Barderas R, San Segundo-Acosta P, et al. Fast electrochemical miRNAs determination in cancer cells and tumor tissues with antibody-functionalized magnetic microcarriers. *ACS Sens*. 2016;1:896–903.
25. Vargas E, Torrente-Rodríguez RM, Ruiz-Valdepeñas Montiel V, Povedano E, Pedrero M, Montoya JJ, et al. Magnetic beads-based sensor with tailored sensitivity for rapid and single-step amperometric determination of miRNAs. *Int J Mol Sci*. 2017;18:2151. <https://doi.org/10.3390/ijms18112151>.
26. Fang CS, Kim KS, Yu B, Jo S, Kim MS, Yang H. Ultrasensitive electrochemical detection of miRNA-21 using a zinc finger protein specific to DNA–RNA hybrids. *Anal Chem*. 2017;89:2024–31.
27. Fang CS, Kim K, Ha DT, Kim M, Yang H. Washing-free electrochemical detection of amplified double-stranded DNAs using a zinc finger protein. *Anal Chem*. 2018;90:4776–82.
28. Ren Y, Deng H, Shen W, Gao ZA. Highly sensitive and selective electrochemical biosensor for direct detection of microRNAs in serum. *Anal Chem*. 2013;85:4784–9.
29. Campuzano S, Yáñez-Sedeño P, Pingarrón JM. Electrochemical biosensing of microribonucleic acids using antibodies and viral proteins with affinity for ribonucleic acid duplexes. *Electrochim Acta*. 2017;230:271–8.
30. Camenisch TD, Brilliant MH, Segal DJ. Critical parameters for genome editing using zinc finger nucleases. *Mini-Rev Med Chem*. 2008;8:669–76.
31. Klug A, Schwabe JW. Protein motifs 5. Zinc fingers. *FASEB J*. 1995;9:597–604.
32. Kim MS, Stybayeva G, Lee JY, Revzin A, Segal DJ. A zinc finger protein array for the visual detection of specific DNA sequences for diagnostic applications. *Nucleic Acids Res*. 2011;39:e29. <https://doi.org/10.1093/nar/gkq1214>.
33. Hiraoka D, Yoshida W, Abe K, Wakeda H, Hata K, Ikebukuro K. Development of a method to measure DNA methylation levels by using methyl CpG-binding protein and luciferase-fused zinc finger protein. *Anal Chem*. 2012;84:8259–64.
34. Takano E, Shimura N, Akiba T, Kitayama Y, Sunayama H, Abe K, et al. Pipette tip biosensors for bacterial double-stranded DNA using bioluminescence induced by zinc finger luciferase. *Microchim Acta*. 2017;184:1595–601.
35. Lee J, Tatsumi A, Abe K, Yoshida W, Sodea K, Ikebukuro K. Electrochemical detection of pathogenic bacteria by using a glucose dehydrogenase fused zinc finger protein. *Analyst*. 2014;6:4991–4.
36. Lee J, Yoshida W, Abe K, Nakabayashi K, Wakeda H, Hata K, et al. Development of an electrochemical detection system for measuring DNA methylation levels using methyl CpG-binding protein and glucose dehydrogenase-fused zinc finger protein. *Biosens Bioelectron*. 2017;93:118–23.
37. Kim MS, Kim J. Multiplexed detection of pathogen-specific DNA using engineered zinc finger proteins without target amplification. *Anal Methods*. 2016;8:6696–700.
38. Noh S, Ha DT, Yang H, Kim MS. Sensitive and direct electrochemical detection of double-stranded DNA utilizing alkaline phosphatase-labelled zinc finger proteins. *Analyst*. 2015;140:3947–52.
39. Iuchi S. Three classes of C₂H₂ zinc finger proteins. *Cell Mol Life Sci*. 2001;58:625–35.
40. Zouari M, Campuzano S, Pingarrón JM, Raouafi N. Competitive RNA–RNA hybridization-based integrated nanostructured disposable electrode for highly sensitive determination of miRNAs in cancer cells. *Biosens. Bioelectron*. 2017;91:40–5.
41. Vargas E, Povedano E, Ruiz-Valdepeñas Montiel V, Torrente-Rodríguez RM, Zouari M, Montoya JJ, et al. Single-step incubation determination of miRNAs in cancer cells using an amperometric biosensor based on competitive hybridization onto magnetic beads. *Sensors*. 2018;18:863. <https://doi.org/10.3390/s18030863>.

42. Kilic T, Topkaya SN, Ariksoysal DO, Ozsoz M, Ballar P, Erac Y, et al. Electrochemical based detection of microRNA, mir21 in breast cancer cells. *Biosens Bioelectron.* 2012;38:195–201.
43. Eguílaz M, Moreno-Guzmán M, Campuzano S, González-Cortés A, Yáñez-Sedeño P, Pingarrón JM. An electrochemical immunosensor for testosterone using functionalized magnetic beads and screen-printed carbon electrodes. *Biosens Bioelectron.* 2010;26:517–22.
44. Conzuelo F, Gamella M, Campuzano S, Reviejo AJ, Pingarrón JM. Disposable amperometric magneto-immunosensor for direct detection of tetracyclines antibiotics residues in milk. *Anal Chim Acta.* 2012;737:29–36.
45. Gamella M, Campuzano S, Conzuelo F, Reviejo AJ, Pingarrón JM. Amperometric magnetoimmunosensors for direct determination of D-dimer in human serum. *Electroanalysis.* 2012;24:2235–43.
46. Moyo M. Horseradish peroxidase biosensor to detect zinc ions in aqueous solutions. *Open J Appl Biosens.* 2014;3:1–7.
47. Li F, Peng J, Wang J, Tang H, Tan L, Xie Q, et al. Carbon nanotube-based label-free electrochemical biosensor for sensitive detection of miRNA-24. *Biosens Bioelectron.* 2014;54:158–64.
48. Jin J, Cid M, Poole CB, McReynolds LA. Protein mediated miRNA detection and siRNA enrichment using p19. *BioTechniques.* 2010;48:xvii–xxiii.
49. Gillespie P, Ladame S, O'Hare D. Molecular methods in electrochemical microRNA detection. *Analyst.* 2019;144:114–29.
50. Li L, Feng J, Liu H, Li Q, Tong L, Tang B. Two-color imaging of microRNA with enzyme-free signal amplification via hybridization chain reactions in living cells. *Chem Sci.* 2016;7:1940–5.
51. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA.* 2008;14:2348–60.

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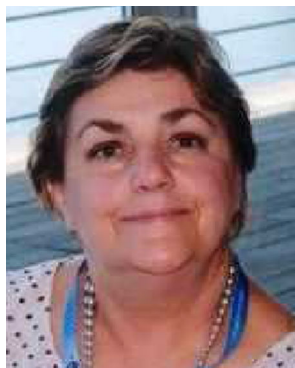


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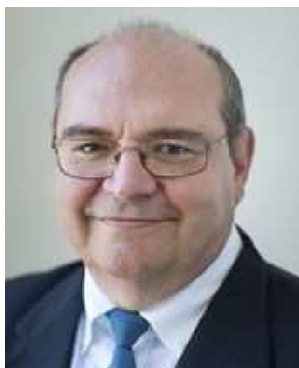


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