Cancer MicroRNAs

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Magnetobiosensors Based on Viral Protein p19 for MicroRNA Determination in Cancer Cells and Tissues**

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Abstract: *MicroRNAs (miRs) have emerged as important clinical biomarkers with both diagnostic and prognostic value for relevant diseases, such as cancer. MiRs pose unique challenges for detection and are currently detected by northern blotting, real-time PCR, and microarray techniques. These expensive, complicated, and time-consuming techniques are not feasible for on-site miR determination. In this study, amperometric magnetobiosensors involving RNA-binding viral protein p19 as a selective biorecognition element were developed for miR quantification. The p19-based magnetosensors were able to detect 0.4 fmol of a synthetic target and endogenous miR-21 (selected as a model for its role in a wide variety of cancers) in only 2 h in total RNA extracted from cancer cells and human breast-tumor specimens without PCR amplification and sample preprocessing. These results open up formidable perspectives for the diagnosis and prognosis of human cancers and for drug-discovery programs.*

MiRs are a class of endogenous small (18–25 nucleotides) noncoding RNAs, which play important roles in various cellular processes. Alterations in miR expression are involved in the initiation and progression of human cancers.[1] Cancer-specific fingerprints of miRs are of great importance, since they can provide information about potential roles of miRs in tumor initiation, the progression of invasion, and metastasis.[2] Circulating miRs can be utilized as minimally invasive biomarkers for the diagnosis of human cancers.[3] These findings highlight the need to make miR testing a routine part of medical care with respect to cancer diagnosis, progression, prognosis, and response to treatment.[4] Therefore, there is an urgent need to develop methods designed to measure miRs in a reliable manner with high specificity and sensitivity. Herein, we describe a novel electrochemical biosensor platform that holds promise as an in situ and effective system for miR determination. MiR-21 was selected as the model target, as it has been identified as the only miR overexpressed in a wide variety of cancers, including breast, ovary, cervix, colon, lung, liver, brain, esophagus, prostate, and thyroid cancers, and as a proven oncogen.[5–7]

Figure 1 illustrates conceptually the fundamentals of our approach. Carnation Italian ringspot virus (CIRV) p19 protein, a tombusvirus (RNA plant virus) protein of 19 kDa that functions as a dimer to bind and sequester only small double-stranded (ds)RNAs of 21–23 nucleotides in length with nanomolar affinity in a size-selective and relatively

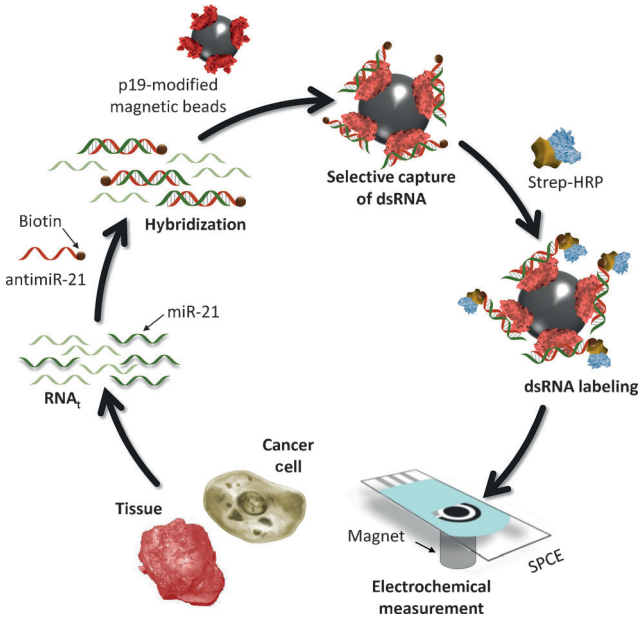

Figure 1. Schematic representation of the p19-based amperometric magnetosensor designed for the determination of miR-21. The components of the magnetosensor are not drawn to scale.

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sequence independent manner,[8,9] was used as the biorecognition receptor. It was immobilized through its terminal chitin-binding domain (CBD) to chitin-functionalized magnetic beads (chitin–magnetic beads) and used as a capture receptor for the miR-21–antimiR-21 duplex (dsRNA) formed in solution by hybridization of the specific and biotinylated antimiR-21 probe to the single-stranded (ss)miR-21 target. Subsequently, the attached biotinylated hybrid was labeled with Strep–HRP polymer (streptavidin protein conjugated to horseradish peroxidase). The electrochemical transduction involved magnetic capturing of the modified magnetic beads on the surface of screen-printed carbon electrodes (SPCEs) and amperometric detection at −0.20 V (versus a Ag pseudo reference electrode) by measurement of the catalytic current upon the addition of H_2O_2 and the use of hydroquinone (HQ) as a redox mediator in solution. Although the use of p19 or magnetic beads for miR detection has been reported previously,[2,10] these previous studies did not involve coupling of the beads to p19 to scavenge the miR duplexes and perform electrochemical detection. In the strategy reported herein, which can be generalized to other research fields, the combined benefits of the use of p19 and magnetic beads enabled the implementation of a very attractive electrochemical methodology for miR detection.

We carried out the experiments in the presence and in the absence of miR-21 to account for any nonspecific binding of p19 or the enzymatic label to the biotinylated antimiR-21 probe on the functionalized magnetic beads (see the Supporting Information for detailed information on all optimization studies). The measured current values were approximately 27 times larger in the presence of 5 nM miR-21, thus confirming that the magnetosensor responses were the result of the selective binding of p19 protein to the RNA duplex. The hybridization between the miR-21 target and the biotinylated antimiR-21 probe sequence generated a blunt-ended 19 bp phosphorylated RNA duplex for which the p19 protein exhibits a high affinity.[11,12] Signals indistinguishable from those of blanks were found when hybridization was carried out with a nonphosphorylated synthetic target, thus confirming that the 5′-monophosphate is critical for tight binding to the p19 protein.[12]

The reproducibility of the amperometric responses obtained with different magnetosensors was evaluated by measuring the current values for 1.0 nM miR-21. The relative standard deviation (RSD) calculated from the measurements made with five different magnetosensors was 3.7%, thus demonstrating the reliability of the procedure for magnetosensor fabrication. The calibration graph constructed for the synthetic miR target under the optimized experimental conditions showed linearity ($r^2 = 0.997$) between 0.14 and 10.0 nM, with a sensitivity of (334 ± 5) nA nM^{-1} and a limit of detection (LOD, estimated as $3 \times s_b/m$, in which s_b is the standard deviation of the blank, and m is the slope of the calibration curve) of 0.04 nM (0.4 fmol in 10 μL of sample), without the use of any amplification technique. Amperometric traces were recorded with the p19-based magnetosensor in the absence of target miR-21 and in the presence of miR-21 at the concentration level corresponding to the detection limit (see Figure S1 in the Supporting Information). Magnetosensors prepared with p19–chitin–magnetic-bead conjugates and stored at 4°C in Eppendorf tubes containing 25 μL of filtered 1 × p19 binding buffer showed no significant decrease in their amperometric response for at least 45 days. The magnetosensor reached a 4000-fold lower LOD than that of a p19-based electrochemical sensor described by Kilic et al., who measured the changes in intrinsic p19 oxidation signals at +0.80 V in an assay time of 160 min.[2] The LOD of our magnetosensor was also 2.5 times lower than that reported for a bioluminescence enzyme assay with p19 and magnetic beads.[9] As compared with the very sensitive three-mode electrochemical sensor reported by Labib et al.,[10] our magnetosensor exhibited a much shorter assay time (approximately 140 min versus 7 h).

The selectivity of the developed methodology was evaluated by comparing the signal produced by the miR-21 target with those of 1-mismatch (1 m) and noncomplementary (NC) sequences (see Figure S2). MiR-192 was tested as a fully noncomplementary sequence because it is also upregulated in breast-cancer patients,[13] but its sequence differs markedly from that of miR-21. Moreover, a synthetic sequence with only one central base mismatched with respect to miR-21 (base underlined in Table S1) was tested (1 m). The current values measured for the NC sequence were similar to that of the blank. Moreover, if 100% complementary hybridization efficiency was assumed for miR-21, this efficiency dropped to approximately 83% for 1 m. This result confirmed an acceptable specificity for profiling miRs with the developed magnetosensor. Notably, the mismatched single base tested is centrally located in the sequence. According to a previous study,[10] a terminal-base mismatched sequence would enable better discrimination. Also, the probability of the simultaneous presence of a target miR and the corresponding sequence with a mismatched central single base is rather low.[14]

Nevertheless, the hybridization selectivity towards the 1 m sequence tested was improved by performing the hybridization step at a higher temperature. The melting temperature was estimated by calculation (http://www.genscript.com/cgi-bin/tools/primer_calculation) for the perfect (PD) and single-mismatch (SM) duplexes with miR-21 as 52 and 48°C, respectively. Therefore, a temperature of 52°C was used in the hybridization step (instead of 25°C). Under these operational conditions, the signal for sequence 1 m was reduced to 49% of that corresponding to the target miR (see Figure S2), thus confirming that simple optimization of the procedure led to remarkably improved discrimination efficiency.

The p19-based magnetosensor was evaluated as an in situ testing system for RNA samples extracted from breast-cancer cells and tissues. The determination of miR-21 from cell lysate is challenging because of the high sensitivity required, since total RNA includes a small amount of the target miR as well as other types of miRs.[15] We employed two different metastatic breast-cancer cell lines (MCF-7 and MDA-MB-231 cells) to determine the miR-21 levels. MCF-7 and MDA-MB-231 cells are well-characterized estrogen-receptor-positive and -negative control cell lines, respectively, and therefore useful cell-based models of breast-cancer cells, in which the levels of miR-21 have been shown to be increased.[16] The

results obtained with these two tumor cell lines were compared with those obtained with MCF-10A cells, which are primary epithelial nontumorigenic cells,[16] and HeLa cells, a human cervix-adenocarcinoma cell line, both of which contain no detectable amount of miR-21.[6]

For these experiments, we used 1–5 μg of total raw RNA (RNA_t) isolated from these cells. The magnetobiosensor response was significantly different with respect to the blank for MCF-7 and MDA-MB-231 RNA_t, thus indicating that miR-21 is highly expressed only in the metastatic cell lines (MCF-7 and MDA-MB-231), and not in the MCF-10A and HeLa cell lines (Figure 2). Consistent with previous findings,[6] miR-21 was overexpressed in MCF-7 and MDA-MB-231 cell lines by a factor of 4–6, as compared to expression in the MCF-10A cell line. No matrix effect was apparent for miR-21 when the amount of RNA_t was lower than 1 μg; therefore, the target miR could be determined by simple interpolation of the amperometric signals measured for 0.5 μg MCF-7 RNA_t extracts into the calibration graph constructed with standard miR-21. The result obtained for five different RNA_t extracts was (18 ± 2) fmol of miR-21 per microgram of RNA_t extracted from MCF-7 cells ($RSD_{n=5} = 10.6\%$), thus demonstrating an acceptable reproducibility of the whole method, including the RNA_t-extraction protocol and the electrochemical magnetosensor performance. We also evaluated the discrimination efficiency towards the tested sequence 1 m by spiking RNA_t (1.0 μg) extracted from MCF-10A cells with the synthetic target (2 nM; ca. 18 fmol) and 1 m oligonucleotides (2 nM; ca. 18 fmol). The amperometric response obtained after hybridization at 25 and 52 °C for the 1 m sequence was 70 and 39 % of that of the target miR (see Figure S3). This result confirmed the enhanced selectivity for 1 m at a selected hybridization temperature even in complex mixtures. This selectivity was higher in the MCF-10A cell lysates than in buffer solutions owing to the more stringent hybridization conditions in complex samples.

We wanted to identify the minimum concentration of detectable miR-21 in the lowest amount of extracted RNA_t. Figure 3 shows the variation in the amperometric responses obtained with the magnetobiosensor as a function of the amount of RNA_t extracted from MCF-7 cells. It was possible to detect miR-21 accurately in only 100 ng of extracted RNA_t (ca. 3.0×10^4 MCF-7 cells). This low RNA_t quantity required constitutes an important advantage with respect to other competitive analytical methodologies. Moreover, the ability of the magnetobiosensor to determine target miR-21 at low concentration in the presence of other nucleic acids was verified by the construction of a calibration plot for miR-21 in the presence of RNA_t (5 μg) extracted from MCF-10A cells. Although the value of the slope of this calibration plot was three times lower than that found when the amount of RNA_t was lower than 1 μg, the range of linearity was similar to that shown in Figure 3, thus demonstrating the ability of the developed sensor to detect miR-21 in the presence of a 30000-fold excess of RNA_t and other miR sequences.

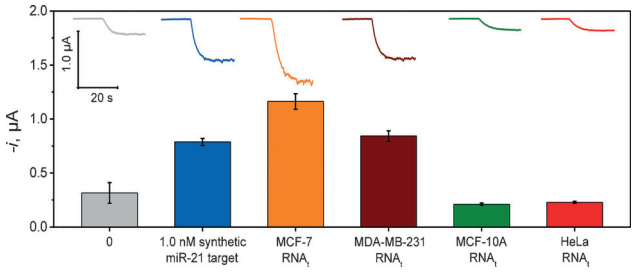

Figure 2. In situ detection of miR-21 in cell lines. Amperometric response measured with the p19-based magnetosensor for RNA_t (0.5 μg) isolated from different cell lines and the synthetic miR-21 target (10 fmol) used as a positive control. Error bars were estimated as triple the standard deviation ($n = 3$).

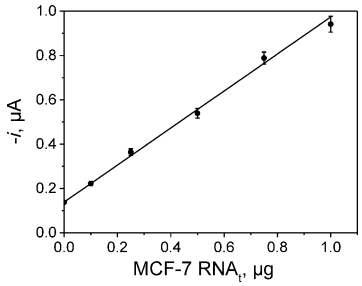

Figure 3. Dependence of the amperometric response on the quantity of RNA_t extracted from MCF-7 cells. Error bars were estimated as triple the standard deviation ($n = 3$).

To expand the applicability of the proposed approach to the detection of miR-21 expression in the RNA_t from breast tumors, and because of the broad interest for the scientific community, we also explored the feasibility of the methodology to detect the target miR in RNA_t extracted from human breast tissues and cytology specimens. This investigation is relevant for delineating the role of miRs in cancer pathology. RNA_t was extracted from breast-cancer (T) tissues and paired normal adjacent (NT) tissues. The responses provided by the magnetosensor discriminated between the miR-21 expression levels in T and NT tissues for each patient (Figure 4). The low level of miR-21 detected in T1 was attributed to the fact that this patient had been treated with chemotherapy before surgery. These results suggest that the measurement of the target-miR level might be used as a prognostic biomarker of breast cancer, and could even enable patient stratification into good and bad responders. These results, in agreement

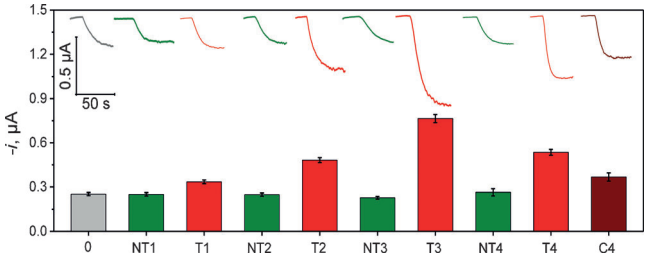

Figure 4. In situ detection of miR-21 in breast tissue and cytology specimens. Amperometric response measured with the p19-based magnetosensor for RNA_t (1 μg) isolated from NT and T tissues of four women diagnosed with breast cancer. Error bars were estimated as triple the standard deviation ($n = 3$). C: Breast cytology.

with the negligible amperometric response obtained with the NC sequence (see Figure S2), demonstrated the high selectivity of the approach for the target miR over other nontargeted miRs when applied to raw RNA_t extracted from cell lines and breast-cancer tissue samples, in which other miRs are present to a large extent.

The magnetobiosensor was also able to detect the target miR level in cytology specimens from human breast cancer (bar C4 in Figure 4). This result is particularly relevant, since cytology provides a sample from the entire cut surface of tumors, thus avoiding difficulties related to tumor heterogeneity or limited size. To the best of our knowledge, there have been no previous reports on the use of electrochemical biosensors for the determination of miRs in human breast-tumor tissue or cytology specimens.

In summary, we have demonstrated that the implemented electrochemical magnetosensor can be employed as a user-friendly, cost-effective on-site device at room temperature, with the convenience of commercial screen-printed electrodes, for the selective (in the presence of nontarget miRs) and sensitive direct determination of miRs in RNA_t raw samples without any amplification, preconcentration, purification, or labeling step. The excellent results obtained in the analysis of a series of RNA_t samples extracted from breast-cancer cell lines and human breast-tumor specimens demonstrate its potential for discriminating upregulated mature miR levels (i.e. in some human malignancies) from normal levels, while avoiding the pitfalls associated with RNA amplification and the requirement of large amounts of RNA_t. It is envisaged that this approach will enable the large-scale production of disposable one-shot miR diagnostics. Routine detection in both clinical and research settings would be feasible with this magnetosensor, which should also allow studying miRs involvement in cancer pathologies with a prognostic and/or diagnostic value. Furthermore, the methodology developed in this study can be readily translated to the determination of other target miRs or multiplexed merely by the use of specific antimiR probes modified with different labels. It could therefore be applied to the fabrication of an array of sensors on a single electrochemical substrate for the sensing of miR expression profiles. Although the concept has been illustrated for the detection of cancer-related miRs, it can be broadened to the diagnosis/prognosis of other relevant illnesses or to completely different disciplines, for example, the targeting of miRs as useful markers for the detection of food fraud (e.g. in olive oil).

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