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Simultaneous detection of two breast cancer-related miRNAs in tumor tissues using p19-based disposable amperometric magnetobiosensing platforms

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Abstract

A novel magnetobiosensing approach for the rapid and simultaneous detection of two breast cancer-related miRs (miR-21 and miR-205) is reported. It involves the use of anti-miR-21 and anti-miR-205 specific probes, chitin-modified magnetic beads (Chitin-MBs), the p19 viral protein as capture bioreceptor and amperometric detection with the H_2O_2 /hydroquinone (HQ) system at dual screen-printed carbon electrodes (SPCEs). The use of dual SPCEs allows the simultaneous independent amperometric readout for each target miR to be measured. The magnetosensor exhibited sensitive and selective detection of both target miRs with a dynamic range from 2.0 to 10.0 nM and detection limits of 0.6 nM (6 fmol) for both target miRs without any amplification step in less than 2 h. The usefulness of the approach was evaluated by detecting the endogenous levels of both target miRs in total RNA (RNA_t) extracted from metastatic breast cancer cell lines and human tissues.

Keywords: miR-21, miR-205, p19, simultaneous detection, Magnetic beads, SPdCEs, cancer samples

1. Introduction

Breast cancer is the most prevalent cancer and the second leading cause of cancer death in women. Like many cancers, breast cancer is a heterogeneous disease that differs molecularly, biologically and clinically. Breast cancer is commonly classified by the estrogen/progesterone (ER/PR) receptor status and the HER2 (ERBB2) amplification status. Breast cancers that are ER+ and or PR+ are treatable with hormonal therapies, while patients having HER2 amplification respond to receptor tyrosine kinase inhibitors. Triple-negative breast cancer (TNBC) represents around 10-20% of breast cancers and is defined by the absence of ER and PR expression and HER2 amplification. The TNBC patients have the worse prognosis because of the lack of effective targeted therapeutics. Successful treatments for these patients

may be possible once the molecular basis of this aggressive phenotype is better understood (Elgamal et al., 2013). Moreover, although tumor markers greatly improve cancer diagnosis, the invasive, unpleasant, and inconvenient nature of current diagnostic procedures limits their application. Hence, there is a great need for identification of novel non-invasive biomarkers for early tumor detection (Chen et al., 2008).

miRNAs (or miRs) are endogenously processed non-coding RNAs of 19-25 nucleotides in length, which control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation. Since their discovery, they have been found to play a role in almost all critical biological processes in cells such as differentiation, proliferation, stress response or apoptosis. Growing evidences suggest that miRs could emerge as revolutionary biomarkers in the cancer field for the diagnosis, prognosis or treatment efficacy. High-throughput profiling of miRs from cancer patients in several types of cells and tissues found that signature groups or 'clusters' of miRs have consistently increased or decreased expression levels when compared to normal tissues, exerting oncogenic or tumor suppressor activity (Bartosik et al., 2014). Although single miRs have been associated with a wide variety of disease conditions and can target multiple mRNAs, several miRs may be simultaneously involved in disease progression and development (Ban et al., 2013). The profiling of different miR types in parallel is important for a comprehensive cancer classification, especially in the case of very heterogeneous cancers—like lymphomas. Consequently, the simultaneous detection of different miRs expression at a single time might allow a more accurate characterization of different tumor subtypes (Zhang et al., 2007) (Weishaupt et al., 2013). However, several intrinsic characteristics of miRs such as their short sequence length, low abundance and high sequence similarity between miR family members, make their detection a challenging task (Dong et al., 2014). Currently, miRs are detected by expensive, complicated and time consuming techniques such as Northern blot, reverse

transcription polymerase chain reaction (RT-PCR), microarrays and RNA sequencing (Lusi et al., 2009) which are not ideally compatible with portable *point-of-care* devices. Therefore, the development of methods capable to measure miRs in a reliable manner with high specificity and sensitivity but also complying the requirements for minute sample volume, cost-effectiveness and multiplexing capability, are highly desired.

In this context, electrochemical nucleic acid biosensors have emerged as particularly attractive options for miRs detection in terms of sensitivity, simplicity of use and automation, short assay time, low detection limit, small amount of sample required, non-toxic experimental steps and adaptability to *point-of-care* testing, (Ramnani et al., 2013).

As miRs represent only a very small fraction (ca. 0.01%) of total RNA mass (Krol et al., 2010), it is important to note that any hybridization based-methods bear the limitation of potential hybridization of the probe with other RNA. Possible interferences in the detection of a mature miR include pri-miR, pre-miR and the longer intermediates of miR biogenesis which possess the sequence motifs of the mature miR. One ingenious way to overcome this problem has been reported through the use of electrochemical sensors based on p19 RNA binding protein (Kilic et al., 2013), (Labib et al., 2013^a), (Campuzano et al, 2014), (Torrente-Rodríguez et al, 2014) which behaves like a molecular caliper of small double-stranded RNA (21–23 base pairs) and sequesters miRs in a size dependent, sequence-independent manner. Thus besides ruling out other nucleotides including single stranded RNA (ssRNA), ssDNA, and dsDNA, a size selectivity for dsRNA in the size range of miRs is achieved. Magnetobiosensors have demonstrated also to be promising approaches for the detection of specific miRs (Betazzi et al., 2013), (Bartosik et al., 2014), (Campuzano et al., 2014), (Torrente-Rodríguez et al., 2014). Despite the increasing number of promising electrochemical sensors described for miRs determination, to our knowledge, none of them allows the simultaneous detection of different miRs in a single test.

Recently two relevant miRs have been shown to be associated with breast cancer: miR-21 and miR-205. In particular, miR-21 acts as a non-specific oncogene and affects tumor invasion and inhibits tumor cell colonization. MiR-205 is a breast cancer specific tumor suppressor involved in angiogenesis (Markou et al., 2014). MiR-205 is also a direct target of the tumor suppressive transcription factor p53, and decreased expression of miR-205 is common in TNBC cells that are frequently p53 deficient (Wang and Lin, 2013) and in cells that had undergone epithelial–mesenchymal transitions (EMT). Studies carried out by Markou et al. (Markou et al., 2014) demonstrated that overexpression of miR-21 and underexpression of miR-205 are clearly associated with shorter disease-free-interval in all early breast cancer patients, whereas miR-205 underexpression is associated with overall patient survival. Furthermore, recent studies have demonstrated that while miR-21 does not discriminate between the different receptor statuses in breast tumors, miR-205 is only significantly downregulated in TNBC patients (Savad et al., 2012). These findings highlight that simultaneous interrogation of miR-21 and miR-205 will allow the unequivocal identification of breast cancer groups (not possible with individual detection of miR-205) making it possible the discrimination between the TNBC group and the others.

In this work, we describe the first electrochemical magnetosensor to simultaneously detect the expression of two different miRs in one single experiment. The implemented methodology involved the use of magnetic beads, specific biotinylated anti-miR probes, the p19 viral protein and amperometric detection at dual SPCEs using the HQ/HRP/H₂O₂ system. Once the dual magnetosensor performance was evaluated, it was successfully applied to the detection of both endogenous miRs in cultured breast cancer cells and human tissues from real breast cancer patients.

2. Materials and Methods

Details about apparatus, electrodes, reagents and solutions and all the protocols used (hybridization, MBs modification, sample analysis and amperometric measurements) are described in detail in the Supporting Information.

3. Results and discussion

The fundamentals of the simultaneous determination of miR-21 and miR-205 using the disposable dual electrochemical magnetosensor are displayed in Fig. 1a. In brief, p19 was immobilized through its terminal CBD to chitin-MBs and used as capture receptor for the miR-21-antimiR-21 or miR-205-antimiR-205 duplexes (dsRNAs) previously formed in solution by homogeneous hybridization of the specific and biotinylated antimiR probe with the single-stranded (ss) target miR. Subsequently, the captured biotinylated hybrid was labeled with Strep-HRP polymer (Fig. 1a). The MBs bearing the dsRNA/p19 complex for each target miR were magnetically captured on the corresponding working electrode (WE 1 and WE 2) of the dual SPCE and amperometric detection at -200 mV of the catalytic current produced upon H_2O_2 addition using HQ as redox mediator in solution was employed to monitor the target miRs concentration. It is important to note that this methodology implied that the dual SPCEs acted only as the electrochemical transducer while all the affinity reactions occurred on the surface of the MBs, thus minimizing unspecific adsorptions of the bioreagents on the electrode surfaces.

The working variables used (summarized in Table S2 in the Supporting Information) were the same than those optimized for the magnetosensor constructed for the individual detection of miR-21 (Campuzano et al., 2014). It is worth to mention that hybridization between the specific biotinylated antimiR probe and the target miR was carried out at 25°C and only for

30 min which constitute important practical advantages with respect to other reported methodologies.

The possible cross-talking between the adjacent working electrodes is considered as a potential major drawback to be solved in the design of electrochemical multisensory platforms because it would lead to false results (Escamilla-Gómez et al., 2009). Fig. 2 shows the amperometric measurements obtained with the dual magnetosensor in solutions containing different miR-21 and miR-205 mixtures. As it can be deduced, no significant cross-talking was apparent even in the presence of a high concentration of the other target miR which endorsed the viability of the dual magnetosensor for the suitable simultaneous detection of both miRs.

These results demonstrated also the feasibility of the proposed assay design as a result of comparing the obtained amperometric responses in the presence and in the absence of the target miRs. This latter current was taken as the negative control to account for any nonspecific binding of the biotinylated RNA probe or the enzymatic label on the functionalized MBs. The results shown in Fig. 2 indicated that the magnetosensor responses were mostly due to the selective binding of the RNA duplexes to the p19 protein, thus demonstrating the potentially of the approach for miR determination.

3.1 Analytical characteristics

The reproducibility of the responses obtained for 5 nM of both miRs with different dual magnetosensors constructed using the same protocol was tested. Amperometric measurements made with 8 dual magnetosensors yielded relative standard deviation (RSD) values of 6.3 and 6.4%, for miR-21 and miR-205, respectively. These values confirmed that the whole dual magnetosensor fabrication procedure, including MBs modification, their magnetic capture on the surface of each SPCE working electrode and the amperometric measurements, was

reliable and that reproducible amperometric responses can be obtained with different biosensors constructed in the same manner.

The analytical performance of the dual magnetosensor was evaluated by constructing calibration curves for both miRs. The corresponding analytical characteristics are summarized in Table S3 in the Supporting Information. Low detection limits (LODs) of 0.61 and 0.62 nM (6.1 and 6.2 fmol in 10 μ L) were calculated according to the $3 \times s_b/m$ criterion, where s_b was estimated as the standard deviation for 10 blank signal measurements and m is the slope value of the calibration plot. It is important to note that these values were achieved without any amplification technique or strategy. Although the analytical characteristics for miR-21 are slightly worse than those reported with the magnetosensor used for the individual determination of this miR (Campuzano et al., 2014), this can be attributed to the remarkably smaller active surface area of the commercial dual SPCEs working electrodes when compared with the single SPCEs (6.3 vs. 12.6 mm²). However, it is important to remark that the LODs achieved with the dual magnetosensor are sufficient to allow the detection of target miRs in tumor breast cells and tissues as it will be shown below.

In addition, the analytical characteristics provided by the dual magnetosensor were also compared with the reported data for other electrochemical sensors used for miRs determination (Table 1). It can be deduced that the achieved LODs are higher than those reported using approaches involving multiple reagents and complex and time consuming working protocols including amplification strategies (Pöhlmann and Sprinzl., 2010), (Yin et al., 2012), (Labib et al., 2013^b), (Betazzi et al., 2013), (Meng et al., 2013), (Wen et al., 2013), (Li et al., 2014), (Yang et al., 2014). The LODs are also higher than that reported by Betazzi et al. for miR-222 involving biotinylated RNA capture probes immobilized on streptavidin-coated paramagnetic beads and previous labeling with biotin (Betazzi et al., 2013), but are better than that reported by Bartosik et al. for miR-522 using a similar strategy with labeling

of the target miR with an electroactive osmium(VI) complex (Bartosik et al., 2014). Besides the LOD values, it is important to mention that the approach reported here did not require previous target labeling to perform the determination which constitutes a very important practical advantage.

3.2 Selectivity

Due to the sequence homology of miR family members, a significant challenge in their analysis is achieving differentiation of the target miRs from other miRs and detect them selectively even in complex mixtures. The selectivity of the dual amperometric magnetosensor was investigated by comparing the current values measured for the blank, synthetic targets, sequences with only one central base mismatched (1-m) with respect to the target miRs (based underlined in Table S1), fully non-complementary (NC) sequences and mixtures of the NC sequences containing or not the synthetic targets (each at a concentration of 5 nM). Apart from miR-21 in the case of miR-205 detection and vice versa, miR-192 was assayed as the NC sequence because it also appears up regulated in breast cancer patients (Wu et al., 2011) but its sequence differs markedly from that of the target miRs. Fig. 3 shows the obtained results. When the NC sequences, or a mixture of them, were assayed, current values similar to that of the blank were measured (red, black and yellow bars in Fig. 3). Moreover, an acceptable discrimination between the target miRs and the corresponding 1-m sequences was also observed. Assuming 100 % complementary hybridization efficiency for each target miR, this efficiency dropped to approximately 86 % for the 1-m sequences (green bars in Fig. 3). Additional studies showed that discrimination towards the corresponding 1-m sequences could be improved reducing their signal to 62 % of that corresponding to the target miRs by performing the hybridization at the melting temperature of each target miR, which is in agreement with data reported previously (Bartosik et al., 2014). On the other hand, the

mixtures containing the target miR and the NC sequences (blue bars in Fig. 3) provided amperometric responses similar to those measured for the target miR alone. These results demonstrated the high selectivity of the approach to discriminate the target miR over other non-targeted miRs, which allowed their detection in raw RNA_t extracted from cell lines and breast-cancer tissue samples where other non target miRs are present in a large extent.

3.3 Detection of miR-21 and miR-205 in RNA_t extracted from breast cancer cells and tumor tissues

Given the undeniable value of detecting miR-21 and miR-205 expression levels in breast cancer tissues, the usefulness of the developed methodology for the simultaneous detection of these target miRs in RNA_t extracted from breast-cancer cells and human tissues was verified.

The methodology was applied to three different metastatic breast-cancer cell lines: MCF-7 (an ER+/PR+ cell line), SK-BR-3 (an ER-/PR- cell line) and MDA-MB-231 (a TNBC cell line) as well as to primary epithelial nontumorigenic cells (MCF-10A) with no detectable amount of miR-21 (Ribas et al., 2013), (Elgamal et al., 2013).

Fig. 4 displays as the results provided with the magnetosensor showed over-expression of miR-21 and reduced expression of miR-205 in the metastatic breast cell lines compared to the normal-like MCF-10A cell line, which was in good agreement with that reported earlier (Ribas et al., 2013), (Campuzano et al., 2014), (Elgamal et al., 2013). Moreover, the magnetosensor response to miR-205 was higher for MCF-7 than for MDA-MB-231 and SK-BR-3 cells which is in agreement with the specificity of miR-205 to ER/PR status, showing higher expression in ER α + / PR+ with respect to ER-/PR- receptor profile (Mattie et al., 2006). The same methodology was also applied to the detection of both miRs in RNA_t extracted from human breast tissues. RNA_t was extracted from tumor (T) and paired normal

adjacent tissues (NT) from different breast cancer patients. Similarly (Fig. 4a, right), and in agreement also with previous findings, while miR-21 was highly overexpressed in breast tumor tissues compared to the matched normal breast tissues (Si et al., 2007), miR-205 was underexpressed in T tissues (Singh and Mo, 2013). It is worth to mention that Fig. 4a shows only one datum for normal tissues because there were not significant differences between the current values measured for both miRs in this kind of samples. Moreover, the miR-21 overexpression levels observed in tumor tissues were, in all cases, in good agreement with the overexpression ratios range (1.557–2.449) reported by Savad et al. (Savad et al., 2012) in tissues from different breast cancer groups.

Lastly, in order to evaluate the possibility of reducing drastically the assay time, the performance of the methodology was tested by shortening considerably each step involved, i.e., 1) homogeneous hybridization between the target miRs and the anti-miR probes (30 min), 2) capture of the dsRNAs by the p19-MBs (60 min) and 3) labeling of the captured dsRNA with Strep-HRP (15 min). The three steps were shortened to only 5 min, thus reducing the total assay time from 105 to 15 min. Interestingly, Figure 5 shows that although, as expected, the sensitivities were smaller (~2 times lower for both miRs), the obtained amperometric responses were still sufficiently large to discriminate clearly between up regulated and normal mature miR levels. This relevant result outlined the potentiality of the developed methodology to be employed as a rapid method for alarm or screening purposes able to discriminate tumoral vs. non-tumoral tissues.

4. Conclusions

We report in this work the first disposable amperometric magnetosensor for the simultaneous detection of miR-21 and miR-205 making it possible to match the clinically relevant concentration ranges of both cancer biomarkers. The dual amperometric magnetosensor

exhibits excellent analytical performance and was employed successfully to measure the endogenous levels of both target miRs directly in RNA_t raw samples extracted from breast-cancer cell lines and human breast-tumor specimens without prior amplification, preconcentration or purification steps in about 2 h. These results suggest that the simultaneous detection of multiple miRNAs is possible through a combination of multiplexed detection and different RNA probes. Although this paper is focused only on two specific miRNAs, the presented methodology could be easily extended to the simultaneous profiling of multiple miRNAs. The simple magnetosensor design, avoiding complicated temperature control, and the relatively low cost of the detection platform may open a new avenue for multi-miRs bioanalysis with significant potential applications in cancer research and clinical diagnosis.

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Legends to Figures

Fig. 1. Schematic display of the fundamentals involved in the development of the disposable dual magnetosensor for the simultaneous determination of miR-21 and miR-205 (a). Picture showing the dual SPCE and the homemade magnet holding block (up), and the modified MBs on the dual SPCE assembled on the magnet holding block and in the specific cable connector (down) (b).

Fig. 2. Simultaneous amperometric responses measured with the dual magnetosensor for miRs mixtures containing: 0 nM of both miRs (1); 10 nM miR-205 and no miR-21 (2); 10 nM miR-21 and no miR-205 (3); 10 nM of both miRs (4). $E_{app} = -0.20$ V vs. Ag pseudo-reference electrode. Error bars estimated as a triple of the standard deviation ($n=3$).

Fig. 3. Selectivity tests with the dual amperometric magnetosensor. Current values were measured in the absence (blank signal, white bar) or in the presence of synthetic target (grey bar), 1-m (green bar) and NC sequences alone (NC1: miR-205 or miR-21, red bar; NC2: miR-192, black bar) and mixtures of the NC sequences in the absence (yellow bar) and in the presence (blue bar) of the target miR. All miRs were assayed at a 5 nM concentration. Error bars estimated as a triple of the standard deviation ($n=3$).

Fig. 4. Simultaneous detection of miR-21 and miR-205 in RNA_t extracted from different cell lines (a) and breast tissues from breast cancer patients (b). Error bars estimated as triple of the standard deviation (n=3). Amperometric traces recorded with the dual magnetosensor for the samples indicated.

Fig. 5. Detection of miR-21 and miR-205 in RNA_t extracted from human breast tissues in a total assay time of 15 min. Error bars estimated as triple of the standard deviation (n=3).

Table 1.- Electrochemical genosensors reported for miRs determination.

Methodology	Technique	Sample	Concentration Range	LOD	Assay time	Reference
Gap hybridization assay based miR-21 and miR-16 determination based on four components DNA/RNA hybridization and electrochemical detection using esterase 2-oligodeoxynucleotideconjugates	Potential of the working electrode is pulsed (250 ms, +200 mV; 750 ms, -200 mV), and measuring phases and relaxation phases are alternately produced	RNA _t isolated from MCF-7 cells	200-0.002 nM miR-16	2 pM (2 amol)	~1 h	(Pöhlman and Sprinzl, 2010)
Locked nucleic acid-integrated nucleic acid hairpin probe, a biotin-labeled bridge DNA–AuNPs–bio-barcode signal amplification unit and enzymatic signal amplification for determination of miR-21	Cyclic voltammetry (CV) and chronoamperometry (CA)	RNA _t extracted from human hepatocarcinoma cells BEL-7402	0.01–700 pM	6 fM	~147 h	(Yin et al., 2012)
Covalent immobilization of the capture probes and/or cell lysates with EDC and NHS onto pencil graphite electrode(PGE), hybridization with the biotinylated complementary target and labelling the resultant hybrid with extravidin labeled alkaline phosphatase (Ex-Ap) for the determination of miR-21	DPV (guanine and alpha naphthol oxidation)	RNA isolated from breast cancer cell lines	—	1 μM (6 pmol)	~1 h 40 min	(Kilic et al., 2012)
DNA four-way junction based electrochemical sensor (4J-SENS)	SWV in K ₃ [Fe(CN) ₆] and [Ru(NH ₃) ₆]Cl ₃	Serum samples	10 aM-1 fM	2 aM	~5 days 1 h and 35 min	(Labib et al., 2013a)
Magnetic bead-based bioassay for the detection of miR-222 and enzyme amplification	DPV	RNA samples from non-small-cell lung cancer and glioblastoma cell lines	7 pM-2.5 nM	7 pM	~100 min	(Betazzi, el al., 2013)
Biosensor for miR-21 detection based on DNA-Au bio bar code and G-quadruplex-based DNAenzyme	DPV	miR-21 extracted from human hepatocarcinoma BEL-7402 cells and human mastocarcinoma MCF-7 cells total RNAs	0.01–500 pM	0.006 pM	~32 h	(Meng et al., 2013)
Tetrahedral nanostructure-based electrochemical miR biosensor for miR-21 and let-7d	Amperometry	miR-21 extracted from tumor and normal tissues of human liver, lung and prostate	10 fM to 10 nM (miR-21)	10 fM (miR-21)	~18 h	(Wen et al., 2013)
Detection of miR-21–antimiR-21 hybrid structure through the changes of intrinsic p19 oxidation signals at +0.80 V	DPV	Total RNA extract of PBS1 cell line	—	0.16 μM (1.6 pmol)	~160 min	(Kilic et al., 2013)
Three-mode electrochemical sensor: hybridization, p19 protein and protein displacement for determination of miRs	SWV	three endogenous miRs: hsa-miR-21, hsa-miR-32, and hsa-miR-122 in human serum	10 aM to 1 μM	5 aM	~7 h	(Labib et al., 2013b)
Immobilization of synthetic DNA probes onto the surface of MWCNT-modified glass carbon electrodes by covalent cross-linking for determination of miR-24	DPV	Recovery studies in total RNA samples	1 pM-1 nM and 1-10 nM	1 pM	~27 h	(Li et al., 2014)

Biotinylated inosine substituted DNA probe immobilized onto Strep-MBs	DPV	—	—	1.4 μ M (4.3 pmol in 3 μ L samples)	~45 min	(Erdem and Congur, 2014)
Magnetic bead-based bioassay for the detection of miR-522 after labelling the target miR with and electroactive complex of osmium(VI) and 2,2'-bipyridine (Os(VI)bipy)	DPV	RNA _i extracted from cancer cell lines	10-200 nM	10 nM	~70 min	(Bartosik et al., 2014)
Hybridization between target miR-21 and a specific and biotinylated detector probe, capture of the resultant hybrid by p19-MBs and labelling with the Strep-HRP polymer	Amperometry	RNA _i extracted from cancer cell lines, tumour tissues and breast cytologies	0.14-10.0 nM	40 pM (0.4 fmol)	~140 min	(Campuzano et al., 2014)
Thiol-modified, redox species-labeled hairpin probes self-assembled on gold electrodes and duplex specific nuclease-assisted target recycling signal amplifications	SWV	Cancer cell lysates	5.0 fM-50 pM	3.0 fM	~6.5 h	(Yang et al. 2014)
Hybridization between the target miR and its specific and biotinylated detector probe, capture of the resultant hybrid by p19-MBs and labelling with the Strep-HRP polymer	Amperometry	RNA _i extracted from cancer cell lines and tumour tissues	2.0-10.0 nM	0.61 nM	~2 h	This work

Highlights:

- First amperometric magnetosensor for simultaneous detection of two miRs.
- Detection limits of 0.6 nM (6 fmol) without any amplification step in less than 2 h.
- Successful detection in breast cancer cell lines and human tissues.
- Methodology easily extended to the simultaneous profiling of multiple miRs.
- Potential applications in cancer research and clinical diagnosis.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Graphical Abstract

Disposable amperometric dual magnetosensor using specific biotinylated RNA probes and p19 as biorecognition elements for the simultaneous detection of miR-21 and miR-205.