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An amplification-free electrochemical detection of exosomal miRNA-21 in serum samples

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Abstract

Recent evidence suggests that small non-coding RNAs or microRNA (miRNA)s encapsulated in exosomes represent an important mechanism of communication between the cells. Exosomal miRNAs play an important role in carcinogenesis via enhancing cell to cell communication and targeting cell growth molecular pathways which in turn facilitate metastasis in cancers. Despite progressive advances, current methods for exosomal miRNA detection most rely on labor intensive sequencing approaches which are often prone to amplification bias and require costly and bulky equipment. Herein, we report an electrochemical approach for detection of cancer-derived exosomal miRNAs in human serum samples by selectively isolating the target miRNA using magnetic beads pre-functionalized with capture probes and then directly absorbing the targets onto the gold electrode surface. The level of adsorbed miRNA is detected electrochemically in the presence of the [Fe(CN)₆]⁴⁻/₃⁻ redox system. This method enabled an excellent detection sensitivity of 1.0 pM with a relative standard deviation (RSD) of <5.5% in cancer cells and serum samples (n = 8) collected from patients with colorectal adenocarcinoma. We believe that our approach could be useful in clinical settings for the quantification of exosomal miRNA in cancer patients.
Introduction

Exosomes are lipid bilayer encased extracellular vesicles ranging in size from 30-150 nm and are secreted by most cell types. Exosomes encapsulate a discrete set of transferable functional biomolecules like proteins, lipids, and nucleic acids providing a unique way of cell-cell communication, horizontal transfer of genetic information, and modulation of recipient cell transcriptome. Thus, these nano-vesicles play vital functions in many biological processes such as; T-cell activation and antigen presentation, coagulation, inflammation, and angiogenesis. Tumor secreted exosomes have been shown to be involved in cancer promoting and pro-metastatic functions indicating their potential as targetable entities for development of novel cancer therapies. Moreover, as exosomes can be found in almost all body fluids, they hold a great potential as minimally invasive disease biomarkers especially for diagnosis, prognosis, and therapy response evaluation in cancer patients.

MicroRNAs (miRNAs), a family of small non-coding RNA molecules (~22 nucleotides), act as critical post-transcriptional regulation of gene expressions in the pathogenesis of many chronic diseases including cancer. MiRNA expression patterns are found to be altered in almost all cancer types suggesting that monitoring the expression of individual miRNAs may serve as reliable cancer biomarkers. For instance, the high expression level of miRNA-21 is associated with poor therapeutic outcome and low survival rate in patients diagnosed with colorectal carcinoma. Recent studies have shown that miRNAs also constitute a key component of exosomal cargo and exosome delivered tumor miRNAs have been implicated in tumor progression via modulation of microenvironment, angiogenesis, immune evasion, as well as metastasis. Moreover, due to the outer lipid membrane of exosome, exosomal miRNAs are generally protected from degradation by RNAase, therby offering a stable source of miRNA in
the peripheral blood. This inherent property of exosomes could be useful for developing miRNA-based liquid biopsy biomarkers.\textsuperscript{12b,13,14}

Over the past decades, plenty of approaches such as quantitative real-time PCR (qRT-PCR), microarrays, Northern blotting, and massively parallel sequencing (MPS) have been used for miRNA analysis.\textsuperscript{15,16} Each of these methods have their own advantages and drawbacks. For instance, qRT-PCR provides an excellent sensitivity and flexibility and is considered as a gold standard for miRNA analysis.\textsuperscript{17,18} Nevertheless, amplification bias and artefacts are among the biggest pitfalls of qPCR based miRNA quantification methods. Furthermore, being short-sized molecules, miRNAs require poly(A) tailing to be detectable through routine PCR assays. However, the efficiency of E. Coli poly(A) polymerase is strongly dependent on terminal nucleotide (A = G > C > U) indicating the inherent bias of this approach towards certain sequences.\textsuperscript{19a} Another alternative way to avoid primer size match with miRNA is the use of specially designed stem-loop reverse transcriptase primer that lengthens the target cDNA. A forward PCR primer increases the length of the target sequence while the reverse primer destabilizes stem loop structure. The assay functionality can further be enhanced using specific hybridization between fluorogenic-labeled probes, which generates a fluorescent signal. Although this assay significantly reduces background and false positives, the overall protocol is expensive due to the requirement of multiple primers and probes.\textsuperscript{19b} MPS is cost and labor intensive, requires substantial bioinformatics support and is best suited for discovery of novel miRNAs.\textsuperscript{20} On the other hand, microarray platforms are less specific when compared to qPCR or sequencing. Other shortcomings of molecular biology based approaches that limit their application for routine clinical analysis include; inability to quantify miRNAs in absolute terms and reliance on expensive sophisticated instruments.\textsuperscript{16}
Electrochemical biosensors represent promising candidates for the development of fast, cost-effective, and amplification free miRNA detection platforms.\(^21,22\) Furthermore, multiplexing and miniaturization capabilities of these devices make them particularly suitable for point-of-care applications. Consequently, a large number of electrochemical miRNA detection methods have been devised so far.\(^23,24\) However, although highly sensitive, these electrochemical genosensing platforms also have certain limitations. For example, complicated fabrication processes are involved in the generation of nanomaterials based devices, while high level of non-specific binding to the electrode surface is observed in platforms in which miRNA capture probes are adsorbed directly onto the electrode surface.\(^25\) Further, signal amplification strategies based on redox reporters or chemical ligations sometimes lead to a loss of dynamic range, which may be a particularly crucial consideration in clinical settings where a high degree of inter-sample variability in levels of target analyte/s is observed.\(^26\) Over the last several years, a number of amplification-free approaches have been developed to overcome these shortcomings.\(^27-30\) Recently, our group has developed an amplification free electrochemical miRNA detection method. Exploiting a range of superior characteristics that superparamagnetic gold-loaded nanoporous iron oxide nanomaterials offer, our method achieved miRNA detection sensitivity down to 100 fM in complex biological samples such as esophageal squamous-cell carcinoma tissue samples.\(^31a\) Despite the high level of sensitivity that was achieved in this report, sensor fabrication and particle synthesis steps add to the complexity and cost of device manufacturing. Therefore, a simple and inexpensive method for miRNA quantification is required which can overcome these challenges and is at the same time able to achieve a clinically relevant sensitivity. Furthermore, to the best of our knowledge, no method for electrochemical detection of exosomal miRNAs has so far been reported.

Herein, we report an-amplification free assay for electrochemical detection of exosomal miRNAs in complex biological samples. Our approach relies on the capture of target miRNA
by hybridization with biotinylated complimentary probes attached to commercially available
streptavidin coated magnetic beads. Post-hybridization, captured miRNA species were heat
released from the hybrid and their level was subsequently monitored by the direct adsorption
onto SPE-Au via RNA-gold affinity interaction followed by the differential pulse voltammetry
(DPV) response in the presence of [Fe(CN)$_6$]$^{4-/3-}$ redox system. We selected miRNA-21 as a
model biomarker considering its well-known regulatory function in a variety of cancers and
subsequently demonstrated its detection in exosomes isolated from SKBR-3 (breast), HKESC-
1 (esophageal) and SW-48 (colon) cancer cell lines. We also tested the clinical applicability
of our assay in serum samples collected from patients with colorectal adenocarcinoma.

Experimental

Reagents and materials

Unless otherwise stated, all the reagents and chemicals used in this study were of
analytical grade and purchased from Sigma Aldrich (Sydney, NSW, Australia). DNase/RNase-
free distilled water (Invitrogen, Australia) was used for preparing all aqueous solutions. Gold
screen printed electrodes Au-SPE (DRP-C250BT) were purchased from Dropsens (Spain).
Synthetic miRNA, capture probe and primers were purchased from Integrated DNA Technologies
(Coralville, IA, USA) and sequences are shown in Table 1.

Cell Culture, isolation of exosomes and exosomal RNA

Patient derived cancer cell lines (n=3) from breast (SKBR-3), colon (SW-48) and
oesophageal (HKESC-1) cancers were used. SKBR-3 and SW48 were purchased from ATCC
(USA). HKESC-1 was from a patient with esophageal squamous cell carcinoma and with the
characterization published.²⁷b SKBR-3 and SW-48 cell lines were cultured in RPMI-1640 while
minimum essential medium alpha (MEMa) growth medium was used for HKESC-4 cells (Life
Technologies, Australia). Cell culture media were supplemented with 10% exosome depleted fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Australia). The cells were grown in 25 cm\(^2\) culture flask (Corning, Australia) and maintained in a humidified incubator containing 5% CO\(_2\) at 37ºC. The conditioned medium was collected after 60 h and centrifuged at 2000×g for 30 min to eliminate contaminants (e.g., cells and debris). Exosomes were isolated using total exosome isolation reagent (Life Technologies, Australia) as per manufacturer’s instructions. Briefly, the cell-free conditioned medium was transferred to a new tube and mixed with the isolation reagent in 2:1 ratio. The samples were incubated overnight at 4ºC followed by centrifugation at 10000×g for 1 h to obtain exosome pellets. Exosome pellets were subsequently resuspended in 50 μL phosphate buffered saline (PBS) (10 mM, pH 7.0) and stored at -20ºC until further analysis. RNA was extracted from exosomes using total exosome RNA & protein isolation kit (Life Technologies, Australia) as per manufacturer’s instructions.

**Clinical samples**

Serum samples from eight patients diagnosed with colorectal adenocarcinoma were obtained from the patients underwent resection for the cancer. The study was conducted after approval from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC). Mean age of the patients (3 males; 5 females) was 72 years, ranging from 60-85 years. These patients diagnosed with colorectal adenocarcinomas were of different pathological stages at the time of sugery (stage I to IV). Exosomes were isolated from the serum samples using total exosome isolation reagent (Life Technologies, Australia) as per manufacturer’s instructions. Briefly, the samples were centrifuged at 2000×g for 30 mins to remove the sedimented cells and debris. The supernatant was transferred to a new tube and mixed with the isolation reagent in the ratio 0.2:1.0. The samples were incubated for 30 min at 4ºC followed by the centrifugation at 10000×g for 10 min to obtain exosome pellets. Exosome pellets were re-suspended in 100 μL PBS (10 mM, pH 7.0) and stored at -20ºC. RNA was extracted
from exosomes using total exosome RNA & protein isolation kit (Life Technologies, Australia) as per manufacturer’s instructions.

**Magnetic isolation of miRNA and adsorption on electrode surface**

20 μL of streptavidin-labelled Dynabeads MyOne™ Streptavidin C1 (Invitrogen, Australia) magnetic beads were washed with 2x binding and wash (B&W) buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl) and resuspended in 25 μL of 2X B&W buffer. Next, 25 μL of 10 μM biotinylated capture probes was added, followed by incubation on a thermomixer for 20 mins at room temperature to facilitate the functionalization of magnetic beads with capture probes. Afterwards, the functionalized magnetic beads were washed three times and mixed in 10 μL of 5X saline sodium citrate (SSC) buffer (pH 7.0).

For specific miRNA capture, 50 ng of extracted total RNA from cell lines and clinical samples or various concentrations of synthetic miRNA-21 sequences were adjusted up to 10 μL with RNase-free water and then mixed with 10 μL of capture probes-functionalized magnetic beads. The mixture was incubated on a thermomixer for 20 mins at room temperature to allow the hybridization of capture probe and target, followed by magnetic separation of beads with attached miRNA targets. Beads were washed twice before being resuspended in 9 μL of RNase-free water. Beads were subsequently heated at 95°C to release captured miRNA targets, and the supernatant containing released miRNAs was separated magnetically from the beads. Collected supernatant was diluted with 5X SSC buffer (pH 7.0) up to a final volume of 20 μL and appropriate sample volume was loaded directly onto the working surface of an SPE-Au before being incubated for 20 min. The electrodes were washed with 10 mM phosphate buffer (137 mM sodium chloride, 2 mM potassium chloride, pH 7.4) before electrochemical readout.
Cryo-Transmission Electron Microscopy (Cryo-TEM)

For cryo-TEM, 4 μL of exosome preparations were directly adsorbed onto lacey carbon grids (Quantifoil, Germany) and plunged into liquid ethane, using an FEI Vitrobot Mark 3 (FEI Company, The Netherlands). Grids were blotted at 100 % humidity at 4 °C for about 3−4 s. Frozen/vitrified samples were imaged using a Tecnai T12 transmission electron microscope (FEI Company) operating at an acceleration voltage of 120 kV. Images were taken at 30000× magnification (approximate dose of 13.6 electrons/Å2) using an FEI Eagle 4k CCD (FEI Company) and SerialEM image acquisition software.  

Electrochemical Detection

All electrochemical measurements were performed on a CH1040C potentiostat (CH Instruments, USA) with the three-electrode system (gold working, platinum counter and silver reference electrode) on each screen-printed gold electrode. Differential pulse voltammetric (DPV) responses were recorded at -0.3 V to 0.5 V with a pulse amplitude of 50 mV and a pulse width of 50 ms in 10 mM PBS solution containing 2.5mM [K₃Fe(CN)₆] and 2.5mM [K₄Fe(CN)₆] electrolyte solution. 5 μL of miRNA-captured samples (diluted in the 5X SSC buffer) was adsorbed onto the SPE-Au surface. Cyclic voltammetry responses were recorded at -0.2 to 0.6 V with a scan rate of 100 mV/s in 10 mM PBS solution containing 2.5mM [K₃Fe(CN)₆] and 2.5mM [K₄Fe(CN)₆] electrolyte solution. The electrodes were washed three times with PBS prior to DPV measurements. Relative DPV current changes (i.e., %I_{Relative}, percent difference of the DPV signals generated for captured mRNA (I_{Sample}) with respect to the baseline current (I_{Baseline})) due to the adsorption of mRNA were then determined by using following equation...
where $i_{\text{Baseline}}$ and $i_{\text{Sample}}$ are current changes for bare electrode and electrode after sample adsorption respectively.

qRT-PCR validation

MiScript reverse transcription kit (Qiagen, Hilden, NRW, Germany) was used for the conversion of cDNA from RNA following the protocols as previously noted. The cDNA samples were aliquoted at 30 ng/µL and stored at -20°C until further use for RT-qPCR analysis. The expression level of miRNA-21 was quantified using RT-qPCR. The reaction mixture was in a total volume of 10 µL comprising 5.0 µL of 2XSensiMix SYBR No-ROX master mix (Bioline, London, UK), 1.0 µL each of 1.0 µmole/µL primer, 1.0 µL of cDNA (30 ng/µL) and 2.0 µL of Nuclease-free water. Thermal cycling was initiated with a first denaturation step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s (extension). The expression levels of miRNA-21 were normalized using the endogenous reference gene, RNU6(Qiagen), which was amplified in the same run and following the same procedure described above. Assays were accomplished in triplicate to verify the results and a non-template control was included in all the experiments. MiRNA-21 expression analysis was achieved as previously described.

Results and discussions

Our assay for electrochemical detection of exosomal miRNAs is schematically depicted in Fig. 1. The assay involves following steps: (i) exosome extraction from cancer cell lines and serum
samples, (ii) isolation of exosomal miRNA, (iii) magnetic capture of target miRNA-21, (iv) heat release of captured miRNA, (v) direct adsorption of exosomal miRNA onto Au-SPE. Exosomes extracted from cancer cell lines/serum samples were initially characterized with cryo-transmission electron microscopy (TEM) which confirmed the presence of double-walled lipid membrane layers in the vesicles (see Figure S1). Upon characterization, a pool of miRNAs was extracted from exosomes. Target miRNA was captured by hybridization with biotinylated complementary probes attached to streptavidin-coated magnetic beads through biotin-streptavidin interaction. Captured miRNA targets were magnetically purified and released from the capture probes through heating. Finally, the released miRNAs were adsorbed onto the bare gold surface of screen-printed electrodes. The level of adsorbed miRNA was detected electrochemically by measuring the interfacial electron transfer reaction of $[\text{Fe(CN)}_6]^{3+/4-}$ redox system on the gold electrode surface (see Fig. S2). Previously, several electrochemistry-based methods have been developed for nucleic acids detection. $^{33,31b,24,34}$ Most of these methods rely on hybridization of the target sequence with a surface bound receptor probe and the use of the redox system (e.g., $[\text{Ru(NH}_3)_6]^{3+/2+}$). $^{35,36}$ Usually $[\text{Fe(CN)}_6]^{3-}$ redox system is coupled with $[\text{Ru(NH}_3)_6]^{2+/3+}$ system to improve the assay sensitivity. However it has also been shown that $[\text{Fe(CN)}_6]^{3-}$ system alone can overcome coulombic repulsion from negatively-charged RNA to access the electrode surface. $^{37,38}$ Zhang and co-workers have shown that under the conditions where density of DNA on the gold electrode surface is adequately low (partial blocking), ferricyanide ions are able to overcome coulombic repulsion of negatively charged DNA and approach the electrode surface for generating a detectable Faradaic current response. $^{39}$ Faradaic current signal thus obtained is significantly lower in comparison with the signal on a bare electrode and is proportional to the amount of adsorbed nucleotides. Recently, our group have shown that other nucleotides, including miRNA exploits the same principle
where the level of adsorbed miRNA correlates with the Faradaic signal generated by the $[\text{Fe(CN)}_6]^{3-/4-}$ system at the gold electrode.\textsuperscript{24,34,40}

In order to achieve optimal analytical performance of the biosensor, as well as maximize the signal/noise ratio, several parameters known to influence RNA-gold adsorption kinetics, such as adsorption time and pH of the electrolyte solution were carefully optimized. Optimization of incubation time is critical as longer incubation times may lead to the saturation of electrode surface. We measured the change in relative current at various time points (5, 10, 20, 25 and 30 min) after adsorption of 1.0 nM of synthetic miRNA-21 onto the electrode surface. Gradual increase in the relative current change was observed with the increasing of incubation time. However, no significant change in current signal was seen after 20 mins of incubation, possibly indicating the saturation of electrode surface. Thus, 20 min was chosen as optimum incubation time with a maximum current response change of 60\% (data not shown). Next, we estimated the effect of pH of electrolyte solution on the adsorption of target miRNA using 5 different buffer pH (pH 3, 5, 7, 9, and 11). Among acidic, basic and neutral pH range, a maximum current response change ($\%i_r=50$) was spotted at neutral pH (pH 7.0). Increase of current response change level being was noticed with increasing acidic pH up to pH 7 but thereafter decreased at even higher basic pH. These results might be due to influence of miRNA stability (miRNA degradation) at the basic pH and working electrode surface damage at lower pH. Moreover, these observations indicate that pH of the buffer solution influence the competition between miRNA and gold electrostatic forces. Thus, we selected pH 7.0 as an optimal pH for our bioassay.\textsuperscript{24}

To evaluate the assay specificity, a set of control experiments under same conditions were performed. First, no-target/template (NoT) control experiment was carried out by using PBS buffer instead of target miRNA sample. As expected, negligible current response ($\%i_r=4$) was observed in the NoT control experiment. Further, the target miRNA-21 sequence was
replaced with a set of unrelated and non-complementary sequences such as miRNA-107, miRNA-9-2, miRNA-338-3p (starting concentration 1 nM each) and the resulting relative current response was compared with that obtained for same concentration (1 nM) of target miRNA-21. As can be seen in Fig 2, %i_r for non-complementary miRNAs was calculated to be approximately 9%, 6% and 6% for miRNA-107, miRNA-9-2 and miRNA-338-3p respectively. These responses are approximately 6-, 9- and 9-fold lower when compared to DPV response obtained for the target miRNA-21. Notably, the response obtained from these non-complementary targets were similar to the NoT control (%i_r=6 vs %i_r=4 respectively). These results demonstrated that our assay possess a good specificity for the detection of miRNA-21.

The sensitivity of the assay was determined by analyzing the serially diluted synthetic miRNA-21 with designated concentration ranging from 1.0 pM to 10 nM. Increasing the amount of target miRNA-21 being adsorbed onto electrode surface creates more coulombic repulsion of [Fe(CN)_6]^{4-/3-} during the interaction with negatively charged miRNA. Consequently, it leads to lower Faradic current and higher relative current response in comparison with bare electrode. Therefore, as can be seen in Fig. 3, an increment in relative DPV current change (%i_r) was observed with increasing concentration of target miRNA-21. Additionally, our assay showed good reproducibility with <5.5% relative standard deviation (% RSD) of the measurements for three independent experiments. The linear regression equation was estimated to be y (% current response change, %i_r) =15 (miRNA concentration) +1.4 (C) with a correlation coefficient (R^2) of 0.97. It has previously been shown that concentration level of miRNA species in clinical samples ranges from 0.2 fM to 20 pM. Therefore we believe that our assay with a LOD of 1pM could be an alternative tool for the detection of exosomal miRNA from the clinically relevant analyte concentration.

To further demonstrate the applicability of our method in more complicated biological systems, we applied our assay to total exosomal RNA isolated from cultured human cell lines.
High levels of miRNA-21 could be detected in exosomes obtained from all three cancer cell lines (Fig 4). These findings are in good agreement with literature. Additionally, we analyzed miRNA-21 levels in eight serum samples derived from CRC patients. One non-cancerous healthy serum sample (Healthy) was also included as a control. As can be seen in Fig 5A, all eight cancerous samples showed about 40-80% current response change as compared with a bare electrode which was significantly higher compared to non-cancerous healthy sample. The obtained data indicated that exosomal miRNA-21 are over expressed in all cancer samples. Electrochemical detection data was further validated by qRT-PCR analysis. Using specific primers, miRNA-21 expression was measured in cancerous samples as well as healthy controls. miRNA expression level was performed as a fold change relative to a housekeeping gene. As shown in Fig 5B qRT-PCR data is in good agreement with the results obtained from our electrochemical detection platform demonstrating that our assay is suitable for analyzing exosomal miRNA in cancer samples.

Despite large number of biosensors that have been developed recently for miRNA detection, clinical applicability of these platforms is downplayed by the fact that they either require complex and tedious amplification processes, such as enzymatic polyadenylation or involve expensive biomaterials as well as time-consuming and laborious procedures. Majority of these biosensors are constructed with multiple complex fabrication steps where complicated chemistries underlying each recognition and transduction layer of the sensor substantially complicate the assay design. For example, an electrochemical platform for miRNA detection has recently been reported by Li et al. where authors demonstrated 1 pM LOD, similar to our method. However their approach requires modification of glassy carbon electrodes with multi-walled carbon nanotubes (MWCNTs). This sensor fabrication step adds to the complexity and cost of the procedure. Zhang and co-workers also reported an efficient miRNA detection method using non-enzymatic amplification by the mismatched catalytic
hairpin assembly (CHA) and achieved an LOD of 1 pM.\textsuperscript{44} However, design flexibility and connectivity of CHA amplifier circuits are relatively low.\textsuperscript{45} Furthermore, this method used capture probes immobilized to the electrode surface.

In contrast, our assay offers several advantages. First, the use of exosomal miRNA instead of naked cell-free circulating miRNA provides a high level of stability and integrity to biological molecules. Thus, targeting exosomal miRNA may prove to be a great advantage for the development of minimally invasive and reliable cancer diagnostic and therapeutic approaches. Second, unlike many of the conventional biosensors which are based on hybridization of target sequences to the surface bound complementary receptor probes, our assay relies on the selective capture of target miRNAs by magnetic bead- based intimate mixing, washing and purification steps. Compared to the conventional way of capturing targets on two-dimensional electrode surface, our three-dimensional diffusion of both RNA sequences and capture probes in a microcentrifuge tube can thus drastically reduce the assay time, and avoid matrix effect of complex biological samples allowing negligible non-specific interference. Third, taking advantage of the gold- RNA affinity interaction, our assay employs the direct adsorption of captured target miRNA onto the electrode surface. This aids to avoid tedious conjugation chemistries commonly involved in electrode surface modification procedure. Moreover, by employing disposable and inexpensive screen-printed gold electrodes (Au-SPE), our assay not only eliminates the use of electrochemical cells and tedious cleaning steps associated with the conventional disk electrodes, it also offers a major thrust towards decentralized miRNA analysis in point-of-care settings.

**Conclusions**

In conclusion, we have developed a simple, amplification-free method for electrochemical detection of exosomal miRNA. Our method achieved a good analytical performance ((i.e.,
reproducibility, % RSD = <5.5), sensitivity (LOD = 1 pM), dynamic range (1 pM - 100 nM))
in analyzing exosomal miRNA derived from various cancer cell lines and a cohort of serum
samples collected from colorectal adenocarcinoma patients. The developed method offers
several key advantages such as enhanced capture, reduced assay time and matrix effect, as well
as simplified and inexpensive device manufacturing for point-of-care applications. This
approach has potential implications in cancer screening, predicting patient prognosis and
therapeutic applications. Moreover, through capture probe adaptation our method can be easily
refashioned to suit detection of a variety of exosomal biomarkers in diverse cancer types.

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Figure Captions

**Fig 1.** Schematic representation of the assay for the detection of exosomal miRNA-21 in cancer serum samples.

**Fig 2.** Specificity of the assay. DPV current changes are corresponding to different non-complementary miRNAs used as controls. Error bars represent the standard deviation of three independent experiments (%RSD = <5.5%, n = 3).

**Fig 3.** Sensitivity of the assay. DPV current changes are corresponding to different concentrations of miRNA-21. The inset shows the corresponding linear calibration plot. Error bars represent the standard deviation of three independent experiments (%RSD = <5.5%, n = 3).

**Fig 4.** DPV current changes corresponding to the different level of the exosomal miRNA-21 present in breast (BT474), esophageal (HKESC1) and colon (SW-48) cancer cell lines. Error bars represent the standard deviation of three independent experiments (%RSD = <5.5%, n = 3).

**Fig 5.** Clinical application of the assay. (A) DPV current changes are corresponding to the different level of exosomal miRNA-21 derived from patients with colon cancer. (B) Representative miRNA expression analysis data (fold change) of exosomal miRNA-21 present in samples from patients with colon cancer. Error bars represent the standard deviation of three independent experiments.
Table 1. Oligonucleotide sequences used in this study.

<table>
<thead>
<tr>
<th>Oligo sample</th>
<th>Oligonucleotide sequences (5′--3′)</th>
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</thead>
<tbody>
<tr>
<td>Synthetic miRNA-21*</td>
<td>UAGCUUAUCAGACUGAUGUUGA</td>
</tr>
<tr>
<td>Biotinylated miRNA-21 capture probe</td>
<td>TCAACATCAGTCTGATAAGCTA-Biotin</td>
</tr>
<tr>
<td>RT-qPCR Forward primer</td>
<td>CGGCGGTAGCTTATCAGACTGA</td>
</tr>
<tr>
<td>RT-qPCR Reverse primer</td>
<td>GTGCAGGGTCCGAGGT</td>
</tr>
</tbody>
</table>
* (miRBASE accession number: MIMAT0000076)
Figures

Fig 1
Fig 3

- The bar chart shows the percentage of fluorescent signal intensity (%IR) for different concentrations of miRNA.
- The red line indicates the linear relationship between miRNA concentration and %IR.
- The equation $y = 15x + 1.4$ is given, where $R^2 = 0.97$.
Cancer cell line

Fig 4
Fig 5