Published in final edited form as:

J Phys Chem C Nanomater Interfaces. 2016 September 22; 120(37): 21047–21050. doi:10.1021/acs.jpcc.6b03299.

Multiplexed Detection of MicroRNA Biomarkers Using SERS-Based Inverse Molecular Sentinel (iMS) Nanoprobes

Hsin-Neng Wang^{†,‡}, Bridget M. Crawford^{†,‡}, Andrew M. Fales^{†,‡}, Michelle L. Bowie[§], Victoria L. Seewaldt^{||,‡}, and Tuan Vo-Dinh^{*,†,‡,⊥}

[†]Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, United States

[‡]Fitzpatrick Institute for Photonics, Duke University, Durham, North Carolina 27708, United States

[⊥]Department of Chemistry, Duke University, Durham, North Carolina 27708, United States

[§]Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710, United States

^{||}Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, United States

Abstract

MicroRNAs (miRNAs) have demonstrated great promise as a novel class of biomarkers for early detection of various cancers, including breast cancer. However, due to technical difficulties in detecting these small molecules, miRNAs have not been adopted into routine clinical practice for early diagnostics. Thus, it is important to develop alternative detection strategies that could offer more advantages over conventional methods. Here, we demonstrate the application of a “turn-on” SERS sensing technology, referred to as “inverse Molecular Sentinel (iMS)” nanoprobes, as a homogeneous assay for multiplexed detection of miRNAs. This SERS nanoprobe involves the use of plasmonic-active nanostars as the sensing platform. The “OFF-to-ON” signal switch is based on a nonenzymatic strand-displacement process and the conformational change of stem-loop (hairpin) oligonucleotide probes upon target binding. This technique was previously used to detect a synthetic DNA sequence of interest. In this study, we modified the design of the nanoprobe to be used for the detection of short (22-nt) miRNA sequences. The demonstration of using iMS nanoprobes to detect miRNAs in real biological samples was performed with total small RNA extracted from breast cancer cell lines. The multiplex capability of the iMS technique was demonstrated using a mixture of the two differently labeled nanoprobes to detect miR-21 and miR-34a miRNA biomarkers for breast cancer. The results of this study demonstrate the feasibility of applying the iMS technique for multiplexed detection of short miRNAs molecules.

Graphical Abstract

*Corresponding Author: tuan.vodinh@duke.edu.

#Present Address

Department of Population Sciences, City of Hope, Duarte, CA 91010, U.S.A.

Notes

The authors declare no competing financial interest.

INTRODUCTION

MicroRNAs (miRNAs) are small, noncoding RNAs of approximately 20–25 nucleotides in length that bind to perfect or near-perfect complementary sequences in the untranslated regions (UTRs) of mRNA targets, thereby regulating gene expression at the post transcriptional level.¹ Dysregulation of miRNAs is often observed in a wide range of cancers, including breast cancer. It has been indicated that miRNAs transcriptionally suppress expression of oncogenes and loss of miRNA expression results in oncogene activation.² Recent studies also show that miRNAs regulate not just single oncogenes but entire signaling networks.^{3–5} For instance, while miR-21 miRNA is overexpressed and plays an important oncogenic role, miR-34a is downregulated in triple-negative breast cancers (TNBC), which has been recognized as the most aggressive subtype of breast cancer.^{6,7} Therefore, miRNAs hold great potential to serve as an important class of biomarkers not only for early diagnosis of cancer, but also for investigation of cancer initiation and progression.^{8–10} However, these small molecules have not been adopted into clinical practice for early diagnostics because of the technical difficulties arising from the intrinsic characteristics of miRNAs, such as the short sequence lengths, low abundance, high sequence similarity, and a wide range of expression levels that could span over 4 orders of magnitude.¹¹ It is noteworthy that miRNA biomarkers also exhibit significance in nonmedical application areas as well. For instance, recent studies in plants indicated that miRNAs can target squamosa promoter binding protein-like (SPL) genes and define a separate endogenous flowering pathway,¹² which is important in biofuel research as the timing to flower is one of the key determinants to plant biomass accumulation and agricultural yields.

Currently, Northern blotting, microarrays, and quantitative reverse transcriptase PCR (qRT-PCR) are often employed as the conventional miRNA detection methods, which involve elaborate, time-consuming, and expensive processes that require special laboratory equipment.^{13,14} While Northern blotting is the only technique that allows for the quantitative visualization of miRNAs, it has low detection efficiency and requires complex methods that cantamination.¹⁵ Microarrays, which allow simultaneous detection of several hundred miRNAs, are only semiquantitative making them most suitable for comparing relative expression levels of miRNA between different cellular states.¹⁶ Microarrays therefore require an additional form of validation, such as qRT-PCR, to quantify expression. qRT-PCR, the gold standard among miRNA detection techniques, allows detection of low-abundance miRNA species but is restricted by the short length of miRNAs.¹⁷ Moreover,

these methods often require either fluorescent or radioactive dyes, which bring about limitations on reliability in that fluorescent dyes photobleach and radioactive probes suffer from difficulties in handling and disposal. Thus, there is a need to develop alternative diagnostic strategies that could offer more advantages over these conventional methods.

Recently, various nanoparticles (NPs) have been applied within few studies for miRNA detection, such as gold nanoparticles (AuNPs),¹⁸ magnetic nanoparticles,¹⁹ silver nanoculsters,²⁰ quantum dots,²¹ and more. Mirkin et al. furthered the DNA functionalized gold nanoparticle as a reporter of miRNA hybridization in developing a Scanometric miRNA array using spherical nucleic acid-gold nanoparticle conjugates.²² This method required sequential metal deposition post hybridization to tune the dynamic range in order to detect low abundance miRNAs. A platform named the biobarcode gel (bio-BaGel) assay, reported by Nam et al., utilized sandwich-like hybridization of target miRNA between the gold nanoparticle barcode probe and the magnetic particles to provide target isolation and enrichment.²³ Surface plasmon resonance biosensors have also been studied as potential miRNA detection platforms as they can offer rapid, sensitive, and point-of-care analysis.^{24,25}

With the advance in nanotechnology, surface-enhanced Raman scattering (SERS) has opened up new and exciting possibilities for a large number of biosensing applications.^{26–28} The origin of the enhancement of Raman scattering mainly comes from surface plasmon excitation in metallic nanostructures with sizes in the order of tens of nanometers. When the surface of a metallic nanostructure is excited by an incident light, an intense localized electromagnetic field (“surface plasmon”) is generated that can interact with molecules adsorbed on or near the metallic surface.^{29,30} Through this surface plasmon effect, the intensity of the normally weak Raman scattering process can be increased by factors of 10^6 – 10^7 , or even up to 10^{15} enhancement at “hot spots”.^{31–33} Due to the sharp, molecularly specific Raman peaks, SERS allows for easy discrimination of many targets at once. The multiplexed nature of SERS offers significant advantages over other methods, such as fluorescence, chemiluminescence, and so on, that exhibit broader emission bands.³⁴

A SERS method was first developed in our laboratory to detect nucleic acid targets.³⁵ SERS-based analysis in the detection of miRNA was demonstrated by Tripp et al. in an assay depending on the molecular fingerprints of individual or mixed miRNA analytes.³⁶ Zhao et al. reported the use of silver nanorod arrays along with a straightforward least-squares technique for SERS detection of miRNA based on quantitative determination of the relative ratios of the four nucleotide components before and after hybridization of the target miRNA with single-stranded probes.³⁷ Ozsoz et al. developed a SERS-based assay for miRNA detection using gold slides and DTNB-labeled, rod-shaped nanoparticles as SERS labels.³⁸

For the past years, we have been investigating the strength of the SERS effect as part of a nucleic-acid sensing nanoprobe. Particularly, we have demonstrated that highly sensitive, specific and multiplexed detection of nucleic acids can be achieved by utilizing SERS-active silver nanoparticles.^{39–41} Recently, we have also reported a novel “turn-on” plasmonics-based nanobiosensor, referred to as “inverse Molecular Sentinel (iMS)” nanoprobe, for the detection of DNA sequences of interest with an “OFF-to-ON” signal switch.⁴² The term “inverse” is used to distinguish this new technology from our previously developed “ON-to-

“OFF” Molecular Sentinel (MS) nanoprobe.^{39,40} This promising type of plasmonic nanobiosensor involves the use of a unique type of SERS-active nanoprobe, that is, silver-coated gold nanostars (AuNS@Ag), as the sensing platform. Nanostars have emerged as one of the best geometries for producing strong SERS in a nonaggregated state due to their multiple sharp branches, each with a strongly enhanced electromagnetic field localized at its tip.⁴³ AuNS@Ag is a new hybrid bimetallic nanostar platform that exhibits superior resonant SERS properties.⁴⁴ Compared to uncoated gold nanostars (AuNS), AuNS@Ag was demonstrated to offer over an order of magnitude of signal enhancement, rendering it an excellent SERS substrate.

The iMS nanobiosensor employs a nonenzymatic DNA strand-displacement process and the conformational change of stem-loop (hairpin) DNA probes for specific target identification and signal switch. As shown in Figure 1, a DNA probe, having a Raman label at one end, is immobilized onto a nanostar via a metal-thiol bond. The probe is designed with a “stem-loop” or “hairpin” structure in order to produce a strong SERS signal when the loop is closed to bring the Raman label to the surface of nanostars. A single-stranded DNA is then served as a “placeholder” strand that can hybridize to the stem-loop probe via a placeholder-binding region. This probe-placeholder duplex disrupts the stem-loop structure and keeps the label away from the nanostar surface. In this “open” configuration, the nanobiosensor exhibits low SERS intensity (“OFF” state) as the SERS enhancement decreases exponentially with increasing the separation between the label and the metallic surface. Upon exposure to a target nucleic acid, the placeholder strand leaves the surface following a nonenzymatic strand-displacement process:^{45,46} the target first binds to the overhang region (called “toehold”) of the probe-placeholder duplex and begins displacing the stem-loop probe from the placeholder through a branch migration process and finally releases the placeholder from the nanobiosensor. This allows the stem-loop structure to “close” and moves the Raman label onto the plasmonics-active nanostar surface, yielding a strong SERS signal (“On” state).

The performance evaluation of the iMS nanoprobe including detection specificity and sensitivity has been reported elsewhere.^{42,47} In this work, we further demonstrated the feasibility of using this nanostar-based iMS SERS sensing technology as a useful homogeneous assay for multiplexed detection of miRNAs. This paper describes a detailed design strategy to overcome the detection challenge posed by the short length of miRNA sequences. The investigation of using iMS nanoprobe to detect miRNAs in real biological samples was performed with total small RNA extracted from breast cancer cell lines. The multiplex capability of the iMS technique was demonstrated using a mixture of the two differently labeled nanoprobe to detect miR-21 and miR-34a miRNAs, which have been recognized as critical biomarkers for breast cancer.^{48–51}

EXPERIMENTAL SECTION

Oligonucleotide Sequences

The Cy5- and Cy5.5-labeled thiolated stem-loop oligonucleotides used for miR-21 and miR-34a detection are 5'-thiol-AAAAAGTCTGTATTAAAAA-TAGCTTATCAGAC-Cy5-3' and 5'-thiol-AAAACTA-AGAAAAAAAATGGCAGTGTCTTAG-Cy5.5-3',

respectively. The placeholder-0T, -4T, -5T, and -6T strands used for miR-21 detection are 5'-TCAACATCAGTCTGATAAG-CTA-3', 5'-TCAACATCAGTCTGATAAGCTATTTT-3', 5'-TCAACATCAGTCTGATAAGCTATTTT-3' and 5'-TCAA-CATCAGTCTGATAAGCTATTTT-3', respectively. The placeholder used for miR-34a detection is 5'-ACAACCAG-CTAAGACACTGCCATTTT-3'. The synthetic miR-21 DNA and RNA targets are 5'-TAGCTTATCAGACTGATGTTGA-3' and 5'-UAGCUUAUCAGACUGAUGUUGA-3', respectively. The synthetic miR-34a RNA target is 5'-UGGCAGUG-UCUUAGCUGGUUGU-3'. The synthetic noncomplementary negative control DNA for demonstration of miR-21 detection is 5'-TAGCAGCACGTAAATATTGGCG-3', and the synthetic noncomplementary negative control RNA for demonstration of miR-34a detection is 5'-GUUCUGCUACUGACAGUAAG-UGAAGAUAAAGUGUGUCUGA-3'. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Preparation of Gold Nanostars

The gold nanostars (AuNS) were prepared as described previously using a seed-mediated method.⁴³ Briefly, 12 nm citrate gold seed solution was first prepared using a modified Turkevich method. Gold nanostars were then synthesized by the simultaneous addition of 50 μL of 2 mM AgNO_3 and 50 μL of 0.1 M ascorbic acid to a solution containing 10 mL of 0.25 mM HAuCl_4 , 10 μL of 1 N HCl, and 100 μL of the 12 nm gold seed solution under stirring at room temperature. The process was completed in less than a minute along with color change from a light orange to dark blue within 10 s, indicating formation of gold nanostars. The stock concentration of nanostars is approximately 0.1 nM, as determined by nanoparticle tracking analysis (NTA).

Preparation of Silver-Coated Gold Nanostars

The silver-coated gold nanostars (AuNS@Ag) were prepared as previously described.⁴⁴ To the as-prepared AuNS solution, 50 μL of 0.1 M AgNO_3 was added, followed by 10 μL of 29% NH_4OH to initiate the silver coating reaction. The color of the solution changed from blue to purple to dark brown over the course of about 5 min. The obtained solution was used for further functionalization without purification.

Synthesis of SERS iMS Nanoprobes

The iMS nanoprobes were synthesized according to our previous report.⁴² Briefly, 10 μL of 10 μM stem-loop DNA probe solution was added to a 0.9 mL of 0.1 nM AuNS@Ag solution followed by the addition of 0.1 mL of 2.5 mM MgCl_2 solution. The mix solution was then allowed to be incubated overnight at room temperature. To stabilize the nanoprobes, 1 μM of *O*-[2-(3-mercaptopropionylamino)ethyl]-*O'*-methylpolyethylene glycol (mPEG-SH, 5000) was added to the solution for 30 min. The solution was then centrifuged at 7000 rpm for 10 min and resuspended in 1 mL of Tris-HCl buffer (10 mM, pH 8.0) containing 0.01% Tween-20. The metallic surface of nanostars was then passivated using 0.1 mM 6-mercaptop-1-hexanol (MCH). The functionalized nanoprobes were washed with Tris Tween-20 buffer using repeated centrifugation at 7000 rpm for 10 min. The purified nanoprobes were finally redispersed in Tris-HCl-Tween-20 buffer.

SERS iMS Assay Procedure

To turn of the iMS SERS signal, the iMS nanoprobe (0.1 nM) were incubated with 0.1 μ M placeholder strands in PBS buffer solution containing 0.01% Tween-20 overnight at 37 °C. The excess placeholders were removed using repeated centrifugation at 7000 rpm for 10 min, and redispersed in PBS Tween 20 buffer. The “OFF” iMS nanoprobe (0.05 nM) was then incubated with analytes at 37 °C for 1 h followed by SERS measurements using a Renishaw InVia confocal Raman microscope equipped with a 632.8 nm HeNe laser. The light from the laser was passed through a laser line filter, and focused into the sample solution with a 10x microscope objective. For tests using total small RNA extracted from AU565 and SUM149 breast cancer cells, 2 μ L of the iMS solution (0.01 nM) was incubated with 200 ng of total small RNA samples in PBS Tween-20 buffer at room temperature for 30 min. Mineral oil was added to the mixture to prevent evaporation. After the reaction, the mixture was transferred to a glass capillary tube for the SERS measurement. For each experiment, three SERS measurements were performed per sample and averaged into a single spectrum. All SERS spectra reported here were background subtracted and smoothed using a Savitsky-Golay filter (five point window and first-order polynomial).

Cell Culture

MCF-10A cells (a kind gift of Dr. Gerald Blobe at Duke University, Durham, NC) were grown in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 μ g/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μ g/mL insulin. SUM149PT cells were originally obtained from Dr. Stephen Ethier (Karmanos Cancer Institute, Detroit, MI) and are commercially available (Asterand, Detroit, MI). SUM149PT cells were maintained in Ham's F12 supplemented with 5% FBS, 5 mg/mL insulin, and 1 mg/mL hydrocortisone. AU565 cell line was obtained from ATCC and was maintained in RPMI 1640 supplemented with 10% FBS and 2 mmol/L L-glutamine.

RNA Extraction and qRT-PCR

Total RNA was extracted using miRVana miRNA Isolation kit (Thermo Scientific) following manufacture's protocol. Quantitative RT-PCR was performed using 80 ng tRNA input into the miRCURY LNA Universal RT microRNA PCR system (Exiqon, Woburn, MA). cDNA was diluted 1:80 and ran in duplicate on Cancer Focused microRNA PCR panel plates (Exiqon) using the LightCycler 480 thermocycler (Roche, Indianapolis, IN). Raw Cp values were adjusted for interplate calibration followed by normalization to 4 of the most stable miRNAs across all plates (miR-101, miR-106a, miR-17, miR-210), as determined by GenEx software. Relative miRNA expression was calculated by the 2^{-C_T} method⁵² using MCF-10A miRNA levels as a relative control.

RESULTS AND DISCUSSION

As a proof of concept, we have designed an iMS nanoprobe labeled with Cy5 to detect the mature human miR-21 miRNA, which has been recognized as an important biomarker in a variety of cancers, including breast cancer.^{48–50} In our previous reports, the physical length of the Raman-labeled stem-loop probes was designed to be around 10 nm for a low background SERS signal in their OFF state.^{42,47} However, the short sequence lengths of

miRNAs would make it difficult to design stem-loop probes with the desired length. To overcome this challenge, a spacer sequence of around 10 nucleotides can be added between the loop and the 5'-end stem (Figure 2A). This spacer is used to increase the physical length of the probe in order to keep the Raman label at an appropriate distance away from the nanostar surface while hybridizing to a placeholder strand. This "internal" spacer, however, does not affect the generation of a strong SERS signal when the probe is in a closed stem-loop configuration (Figure 2B).

As for the "OFF-to-ON" sensing technique, the iMS nanoprobe should have a low SERS background signal in the "OFF" state through the formation of stable probe-placeholder duplexes. In this study, different placeholder strands were investigated for their capability to turn off the SERS signal of the iMS nanoprobe. A short poly(T) tail (4–6 thymine bases) was added to the 3'-end of the placeholder strand; as such, it can hybridize to a portion of the internal spacer in the probe. The gray bars in Figure 3A show the SERS background signal (peak height at 558 cm^{-1}) from the iMS nanoprobe in their OFF state with different placeholder strands. Without the poly(T) tail (placeholder-0T), a strong SERS background signal was observed indicating that the placeholder-0T strand did not effectively turn off the iMS SERS signal. This is due to the fact that this probe-placeholder-0T duplex with melting temperature (T_m) of $38\text{ }^{\circ}\text{C}$ is thermodynamically unstable at the reaction temperature of $37\text{ }^{\circ}\text{C}$. In this case, the probe (with the hairpin stem T_m of $44.1\text{ }^{\circ}\text{C}$) has a high tendency to fold into a stem-loop structure, yielding a strong SERS background signal. In contrast, with short poly(T) tails (i.e., placeholder-4T containing 4 thymine bases, placeholder-5T containing 5 thymine bases, and placeholder-6T containing 6 thymine bases), low background signals were observed indicating that stable probe-placeholder duplexes were formed. The T_m s of these probe-placeholder duplexes are estimated to be 44.7 , 46.1 , and $47.4\text{ }^{\circ}\text{C}$ for placeholder-4T, -5T, and -6T, respectively, which are higher than the reaction temperature, resulting in a higher thermal stability.

To turn on the nanobiosensor, the placeholder strands need to be released from the system upon target binding. We next investigated whether the additional poly(T) tail affects the dissociation of the placeholders from the iMS nanoprobe. The red bars in Figure 3A represent the peak height SERS intensity at the 558 cm^{-1} peak from the iMS nanoprobe after 1 h incubation of $1\text{ }\mu\text{M}$ of synthetic DNA targets at $37\text{ }^{\circ}\text{C}$. Figure 3B shows an example of the SERS spectra for the detection of miR-21 targets using the placeholder-6T. In the presence of $1\text{ }\mu\text{M}$ miR-21 targets, the SERS intensity was significantly increased ("signal ON"), indicating that the hybridization between targets and placeholders enabled the formation of the stem-loop structure of the probes by triggering the strand displacement reaction and releasing the placeholder strands. Thus, because of the thermal instability of the short poly(A)-poly(T) duplexes ($T_{m(\text{polyT})} = 0\text{ }^{\circ}\text{C}$), the additional short poly(T) tail can spontaneously dissociate from the probe following the branch migration process at an elevated temperature (Figure 3C). However, it was also observed that, in the presence of targets, the intensity decreases slightly with increasing the length of the poly(T) tail. Thus, the placeholder needs to be designed carefully so that it still can be released from the system following the branch migration process. Nevertheless, the results demonstrated that a proper poly(T) tail could be added to the placeholder in order to minimize the background signal without affecting the sensor functionality.

To demonstrate the possibility of detecting miR-21 in real samples, preliminary experiments were performed using total small RNA extracted from two breast cancer cell lines (AU565 and SUM149) that have been shown to exhibit different expression levels of miR-21 in our qRT-PCR experiments. Each sample (200 ng of enriched small RNA) was incubated with iMS nanoprobes at room temperature for 30 min followed directly by SERS measurements. Figure 4A shows the blank-corrected SERS spectra of the iMS nanoprobes in the presence of the total small RNA samples. The spectrum a is the SERS signal measured when the iMS nanoprobes were mixed with the RNA sample extracted from AU565 cells while the spectrum b shows the SERS signal detected from the RNA sample extracted from the SUM149 cells. The bar graph (Figure 4B) shows that the SERS signal of the major SERS peak at 558 cm^{-1} measured with RNA extracted from SUM149 cells was significantly higher than that extracted from AU565 cells. The SERS data were consistent with the qRT-PCR data showing that the miR-21 expression in SUM149 cells was higher than that in AU565 cells (Figure 4C). These results demonstrate the possibility to detect and evaluate the expression levels of miRNAs from cell lysates that contain different amounts of miRNAs and provide the foundations for translating the detection of miRNAs into clinical practice.

The dose response of the iMS technique was assessed by using serial dilutions of total small RNA enriched from human breast adenocarcinoma MCF-7 total RNA (Thermo Fisher Scientific Inc., Waltham, MA). The MCF-7 RNA was used because it has been found that miR-21 is highly expressed in this cell line.⁵⁵ As shown in Figure 5, the assay was linear in the range of 10 to 500 ng total small RNA sample ($R^2 = 0.9949$), and as little as 10 ng small RNA can be used if the target of interest is of sufficient abundance.

The multiplexing capability of SERS is an important feature due to the narrow Raman bandwidths. To demonstrate the multiplexing capability of the iMS technique, a second iMS nanoprobe labeled with a different Raman dye, Cy5.5, was designed to target miR-34a. Figure 6 shows the blank corrected SERS signal of the miR-34a iMS nanoprobes in the presence or absence of target molecules. In the presence of $1\text{ }\mu\text{M}$ miR-34a synthetic RNA targets (spectrum b), the SERS intensity was significantly increased, comparing with the negative control in the presence of $1\text{ }\mu\text{M}$ noncomplementary sequences (spectrum a), indicating that the miR-34a iMS nanoprobes were turned on upon target binding. It is noticed that the SERS spectrum of the Cy5.5-labeled miR-34a nanoprobes exhibits multiple unique Raman peaks, which are significantly different than that from the Cy5-labeled miR-21 nanoprobes. Thus, the SERS sensing modality provides specific spectral “fingerprints” with very sharp peaks allowing sensing multiple targets simultaneously in a single assay platform.

Demonstration of the multiplex capability of the iMS technique was first performed with a mixture of the two iMS nanoprobes for simultaneous detection of synthetic miR-21 and miR-34a miRNAs. The experiments were carried out by mixing different ratios of miR-21 and miR-34a nanoprobes (1:1, 1:2, and 1:4) while keeping the concentration of miR34a nanoprobes constant at 10 pM in a $10\text{ }\mu\text{L}$ assay volume. Figure 7A shows the blank-corrected SERS spectra from the mixtures in the presence of $1\text{ }\mu\text{M}$ of both miR-21 and miR-34a synthetic targets. To identify the SERS signal of each nanoprobe from the mixture, the spectra were analyzed using a spectral decomposition method. As previously

described,⁵⁶ the spectral decomposition procedure, which was adapted from Lutz et al.,⁵⁷ was processed using Matlab (R2015b, MathWorks, MA). The decomposition is based on the assumption that the multiplex spectrum is comprised of the reference spectra and an unknown polynomial. SERS spectra were collected for each probe alone (reference spectra) and for the mixture. The acquired SERS spectra were background subtracted and smoothed using a Savitsky-Golay filter (five point window and first-order polynomial). The entire spectra ranging from 400 to 1800 cm^{-1} , which contains the distinctive Raman signature of each dye, were loaded into Matlab's workspace. The decomposition processes utilized Matlab functions `lsqnonneg` and `fmincon` to determine the best fit of the reference spectra to the mixture spectrum. A free-fitting polynomial was introduced to reduce the fitting error.⁵⁷ Matlab analysis subsequently generated the minimally constrained coefficients (i.e., the fractions) for each reference spectrum, which were normalized to 1. As can be seen in Figure 7B, the SERS spectra of the mixture can be decomposed into the contributions of the two distinct reporters, Cy5 (miR-21 nanoprobe) and Cy5.5 (miR-34a nanoprobe). The signal fractions were in good agreement with the predetermined ratios of the two nanoprobe.

To demonstrate the detection of the two real miRNA targets in RNA extracts, different amounts (250 ng, 500 ng, and 1 μg) of total small RNA extracted from the MCF-7 breast cancer cell line were added to a 10 μL nanoprobe mixture containing 5 pM of miR-21 nanoprobe and 10 pM of miR-34a nanoprobe. Figure 8A shows a typical SERS spectrum of the mixture in the presence of 1 μg of total small RNA sample. The unique SERS peaks of the miR-21 nanoprobe at 558 and 797 cm^{-1} , and of the miR-34a nanoprobe at 1523 and 1626 cm^{-1} were indicated by the arrows. The integrated SERS intensity of these peaks shown in Figure 8B,C were given by the area under the curve over the spectral range of 540–569, 780–810, 1512–1530, and 1615–1640 cm^{-1} for peaks at 558, 797, 1523, and 1626 cm^{-1} , respectively. The SERS intensity of the miR-21 nanoprobe at 558 and 797 cm^{-1} (Figure 8B) was found to increase significantly with increasing amounts of small RNA input. For miR-34a SERS signal, a slight increase in the intensity at both 1523 and 1626 cm^{-1} was observed only when 1 μg of the RNA sample was added (Figure 8C). Due to the significant difference in the amounts of RNA samples required for a detectable signal change, the abundance of miR-21 in MCF-7 cells was found to be higher than that of miR-34a. This result was consistent with that reported by Fix et al.⁵⁵ using microarray. The results of this study demonstrate the feasibility of using the iMS technique for multiplexed detection of miRNAs in real biological samples. Note that the SERS measurements were performed immediately following the incubation of target molecules without any washing steps, which greatly simplifies and accelerates the assay procedure.

CONCLUSIONS

In conclusion, we have demonstrated the feasibility of applying the “OFF-to-ON” SERS iMS nanoprobe as a homogeneous assay for multiplexed detection of miRNAs in a single sensing platform. As it does not require target labeling and any subsequent washing steps, the positive-readout iMS biosensing platform will provide a versatile and powerful tool for a wide variety of applications. As illustrated in this study, the iMS can be used for early diagnostics of cancer. For nonmedical areas, the technique could be used as analytical

monitors to investigate plant development to increase biomass for biofuels and for agricultural improvements. Further investigation will involve comparison of quantitative detection limit of the iMS technique with other methods.

Acknowledgments

This material is based on work supported by the National Institutes of Health (1R21CA196426), by the U.S. Department of Energy Office of Science, under Award Number DE-SC0014077, and by the Duke Faculty Exploratory Research Fund. This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency, thereof.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism and function. *Cell*. 2004; 116:281–297. [PubMed: 14744438]
2. Lee YS, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol: Mech Dis*. 2009; 4:199–227.
3. Ma X, Buscaglia LEB, Barker JR, Li Y. MicroRNAs in NF- B signaling. *J Mol Cell Biol*. 2011; 3:159–166. [PubMed: 21502305]
4. Schepeler T. Emerging roles of microRNAs in the Wnt signaling network. *Crit Rev Oncog*. 2013; 18:357–371. [PubMed: 23614621]
5. Bischoff A, Bayerlová M, Strotbek M, Schmid S, Beissbarth T, Olayioye MA. A global microRNA screen identifies regulators of the ErbB receptor signaling network. *Cell Commun Signaling*. 2015; 13:1.
6. D'Ippolito E, Iorio MV. MicroRNAs and triple negative breast cancer. *Int J Mol Sci*. 2013; 14:22202–22220. [PubMed: 24284394]
7. Dong G, Liang X, Wang D, Gao H, Wang L, Wang L, Liu J, Du Z. High expression of miR-21 in triple-negative breast cancers was correlated with a poor prognosis and promoted tumor cell in vitro proliferation. *Med Oncol*. 2014; 31:1–10.
8. Di Leva G, Garofalo M, Croce CM. MicroRNAs in Cancer. *Annu Rev Pathol: Mech Dis*. 2014; 9:287–314.
9. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med*. 2014; 20:460–469. [PubMed: 25027972]
10. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Ebert BL, Mak RH, Ferrando AA, Downing JR. MicroRNA expression profiles classify human cancers. *Nature*. 2005; 435:834–838. [PubMed: 15944708]
11. Wang H, Ach RA, Curry B. Direct and sensitive miRNA profiling from low-input total RNA. *RNA*. 2007; 13:151–159. [PubMed: 17105992]
12. Wang JW, Czech B, Weigel D. miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell*. 2009; 138:738–749. [PubMed: 19703399]
13. Degliangeli F, Pompa PP, Fiammengio R. Nanotechnology-based strategies for the detection and quantification of MicroRNA. *Chem - Eur J*. 2014; 20:9476–9492. [PubMed: 24989446]
14. Johnson BN, Mutharasan R. Biosensor-based microRNA detection: techniques, design, performance, and challenges. *Analyst*. 2014; 139:1576–1588. [PubMed: 24501736]
15. Koscińska E, Starega-Roslan J, Czubala K, Krzyzosiak WJ. High-resolution northern blot for a reliable analysis of microRNAs and their precursors. *Sci World J*. 2011; 11:102–117.
16. Liu CG, Calin GA, Meloon B, Gamliel N, Sevignani C, Ferracin M, Dumitru CD, Shimizu M, Zupo S, Dono M, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A*. 2004; 101:9740–9744. [PubMed: 15210942]

17. Lao K, Xu NL, Yeung V, Chen C, Livak KJ, Straus NA. Multiplexing RT-PCR for the detection of multiple miRNA species in small samples. *Biochem Biophys Res Commun*. 2006; 343:85–89. [PubMed: 16529715]
18. Degliangeli F, Kshirsagar P, Brunetti V, Pompa PP, Fiammengio R. Absolute and direct microRNA quantification using DNA-gold nanoparticle probes. *J Am Chem Soc*. 2014; 136:2264–2267. [PubMed: 24491135]
19. Hwang DW, Song IC, Lee DS, Kim S. Smart magnetic fluorescent nanoparticle imaging probes to monitor microRNAs. *Small*. 2010; 6:81–88. [PubMed: 19911390]
20. Shah P, Thulstrup PW, Cho SK, Bhang YJ, Ahn JC, Choi SW, Bjerrum MJ, Yang SW. In-solution multiplex miRNA detection using DNA-templated silver nanocluster probes. *Analyst*. 2014; 139:2158–2166. [PubMed: 24616905]
21. Zeng YP, Zhu G, Yang XY, Cao J, Jing ZL, Zhang CY. A quantum dot-based microRNA nanosensor for point mutation assays. *Chem Commun*. 2014; 50:7160–7162.
22. Alhasan AH, Kim DY, Daniel WL, Watson E, Meeks JJ, Thaxton CS, Mirkin CA. Scanometric microRNA array profiling of prostate cancer markers using spherical nucleic acid-gold nanoparticle conjugates. *Anal Chem*. 2012; 84:4153–4160. [PubMed: 22489825]
23. Lee H, Park JE, Nam JM. Bio-barcode gel assay for microRNA. *Nat Commun*. 2014; 5:3367. [PubMed: 24569571]
24. Fang S, Lee HJ, Wark AW, Corn RM. Attomole microarray detection of microRNAs by nanoparticle-amplified SPR imaging measurements of surface polyadenylation reactions. *J Am Chem Soc*. 2006; 128:14044–14046. [PubMed: 17061884]
25. Šípová H, Zhang S, Dudley AM, Galas D, Wang K, Homola J. Surface plasmon resonance biosensor for rapid label-free detection of microribonucleic acid at subfemtomole level. *Anal Chem*. 2010; 82:10110–10115. [PubMed: 21090631]
26. Cao YC, Jin R, Mirkin CA. Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science*. 2002; 297:1536–1540. [PubMed: 12202825]
27. Graham D, Smith WE, Linacre AMT, Munro CH, Watson ND, White PC. Selective detection of deoxyribonucleic acid at ultralow concentrations by SERRS. *Anal Chem*. 1997; 69:4703–4707.
28. Jin R, Cao YC, Thaxton CS, Mirkin CA. Glass-Bead-Based parallel detection of DNA using composite Raman labels. *Small*. 2006; 2:375–380. [PubMed: 17193054]
29. Campion A, Kambhampati P. Surface-enhanced Raman scattering. *Chem Soc Rev*. 1998; 27:241–250.
30. Wolkow R, Moskovits M. Enhanced photochemistry on silver surfaces. *J Chem Phys*. 1987; 87:5858–5869.
31. Kneipp K, Wang Y, Kneipp H, Perelman LT, Itzkan I, Dasari RR, Feld MS. Single molecule detection using surface-enhanced Raman scattering (SERS). *Phys Rev Lett*. 1997; 78:1667–1670.
32. Nie S, Emory SR. Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science*. 1997; 275:1102–1106. [PubMed: 9027306]
33. Vo-Dinh T. Surface-enhanced Raman spectroscopy using metallic nanostructures. *TrAC, Trends Anal Chem*. 1998; 17:557–582.
34. Lutz BR, Dentinger CE, Nguyen LN, Sun L, Zhang J, Allen AN, Chan S, Knudsen BS. Spectral analysis of multiplex Raman probe signatures. *ACS Nano*. 2008; 2:2306–2314. [PubMed: 19206397]
35. Vo-Dinh T, Houck K, Stokes DL. Surface-enhanced Raman gene probes. *Anal Chem*. 1994; 66:3379–3383. [PubMed: 7978314]
36. Driskell JD, Seto AG, Jones LP, Jokela S, Dluhy RA, Zhao YP, Tripp RA. Rapid microRNA (miRNA) detection and classification via surface-enhanced Raman spectroscopy (SERS). *Biosens Bioelectron*. 2008; 24:917–922.
37. Abell JL, Garren JM, Driskell JD, Tripp RA, Zhao Y. Label-free detection of micro-RNA hybridization using surface-enhanced Raman spectroscopy and least-squares analysis. *J Am Chem Soc*. 2012; 134:12889–12892. [PubMed: 22788749]
38. Guven B, Dudak FC, Boyaci IH, Tamer U, Ozsoz M. SERS-based direct and sandwich assay methods for mir-21 detection. *Analyst*. 2014; 139:1141–1147. [PubMed: 24418951]

39. Wabuyele MB, Vo-Dinh T. Detection of human immunodeficiency virus Type 1 DNA sequence using plasmonics nanoprobes. *Anal Chem.* 2005; 77:7810–7815. [PubMed: 16316192]
40. Wang HN, Vo-Dinh T. Multiplex detection of breast cancer biomarkers using plasmonic molecular sentinel nanoprobes. *Nanotechnology.* 2009; 20:065101. [PubMed: 19417369]
41. Vo-Dinh T, Liu Y, Fales AM, Ngo H, Wang HN, Register JK, Yuan H, Norton SJ, Griffin GD. SERS nanosensors and nanoreporters: golden opportunities in biomedical applications. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2015; 7:17–33. [PubMed: 25316579]
42. Wang HN, Fales AM, Vo-Dinh T. Plasmonics-based SERS nanobiosensor for homogeneous nucleic acid detection. *Nanomedicine.* 2015; 11:511–520. [PubMed: 25510735]
43. Yuan H, Khoury CG, Wang HH, Wilson CM, Grant GA, Vo-Dinh T. Gold nanostars: surfactant-free synthesis, 3D modelling, and two-photon photoluminescence imaging. *Nanotechnology.* 2012; 23:075102. [PubMed: 22260928]
44. Fales AM, Yuan H, Vo-Dinh T. Development of hybrid silver-coated gold nanostars for nonaggregated surface-enhanced Raman scattering. *J Phys Chem C.* 2014; 118:3708–3715.
45. Bath J, Turberfield AJ. DNA nanomachines. *Nat Nanotechnol.* 2007; 2:275–284. [PubMed: 18654284]
46. Zhang DY, Seelig G. Dynamic DNA nanotechnology using strand-displacement reactions. *Nat Chem.* 2011; 3:103–113. [PubMed: 21258382]
47. Ngo HT, Wang HN, Fales AM, Nicholson BP, Woods CW, Vo-Dinh T. DNA bioassay-on-chip using SERS detection for dengue diagnosis. *Analyst.* 2014; 139:5655–5659. [PubMed: 25248522]
48. Selcuklu SD, Donoghue MT, Spillane C. miR-21 as a key regulator of oncogenic processes. *Biochem Soc Trans.* 2009; 37:918–925. [PubMed: 19614619]
49. Krichevsky AM, Gabriely G. miR-21: a small multi-faceted RNA. *J Cell Mol Med.* 2009; 13:39–53. [PubMed: 19175699]
50. Weiland M, Gao XH, Zhou L, Mi QS. Small RNAs have a large impact: circulating microRNAs as biomarkers for human diseases. *RNA Biol.* 2012; 9:850–859. [PubMed: 22699556]
51. Mackiewicz M, Huppi K. Identification of the receptor tyrosine kinase AXL in breast cancer as a target for the human miR-34a microRNA. *Breast Cancer Res Treat.* 2011; 130:663–679. [PubMed: 21814748]
52. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-C_T} method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
53. Markham NR, Zuker M. DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res.* 2005; 33:W577–W581. [PubMed: 15980540]
54. Markham NR, Zuker M. UNAFold: software for nucleic acid folding and hybridization. *Methods Mol Biol.* 2008; 453:3–31. [PubMed: 18712296]
55. Fix LN, Shah M, Efferth T, Farwell MA, Zhang B. MicroRNA expression profile of MCF-7 human breast cancer cells and the effect of green tea polyphenon-60. *Cancer Genomics-Proteomics.* 2010; 7:261–277. [PubMed: 20952761]
56. Yuan H, Liu Y, Fales AM, Li YL, Liu J, Vo-Dinh T. Quantitative surface-enhanced resonant Raman scattering multiplexing of biocompatible gold nanostars for in vitro and ex vivo detection. *Anal Chem.* 2013; 85:208–212. [PubMed: 23194068]
57. Lutz BR, Dentinger CE, Nguyen LN, Sun L, Zhang J, Allen AN, Chan S, Knudsen BS. Spectral analysis of multiplex Raman probe signatures. *ACS Nano.* 2008; 2:2306–2314. [PubMed: 19206397]

Figure 1.
Detection scheme of the SERS iMS nanoprobe.

Figure 2. Design of the iMS nanoprobe for miRNA detection. (A) The sequence structure of the iMS stem-loop probe with an internal spacer for miR-21 detection. (B) The stem-loop configuration of the probe showing the internal spacer is located within the loop region. The stem-loop structure was predicted using the two-state folding tool on the DINAMelt server.^{53,54}

Figure 3.

(A) Investigation of the use of different placeholders with a short poly(T) tail (4–6 thymine bases) for turning of the iMS SERS signal. Gray bars represent the peak height SERS background signal at 558 cm⁻¹ from the “OFF”-state iMS nanoprobe when using different placeholder strands. Red bars represent the peak height SERS intensity at 558 cm⁻¹ from the “ON”-state iMS nanoprobe after 1-h incubation of 1 μM of synthetic DNA targets at 37 °C. Placeholder-0T, -4T, -5T, and -6T contain 0, 4, 5, and 6 thymine base, respectively. (B) SERS spectra of the Cy5-labeled iMS nanoprobe with placeholder-6T in the presence or absence of miR-21 synthetic target: spectrum a, blank; spectrum b, in the presence of 1 μM noncomplementary DNA with sequences corresponding to miR-16 miRNA; spectrum c, in the presence of 1 μM synthetic miR-21 target DNA. (C) Schematic diagram showing the spontaneous dissociation of the short poly(T) tail of the placeholder from the probe following the branch migration process.

Figure 4. SERS detection of miR-21 from 200 ng total small RNA extracted from AU565 and SUM129 breast cancer cell lines. (A) Blank-corrected SERS spectra of the iMS nanoprobe in the presence of the RNA samples. (B) Bar graph showing the SERS signal of the major SERS peak at 558 cm⁻¹ in the presence of the RNA samples. (C) QRT-PCR showing miR-21 expression in AU565 and SUM149 cell lines relative to MCF-10a. Values represent average $\pm$ s.d.

Figure 5.

Evaluation of the dose response of the iMS technique using serial dilutions of total small RNA (10, 25, 50, 100, 250, and 500 ng in a 10 μ L assay volume) enriched from breast adenocarcinoma MCF-7 total RNA.

Figure 6.

SERS spectra of the Cy5.5-labeled iMS nanoprobe in the presence or absence of miR-34a synthetic target: spectrum a, in the presence of 1 μ M noncomplementary synthetic RNA with sequences corresponding to SNORD38B small RNA; spectrum b, in the presence of 1 μ M synthetic miR-34a RNA target.

Figure 7.

(A) SERS spectra measured from mixtures of different ratios of Cy5-labeled miR-21 and Cy5.5-labeled miR-34a nanoprobes (1:1, 1:2, and 1:4) in the presence of 1 μ M of both miR-21 and miR-34a synthetic targets. The concentration of miR34a nanoprobes was kept constant at 10 pM in a 10 μ L assay volume. (B) SERS signal fractions obtained from the spectral decomposition procedure. The mixture spectra were decomposed into contributions of the two distinct reporters, Cy5 (miR-21 nanoprobes) and Cy5.5 (miR-34a nanoprobes).

Figure 8.

Multiplexed detection of miR-21 and miR-34a targets in different amounts (250 ng, 500 ng, and 1 μ g) of MCF-7 total small RNA extracts using a 10 μ L nanoprobe mixture containing 5 pM of miR-21 nanoprobe and 10 pM of miR-34a nanoprobe. (A) SERS spectrum of the mixture in the presence of 1 μ g of total small RNA sample. The arrows indicate the unique SERS peaks of the miR-21 nanoprobe at 558 and 797 cm⁻¹ and miR-34a nanoprobe at 1523 and 1626 cm⁻¹. (B) The integrated SERS intensity of the peaks at 558 and 797 cm⁻¹ contributed from miR-21 nanoprobe. (C) The integrated SERS intensity of the peaks at 1523 and 1626 cm⁻¹ contributed from miR-34a nanoprobe.