Development of microfluidic concentrator using ion concentration polarization mechanism to assist trapping magnetic nanoparticle-bound miRNA to detect with Raman tags

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding single-stranded ribonucleic acid molecules. This type of endogenous oligonucleotide could be secreted into the circulation and exist stably. The detection of specific miRNAs released by cancer cells potentially provides a noninvasive means to achieve early diagnosis and prognosis of cancers. However, the typical concentration of miRNAs in blood is below the ultratrace level. This study uses a simple thermoplastic microfluidic concentration device based on an ion concentration polarization mechanism to perform enrichment and cleanup and Raman sensing beads to determine miRNA quantitatively. One sample solution containing target miRNA molecules having been hybridized with two nucleotide probes, where one probe is on a Raman tag of a nanoaggregate embedded bead (NAEB) and the other probe is on a magnetic nanoparticle (MNP), is first filled into the device. When an external field is applied across a cation exchange membrane stationed in the middle conduit of the device, the MNP-miRNA-NAEB complexed particles are enriched near the membrane edge of the cathode side. The concentrated complexed particles are further trapped using an external magnet to perform washing steps to remove excess noncomplexed NAEBs. When cleanup steps are accomplished, the remaining complexed particles are loaded into one detection capillary to acquire Raman signals from the sensing beads. Compared with that using a conventional magnetic trapping device, the cleanup time is shortened from nearly an hour to less than 10 min. Sample loss during the washing steps becomes more controllable, resulting in adequate standard curve linearity ($R > 0.99$) ranging from 1 to 100 pM.

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I. INTRODUCTION

MicroRNAs (miRNAs) are endogenous single-stranded oligonucleotides, typically composed of 18–24 ribonucleic acid (RNA) units. In eukaryotic cells, these noncoding RNAs are responsible for regulating protein expression. Recent studies indicate that miRNAs play a critical role in different cellular processes such as proliferation, differentiation, apoptosis, and metastasis.¹ MiRNAs have also been demonstrated to be crucial for cancer initiation, progression, and dissemination as well as being ideal biomarkers for

cancer diagnosis and prognosis of cancer treatment.² In addition, miRNAs have been found to be extremely stable in body fluid; this presents a novel opportunity to use miRNAs in the blood as an early predictor of cancer as well as a marker of response to therapy,³ thus providing a noninvasive means to achieve early diagnosis and prognosis.

Quantification of miRNAs in bodily fluids is challenging due to their short and highly homologous sequences and low abundance in bodily fluids. Like determining other nucleotides of low

abundance, amplification procedures are often required prior to detection. Although many miRNA detection methods have been developed, quantitative reverse transcription polymerase chain reaction (qRT-PCR) has been dominantly used for detecting an ultra-low amount of miRNAs.⁴ However, qRT-PCR has disadvantages including its complex, expensive, time-consuming, and labor-intensive procedure, and a limitation in multiplex detection of short targets of similar lengths such as miRNA. The short primer sequences and low temperature used in the PCR process for targets may cause limited primer hybridization efficiency, nonspecific hybridization, and erroneous results, resulting in a difficulty to differentiate different miRNAs.⁵ Thus, alternative rapid multiplex detection strategies for miRNAs are urgently needed.

Using various types of suitable surface functionalities, magnetic particles have the combined ability to directionally respond to the applied magnetic field and specifically recognize biomolecules (e.g., protein biomarkers and nucleic acids) and pathogens.^{6–8} Emerged as well-established nanomaterials, magnetic nanoparticles (MNPs) have been popularly utilized to accomplish the enrichment and separation of target analytes and coupling with various signal transduction mechanisms such as fluorescence emission and plasmonics-assisted light scattering to develop highly sensitive and selective assays.^{9,10}

In particular, when a nucleotide analyte (DNAs/RNAs) is to be detected, the target DNA/RNA is hybridized with both segments of complementary sequences anchored on a MNP and the other complementary sequence linked with a signal reporter to become a sandwichlike complex particle.^{11–14} The complex particles can be separated and enriched using a magnetic trapping device in a straightforward manner. The low amount of the trapped analyte can be successfully detected using the acquired signal intensities from reporters with significantly enhanced sensitivity because of the aforementioned analyte enrichment step prior to concentration determination. Various spectroscopic signal reporting methods, including fluorescence emission, plasmonics-based colorimetry, and surface-enhanced Raman scattering (SERS), have been used in sandwich assays to determine DNA and RNA below the nanomolar level.

We have invented nanoaggregate embedded beads (NAEBs) as surface-enhanced Raman scattering (SERS) tags.^{15–17} This sensing bead is made of a silica encapsulated aggregate of gold nanoparticles (AuNPs) absorbed with SERS reporter molecules. NAEB SERS tags can generate strong SERS signals because the reporter presents in the plasmonic hot spots at the gap between AuNPs. When a molecular probe of high biospecificity is conjugated on the bead surface, each NAEB works as a nanosensor to detect the target entity with high sensitivity. In addition, multiple Raman reporters produce distinct spectral features to provide multiplex detection capability.^{18–20}

The conventional trapping device employing magnetic particles is composed of a sample vial of ~1 ml and one permanent magnet with ~1 T strength placed next to the sample vial. Typically, the attracted magnetic particles migrate under a weak magnetophoretic field gradient, tens of Tesla per meter, up to a distance of 0.5 mm to accumulate on the sidewall of the sample vial next to the magnet. Although the trapping of magnetic particles at a submicrometer size can be efficiently accomplished in a couple of minutes,²¹ nanoparticles smaller than 100 nm of higher surface

areas are often employed for the convenience to immobilize molecular probes and to avoid sedimentation. To perform hybridization sandwich assays, the NMPs also have to be small enough to avoid steric hindrance to ensure the successful binding of the target, especially when the size of the signal reporter NAEB tags is considerable. Besides, because of the unique superparamagnetic properties of iron oxide nanoparticles smaller than 20 nm, these small nanoparticles provide a quick magnetophoretic response but do not agglomerate after removal of the magnetic field.²²

Although tiny particles possessing larger surface areas are more convenient to derive recognition moieties, nanoparticles smaller than 20 nm encounter significant viscous dragging friction during the magnetophoretic migration process. The trapping time of MNPs in the conventional trapping device is lengthened from two or three minutes to half an hour.²¹ In addition, when repetitive washing steps are required prior to detection, the whole assay procedures finally become too tedious to finish in one hour.

Because the strength of the magnetic field quadratically decreases with distance away from a cylindrical magnet,²³ the magnetic trapping time of nanoparticles can be significantly shortened in a microfluidic channel of which the width or height is at least one order smaller than the width of a sample vial. In other words, when passing through one microchannel, these nanoparticles can be trapped by magnetophoretic force within minutes.²⁴ However, the flow rate of this liquid has to be adequately controlled. Otherwise, these nanoparticles are passing through the strong magnetic field region too fast to be efficiently trapped.²⁵ On the other hand, a slow flow rate probably results in limited throughput.

Nanochannels possessing charged functional groups on the wall can work as ion perm-selective materials because only counterions with the opposite polarity remaining in the electrical double layer inside the nanochannel migrate through while the coions are repelled by surface charges to screen out and off the nanochannel. When an electric field is applied across the substrate containing nanopores, e.g., the ion exchange membrane or granule, the counterion flux keeps flowing through the substrate pores but the coion flux is finally blocked at the nanochannel junctions. Therefore, ion concentration polarization (ICP), ion enrichment, or ion depletion arises at either side of the ion exchange membrane placed between two electrodes. For a cation exchange membrane, ion depletion occurs on the high voltage side, while ion concentration occurs on the low voltage side because of mass conservation and electrical neutrality constraints. ICP mechanisms have been explored extensively more than one decade to develop a variety of microfluidic concentration devices to improve detection sensitivity.^{26,27} Specifically when ion exchange resin is used to focus electric field lines on the ion enrichment zone because of its spherical shape, nearly one million folds of the superconcentration effect can be achieved.^{28,29} Because a small piece of the ion exchange membrane can be glued inside the microchannel made of affordable materials such as paper and thermoplastics in a straightforward manner, a few disposable microconcentrators using ICP mechanisms have been reported.^{30,31}

In this work, we fabricate one ICP-based microconcentrator to assist magnetic trapping procedures when Raman tags are used to determine miRNAs. This simple device made of inexpensive thermoplastics contains one flow channel, of which one piece of a

cation exchange membrane is fixed on the middle part of the bottom. One aliquot containing the target miRNA molecules, MNPs conjugated with a segment of a complementary sequence (MNP@DNA¹), and NAEBs conjugated with the other segment of a complementary sequence (NAEB@DNA²) is first filled into the sample conduit of this device to remain static. When the external field is applied across the channel, the hybridized MNP-miRNA-NAEB sandwichlike complex particles are enriched near the membrane edge of the cathode side. The concentrated MNP-miRNA-NAEB complex particles are further trapped using an external magnet. Excess noncomplexed NAEBs, not attracted by the magnet, can be washed away to minimize their nonspecific adsorption. When these cleanup steps are accomplished, the remaining MNP-miRNA-NAEB complex particles in the sample channel are transferred using a pipet into a detection capillary to acquired Raman scattering signals from the sensing beads. Assisted with ICP-based concentration in a microchannel, fast magnetic trapping can be accomplished with high efficiency. The assay time of performing the sandwich hybridization method is expected to be significantly shortened. Because of sufficient washing to remove nonspecific adsorbed NAEBs, the assay precision is presumed to be improved.

II. EXPERIMENTAL

A. Materials

Hydrogen tetrachloroaurate trihydrate (HAuCl₄ · 3H₂O, 99.9%) and sodium citrate tribasic dihydrate (99%) were obtained from Showa to synthesize AuNPs. Raman reporter Safranin O was obtained from Sigma-Aldrich. The following chemicals were purchased to be used as received to prepare carboxylated NAEBs: tetraethylorthosilicate (TEOS, 99.0%); (3-aminopropyl) triethoxysilane (APTES, 99%); carboxyethylsilanetriol (99%) from Sigma-Aldrich; and (3-mercaptopropyl)-trimethoxysilane (MPTMS, 95%) from ACROS. The solution of carboxyl-terminated magnetic nanoparticles made of Fe₃O₄ was from Taiwan Advanced Nanotech.

To derive linker moieties on MNPs and NAEBs to conjugate nucleotide probes, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich. Ethanolamine from Janssen was used to cap the unreacted carboxylate groups on the nucleotide probe-conjugated particles.

The nucleotide probes containing locked nucleic acid (LNATM) units, of which the sequences were 5'-/5AmMC6/ACATCGT + TACC-3' (LNATM-probe 1) and 5'-AGACAGTGT + TA/3AmMO/-3' (LNATM-probe 2), to conjugate on MNPs and NAEBs, respectively, were purchased from Qiagen. The miRNA standard (miR-200a-3p) with the sequence 5'-UAACACUGUCU GGUAACGAUGU-3' was obtained from Genewiz.

2-(N-morpholino)ethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane from Sigma-Aldrich and nitric acid and sodium hydroxide from Showa were used to prepare pH buffer solutions.

Copolyester thermoplastic sheet (VivakTM) purchased from DSM Engineering Plastic Product was used to fabricate microfluidic chips. The cation exchange membrane [RALEX[®] CM(H)-PES] using the polyethylene substrate was from MEGA. The cylindrical

magnet containing 4000 Gauss magnetic induction was obtained from U. A. Cultural Enterprises, Taiwan. The diameter and height of this magnet are 2 cm and 1 cm, respectively.

B. Fabrication of magnetic trapping device

The homemade conventional magnetic trapping device is shown in the photograph of Fig. S-IA in the [supplementary material](#). This device was composed of one series of stacked acrylic chips. The size of each partially transparent chip was 4.0 × 3.0 cm², of which the thickness is 0.4 cm. In addition to four screw holes close to each chip corner, one rectangular slot and one circular hole of larger size (1.1 cm i.d.) were drilled. On the other side of the slot opposite to the large circular hole, another large circular hole of the same size was drilled but at a position to make part of the perimeter over the edge so that part of the cylindrical surface was removed, leaving an open semicylindrical slot. Seven pieces of these crafted chips were well-aligned to stack on a white color chip in the bottom to assemble the device by fixing four bolts into the screw holes and tightening with nuts. The rectangular slot was used to contain the trapping magnet. The cylindrical slot served as one of the vial holders. The semicylinderlike open slot served as another vial holder for the convenience to observe trapped particles. The magnetic trapping processes were observed by concentrating MNPs in a solution containing MNPs conjugated with a capture probe containing 10 repeating adenine monomers and Probe T of single-strand DNA containing 22 repeating thymine monomers. The results in the first three minutes are illustrated in the photographic images of Fig. S-IB in the [supplementary material](#).

As shown in the imbedded photograph on the upper right corner in Fig. 1, the microfluidic magnetic trapping device was composed of two copolyester slides, of which the dimensions are 7.5 cm (length) × 2.5 cm (width) × 0.1 cm (thickness). The cover plate contained one microfluidic channel and two side reservoirs, which were fabricated by CO₂ laser ablation processes. The two side reservoirs had a diameter of 0.4 cm. The width and total length of the microchannel are 0.2 cm and 3 cm, respectively. Clamped together with binding clips, these two slides were placed in an 85 °C oven for 15 min to seal. Afterward, one pair of platinum electrodes was fixed into these two side reservoirs by gluing. One small piece (0.2 × 0.2 cm²) of the cation exchange membrane to produce the ICP effect was stationed on the bottom of the middle part of the channel.

C. Preparation of miR-200a-3p RNA samples

1. The procedures of using the conventional magnetic trapping device

The standards of target miR-200a-3p were prepared as 1 ml solutions by diluting the stock solution with Tris buffer (1.25 mM) to the following concentrations: 10⁻¹⁰ M, 5 × 10⁻¹¹ M, 10⁻¹¹ M, 5 × 10⁻¹² M, and 10⁻¹² M. Each of the standards of target RNA in a 1.7 ml vial was spiked with a 10 μl solution of LNATM probe 1-functionalized NAEBs (about 10 nM) and a 10 μl solution of LNATM probe 2-functionalized MNPs (about 0.6 μM) and shaken for 30 min. Probe-functionalized NAEBs and MNPs were prepared using the procedures in the [Appendix](#). The vial was then placed in

FIG. 1. (a). The schematic illustration of the microfluidic magnetic trapping system. I and II: platinum electrode; III: flow channel; IV: ion exchange membrane; V: magnet. The electrodes are connected with one power supply to apply the external electric field. (b). Photographic image of the microfluidic device.

the magnetic trapping device for 20 min. When the sandwichlike NAEB-RNA-MNP complex particles were attracted by the magnet and collected on the bottom of the vial, the supernatant liquid was removed and the remaining complex particles were resuspended with 1 ml of Tris buffer. The same trapping and washing steps were repeated for another two times. Finally, the particles remained in the vial bottom were reconstituted using 20 μ l Tris buffer to acquire Raman scattering spectra.

To verify assay specificity, control RNA sample miR-484 containing a noncomplementary sequence (5'-UCAGGCUCAGU CCCCUCGGAU-3') with the probes was used to perform comparison experiments.

2. The procedures of using a microfluidic magnetic trapping device

The experimental setup to perform microfluidic magnetic trappings is illustrated in Fig. 1. The voltage source was provided by a power amplifier (Model 10/10, Trek) to apply an external field across the electrodes in the side reservoirs of the microfluidic device to produce the ion concentration polarization effect near the cation exchange membrane glued in the middle of the flow channel. The magnet was placed underneath the device close to the center area to trap the concentrated solutes.

The standards of target miR-200a-3p were prepared as 1 ml solutions by diluting the stock solution with Tris buffer (1.25 mM) to the following concentrations: 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, and 10^{-12} M. Each of the standards of target RNA in a 1.7 ml vial was spiked with a 10 μ l solution of LNATM probe1-functionalized NAEBs (about 10 nM) and a 10 μ l solution of LNATM probe2-functionalized MNPs (about 0.6 μ M) and shaken for 30 min. The vial was then placed in the conventional magnetic trapping device for 20 min. When the sandwichlike NAEB-RNA-MNP complex particles were attracted by the magnet and collected on the bottom of the vial, the supernatant liquid was removed and the remaining complex particles were resuspended with 35 μ l of Tris buffer. The reconstituted particle solution was transferred into the channel segment close to the cathode of the microfluidic device. Tris buffer of the same volume (35 μ l) was filled into the segment close to the anode. One external electric field of 100 V/cm was applied across the whole channel for about one minute to concentrate the sandwichlike complex particles. One magnet was placed underneath the sample concentration zone to trap the sandwichlike complex particles in the sample. The polarity of electrodes was then reversed for another 1 min to disperse the excess NAEBs toward the anode. The supernatant liquid was drained to dry using a pipet. The remaining particles were reconstituted with 20 μ l Tris buffer to acquire Raman scattering spectra.

F. Acquisitions of Raman scattering spectrum

The final solutions of buffer-reconstituted samples were loaded through one open end of a capillary, made of the tip of a glass Pasteur pipet, while the other end was sealed. This capillary holding the liquid with its surface force was placed on the top of a magnet to attract the MNP-miRNA-NAEB complex particles to one spot. Finally, this capillary was transferred to the stage of a Raman microscope to acquire SERS spectra from NAEBs.

III. RESULTS AND DISCUSSION

First, we used a conventional magnetic trapping device to enrich the complex particles of the MNP-RNA-NAEB as a reference point. Mixed with functionalized MNPs and NAEB tags, one aliquot of the miR-200a-3p RNA sample or blank was filled into the vial of the conventional magnetic trapping device. Although the graphs of Fig. S-IB in the [supplementary material](#) show that most hybridized products of NMPs were enriched by the trapping device in three minutes, the complex particles of the NMP-RNA-NAEB were expected to encounter a much stronger viscous dragging force during the magnetophoretic process because the size of complex particles (~ 100 nm) was significantly larger than that of MNPs (~ 10 nm). The trapping step was, therefore, set as 20 min to collect the complex particles.

The supernatant liquid after magnetic trapping was then siphoned out to wash out excess noncomplexed NAEBs. After the sample vial was removed from the device, the remaining complex particles were resuspended with Tris buffer. The sample vial was then returned to the device to repeat the magnetic trapping step. The aforementioned trapping and cleanup cycles were repeated

twice. The whole procedure, including three cycles of repeated magnetic trapping and cleanup, was accomplished in more than one hour. The trapped sample was finally reconstituted with 20 μ l Tris buffer to fill into the detection capillary prior to Raman spectrum acquisitions.

Figure 2 shows the Raman scattering spectra of the Safranin O molecule from NAEB tags in samples of a fixed concentration of LNATM probe2-functionalized MNPs and RNA standards at the concentrations of 50 pM, 5 pM, 1 pM, and 0 pM (blank), respectively. Using the peak intensities of characteristic wavelengths of Safranin O at 615 cm^{-1} , 1378 cm^{-1} , 1557 cm^{-1} , and 1638 cm^{-1} , in the concentration range from 1 pM to 100 pM, we obtained the standard curves at each wavelength, of which the correlation coefficients (R) are 0.89, 0.98, 0.97, and 0.96, respectively. Although the R values of some of these curves are acceptable, the residual standard error (~ 103 counts) is 16% of mean signal intensity (~ 600) in the curve in Fig. 2. Hence, the precise quantification of miRNAs in the samples using these curves cannot be obtained. In addition, the whole procedure is too tedious to accomplish within one hour. (The signal intensities of 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, and 10^{-12} M samples were 1560, 1730, 1965, 2070, and 2380 counts, respectively, of which the average value was 1940 counts. The intensity of the blank was 1340 counts.)

To verify assay specificity, comparison experiments using the control RNA sample miR-484 containing the noncomplementary sequence with the probes were carried out. While the average signal intensity of blank was 31% ($\pm 13\%$) of the mean

intensity of the 100 pM target sample, the average intensity of the control sample was 36% ($\pm 1\%$) of the aforementioned intensity. Because the difference between control sample intensity and blank intensity (5%) is lower than pooled variations (9%), this indistinguishable difference indicates that the studied assay is highly specific to miR-200a-3p. (The spectra of the control sample and the target sample are in Fig. S-III of the supplementary material.)

It should be noted that if the number of trapping and cleanup cycles is reduced to less than three, the removal of excess noncomplexed NAEBs became insufficient. In Fig. S-IIA in the supplementary material, the peak intensity of 1 pM standard is not higher than that of the blank when the trapping and cleanup cycle was only performed once. In comparison, the spectrum of the 1 pM standard with two trapping and cleanup cycles, as shown in Fig. S-IIB in the supplementary material is distinguishable from that of the blank.

Next, we filled a NAEB solution into the flow channel to demonstrate the concentration effect using the ion exchange membrane in the microfluidic device. The photographic images in Fig. 3 show the evolutions of NAEBs concentrated on the cathodic side of the cation exchange membrane in the middle of the flow channel in the microfluidic device because of the ICP effect when the external voltage was applied. When the voltage polarity was reversed, NAEBs were dispersed toward the anode. The untreated copolyester channel surface possessed only a few charged sites to generate substantial electro-osmotic flow (EOF).³² When ICP occurred at the positive electrode side, unlike the channel of the polydimethylsiloxane-made concentration polarization device to sustain strong EOF,³³ there was no clear pinching effect to concentrate particles in our device.

As a further demonstration of the concentration and cleanup effects using ICP, when the MNP-RNA-NAEB complex particles in the samples were first pre-enriched in the vial of the conventional magnetic trapping device, the concentrated complex particles was reconstituted in 35 μ l Tris buffer and loaded into the flow channel segment close to the cathode of the microfluidic concentration device to perform trapping and washing cycles. In about one minute, the loaded sample solution was further concentrated in the cathode side of the membrane to be trapped using a magnet underneath the microfluidic device. When the voltage polarity was reversed, these complex particles were still stationed because of magnetic attractions. The excess noncomplexed NAEBs, not bound with the MNPs via linking with miRNAs, were washed out to disperse toward the anode because these LNATM probe-conjugated beads are negatively charged. When the washing step was accomplished, the remaining liquid was siphoned to remove completely. The trapped MNP-miRNA-NAEB complex particles near the membrane were collected using a pipet to resuspend into 20 μ l Tris buffer to acquire Raman scattering spectra. Because the flushing step by electrode polarity reversal drove most excess noncomplexed NAEBs into the reservoir, the trapped MNP-RNA-NAEB complex particles serving as the signal reporter were effectively separated with the aid of electromigration to remove the noncomplexed NAEBs. As a result, sufficient removal of excess NAEBs was accomplished with just a single trapping and washing cycle using the microfluidic device.

FIG. 2. The SERS spectra acquired from the MNP-miRNA-NAEB complex particles containing the Safranin O reporter when the samples are enriched using the homemade conventional magnetic trapping device. The black, red, and green traces are using miRNA samples at the concentration of 50 pM, 5 pM, and 1 pM, respectively. The blue trace is using a blank containing functionalized MNPs and NAEBs. The imbedded graph at the upper right corner is one standard curve of scattering intensity vs the logarithm value of the sample concentration using the spectrum peak at 1557 cm^{-1} . The correlation coefficient of this curve is 0.97.

FIG. 3. The photographic images showing enrichment and dispersion processes of NAEBs using the microfluidic magnetic trapping device with the cation exchange membrane. (a) A purple solution containing NAEBs is loaded into the flow channel of the microfluidic device prior to the application of electrode voltages. The cation membrane [RALEX[®] CM(H)-PES] is indicated with a red arrow. (b) When the external field is applied for 60 s, NAEBs are concentrated at the cathode side of the membrane. The concentration area is indicated using a red dotted circle. (c) When the electrode polarity is reversed, the concentrated particles gradually leave the membrane toward the anode side. (d) In 60 s, most particles are dispersed into the anodic reservoir.

Figure 4 shows the Raman scattering spectra of the Safranin O molecule from NAEB tags in samples of a fixed concentration of LNA[™] probe2-functionalized MNPs and RNA standards at the concentrations of 50 pM, 5 pM, 1 pM, and 0 pM (blank), respectively.

FIG. 4. The SERS spectra acquired from MNP-miRNA-NAEB complex particles containing the Safranin O reporter when the samples are enriched using the microfluidic magnetic trapping device developed in this study to carry out the washing steps for one cycle. The black, red, and green traces are using miRNA samples at the concentration of 50 pM, 5 pM, and 1 pM, respectively. The blue trace is using a blank containing functionalized MNPs and NAEBs. The imbedded graph at the upper right corner is one standard curve of scattering intensity vs the logarithm value of sample concentration using the spectrum peak at 1557 cm^{-1} . The correlation coefficient of this curve is 0.99.

Using the peak intensities of characteristic wavelengths of Safranin O at 615 cm^{-1} , 1378 cm^{-1} , 1557 cm^{-1} , and 1638 cm^{-1} , in the concentration range from 1 pM to 100 pM, we obtained standard curves with high linearity, of which the correlation coefficients (R) are greater than 0.99. These validated assays using the microfluidic magnetic trapping device, accomplished within half an hour, can determine miRNAs in samples with adequate precision and accuracy. In addition, unlike the spectra of 1 pM standard and blank using the conventional magnetic trapping device, as shown in Fig. 2, the spectrum of 1 pM standard using the microfluidic magnetic trapping device is clearly distinguished from that of the blank, as shown in Fig. 4. These results indicate that the microfluidic device provides more efficient cleaning to wash out more noncomplexed NAEBs.

When the aforementioned trapping and washing cycle was carried out one more time in the microfluidic device, the Raman scattering spectra of collected samples are shown in Fig. 5. The intensities of Raman scattering peaks of these traces are weaker than the peaks in Fig. 4, possibly because more complexed particles were lost during repetitive siphoning steps. In addition, the linearity of standard curves using the aforementioned characteristic wavelengths of Safranin O was also somewhat worsening. The correlation coefficients of some of these curves were below 0.99.

The spectra in the upper imbedded frame of Fig. 5 indicate that in some cases repeated washing would improve the removal of noncomplexed NAEBs because the peak intensities of the blank are only nearly half of the peak intensities of the 1 pM standard. However, the lower imbedded frame of Fig. 5 shows that in other cases the recoveries would be so inconsistent after repeated siphoning steps that the spectra of the 1 pM standard and the blank are not distinguishable. Various cleanup buffer solutions could be tested in the future to obtain higher removal efficiency without repeated washing steps.

FIG. 5. The SERS spectra acquired from MNP-miRNA-NAEB complex particles containing the Safranin O reporter when the samples are enriched using the microfluidic magnetic trapping device to carry out the washing steps for two cycles. The black and red traces are using miRNA samples at the concentration of 50 pM and 5 pM, respectively. The blue trace is using a blank containing functionalized MNPs and NAEs. In the imbedded spectrum frames, the green traces are the spectra using the 1 pM miRNA sample. The blue trace is using the blank.

IV. CONCLUSION

We successfully develop one microfluidic magnetic trapping device assisted with the ICP concentration effect to detect miRNAs at the picomolar level when miRNA is sandwiched between MNPs with a capture probe and NAEs with a detection probe. Using the ICP mechanism occurring near one cation exchange membrane fixed in the flow channel through external voltage application, the sandwiched complex particles of miRNA can be concentrated by trapped particles using one magnet underneath the microfluidic device in one minute. Assisted with electrophoretic forces to efficiently separate noncomplexed NAEs from the trapped beads, the washing step can be finished in one minute by reversing the voltage polarity. Therefore, although the initial trapping step of 20 min using the conventional magnetic trapping device to reduce sample volume is still needed, the whole assay procedures take less than 30 min to accomplish.

Using the conventional magnetic trapping device, on the other hand, the whole tedious procedures take more than one hour to finish because each trapping and cleanup cycle requires 20 min to accomplish. In addition to the initial trapping and washing cycle, two more cycles are needed to sufficiently remove noncomplexed NAEs. The sample recovery is still not reproducible enough, resulting in nonideal standard curve linearity. The correlation coefficients range from 0.89 to 0.98.

In comparison, our microfluidic method provides superior and efficient cleanup and sample recovery as compared to the conventional magnetic trapping assay. The linearity of the standard curve using the microfluidic device is adequate ($R > 0.99$) to validate accurate and precise quantitation at the concentration range from 1 pM to 100 pM.

SUPPLEMENTARY MATERIAL

The supplementary material file contains Fig. S-I showing the image of the homemade conventional magnetic trapping device and the process of attracting the complex particles composed of magnetic nanoparticles (MNPs) and Probe T of single-strand DNA; Fig. S-II showing the SERS spectra acquired from enriched sample complex particles using the homemade conventional magnetic trapping device with one or two cleanup steps; and Fig. S-III showing the SERS spectrum acquired from enriched sample and control.

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APPENDIX I: PREPARATION OF LNATM PROBE-FUNCTIONALIZED MNPs

One 25 μ l aliquot of carboxyl-terminated iron oxide nanoparticles was mixed with 975 μ l MES buffer in a vial of 1.7 ml. (The resulting nanoparticle concentration was estimated as 0.6 μ M.) This vial remained static in one magnetic trapping device for 15 min to collect nanoparticles. When the supernatant liquid was removed, the nanoparticles were resuspended in MES buffer. The vial was returned to the trapping device to repeat the previous nanoparticle collection and washing procedure.

When the supernatant was removed, MES buffer (500 μ l) and EDC/NHS solution (500 μ l, 40 mM/100 mM) were added into the vial to be shaken for 30 min. Similar nanoparticle collection and washing procedures were repeated twice. The supernatant liquid was then removed to be replaced with MES buffer (900 μ l) and the solution of inaccessible RNA, LNATM-probe 1 (100 μ l, 1 μ M), of which the nucleotide end was modified with amine groups, was added and shaken for 30 min. Finally, ethanolamine solution (200 μ l, 1.2 M) was added to block the unreacted carboxylate groups remaining on the surface of nanoparticles. Inaccessible RNA, known as locked nucleic acid (LNATM), is a modified RNA nucleotide in which the ribose moiety is derived with a methylene bridge connecting the 2' oxygen and 4' carbon. This structural change ensures the ribose firmly remaining in the 3'-endo conformation to significantly increase the binding affinity of the LNATM probe with short RNA or DNA nucleotides of complementary sequences.

The vial was again returned to the trapping device, remaining static for another 15 min to collect nanoparticles. When the supernatant liquid was removed, these nanoparticles were washed twice with 1.25 mM Tris buffer prior to storage in a refrigerator at 4 $^{\circ}$ C.

APPENDIX II. PREPARATION OF LNATM PROBE-FUNCTIONALIZED NAEBS

The procedures of producing NAEBS containing Raman reporter molecules followed the procedures in previous papers. The NAEB suspension was added into a solution of carboxyethylsilane-triol in ethanol to modify the bead surface with carboxylate groups. One milliliter of carboxylate-derived NAEB particles containing Safranin O reporter molecules were loaded into a centrifuge vial of 1.7 ml to centrifuge for 15 min at 8000 rpm. Using the absorbance of encapsulated gold nanoparticles, the concentration of NAEB suspension was estimated as 10 nM. The supernatant liquid was removed and the particles were resuspended with MES buffer. Then, the suspension was centrifuged again for 15 min at 8000 rpm. When the supernatant liquid was siphoned out again, MES buffer (500 μ l) and EDC/NHS solution (500 μ l, 40 mM/100 mM) were filled into the vial and shaken for 30 min. Similar centrifuge and washing procedures were repeated twice. The supernatant liquid was then removed and the particles were resuspended with MES buffer (900 μ l). Then, a solution of LNATM-probe 2 (100 μ l, 1 μ M), of which the nucleotide end was modified with amine groups, was added and shaken for 30 min. Finally, ethanolamine solution (200 μ l, 1.2 M) was added to block the unreacted carboxylate groups remaining on the surface of NAEB particles. The solution was again centrifuged for another 15 min. When the

supernatant liquid was removed, these particles were washed twice with 1.25 mM Tris buffer prior to storage in a refrigerator at 4 $^{\circ}$ C.

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