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Highly Sensitive Biological Analysis Using Optical Microfluidic Sensor

Sangyeop Lee, Lingxin Chen, and Jaebum Choo*

Department of Applied Chemistry, Hanyang University, Ansan 426-791, South Korea

Eun Kyu Lee

Department of Chemical Engineering, Hanyang University, Ansan 426-791, South Korea

Sanghoon Lee

Department of Biomedical Engineering, Korea University, Seoul 152-703, South Korea

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Lab-on-a-chip technology is attracting great interest because the miniaturization of reaction systems offers practical advantages over classical bench-top chemical systems. Rapid mixing of the fluids flowing through a microchannel is very important for various applications of microfluidic systems. In addition, highly sensitive on-chip detection techniques are essential for the in situ monitoring of chemical reactions because the detection volume in a channel is extremely small. Recently, a confocal surface enhanced Raman spectroscopic (SERS) technique, for the highly sensitive biological analysis in a microfluidic sensor, has been developed in our research group. Here, a highly precise quantitative measurement can be obtained if continuous flow and homogeneous mixing condition between analytes and silver nano-colloids are maintained. Recently, we also reported a new analytical method of DNA hybridization involving a PDMS microfluidic sensor using fluorescence energy transfer (FRET). This method overcomes many of the drawbacks of microarray chips, such as long hybridization times and inconvenient immobilization procedures. In this paper, our recent applications of the confocal Raman/fluorescence microscopic technology to a highly sensitive lab-on-a-chip detection will be reviewed.

OCIS codes : 300.2530, 300.6450

I. INTRODUCTION

Lab-on-a-chip technology has recently been developed to perform a variety of biological analyses [1,2]. This has several advantages compared with conventional techniques, such as minimal sample requirement, reduced reaction time, ease-of-use, improved product conversion, and reduced waste generation [3,4]. Fig. 1 shows a schematic view of a Poly (dimethylsiloxane) (PDMS) microfluidic sensor. However, a highly sensitive detection method is required for monitoring the biological analysis in a chip since the detection volume in the channel is extremely small. Off-chip detection methods, such as HPLC or mass spectrometry, have been widely used to detect the small volumes of chemical species in a microfluidic chip. However, off-chip detection is very inconvenient for continuous monitoring of chemical reactions since the sample must be removed from the reservoir of a chip at each detection occasion.

On the other hand, spectroscopic detection methods, such as laser-induced fluorescence (LIF) [5-7], UV/Vis absorption [8], chemiluminescence [9] and thermal lens microscope (TLM) [10,11] have been used for on-chip

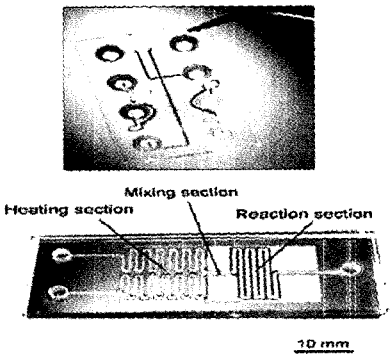


FIG. 1. A schematic view of a PDMS microfluidic sensor.

detection. Among these spectroscopic methods, the LIF detection method has been the most widely used because of its high sensitivity and low detection limits for biologically relevant species. However, the LIF detection technology has some disadvantages. Many chemical species do not fluoresce and so need to be treated with fluorescent tags to allow on-chip detection. Furthermore, fluorescence measurement does not provide detailed structural information and photo bleaching often limits detection sensitivity.

Raman spectroscopy is a well-known analytical method, which offers great advantages for probing the biological and structural properties of a compound on a microscopic scale. The detection and identification of a non-fluorescent sample is possible using this technique. Photodecomposition is reduced compared with fluorescent samples, since the excited states are rapidly quenched and the excitation energy does not have to be in resonance with electronic transitions. However, Raman scattering is an extremely inefficient process with low scattering cross sections that are approximately fourteen orders of magnitude smaller than the absorption cross sections of fluorescent dye molecules. In order to achieve a high sensitivity for a biological sample, the scattering intensity should be greatly increased. Surface enhanced Raman spectroscopy (SERS), using silver nanoparticles, has shown promises in overcoming the low sensitivity problems inherent in Raman spectroscopy. Using the SERS technique, the detection sensitivity is enhanced up to 6-10 orders of magnitude over conventional Raman spectroscopy. As a result, the SERS technique provides a comparable sensitivity with the fluorescence detection [12,13]. Furthermore, with SERS it is possible to simultaneously detect multiplex analytes since its signals have much narrower than fluorescence bands.

However, the quantitative application of SERS is known to be very difficult because it is very hard to control the experimental conditions such as the degree of aggregation, the particle sizes of metal colloids, and the inhomogeneous distributions of molecules on the metal surface. As a result, under ordinary sampling conditions, the precision expected from a SERS experiment is very poor. However, a highly precise quantitative measurement can be obtained if continuous flow and homogeneous mixing conditions between analytes and silver nano-colloids are maintained. For this purpose, an alligator teeth-shaped PDMS channel has been fabricated and the SERS detection method has been used to achieve highly sensitive analyte detection. In our research group, the SERS detection, in combination with lab-on-a-chip technology, has been applied to the trace analysis of cyanide ion [14], methyl parathion [15] and duplex DNA mixtures [16]. Compared with other methods for the trace analysis, the detection sensitivity was enhanced by several orders of magnitude. We expect this analytical technique to be successfully applied to highly sensitive bioanalysis as well as other trace analyses [17-21].

Recently, we also reported a new analytical method of DNA hybridization involving a PDMS microfluidic sensor using fluorescence energy transfer (FRET) [22-24]. Hybridization analysis of DNA plays an important role in the detection of genetic diseases and gene expression profiling. Today, one of the most popular approaches to DNA hybridization analysis is the use of a microarray chip where probe DNA sequences are immobilized on a solid phase surface and incubated with a mixture of the unknown target DNA. Detection of hybridized sequences generally involves the covalent labelling of target DNA with a fluorescence dye prior to hybridization. The fluorescence is caused by an interaction between a target analyte and an immobilized probe element, and provides valuable information on the presence of a target DNA. Although microarray technology provides a cost-effective method for microscale bioassays, it has several drawbacks. First, immobilization schemes using a spotter and an arrayer need to be adopted for the hybridization. Second, a relatively long time (about 1-2 h) is required for complete hybridization because of the diffusion-limited hybridization kinetics. However, a restriction on molecular diffusion near to the surface makes the kinetics of DNA hybridization assays slower than for a microarray system. These drawbacks make the microarray technique unsuitable for high throughput applications.

To resolve these problems, a quick and accurate DNA analysis technique using microfluidic devices has been developed. This microfluidic analysis method does not use an immobilization procedure; instead, it uses a simple syringe pumping system. By injecting target and probe DNA solutions, it is possible to detect the sequence-specific hybridization of both the probe and target DNA, and the simple operation enables a highly accurate DNA analysis to be performed. Microfluidic devices also overcome the slow hybridization problem caused by the diffusion-limited kinetics on a microarray chip, since the hybridization occurs in solution. As a result, the hybridization time is greatly reduced to less than a few seconds if a properly designed channel to obtain optimized mixing performance is utilized.

However, a new detection method in a microfluidic device is required for the identification of the change in fluorescence on hybridization, since nonhybridizing fluorescence oligonucleotides cannot be washed out inside the channel. Therefore, we used fluorescence resonance energy transfer (FRET) for this purpose, where two types of DNA oligonucleotides, which share complimentary base sequences, were prepared. Each DNA oligonucleotide was labelled with a different fluorescence dye at the 5'- or 3'- terminus. Here, one oligonucleotide is the fluorescence donor and the other

is the acceptor. When the two fluorescent oligonucleotides hybridize to form adjacent sequences in the microfluidic channel, the distance between two fluorophores on the new hybrid becomes close enough for FRET to occur. FRET is caused by an interaction between the donor and acceptor fluorescence dyes located within a distance of < 8 nm from each other. The excited state energy of the donor molecule is transferred nonradiatively to the acceptor molecule, and FRET results from a quenching of the donor fluorescence and an enhancement of the acceptor fluorescence intensity [25-27]. By monitoring the change in fluorescence intensity between the donor and acceptor DNA oligonucleotides, it is possible to accurately detect their hybridization process. In this work, we used FRET in the sensitive detection of DNA hybridization in a solution phase. For this purpose, an alligator teeth-shaped PDMS microfluidic channel was used to obtain efficient mixing between the probe and target DNA oligomers. For an optimum efficiency mixing channel and flow velocity, the quantitative changes in FRET signal for DNA hybridization could be successfully observed. This method does not require either an immobilization procedure or an amplification procedure for DNA analysis. The detection time was also very fast when the microfluidic technique was applied to the analysis of DNA hybridization. These features are suitable for a high throughput bio-analysis method. In this paper, our recent applications of the confocal Raman/fluorescence microscopic technology to a highly sensitive lab-on-a-chip detection will be reviewed.

II. METHOD AND EXPERIMENT

1. Fabrication of the alligator teeth-shaped PDMS channel

Microfluidic channels were fabricated by stacking two PDMS layers that have upper and lower teeth patterns. These layers were produced by the pattern replication from mould masters. Two epoxy-based photoresist (EPON) mould masters, including upper and lower teeth patterns, were fabricated using a previously reported procedure [16]. By pouring the mixture of PDMS prepolymer and curing agent (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI) in a 10:1 ratio onto the lower mould master and by curing for 2 h on the hot plate at 80℃, the patterned thick layer (thickness: 1.0 cm) with a lower pattern was constructed. The layer with upper teeth pattern was fabricated by the compression micro-moulding of PDMS elastomer. The PDMS pre-polymer was poured onto the mould master and compressed with transparent film and an aluminium disk. Then it was cured for 2 h. The thin upper layer (thickness: 200 μm) was produced by separating it from the mould master. For the bonding of upper and lower layers, the surfaces of both layers were activated in an oxygen plasma. Then it was aligned using our house-made aligner. Methanol was used as a surfactant between both layers. Finally, the cover glass was stacked onto the upper layer. Fig. 2 shows a typical fabrication process of PDMS microfluidic sensors.

2. Preparation of silver colloids

Silver colloids were prepared by the method recently reported by Leopold and Lendl [28]. Here silver nitrate was reduced by hydroxylamine hydrochloride. The advantages of the hydroxylamine hydrochloride-reduced silver colloid are in its fast preparation at room temperature and its immediate applicability for SERS. First, 5 mL of hydroxylamine hydrochloride (3.0×10^{-2} M) was dissolved in 84 mL of triply distillated water and then 1 mL of sodium hydroxide (1.7×10^{-3} M) was added to maintain an alkaline pH condition. Next, 10 mL of silver nitrate solution (1.0×10^{-3} M) was added dropwise to the solution with continuous stirring. The solution was continuously stirred for additional 20 minutes. UV/Vis spectroscopy and TEM were used to characterize the particle size of produced colloids. The average particle size was determined to be between 65 and 70 nm. Polyamine spermine tetrahydrochloride was used as the agent to neutralize the negatively charged phosphate backbone of DNA oligonucleotides [29,30]. For spermine optimization, 20 μL of 10^{-10} M DNA oligonucleotide was premixed with 20 μL of 10^{-2} M

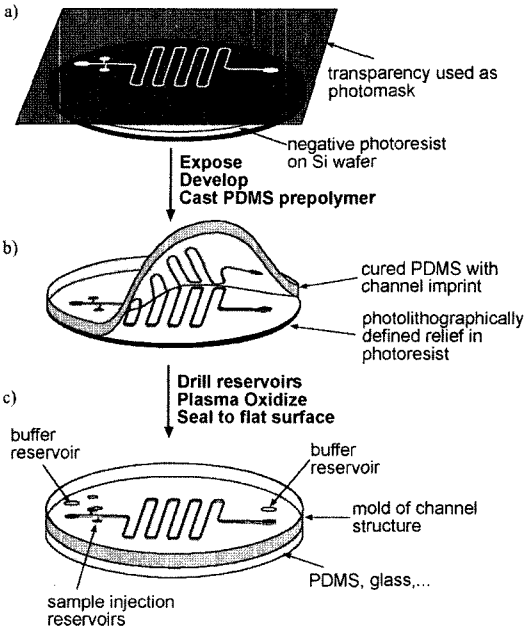


FIG. 2. Fabrication process of PDMS microfluidic sensor.

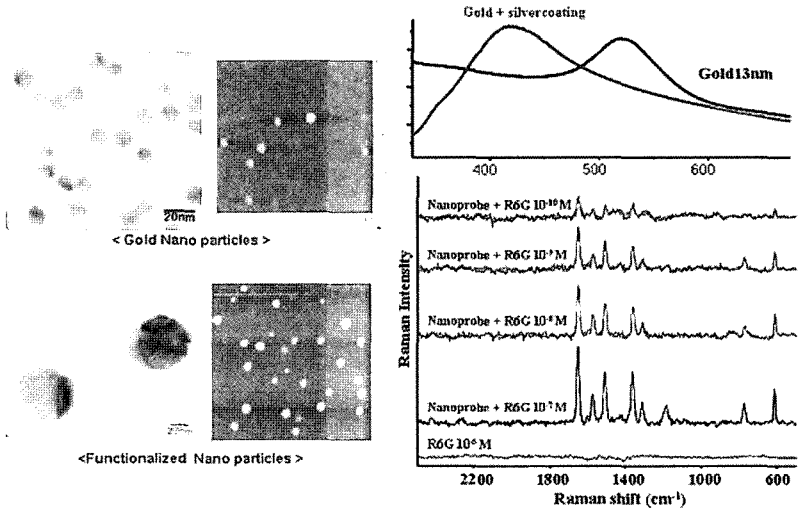


FIG. 3. TEM, AFM images and UV/Vis absorption and surface enhanced Raman spectra of SERS-active colloidal nanoparticles.

spermine. This solution was mixed with silver nanocolloids in the microfluidic channel. As a result, the oligonucleotides were effectively adsorbed on silver nanoparticles. Fig. 3 shows the TEM, AFM images and UV/V is absorption and surface enhanced Raman spectra of SERS-active colloidal nanoparticles.

3. Confocal surface enhanced Raman measurements.

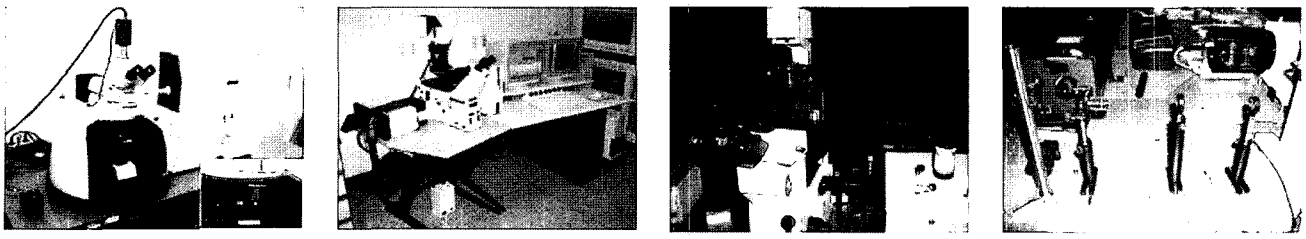
Confocal Raman measurements were performed using a Renishaw 2000 Raman microscope system. A Spectra Physics argon ion laser operating at λ=514.5 nm was used as the excitation source with a laser power of approximately 20 mW. The Rayleigh line was removed from the collected Raman scattering by a holographic notch filter located in the collection path. Raman scattering was detected using a charge-coupled device (CCD) camera at a spectral resolution of 4 cm^{-1}. An additional CCD camera was fitted to an optical microscope to obtain optical images. A two-slit confocal arrangement was used to reduce the background Raman scattering from the unfocused laser beams. Although the laser beam was focused on the middle of the micro channel, i.e., the section between the top and the bottom in the z direction, the Raman signal from a small volume of chemicals in the micro channel could not be completely separated from the signals originating from the surrounding PDMS material. To resolve this problem, all the Raman spectra were measured in the confocal mode. In the Raman system, the function of the pinhole was replaced by the cooperation of the entrance slit and the pixels in the CCD detector. The first confocal slit was set to 15 μm width. The signal was then collected from only two pixel rows on the CCD detector, creating a virtual second slit that was aligned perpendicular to the spectrometer slit. Each spectrum was accumulated for 30 seconds. Using the confocal technique, the background stray light, due to any out-of-focus regions of the PDMS, was effectively removed. Experiments were conducted to investigate the feasibility of confocal SERS as a sensitive detection tool for the evaluation of the mixing behaviour of non-fluorescent confluent streams in a PDMS microfluidic channel.

4. Confocal fluorescence measurements

The fluorescence emission spectra were measured using a Leica TCS SP confocal fluorescence microscope. The confluent mixing streams of the molecular beacon and target DNA were analysed using two-dimensional confocal fluorescence images in the x-y plane located perpendicular to the optical axis using a 10× water-immersion objective lens, the resolution of which was estimated to be 1 μm. The laser excitation of FAM occurs at λ= 488 nm, and the emitted fluorescent light is detected between λ=500 and 640 nm. The image size was 512×512 pixels, and the width of each pixel was 0.49 μm. The fluorescence spectra were also measured using the λ-scanning mode of the confocal laser scanning microscope [31,32] to quantitate the change in fluorescence intensity during the hybridization of the MB and the target DNA. Fig. 4 shows the optical fluorescence/Raman systems used in this study.

5. Preparation of the molecular beacon

All the molecular beacon probe (MB-probe) and target DNA oligonucleotides were purchased from Bio Basic Inc. (Canada), and used without further purifi-



(a) (b) (c)

FIG. 4. Optical detection systems for microfluidic sensors: (a) confocal Raman microscope, (b) confocal laser scanning fluorescence microscope, and (c) confocal Raman/fluorescence microscope.

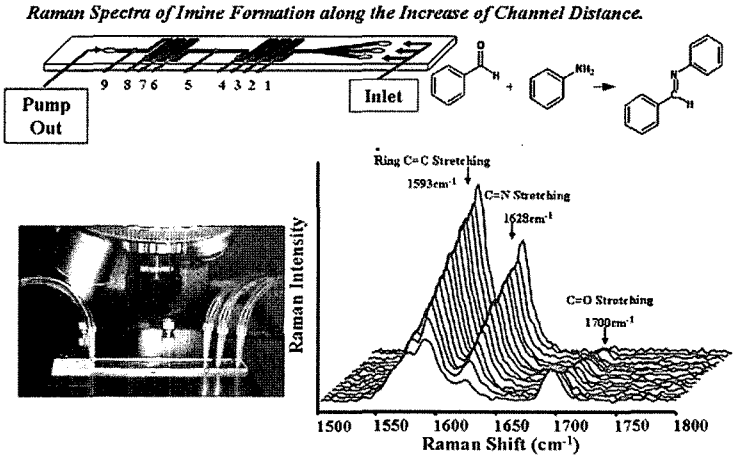


FIG. 5. In situ Raman monitoring of imine formation reaction in a glass microfluidic sensor. The micro channels are 400 μm wide and 20 μm deep. The change in Raman spectra corresponds to the peak changes caused by the imine formation at various points along the channel. The flow rate was 2.7 μL/min.

cation. In this work, the molecular beacon, labelled with a fluorophore (FAM) and a quencher (DABCYL) attached to opposite ends of the stem, was used as the probe DNA oligonucleotide. The base sequence of the MB was (FAM)-5′-TACGAGGTAAAAGGCTCTCTC CCTGTCGTA-3′-(DABCYL). The base sequence of the target DNA oligonucleotide was 3′-CCATTTTC CGAGAGAGGGAC-5′. This DNA oligonucleotide is known to be closely related to azoospermia. A significant percentage of patients with non-obstructive azoospermia test positive for small deletions in the DNA of the Y chromosome, at the locus DYS 209, and those gene sequences are closely associated with the deleted segments. For comparison purposes, the hybridization of non-complementary target DNA (mismatched base sequence: 3′-CTCTAGACTTAGTCTCGCAG-5′) with the MB was also investigated.

III. APPLICATION EXAMPLES

1. In-situ monitoring of chemical reaction using Raman microscopy [20]

In recent years, there has been a great progress in the development of microfluidic chips for use in synthetic organic chemistry. This development in micro-scale devices has been driven by its advantages over conventional bench top glassware synthesis. These include increased efficiencies, reduced mixing times, high selectivity, improved product conversion and reduced consumption of reagents. It has long been realized that the system used for detection of the progress of the reaction is a key issue in determining the applicability of a microfluidic system. Because of the extremely small volume within a microchip, a highly sensitive detection method is essential in order to monitor the progress of the chemical reaction. To resolve this problem, laser-induced Raman microscopy has been used to illustrate its applicability for the in-situ monitoring of imine formation reaction in a glass microfluidic chip.

In Fig. 5, the Raman peaks at 1628 cm^{-1} and 1700 cm^{-1} are the C=N stretching and C=O stretching modes, respectively. Here, it is important to notice that the C=N stretching peak of the product becomes steadily stronger and the C=O stretching peak of the reactant becomes weaker along the increase of channel distance from the inlets. This change indicates that the concentrations of the reactants and the product are

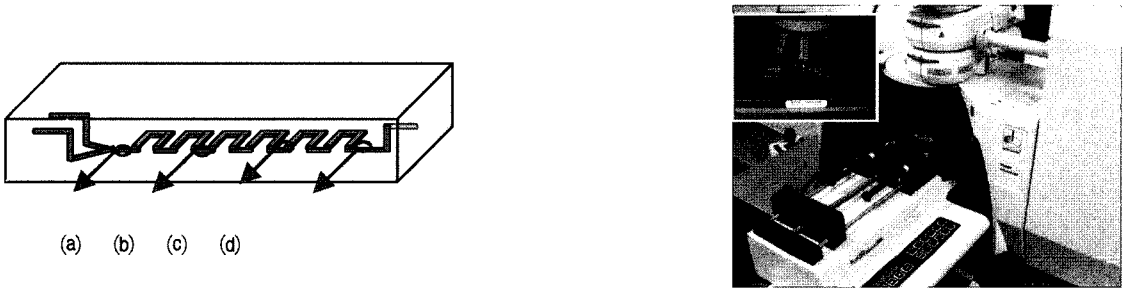
changed by a diffusion mixing during the continuous flowing process. Laser-induced Raman microscopy is considered as a very sensitive detection technique with a high spatial resolution for the in-situ monitoring of organic reaction in a microfluidic chip system.

2. Analysis of passive mixing behavior in a PDMS microfluidic channel using confocal fluorescence and Raman microscopy [19]

A rapid mixing of the fluids flowing through a microchannel is very important for various applications of microfluidic systems, and a number of devices have been designed to enhance the mixing on the microscale. To use this channel as a microchemical reactor, it is very important to continuously monitor the chemical changes of reaction mixtures in the channel. Confocal Raman Microscopy (CRM) offers a promising route to achieve this goal, since it provides a direct measurement of the conversion process from reactant to product. In this work, the applicability of CRM for effective evaluation of the mixing efficiency in a recently developed 3D serpentine channel has been studied.

Fig. 6 (a) shows a schematic view of a 3D serpentine PDMS channel and four measurement spots along the channel distance. Fig. 6 (b) shows the experimental set-up for the confocal Raman measurements on the confluent streams in the microfluidic channels. These two streams were introduced into the PDMS channel from micro syringes connected by tubes to the inlet pipettes. The flow rates were controlled simultaneously using a KD Science micro syringe pump. For the confocal Raman spectroscopic measurements, no fluorescence dye was used during the signal measurements.

Fig. 7 shows the changes of profiling Raman spectra along the channel distance in the 3D serpentine chan-



(a) (b)

FIG. 6. Schematic diagram of the experimental set-ups comprising a PDMS microfluidic channel and a microstage: (a) A schematic view of a 3D serpentine PDMS channel and four measurement spots along the channel distance: (a) at 0.15 mm, (b) at 28.5 mm, (c) at 68.4 mm, and (d) at 105.7 mm. (b) the optical arrangements for focusing the laser on the PDMS channel on the stage of confocal Raman microscope.

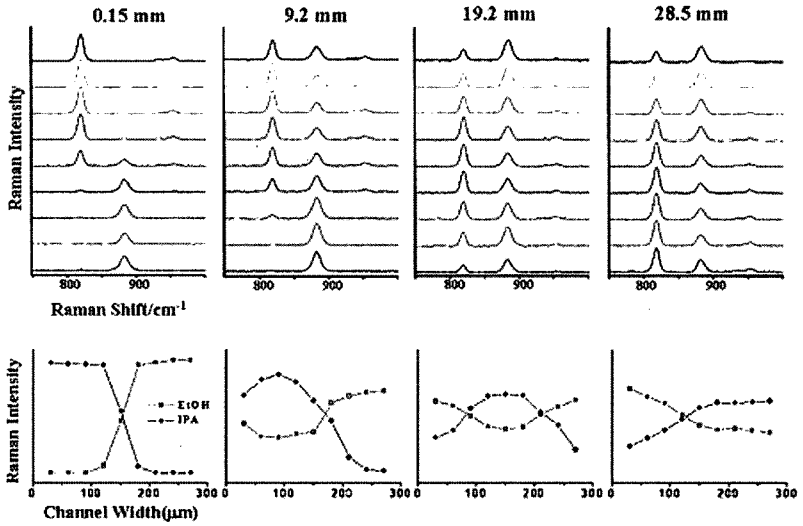


FIG. 7. Raman profiling spectra and corresponding peak area changes for the C-O/C-C stretching modes of an ethanol-isopropanol mixture in a 3D serpentine channel. The upper number denotes the channel distance beyond the T-junction spot of the channel.

nel. Etanol and isopropanol were used for the test of mixing process in the Raman spectroscopy. The results show that the fast mixing behavior of the confluent laminar streams in the 3D serpentine channel that are driven by chaotic advection can be successfully evaluated using the confocal Raman profiling technique.

3. Ultra-sensitive trace analysis of cyanide ion water pollutant in a PDMS microfluidic channel using surface-enhanced Raman spectroscopy [14]

Cyanide ion is one of the most hazardous toxic pollutants in ground waters. It is mainly discharged from industries such as gold mining, electroplating, printing, textiles and leather manufacturing. Thus, it is very important to develop a highly sensitive detection system for monitoring trace amounts of cyanide ion in ground waters. We used the surface enhanced Raman spectroscopic technique for a rapid and sensitive trace analysis of cyanide ion water pollutant in an alligator-teeth shaped PDMS microfluidic channel.

Fig. 8 (a) shows a schematic illustration of the alligator teeth-shaped microfluidic channel. The confluent streams of silver colloids and trace analytes were effectively mixed in the channel through the triangular structures, which are located on the upper and lower surfaces of the channel in a zigzag manner. The flow rate was 5 μL/min. Fig. 8 (b) shows the SER spectra for increasing concentrations of cyanide ion in the microfluidic channel: (a) 0 ppb, (b) 1 ppb, (c) 5 ppb, (d) 50 ppb, (e) 100 ppb, (f) 200 ppb, and (g) 300 ppb. In this work, an alligator teeth-shaped PDMS channel has been fabricated and the SERS detection method has been used to achieve highly sensitive analyte detection. SERS detection, in combination with lab-on-a-chip technology, has been applied to the trace analysis of cyanide ion water pollutant. Compared with other methods for the trace analysis of cyanide ions, the detection sensitivity was enhanced by several orders of magnitude.

4. Quantitative nalysis of methylparathion pesticides in a PDMS microfluidic channel using confocal surface enhanced Raman spectroscopy [15]

A fast and ultra-sensitive trace analysis of methyl parathion pesticides in a PDMS microfluidic channel was investigated using confocal surface enhanced Raman spectroscopy (SERS). A three-dimensional PDMS-based passive micromixer was fabricated for this purpose. This PDMS micromixer showed a high mixing efficiency, since a strong chaotic advection was developed by the simultaneous vertical and transverse dispersion of the confluent streams. The confocal SERS signal was measured after methyl parathion pesticides were effectively adsorbed onto silver nano-particles while flowing along the upper and lower alligator teeth-shaped PDMS channel. A quantitative analysis of the methyl parathion pesticides was performed based on the measured peak height at 1246 cm^{-1}. Our method has a detection limit of 0.1 ppm. This value satisfies the requirement recommended by the Collaborative International Pesticides Analytical Council (CIPAC) for the determination of methyl parathion in pesticide formulations. This study demonstrates the feasibility of using confocal SERS for the highly sensitive detection of methyl parathion pesticides in a PDMS microfluidic

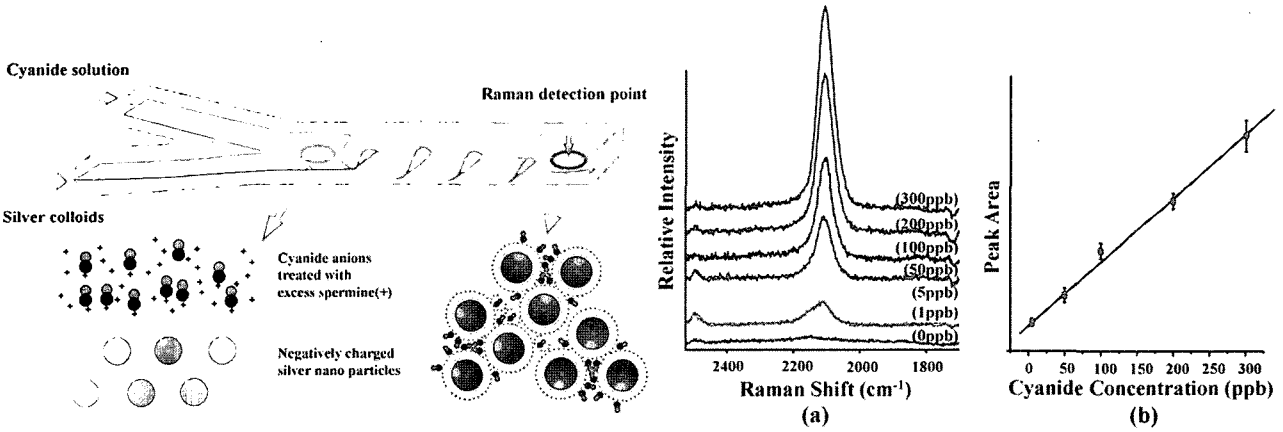


FIG. 8. Schematic illustration of the alligator teeth-shaped microfluidic channel. The confluent streams of silver colloids and trace analytes were effectively mixed in the channel through the triangular structures, which are located on the upper and lower surfaces of the channel in a zigzag manner. The flow rate was 5 μL/min. (a) SERS spectra for increasing concentrations of cyanide ion in the microfluidic channel: (a) 0 ppb, (b) 1 ppb, (c) 5 ppb, (d) 50 ppb, (e) 100 ppb, (f) 200 ppb, and (g) 300 ppb. (b) Variation of C≡N stretching peak area as a function of cynide ion concentration. (correlation coefficient, R=0.991).

channel.

Raman peak, centered at 1246 cm^{-1}, was used as a quantitative evaluation of methyl parathion pesticides. Its variation in peak area and the corresponding calibration curve are shown in Fig. 9, where the error bars indicate the standard deviation derived from a total of eight measurements. A very good linear response was found in the concentration range 0.1-1 ppm. The limit of detection (LOD) was determined to be 0.1 ppm assessed from five standard deviations above background. Compared to other methods for the trace analysis of methyl parathion pesticides, the LOD was enhanced by several orders of magnitude. We expect this analytical technique can be successfully applied to highly sensitive bioanalysis as well as to other trace analysis.

5. Quantitative analysis of duplex dye-labelled DNA oligonucleotides in a PDMS microfluidic chip using confocal surface-enhanced Raman spectroscopic study [16]

Rapid and highly sensitive detection of duplex dye-labelled DNA sequences in a PDMS microfluidic channel was investigated using confocal surface enhanced Raman spectroscopy (SERS). This method does not need either an immobilization procedure or a PCR amplification procedure, which are essential for a DNA microarray chip. Furthermore, Raman peaks of each dye-labelled DNA can be easily resolved since they are much narrower than the corresponding broad fluorescence bands. To find the potential applicability of confocal SERS for sensitive bio-detection in a microfluidic channel, the mixture of two different dye-labelled (TAMRA and Cy3) sex determining Y genes, SRY and SPGY1, was adsorbed on silver colloids in the alligator-teeth shaped PDMS microfluidic channel and its SERS signals were measured under flowing conditions. Its major SERS peaks were observable down to the concentration of 10^{-11} M. In the present study, we explore the feasibility of confocal SERS for the highly sensitive detection of duplex dye-labelled DNA oligonucleotides in a PDMS microfluidic chip.

Fig. 10 (a) shows the confocal SER spectra of 1:1 DNA oligomer mixture adsorbed on colloidal silver at the constant flow velocity of 74.08 mm/sec. According to our spectral data, the LOD of duplex oligomer mixture is estimated to be 10^{-11} M. Fig. 10 (b) illustrates the confocal SER spectra for different molar ratios of two oligonucleotides in a microfluidic channel. Different amounts of Cy3-labeled SRY (from 1.65×10^{-10} to 1.5×10^{-9} M) were added to the constant concentration of TAMRA-labelled SPGY1 (5.0×10^{-10} M) to control the molar ratio between SRY and SPGY1 to be 1:3, 1:2, 1:1, 2:1, and 3:1. The TAMRA peak at 1650 cm^{-1} was used as an internal standard and the variations of Cy-3 Raman peaks at 1588, 1469 and 1393 cm^{-1} were monitored for their different molar ratios. The intensities of those Raman peaks increase concomitantly with the increase in the concentration of Cy3-labeled SRY. In particular, the Raman peak at 1469 cm^{-1} can be used for the quantitative evaluation of SRY since it does not overlap with any Raman peaks of SPGY1. The insert in Fig. 10 (b) shows the linear response of peak area ratio (I_{1469}/I_{1560}) with the changes in the molar ratio of duplex DNA oligonucleotides. This means that the highly sensitive quantitative de-

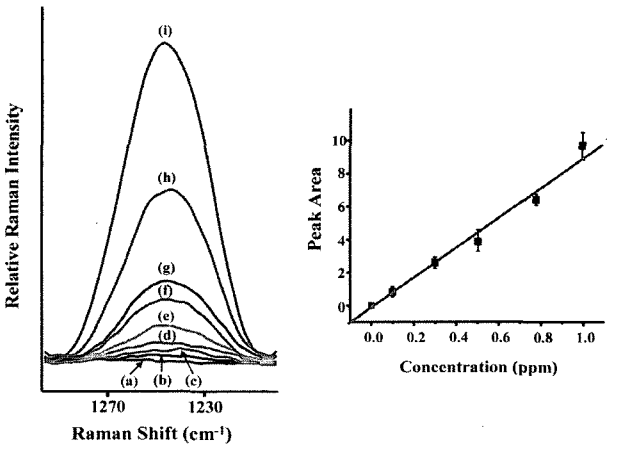


FIG. 9. (a) Confocal SERS spectra of methyl parathion standards acquired during calibration in the range 1080-1200 cm^{-1}: (a) 0, (b) 0.1, (c) 0.3, (d) 0.5, (e) 0.8, (f) 1.0, (g) 5, (h) 10, and 30 ppm. (b) The variation in Raman peak area as a function of methyl parathion concentration. (Correlation coefficient, R, = 0.991)

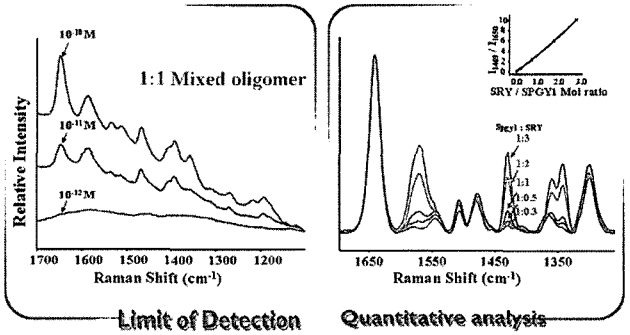


FIG. 10. (a) Confocal SERS spectra of 1:1 duplex DNA oligomer mixtures (Cy3-labeled SRY and TAMRA-labelled SPGY1) measured at different concentrations. The flow velocity was 74.08 mm/sec. (b) Confocal SERS spectra of different molar ratios of duplex DNA oligomer mixtures. The molar ratio between Cy3-labeled SRY and TAMRA-labelled SPGY1 is (a) 1:3, (b) 1:2, (c) 1:1, (d) 2:1, and (e) 3:1. The flow velocity was 74.08 mm/sec. The insert shows the variation of peak area ratio (I_{1469}/I_{1650}) as a function of SRY/SPYG1 molar ratio.

tection of duplex DNA oligonucleotide mixtures in a microfluidic channel is possible using the confocal SERS technique.

6. Fast and sensitive analysis of DNA hybridization in a PDMS microfluidic channel using fluorescence resonance energy transfer [22]

A quick and accurate DNA analysis technique using microfluidic devices has been developed. This microfluidic analysis method does not use an immobilization procedure; instead, it uses a simple syringe pumping system. By injecting target and probe DNA solutions, it is possible to detect the sequence-specific hybridization of both the probe and target DNA, and the simple operation enables a highly accurate DNA analysis to be performed. Microfluidic devices also overcome the slow hybridization problem caused by the diffusion-limited kinetics on a microarray chip, since the hybridization occurs in solution. As a result, the hybridization time is greatly reduced to less than a few seconds if a properly designed channel to obtain optimized mixing performance is utilized. In this work, we used FRET in the sensitive detection of DNA hybridization in a solution phase. For this purpose, an alligator teeth-shaped PDMS microfluidic channel was used to obtain efficient mixing between the probe and target DNA oligomers. For an optimum efficiency mixing channel and flow velocity, the quantitative changes in FRET signal for DNA hybridization could be successfully observed. This method does not require either an immobilization procedure or an amplification procedure for DNA analysis. The detection time was also very fast when the microfluidic technique was applied to the analysis of DNA hybridization. These features are suitable for a high throughput bio-analysis method.

An experiment was carried out to establish if it was possible to monitor the solution-based DNA hybridization reaction using our microfluidic system. Figure 11 (a) shows a schematic view of our alligator-teeth shaped PDMS channel and the mixing process of the confluent streams. The probe DNA (TET-P) and target DNA (TAMRA-T) were introduced into the PDMS channel from microsyringes connected via tubes to the inlet pipettes. The fluorescence emission spectra were measured using a Leica TCS SP confocal fluorescence microscope. The fluorescence spectra were also measured using the λ-scanning mode of the confocal laser scanning microscope to quantitatively investigate the fluorescence energy transfer through the hybridization process between the two DNA oligomers. Figure 11 (b) shows the fluorescence spectra measured at seven different points along the channel at a constant flow rate of 1.0 μL/min. A 1:1 molar ratio of donor:acceptor was used, and a complete mixing of both solutions was obtained after passing the saw-shaped channel. The fluorescence energy transfer between the donor TET-P and the acceptor TAMRA-T successfully occurred on DNA hybridization. Figure 11 (c) shows a calibration curve indicating the relative fluorescence intensity at

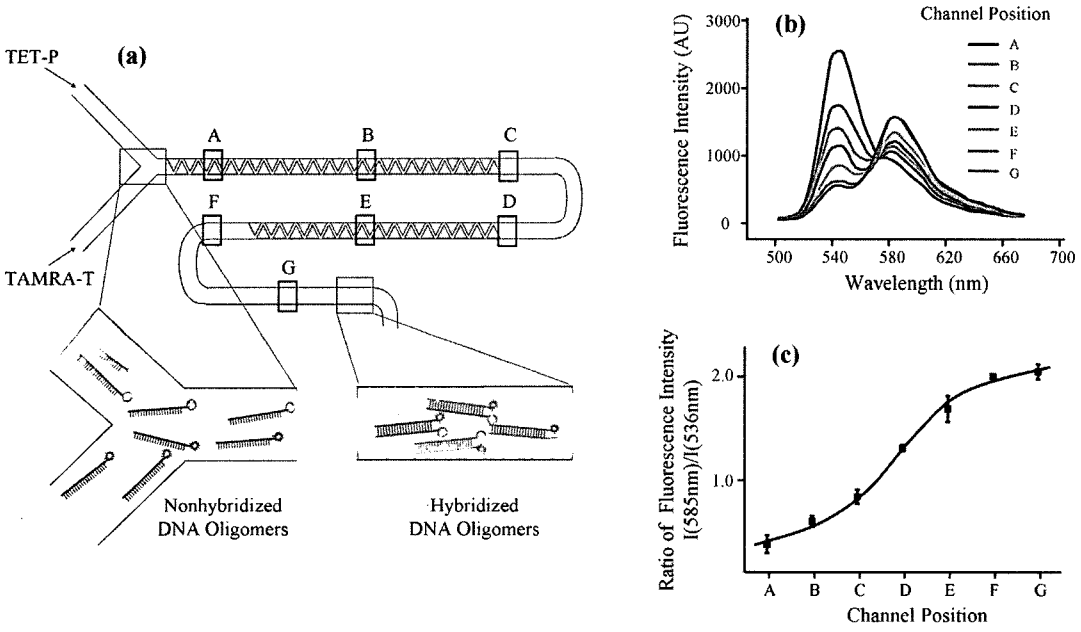


FIG. 11. (a) Schematic illustration of a PDMS microfluidic channel for the FRET detection of DNA hybridization. The seven blue boxes denote the FRET measurement areas. (b) The corresponding fluorescence spectra for each channel position. (c) A calibration curve showing the relationship between the ratio of the fluorescence intensity at 585 nm and at 536 nm in the spectra with channel distance. The flow rate was 1 μL/min.

585 and 536 nm with distance along the channel. This result shows that DNA hybridization between the donor and acceptor fluorophores increases steadily with increasing distance along the channel. In addition, similar fluorescence band intensities in the channel at Points f and g indicate that the hybridization reaction was almost complete just after the confluent streams passed through the alligator teeth-shaped saw channel. This three-dimensional PDMS channel shows a high mixing efficiency, since a strong chaotic advection is developed by the simultaneous vertical and transverse dispersion of the confluent streams. DNA hybridization analysis using microfluidic technology overcomes many of the drawbacks of microarray chips, such as the long hybridization time and inconvenient immobilization procedures.

7. Rapid DNA hybridization analysis using PDMS microfluidic sensor and molecular beacon [23]

The DNA detection method in 3-6 overcomes many of the drawbacks of microarray chips, such as long hybridization times and inconvenient immobilization procedures. However, there are some limitations in its application to real DNA samples because the target DNA must be labelled with a suitable fluorescent dye. To resolve this problem, a new DNA microfluidic sensor using a molecular beacon was developed in this study. In the molecular beacon, a fluorescent moiety is attached to the end of one arm and a non-fluorescent quencher is attached to the end of the other arm. By monitoring the change in the restored fluorescence intensity along the channel length, it is possible to rapidly detect the hybridization of the molecular beacon to the target DNA. In this case, the target DNA need not be labelled.

In Fig. 12 (a), molecular beacon (MB) has a hairpin structure, in which the fluorophore, FAM, and the quencher, DABCYL, are in close proximity, so that energy from the fluorophore is transferred to the quencher molecule. As a result, the energy is absorbed by DABCYL and no fluorescent signal is observed. In the presence of target DNA, the loop section of the MB hybridizes with the complementary target DNA. Because the hybridization between the loop and the target is stronger than that within the stem, the MB undergoes a conformational change and the fluorophore and quencher molecules are separated from each other. Consequently, the fluorescent signal is restored. By monitoring the change in the restored fluorescence intensity in a microfluidic channel, it is possible to quantitatively evaluate the unlabelled target DNA. Figure 12 (b) shows a schematic drawing of the alligator-teeth-shaped PDMS micromixer. The triangular structures are located on the lower and upper surfaces of the channel, within a rectangular duct, in a zigzag manner. The mixing of the MB and the target DNA proceeds in the alligator-teeth-shaped PDMS microfluidic channel. The MB and specific target DNA are introduced into the channel using microsyringes connected by tubes to inlet pipettes. The seven rectangles denote the fluorescence measurement areas.

As shown in Fig. 13 (a), the relative fluorescence in-

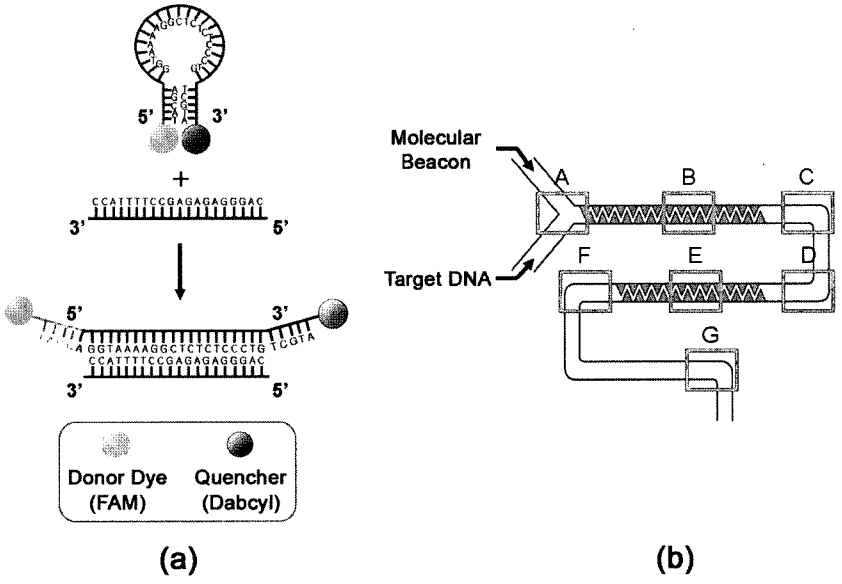


FIG. 12. (a) A schematic representation of the molecular beacon and its operating principle. Target hybridization leads to the separation of the fluorophore (FAM) and quencher (DABCYL) and a consequent fluorescent signal. (b) A schematic representation of an alligator-teeth-shaped PDMS microfluidic channel. The seven boxes denote the fluorescence measurement areas. The MB and the target DNA are injected into the channel using a microsyringe pump.

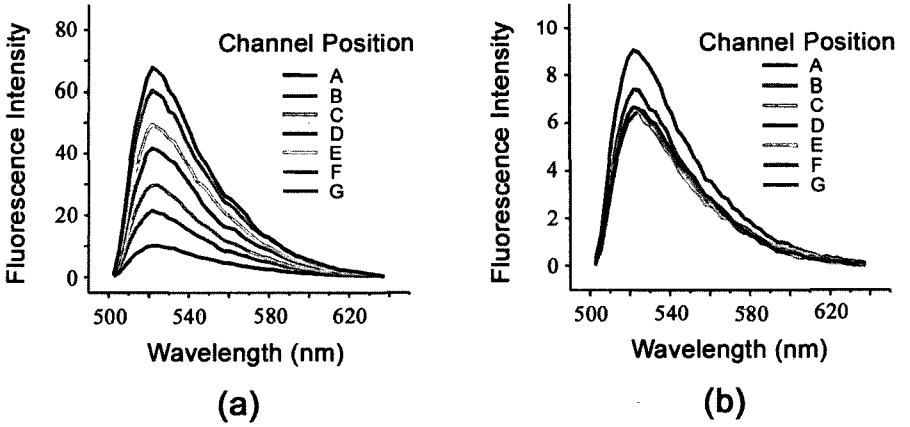


FIG. 13. Changes in the fluorescence emission intensity corresponding to the measurement areas denoted in Fig. 1. (a) Fluorescence emission spectra for the hybridization of the MB and the complementary target DNA. (b) Fluorescence emission spectra for the hybridization of the MB and a non-complementary target DNA.

tensity of the complementary target DNA gradually increased with increasing distance along the channel. This means that the fluorescence intensity of FAB was restored by the hybridization between the MB and the target DNA. In other words, the stem-loop configuration of the MB was disrupted by the hybridization and the fluorescence intensity was gradually restored with increasing distance along the channel. For comparison purposes, the fluorescence emission spectra for the hybridization between the MB and a non-complementary target DNA were also measured. In this case, no change in fluorescence intensity was observed along the channel length. Only a small change in intensity from channel positions A to C was observed due to the dilution effect of the phosphate-buffered saline buffer solution. The changes in relative fluorescence intensity along the channel length for the complementary and non-complementary target DNAs are plotted in Fig. 13.

In this study, a new hybridization analysis technique using a molecular beacon (MB) and the PDMS microfluidic channel has been developed. The MB is a single-stranded oligonucleotide that forms a stem-loop structure. In the MB, a fluorescent moiety is attached to the end of one arm and a non-fluorescent quencher is attached to the end of the other arm. By monitoring the change in the intensity of the restored fluorescence along the channel length, it is possible to rapidly detect the hybridization of the MB with the target DNA. In this technique, the target DNA need not be labelled. Our experimental results demonstrate that the MB-based DNA analysis technique, combined with the continuous flow of the microfluidic device, is a promising diagnostic tool that can be applied to high-throughput bioanalyses.

IV. CONCLUSIONS

In this paper, we reviewed our recent applications of the confocal Raman/fluorescence microscopic technology to a highly sensitive lab-on-a-chip detection. Recently, a rapid and highly sensitive detection technique is required for the detection of genetic diseases and gene expression profiling. In DNA microarray chip technologies, DNA samples should be amplified using the PCR technique before the detection since DNA is present in very low concentrations. Then the samples are immobilized on a slide glass using a microarray spotting machine. Finally, the amplified signals are detected by the fluorescence or chemiluminescence detection method. In the present study, the lab-on-a-chip technology, combined with confocal SERS technique, has been applied for the analysis of duplex dye labelled DNA oligonucleotides. This does not need an immobilization procedure as the microarray DNA chip does, since DNA signals can be detected under the flowing condition in a microfluidic channel. It does not need an amplification procedure as the low sensitivity problem, evident in conventional Raman spectroscopy, can be solved using SERS technique and the adsorption process of dye-labelled oligonucleotides onto silver nanoparticles. This detection technique can be also applied to non-fluorescent, as well as fluorescent samples. We also applied this optical detection technique using metal nanoprobes to the trace analysis of toxic material and pesticides.

In recent study, we also developed a new hybridization analysis technique using microfluidic sensors and FRET. This technique overcomes the slow hybridization problem caused by the diffusion-limited kinetics on a microarray chip, since the hybridization occurs in solution. As a result, the hybridization time is greatly

reduced to less than a few seconds if a properly designed channel to obtain optimized mixing performance is utilized. We expect this confocal SERS/FRET detection, in combination with a lab-on-a-chip technology, can be successfully applied to micro-environmental analysis as well as other highly sensitive bio-analysis.

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* Corresponding author : jbchoo@hanyang.ac.kr

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