공학석사 학위논문

동형 2기 이미도에스터를 이용한 효율적인 샘플 전처리 기술 연구 및 자동화 시스템 구축

A study on molecular diagnostic sample preparation combining modified materials with homobifunctional imidoester (HI)

울 산 대 학 교 대 학 원

의 과 학 과

김 명 규

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2022년 02월

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ABSTRACT

Infectious diseases caused by various pathogens such as *Brucella ovis, Escherichia coli* and coronavirus remain a major public health emergency of worldwide. Therefore, accurate, rapid, and robust diagnostic tests for infectious pathogens are crucial to prevent further spread of the infection and ensure timely therapeutic intervention. Particularly, the pathogen enrichment and biomolecules isolation are an essential step for both diagnostic and analytical purposes. However, conventional biomolecule isolation techniques are intensive, laborious, and require expensive reagents and multiple instruments. In addition, sample separation methods that can only use a limited amount of sample still have difficulties in detecting low amounts of pathogens.

Homobifunctional imidoesters (HI) are a cross-linker with amine groups on both sides, and can capture nucleic acids (NAs) by binding to pathogens through electrostatic interaction and recognizing amine groups at the ends of NAs. Hence, in this study, we investigated the efficient processes for pathogen enrichment and NAs isolation using modified materials (Microfluidic chip, Diatomaceous earth, BP) with HI.

First, we developed a pathogen enrichment and nucleic acid extraction method using a microfluidic chip and HI. It was found that using the microfluidic chip system, even a low concentration of pathogens can be detected using a larger amount of sample than the conventional method. Moreover, we confirmed the possibility of isolating Extracellular vesicles (EVs), which are attracting attention as new biomarkers in the field of molecular diagnostics. The conventional typical EVs isolation method is using an ultracentrifuge. However, it has the disadvantage that it requires large equipment and a lot of time. Here, we can isolate EVs faster and easier than conventional methods using microfluidic chips and HI.

Second, we developed a technology for simultaneously extracting nucleic acids from multiple samples in a 96-well filter/membrane plate using amine surface-treated diatomaceous earth (DE) and HI. Commercially available nucleic acid extraction kits require a lot of time due to complicated procedures, and it is difficult to process multiple samples at once. However, we can extract nucleic acids from 96 samples at once.

Third, we developed a simple nucleic acid extraction method using black phosphorus (BP) and HI. Two-dimensional materials: black phosphorus (BP) which has attracted significant interest employing for the diagnosis and treatment of diseases due to its high charge carrier mobility, strong optical absorption, excellent bioactivity. With the characterization of BP, we have built an optimized nucleic acid extraction system. We found that the purity and quantity of nucleic acid could arrive 2.5 times higher than the extraction form commercial kit.

The sample preparation method developed in this study solved problems of existing methods such as complex processes, sample contamination, and high price. In addition, in this study, a sample preparation method was established with an automated system. Sample preparation using an automated system is all done in one equipment without other equipment, and anyone can use it easily. These techniques are sensitive and allow for easy and fast sample preparation. Furthermore, since this sample pretreatment method does not require large-scale equipment, it is expected that it can be combined with RPA (Recombinase Polymerase Amplification), LFA (Lateral Flow Immunoassay), LAMP (Loop-Mediated Isothermal Amplification) and bio-optical sensors to create a miniaturized point-of-care-test (POCT) platform.

Keywords: Sample preparation, Extracellular vesicles, Nucleic acid, Automation device

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ABBREVIATIONS

APDMS	3-Aminopropyl(diethoxy)methylsilane
HI	Homobifunctional Imidoester
DMP	Dimethyl Pimelimidate
DMS	Dimethyl Suberimidate
DW	Distilled Water
Ct	Cycle Threshold
FE-SEM	Field-Emission Scanning Electron Microscopy
DE	Diatomaceous Earth
NA	Nucleic Acid
POC	Point-Of-Care
qPCR	Real-time PCR
RT-qPCR	Reverse Transcription real-time PCR

1. INTRODUCTION

Currently, infections caused by highly contagious pathogens are spreading around the world, and in particular, new mutant infectious pathogens can cause huge social losses(1). In order to prevent the spread of these diseases, it is necessary to isolate and treat patients through rapid diagnosis(2). Therefore, many methods for isolating nucleic acids (NAs)(3), proteins(4), and extracellular vesicles(5)(EVs), which are currently used in molecular diagnostics, are being studied(6). In particular, different approaches toward NAs extraction have rapidly developed in the last century and been applied in disease diagnosis(7). At present, the main methods for NAs extraction are the concentrated salt method, anion decontamination method, water extraction method, phenol extraction method, and enzymatic hydrolysis method(8). Today, NAs extraction is considered a reasonably routine process; however, these methods still have many problems(9). For example, expensive extraction kits and equipment, time consuming due to complex protocols, limited sample volume, and inaccurate results due to external contamination(10). In this study, to overcome these problems, we developed three sample pretreatment methods using homobifunctional imidoester (HI) and modified materials (Microfluidic chip, Diatomaceous earth, BP). HI is a cross-linker reagent that forms amidine by covalently bonding to a biological material through a reaction between two imidoester groups and a primary amine group(11). It has been proven in various studies to be a suitable reagent that can capture nucleic acids by binding to pathogens through electrostatic interaction and recognizing amine groups at the ends of NAs(12).

First, we studied pathogen enrichment and nucleic acid extraction methods using microfluidic chip and HI.(13). The microfluidic chip was surface amine using APDMS. When used in conjunction with HI, pathogen enrichment and nucleic acid extraction are possible. As a

result, the low concentration pathogen was successfully isolated from a large amount of sample through sample enrichment. This compensates for the existing disadvantage that only a limited volume can be used, allowing for more sensitive detection. Moreover, we successfully isolated Extracellular vesicles (EVs) using microfluidic chips. Extracellular vesicles (EVs) are substances released from cells(14). EVs are largely divided into exosomes (30-150 nm in diameter), microvesicles (100-1000 nm in diameter) and apoptotic bodies (1-4 μm in diameter)(15) which would be found in many body fluids such as blood, saliva, urine, and breast milk(16). In particular, among EVs, exosomes are known to play an important role as non-invasive biomarkers for early detection of diseases(17). The currently used EVs isolation method requires an ultracentrifuge and takes more than 3 hours(5). In this study, EVs were isolated from the cell culture media (CCM) HCT116 [colorectal, ATCC CCL-247] using a microfluidic chip(18). Negatively charged extracellular vesicles (EVs) bind to HI by electrostatic interactions. The whole process is done within 80 min. As a result, unlike conventional methods, we successfully isolated EVs from CCMs quickly and without large-scale equipment.

Second, Diatomaceous earth(DE) refers to the soil formed by the deposition of fine gray silicate components composed of silicate residues composed of silicates (Si(OH)4)(19). It is known to have excellent adsorption capacity, withstand high temperatures, and have many small pores(20). It is used in many research fields in combination with various materials using the advantage of a large surface area(21). In this study, multi-sample preparation analysis was developed by combining DE with 96well filter/membrane plate(22). DE treated with amines using APDMS concentrates nucleic acids using HI, a cross-linker. Then, the diatom is filtered based on the size using the PVDF membrane of the 96-well filter/membrane plate. E.B with a pH of 10 or higher degraded HI and successfully isolated nucleic acids.

These systems can process at least six samples simultaneously, and real-time PCR analysis confirmed higher sensitivity than commercially available kits.

Third, as a new star of the 2D materials family, black phosphorus(BP) has attracted considerable attention since its first mechanical exfoliation from bulk BP in 2014(23). BP consists of corrugated planes of P atoms with strong in-plane covalent bonding and weak interlayer van der Waals interactions(24), which benefit its bilayer structure along the zigzag direction and puckered lattice configuration along the armchair direction(25). Thereby, BP is superior due to its much higher surface-to-volume ratios(26), better molecular adsorption energy(27), and modified biocompatibility(28), having wide applications in biochips(29), drug treatments(30), nano-bioprobes, and biological applications(31). We proposed the use of the photo-thermal properties of BP for the purpose of improving the enzymatic digestion method of a spin-column kit to extract DNA from the pathogen *E. coli*(32). The BP-near-infrared(NIR)-homobifunctional imidoester(HI) system for DNA extraction was designed based on our optimized nucleic acid protocol. Thereafter, enriched nucleic acids were simply spun down and eluted using our prepared elution buffer. Nucleic acids released from BP nanosheets were collected, and the quantity and purity of the extracted nucleic acid was checked through real-time PCR and nanodrop to validate the function of the BP-NIR-HI system. Meanwhile, we studied the possible mechanisms of the BP-NIR-HI system through a variable control experiment. As a result, the chemical characterizations(surface potential charge and reactive oxygen species (ROS) release) supported our hypothesis(29).

Finally, through this study, various methods were introduced to solve the problems of the existing sample preparation process. It is easy, fast, and provides a method necessary for diagnosis without other equipment. Furthermore, we have established an automated system for this sample preparation process. In this system, 5 syringe pumps each inject air, sample,

lysis buffer, and elution buffer into the chip in order. The order and speed can be modified through the touch screen connected to the equipment. This automated system can process samples up to 2.5ml at a time, which can detect high concentrations of nucleic acids in large volumes of samples. Since the sample preparation process using automated equipment is done inside the chip, there is no external contamination and it is completed within 1 hour 20 minutes. This study developed various sample preparation methods for molecular diagnostics. However, molecular diagnosis is very important not only for sample preparation, but also for research on detection methods. Therefore, it is necessary to study the detection methods that can be used in conjunction with the techniques introduced above. Ultimately, we aim to create a molecular diagnostic platform that is easy and fast and that anyone can use.

2. MATERIALS AND METHODS

2-1 Microfluidic chip Production

Microfluidic chip system consists of microfluidic chambers for EVs and NAs enrichment. The microfluidic chamber consisted of several slot-type microwells connected to each other with a flow path in the chamber to enrichment of EVs and NA(13). The chips consist of a total of five layers. The top and bottom layers are covered by films with hydrophilic surfaces (Kemafoil hydrophilic film, HNW-100, COVEME, Italy), and the middle layer is made by attaching a film of thin film between the two double-sided tapes (Adhesive 300LSE-9495LE, 3 M, Minnesota, US). The middle layer was processed using a laser cutting machine (VLS3.50 (610 × 305 mm); Universal Laser Systems, Scottsdale, AZ)(33). As reported earlier, it cast acrylic sheets, Tigon tubes and epoxy to control the reaction of the microfluidic chip system Pre-treatment process is needed to concentrate samples by using microfluidic chip system(4). First, the chip was processed for 10 minutes using oxygen plasma (Covance Model, Femtoscience, Korea). Second, mix APDMS (APDMS; Sigma-Aldrich, St. Louis, MO) with deionized water and put it into the chip and conduct incubation at 65°C for one hour. After incubation, the chips are washed using deionized water and stored at room temperature.

2-2 Pathogen enrichment and Nucleic Acids isolation method using microfluidic chip

To determine the optimal conditions for pathogen enrichment with the microfluidic system, HI (DMP, Sigma, (One of the HI))) was evaluated for their ability to directly bind pathogens. HI (100 mg/mL in DW) was added in an amount of 10% to the sample volume. The mixed sample was injected into the microfluidic chip at a speed of 200 μL /min. Incubated for 20

min at room temperature to allow the HI and pathogen to bind to the amine-modified surface. Lysis Buffer (D.W, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecyl sulfate, 10% Triton X-100 and 20 μL Proteinase K) and HI added to obtain nucleic acids from pathogens. After removing the sample through air, lysis buffer was added and nucleic acid was obtained from the pathogen through incubation in an incubator at 56 ℃. Next, the microfluidic system was washed thoroughly with PBS 1ml at a flow rate of 100 μL/min to remove cellular debris and residual contaminants. The DNA was then extracted using an elution buffer (10 mM sodium bicarbonate, pH > 10, adjusted by NaOH, pH>10) with the pump at a flow rate of 50 μL/min.

2-3 Extracellular vesicles (EVs) extraction method using microfluidic chip

Microfluidic chip and HI were used to separate EVs from cell culture media (CCM, HCT116). HI was used at a concentration of 100 mg/mL (DMP), and 10% of the total amount of the sample was added. The solution mixed with CCM and HI was rotated at 60 RPM for 15 minutes. This is so that HI and EVs can be sufficiently combined. Thereafter, the sample was transferred to a syringe and injected into the microfluidic chip at a rate of 400 μL/min using a syringe pump (KD Scientific, Holliston, MA). After sample injection, incubate at room temperature for 15 minutes. Afterwards, wash with 1ml of PBS. Separate the chip and EVs using 200 μL elution buffer (10 mM sodium bicarbonate, pH > 10, adjusted by NaOH, pH>10, 50 μL / min). The C_T value was confirmed using reverse transcription qPCR by extracting miRNA from the isolated EVs. miRNA was extracted commercial kit (Total Exosome RNA and Protein Isolation Kit, Invitrogen) according to the manufacturer's protocol.

2-4 Filter/Membrane-Based Pathogen Extraction NAs

D-APDMS and HI were used as the enrichment and extraction matrices for PBS with samples. First, the Multiscreen Solvinert 96-well filter plate and the 96-well cell culture plate (F-Type) are combined. Twenty microliters of D-APDMS suspension (50 mg/mL in DW) and 50 μL of DMP solution (100 mg/mL in DW) were pipetted into a sample solution. The NAs isolation was subsequently performed in the same PTFE membrane plate. Subsequently, 20 μL of Proteinase K, 150 μL of internal lysis buffer, 30 μL of lysozyme solution (50 mg/mL in DW) were added separately. After mixing, the PVDF membrane plate was incubated in a dry oven for 30 min at 56 °C for DNA extraction. Operating the 500 relative centrifugal force (RCF) centrifuges for 2 min will allow it to exit the PTFE membrane plate with the exception of the NAs template fixed to the ADE through the HI crosslinking. Using a multi-pipette, the remaining pellets in each hole were washed twice with a 300 μL PBS. For reverse crosslinking, 100 μL of elution buffer (10 mM sodium bicarbonate, pH > 10, adjusted by NaOH) was inserted into each hole. The PTFE membrane plate was kept at RT for 1 min and centrifuged for 2 min in the 500 RCF. The NAs template that eluted to the 96-well cell culture plate was moved to the tube using a pipette. After the supernatant containing the isolated DNA was stored at −20 °C until needed. Evaluation and optimization of the NAs extraction experiments using DNA absorbance and real-time PCR was performed.

2-5 Nucleic acid extraction method using microfluidic chip-based automated devices

Nucleic acid extraction using an automated system is divided into 4 steps (1.Pathogen enrichment 2.Lysis 3.PBS Wash 4.Elution). There are five syringe pumps for each step and air injection. (#1Air, #2Sample, #3Lysis buffer, #4 PBS, #5 Elution buffer). The solution from each pump is injected into the microfluidic chip through the mainfold. The microfluidic chip stage is temperature controlled. First, the sample is mixed with 10% dimethyl pimelimidate dihydrochloride solution (DMP, Sigma, 100 mg/ml in DW (One of the HI)) and injected into the chip through pump #2. Samples were injected at a rate of 200 μL / min, respectively, to conduct a comparative experiment according to the sample injection rate. incubation at room temperature to allow pathogens and chips to bind. The sample is removed from the inside of the chip through air (pump #1). Inject 200 μL DMP solution (100 mg/ml in DW) and 580 μL Lysis Buffer (D.W, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% sodium dodecyl sulfate, 10% Triton X-100 and 20 μL Proteinase K)through the #3 syringe pump. Incubate for 15 minutes to isolate nucleic acids (DNA 56 °C, RNA room temperature). After washing with 1ml PBS (100 μL/min, syringe pump #4), the chip and nucleic acid are separated using 100 μL elution buffer (10 mM sodium bicarbonate, pH > 10, adjusted by NaOH, pH>10, 50 μL/min, syringe pump #5). The nucleic acids extracted by the automation system were optimized based on real-time PCR results.

2-6 Biological samples

B. ovis (ATCC 25840) and *Escherichia coli* (ATCC 25922) were used to assess pathogen diagnosis. After blending Brucella Broth Powder, BactoTM Agar powder, and DW, *B. ovis* was grown in a medium containing 5% defibrinated sheep's blood. The medium was incubated 48 to 72 h at 37 °C in an incubator that maintains CO2 atmosphere. E. coli was cultured at 37 °C in a medium mixed with Nutrient Broth Powder, BactoTM Agar Powder, and DW. After cultivating bacterial suspension, it was quantified in a medium mixed with BactoTM Agar powder and DW and diluted to a different concentration using PBS, 10 X, pH 7.4. The eukaryotic cells HCT116 (colorectal cancer cells, ATCC_CCL-247) were maintained in plastic culture dishes with high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Life Technology), supplemented with 10% fetal bovine serum (FBS), in a 37 °C humidified incubator with 5% ambient CO2. After culturing, the cell culture media were used for EVs isolation with the microfluidic chip.

3. RESULTS

CHAPTER 1 – Sample preparation method using microfluidic chips and HI

1.1 Pathogen enrichment and Nucleic Acid Extraction using microfluidic chip

Experiments were conducted using *B. ovis* for pathogen enrichment and nucleic acid extraction using the microfluidic chip. The amino group of HI binds through electrostatic interactions with negatively charged pathogens. Nucleic acids are separated from pathogens through lysis. Nucleic acids are bound to HI by covalent and electrostatic bonds. This process is shown in Figure 1.1 A. To confirm pathogen enrichment, 10^5 CFU of *B.ovis* bacteria was diluted in 1ml PBS and injected into the microfluidic chip along with 200ul of HI. After receiving the injected sample from the chip, DNA was extracted with the spin column kit. As a result, higher C_T values were obtained from after enrichment. This indicates that the microfluidic chip cans successfully enrichment pathogens (figure 1.1 B). As a result of isolating nucleic acids from samples of the same concentration, similar C_T values were confirmed in the microfluidic chip and kit. However, the microfluidic chip technology has the advantage of being simpler than the existing method and having no external contamination (figure 1.1 C)

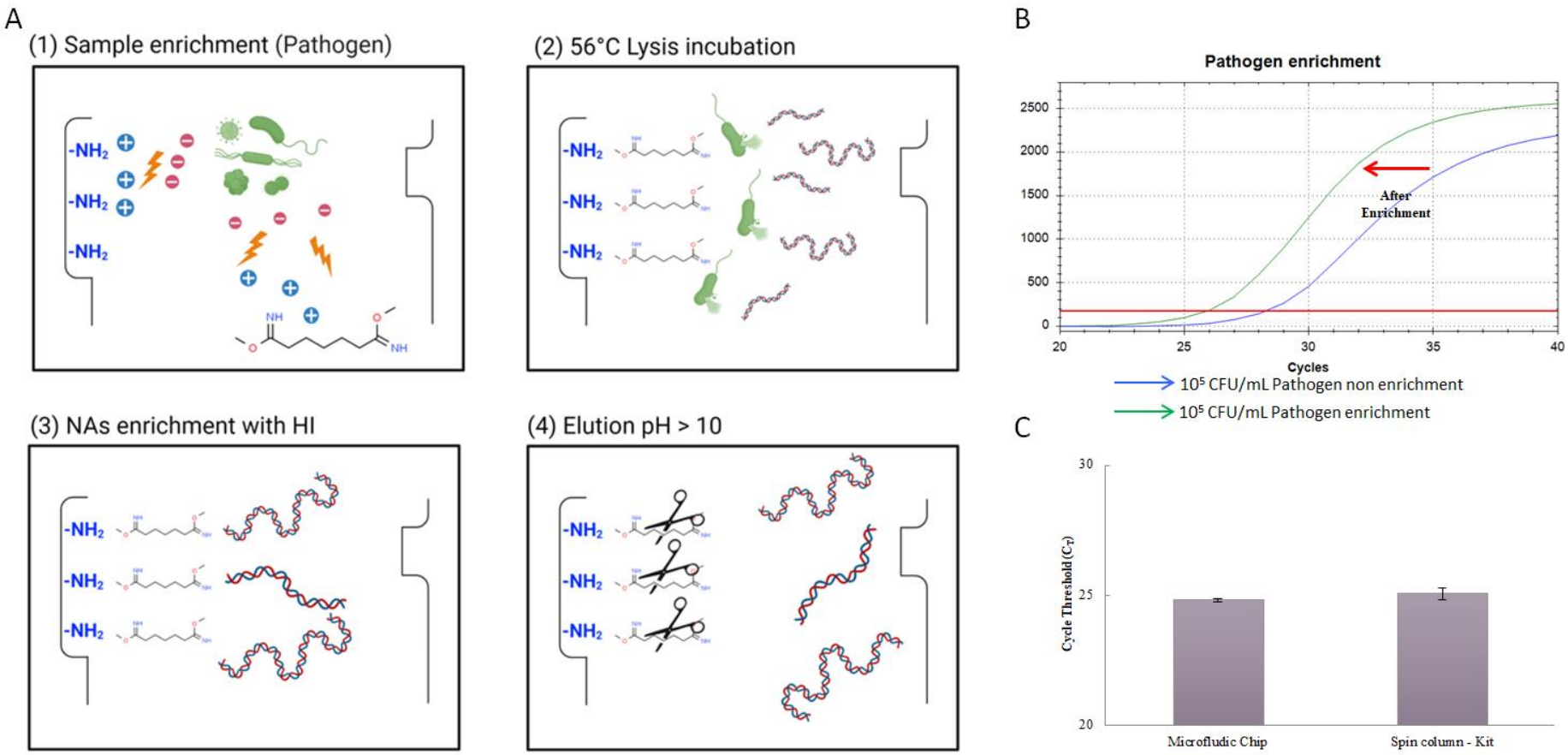

Figure 1.1 Schematic and results of pathogen enrichment method using microfluidic chip. (A) Pathogen enrichment and NA extraction process using surface amine-treated microfluidic chip and HI. (B) The result of extraction of nucleic acids from the sample enrichment was completed. Higher C^T values than before enrichment. (C) Microfluidic chip and spin column kit nucleic acid extraction results from samples of the same concentration.

1-2 Characterization of microfluidic chip for EVs isolation

As reported in the previous study, various materials were separated through various surface treatments on the microfluidic chip(34). In this paper, we focused on the fact that the inside of the chip has a strong negative charge when using the conventional amine-treated microfluidic chip and HI. As a result, it shows the possibility of separating and concentrating positively charged EVs inside the chip. In addition, the microfluidic chip has the advantage of being able to process a large amount of sample, which shows that even a small concentration sample can be concentrated. The cross-linker HI binds to the chip surface where the amine group is exposed the amine group of HI on the other side can bind to the EVs by electrostatic interaction. These coupling mechanisms are shown in Figure 1.2. The binding of HI is readily cleaved at pH > 10, and this property was used to successfully enrich and isolate positively charged EVs in CCM. Here, we developed an easier and faster separation method than the ultracentrifugation method used as the existing EVs enrichment method.

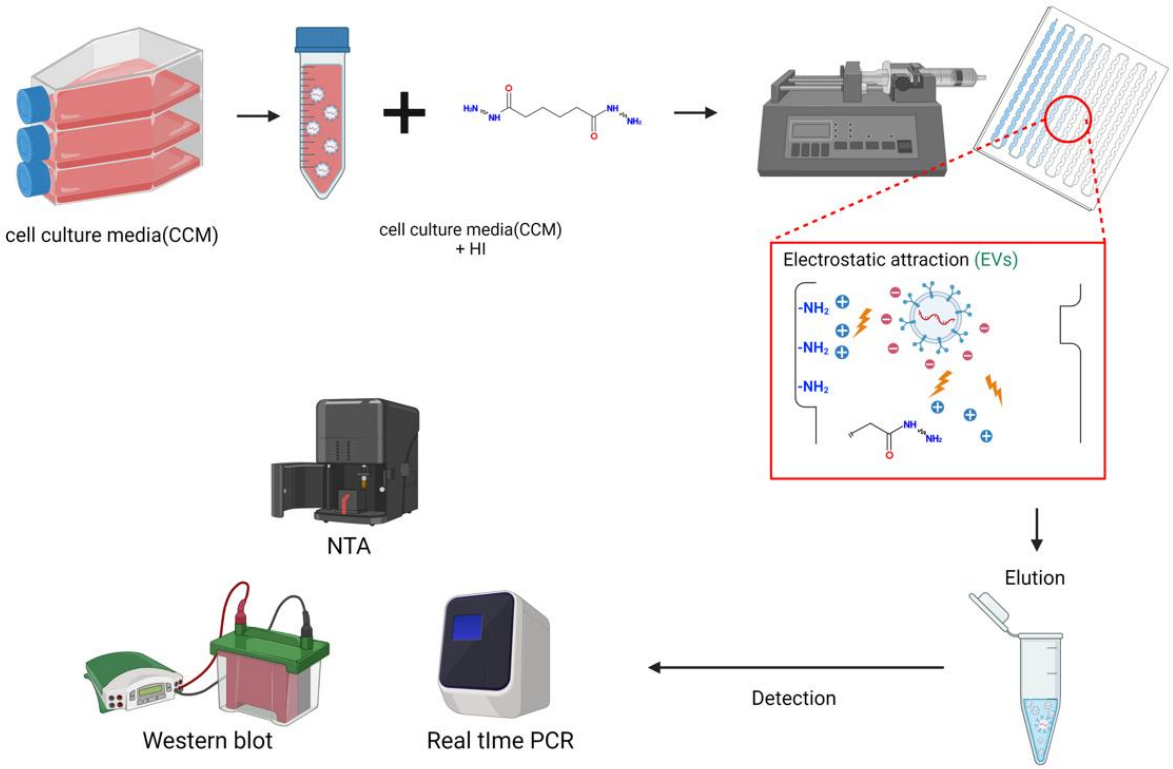

Figure 1.2 Schematic diagram of Extracellular vesicle (EVs) enrichment method using microfluidic chips. The microfluidic chip surface was modified with 3-aminopropyl-methyl-diethoxysilane (APDMS). With the help of the cross-linker HI, the EVs in the sample can be interconnected with D-APDMS to form electrostatic interactions. Surface-adhered EVs can be collected by elution buffer (pH > 10).

1-2 EVs isolation by microfluidic chips for optimization

An experiment was conducted to optimize the method of separating EVs using a microfluidic chip CCM used the media of HCT116 cell. CCM used media from HCT116 cells. Media obtained CCM when cells were over 80% grown in cell culture dishes. In order to proceed with the experiment under the same conditions, all CCMs were combined and then divided and used for the experiment. As a result of Figure 1.3, miRNA was extracted from EVs separated by a microfluidic chip and C_T values were confirmed using RT-qPCR. Results were performed using the miRNA-21 primer, which is most commonly detected in cancer cell EVs. First, a comparative experiment was performed according to the HI concentration. 10ml of CCM was used and 1ml of different concentrations of HI was mixed (Figure 1.2 A). Subsequent experiments were conducted using an HI concentration of 50 mg/ml. Various rates were used during sample injection using the syringe pump. These results show that the slower the speed, the more likely EVs to attach to the chip surface (Figure 1.2 B). After sample injection, EVs can bind to the chip surface with HI as a function of incubation time (Figure 1.2 C). Subsequent experiments were conducted based on these results.

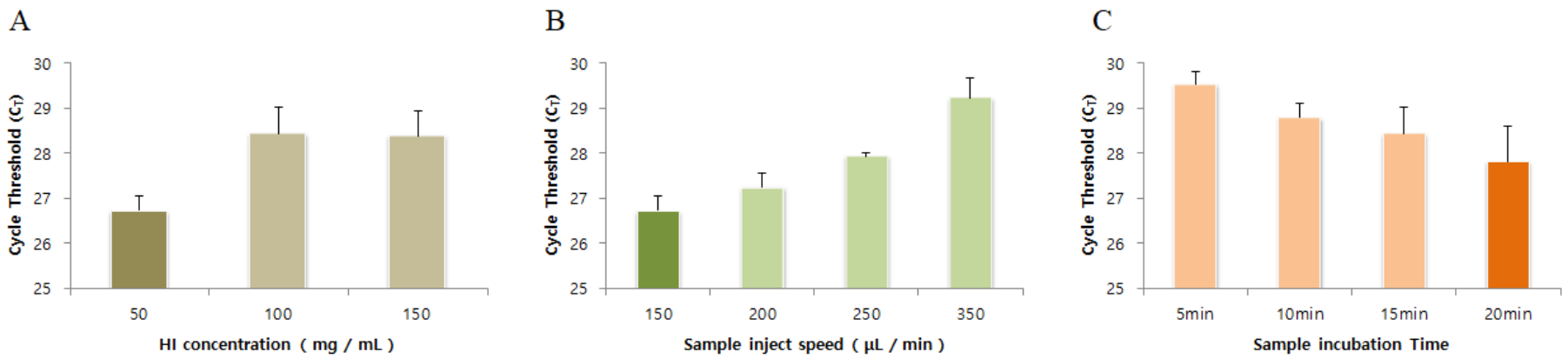

Figure 1.3 EV isolation RT-qPCR (miRNA-21, C_T value) results by microfluidic chip for optimization. All experimental results used CCM (HCT116) cultured under the same conditions. RT-qPCR results were performed using the miRNA-21 primer, which is most commonly detected in cancer cell EVs. (A) EVs Isolation Results According to HI Concentration. (B) EVs Isolation results according to sample injection rate. It can be seen that the slower the injection rate, the more EVs are Isolated. (C) Results of incubation after sample injection.

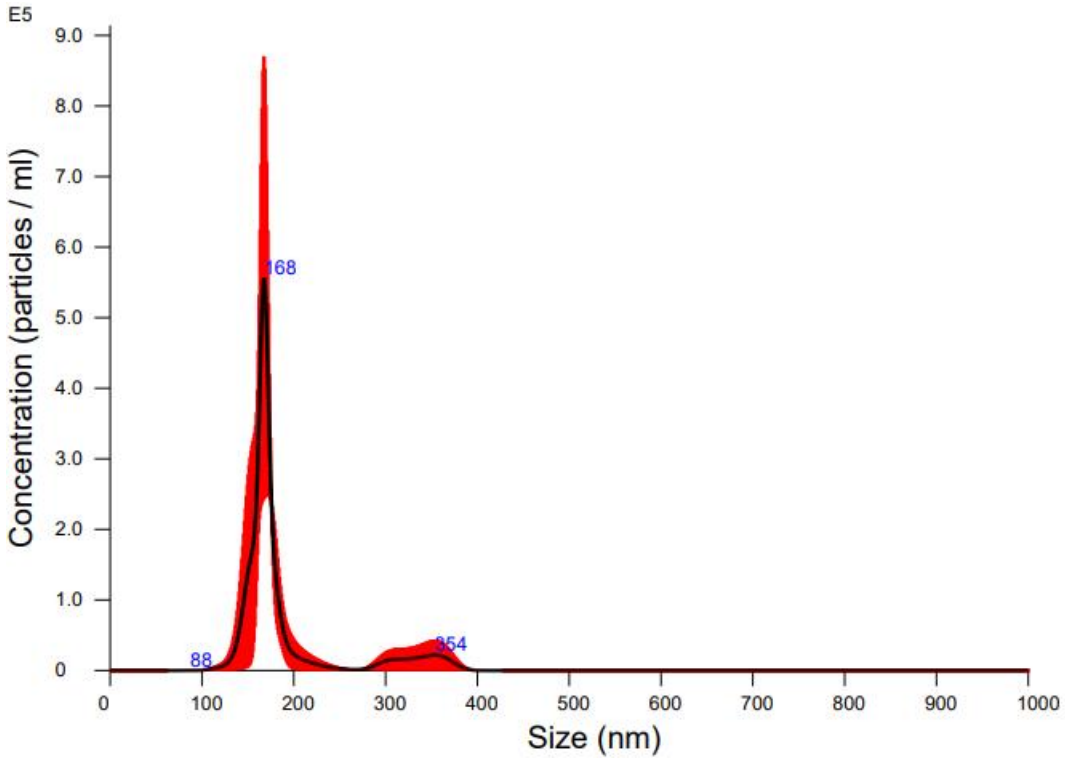

Figure 1.4 EV isolation Nanoparticle Tracking Analysis (NTA) result by microfluidic chip. Results of NTA measurement of EVs isolated based on the results of optimization. Through NTA analysis, the size of EVs isolated using a microfluidic chip was measured. The average size of EVs is 150nm ~ 200nm. EVs isolated using microfluidic chips can be used for various molecular diagnostics.

CHAPTER 2 - Nucleic acid extraction method for multi sample with Diatom and 96well filter/membrane plate

2-1 Development of the DE and 96well filter/membrane plate extraction system

As reported in previous studies, DEs with numerous qualities and different morphologies are suitable candidates for many biotechnology applications and show strong host-guest interactions to support NAs enrichment and extraction(19). Here, we fabricated DE into delicate micro three-dimensional (3D) structures through APDMS processing using a special lattice-shaped micro DE frame. Because APDMS increases the surface activity of DE, it can support cell capture, NAs enrichment and extraction through strong interactions and large surface area. The schematic diagram of pathogen enrichment with the micro lattice-like D-APDMS and HI reagent, in the plate is shown in Figure 2.1. The imidoesters at both ends of HI help to form amide bonds with the amine groups exposed on the D-APDMS surface, which also supports binding to pathogens or NAs. Pathogens bound to D-APDMS dissolve nucleic acids through a lysis process. Lysed nucleic acids bind with D-APDMS and HI. This process is performed on one plate and can be used simultaneously with up to 96 samples using a multi-pipette.

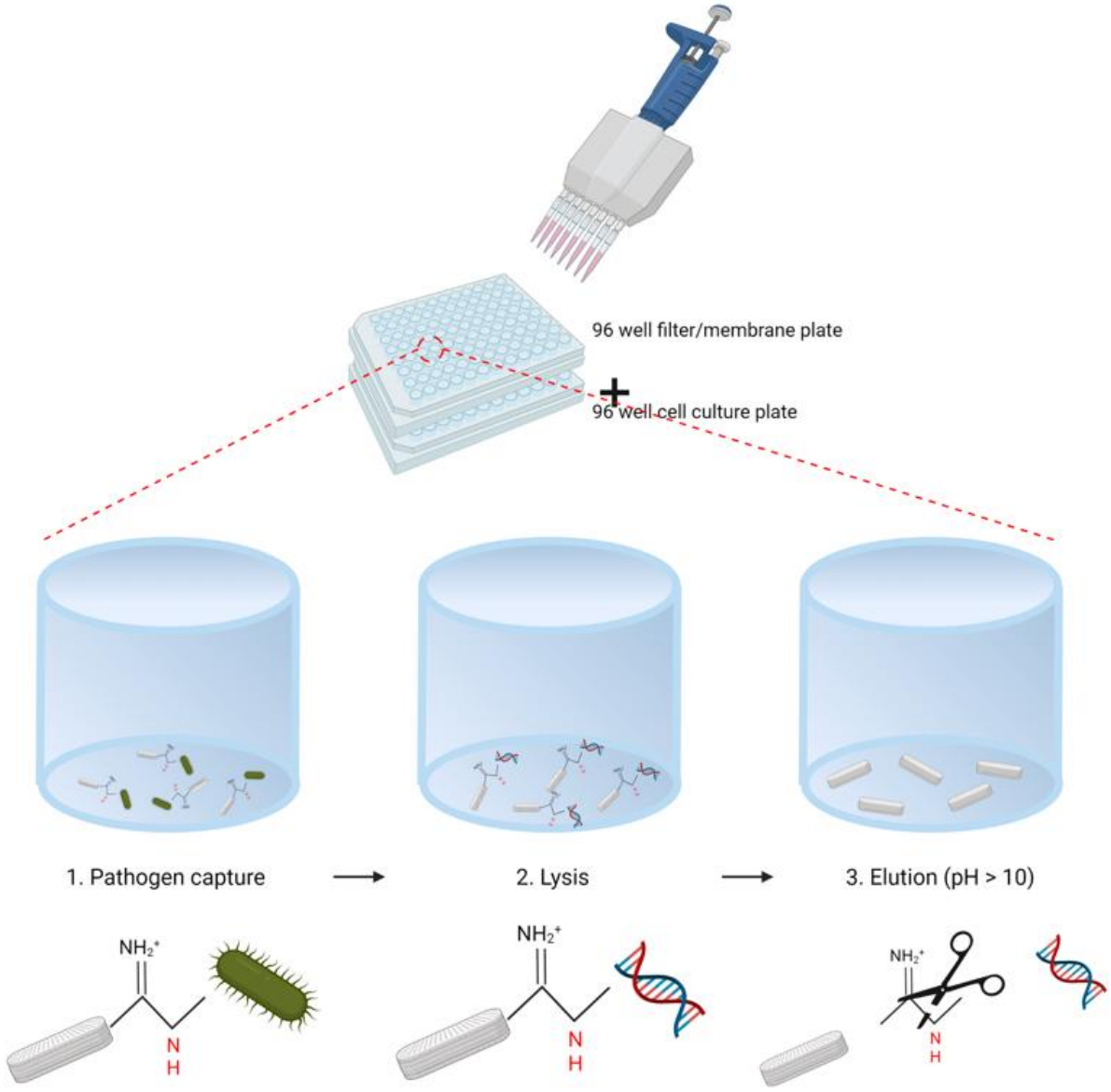

Figure 2.1 Schematic diagrams of nucleic acids (NAs) enrichment and extraction via a filter/membrane plate system. Washed diatomaceous earth (DE) was modified with 3-aminopropyl-methyl-diethoxysilane (APDMS). With the help of the cross-linker HI, NAs of lysed pathogen samples can be interconnected with D-APDMS to form a solid covalent bond. The formed complex could collect NAs by using elution buffer (pH > 10)

2-2 Accuracy and Suitability of Multiple Diagnostic Systems that enrichment and Extract Multiple Samples at a Time

To proceed with NAs enrichment and extraction, 1 mL of pathogen samples should be concentrated with the D-APDMS and HI in a tube, and then transferred to a plate repeatedly using the centrifuge. A total of six *E. coli* 1 mL (in PBS, 10^5 CFU) samples were used for NA enrichment and extraction experiments at the same time, and the results were confirmed using qPCR. (Figure 2.2 A). There was almost no difference in C_T values between the six enriched and extracted samples, ensuring the accuracy of diagnosis. To check the enrichment and extraction efficiency of the 96-well filter/membrane plate system, seven serial dilution *E. coli* (in PBS, 10^7–10^1 CFU) samples added with D-APDMS and HI are simultaneously enriched, and the extraction experiment was conducted. When using the 96-well filter/membrane plate system, it was confirmed that the detection limit was 10^1 CFU. In the same type of serially diluted samples, changes in uniform C_T values from low to high concentrations were confirmed (Figure 2.2 B). Our 96-well filter/membrane plate system can efficiently extract and enrichment a large number of samples at the same time.

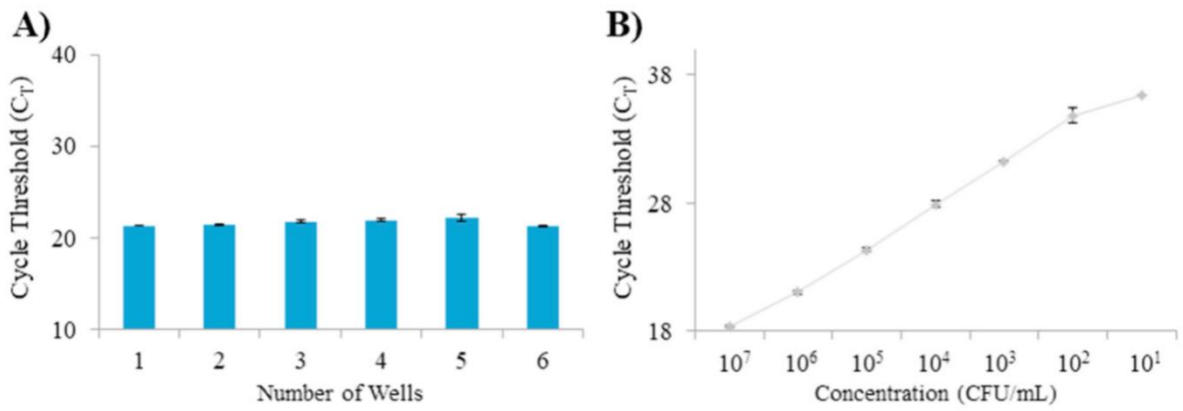

Figure 2.2 Evaluation of enrichment and extraction of several samples simultaneously in the 96-well filter/membrane plate (0.45 µm pore size, Hydrophilic PVDF membrane). (A) The cycle threshold (CT) values of the DNA enriched and extracted using the AD–DMP filter plate (0.45 µm pore size, Hydrophilic PVDF membrane) system for a 1 mL sample of the same concentration. (B) Evaluation of the efficiency of the AD–DMP filter plate (0.45 µm pore size, hydrophilic PVDF membrane) system for samples diluted (*E. coli* in PBS, 10^7–10^1 CFU/mL). Error bars indicate standard deviation from the mean, based on at least three independent experiments.

CHAPTER 3 - Homobifunctional imidoester (HI) combined black phosphorus nanosheets used as cofactors for nucleic acid extraction

3-1 Characterization of the black phosphorous multilayer and nanosheets

We proposed a new pathogen DNA extraction strategy called the BP-NIR-HI system. First, we analyzed the morphology of the BP materials through SEM. The untreated BP mass showed multilayering with a size of around 3~5 μm (Figure 3.1 A). According to the assisted EDX spectrum of BP mass shown in Figure 3.1 B, the high content ratio of P (86.96%) confirmed the properties and purity of the commercial BP mass(35). Meanwhile, the exited O (13.04%) indicated that the activated surface of BP would be oxidized by the O quickly in air. After protected sonication, the fragmentation of BP showed dispersion sheets with sizes of less than 100 nm (BP nanosheets, Figure 3.1 C). Secondly, we studied the surface charge of our materials through the zeta potential. We set two factors in one studied group, BP multilayer vs. BP nanosheets, and the stability after maintaining for 30 days (Figure 3.1 D). We found that the real-time detection of the BP multilayer (surface charge: |-5|) demonstrated the considerable activity of the BP mass surface(36). This result also echoes that of the O in the EDX spectrum. Moreover, the surface charge of the BP nanosheets showed that it had less activity than the BP multilayer. We inferred that the oxygenation BP materials were related to the quantity of P. In addition, the BP nanosheets showed higher stability than BP mass in solution. In another studied group, we detected the irradiation time effect on the stability of the BP nanosheets solution. The results (Figure 3.1 E) showed that the surface charge remained in the range of |± 20~± 40|, thus, it was relatively stable. We detected the irradiation time effect on the stability of the BP nanosheets solution. The results (Figure 3.1 E) showed that the surface charge remained in the range of |± 20~± 40|, thus, it was relatively stable

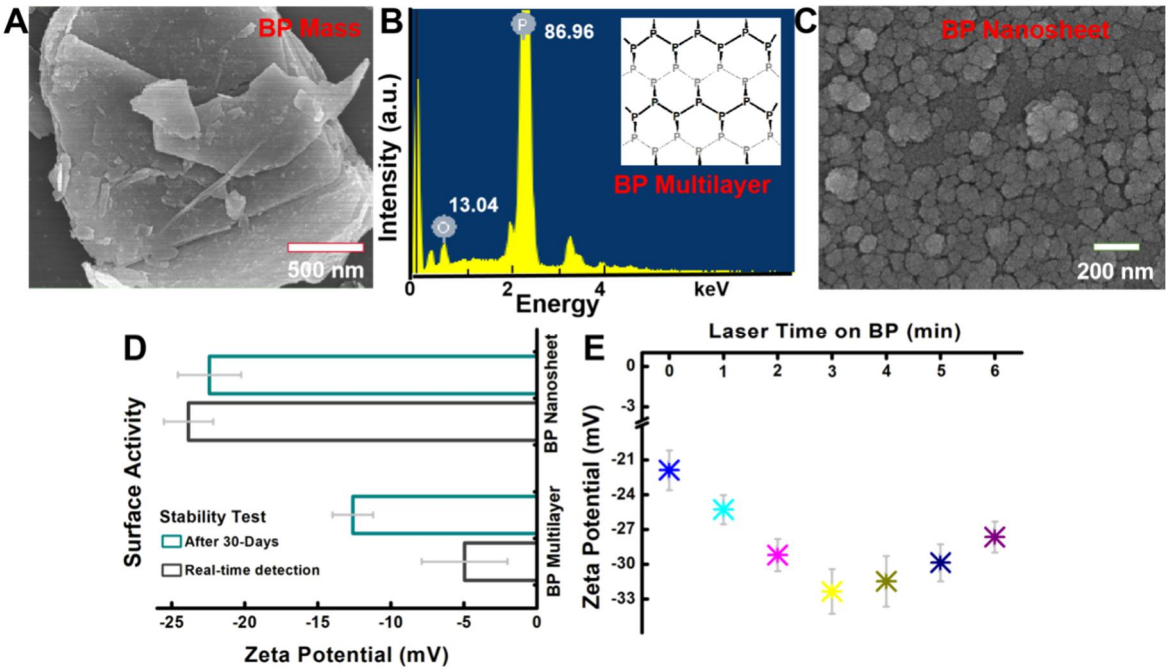

Figure 3.1 Characterization of black phosphorous (BP) multilayer and nanosheets after fragmentation. (A) Scanning electron microscope (SEM) image of commercial solid BP. (B) Energy-dispersive X-ray spectroscopy (EDX) spectra of the solid BP and the inside molecular diagram of the BP multilayer. (C) SEM image of BP nanosheets after 4 h in an ultrasonic water bath (size around 50–100 nm). (D) Stability of the BP multilayer and nanosheet through testing the zeta potential value of instant and water-preserved samples. (E) Effect of laser irradiation on the stability of BP nanosheets. Error bars indicate standard deviation from the mean based on at least three independent experiments.

3-2 Mechanism study of the BP-NIR-HI system for nucleic acid extraction

For the study of the mechanism of DNA extraction of the BP-NIR-HI system, we explored the possible factors through the qualitative method. Except the obvious (a) thermal effect, which we have explained in the laser-plasmonic thermal performances of BP nanosheets, the related chemical effects and the effect activity of BP nanosheets by HI crosslinking have been provided (Figure 3.2 A). (b) Studies have shown that BP causes an effective antioxidant to be present in the solution. Here, we tested the intensity of the ROS of BP nanosheets by the dichlorodihydrofluorescein diacetate (DCFDA) ROS kit(37). We observed a sustaining ROS release from the degradation of DCFDA. Moreover, as we used longer irradiation, the amount of released ROS increased, which is a good sign that it helped the nucleic acid release in the system. We added 200 μL of BP nanosheets (500 μg/mL) to 200 μL of E. coli in a sample tube and used 3 A laser irradiation for different lengths of time (1–5 min). The OD value of 560 nm was set for the detection. The experimental results are shown in Figure 4.2 B. With the gradual prolongation of irradiation time, the content of ROS production increased and reached about 192% of the blank control group at 5 min. Obviously, the production of a large amount of ROS is conducive to destroying the cell membrane, cytoplasm, and other components of the bacteria, as well as to the release of more DNA(38). (c) The principle of how HI works on the biomaterial crosslinking has been studied for many applications. Here, we added the HI to activate the BP nanosheets and facilitate the linking of the nucleic acids to the surfaces of the BP nanosheets. In particular, we designed a study based on the processed composites during the extraction system where we used commercial chains of DNA to test whether the HI linked the BP and nucleic acids. We found that the BP-HI composites showed some activity and the BP-HI-DNA showed mild stability (Figure 3.2 C) even after washing several times. We believe that this could be strong evidence for the crosslinking principle.

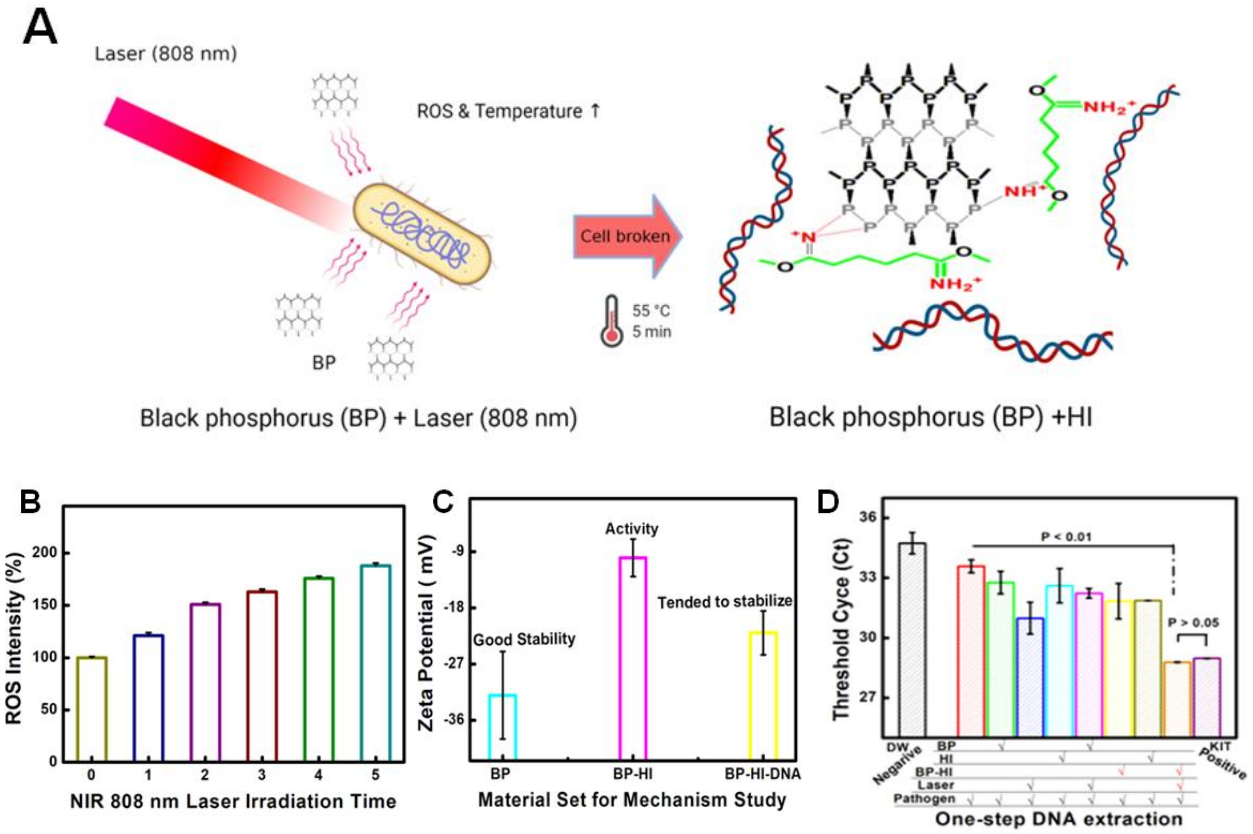

Figure 3.2 Mechanism study of the black phosphorus-near infrared-homobifunctional imidoester (BP-NIR-HI) system. (A) Scheme of the bio-performances of BP under laser (NIR) plasmonic irradiation. (B) Reactive oxygen species (ROS) detection assay of BP with E. coli, Dichlorodihydrofluorescein diacetate (DCFAD) kit in 485/535 nm. (C) Stability study of the possible surface linking of DNA extraction between BP-HI and DNA. (D) One-step BP-NIR-HI system design through the C_T value comparison of the BP-NIR-HI group with the single-factor group BP, HI, and laser and the double-factor group BP-HI, BP-laser, HI-laser, and control group DW and Spin column kit group after DNA amplification by real-time PCR.

CHAPTER 4 - Nucleic acid extraction method using microfluidic chip-based automated devices

4-1 Development of the microfluidic chip-based automated devices

As reported in previous studies, we confirmed the possibility of a sample pretreatment process using a microfluidic chip(3). The surface of the microfluidic chip was successfully treated with an amine group using APDMS and extracted nucleic acids using HI, a cross linker(39). In this paper, we developed this process as an automation system. The schematic diagram of the automation system is shown in Figure 4.1. The five syringe pumps move according to each step (1. Pathogen enrichment 2. Sample incubation 3. Lysis 4. PBS wash 5. Elution). The solution comes out of the syringe pump and moves to the main fold, with five inlets and one outlet. In the pathogen enrichment process, HI and the pathogen are bound by electrostatic interaction. As the sample flows through the chip, the amine group of HI binds to the amine group on the chip surface. HI is a cross-linker that binds to the pathogen on one side and the chip surface on the other. Nucleic acids released from pathogens during the lysis process bind to HI contained in the lysis buffer. Through the PBS washing process, other wastes other than nucleic acids bound to the chip surface are expelled out of the chip. Finally, the nucleic acid is separated from the chip with elution buffer (pH > 10). The tablet allows you to set the injection rate and sequence for each syringe pump. All of these processes will be completed in 80 minutes.

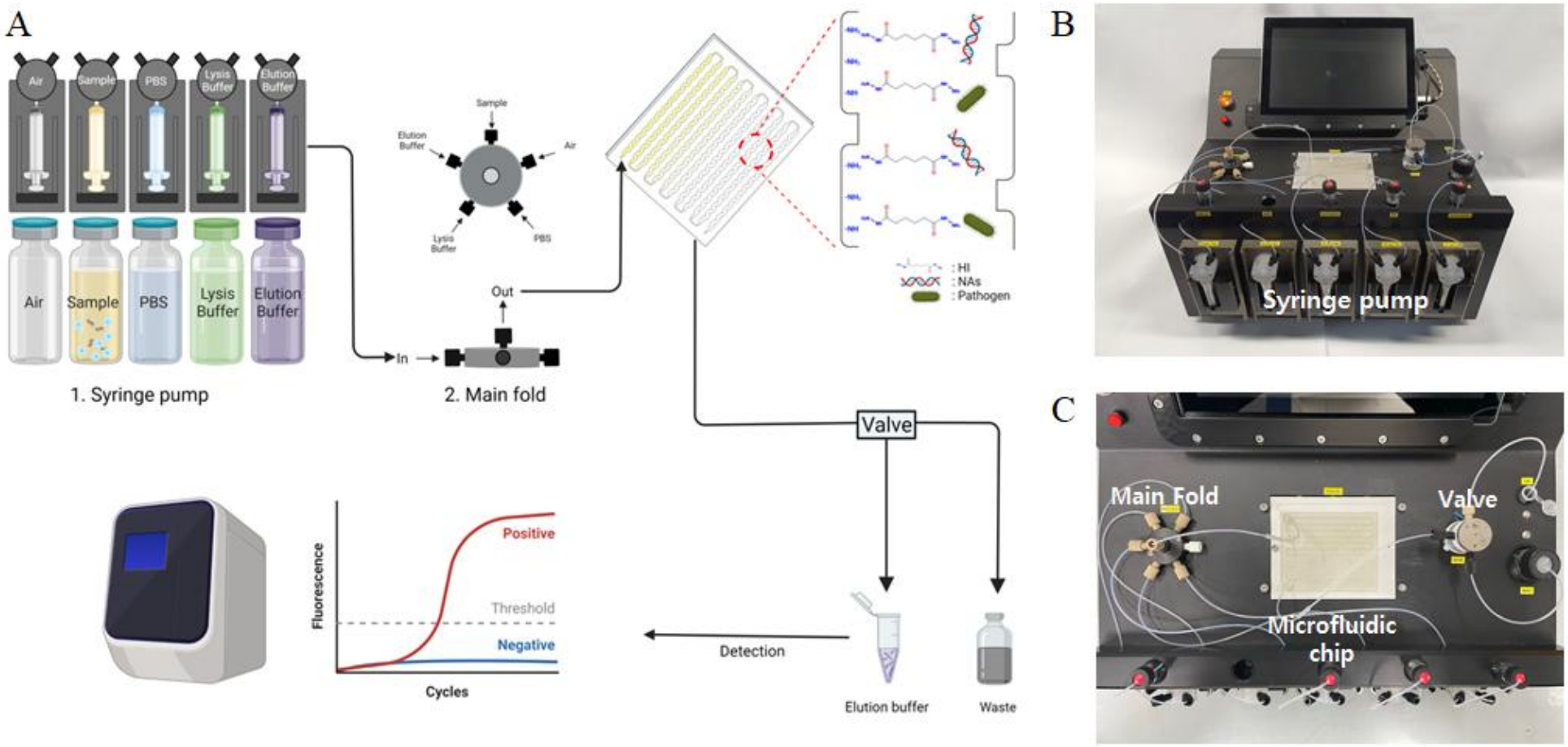
A
Air
Sample
PBS
Lysis Buffer
Elution Buffer
1. Syringe pump
2. Main fold
In
Out
: HI
: NAs
: Pathogen
Valve
Detection
Elution buffer
Waste
Positive
Threshold
Negative
Fluorescence
Cycles
B
Syringe pump
C
Main Fold
Valve
Microfluidic chip

Figure 4.1 Schematic diagram and actual equipment pictures of the automatic sample preparation platform. (A) The five syringe pumps are connected to the main fold. Through the main fold, each solution is injected into the chip that has been surface-treated with APDMS. Inside the microfluidic chip, APDMS, pathogens, and NAs combine. Nucleic acid on the surface of the chip is extracted using an elution buffer (pH > 10). (B) The entire picture of the automation system, all systems can be controlled by a central tablet PC. (C) This is a picture of the sample concentration and extraction part of the automation system. The solution of the syringe pump flows through the main fold to the microfluidic chip.

4-2 DNA extraction by the optimized microfluidic chip-based automated devices

In this study, in order to optimize the nucleic acid separation efficiency in the automation system, comparative experiments were conducted on sample injection speed, sample incubation time, Lysis incubation time, and sample volume under different conditions. (Figure 4.2) Sample injection rates were 400 μL, 350 μL, 300 μL, 250 μL, and 200 μL, respectively, and 10^5 CFU *b. ovis* bacteria of the same 1 ml were injected as microfluidic chips. It was confirmed that the lower the sample injection rate, the lower the Cycle threshold (C_T) value. Accordingly, all experiments allowed samples to be injected at a rate of 200 μL (Figure 4.2B). After the sample injection, incubation was performed at room temperature from 1 to 15 minutes so that the amine group could sufficiently bind the pathogen to the surface of the microfluidic chip using HI. The concentration and HI concentration of all samples were the same, and it can be seen that the C_T value was the lowest in 15 minutes (Figure 4.2 C). After the pathogen concentration, HI was added to the Lysis buffer and injected into the microfluidic chip. This is to enable the binding of nucleic acids separated from pathogens to the chip surface. This process was incubated at 56 degrees. Similar C_T values were obtained at 15 minutes and 20 minutes, and 15 minutes were required during the lysis process. (Figure 4.2 D) Finally, the same concentration of samples was diluted with PBS to confirm the pathogen concentration efficiency. Since we used samples with the same concentration (Figure 4.2 E), we were able to obtain similar C_T values at all doses. Based on this experiment, the optimization of the automation device was completed. The system can be used for pathogen concentration and nucleic acid extraction from up to 2.5 ml of samples at a time. The optimized workflow is shown in Table.

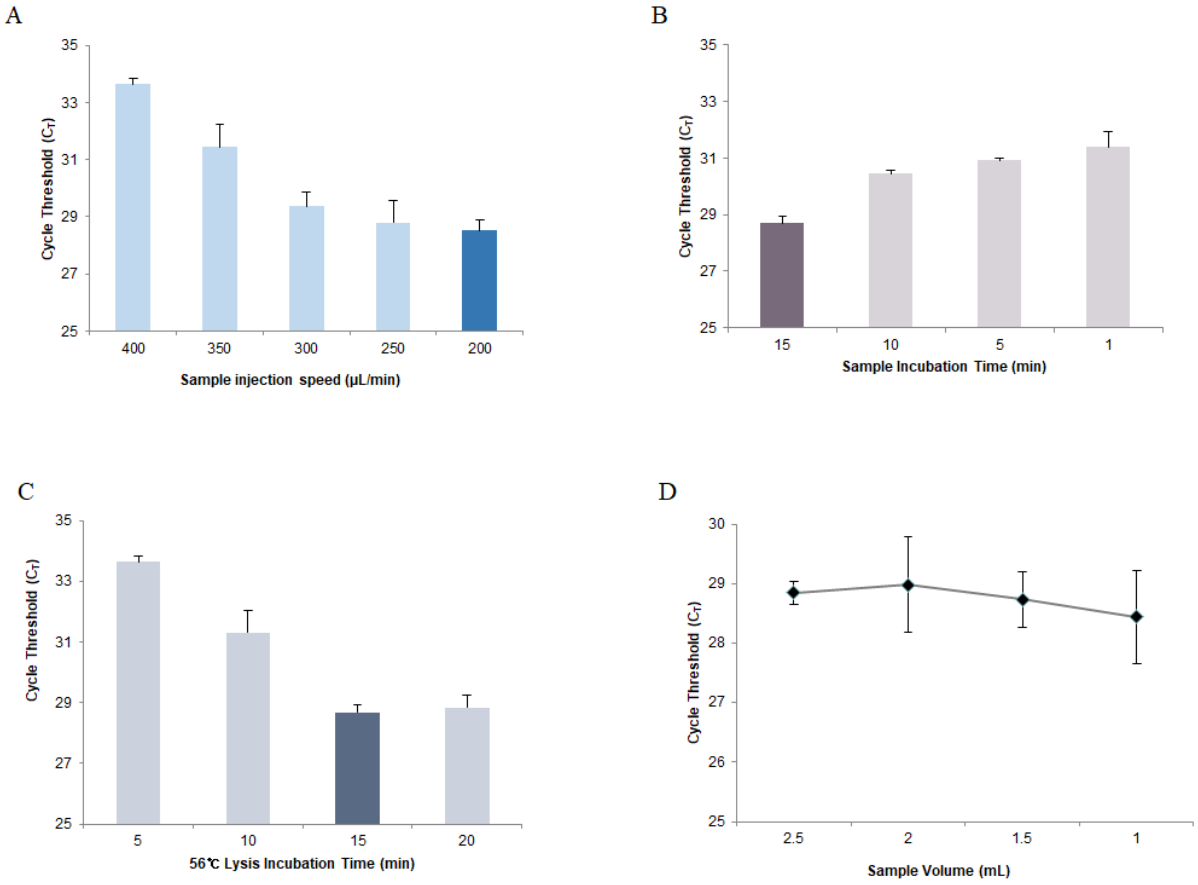

Figure 4.2 Microfluidic chip automation device extraction process and DNA extraction real-time PCR test results for optimization. The solution from the syringe pump is injected into the microfluidic chip through the main fold. In this process, pathogens and NAs are attached to the chip surface by electrostatic interaction. (A) DNA extraction results according to sample (*B. ovis* bacteria 10^5 CFU in PBS 1mL) injection rate. (B) DNA extraction results according to sample (*B. ovis* bacteria 10^5 CFU in PBS 1mL) incubation time. (C) DNA extraction results according to 56°C lysis incubation time. (D) DNA concentration and extraction test results by diluting the same concentration of *B. ovis* bacteria 10^5 CFU in different amounts of PBS (1mL to 2.5 mL) Error bars indicate standard deviation from the mean, based on at least three independent experiments.

Table 1. Automation system workflow (DNA)

	Automation workflow (pathogen enrichment and DNA extraction)	**Volume [µl]**	**Speed [µl / min]**
1	Sample + HI	1000	**200**
2	Air	120	200
3	Incubation for sample enrichment	**15min**	
4	Air	1000	200
5	Lysis buffer + HI	1000	300
6	Heater	**56 (15min)**	
7	PBS washing	1200	100
8	Air	1000	200
9	Elution buffer	100	200
10	Air	1250	50
	Operational Time : 1h 20min		

4-3 Limit of nucleic acid detection in microfluidic chip automation system

Based on the previously optimized data, the nucleic acid detection limitations of the automation system were measured. The detection limit was measured using real time PCR. All DNA and RNA templates were extracted for automation system. DNA was extracted from *Brucella ovis*. The sample concentration was serial diluted from 10^6 CFU to 10^0 CFU. As a result, DNA could be successfully separated from bacteria up to 10^2 CFU. As a result, DNA could be successfully concentrated and extracted from bacteria up to 10^2 (Figure 4.3 A). RNA was extracted from SARS-CoV-2 VeroE6 cells. The sample concentration was serial diluted from 10^5 PFU to 10^0 PFU. RNA can also be detected up to 10^2 PFU (Figure 4.3 B).

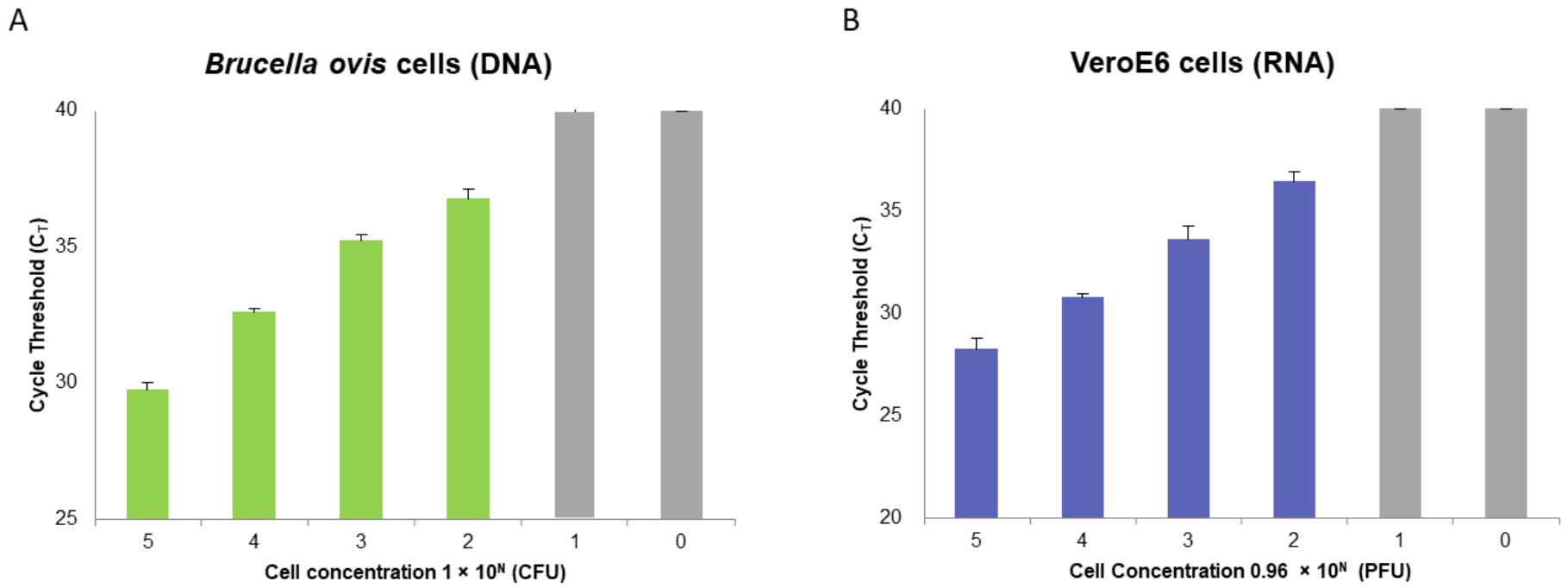

Figure 4.3 DNA and RNA extraction real-time PCR result for limit of detection measurement of microfluidic chip automation system.

(A) Microfluidic Chip Automation System DNA Detection Limit real time PCR Results (C_T value). Samples were used with 10^6 CFU to 10^0 CFU serial dilutions of *B. ovis* bacteria in 1 ml of PBS. (B) Microfluidic Chip Automation System RNA Detection Limit real time PCR Results (C_T value). Samples were used with 10^5 CFU to 10^0 CFU serial dilutions of SARS-CoV-2 VeroE6 cells in 1 ml of PBS. Error bars indicate standard deviation from the mean, based on at least three independent experiments.

4. DISCUSSION

Fast and accurate molecular diagnostic methods have been studied for decades in the field of biomedical engineering. However, there are still many problems with sample preparation methods for molecular diagnostics. The complex procedure is time consuming and costly. In addition, since there is no technology to enrichment the pathogen at a low concentration, it is difficult to diagnose the disease at an early stage. In this study, our sample preparation technologies were developed with several features. First, a fast and low-cost sample enrichment process to detect even a small concentration of pathogens. Second, it is a low-cost procedure that can process multiple samples at once. Third, an automated sample preparation system that anyone can use easily. To develop the technologies including these features, we used Homobifunctional imidoesters (HI). Because the amine group of HI can bind to pathogens through electrostatic interactions. Using amine-treated microfluidic chip and HI, pathogen enrichment, nucleic acid extraction, and even EVs were isolated. D-APDMS and HI, 96-well filter/membrane plate, we successfully enriched pathogens and extracted nucleic acids from multiple samples simultaneously. It has higher sensitivity than commercially available kits because pathogens can be concentrated in the sample. BP-NIR-HI system successfully separated nucleic acids using only BP and laser without any other equipment. Furthermore, we have developed automated sample preparation equipment that anyone can use easily.

In molecular diagnostics, besides sample preparation, the detection method is one of the important tasks. We plan to conduct research on detection methods that can be used together with the methods studied in this paper. Based on research using bio-optical sensors, LFA, RPA, LAMP, etc., we would like to finally develop a system that allows sample preparation and detection in one device.

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6. ABSTRACT (KOREA)

다양한 병원체에 의한 감염은 전 세계적으로 공중보건에 대한 문제점으로 남아있습니다. 따라서 감염성 병원체에 대한 신속하고 정확한 진단은 추가적인 확산을 막을 뿐만 아니라 초기에 질병을 치료하는데 매우 중요합니다. 특히, 병원체 농축 및 핵산 추출 기술은 분자진단을 위한 필수적인 단계입니다. 하지만 기존의 샘플 전처리 과정은 복잡한 절차로 인해 많은 시간을 필요로 하며 비싼 가격과 많은 장비를 필요로 하는 단점이 있습니다. 또한 제한된 양의 샘플만 처리가 가능하기 때문에 낮은 농도의 병원체를 검출하지 못할 수 도 있습니다. 동형 2기 이미도에스테르는 양쪽에 아민기가 있는 크로스링커 입니다. 정전기 적 결합을 통해 병원체에 결합 가능하며, 아민기의 결합으로 인해 핵산과 결합할 수 있습니다. 따라서 본 연구에서는 동형 2기 이미도에스테르와 다양한 물질을 결합하여 병원체 농축 및 핵산 추출 방법에 관한 연구를 진행했습니다.

첫 번째, 아민표면 처리된 미세유체칩과 HI를 이용하여 병원체 농축 및 핵산 추출 시스템을 개발했습니다. 미세유체칩을 이용한 샘플 전 처리는 많은 양의 샘플을 한번에 처리 할 수 있기 때문에 낮은 농도의 샘플도 추출이 가능합니다. 또한 모든 과정이 칩 내부에서 일어나기 때문에 외부 오염으로부터 자유로운 장점이 있습니다. 우리는 이러한 장점을 이용하여 분자진단 분야에서 새로운 바이오마커로 주목 받고 있는 EVs의 분리 가능성을 확인 했습니다. 기존의 대표적인 EVs 분리 방법은 초원심분리기를 이용한 방법입니다. 하지만 이는 대형 장비와 많은 시간을 필요로 하는 단점이 있습니다. 여기서 우리는 미세유체칩과 HI를 결합한 시스템을 이용하여 기존 방법보다 쉽고 빠르게 EVs를 농축할 수 있습니다.

두 번째, 아민표면처리된 규조토와 HI를 이용하여 96well filter/membrane plate에서 여러 개의 샘플에서 동시에 핵산을 추출 하는 기술을 개발했습니다. 상용화된 핵산 추출 키트는 복잡한 절차 때문에 많은 시간을 필요로 하며 한번에 여러 개의 샘플을 처리하기에는 많은 어려움이 있습니다. 하지만 이 기술은 한번에 96개의 샘플을 동시에 처리할 수 있고 추가적인 장비 없이 핵산을 추출

할 수 있습니다.

세 번째, 2차원 물질인 BP 와 HI를 이용하여 간단한 핵산 분리 방법을 개발했습니다. BP는 강력한 광 흡수, 생체 활성 등으로 인해 질병의 진단 및 치료에 사용되고 있습니다. BP의 특성화를 통해 최적화된 핵산 추출 시스템을 개발 하였고 이는 상용화된 기존의 키트보다 2.5배 더 높은 민감도를 보여줍니다. 또한 다른 장비 없이 레이저만으로도 세포를 용해시켜 핵산을 방출 시킬 수 있습니다.

본 연구를 통하여 개발된 샘플 전처리 기술은 현재 상용화된 키트의 문제점인 복잡한 과정, 샘플 오염, 비싼 가격의 문제를 해결했습니다. 더 나아가 본 연구에서는 샘플 전처리 기술을 자동화 시스템으로 만들었습니다. 자동화 시스템을 이용한 전처리 과정은 다른 장비 없이 하나의 장비에서 모든 과정이 이루어지며, 이는 누구나 쉽게 사용할 수 있습니다. 자동화 시스템을 사용하여 다양한 감염성 질환 진단을 위한 쉽고 빠른 샘플 전처리 기술로 사용 될 수 있습니다. 이러한 기술들은 대형 장비 없이 샘플 전처리가 가능합니다. 그렇기 때문에 분자진단에서 검출방법에 대한 연구를 추가로 진행할 예정입니다. 바이오 옵티컬 센서, LFA, RPA, LAMP 등의 검출 기술과 접목하여 민감도 높은 하나의 진단 플랫폼을 개발하고자 합니다.

Keywords: Sample preparation, Extracellular vesicles, Nucleic acid, Automation device