공학석사 학위논문

바이오 실리카를 이용한

병원체 핵산 분리 기술 연구

Study for a novel technique using combination of filter/membrane and bio-silica to isolate nucleic acids from various pathogens in human specimens

울 산 대 학 교 대 학 원

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지 도 교 수　　신　　용

이 논문을 공학석사 학위 논문으로 제출함

2020 년 8 월

울 산 대 학 교 대 학 원

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ABSTRACT

Diseases caused by pathogens are an important problem worldwide, and infectious diseases caused by zoonosis are increasing the health risks of people in low-income countries. Moreover, diseases diagnosis usually requires complicated pretreatment processes and is hard to obtain Nucleic acid (NA) due to low concentrations and expensive equipment. In this study, we developed a more efficient system for the enrichment of pathogens and isolation of NA by amine-functionalized the surface of the biocompatible nanomaterial diatomaceous earth (DE) and combining it with other molecules to make new compounds or adding chemicals with amine-functionalized diatomaceous earth (ADE).

First, a pumpkin-shaped molecular cucurbituril (CB) coating ADE composite (ADE-CB) was fabricated to support effective sample enrichment and NA isolation from enriched pathogens and cells. Using ADE-CB, enrichment of pathogen samples was confirmed to have a concentration efficiency of 90% or more in a short time by simply shaking the hand without any equipment. Next, a syringe filter system was used together with ADE to conveniently and easily enriched pathogens to develop a technology that isolates nucleic acids without equipment in a short period of time. In addition, we developed a technology that can extract multiple samples at once by combining with 96-well filter/membrane plates. Our technology is 10 to 100 times more sensitive than commercial kits and inexpensive due to no need additional experimental equipment.

This effective method for capturing pathogen and nucleic acid might be useful for preparing samples for diagnostic systems and several other benefits include the simplicity of producing its components and its ease of operation, and it can be readily integrated with other assays for point-of-care test (POCT).

Keywords: Pathogen, Nucleic acids, Amine-functionalized diatomaceous earth, Cucurbituril, filter, Point-of-care test

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ABBREVIATIONS

ADE	Amine-functionalized diatomaceous earth
ADE-CB	Cucurbituril (CB) coating amine-functionalized diatomaceous earth composite
APDMS	3-aminopropyl(diethoxy)methylsilane
CB	Cucurbituril
C_T	Cycle threshold
DE	Diatomaceous earth
DMP	Dimethyl pimelimidate
DMS	Dimethyl suberimidate
DW	Distilled water
HI	Homobifunctional imidoester
NA	Nucleic acid
NAT	Nucleic acid testing
POCT	Point-of-care test
qPCR	Quantitative real-time PCR
RT	Room temperature
RT-qPCR	Reverse transcription real-time PCR

1. Introduction

Pathogenic infections result in diseases caused by toxins released by pathogenic organisms, and such infections are a growing threat to human health and public health worldwide (1-3). Currently, pathogen identification and diagnosis approaches play important roles in controlling infections.

Various methods have been developed for diagnosing pathogenic diseases, including blood chemistry (4), blood culture (5), immunoassays (6), flow cytometry (7), and nucleic acid (NA) testing (8). Although bacterial blood cultures are widely used for clinical diagnosis, this approach is time-consuming (usually taking several days), expensive, uses species-specific protocols, and requires laboratory facilities (9). As an alternative to these problems, the NA testing (NAT) has been the subject of increasing attention; its techniques are relatively fast and universal and it is applicable to diverse applications for diagnostic testing, including window period diagnosis, immunovariant virus detection and infection identification (10-12). However, existing NA-based assays are generally complex both for commercialized products and for laboratory-developed assays (13). They typically involve intricate pre-treatments (such as pre-fabrication or sample centrifugation), which require laboratory-based procedures involving multiple steps, skilled technicians, and specific instruments (14). These drawbacks limit the application of NAT to point-of-care test (POCT), especially in resource-limited settings. There are three main aspects to NA-based POCT: sample preparation, template amplification, and signal detection (15). Efforts to improve NA-based POCT assays have been directed at developing simple and effective amplification and detection methods; few researchers have focused on the primary sample preparation step (16). Sample preparation, including NA isolation from biological matrices, is critical because high-quality NA is the foundation for all the downstream analysis (13). In particular, it is required for sample preparation to be adaptable to an open environment rather than restricted to a laboratory to eliminate the need for clean rooms in resource-limited settings (14).

Focus on these areas, ‘pathogen enrichment and NA extraction technology' plays an increasingly important role in sample preparation and sensor diagnostic amplification (17-19). Diatomaceous earth (DE), Biocompatible silica that can be used as nanosorbents and activators for sample preparation have received considerable attention in various applications. The main advantage of this biocompatible silica DE is its usefulness as an adsorbent. Their

large surface area and the potential to modify their surfaces with various special reactive groups can increase their chemical affinity to target compounds (20-22). A supermolecular modified DE composite platform has been used for molecular encapsulation in water treatment and in a broad range of biological systems that require rapid results and high stability (21, 23). Furthermore, the extraordinary three-dimensional porous structure of DE has been shown to supply a massive surface area. Using these advantages of DE, we studied to improve the enrichment of pathogens by synthesizing cucurbituril (CB) on the surface of amine-functionalized DE (ADE). It introduced to enhance the ability of cucurbituril (CB) coating of amine-functionalized diatomaceous earth (ADE-CB) composites to rapidly and efficiently adsorb molecules. The efficiency of the ADE-CB composite to enrich for pathogens and cells was examined using two approaches. First, the morphology of the composites precipitated by the ADE-CB system showed that many eukaryotic cells adhered to the surface of the ADE-CB. Second, we also compared the performance of quantitative real-time PCR (qPCR) using amplified DNA, samples collected from the ADE-CB enrichment system, and DNA extracted via a commercial column system as templates. The results showed an approximately 4-fold increase in the early amplification signal. In summary, we have confirmed that this ADE-CB composite system can provide improved performance for bio-sample preparation for early diagnosis.

In addition, we developed fast, simple and universal technology by combining ADE and homobifunctional imidoester (HI) to enrich pathogens with syringe filter. HI has imidoesters at both ends, so it forms an amide bond with the amine group exposed on the surface of the treated ADE. The other imidoester was used to bind pathogens or NAs. In the presence of HI, which reacts with the amine groups of ADE, more positively charged groups are formed, resulting in enhanced pathogen enrichment (18). In this way, negatively charged pathogens can be directly and rapidly absorbed onto the DE from small sample volumes (1 mL) or absorbed with only a short incubation for large sample volumes (50 mL). Furthermore, the HI cans reversibly crosslink amine groups on proteins and NAs, as well as NAs to the DE (24). This reaction can be reversed by changing the pH, so the preparation of NA samples for diagnostics can easily be achieved by the injection of different buffers. Next, we combined the NA separation technology used in the ADE, syringe filter with a 96-well filter plate. The technology using ADE and 96-well filter/membrane plate allows multiple samples to be extracted simultaneously, and the isolated DNAs were amplified with fine uniformity.

For these reasons, this method is suitable for adoption into a convenient-effective NA

isolation process for various samples. This assay has a number of advantages: ( ) it enriches pathogens effectively and isolates NA, subsequently for downstream diagnostic analysis; ( ) it simplifies procedures of test manufacturing and operation; ( ) it is a robust method.

2. MATERIALS AND METHODS

2.1 Preparation of amine-functionalized diatomaceous earth (ADE) and characterization of cucurbituril (CB) coating ADE composite (ADE-CB)

Amine-functionalized diatomaceous earth (ADE) was utilized as the matrix in both the enrichment and extraction processes and was prepared as follows. Diatomaceous earth (DE, calcined powder, Sigma-Aldrich) was washed with distilled water (DW) for 30 min with vigorous stirring. The sediment containing impurities was removed after a short period of settling under gravity. 3-aminopropyl(diethoxy)methylsilane (APDMS, 97%, Sigma-Aldrich) were used to prepare the ADE. Briefly, 5 mL of APDMS was pipetted dropwise into 100 mL 95% (v/v) ethanol solution, which was acidified with acetic acid (pH 5). Then, 2 g DE was added with vigorous stirring. The reaction was maintained at room temperature (RT) for 4 h. The ADE was washed with ethanol and then dried under vacuum overnight, and the dried ADE was stored at RT until further analysis. Preparation of the ADE-CB was performed via the microwave method. Briefly, 50 mg of ADE was dissolved in 1 mL of DW to form a 50 mg/mL ADE solution. Cucurbituril (CB) (25 mg) (23) was added to 1 mL of DW, and this solution was then sonicated for 1 minute using an ultrasonic instrument. Subsequently, 20 μL of the 25 mg/mL CB solution was added dropwise into 2 mL of prepared ADE solution, followed by heating in a microwave oven for 1 minute. The double-functionalized ADE-CB was washed and collected by centrifugation and then dried in a vacuum overnight at RT. The ADE-CB powder was stored in a conical tube

In the study of filter / membrane plate, a new DE (flux-calcined powder, Sigma-Aldrich) was used to obtain a uniform diameter of particle more than the existing DE (calcined powder, Sigma-Aldrich), and a new ADE process was studied accordingly. The DE (flux-calcined powder, Sigma-Aldrich) was washed at RT for 10 minutes with DW as a dynamic stirring. After the stirring, wait at RT for 1 minute. Carefully move the supernatant except sediment to the new tube to obtain clean, even diatom. Once again, DE was cleaned at RT with ethanol (EtOH, 99%) with dynamic stirring for 10 minutes. The washed DE is moved to a new tube and stored in a 65 dry oven for one day. 3 mL of APDMS was pipetted dropwise into 50 mL 95% (v/v) ethanol solution. Then, washed 1 g DE was added with dynamic stirring. The

reaction was maintained at RT for 4 h. The ADE was washed with DW and then dried in a 65 dry oven overnight.

2.2 Pathogen and cell enrichment and nucleic acid capturing using cucurbituril (CB) coating amine-functionalized diatomaceous earth composite (ADE-CB)

The morphologies of the DE and ADE-CB were characterized using a field-emission scanning electron microscopy (FE-SEM, JEOL JSM-7500F) to confirm a uniform size distribution and decoration of the DE with CB. The zeta potentials of the materials were acquired via dynamic light scattering (DLS, DynaPro NanoStar, Wyatt). A Libra 22 UV/visible spectrophotometer was used to estimate the effectiveness of the composites for pathogen enrichment by measuring the absorbance of the cell and pathogen solutions at 600 nm. A vortex mixer (T5AL, 60 Hz, 30 W, 250 V) was used for mixing the sample wells in the enrichment system. A CF-5 centrifuge (100–240 Vas, 50/60 Hz, 8 W) was used for sample collection. QIAamp DNA (DNA mini kit, Sigma-Aldrich) were used as spin column-based methods for isolating nucleic acids (following the manufacturer's instructions) from both the supernatant and precipitate from the ADE-CB enrichment system. An AriaMx quantitative real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) was used to confirm and estimate the enrichment efficiency.

2.3 Single tube based pathogen enrichment and RNA extraction

ADE and dimethyl suberimidate (DMS, one kind of Homobifunctional imidoester (HI)) were used as the enrichment and extraction matrices for PBS, urine, and serum sample. The urine samples were collected daily. The human serum sample was purchased from Sigma-Aldrich (H4522-100ML). First, 80 μL of ADE suspension (50 mg/mL in DW) and 100 μL of DMS solution (100 mg/mL in DW) were pipetted into a sample solution. With the 1 mL samples, the pathogens were collected with ADE after 1 min of settling under gravity at RT, whereas the 50 mL samples were incubated on a rotating mixer (Topscien Instrument Co., Ltd., Ningbo, China) for 30 min at 99 rpm. After washing with 1 mL of PBS, the enriched pathogens were harvested by centrifugation. NA isolation was subsequently performed in the same tube. Briefly, 20 μL of proteinase K, 150 μL of in-house lysis buffer (100 mM Tris-HCl

[pH 8.0], 10 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate, and 10% Triton X-100), 30 μL of lysozyme solution (30 mg/mL in DW), and 10 μL of RNase-Free DNase solution were added separately. After mixing, the RNA extraction incubated the tube for 10 minutes at RT. NA templates from lysed cells were immobilized on ADE through DMS crosslinking. The supernatant was removed with a short centrifugal pulse and the pellet washed twice with 200 μL of PBS. For reverse crosslinking, 100 μL of elution buffer (10 mM sodium bicarbonate, pH >10, adjusted by NaOH) was added and incubated for 1 min at RT. After brief centrifugation, the supernatant containing the isolated RNA was removed and stored at −20°C until needed. As a positive control, the same samples were subjected to NA extraction using commercial kits (QIAamp DNA Mini Kit and RNeasy Mini Kit, Qiagen) following the manufacturers' protocols. The maximum capacity of these kits was considered to be 200 μL of a 1 mL sample.

2.4 Syringe filter based pathogen enrichment and RNA extraction

As with the tube-based procedure, 80 μL of ADE suspension (50 mg/mL in DW) and 100 μL of DMS solution (100 mg/mL in DW) were pipetted into a sample solution. With the 1 mL samples, the mixture was loaded into a 5 mL syringe and inverted twice. No extra incubation time was needed. An incubation time of 30 min was used for larger volume samples (5 to 50 mL). During incubation, the mixture was occasionally shaken by hand to disperse the sample. For the 50 mL samples as an alternative option, a rotating mixer (Topscien Instrument Co., Ltd., Ningbo, China) set to 99 rpm was used, with incubation for 30 min. After incubation, the mixture was passed through a polytetrafluoroethylene (PTFE) syringe filter with 1.0 μm pores (Whatman, USA) and then washed with 1 mL of PBS, also using the syringe. The remaining solution in the filter unit was removed by pumping air through it with the syringe; this was repeated twice. NAs were extracted from the bacteria trapped on the filter by injecting a mixture of 20 μL proteinase K, 150 μL of in-house lysis buffer, 30 μL of lysozyme solution and 10 μL of RNase-Free DNase solution. The mixture could just fill most of the filter. And then, the filter was inverted and incubated for 10 min at RT for RNA extraction. An empty syringe was used to pump air into the filter and the mixture was naturally removed from the filter. The filter was then washed twice with 1 mL PBS and then air. Finally, 100 μL

of elution buffer was injected onto the filter and, after 1 min of incubation at RT, the elution buffer containing NAs was collected by pumping air and stored at −20°C until needed.

2.5 Filter/membrane plate based pathogen DNA extraction

ADE and dimethyl pimelimidate dihydrochloride (DMP, powder, Sigma-Aldrich) were used as the enrichment and extraction matrices for PBS with samples. First, the MultiScreen Solvinert 96 Well Filter Plate (0.45 μm pore size, Hydrophobic Polytetrafluoroethylene (PTFE), Darmstadt, Germany) and the 96 well cell culture plate (F-Type, SPL, Korea) are combined. With the 100 μL samples, 20 μL of ADE suspension (50 mg/mL in DW) and 50 μL of DMP solution (100 mg/mL in DW) were pipetted into a sample solution. NA isolation was subsequently performed in the same the PTFE membrane plate. Simply, 20 μL of Proteinase K, 150 μL of lysis buffer, 30 μL of lysozyme solution (50 mg/mL in DW) were added separately. After mixing, the PTFE membrane plate was incubated in a dry oven for 30 min at 56°C for DNA extraction. Operating the 500 RCF Centrifuge for 2min will allow it to exit the PTFE membrane plate with the exception of the NA template fixed to the ADE through the DMP 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, then centrifuge for 2 minutes at 500 RCF. The NA template that eluted to the 96 well cell culture plate (F-Type) was moved to the tube using a pipette. The same sample (*E.coli* in PBS, 10^5 CFU) was taken as a positive control group using a commercial kit (QIAamp DNA Mini Kit, Qiagen) according to the manufacturer's protocol. The maximum capacity of this commercialized kit was considered 200 μL. Evaluation of NA extraction experiment using quantitative real-time PCR (qPCR)

2.6 Biological samples

The eukaryotic cells (HCT-116 colorectal cancer cells) were maintained in plastic culture dishes in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum in a 37 °C humidified incubator with 5% ambient CO_2. After culturing, genomic DNA was extracted from the cells using a spin column-based

kit (QIAamp DNA mini kit, Sigma-Aldrich) and a nano-composite method. *Brucella ovis* (*B.ovis*, ATCC 25840) was used to evaluate the pathogen diagnostic method. *B. ovis* was grown in Brucella agar (MB Cell) containing 5% defibrinated sheep blood (Kisan Bio) and incubated at 37°C in an atmosphere of 5% CO_2 for 48 h. *Escherichia coli* (ATCC 25922) was inoculated in nutrient broth (NB, BD Difco) medium and incubated overnight at 37°C under shaking conditions. After culturing, the bacterial suspension was quantified by the agar plate method and subsequently diluted to different concentrations in PBS.

2.7 Quantitative real-time PCR (qPCR) and reverse transcription real-time PCR (RT-qPCR)

We performed quantitative real-time PCR (qPCR) to determine the quality of the isolated DNA and reverse transcription real-time PCR (RT-qPCR) to determine the quality of the extracted RNA. The primers used are listed (Table 1). The real-time PCR and real-time RT-qPCR procedures were modified from the AriaMx real-time PCR Instrument protocol (Agilent Technologies, Santa Clara, CA, USA). Briefly, 5 μL of isolated DNA was amplified in a total volume of 20 μL containing 2× Brilliant III SYBR Green QPCR master mix, 25 pM of each primer, and DW. An initial pre-denaturation at 95°C for 10 min was followed by 40 cycles at 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s, and then by a cooling step at 40°C for 30 s. For the real-time RT-qPCR, 5 μL of isolated RNA was amplified in a total volume of 20 μL containing 2× Brilliant III SYBR Green QRT-PCR master mix, 25 pM of each primer, 0.2 mM of dithiothreitol, 1 μL of RT/RNase block and DW. The following thermal profile was used for the real-time RT-PCR: 20 min reverse transcription at 50°C; 15 min pre-denaturation at 95°C; 40 cycles of 10 s at 95°C, 20 s at 58°C, and 20 s at 72°C; and a cooling step at 40°C for 30 s. The SYBR Green signals of the amplified products were acquired using AriaMx real-time PCR (Agilent Technologies).

Table 1. Primer sequences sets for RT-qPCR and qPCR.

Samples	Targets	Seuqence (5' → 3')
B. ovis	*O 223*	F: TGG CTC GGT TGC CAA TAT CAA
		R: CGC GCT TGC CTT TCA GGT CTG
E.coli	*rodA-105*	F: GCA AAC CAC CTT TGG TCG
		R: CTG TGG GTG TGG ATT GAC AT

3. RESULTS

CHAPTER 1 - Biomedical applications using biocompatible composites

1.1 Preparation and characterization of biocompatible composite

A schematic representation of cucurbituril (CB) coating amine-functionalized diatomaceous earth composite (ADE-CB) is shown (Fig 1.1A). The two free chains of the APDMS on the substrate are encapsulated in the cavity of the CB, which is a key property of this second DE surface modification. Chemical-optical spectrum analysis of the composites was performed to assess the modification status. SEM images were used to assess the morphology of the DE and ADE-CB, as shown (Fig 1.1B). The pores on the DE surface are open, and the pore diameter is less than 100 nm (Fig 1.1B, top). However, the ADE-CB is rougher with blocked pores on the surface (Fig 1.1B, bottom). To assess the electrostatic properties of the ADE-CB in solution, the zeta potentials of the ADE-CB were measured (Fig 1.1C). Because of the uniform size of the DE particles, we ignored the size effect of the surface charge. Notably, the zeta potential of the ADE-CB composite was higher than that of the ADE, likely reflecting the diverse anchor bindings between the ADE and CB, which may include the reported possible anchor linking/ion-dipole interaction between the carbonyl groups of the CB portals. Furthermore, the positively charged amine groups in the ADE-CB composite could enhance the absorbency efficiency of the ADE-CB conjugate during its interaction with other molecules via enhanced covalent bonding, physical adsorption, electrostatic interaction, and heterogeneous surface binding (25-27).

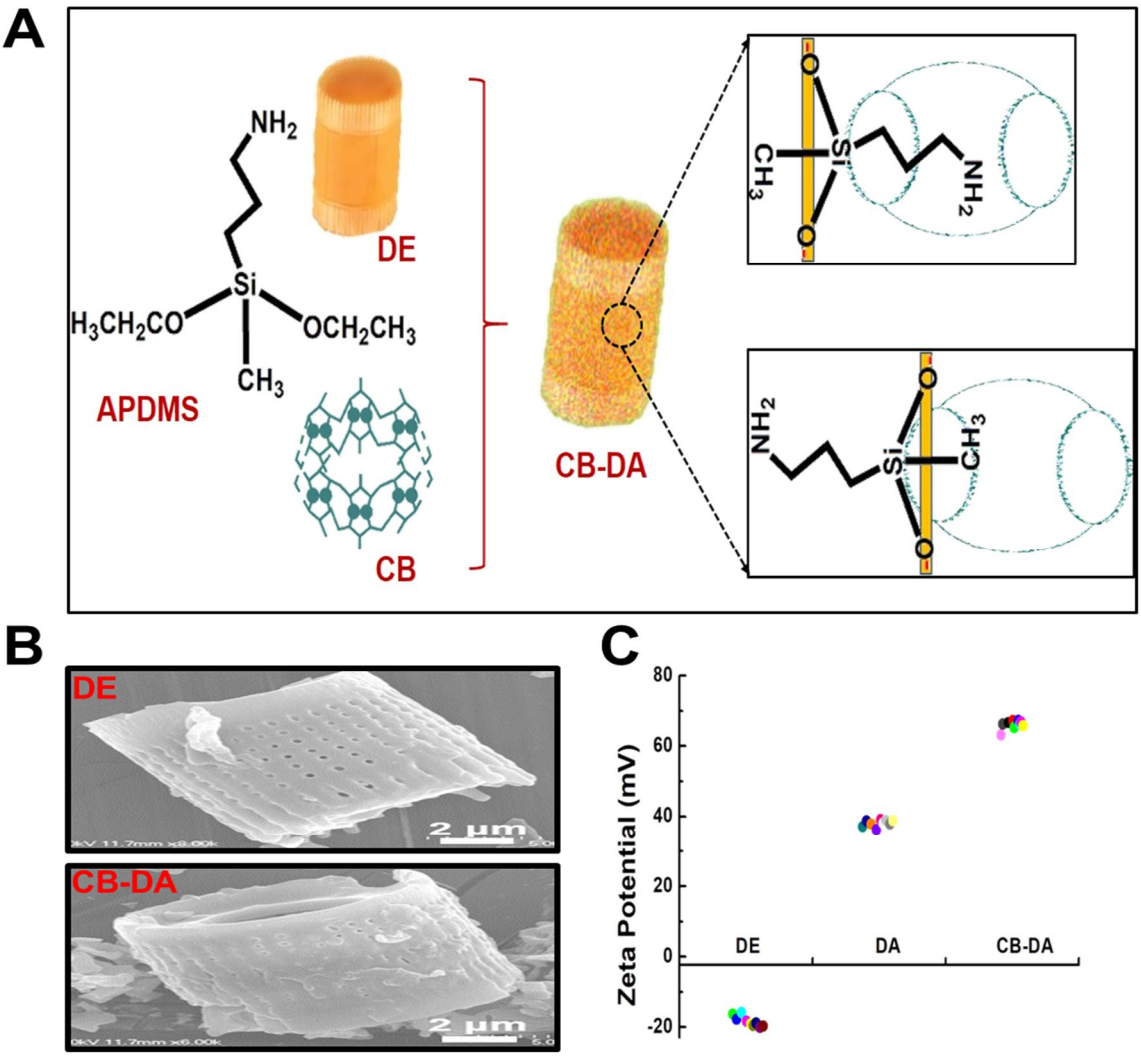

Figure 1.1 Schematic of the process flow for cucurbituril (CB) coating amine-functionalized diatomaceous earth composite (ADE-CB). (A) Schematic representation of the CB coating of ADE-CB. The two free chains of APDMS on the substrate are encapsulated in the cavity of the CB. (B) Scanning electron microscopy (SEM) images of DE and ADE-CB. (C) Zeta potentials of the prepared materials: pure DE, amino-functionalized diatomaceous earth (ADE), and cucurbituril (CB) coating amine-functionalized Diatomaceous earth composite (ADE-CB).

1.2 Pathogen and cell enrichment using the biocompatible composite

A schematic of the pathogen enrichment process is shown (Fig 1.2). Electrostatic interactions between the positive surface of the ADE-CB and the negative charge of the cell membrane form bridges that facilitate absorption (Fig 1.2A). The pathogen-composite complex precipitates easily. To assess the enrichment capacity, a UV spectrophotometer (Libra 22 UV) was used to measure the absorbance of the supernatants from the tested pathogen samples containing *E. coli* (10^6 CFU, 2 mL) after treatment with ADE alone or after the ADE-CB enrichment process. As shown (Fig 1.2B), the lower absorbance of the supernatant following ADE-CB enrichment indicates that the ADE-CB composite achieved a 90% capture efficiency within 3 minutes at an *E. coli* concentration of 10^6 CFU/ml. Furthermore, SEM images of HCT-116 cells bound to the ADE-CB surface are shown (Fig 1.2C). These experiments confirm that ADE-CB is useful for biocompatible enrichment of pathogens and cells.

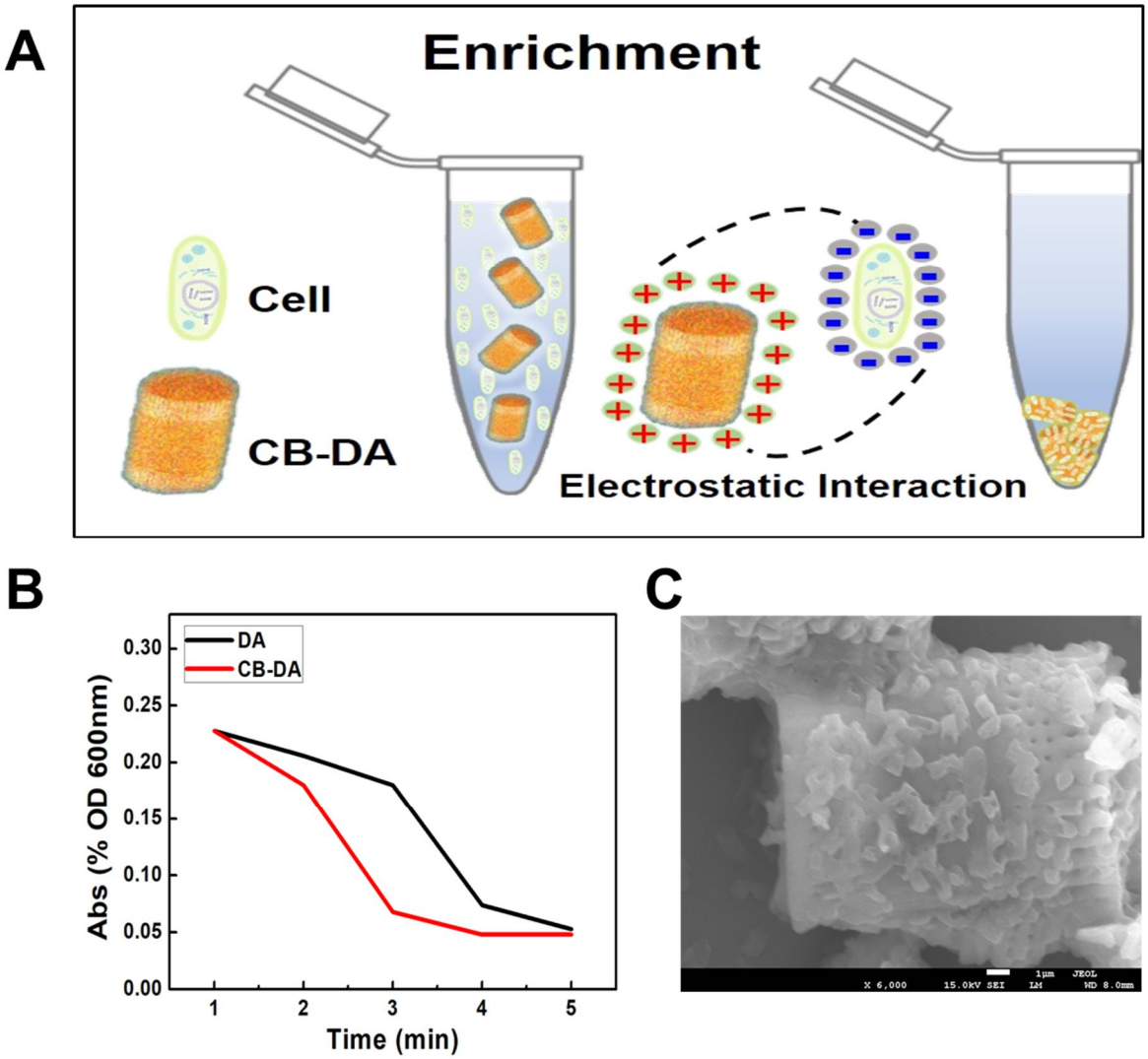

Figure 1.2 Pathogen enrichment schematic and demonstration. (A) Enrichment schematic; the electrostatic interaction between the positive surface of the ADE-CB and the negative charge from the cell membrane. (B) The supernatant absorbances of the tested pathogen samples after ADE and ADE-CB *E. coli* enrichment (CFU 10^6, 2 mL). (C) Cell enrichment demonstration. SEM images of the HCT-116 cells adhered to the surface of ADE-CB.

1.3 Nucleic acid (NA) isolation using the biocompatible composite

To further confirm that the ADE-CB composite rapidly and effectively adsorbed the bacteria, the fluorescence signals from qPCR analyses of amplified DNA extracted from the supernatant and precipitate (Fig 1.3A) of the E. coli (CFU 10^4, 1 mL) enrichment using a Qiagen kit (100 µL of tested sample) are shown (Fig 1.3B). The inside figure shows the melting-curve plots, which represent the amplification products from the systems (black line: 10^4, 100 µL as a positive control; red line: supernatant from the ADE-CB-treated sample, 100 µL; blue line: enrichment with ADE-CB, 100 µL of precipitate; green line: DW as a negative control). We observed that the ADE-CB enrichment system was 4-fold more effective for pathogen enrichment, suggesting that it could be used for sample preparation in diagnostic systems. The reduction in the surface charge of the ADE-CB-DNA indicates that the ADE-CB composite is more effective at capturing the nucleic acid, and this effect may be due to the previously reported covalent bonding, physical adsorption, electrostatic interactions, and heterogeneous surface binding intrinsic to supermolecular family members (28, 29).

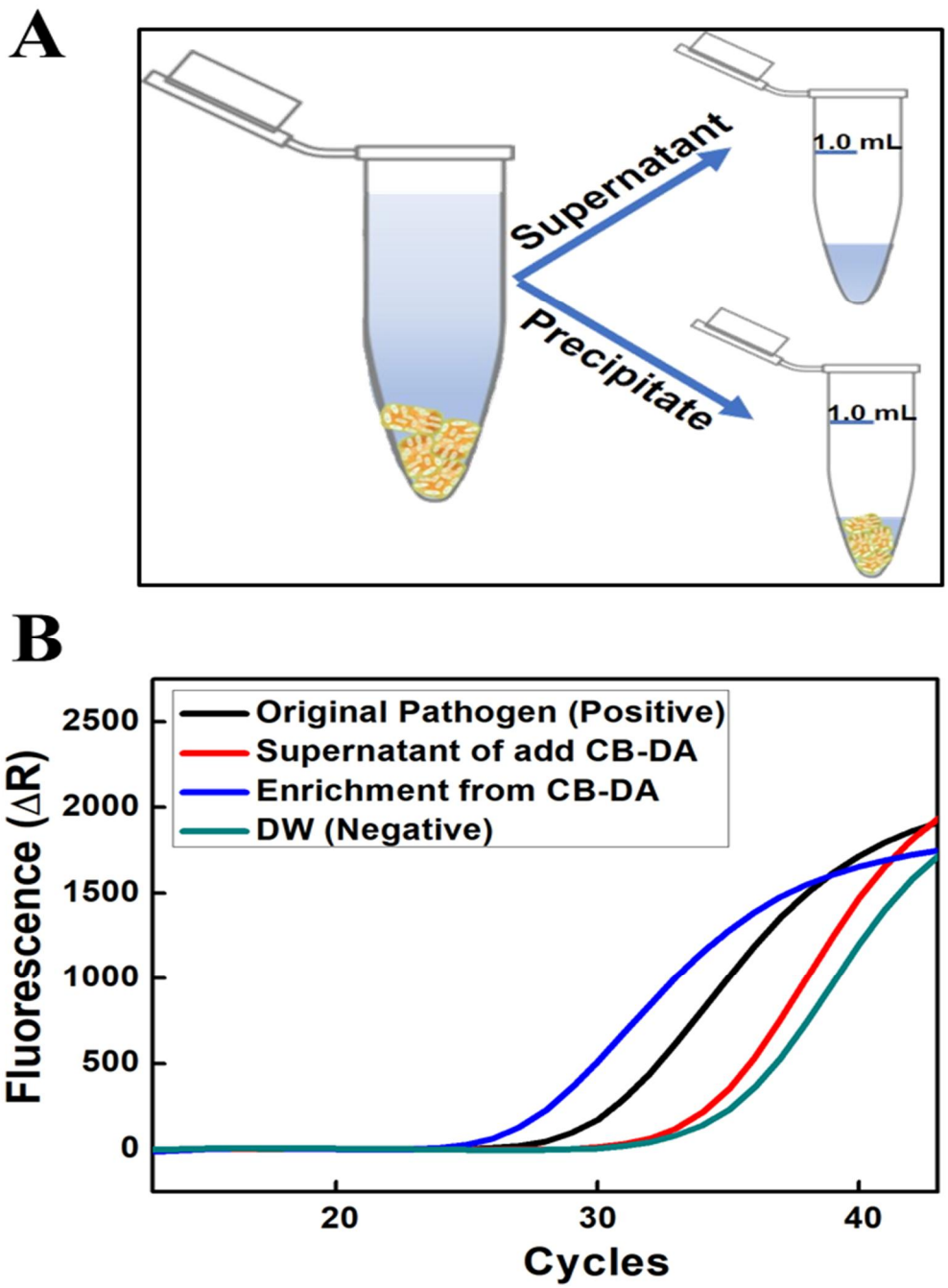

Figure 1.3 Schematic depiction of pathogen enrichment using the cucurbituril (CB) coating amine-functionalized diatomaceous earth composite (ADE-CB). (A) diagram of the supernatant and precipitate from the enrichment system. (B) Fluorescence signals from qPCR analyses of amplified DNA extracted from the supernatant and precipitate following *E. coli* enrichment (CFU 10^4, 1 mL) using a Qiagen kit (100 μL of the tested sample). The inside figure shows the melting-curve plots representing the amplification products

CHAPTER 2 – Sample preparation for nucleic acid (NA) isolation using single tube and syringe filter

2.1 Principle of the all-in one hand-held extraction method

The analysis used to collect NA templates from the various types of samples is based on an amine-functionalized diatomaceous earth (ADE) modified commercial PTFE filter in combination with a syringe (Fig 2.1, top left). The enhanced pathogen enrichment process is achieved via the use of dimethyl suberimidate (DMS, one kind of HI) and ADE (Fig 2.1, top middle). As a HI reagent, DMS reacts with the amine groups of ADE and generates more positively charged amidine bonds, which then directly attract negatively charged pathogens (Fig 2.1, bottom left). In this case, the saline-functionalized amine groups are the basis of this all-in-one hand-held extraction approach. Furthermore, ADE's nano-porous structure gives it strong absorption capability and it has an ultra-high reaction area, which also contributes to its enhanced performance (30). A syringe filter was utilized to trap ADE and its attached pathogens, so the enrichment process could easily be performed by mixing the DMS and ADE with pathogens in solution and then injecting this onto the filter. After washing, the trapped pathogens were lysed in situ (Fig 2.1, top right). Subsequently, the NAs were isolated chemically via a reversible crosslinking reaction. The DMS serves as a cross linker between amine groups of NAs and ADE (Fig 2.1, bottom right). This reaction is reversed at pH 10 (24). So the NAs released upon lysis are crosslinked to the surface of ADE in the presence of reaction buffer at pH 8 and can then be released by the elution buffer at pH 10. Therefore, it is easy to extract NA templates from a sample on the filter by controlling the pH of the injected buffers. This method does not require sample pretreatment to remove the RNase, nor any electrical apparatus such as centrifuge, vortex, or pump, which may not be options in remote areas. The method could be readily adapted for use in a POCT system.

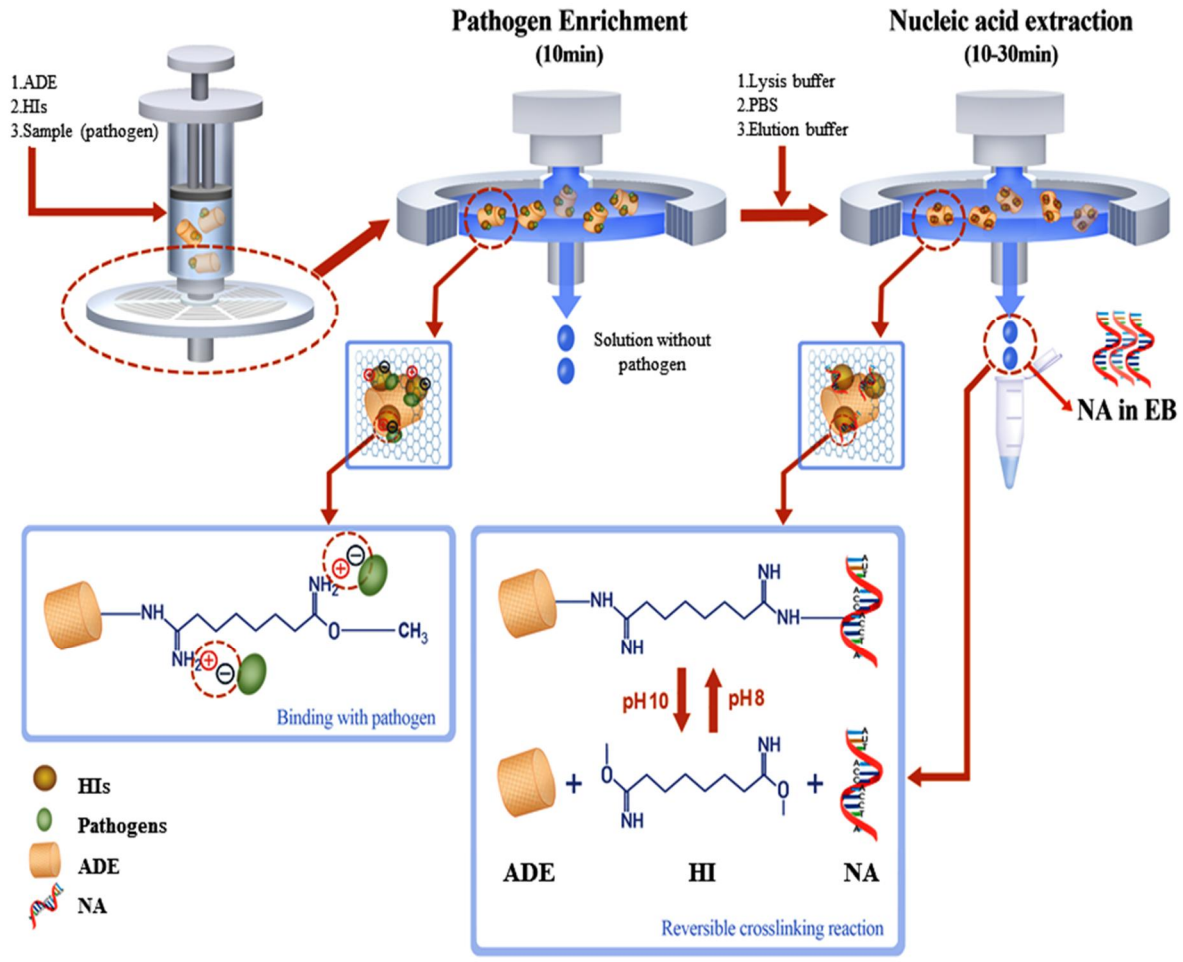

Figure 2.1 Schematic diagram illustrating nucleic acid (NA) isolation using the syringe filter method based on amine-functionalized diatomaceous earth (ADE) in conjunction of homobifunctional imidoesters (HI). Pathogen enrichment (top middle) and NA extraction (top right) can be completed with this hand-held system without the use of any chaotropic agents or instruments within as little as 20 min for 1 mL samples and 40 min for 50 mL samples. Shown by the dashed line from the pathogen enrichment process, the pathogens are absorbed onto the surface of the ADE; this is assisted by DMS via its positively charged amidine bonds, which can interact electrostatically with negatively charged pathogens (bottom left). The principle of NA isolation is shown by the dashed lines from the NA extraction process: DMS crosslinks the pathogen NA to ADE via a covalent bond in the reaction buffer (at pH 8); this can be broken by injecting a high-pH elution buffer (pH 10) into the filter, allowing the NA to be collected (bottom right).

2.2 Performance of the single tube-based method

To evaluate the capability of our proposed system for on-site sample preparation, a dilution series of pathogenic *B. ovis* bacteria in 1 mL PBS was prepared with concentrations of 10^0 to 10^4 CFU/mL. RNA templates were prepared using our method and commercial kits, followed by qPCR analysis for pathogen detection. This showed the detection limit for our system to be 1 CFU/mL (Fig 2.2A), which was 100-fold more sensitive than those of the Qiagen kit and Pathogen-specific kit (10^2 CFU/mL). Clinical samples are commonly collected in relatively large volumes, varying from several to dozens of mL, depending on the source (e.g., blood or urine). However, only part of the collected clinical samples is used due to the limitations of the capacity of sample handing. Although, conventional NA extraction kits (Qiagen Midi and Maxi Kits) can deal with up to 15 mL of sample, its sensitivity was much low (Fig 2.2B). Especially, large volume extraction kits require additional equipment such as conical tube centrifuge system. What's more, the 15 mL extraction kit is much more expensive. Thus, the typical kits with a sample volume capacity of 200 μL (eg: Qiagen Mini, 200 μL volume) are more widely used in the clinical diagnosis (31-32). Therefore, to evaluate our proposed assay, we used Qiagen Mini Kit as standard control rather than the Qiagen Midi or Maxi Kit. Our method can accommodate samples of up to 50 mL from various matrices, such as PBS, urine, and serum (Fig 2.2C). Compared with the absolute references by kit, there were delays of 2.3 and 0.8 cycles in the templates from 10mL PBS and serum samples, respectively; however, clear early cycles were observed in all the templates from the urine samples. For the kit-treated samples shown (Fig 2.2B), C_T values for the urine and serum samples were greatly delayed compared with those for the PBS samples, which may have contributed to the RNase and PCR inhibitors in the human samples. It is notable that, for samples extracted by both kit and assay treatment, the serum samples commonly showed much longer delays than the PBS samples. The serum samples contained much more RNase, so the released RNA could easily become degraded when using the standard method; conversely, the earlier cycles observed with the assay treatment suggested the RNA extracted by our assay benefited from covalent attachment to the matrix, inducing resistance to RNase degradation (33).

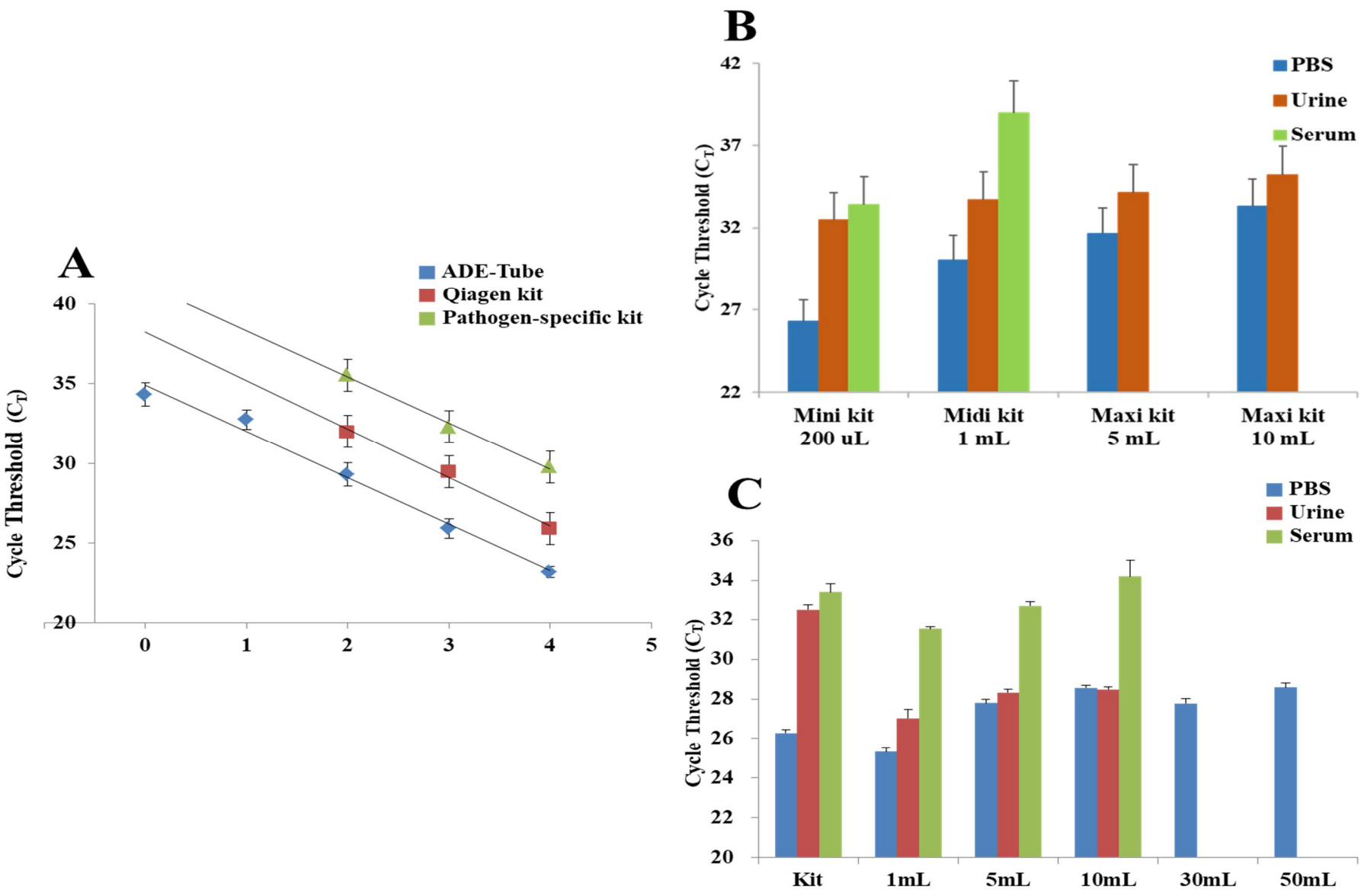
A
ADE-Tube
Qiagen kit
Pathogen-specific kit
Cycle Threshold (C_T)
40
35
30
25
20
0
1
2
3
4
5
B
PBS
Urine
Serum
Cycle Threshold (C_T)
42
37
32
27
22
Mini kit 200 uL
Midi kit 1 mL
Maxi kit 5 mL
Maxi kit 10 mL
C
PBS
Urine
Serum
Cycle Threshold (C_T)
36
34
32
30
28
26
24
22
20
Kit
1mL
5mL
10mL
30mL
50mL

Figure 2.2 Evaluation of the ADE-tube system for RNA isolation performance based on the cycle threshold (C_T) values of quantitative reverse transcription real-time PCR (RT-qPCR). (A) qPCR performance of isolated RNA templates from a dilution series of pathogenic *B. ovis* bacteria in 1 mL PBS was evaluated with concentrations of 10^0 to 10^4 CFU/mL samples using commercial kits (Qiagen Kit and Pathogen-specific Kit) and the tube-based ADE–DMS system (ADE-Tube). (B) Test of the performance of commercial kits for the treatment of large-volume samples. Qiagen Mini, Midi, and Maxi kits were used. The test used 1-mL samples of 10^3 CFU/mL B. ovis in PBS, urine, and serum diluted to 5 and 10 mL using matched original solutions. (C) Evaluation of the ADE-Tube system for large sample volumes in various sample matrices. Here, 1 mL samples of 10^3 CFU/mL *B. ovis* were used. The Kit (Qiagen Mini kit) used 200 µL of the 1 mL samples 10^3 CFU/mL *B. ovis* in PBS, urine, and serum. To evaluate the capture performance of the assay for large volume samples, the 1mL samples were diluted serially to volumes up to 50 mL, using matched original solutions; the urine and serum samples were tested up to 10 mL.

2.3 Evaluation of the Syringe filter method

To achieve complete laboratory independence, we integrated our method into a syringe filter system. This method was powered entirely by hand via a syringe and needed no other devices. It means our sample preparation methods was perfectly suited to POCT applications, especially for the application in resource-limited settings owing to its low cost, as well as ease of use and production. We further tested the performance of this hand-held syringe method in the preparation of RNA templates from a dilution series of pathogens, assessing the performance using qPCR (Fig 2.3A). The performance of the syringe method was comparable to that of the tube-based method, resulting in an ultra-low detection limit of 10^0 CFU/mL, which was 100-fold better than that of the kit system (10^2 CFU/mL). There was no significant difference in pathogen capture efficiency between tube-based and filter-based assays (Fig 2.3B). However, the tube-based assay requires a centrifugal device whereas the filter-based system does not require any specialized instruments and can be completed within 20 min, which is much faster than the tube-based system. In addition, the filter-based method can easily be used with large sample volumes because of the easy loading step using the syringe, allowing samples of up to 50 mL to be processed easily. Our assay also delivered effective enrichment and on-site NA extraction with various volume samples (Fig 2.3C), as well as exhibiting relatively stable performance. We also tested the performance of the method with other biological matrices and found that it performed equally well with urine samples, but less well with serum samples, for which greater variability was observed; however, the difference was not statistically significant (Fig 2.3D).

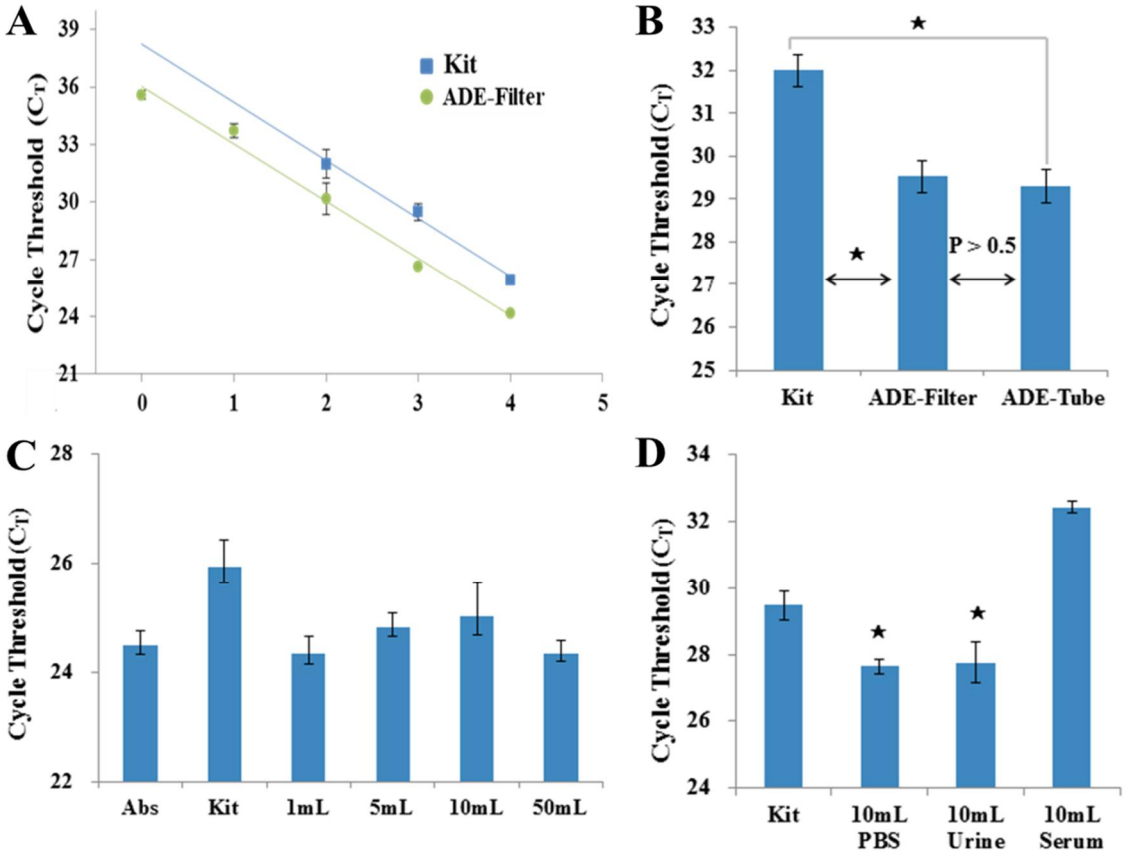

Figure 2.3 Performance evaluation of the ADE-filter system for RNA isolation based on the cycle threshold (C_T) values of quantitative reverse transcription real-time PCR (RT-qPCR). (A) The qPCR performance of isolated RNA templates from both a commercial kit (Kit) and the syringe-based ADE system (ADE-Filter) using *B. ovis* bacteria in 1 mL PBS was evaluated with concentrations of 10^0 to 10^4 CFU/mL samples. (B) Performance of the ADE–DMS system in both of tube and filter formats. 1 mL of 10^2 CFU/mL *B. ovis* in PBS samples were used. (C) RNA isolated by the ADE-filter system from different volume samples (1 mL to 50 mL) containing 10^4 CFU of *B. ovis* in PBS. For the 1 mL (10^4 CFU/mL) samples, the Kit used 200 µL of the 1 mL samples whereas the filter-based assay used the entire 1 mL samples because of its different capacity. "Abs" refers to the absolute NA references extracted from 100 µL (10^5 CFU/mL) samples, which contained the same amount of pathogens as the 1 mL of 10^4 CFU/mL samples. (D) RNA isolated by the ADE-filter system from different sample matrices containing 10^3 CFU of *B. ovis* in all the 10 mL PBS, urine, and serum samples. The total amount of pathogenic bacteria was controlled at the same sample level for all the parallel experiments.

CHAPTER 3 - Combination of filter/membrane plate and biocompatible silica to isolate nucleic acid (NA)

3.1 Development of the filter/membrane extraction method

As reported in previous studies, the DE with numerous qualities and various morphology is a good candidate for many bioengineering applications and show a powerful interaction (34, 35) that supports NA isolation. In order to increase the activity of DE, the APDMS treated (ADE) has been developed and optimized which could support pathogens capturing, NA isolation through powerful interaction and large surface area. The schematic diagram of pathogen isolation with the ADE and HI reagent, in tube has been shown in (Fig 3.1,). The imidoesters at both ends of dimethyl pimelimidate dihydrochloride (DMP, one kind of homobifunctional imidoester (HI)) help to form an amide bond with the amine group exposed on the surface of the ADE and also support to combine pathogens or NA. The 96well Filter Plate system has been used to do the multiplex NAs extraction study. The trapped pathogens were lysed in the 96well filter/membrane plate (Fig 3.1,). Subsequently, the exposed NAs combine with ADE and DMP, then NA could be chemically isolated through reversible crosslinking reaction (Fig 3.1,) (30). For extraction experiments, no need to move the mixture, and easily extract multiple samples at the same time using multi-pipette on the plate. Also there are no needs to exchange tubes repeatedly, and up to 96 samples are available at once.

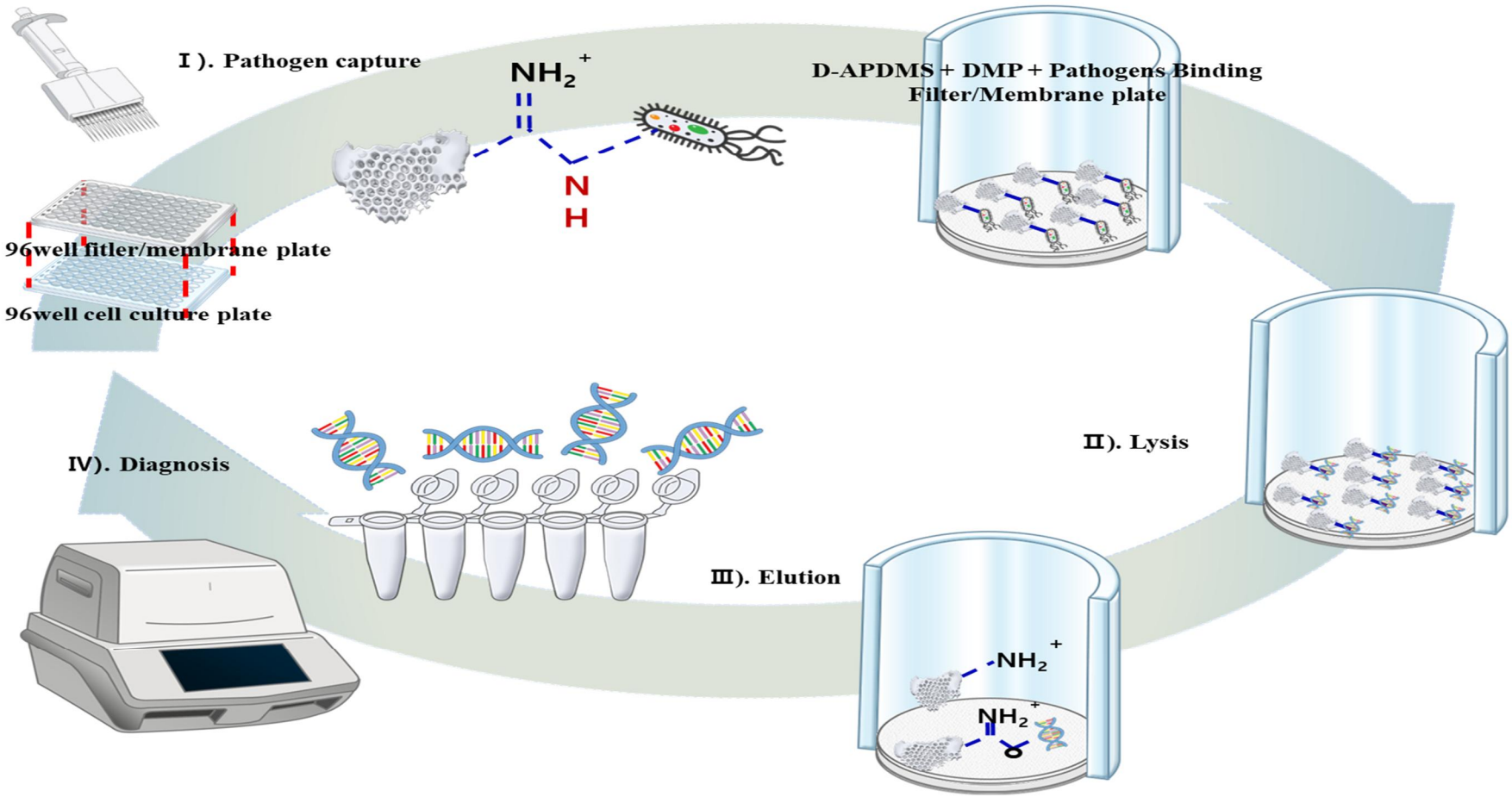

Figure 3.1 Schematic diagram of nucleic acid (NA) isolation via filter/membrane plate. Washed diatomaceous earth (DE) was modified with 3-aminopropyl-methyl-diethoxysilane (APDMS). With the help of the cross-linker dimethyl pimelimidate dihydrochloride (DMP, kind of HI), Nucleic acids of lysed pathogen samples can be interconnected with ADE to form a solid covalent bond. The formed complex could collect nucleic acids by using elution buffer (pH > 10).

3.2 Evaluation of nucleic acid (NA) extraction using the filter/membrane method

Base on the optimization of our convenient -multiple nucleic acid (NA) extraction system, we have tested the MultiScreen Solvinert 96 Well Filter Plate (0.45 μm pore size, Hydrophobic Polytetrafluoroethylene (PTFE)). With the same filtration area (0.28 cm^2) and same pore size (0.45 μm), both of them could carry the DE (2~10 μm) upon the surface of membrane. To evaluate the validity of the filter/membrane plate, different mixtures were separated at the same time (Fig 3.2). When all conditions were equal, the difference in C_T values could be confirmed depending on the presence or absence of ADE and DMP. This once again confirmed that the bonding of ADE of the present invention effectively captures pathogens and is excellent in nucleic acid isolation results. It was also confirmed that the rest of the impurities in the filter/membrane plate, except for the combination of pathogens and nucleic acids of ADE-DMP, were washed well during the washing process and had little effect on the subsequent qPCR. As results, there was little difference compared to DNA extracted using commercialized kits. Next, we applied ADE to a 96 well filter / membrane plate at once for robust sample preparation. When the 8 samples were simultaneously treated using the ADE-96 well filter plate, the DNAs were isolated and amplified with good uniformity (Fig 3.2B). It is possible to add reagents more rapidly and easily by using a multi-pipette on the filter/membrane plate, allowing for faster time diminution. Thereby, the proposed convenient-multiple NA extraction system is likely to be used for point of care testing (POCT) applications due to its great performance and applicability.

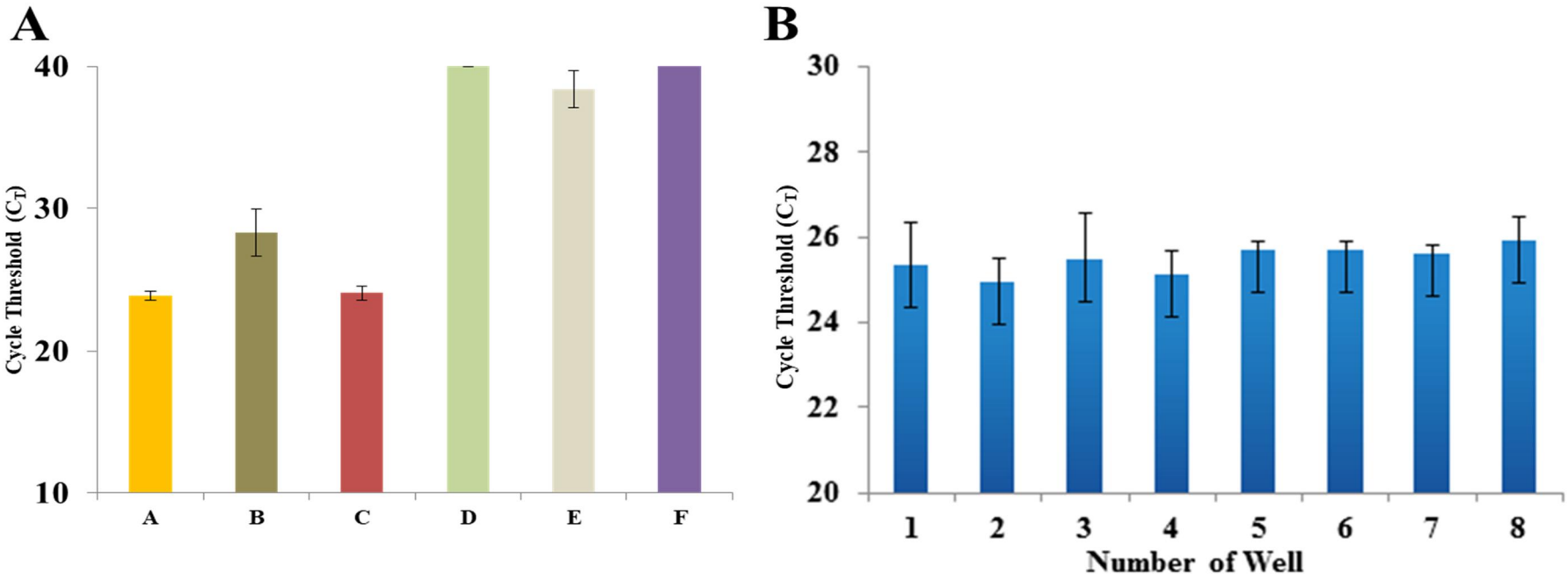

Figure 3.2 Evaluation of the ADE-filter/membrane plate system for DNA isolation based on the C_T values of quantitative real-time PCR (qPCR) (A) The C_T values extracted in the MultiScreen Solvinert 96 Well Filter Plate (0.45 µm pore size, Hydrophobic Polytetrafluoroethylene (PTFE)) from each different mixture in the same way. A; Kit, B; ADE system without ADE, DMP, C; ADE system, D; Lysis buffer of ADE system, E; ADE system without *B. ovis*, F; DW. (B) Robust testing with 96 well filter plates. DNA isolated by the ADE-96 well filter plate from the sample containing 10^3 CFU of *B. ovis* in the 1 mL PBS sample. 8 samples were treated at once. Error bars indicate standard deviation from the mean based on at least three independent experiments. It was done in the same volume using PBS. The C_T values of 40 are samples that did not detect.

4. DISCUSSION

Many technologies for pathogen diagnosis have been developed in the field of infectious diseases, but there are still many difficulties due to the low concentration of pathogens, complicated processes and high costs. In addition, it takes a long time to diagnose and requires skilled technicians, making it even more difficult to approach the field of pathogen diagnosis. In this study, our technologies were developed with several features. ( ) it has excellent concentration efficiency of pathogens and nucleic acids and minimizes time, ( ) convenient and easy and universal technology, ( ) it is easily combined with multiple on-site diagnostic technologies at low cost. To develop the technologies including these features, we used amine-functionalized diatomaceous earth (ADE). Because DE has a large surface and volume ratio and can further improve absorption, the new biocompatible composites of ADE and CB using these characteristics have excellent positive charge properties. Using these features, electrostatic interaction between the positive surface of ADE-CB and the negative charge of the pathogen cell membrane promotes absorption. ADE-CB has been found to have a concentration efficiency of 90% or more in a short period of time with a simple inverting step. ADE and homobifunctional imidoesters (HI) were also used in a single tube and syringe filter. DMS and DMP among HIs acts as a cross-linker between ADE, pathogens and nucleic acids. The amidine bonds formed between ADE and nucleic acid were reversed at high pH (pH>10), then nucleic acid was isolated. When using a syringe filter, the enrichment efficiency of the pathogen sample was more than 98%, and it was easy to concentrate and extract nucleic acid in 50ml without any electrical equipment. The sensitivity of the technique was improved at least 10 to 100 times in several pathogen samples compared to commercialized column-based nucleic acid separation kits. Furthermore, the technology was applied to the 96well-filter/membrane plate for multiple sample preparation at once. The uniform results were obtained by qPCR when the nucleic acid was isolated from several pathogen samples.

This study lays basics for further development of sample preparation techniques for disease diagnostics. The developed filter/membrane system exhibits immense potential for use in NA-based POCT applications, especially in resource-limited settings.

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6. ABSTRACT (KOREA)

병원체로 인한 질병은 전 세계적으로 중요한 문제이며, 인수공통감염병으로 인한 감염병은 저소득 국가의 사람들의 건강 위험을 증가시키고 있다. 또한 병원체에 의한 질병은 보통 복잡한 전처리 과정이 필요하며 농도가 낮고 값 비싼 장비로 인해 핵산 (nucleic acid, NA)을 얻기가 어렵다. 본 연구에서, 우리는 생체 적합성 나노물질인 규조토 (diatomaceous earth, DE)의 표면을 아민 기능화하고 다른 분자와 결합하여 새로운 화합물을 만들거나 아민 기능화된 DE (amine-functionalized Diatomaceous earth, ADE)에 화학물질을 추가하여 병원체 농축 및 핵산 분리를 위한 더 효율적인 시스템을 개발했다.

먼저, 호박 모양의 분자인 cucurbituril (CB)로 ADE 를 코팅 (ADE-CB)하여 농축된 병원체 및 세포로부터의 효과적인 샘플 농축 및 NA 분리를 지원하도록 제작했다. 새로운 생체 적합성 복합물, ADE-CB 를 사용한 병원체 샘플 농축은 장비 없이 간단히 손으로 흔들어주면 단시간에 90 % 이상의 농도 효율을 갖는 것으로 확인되었다. 다음으로, 주사기 필터 시스템을 ADE 와 함께 사용해 편리하고 용이하게 농축 된 병원체를 단기간에 어떠한 장비 없이 핵산을 분리하는 기술을 개발했다. 또한 ADE 및 필터 시스템을 96 well filter/membrane plate 와 결합하여 쉽고 빠르게 한 번에 여러 샘플을 추출 할 수 있는 기술을 개발했다. 우리의 기술은 상용화된 키트보다 10 ~ 100 배 더 민감하며 추가적인 실험 장비 나 도구가 필요하지 않기 때문에 경제적이다.

병원체와 핵산을 포획하는 효과적인 방법은 진단 시스템을 위한 시료를 준비하는 데 유용 할 수 있으며 그 밖의 여러 가지 이점은 구성 요소 생산의 단순성과 작동의 용이성을 포함하며, POCT (Point-of-Care Test)를 위한 다른 검사와 쉽게 통합될 수 있다.

Keywords: Pathogen, Nucleic acids, Amine-functionalized diatomaceous earth, Cucurbituril, filter, Point-of-care test