Master of Engineering

Study for a Novel Diagnostic Performance of Microparticle Diatom-ZnO-APDMS Based on Fungus

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Department of Medical Science

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ABSTRACT

Background and purpose: The expanding spectrum and rising incidence of invasive aspergillosis (IA) makes the early-accurate identification of fungal pathogen a daunting task. Recently, as the nucleic acid-based diagnostics are widely used for clinical applications, the efficiency of DNA extraction of the fungal becomes one of the particular importance developing technologies in medical diagnostic applications. In this research, we first combined the nanotechnology for the fungal DNA extraction and developed a rapid, economical and reliable system to easily extract the genomic DNA from *Aspergillus fumigatus*. Our method took advantage of the diatomaceous earth (DE) to enrich the spores sample to make sure the spores can be captured even in low spores content sample. Meanwhile, the burgeoning nanomaterial ZnO performed the spores membrane breaking process, we synthesized a novel triphase micropartcles DE-ZnO-APDMS to extract the DNA from *Aspergillus fumigatus*. The quality and quantity of the extracted DNA were sufficient for the further diagnostics in polymerase chain reaction (PCR) amplification.

So, this study confirms that the triphase micropartcles DE-ZnO-APDMS is a good candidate for varies bio-applications, especially in the diagnostic performance.

Materials and Methods: Single ZnO nanoparticles and triphase micropartcles DE-ZnO-APDMS were synthesized using the hydrothermal method in an alkaline medium. The morphology of DE, ZnO, and DE-ZnO-APDMS was characterized

using field-emission scanning electron microscopy (FE-SEM) on a JSM-7500F instrument (JEOL) to confirm the DE and ZnO structure as well as the decoration of ZnO on DE. *Aspergillus fumigatus* which is the most common species cause invasive aspergillosis was used in all experiments in this study. In order to confirm the DE-APDMS can enrich the *Aspergillus fumigatus* spores in large Sample volume, the traditional fungal culture method was used. Meanwhile, the DNA binding ability of DE-APDMS and DE-ZnO-APDMS were tested by the adsorption equilibrium experiment with UV-Vis spectroscopy. The *Aspergillus fumigatus* DNA extracted by ZnO and DE-ZnO-APDMS were compared with the DNA extraction from commercial kit. The quality and quantity of the extracted DNA were sufficient for the further diagnostics in polymerase chain reaction (PCR) amplification.

Results: The firstly synthesized triphase micropartcles DE-ZnO-APDMS showed that the multigonal star-shape nanoparticles ZnO (~300nm) grew on the microscale hollow silica structures which have lots of nanoscale pores in the wall. The DE-APDMS can enrich the limited amount *Aspergillus fumigatus* spores in large sample volume. Both the DE-APDMS and DE-ZnO-APDMS had the DNA binding ability because the DNA form the spores was cross-linked by the composite surface modifier APDMS. The synthesized nanoparticles ZnO (~300nm) perform the successful lysis of fungal spores. The unique triphase micropartcles DE-ZnO-APDMS combined the advantages of the each part in the composite, it can extract fungal DNA in this all-in-one assay. Compared with the commercial kit assay,

the fungal DNA isolation assay based on the triphase micropartcles DE-ZnO-APDMS was more sensitive in large sample volume with limited numbers of spores.

Conclusions: In this study, a simple and rapid fungal DNA isolation assay based on synthesized triphase micropartcles DE-ZnO-APDMS for the diagnosis of invasive aspergillosis is presented. With quantitative PCR analysis of the amplified DNA isolated using the DE-ZnO-APDMS-based fungal DNA isolation assay and commercial kit assay, our results show that these uniquely synthesized triphase micropartcles DE-ZnO-APDMS was more effective to extract the fungal DNA from samples with less density. This study introduces a new candidate for diagnostic techniques for human invasive aspergillosis.

Keywords: Invasive Aspergillosis, Diatomaceous Earth, Zinc Oxide, DNA Extraction, Molecular Diagnosis.

CONTENTS

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LIST OF ABBREVIATIONS

APDMS	3-aminopropyl(diethoxy)methylsilane
CT	Computerized Tomography
Ct	Cycle Threshold
CTAB	Cetyltrimethylammonium Bromide
DA	Amino-functionalized Diatomaceous Earth
DE	Diatomaceous Earth
DNA	Deoxyribonucleic Acid
DW	Distilled Water
FE-SEM	Field-Emission Scanning Electron Microscopy
IA	Invasive Aspergillosis
N.D	Negative Diagnosis
PCR	Polymerase Chain Reaction
TEM	Transmission Electron Microscopy

1. INTRODUCTION

Invasive aspergillosis (IA) plays a significant role in the morbidity and mortality of immunocompromised individuals, bone marrow transplant recipients, cancer patients, HIV patients, and patients undergoing treatment with immunomodulators [1-2]. There are more than 300 kinds of genus of Aspergillus, and *A. fumigatus* is the most prominent causative organism of IA, followed by *A. flavus*, *A. niger*, and *A. terreus*. *A. fumigatus* is common in all environments but is difficult to distinguish from certain other molds under the microscope [3]. Currently, the most common tests used to diagnose aspergillosis are the imaging tests chest X-ray or computerized tomography (CT) and specimen tests of sputum, tissue, and blood [4-5]. In respiratory secretion test, the patient's sputum is stained with a dye and checked for the presence of Aspergillus filaments. The specimen is then placed in a culture that encourages the mold to grow to help confirm the diagnosis. Moreover, for the skin tissue test, Aspergillus antigen is injected into the patient's forearm skin. A hard, red bump at the injection site appears if the patient has antibodies [6-7]. However, all these tests are time-consuming and uncomfortable, and additionally, due to the weak and indistinct clinical features of early IA, precious time is wasted in many patients requiring early diagnosis of the infection and prompt medical attention.

In the last two decades, developing molecular techniques have been widely used in the detection of fungal DNA in clinical samples. These molecular methods can identify clinical samples which usually contain limited numbered fungal cells with

high sensitivity [8]. For the DNA samples used in the steps of downstream molecular analysis, besides the optimal primers and PCR conditions, it is exceedingly important to use the most efficient way to extract the DNA to detect the low levels of Aspergillus DNA. Commonly, because of the fungal cell wall, isolating DNA from fungi is more difficult than from bacteria or mammalian cells and requires additional steps involving the use of enzymatic, mechanical, or chemical methods to disintegrate the fungal cell wall [9-10]. Currently, most commercial fungal DNA extraction kits used in clinics and laboratories are based on a standard method comprising the lyophilization of mycelia, disruption of the cell wall by grinding, extraction of DNA in a buffer containing sodium dodecyl sulfate, removal of proteins with a mixture of phenol and chloroform, and precipitation of DNA with 2-propanol [11-13]. Although this method can obtain large amounts of pure fungal DNA, it is time- and labor-intensive and presents with the potential threats of phenol and chloroform pollution. Therefore, a novel fungal DNA isolation assay would be desirable for simple, rapid, and highly sensitive detection of Aspergillus even at low levels.

Here we developed a simple fungal cells DNA extraction assay which included two main parts, fungal cells enrichment by homobifunctional imidoesters and fungal DNA extraction by ZnO nanoparticles. In order to perform the process in single tube, we synthesized an unique triphase micropartcles DE-ZnO-APDMS. Diatomaceous earth (diatomite, DE) in this composite plays the part of groundwork. DE is made from the fossilized remains of diatom in the water and show a microscale hollow silica structures which have lots of nanoscale pores in the wall. And DE has abundant

active hydroxyl groups on its high surface area (200 m^2/g), this excellent characteristic make it easily be performed chemical modification and widely be used in biological applications [14-17]. The composite DE-ZnO was synthesized by the first-used protocol which the nanoscale zinc oxide (ZnO) was grow up on the surface of the DE. Metal nanoparticles, such as Ag, CuO, MgO, and ZnO, have attracted tremendous attention for their use in medical diagnostics [18-20]. ZnO is an emerging semiconductor which has a wide direct band gap (~3.3 eV) near the UV spectral region and large exciton binding energy (60 meV). Due to their unique chemical and physical properties, Zinc (Zn) and its oxide (ZnO) are widely applied in photoelectronics, sensors, electronics, photocatalysts, and antibacterial agents among other applications, and they have become the most interesting hotspot in this regard [21-23]. Actually, some promising studies have revealed that ZnO nanoparticles have an inhibitory effect on microbial growth and metabolism. In our previous study, the nanomaterial ZnO was a good lysis buffer candidate for eukaryotic cells and common bacterial pathogens except fungi due to its limitation of not being able to break the cell membrane. ZnO have three main pathway to lysis the cells, they are physical, chemical and biological ways [24]. Meanwhile, the APDMS (3-Aminopropyldimethylethoxysilane) was used as functionalizing agent for modify the composite DE-ZnO which improve attachment of the triphase micropartcles DE-ZnO-APDMS to the cells, proteins and biomolecules such as the DNA [25-27]. APDMS was selected to work as cross linker because it can silanize the DE-ZnO to increase the hydrophilicity of the composite, and the triphase micropartcles

DE-ZnO-APDMS showed high positive charge because of the divalent electrostatic interaction between the DE-ZnO and APDMS [28-29]. This all-in-one DNA isolation assay based on the triphase micropartcles DE-ZnO-APDMS can be used for fungal cells enrichment and DNA extraction within 45min in a single tube without the complicated operation and large instrument. Combine with the PCR detection technology, this assay can successfully diagnose limited number fungal cells in the larger volume samples. Accordingly, fungal cells DNA extraction assay based on the triphase micropartcles DE-ZnO-APDMS is a novel candidate which is low-cost, environmentally-friendly, easy-to-operate, time-saving and highly sensitive for the clinical invasive aspergillosis diagnosis.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All reagents were of analytical grade and used without further purification. Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, 98%), ammonium hydroxide solution (28% NH_3 in H_2O, 99.99% trace metals basis), 3-aminopropyl(diethoxy)methylsilane 97% (APDMS), biocompatible DE (powder), sodium bicarbonate ($NaHCO_3$), deoxyribonucleic acid (DNA, low molecular weight from salmon sperm, 31149)，commercial zinc oxide (powder, <5 μm, 99.9%; SIGMA-P Code: 205532) and Tween20 (P2287) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cetyltrimethylammonium bromide ($C_{19}H_{42}BrN$ > 98%, CTAB) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Sabouraud dextrose agar with chloramphenicol media (Cat. No.C6781; Lot No.437412) was used for the *A. fumigatus* culture obtained from Santa Maria-USA. Ethanol(99%), Milli-Q water with a resistance greater than 18 MΩ, 99% ethyl alcohol, phosphate-buffered saline (PBS, 10×, pH 7.4) were used in the experiments.

2.2 Instruments and Kits

A commercial fungal DNA isolation kit, YeaStar Genomic DNA KitTM (Cat. No.D2002), was purchased from ZYMO RESEARCH. C-Chip used as disposable hemocytometer was purchased from INCYTO. Feld Emission Scanning Electron

Microscopy (FE-SEM) on a JSM-7500F instrument (JEOL) was used to confirm the reaction and decoration materials. Zeta potentials of materials were measured using dynamic light scattering (DLS) on a DynaPro NanoStar instrument (Wyatt). Vortex mixer (T5AL), a mini manufacturer (LABOGENE 1730R) MSH-30d stirring heater and disposable cuvette for spectroscopy were produced by Daihan Scientific Co., Ltd (Wonju-Si, South Korea) were obtained. Magic mixer (TMM-5) used for rotating the mixture tube during incubation was purchased from TOPSCIEN Instrument Co., Ltd. CFX96 Touch Real-time PCR Detection System was purchased from BIO-RAD. 96-well MultiScreen-HA Filter Plate (0.45μm) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Fungal Samples

Aspergillus fumigatus (ATCC36607) was used in this study. It was grown in Sabouraud dextrose agar at 25°C for 5 days. As the *Aspergillus fumigatus* grew, it produced spores. 1 mL 0.03% Tween 20 in water was used to collect the spores of *A. fumigatus*. After washing with PBS, the *A. fumigatus* spore cells were counted using a hemocytometer.

2.4 Preparation of homogeneous diatomaceous earth

Firstly, the commercial was 500 rpm stirring with distilled water (DW) for 10 min. After 1min gravity settling of the mixture, the supernatant was collect in 50-ml tubes and centrifuge at 1400rpm for short pattern. Remove the top liquid and wash the

precipitate with 99.9% ethanol for 30 min. Then after 30s gravity settling of the mixture, the supernatant was collect in 50ml tubes and centrifuge at 1400rpm for short pattern again. Next washed the precipitate with DW for 10 min and settled for 30s, then collect the precipitate after short centrifugation. This DW wash step was repeated for 3times for remove the uneven DE particles and free ethanol molecule. At last, the DE precipitate was collected and dried in the oven at 56℃ overnight.

2.5 Preparation of triphase micropartcles DE-ZnO-APDMS

Herein, triphase micropartcles DE-ZnO-APDMS were synthesized using the hydrothermal method in an alkaline medium. For this, 0.1 M zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) and 0.1 M cetyltrimethylammonium bromide (CTAB) were mixed in 100 mL of sterilized deionized water (DI) with 0.1g homogeneous diatomaceous earth in a 250-mL flask. The mixture was heated till 90°C for 50 min with continuous stirring (500 rpm). Then, while maintaining the mixture at 90°C with continuous stirring (500 rpm), 2 mL of ammonium hydroxide solution ($NH_3 \cdot H_2O$) was added at a rate of 1 mL/min, and during this period, as we see the color of reaction solution had been changed from brick-red (DE) to pink-white. Subsequently, the flask was transferred into an ice box and cooled down immediately to stop the reaction. The produced pink-white precipitates were collected and washed with DI three times to wash away the residual ions. Then, we dried the pink-white precipitates of triphase micropartcles DE-ZnO-APDMS at 56°C in an oven. For the single ZnO nanoparticles synthesis, this hydrothermal method was carried out without the DE in first step.

2.6 Evaluation of the *Aspergillus fumigatus* spores enrichment

The performance evaluation experiment of the *Aspergillus fumigatus* spores enrichment used functionalized diatomaceous composite DE-APDMS. 1 mL *A. fumigatus* spores was added into the 1.5-mL sample tube, and then, 5 mg (50 mg/mL, 100 μL) functionalized diatomaceous composite DE-APDMS was added. Next, the mixture was put onto the rotator for 30 min rotation. After incubation, the mixture was centrifuged at 10,000 rpm for 1 min. The supernatant (~1000 μL) was transferred into another new 1.5-mL tube. Meanwhile, the precipitate was resuspended by 200 μL PBS. Respectively spread 200 μL supernatant or precipitate sample onto the new Petri dishes containing the solid fungal culture medium. The dishes were move to the culture oven which was constant 37℃ and cultured for 2 days.

2.7 The DNA binding ability of synthesized composite

For testing DNA binding ability of two micropaticles, the adsorption equilibrium experiment was used. 5 mg (50 mg/mL, 100 μL) functionalized diatomaceous composite DE-APDMS and triphase microparticles DE-ZnO-APDMS were respectively added into 1 mL deoxyribonucleic acid (30μg/mL) solution in the 1.5-mL tube. Then the mixture was put onto the rotator for 30 min rotation. After incubation, the mixture was centrifuged at 10,000 rpm for 1 min. Next， the supernatant of each sample was analyzed by UV-Vis spectroscopy on a UV-2550 instrument by measuring the absorbance in the DNA concentration measure mode.

2.8 *Aspergillus fumigatus* spores lysis by ZnO

The synthesized ZnO nanoparticles (ZnO-S-300) and commercial ZnO (ZnO-C-100 and ZnO-C-5000) were used for developing and optimizing the ZnO-based fungal DNA isolation assay. The workflow of the ZnO-based fungal DNA isolation assay is shown at the top in Figure 3. Herein, 100 μL *A. fumigatus* spores was added into the 1.5-mL sample tube, and then, 100 μg (1 mg/mL, 100 μL) ZnO was added. Next, the mixture was put onto the rotator for 30 min rotation. After incubation, the mixture was centrifuged at 10,000 rpm for 1 min. Herein, the white ZnO was at the bottom of the tube. The supernatant was transferred into the Zymo-Spin III column and centrifuged at 10,000 rpm for 1 min. Then, 300 μL of DNA wash buffer was added and centrifuged for 1 min at 10,000 rpm to wash, and this wash step was repeated. Finally, the Zymo-Spin III column was transferred to a new 1.5-mL centrifuge tube, and 60 μL of water or TE was added directly onto the membrane. After waiting for 1 min, DNA was eluted by centrifugation at 10,000 rpm for 10 seconds.

2.9 *Aspergillus fumigatus* DNA isolation by commercial kit

The commercial YeaStar Genomic DNA KitTM was used for comparing the cell lysis properties of the ZnO nanoparticles. The workflow for DNA extraction with the kit is shown at the bottom of Figure 3. For the first procedure, 10 μL *A. fumigatus* spores was added into the 1.5-mL sample tube, and 120 μL of YD Digestion Buffer and 5 μL of R-ZymolyaseTM (RNase A + ZymolyaseTM) were added. These were

mixed well by vortexing and incubated at 37°C for 40–60 min. After incubation, 120 μL of YD lysis buffer was added into the mixture and gently vortexed. Next, 250 μL of chloroform was added and mixed thoroughly for 1 min, and the mixture turned from transparent to milky white. Then, the mixture was centrifuged in a table top centrifuge at 10,000 rpm for 2 min. At this point, the aqueous mixture had two layers, and the supernatant was transferred into the Zymo-Spin III column and centrifuged at 10,000 rpm for 1 min. Then, 300 μL of DNA wash buffer was added and centrifuged for 1 min at 10,000 rpm to wash, and this wash step was repeated. At last, the Zymo-Spin III column was transferred to a new 1.5-mL centrifuge tube and 60 μL of water or TE was directly added onto the membrane. After waiting for 1 min, DNA was eluted by centrifugation at 10,000 rpm for 10 seconds.

2.10 *Aspergillus fumigatus* DNA isolation by the assay based on DE-ZnO-APDMS

The synthesized triphase micropartcles DE-ZnO-APDMS was used for developing the fungal DNA isolation assay. Herein, 1 mL *A. fumigatus* spores was added into the 1.5-mL sample tube, and then, 5 mg (50 mg/mL, 100 μL) triphase micropartcles DE-ZnO-APDMS was added. Next, the mixture was put onto the rotator for 30 min rotation. After incubation, the mixture was centrifuged at 10,000 rpm for 1 min. Herein, the pink-white DE-ZnO-APDMS was at the bottom of the tube. The 300μL supernatant was transferred into one of the 96 well filter plate and centrifuged at 10,000 rpm for 1 min, this step was repeated for 4 times until all the supernatant was flowed through. Then, 300 μL of PBS was added and centrifuged for

1 min at 10,000 rpm to wash, and this wash step was repeated. Finally, the 96 well filter plate was transferred onto a new collection plate, and 100 μL of elution buffer (10mM $NaHCO_3$, pH10.6) was added into the well contain composite. After suspending the composite on the bottom and waiting for 1 min, DNA was eluted by centrifugation at 10,000 rpm for 10 seconds. At last, transferred the DNA elution from the well to 1.5-mL tube.

2.11 Quantitative PCR Condition

To analyze fungal DNA isolation performance, real-time PCR was performed after the isolation process. For the quantitative PCR process, the following procedure was implemented: 5 μL of DNA was amplified in a reaction mixture containing 10 μL of Green QPCR Master Mix (Agilent, #600882), 2.5 pmol of each primer, and 3 μL of DI water, and the total volume was made up to 20 μL. For detecting *A. fumigatus* DNA, we used the following primers: forward (5′-CACCCGTGTCTATCGTACCT-3′) and reverse (5′-ATTTCGCTGCGTTCTTCATC-3′). The PCR reactions were performed at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s, before a final elongation step at 95°C for 10 s.

3. RESULTS

3.1 Characterization of DE-APDMS, ZnO nanoparticles and DE-ZnO-APDMS

For triphase micropartcles DE-ZnO-APDMS used in all the experiments in this study, the optimal synthetic ratio of Zn^{2+} to DE 2:1 was used to present good morphology, perform friendly biocompatibility and take advantage of the surface area of DE-ZnO-APDMS. As the SEM image and schematic of the amino functionalization of diatomaceous earth (DE-APDMS) showed in Figure 1A, the main structure of the composite achieve microscale hollow silica structures which have lots of nanoscale pores in the wall. Two points of the bonding site from APDMS were efficiently bonding on the surface of DE. And the pure synthesized ZnO nanoparticles in Figure 1B were constituted by amounts of ZnO nanodots and had a multigonal star-shape. The firstly synthesized triphase micropartcles DE-ZnO-APDMS in Figure 1C showed that the multigonal star-shape nanopaticles ZnO grew on the surface of the microscale hollow silica structures DE. Next, the surface charges of five materials and composite (reported as the zeta potential) were measured and present in Figure 1D. While the DE display a negative surface charge, after modifying with APDMS, amine groups surrounding the surfaces of the DE make its charge turn positive. As the unique synthesized ZnO showed positive charge, the composite DE-ZnO was neutralized, and after amino functionalization, the triphase micropartcles DE-ZnO-APDMS was highly positive.

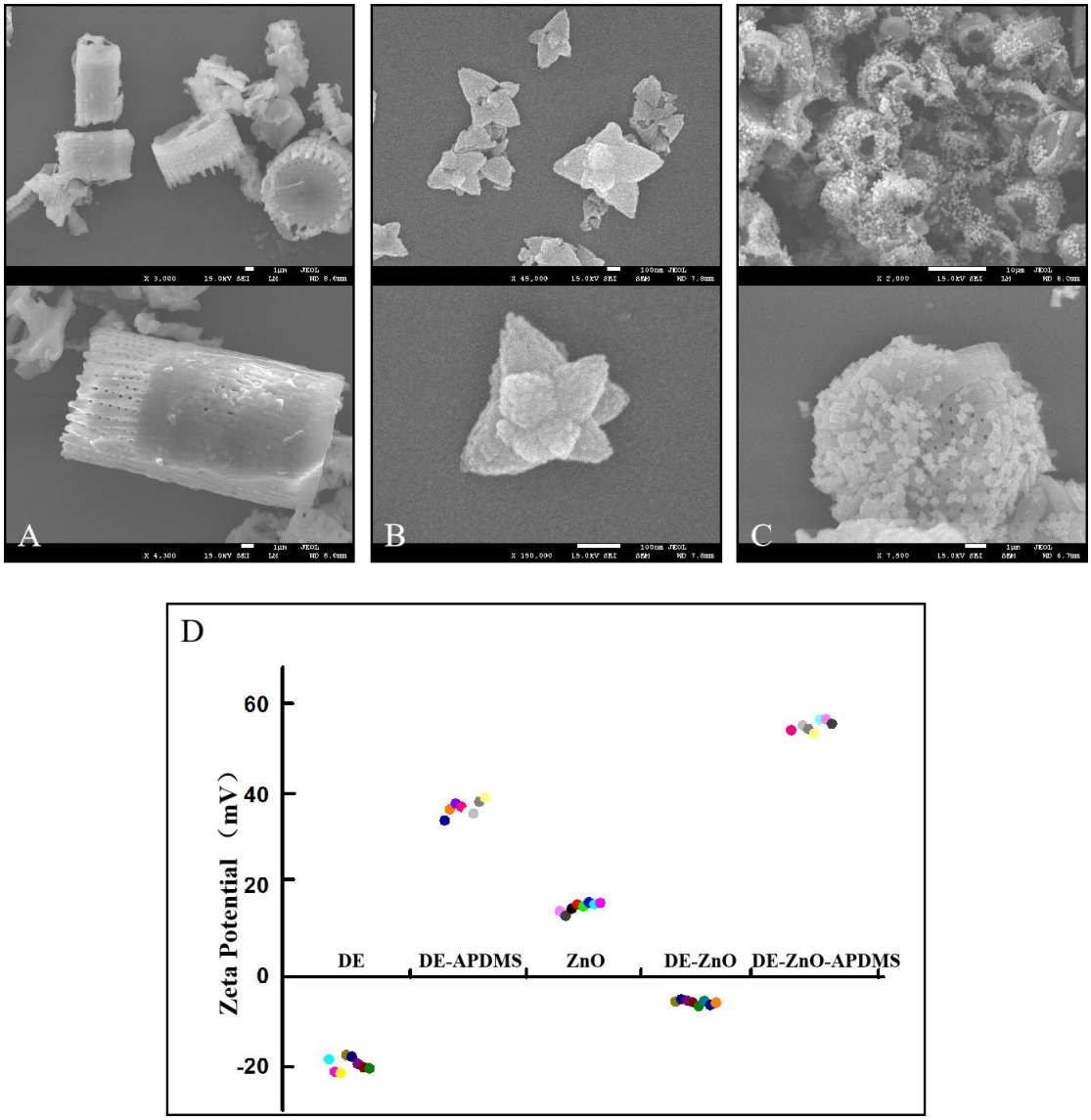


Figure 1. Characterization of DE-APDMS, ZnO nanoparticles and DE-ZnO-APDMS. (A) SEM images of DE-APDMS in measuring bar of 1μm. (B) SEM images of synthesized ZnO nanoparticles in measuring bar of 100 nm. (C) SEM images of synthesized triphase micropartcles DE-ZnO-APDMS in measuring bars of 10 μm and 1 μm. (D) Zeta potential values of the prepared materials: clean DE, amino-functionalized diatomaceous earth (DA), synthesized ZnO nanoparticles, synthesized intermediate composite DE-ZnO and synthesized triphase micropartcles DE-ZnO-APDMS.

3.2 Characterization of *Aspergillus fumigatus* spores enrichment

As the Figure 2A display the schematic and demonstration of the *Aspergillus fumigatus* spores enrichment. The electrostatic interaction between the positive surface of the composite and the negative charge from the *Aspergillus fumigatus* spore cell membrane. The same pathway was applied with negative charged DNA. The real photos results in Figure 2B. Different density of spores was incubated with the DE-APDMS for 30min, the composites enriched the spores onto their surface, then the samples contained different numbers of *Aspergillus fumigatus* spores in the supernatant and precipitate. After culturing the supernatant and precipitate on the fungal solid medium plates, the colonies grew from *Aspergillus fumigatus* spores in the DE-APDMS precipitate and supernatant. Although inevitably the composite left out the spores, obviously the DE modified by APDMS can efficiently enrich the *Aspergillus fumigatus* spores in a large sample volume (1 ml or more).

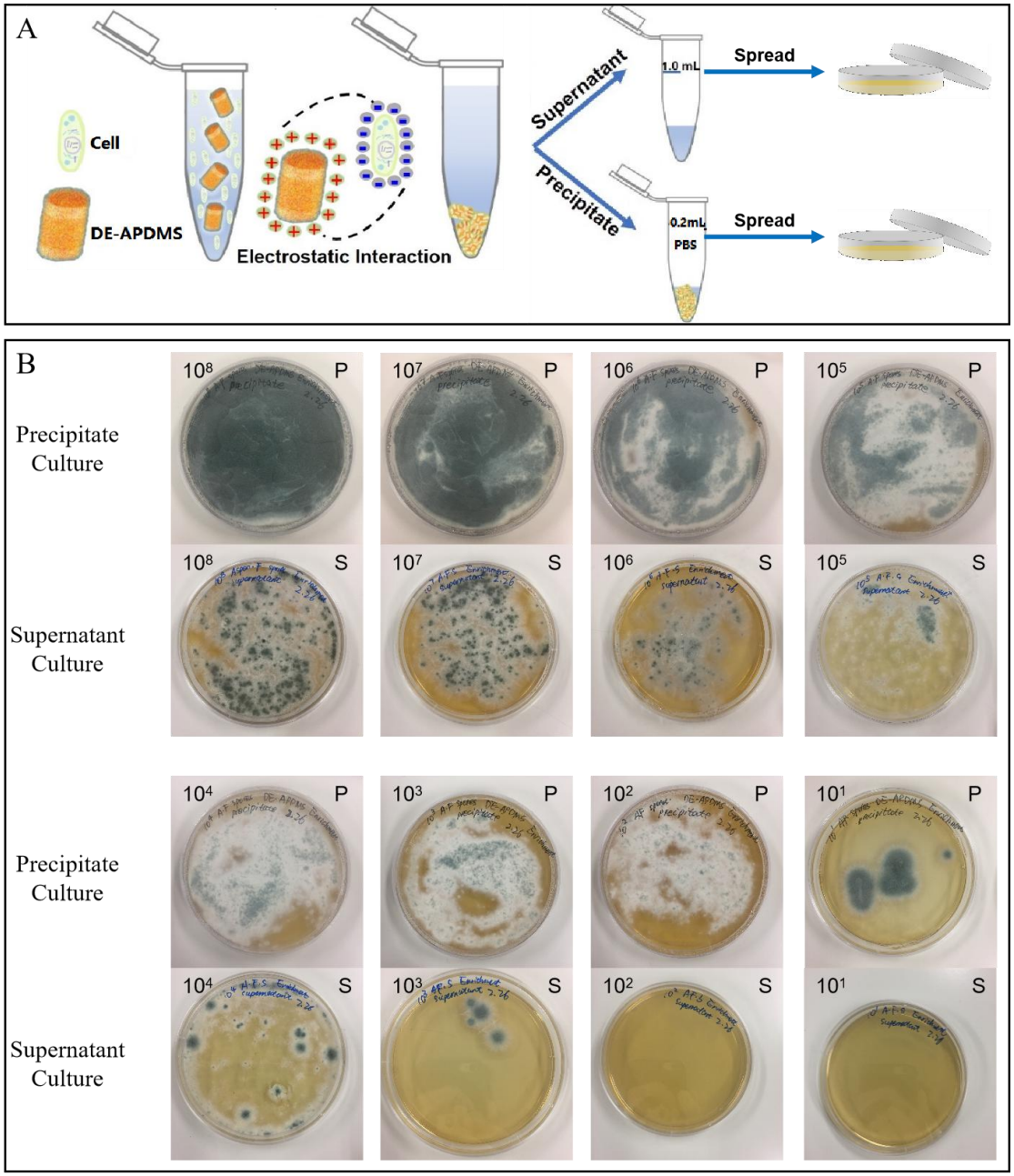


Figure 2. *Aspergillus fumigatus* spores enrichment schematic and demonstration.

(A) Schematic of the *Aspergillus fumigatus* spores enrichment. (B) Colony figure of fungal culture of the precipitate and supernatant.

3.3 DNA strand binding using DE-APDMS and DE-ZnO-APDMS

For the DNA binding test, two measurement methods were used in Figure 3A. Meanwhile, as showed in Figure 3B and 3C, both the UV-vis and zeta potential measurement method represent DNA strands can bind to the composite DE-APDMS and DE-ZnO-APDMS under the action of surface amino functionalization by APDMS. The spores enrichment and DNA binding ability of the DE-APDMS and DE-ZnO-APDMS in large volume samples within 30 min make the composite have the opportunity to be used on the diagnostic application.

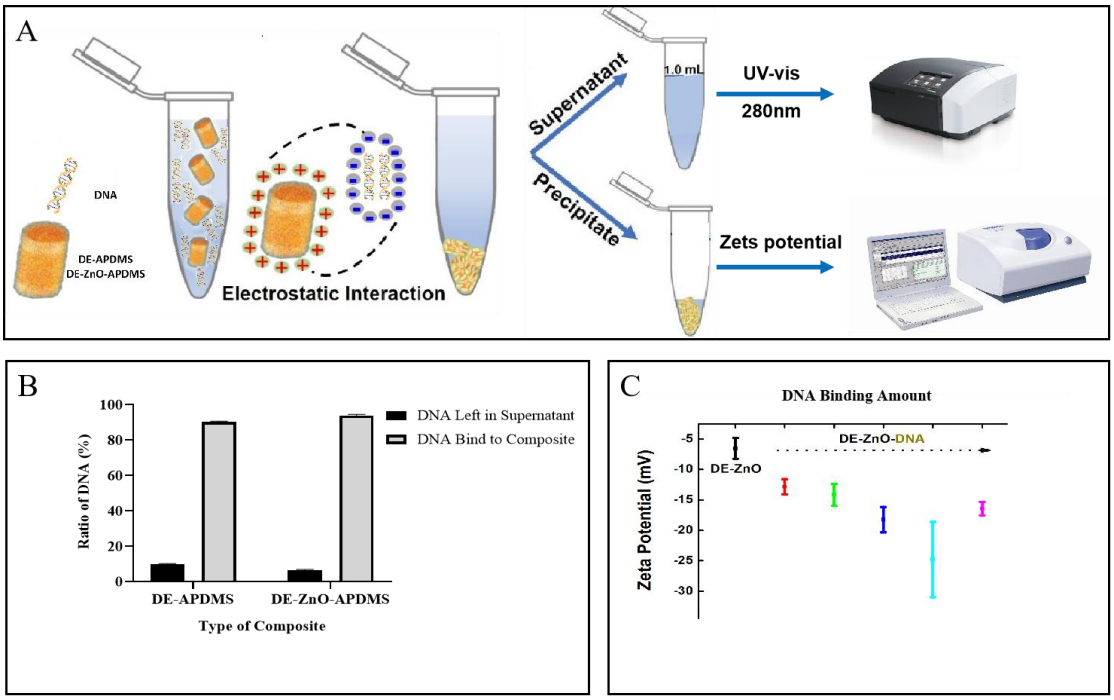


Figure 3. DNA binding schematic and demonstration (A) Schematic of the DNA bind to the composite. (B) Ratio of the UV-vis measurement value compare with the untreated DNA sample. (C) Zeta potential of the DE-APDMS after incubating with different amount of DNA.

3.4 ZnO nanoparticles as a novel *Aspergillus fumigatus* spores lysis substance

3.4.1. Design of the ZnO-based fungal DNA isolation assay

Herein, we used the in-house synthesized ZnO nanomaterials to develop a fungal DNA isolation assay. The synthesized ZnO nanomaterials took the place of the traditional fungal cell lysis buffer and chloroform method to perform the fungal cell lysis process. This is briefly shown in Figure 4. Compared with the process of the commercial kit assay, this ZnO-based fungal DNA isolation assay showed several advantages. First, the whole DNA extraction process was fast and took approximately 45 min; this helps researchers not only save almost half the time but also lessen the workload. In commercial kit assays, after adding the digestion buffer, the mixture needed to be incubated at 37°C for 40–60 min, followed by the addition of more lysis buffer and chloroform. Second, in the ZnO-based fungal DNA isolation assay, the use of ZnO instead of a common cell lysis buffer allows for fungal lysis within few minutes at room temperature without the need for extra thermal instrument. Third, it can also bypass the use of organic pollutants, such as chloroform. Generally, in most existing commercial kits, the input fungal cells are small volume of spores or the mycelia cell pellets after spinning down, one of the reasons is the input sample solution can dilute the digestion buffer and lysis buffer; this approach has a limitation in that a sample with few cells cannot get into a pellet. In comparison, the ZnO-based fungal DNA isolation assay is not limited by the input volume since the amount of ZnO can be increased with the sample volume.

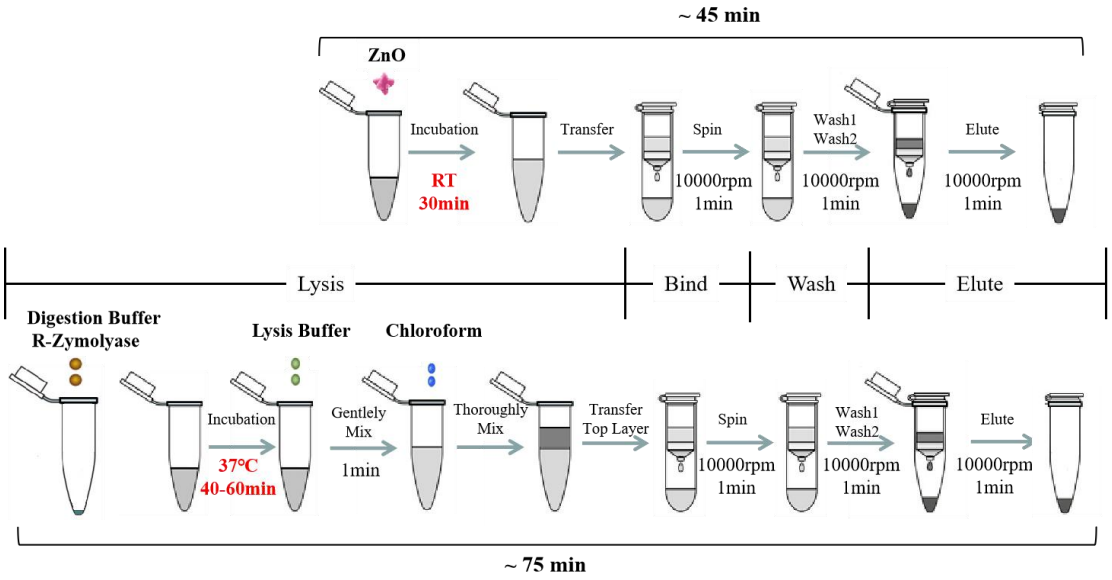


Figure 4. Diagram of the optimized ZnO-based fungal DNA isolation assay. A schematic illustrating nucleic acid (NA) isolation using the lysis buffer replacement method based on ZnO (the ZnO-based fungal DNA isolation assay) instead of using the traditional lysis buffer in the commercial fungal DNA extraction kit. The ZnO-based fungal DNA isolation assay (top) and commercial kit assay (bottom) both have four steps involving different lysis buffers but the same filter column, wash buffer, and elution buffer. For lysis process, in the ZnO-based fungal DNA isolation assay, 100 µL *A. fumigatus* spores was mixed with 100 µg ZnO for 30 min at room temperature. Whereas in the commercial kit assay, 10 µL *A. fumigatus* spores was mixed with 120 µL of YD Digestion Buffer and 5 µL of R-ZymolyaseTM (RNase A + ZymolyaseTM) for 40–60 min at 37°C, then YD lysis buffer and chloroform were added into the mixture. In the binding, washing, and elution steps, the buffer and columns used were the same in both methods. Subsequently, the NA extracted by both methods could be detected in downstream analysis.

3.4.2. ZnO nanoparticles effectively works as lysis buffer

The synthesized ZnO nanoparticles (ZnO-S-300) which we compounded by the special formula showed unique morphology (Figure 5A). SEM images were used to confirm that ZnO nanoparticles affected the fungal spores (Figure 5B) during the incubation process. Figure 5C shows that fungal spore cells were in physical contact with ZnO nanoparticles. The membrane surface of untreated fungal spore cells was plump. After incubating with ZnO nanoparticles, these cells appeared crumpled. As PCR cycle threshold (Ct) shown in Figure 5D, for the extraction by ZnO-based fungal DNA isolation assay with the room temperature and 60min longtime incubation,, the cycle threshold (Ct) is 25.01 cycles, meanwhile, the cycle threshold (Ct) of the extraction by commercial kit assay is 22.92 cycles. Here,the kit method performed better than our method because of the amount of ZnO was not enough for the spores density at 10^8. The DNA product extracted by the commercially available kit and that extracted using the ZnO-based fungal DNA isolation assay had the same appointed melt temperature peak at 81.5°C (Figure 5E).

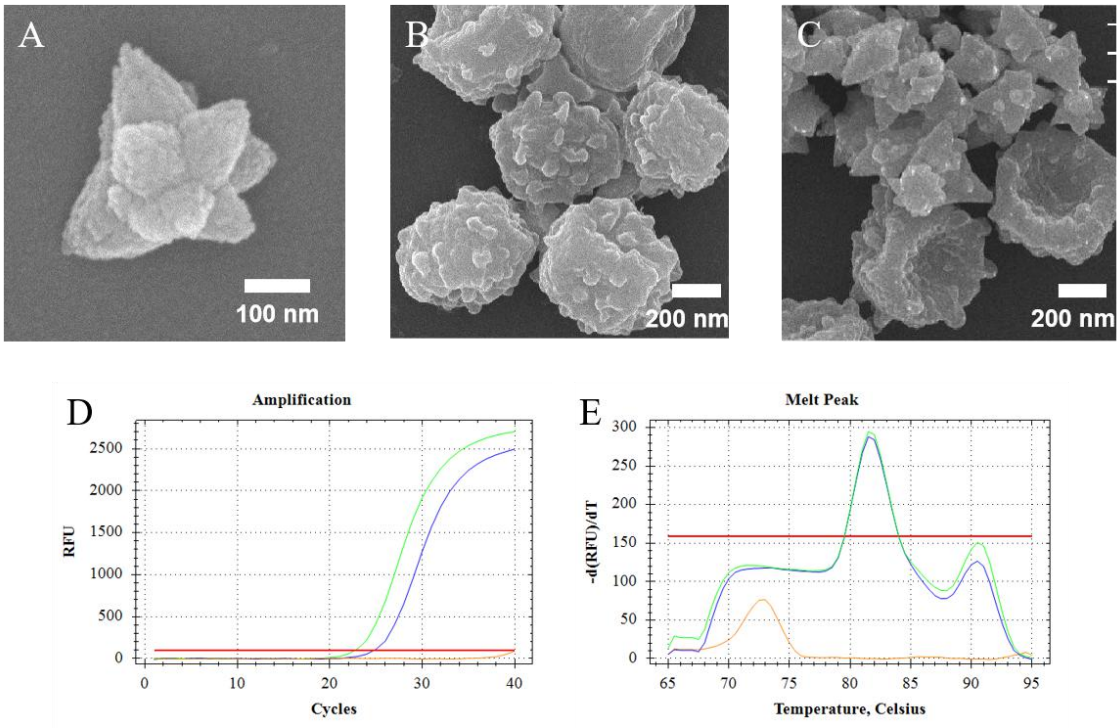


Figure 5. Study on the application of ZnO for the lysis of fungal spore cells. (A) SEM images of synthesized nanoparticles ZnO-S-300; (B) SEM images of untreated *Aspergillus fumigatus* spore cells; (C) SEM image of the lysis process of ZnO-S-300 on *A. fumigatus* spore cells. (D,E) Fluorescence signals and melt data from real-time PCR analyses of amplified DNA extracted by the ZnO-based fungal DNA isolation assay (Blue line) and kit assay (Green line). For the ZnO-based fungal DNA isolation assay, the cells were incubated for 60 min at room temperature. The extracted DNA of *A. fumigatus* spore cells (10^8 cells) was eluted in 100-μL elution buffer. Orange line was the negative control which is pure elution buffer. RFU is Relative Fluorescence Units.

3.4.3. Optimization of synthesized ZnO nanoparticles work conditions in the ZnO-based fungal DNA isolation assay

In foregoing tests, 100 μg (1 mg/mL, 100 μL) of synthesized ZnO nanoparticles was incubated with 10^8 *A. fumigatus* spores in 100 μL of PBS for 60 min at room temperature. It was shown that this ZnO-based fungal DNA isolation assay could successfully extract the fungal DNA. Herein, we evaluated the DNA extraction efficiency of the ZnO-based fungal DNA isolation assay with different incubation temperatures and times using the quantitative PCR cycle threshold (Ct) as an indicator of DNA quality. Furthermore, we introduced two other commercial ZnO variants whose average sizes were ~100 nm (ZnO-C-100, Figure 6A) and ~5000 nm (ZnO-C-5000, Figure 6C). In the kit assay, 37°C was the temperature used for activating and protecting R-Zymolyase. In the ZnO-based fungal DNA isolation assay, we explored the influence of temperature on the extraction process. Results as shown in the Figure 6D revealed that ZnO-S-300 used at 55°C and room temperature can both help procure the maximum quantity of DNA. The benefits of working at room temperature are that the heater step is eliminated and the word does not require large instruments. On comparing the three ZnO types, our synthesized ZnO-S-300 was the most effective in fungal DNA extraction. Furthermore, as effectiveness is another important index in a new operational approach, we checked the outcomes of the extraction by the three kinds of ZnO in at different incubation time periods. As shown in Figure 6D, ZnO-C-5000 had few variations in extraction from 15 min to 60 min

incubation periods, whereas ZnO-C-100 had good performance as lysis buffer at 30 min and 60 min incubation periods; remarkably, our synthesized ZnO-S-300 showed superior performance to the other two kinds ZnO at 30 min and 60 min incubation periods. In addition, for ZnO-S-300, the average Ct values at 30 min (27.07 cycles) and 60 min (26.35 cycles) incubation times have slightly difference. The shortened incubation time improved work efficiency and reduced the burden of work. With regard to the performance of ZnO-S-300 being superior to that of ZnO-C-100 and ZnO-C-5000, one of the most crucial known reasons was that the synthesized ZnO-S-300 showed a special positive charge unlike the common ZnO, which has a negative charge; this property of ZnO-S-300 provided it better affinity to negatively charged fungal spores and allowed for satisfactory lysis work.

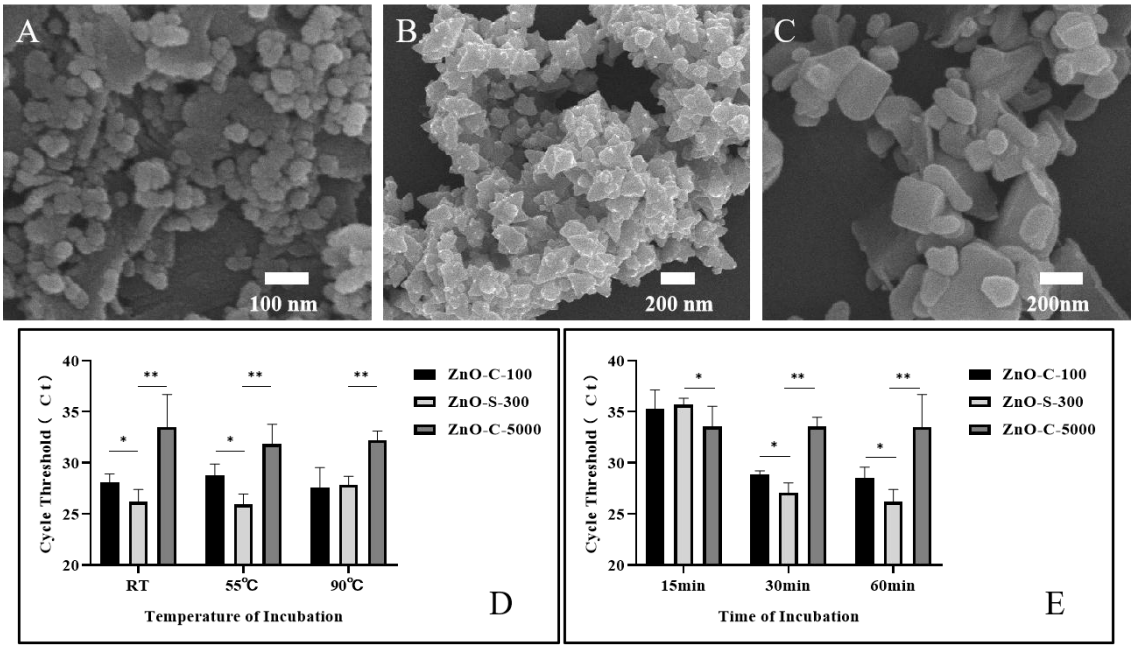


Figure 6. Optimization of the work conditions for ZnO nanoparticles synthesis in the ZnO extraction method. (A) SEM images of commercial nanoparticles ZnO-C-100,(B) SEM images of synthesized nanoparticles ZnO-S-300, (A) SEM images of commercial nanoparticles ZnO-C-5000, (D, E) Performance evaluation of the ZnO lysis buffer DNA extraction method using the synthesized ZnO (~300 nm, ZnO-S-300) nanomaterial at three different incubation temperatures (D) and times (E) by comparing the cycle threshold (Ct) of real-time PCR against two other commercially available ZnO types (~100 nm,~5000 nm). The extracted DNA of *A. fumigatus* spore cells (10^8 cells) was eluted in 100-µL elution buffer. Error bars indicate standard deviation from the mean based on at least three independent experiments. * $p < 0.05$, ** $p < 0.01$.

3.4.4. Performance of ZnO extraction method

The efficiency of both the ZnO-based fungal DNA isolation assay and commercial kit assays in different numbers of *A. fumigatus* spores is presented in Figure 7. In the CFX Maestro™ Software, the threshold of the quantitative PCR results was manually selected at 100 RFU (Relative Fluorescence Units). Both methods can successfully extract DNA from PBS suspensions of *A. fumigatus* spores at a minimum of 10 cells. At higher densities of 10^8 and 10^7 spores, the commercial kit assay showed better performance than the ZnO-based fungal DNA isolation assay, whereas the latter persistently showed more sensitive and stronger signals than the former at densities of 10^6 to 10. The main cause of the ZnO-based fungal DNA isolation assay showing relatively inferior performance with densities of 10^8 and 10^7 is the deficient quantity of ZnO nanoparticles, whereas from the density of 10^6, the proportion of ZnO and spores becomes optimal, and this ZnO-based fungal DNA isolation assay shows its advantages. High sensitivity of the ZnO-based fungal DNA isolation assay in low densities makes it a good candidate for use with early invasive aspergillosis clinical samples which have few pathogens in samples.

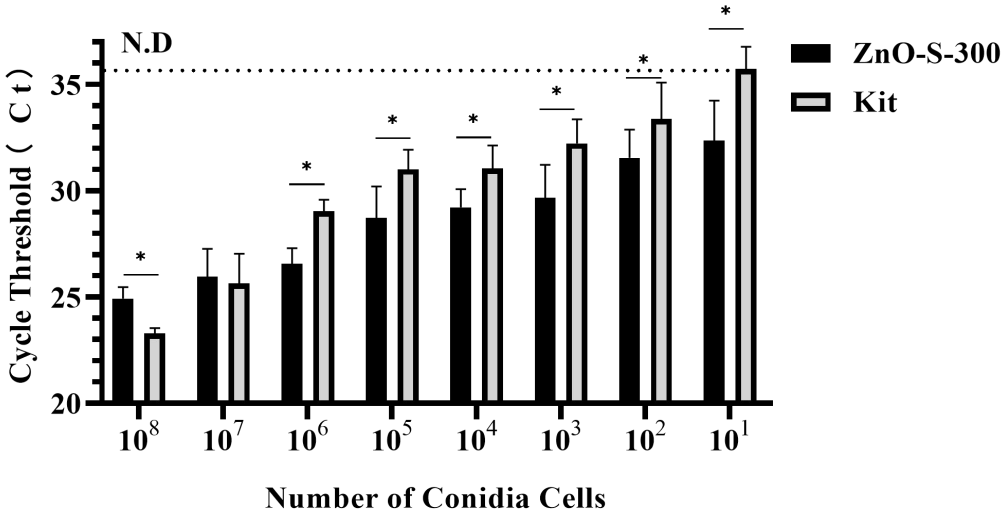
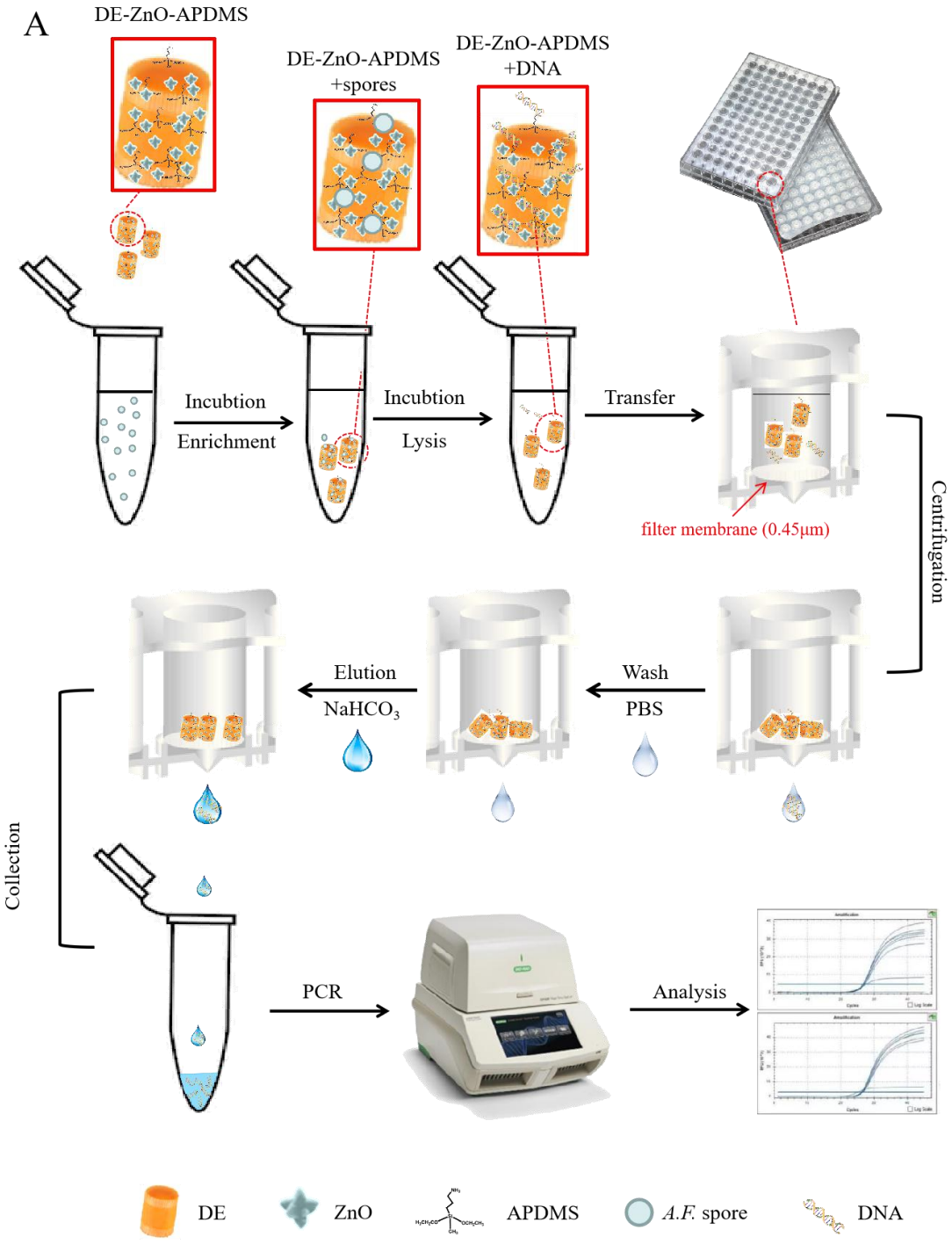


Figure 7. Performance evaluation of the DNA extraction assay using either the ZnO-based fungal DNA isolation assay or the commercial kit assay with *A. fumigatus* spore concentrations ranging from 10 to 10^8 cells/100 µL by comparing the cycle threshold (Ct) of real-time PCR. Error bars indicate standard deviation from the mean, based on at least three independent experiments. Error bars indicate standard deviation from the mean, based on at least three independent experiments. N.D display the negative diagnosis. * $p < 0.05$

3.5 Aspergillus fumigatus DNA isolation assay based on DE-ZnO-APDMS

Because of the *Aspergillus fumigatus* spores enrichment and DNA binding ability of the DE-APDMS, as well as the spores lysis effectiveness of ZnO nanoparticles, we combined the two materials together to triphase micropartcles DE-ZnO-APDMS in order to develop a sample and rapid all-in-one *Aspergillus fumigatus* DNA isolation assay (Figure 8A). *Aspergillus fumigatus* spores in 1 ml of PBS buffer were first enriched by DE-ZnO-APDMS, the ZnO preformed the lysis process when the spores reached the composite, then the amine groups surrounding the composite DE-ZnO-APDMS captured the DNA release from the broken *Aspergillus fumigatus* spores. After washing, the elution was collected and analyzed by PCR. As the quantitative PCR results showed in Figure 8B, comparing with the commercial kit group, the amount of *Aspergillus fumigatus* DNA isolated by the all-in-one assay based on triphase micropartcles DE-ZnO-APDMS distinctly preceded the commercial kit assay from the high density to low density of spores in the samples. And the commercial kit could not detect the *Aspergillus fumigatus* spores in the 10 cell/ml,while the *Aspergillus fumigatus* DNA isolation assay based on DE-ZnO-APDMS still kept the sensitivity. This all-in-one *Aspergillus fumigatus* DNA isolation assay avoided the DNA loss between the too many procedures in commercial kit assay.


A
DE-ZnO-APDMS
DE-ZnO-APDMS
+spores
DE-ZnO-APDMS
+DNA
Incubtion
Enrichment
Incubtion
Lysis
Transfer
filter membrane (0.45μm)
Centrifugation
Wash
PBS
Elution
NaHCO3
Collection
PCR
Analysis
DE
ZnO
APDMS
A.F. spore
DNA

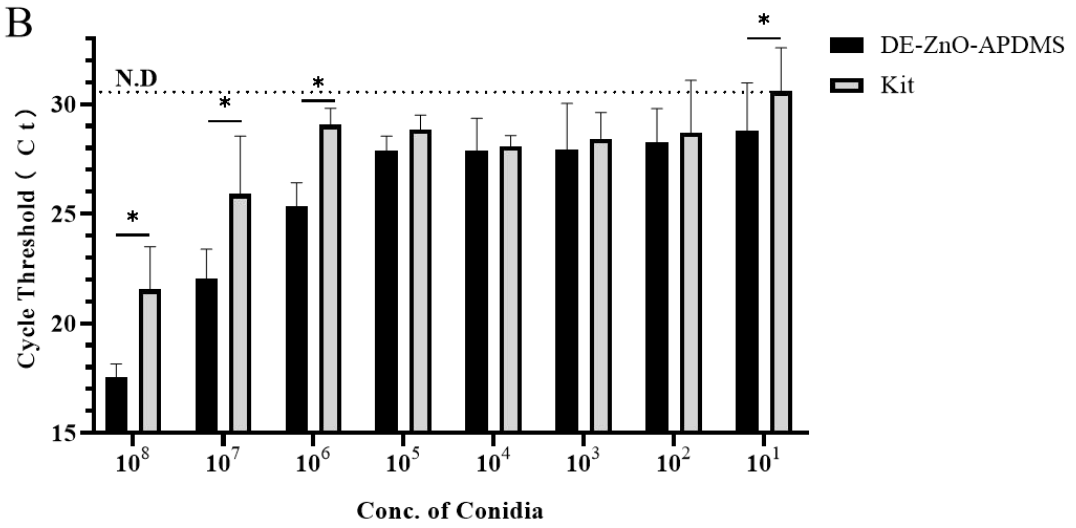


Figure 8. ***Aspergillus fumigatus*** **DNA isolation assay based on DE-ZnO-APDMS.**

(A) A schematic illustrating DNA isolation via assay based on DE-ZnO-APDMS. (B) Performance evaluation of the the DNA extraction assay using either the DE-ZnO-APDMS fungal DNA isolation assay or the commercial kit assay with *A. fumigatus* spore concentrations ranging from 10 to 10^8 cells/100 μL by comparing the cycle threshold (Ct) of real-time PCR. Error bars indicate standard deviation from the mean, based on at least three independent experiments. Error bars indicate standard deviation from the mean, based on at least three independent experiments. N.D display the negative diagnosis. * $p < 0.05$

4. DISCUSIONS

An efficient disease diagnosis method is conducive to the timely diagnosis and treatment of diseases. Instead of the traditional disease diagnosis method, the molecular diagnostic technique based on the developing PCR technique provides new diagnosis method to the disease. However, for the samples such as the fungal cells which are not easily to extract the DNA, this PCR technique faces the challenges. So, optimize the DNA extraction efficiency is the most important task.

ZnO nanoparticles are listed as a safe substance by the United States Food and Drug Administration (21CFR182.8991). Because of their semi-conductive speciality, anticancer, antibacterial, antifungal and antiinflammatory activities, ZnO nanoparticles have been applied to biomedical fields. In the further study, ZnO nanoparticles exhibit the function of cell lysis. There are several hypotheses around the mechanism of the pathway by which ZnO achieved cell split. Regarding physical aspects, the specific shape and distinct positive zeta potential made it impossible for ZnO nanoparticles to get into the cells. Regarding chemical aspects, the excessive Zn^{2+} released from the ZnO got firmly adsorbed on the cell membrane surface by Coulomb's law after reaching the membrane surface, and then, Zn^{2+} further penetrated the cell wall, causing cell wall rupture and the consequent cytoplasmic outflow. Regarding biological aspects, the high-concentration ZnO treatment triggered an ROS reaction in the tube environment, thus leading to apoptotic cell death and the consequent cellular structure collapse(Figure 9A).

Diatomaceous earth (DE) a biocompatible frustule powder containing about 80%-90% silica. In an organic solvent, the ethoxy group of the APDMS molecule directly reacts with Si-OH on the surface of DE to form a Si-O-Si bond (Figure 9B). The APDMS modified DE has the ability to adsorb a variety of inorganic ions, metal ions or inorganic, organic, biological molecules.

Considered the superior characteristics of the ZnO, DE and APDMS, we firstly synthesized the triphase micropartcles DE-ZnO-APDMS. The fungal enrichment and DNA extraction can preform all in one by this composite.

The diagnostic method combined the fungal DNA isolation assay based on DE-ZnO-APDMS and the PCR technique is fast, accurate, cheap, high-throughput, portable, simple to read the results, and easy to operate. It is suitable for the status of clinical diagnosis and treatment, and has great significance for guiding the formulation of correct clinical medication programs.

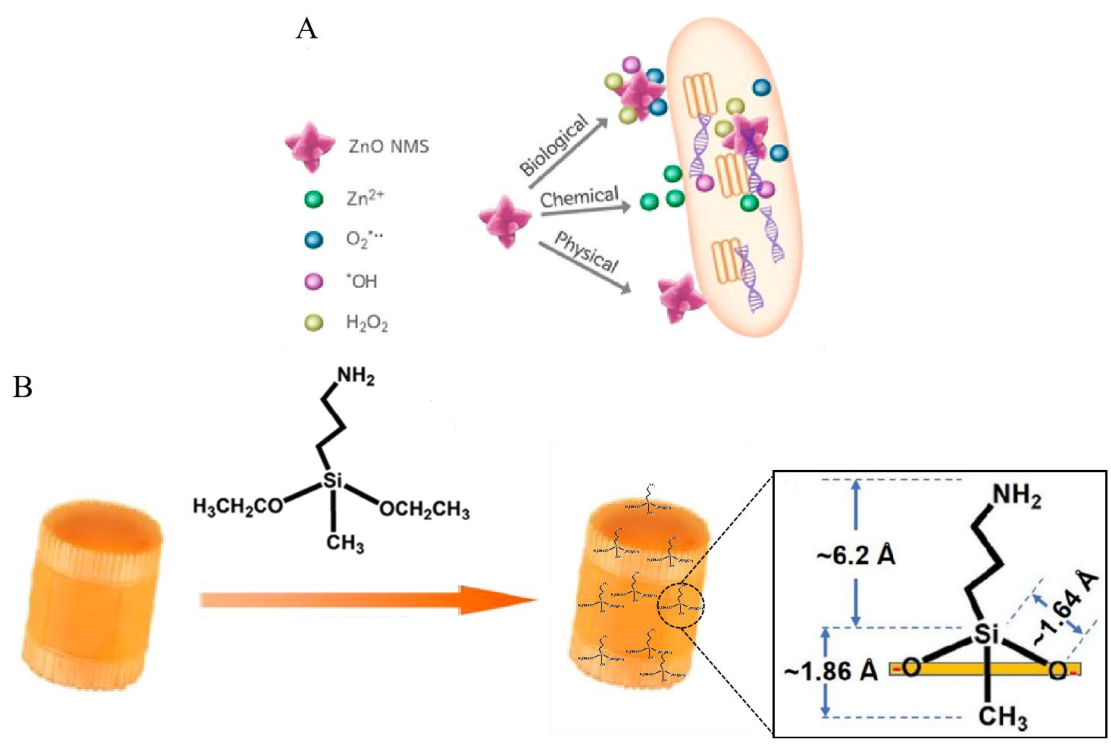


Figure 9. (A) Schematic of ZnO nanopaticles for breaking of cell membrane based on biological, chemical, and physical properties of ZnO. (B) Schematic representation of the APDMS modification of the amino-functionalized diatomaceous earth (DE-APDMS).

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6. ABSTRACT IN KOREAN

국문 초록

배경 및 목적 : 침습성 아스페르길루스증(IA)의 감염 범위가 확대되고 발생률이 높아짐에 따라 진균의 조기 진단이 어려운 과제가 되었습니다. 최근에 핵산 기반 진단 기술이 임상 응용에 널리 사용됨에 따라 진균의 DNA 추출 효율성 증대가 의료 진단 응용기술을 개발하는 데 특히 중요한 요인 중 하나가 되었습니다. 이 연구에서 우리는 먼저 진균의 DNA 추출을 위해 나노 기술을 융합하여 아스페르길루스 푸미가투스에서 DNA 를 쉽게 추출할 수 있고, 빠르고 경제적이며 안정적인 시스템을 개발했습니다. 본 방법은 포자 샘플을 농축하기 위해 규조토 (DE)를 이용하여 포자 함량이 낮은 샘플에서도 포자를 획득하여 농축 할 수 있도록 하였습니다. 한편, 급성장하는 나노 물질인 ZnO 는 포자 막을 파괴하는 작용을 하여 DNA 추출이 가능하게 하였습니다. 아스페르길루스 푸미가투스로부터 DNA 를 추출하기 위해 새로운 3 차원 구조의 미세 입자 DE-ZnO-APDMS 를 합성 하였습니다. 추출 된 DNA 의 양과 질은 중합 효소 연쇄 반응 (PCR) 증폭법을 이용하였을때도 충분하여 진단에 사용이 가능하였습니다.

따라서 이 연구는 3 차원 구조의 미세 입자 DE-ZnO-APDMS 가 다양한 바이오 응용 분야에서, 특히 진단 분야에서 진균을 농축하고 핵산을 추출하는데 적합한 물질로서 응용 가능성이 큽니다.

재료 및 방법 : 단일 ZnO 나노 입자 및 3 차원 구조의 미세 입자 DE-ZnO-APDMS 를 알칼리 매질에서 열 수법을 사용하여 합성 하였습니다. DE, ZnO 및 DE-ZnO-APDMS 의 구조 및 형태는 필드 방사 스캐닝 전자 현미경 (FE-SEM) JSM-7500F 기기 (JEOL)에서 확인하였고 DE 에 부착된 ZnO 을 확인 하였습니다 . 가장 일반적인 아스페르길루스 푸미가투스가 침습적 아스페르길루스증을 유발하는 균종이므로 본 연구의 모든 실험에 사용되었습니다. DE-APDMS 가 아스페르길루스 푸미가투스 포자를 농축 할 수 있음을 확인하기 위해 전통적인 진균 배양법을 사용했습니다. 한편, DE-APDMS 및 DE-ZnO-APDMS 의 DNA 결합 성능은 UV-Vis 분광법을 이용한 흡착 평형 실험으로 테스트하였습니다. ZnO 및 DE-ZnO-APDMS 로 추출한 아스페르길루스 푸미가투스 DNA 를 상용 키트로 추출한 DNA 과 비교했습니다. 추출 된 DNA 의 양과 질은 중합 효소 연쇄 반응 (PCR) 증폭법을 이용하였을 때도 충분하여 진단에 사용이 가능하였습니다.

결과 : 처음에 합성 된 3 차원 구조의 미세 입자 DE-ZnO-APDMS 는 다 각성 별형 나노 입자 ZnO (~ 300nm)가 많은 나노 구조의 공극을 가진 미세한 중공 실리카 구조벽에서 형성되는 것을 보여 주었습니다. DE-APDMS 가 제한된 양의 아스페르길루스 푸미가투스 포자를 풍부하게 농축 할 수 있게 하였습니다. DE-APDMS 및 DE-ZnO-APDMS 는 포자 내부의 DNA 가 복합 표면 개질제 APDMS 에 의해 상호결합되어 있기 때문에 DNA 결합 능력을 가졌습니다. 합성 된 나노 입자 ZnO (~ 300nm)는 진균 포자를 성공적으로 용해합니다. 독특한 3 차원 구조의 미세 입자 DE-ZnO-APDMS 는 각 구성물의 장점을 결합하여 올인원 분석으로 진균의 DNA 를 추출 할 수 있습니다.

상용 키트 분석과 비교하면 3 차원 구조의 미세 입자 DE-ZnO-APDMS 을 이용한 진균 DNA 추출 분석이 제한된 포자수 및 양이 많은 시료에서보다 더 민감 하게 검출 하였습니다.

결론 : 이 연구에서는 침습성 아스페르길루스증 진단을 위하여 합성 3 차원 구조의 미세입자 DE-ZnO-APDMS 를 기반으로하는 간단하고 빠른 진균 DNA 추출 분석법이 제시되었습니다. DE-ZnO-APDMS 기반 진균 DNA 분리 분석과 상용 키트를 사용하여 분리 된 증폭 DNA 의 정량적 PCR 분석을 통해 본 결과 독특하게 합성 된 3 차원 구조의 미세 입자 DE-ZnO-APDMS 가 낮은 밀도의 샘플에서 진균 DNA 추출에 더 효과적이라는 것을 보여줍니다. 이 연구는 인체 침습성 아스페르길루스증 진단 기술에 대한 새로운 시료 전처리 기법으로 소개하였습니다.

키워드 : 침습성 아스페르길루스증; 규조토; 산화 아연; DNA 추출; 분자 진단.