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Copper(II)-MOF Containing Glutarate and 4,4'-Azopyridine and Its Antifungal Activity

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Abstract: Antifungal activities of MOFs (metal organic frameworks) have been demonstrated in studies, and improvement in efficiency of fungal inactivation is a critical issue in the application of MOFs. In this study, we employed 4,4'-azopyridine (AZPY) in the construction of MOF to improve its antifungal activity. Three-dimensional (3D) copper metal organic framework containing glutarate (Glu) and AZPY (**Cu(AZPY)-MOF**) was synthesized by a solvothermal reaction. Glutarates bridge Cu₂ dinuclear units to form two-dimensional (2D) layers, and these layers are connected by AZPY to form a 3D framework. When spores of two fungi, *Candida albicans* and *Aspergillus niger*, were treated with **Cu(AZPY)-MOF** for one day, number of CFU (colony forming unit) was continuously reduced over treated MOF concentrations, and maximum 2.3 and 2.5 log₁₀CFU reductions (approximately 99% reduction) were observed in *C. albicans* and *A. niger*, respectively. Small amounts of Cu^{II} ions and AZPY released from **Cu(AZPY)-MOF** were not critical for fungal inactivation. Our results indicate that the level of antifungal activity of **Cu(AZPY)-MOF** is greater than that of Cu-MOF without AZPY constructed in our previous study, and intercalation of AZPY is able to improve the antifungal activity of **Cu(AZPY)-MOF**.

Keywords: 4,4'-azopyridine; glutaric acid; metal-organic frameworks (MOFs); X-ray crystallography; antifungal activity

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1. Introduction

The preparation, characterization and application of metal organic frameworks (MOFs) have attracted considerable attention in the past two decades [1–6]. The well-known applications of MOFs are gas sorption and separation [7–15], catalysis [16–19], luminescence sensing [20–24] and biomedical usage [25,26]. MOFs can be used as bioactive framework materials (BioMOFs) for efficient antimicrobial agents [25–31]. For example, the water-stable Cu-BTC MOF (BTC = 1,3,5-benzenetricarboxylate) inhibits the rate of growth of *C. albicans* [32], and the other Cu-based HKUST-1 MOF inactivates *Saccharomyces cerevisiae* due to Cu^{II} ions released from its structure [33]. A Co-based MOF (Co-TDM, TDM⁸⁻ = [(3,5-dicarboxyphenyl)-oxamethyl]methane) is highly effective at inactivating *E. coli* [34]. A nontoxic, biocompatible and thermally stable MIL-53(Fe) and its silver(I) nitrate nanocomposite (Ag@MIL-53(Fe)) have been shown to have remarkable antifungal activity against *Aspergillus flavus* [35]. A cerium-based MOF has shown excellent enzymatic activity (peroxidase-like activity) towards fungal cells (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Candida albicans* and *Rhodotorula glutinis*) [36]. In addition, MOFs can be used as a drug delivery system to solve problems such as the resistance of fungi to drugs and the toxicity of a drug to normal human cells. ZIF-8 (ZIF = zeolitic imidazolate framework) has been used as an effective, low-toxicity and pH-responsive antifungal drug

delivery system with fewer side effects [37]. Fe-MIL-100 was used as a carrier for the fungicide azoxystrobin, and azoxystrobin-loaded Fe-MOFs (AZOX@Fe-MIL-100) exhibited good fungicidal activities against two pathogenic fungi (*Fusarium graminearum* and *Phytophthora infestans*) [38].

Recently, the antifungal activities of Cu-MOFs and Co-MOFs have been analyzed [39,40]. A flexible dicarboxylate ligand and glutarate were used to bridge metal ions to form two-dimensional (2D) sheets, and bipyridyl ligands (bpy = 4,4'-bipyridine, bpa = 1,2-bis(4-pyridyl)ethane, bpe = 1,2-bis(4-pyridyl)ethylene, and bpp = 1,3-bis(4-pyridyl)propane) can connect these sheets to form three-dimensional (3D) frameworks, Cu-MOFs [41–46] and interpenetrated 3D and 2D coordination polymers, Co-CPs [39]. Co-CPs can inactivate *C. albicans* cells and *A. niger* spores with maximum 99% and 62% inactivation efficiency, respectively [39]. Cu-MOFs show approximately 50–80% inactivation of both fungi in our experimental conditions [40]. However, these antifungal activities of Cu-MOFs and Co-CPs were acquired after a 4-day treatment. The development of MOFs for upgraded antifungal activity will be continuously explored.

To develop MOFs with improved antifungal activity, we synthesized a modified version of Cu-MOFs that were used in our previous study [40]. 4,4'-azopyridine (AZPY) is employed for the developed 3D **Cu(AZPY)-MOF** while the bpe ligand has been used for connecting 2D sheets in the previous Cu-MOF, [Cu₂(Glu)₂(bpe)] (Scheme 1). The structure of **Cu(AZPY)-MOF** is confirmed by X-ray crystallography, PXRD (powder X-ray diffraction), elemental analysis and TGA (thermogravimetric analysis) technique. The antifungal activity of **Cu(AZPY)-MOF** were examined against *C. albicans* and *A. niger* in this study.

Scheme 1. Chemical structure of glutaric acid, AZPY, and bpe ligands.

2. Materials and Methods

2.1. Instrumentation

TGA (TG 209 F3 Tarsus Instrument, NETZSCH, Germany), Elemental analyzer (Flash EA1112, Thermo Scientific, Waltham, MA, USA), PXRD (Rigaku Miniflex diffractometer, Tokyo, Japan), Scanning electron microscope (FE-SEM, JEOL JSM-6700F, Tokyo, Japan), Inductive coupled plasma mass spectrometry (ICP-MS, Elan DRC II, Perkinelmer, Waltham, MA, USA), UV-Vis spectrophotometer (LAMBDA365, Perkinelmer, Waltham, MA, USA) and single crystal XRD (Bruker APEX-II Diffractometer, Bruker, Ettlingen, Germany) were used for characterization.

2.2. Preparation of [Cu₂(Glu)₂(AZPY)]·3H₂O (Cu(AZPY)-MOF)

A mixture of Cu(NO₃)₂·3H₂O (60 mg, 0.25 mmol, 99%, Sigma-Aldrich Korea, Seoul, Korea), glutaric acid (0.65 mg, 0.5 mmol, 99%, Sigma-Aldrich Korea, Seoul, Korea), and 4,4'-azopyridine (AZPY, 46 mg, 0.25 mmol, 99%, Sigma-Aldrich Korea, Seoul, Korea) in DMF/water (1:1 volume ratio, total 10 mL) was placed in a Teflon-lined high-pressure vessel. The vessel was placed in an oven at 100 °C for 24 h. After cooling to room temperature, turquoise crystalline needles were retrieved by filtration, washed with distilled water three times, and dried in air overnight. The yield was 64.5 mg (45.2%). Elemental analysis involved calculations for the dried Cu₂C₂₀H₂₀O₈N₄ (571.49): C, 42.03; H, 3.53; N, 9.80% and found: C, 41.78; H, 3.81; N, 10.39%. The morphology was examined under a scanning electron microscope (FE-SEM, JEOL JSM-6700F, Tokyo, Japan) with the sample on the carbon tape coated by Pt.

2.3. X-ray Crystallography

X-ray diffraction measurements were performed using a Bruker APEX-II Diffractometer equipped with a monochromator and a Mo K α ($\lambda = 0.71073$ Å) incident beam at the National Research Facilities and Equipment Center (NanoBio-Energy Materials Center) at Ewha Womans University. A crystal was mounted on a glass fibre. The CCD data were integrated and scaled using the Bruker-SAINT software-2019.11 (Bruker, Karlsruhe, Germany) package, and the structure was solved and refined using SHELXTL-2018/3 (George Sheldrick, Georg-August Universität Göttingen, Göttingen, Germany). All hydrogen atoms were placed at the calculated positions. The crystallographic data are listed in Table S1. The bond lengths and angles are listed in Table S2. Structural information was deposited at the Cambridge Crystallographic Data Centre. The CCDC reference number is 2057402 for **Cu(AZPY)-MOF**.

2.4. Stability and Metal Ion Release Test

To test stability of **Cu(AZPY)-MOF**, thermogravimetric analysis (TGA) was performed on a TG 209 F3 Tarsus Instrument under a nitrogen atmosphere. 11.76 mg of the sample was used. The gas flow rate was 20 mL/min and the heating/cooling rate was $-2^\circ/\text{min}$. To confirm the stability of **Cu(AZPY)-MOF**, MOF degradation tests were performed after 1 day in H₂O and PBS by PXRD. PXRD patterns were recorded on a Rigaku Miniflex diffractometer. The X-ray source is Cu K α source, the speed rate was $2^\circ/\text{min}$, the step size was 0.02° and 2θ range was $5^\circ \leq 2\theta \leq 50^\circ$.

H₂O and PBS solutions of MOF were prepared after 1 day and 4 days to measure the amounts of Cu^{II} ion released from **Cu(AZPY)-MOF** by ICP-MS. The solutions were in concentration of $1 \text{ mg} \cdot \text{mL}^{-1}$ in H₂O and PBS.

2.5. AZPY Ion Release Test

Four concentrations of AZPY in PBS were prepared (2.5×10^{-4} , 5×10^{-4} , 2.5×10^{-3} and 2.5×10^{-3} mM). The UV absorbance of those concentrations was measured between 200 nm to 800 nm in the 1 cm glass cuvette. The absorbance was measured on UV/Vis spectroscopy (LAMBDA365, Perkinelmer, Waltham, MA, USA). The molar absorptivity of AZPY was calculated by using Beer's law. The UV absorbance of 0.5 mg of **Cu(AZPY)-MOF** in PBS was measured after 1, 3, 5, 15, 18, 20 and 24 h. The amounts of AZPY released from MOF were calculated by using Beer's law. The absorbance wavelength is 447 nm.

2.6. Fungal Strains and Antifungal Test

Yeast-type (*C. albicans*) and filament-type (*A. niger*) fungi were used for antifungal tests. *C. albicans* (KCTC 7270) and *A. niger* (130708) were generously provided by the Korean Collection for Type Cultures in Korea Research Institute of Bioscience and Biotechnology (Jeongeup-si, Jeollabuk-do, Korea) and Dr. Seong-Hwan Kim's laboratory at Dankook University (Cheonan-si, Chungnam, Korea), respectively. Both fungal strains were maintained in potato dextrose agar (PDA) and propagated in potato dextrose broth (PDB) for experimental use. Culture temperature was 30°C .

For treatment with **Cu(AZPY)-MOF**, cells of *C. albicans* and *A. niger* spores were collected. One or two colonies of *C. albicans* from a PDA culture plate were suspended in 1 mL $1 \times$ PDB solution; then, 10 μL of the suspension was inoculated into new 15 mL PDB media in a flask. After incubation at 30°C with shaking for 20 h, the culture suspension was centrifuged at $3143 \times g$ for 5 min, and the liquid part was discarded. The cell pellet was washed twice with $1 \times$ phosphate buffered saline (PBS) and subsequently resuspended in new PBS. Cell number in 10 μL of suspension was counted under the microscope using hemacytometer (Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) and then, number in 1 mL was calculated. The final cell concentration was then adjusted to 10^8 cells/mL. For *A. niger* culture, several pieces of *A. niger* mycelia were inoculated onto PDA plates, and the plates were incubated at 25°C for 2 weeks. After 2 weeks, approximately 10 mL of $1 \times$ PBS was added to the incubated plates, and fungal mycelia,

including spores, were scraped using a scraper. The scraped suspension was filtered through 4 layers of miracloth, and the filtered suspension was centrifuged at $3143 \times g$ for 5 min. After the liquid part was discarded, the fungal spore pellet was washed once with $1 \times$ PBS and subsequently resuspended in new PBS. As performed in *C. albicans*, spore number per unit volume (mL) was counted and estimated under the microscope using hemacytometer and then the final spore concentration was adjusted to 10^8 spores/mL.

Suspensions of *C. albicans* cells and *A. niger* spores were placed in a 24-well plate (1 mL per well, 10^8 cells or spores per well). A suspension of **Cu(AZPY)-MOF** in PBS was added to each well at final concentrations of 0, 0.125, 0.25, 0.35, and 0.5 mg/mL, and the plate was incubated at room temperature with shaking. After incubation for 1 day, the treated spore suspensions were transferred into a 1.5-mL tube and serially diluted. One hundred microlitres (100 μ L) of the diluted suspension was spread onto a PDA plate, and the plates were incubated at 30 °C for *C. albicans* and room temperature for *A. niger* for 2 days. After incubation, the number of colony forming units (CFUs) was counted from 3 replicate plates, and experiments were repeated 3 times.

2.7. Measurement of the Levels of H_2O_2 and NO_x

The levels of H_2O_2 and NO_x ($NO + NO_2^- + NO_3^-$) were measured in PBS using an assay kit after treatment with **Cu(AZPY)-MOF**. PBS was placed in a 24-well plate (1 mL/well). Then, **Cu(AZPY)-MOFs** were added at final concentrations of 2, 1, 0.5, 0.25, 0.125, and 0 mg/mL. The plate was incubated at room temperature with shaking for 1 day. Then, PBS was transferred to a 1.5 mL tube. The levels of H_2O_2 and NO_x in PBS were measured using an Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR, USA) and QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA), respectively, following the manufacturer's protocols.

3. Results and Discussion

3.1. Structure Description

Cu(AZPY)-MOF has previously been reported on [47], but we newly synthesized it via a solvothermal reaction (see the Experimental section) to investigate its antifungal activities. The structure of **Cu(AZPY)-MOF** is similar to $[Cu_2(Glu)_2(bpe)]$ [40]. Glutarates bridge paddle-wheel Cu_2 dinuclear units to form 2D layers, and these layers are connected by AZPY to form a 3D framework (Figure S1) while bpe was used for connecting 2D sheets for 3D $[Cu_2(Glu)_2(bpe)]$. The morphology of **Cu(AZPY)-MOF** was investigated by scanning electron microscopy (SEM), as shown in Figure 1. The number of solvent water molecules was estimated from thermogravimetric analysis (TGA) (Figure S2). The weight loss of 8.26% was attributed to three solvent water molecules (calc. 8.63%), and **Cu(AZPY)-MOF** is formulated as $[Cu_2(C_5H_6O_4)_2(C_{10}H_8N_4)] \cdot 3(H_2O)$. **Cu(AZPY)-MOF** is stable up to 271°, and it is decomposed with a weight loss of 49.51%, which may attributed to glutarates (calc. 41.6%) [39]. The purity of the as-prepared **Cu(AZPY)-MOF** was confirmed by powder X-ray diffraction (PXRD), as shown in Figure 2. The intensities of diffraction planes of (2 0 0), (3 1 0), (2 2 0), (3 1 -1) and (6 0 1) at $2\theta = 7.22, 12.769, 15.392, 16.099$ and 24.776° , respectively, were maintained in those of samples after 1 day in H_2O and PBS as well as the as-prepared sample. This result indicates that **Cu(AZPY)-MOF** is stable in water and PBS solution.

In addition, MOF degradation tests were performed in H_2O and PBS to confirm the stability of **Cu(AZPY)-MOF**. The amount of Cu^{II} ions released from **Cu(AZPY)-MOF** was measured by inductively coupled plasma mass spectrometry (ICP-MS) after 1 day and 4 days (Figure S3). The amount of Cu^{II} ions released from **Cu(AZPY)-MOF** after 1 day was 2.34×10^{-4} mg/mL, which is negligible compared to the 99% inactivating property of 0.5 mg/mL.

Figure 1. SEM image of Cu(AZPY)-MOF.

Figure 2. PXRD patterns of Cu(AZPY)-MOF. The simulated pattern from the X-ray data (a), the pattern for the as-prepared sample (b), the pattern for the sample after 1 day in H₂O (c) and the pattern for the sample after 1 day in PBS (d).

3.2. Antifungal Activity

The antifungal activity of Cu(AZPY)-MOF was examined on yeast-type (*C. albicans*) and filament-type (*A. niger*) fungi. Cell and spore viability was significantly reduced in both fungi after the 1-day treatment with Cu(AZPY)-MOF (Figures 3 and 4). The decrease in CFU number increases with increasing concentrations of Cu(AZPY)-MOF in both fungi (Figures 3 and 4). In *C. albicans*, an approximately 75% reduction in CFU number (0.6 log reduction) was observed after treatment with 0.125 mg/mL Cu(AZPY)-MOF, and a 98–99.6% reduction (1.8–2.5 log reduction) was observed after treatments with 0.25, 0.35 and 0.5 mg/mL Cu(AZPY)-MOF (Figure 3). In *A. niger*, an approximately 60% reduction in CFU number (0.2 log reduction) was observed after the treatment with 0.125 mg/mL Cu(AZPY)-MOF, and a 97–99.6% reduction (1.5–2.5 log reduction) was observed after

treatments with 0.25, 0.35, and 0.5 mg/mL **Cu(AZPY)-MOF** (Figure 4). Generally, no dramatic difference in inactivation efficiency was observed between *C. albicans* and *A. niger*, although the viability of *C. albicans* cells slightly decreased under treatment with the same concentration of **Cu(AZPY)-MOF** (Figures 3 and 4). Over 99% of cells or spores were inactivated in the treatments with 0.35 and 0.5 mg/mL **Cu(AZPY)-MOF** (Figures 3 and 4), which indicates that these concentrations may be efficient doses to eradicate fungal cells and spores regardless of species.

Figure 3. Antifungal activities of **Cu(AZPY)-MOF** against *C. albicans*. (a) Yeast cell viability indicated as the CFU number, $\text{Log}_{10}(\text{CFU number})$, and relative percentage after 1 day of treatment with **Cu-MOF**. Each value in CFU number was an average of nine replicate measurements. $\text{Log}_{10}(\text{CFU number})$ and relative percentage were calculated using the average value of CFU number. *** $p < 0.001$. (b) Pictures of plates showing CFU. The cell suspension was diluted 10^4 times after treatment with **Cu(AZPY)-MOF** and 100 μL was spread onto the plates.

Figure 4. Antifungal activities of **Cu(AZPY)-MOF** against *A. niger*. (a) Fungal spore viability indicated as the CFU number, $\text{Log}_{10}(\text{CFU number})$, and relative percentage after 1 day of treatment with **Cu-MOF**. Each germinated spore was considered a CFU. Each value in CFU number was an average of nine replicate measurements. $\text{Log}_{10}(\text{CFU number})$ and relative percentage were calculated using the average value of CFU number. *** $p < 0.01$. (b) Pictures of plates showing CFU. The cell suspension was diluted 10^5 times after the treatment with **Cu(AZPY)-MOF** and 100 μL was spread onto the plates.

Our results demonstrate that **Cu(AZPY)-MOF** has much greater antifungal activity than the constructed Cu-MOFs and Co-CPs in our previous studies [39,40]. **Cu(AZPY)-MOF** inactivated fungi faster (1-day treatment in this study vs. 4-day treatment in the previous study) with a lower amount than Cu-MOF and Co-CPs. **Cu(AZPY)-MOF** inactivated over 99% of fungal cells or spores at 0.35–0.5 mg/mL after 1 day, while the previously constructed Cu-MOFs and Co-CPs showed 50–80% and 44–99% fungal inactivation, respectively, at 2 mg/mL after 4 days [39,40].

3.3. Mechanisms of the Antifungal Activity of Cu(AZPY)-MOF

To understand the mechanism(s) for the antifungal activity of **Cu(AZPY)-MOF**, we investigated the effects of released metal and ligands (free Cu^{2+} , glutarate, and 4,4'-azopyridine) into PBS media on the inactivation of fungi. The reason is that the antimicrobial activity of MOFs can be derived from free metal ions or organic ligands released from the frameworks, as demonstrated in previous studies [27,39,40,48]. The released Cu^{2+} from **Cu(AZPY)-MOF** or glutarate (chemical framework) does not seem to be a critical factor in causing fungal inactivation, as shown in Figure 5. The log CFU number and relative percentage of viable cells were not significantly different between the control and $\text{Cu}(\text{NO}_3)_2$ or glutarate (Figure 5).

Figure 5. Effect of $\text{Cu}(\text{NO}_3)_2$ and glutarate. (a) Fungal viability indicated as $\text{log}_{10}(\text{CFU number})$ and relative percentage after treated with each ligand for 1 day. The concentrations of $\text{Cu}(\text{NO}_3)_2$ and glutarate used in the treatment were 0.1 $\mu\text{g/mL}$ and 0.25 mg/mL , respectively. Each value in $\text{log}_{10}(\text{CFU number})$ was an average of three replicate measurements, and relative percentage was calculated using the average value of CFU number. (b) Pictures of plates showing CFUs of *C. albicans* and *A. niger* on the PDA plates 1 day after treatment.

The higher level of antifungal activity of **Cu(AZPY)-MOF** compared to the previous Cu-MOFs may be related to the addition of 4,4'-azopyridine (AZPY) to the MOF struc-

ture. Azopyrine derivatives are known to have antimicrobial activity [49]. We found that a very small amount of AZPY was released from **Cu(AZPY)-MOF** into PBS over the incubation time, which reached approximately 0.0006 mg/mL after 24 h (Figure 6). The released amounts of AZPY from **Cu(AZPY)-MOF** were measured by UV/Vis adsorption spectroscopy. When the maximum released amount of AZPY (0.0006 mg/mL) was applied to fungal cells, the log CFU number and the relative percentage of viable fungal cells did not significantly change compared to the control (Figure 7). This result indicates that the amount of released AZPY is not critical to inactivate fungal cells and spores. We also exposed fungal cells to free AZPY at a half concentration (0.125 mg/mL) of the inactivation concentration (0.25 mg/mL) of **Cu(AZPY)-MOF** and found that the fungal cells were completely inactivated (Figure 7). This result suggests that AZPY intercalated in the framework may be able to contribute to the fungal deactivation. The mechanism by which AZPY intercalated in MOF can cause fungal inactivation requires further investigation. However, our data suggest that **Cu(AZPY)-MOF** containing AZPY is very stable in PBS and can act as a 3D AZPY derivative with antimicrobial activity.

Figure 6. Concentrations of AZPY released from 0.5 mg of **Cu(AZPY)-MOF** in 1 mL of PBS.

Figure 7. Effect of 4,4'-azopyridine (AZPY) on the viability of *C. albicans* and *A. niger*. Fungal viability indicated as Log₁₀ (CFU number) and relative percentage after treated with the amount of AZPY released into media (0.0006 mg/mL) or the half amount (0.125 mg/mL) of inactivation concentration (0.25 mg/mL) of **Cu(AZPY)-MOF** for 1 day. The number of replicate measurements in the analysis was as follows: 9 replicates for *C. albicans* and 12 replicates for *A. niger* in the control and 0.0006 mg AZPY treatment; 3 replicates for both *C. albicans* and *A. niger* in the 0.125 mg AZPY treatment.

Another possible mechanism for the antifungal activity of **Cu(AZPY)-MOF** containing AZPY is the generation of ROS and RNS. Many MOFs are known to produce ROS and RNS, which can cause antimicrobial effects [27,39,40,50]. We measured the levels of H_2O_2 and NO_x generated in PBS after the treatment with **Cu(AZPY)-MOF**. Approximately 3–4 μM H_2O_2 was measured in PBS in the treatments with **Cu(AZPY)-MOF**, whereas no NO_x was detected (Figure 8). This result is different from that observed in the constructed MOFs in previous studies, where NO_x was generated in PBS treated with MOFs [39,40]. As shown in the results, H_2O_2 instead of NO_x might play a role in inactivating fungal cells and spores. However, the H_2O_2 level was not significantly different among the different treated amounts of **Cu(AZPY)-MOF** (Figure 8). This is not sufficient to explain the observed differences in antifungal activity among the applied concentrations of **Cu(AZPY)-MOF**. Interestingly, H_2O_2 (3–4 μM) and NO_x (0–6 μM) were detected in PBS treated with free AZPY (Figure 8b). This result suggests that AZPY intercalated in MOF (although not in free form) may play a role in generating ROS and RNS.

Figure 8. Concentrations of H_2O_2 and NO in 1 mL of PBS treated with **Cu(AZPY)-MOF** (a) and free AZPY (b). * $p < 0.05$, ** $p < 0.01$.

Recently, several studies have demonstrated the antibacterial and antifungal effects of Cu associated nanostructures [51–53]. These studies show that incorporation of additional components, such as lysozyme or plant extract, seems to enhance the antimicrobial efficiency of Cu nanostructures. Similarly in our study, incorporation of AZPY can elevate the effectiveness of fungal inactivation by **Cu(AZPY)-MOF**. Besides fungicidal effect of released Cu ions, shape of nanostructures can cause mechanical damages to fungal cells [52]. This suggests a possibility that structural shape of **Cu(AZPY)-MOF** may be able to cause mechanical damage to fungal cells in our study. Further investigation is needed to clarify this.

4. Conclusions

We developed a 3D Cu-MOF in which AZPY connects 2D Cu-Glu layers in this study. Addition of AZPY in the 3D framework may be able to contribute to improving antifungal activity of Cu-MOF because AZPY is known to produce antimicrobial effect. As demonstrated in our results, antifungal activity of **Cu(AZPY)-MOF** is highly improved (over 98 % of fungal spores inactivated with 0.25 mg/mL **Cu(AZPY)-MOF** after 1 day)

compared to that of MOFs constructed in our previous studies. This improvement in antifungal activity does not seem to be caused by free Cu^{II} ions or AZPY released from Cu(AZPY)-MOF but by intercalation of AZPY in the framework. Our data suggest that intercalated AZPY may be involved in generating ROS. However, further investigations are needed. Nevertheless, our study demonstrates that addition of antimicrobial compounds into MOF structure can enhance the efficiency of fungal inactivation by MOF.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app12010260/s1>, Figure S1: Structure of Cu(AZPY)-MOF, Figure S2: TGA profile for Cu(AZPY)-MOF, Figure S3: Concentrations of Cu^{II} ions released from 1 mg of Cu(AZPY)-MOF in 1 mL of H₂O or PBS, Table S1: Crystallographic data for Cu(AZPY)-MOF, Table S2: The selected bond lengths (Å) and angles (°) for Cu(AZPY)-MOF.

Author Contributions: The original idea was conceived by Y.K. and G.P. Experiments and data analysis were performed by S.Y., M.V., N.Y., W.K. and S.K.; the crystal structure was analyzed by Y.K.; manuscript was drafted by G.P. and Y.K. All authors have read and agreed to the published version of the manuscript.

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