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Article

Investigating the role of shape and size of gold nanoparticles on their toxicities to fungi

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Abstract: The possibility of releasing gold nanoparticles (GNP) into the environment has been rapidly increasing with the wide spread and flourishing application of gold nanoparticles (GNPs) in a wide range of areas. Consequently, environmental effects of GNP, especially toxicities to living organisms have drawn great attention. However, their toxicological characteristics still remain unclear. Fungi, as the decomposers of the ecosystem, interact directly with the environment and critically control the overall health of the biosphere. Thus, their sensitivity to GNP toxicity is particularly important. The aim of this study was to evaluate the role of shape and size of GNPs on their toxicities to fungi, which could help reveal the ecotoxicity of GNPs. *Aspergillus niger*, *Mucor hiemalis* and *Penicillium chrysogenum* were chosen for toxicity assessment, and circular and star/flower-shaped GNPs sized from 0.7 nm to large aggregates of 400 nm have been synthesised. After mixed with GNPs and reacting agents of GNPs accordingly and incubated for 48 hours, the relative survival rates of each kind of fungus was calculated and compared. The results indicated that with similar sizes, star/flower-shaped GNPs are more toxic to fungi than circular-shaped GNPs; the toxicity of star/flower-shaped GNPs increases with smaller sizes. The results also showed that different species of fungus reacts differently to same GNPs, and *Penicillium chrysogenum* was relatively more sensitive under the exposure to GNPs.

Keywords: gold nanoparticle; fungi; nanoparticle shape; nanoparticle size; nanotoxicology

1. Introduction

In the flourishing field of nanotechnology, gold nanoparticles (GNPs) have received a lot of attention, particularly due to potential applications in bio-related areas [1-5]. The unique physical and chemical properties of noble metal nanoparticles such as GNPs, with specific electronic structures different from atoms or bulk states, have long been of interest and have been widely exploited in diversiform areas such as electronics, chemistry, optics, biomedicine [1-5]. One of the most important characteristics of GNP is the localised surface plasmon resonance (LSPR). LSPR phenomena of GNPs are manifest when the dimensions of the GNP are smaller than the extent of the plasmon wavefunction delocalisation, resulting in strong resonances of the surface electronic states with radiation in the visible region of the spectrum. [6,7]. Although silver nanoparticles show stronger LSPR phenomena, with a stronger absorption, GNPs are more commonly used in biological related applications because of their reputed biocompatibility [6-8].

Nevertheless, while silver nanoparticles have been widely used as anti-microbial materials due to their high toxicity [9-11], the toxicity of GNPs has not yet been fully understood and has drawn the attention of researchers. Notably, it has been reported that the toxicity of GNPs on microbes depends strongly on the species of microbe and the physicochemical properties of the GNP [12-14], and it has also been reported that different shapes of GNPs, such as spheres, rods, triangles, hexagons, prisms, and so on, have different cellular uptake mechanisms and elicit different toxic responses [15-18].

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With the rapidly increasing applications of GNPs, the possibility of their release into the environment has grown dramatically [19-21]. Thus, their effects on the environment, especially their ecotoxicity, have drawn increasing attention [22-24]. Therefore, the toxicity of GNPs to organisms that strongly interact with their direct environment, such as fungi and plants, is of critical importance [23,24].

Fungi are found in most terrestrial, marine and freshwater environments and are the dominant decomposers within the ecosystem. They play a key role in energy cycling and ensuring that nutrients are released from dead and dying plants and animals, thereby ensuring these nutrients remain in circulation within the biosphere [25]. Bioaccumulation of contaminating heavy metals has been observed in terrestrial and water-borne fungi due in part to the large surface area of fungal mycelia, their role as decomposers of dead organic matter and their ecological niche to extract, concentrate and recycle nutrients and minerals back into the biosphere [26]. Organisms within an ecosystem that accumulate toxicants are typically more likely to suffer adverse effects at lower environmental concentrations, thus, the sensitivity of common fungi to GNP exposure is critical, as it may impact negatively on the biosphere's capacity to recycle organic and inorganic materials. In this study, 3 kinds of fungi, namely *Aspergillus niger*, *Mucor hiemalis* and *Penicillium chrysogenum*, are chosen for toxicity assessment, since they are common fungi species, wide-spread in the environment [27-29]. Comparing to the standard synthesis method of HEPES reduced GNPs, which only use HEPES and chloroauric acid [30,31], circular-shaped GNPs with different sizes are also synthesised by adding different concentrations of monosodium phosphates. Similarly, star/flower-shaped GNPs of various sizes are synthesised by adding different concentrations of disodium phosphates. The toxicities of these GNPs on the 3 chosen fungi species are examined and compared to determine the role of size and shape on GNP toxicity.

2. Materials and Methods

2.1. Materials

Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$) was purchased from Fisher Chemical, Ireland. N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer was purchased from Hampton Research, US. Sodium hydroxide (NaOH), hydrochloride acid (HCl), sodium phosphate monobasic (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma Aldrich, Ireland. *Aspergillus niger* ATCC 16404, *Mucor hiemalis* LZB 130, *Penicillium chrysogenum* LZB 141 strains were purchased from Blades Biological Ltd., UK. Potato dextrose agar (PDA) was purchased from Lab M, UK.

Sterilised deionised water, deionised using Elix® Reference Water Purification System from Millipore, Ireland, and sterilised using Autoclave SX-500E from Mason Technology, Ireland, was used for all experiments and solution preparations.

2.2. Synthesis of colloidal GNPs

A 1 mM stock solution of chloroauric acid was made by dissolving hydrogen tetrachloroaurate in water. The 10 mM HEPES buffer stock solution was made by diluting 1 mM HEPES buffer, purchased from Sigma Aldrich (Ireland), with water. Different concentrations of monosodium phosphate and disodium phosphate solutions were prepared by dissolving in water, accordingly.

In the standard synthesis of GNPs, which will be referred to as 'Standard' in the following sections, 200 µL of 1 mM chloroauric acid were mixed with 200 µL of 10 mM HEPES buffer and 600 µL of water. After 10-15 minutes, the colour of the solution changed from pale yellow to colourless, then eventually changed to pink, indicating the formation of GNPs

In the synthesis of GNPs using phosphates, a similar procedure was followed, with the exception that 200 µL of 1 mM chloroauric acid were mixed with 200 µL of 10 mM HEPES buffer and 600 µL of monosodium phosphate or disodium phosphate solution, of systematically varied concentrations.

For all GNP samples, the mass concentration of gold is 39.394 mg/L.

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2.3. Characterisation of GNPs

The absorption spectrum of the GNPs in the ultraviolet-visible spectral region was measured using a Perkin Elmer Lambda 900 UV/VIS/NIR Spectrometer. The absorption spectrum was used to monitor the formation of GNPs, and to characterise and discriminate different samples.

The formation time of GNPs was recorded, defined as the amount of time from the start of the reaction to the time point at which the ratio of the light absorption of GNPs at 540 nm (A_{540}) and the absorption at 450 nm (A_{450}) was higher than 1 [32-34].

A Hitachi SU6600 FESEM instrument was used to record images of different kinds of GNPs synthesised with or without phosphates. Three representative formulations of GNPs were prepared, named as, Standard, 6mM of monosodium phosphate and 6mM of disodium phosphate. In the synthesis of GNPs using phosphates, 200 μL of 1 mM chloroauric acid were mixed with 200 μL of 10 mM HEPES buffer and 600 μL of 6mM of monosodium phosphate and 6mM of disodium phosphate solution were used respectively. After reaction for 1.5 hours, the samples were dropped onto silicon wafers and spun for 2.5 minutes to dry in the air. The samples on silicon substrates were then observed under the scanning electron microscope within 2 days. An accelerating voltage of 20 kV was used for all samples.

The hydrodynamic particle sizes and zeta potentials of different samples were also measured, using a Zetasizer Nano ZS Analyser from Malvern Instruments, Worcestershire, UK.

The pH values of the GNP synthesis systems with different concentrations of monosodium phosphates or disodium phosphates added were measured using Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter.

2.4. Toxicity test of GNPs on fungi

The toxicity of the Standard GNP and GNPs synthesised using phosphates was tested. Three species of fungus, *Aspergillus niger*, *Mucor hiemalis* and *Penicillium chrysogenum* were used in the toxicity test. The fungi samples were prepared by inoculating each in a bottle of sterilised and deionised water and then examined as prepared, and the survival and growth of fungi was measured using the plate count method as described previously [35].

Briefly, for each species of fungus, a comparison group and a test group were prepared and incubated at the same time. In the comparison group, mixtures of 10 mM HEPES buffer and deionised water or different concentrations of monosodium or disodium phosphates at a ratio of 1:3 were prepared. Then these mixtures were adjusted using sodium hydroxide and/or hydrochloride acid until the pH values were 7. Then the fungus sample was mixed with each mixture at a ratio of 1:1 and each spread on the potato dextrose agar (PDA) plates. In the test group, the GNPs were synthesised using phosphates with the same concentrations as in the comparison group, and the pH values were adjusted to 7 with sodium hydroxide and hydrochloride acid, then mixed with fungus samples at a ratio of 1:1 and spread on the PDA plates. The GNP exposure dose for all samples is 19.697 mg/L gold.In total, for each kind for fungi, 8 pairs of comparison group and test group were prepared, which includes Standard GNP, 3 kinds of GNPs synthesised adding monosodium phosphate, and 4 kinds of GNPs synthesised adding disodium phosphate.

All chemicals used in the comparison and test groups were sterilised in advance, and triplicates were measured for all test plates. All plates were incubated at 28℃ for 48 hours, and then the colony-forming units (CFUs) were counted.The CFU result of each test group was divided by the CFU result of corresponding comparison group, indicating relative survival rate.During the process of pH adjustment, for all samples, to minimise the change in volume, high concentrations of sodium hydroxide and hydrochloride acid, namely 1 M and 5 M NaOH stock solution and 1 M and 5 M HCl stock solution, were used. For each 3 mL sample solution, less than 20 μL base or acid was added. Samples with base/acid added was shaken for 1 minute to mix properly, then measured with pH meter. After pH value adjusted to 7, samples were sterilised and ready for mixing with fungi sample.

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2.5. Statistical Analysis

A paired t-test with two-tailed P value and 95% confidence interval was employed to evaluate the difference between the survival rates of the comparison group and the experimental group for the evaluation of toxicity. All statistical analyses are carried out using the software GraphPad Prism 7.

3. Results

3.1. GNP formation time

As the formation of GNPs progresses, the colour changes from colourless to pink/purple/blue and the absorption spectrum of GNPs evolves, most significantly in terms of the relative absorption at 540 nm and at 450 nm. Thus, the ratio between these two absorptions (A_{540}/A_{450}) has been selected to define the formation time of GNPs.

The ratio A_{540}/A_{450} changes as the formation of GNP progresses, along with (see **Figure 1**). A dramatic increase, within 3 minutes or less, is observed for the synthesis using phosphate, during which time the colour changes from colourless into pink/blue; for the standard synthesis (Standard), the absorption ratio A_{540}/A_{450} increases much slowly as the colour changes slowly from colourless to pale pink and eventually into pink.

Since the ratio changes rapidly from less than 1 into more than 1, 1 is defined as the critical point of GNP formation. Once A_{540}/A_{450} is higher than 1, the formation of GNP is considered complete, and the time from the start of the reaction to the time point when A_{540}/A_{450} equalled 1 was recorded accordingly as the GNP formation time.

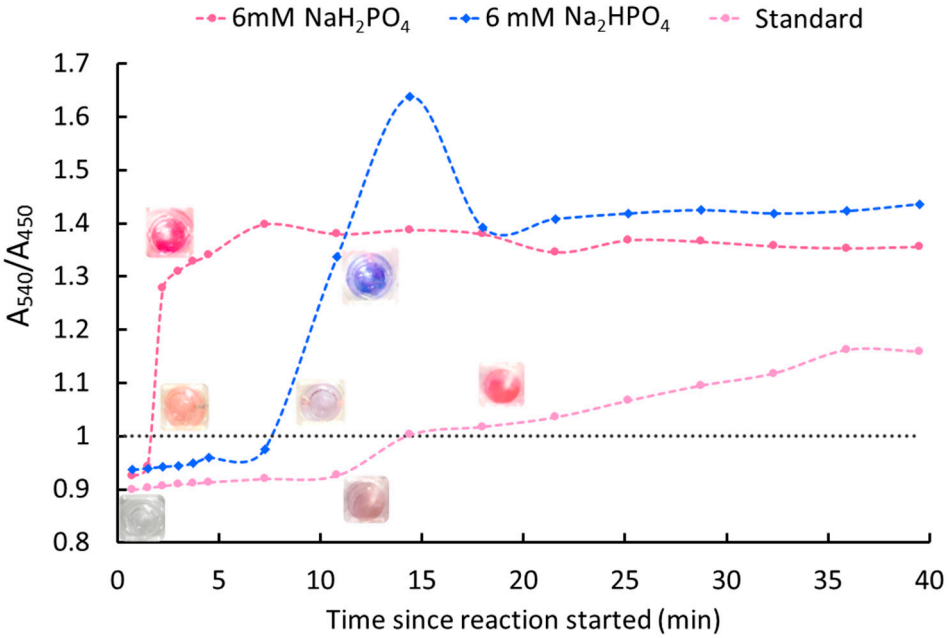

Figure 1. The ratio of the light absorption of GNPs at 540 nm to that at 450 nm changes as a function of reaction time during GNP formation. The colour changes of GNPs have also been presented accordingly.

3.2. Characterisation of GNPs

The measured physicochemical characteristics of each kind of GNP are shown in **Table 1**.

For GNPs synthesised by adding monosodium phosphates, as the concentration of monosodium phosphate increases, the GNP formation time shortens, and the absolute value of zeta potential increases.

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In contrast, for GNPs synthesised by adding disodium phosphates, both the formation time and absolute value of zeta potential decrease as the concentration of disodium phosphate increases.

While the average hydrodynamic size of Standard GNP is ~62 nm, the GNPs synthesised by adding monosodium phosphate reach the smallest size of ~4.6 nm in diameter when 6 mM monosodium phosphate are added, and higher or lower concentration of phosphate leads to larger particles. The diameters of GNPs synthesised adding disodium phosphates remain smaller than 53 nm when the concentration of phosphate is lower than 100 mM, and particle sizes grow larger with the increase of disodium phosphate concentration after that.

Table 1. Physicochemical characteristics of the GNPs synthesised using different phosphates.

Sample [1]	Shape	Concentration of phosphate added (mM)	Formation time (min ± SD)	LSPR peak (nm)	Diameter (nm ± SD)	Zeta potential (mV ± SD)
Standard	Circular	0	15.00 ± 0.53	558.8	61.69 ± 22.35	-23.50 ± 0.21
NaH_2PO_4 added	Circular	2	6.50 ± 0.25	565.0	82.33 ± 35.86	-26.43 ± 0.13
		6	1.50 ± 0.08	531.2	4.60 ± 1.32	-29.67 ± 0.13
		40	0.02 ± 0.01	532.2	634.54 ± 224.30	-36.87 ± 0.45
Na_2HPO_4 added	Star-shaped/ Flower-shaped	1	12.50 ± 0.41	625.8	52.26 ± 14.94	-40.80 ± 0.24
		6	8.25 ± 0.27	587.6	1.42 ± 0..32	-34.97 ± 0.35
		50	2.83 ± 0.14	543.4	0.74 ± 0.25	-23.73 ± 0.60
		240	0.83 ± 0.01	664.0	391.05 ± 153.47	-22.13 ± 0.27

[1] Sample refers to the GNP synthesis methods. "Standard" refers to the GNPs synthesised using standard synthesis protocol. "NaH_2PO_4 added" and "Na_2HPO_4 added" refers to the GNPs synthesised adding different concentrations of monosodium and disodium phosphates accordingly.

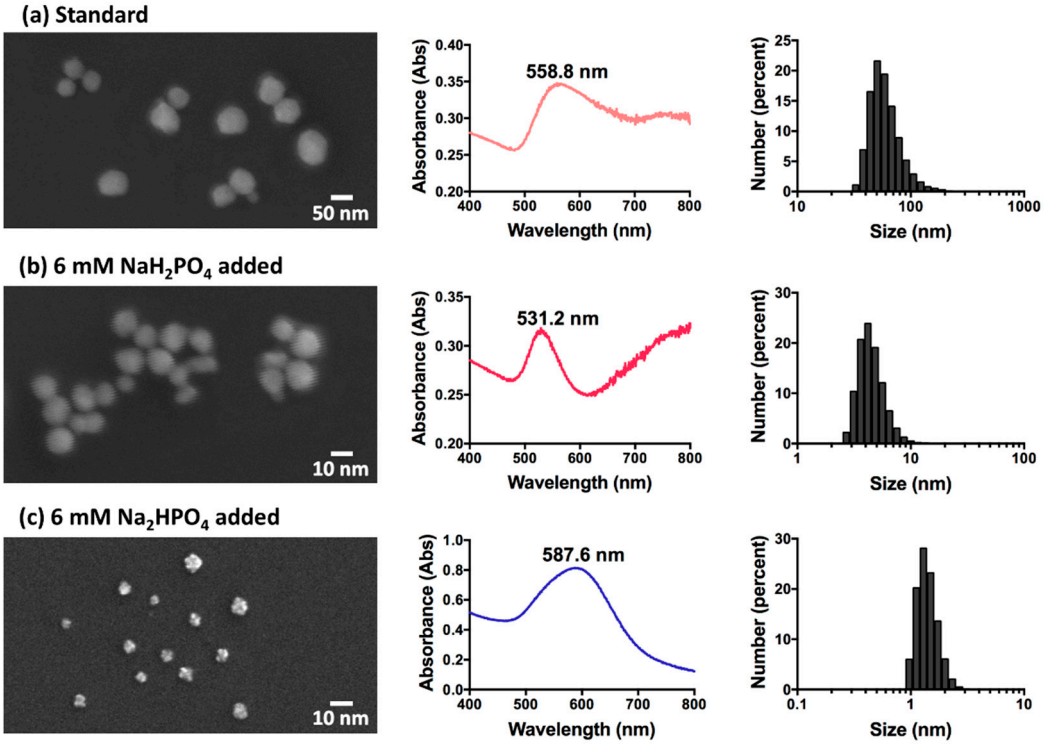

Figure 2. SEM images of GNPs with different sizes and shapes, and the corresponding UV-VIS spectra and size number distribution histogram (from left to right). **(a)** Standard GNPs synthesised with only chloroauric acid and HEPES buffer. **(b)** GNPs synthesised with chloroauric acid, HEPES buffer and 6 mM monosodium phosphate. **(c)** GNPs synthesised with chloroauric acid, HEPES buffer and 6 mM disodium phosphate.

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The shape and size differences of the GNPs were analysed under SEM. The Standard and those synthesised with monosodium phosphate are all circular-shaped, as shown in **Figure 2 a** and **b**. However, even though they are both gold nanospheres, the diameter of the Standard GNPs is about 30-100 nm, while the diameter of those synthesised with 6 mM monosodium phosphate is much smaller, ~2-10 nm. The GNPs synthesised by adding higher concentrations of monosodium phosphate tend to aggregate into large clusters, which results in a UV-VIS peak at 800nm, and a large scattering background to the UV-vis spectra.

In **Figure 2 c**, it can be inferred that the GNPs synthesised with 6 mM disodium phosphate are star-shaped or flower-shaped, which explains the phenomenon that, although most GNPs with an LSPR peak of more than 580 nm and a blue colour are aggregated or large sized, these appear to be rather smaller, with diameters of only 1-3 nm.

3.3. Toxicity test of GNPs with different sizes and shapes on fungi

After mixing pH adjusted HEPES and Standard GNP with *A. niger*, *M. hiemalis* and *P. chrysogenum* and incubating for 48 hours, the fungal growth on each plate was compared by calculating relative survival rates (**Figure 3**). It can be seen that *P. chrysogenum* shows the lowest relative survival rate, followed by *A. niger*, and *M. hiemalis* has the highest relative survival rate at ~1, which indicates that Standard GNP (19.697 mg/L gold) inhibits the growth of *P. chrysogenum* and *A. niger*, while is not toxic to *M. hiemalis*.

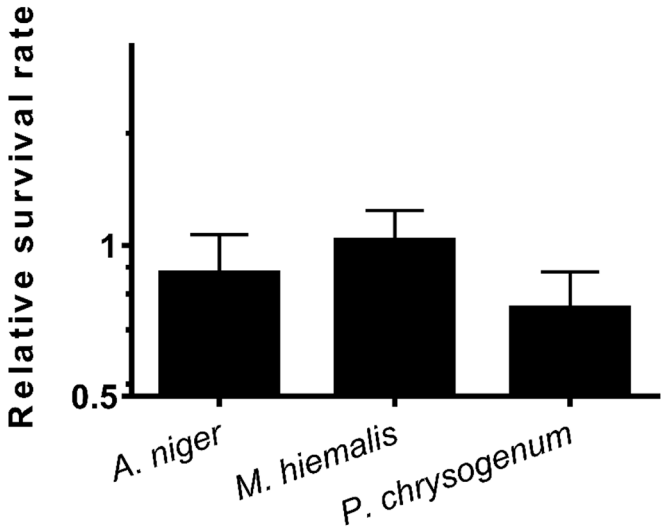

Figure 3. Toxicity of Standard GNP to fungi. Comparisons between the relative survival rates of *A. niger*, *M. hiemalis* and *P. chrysogenum* are shown.

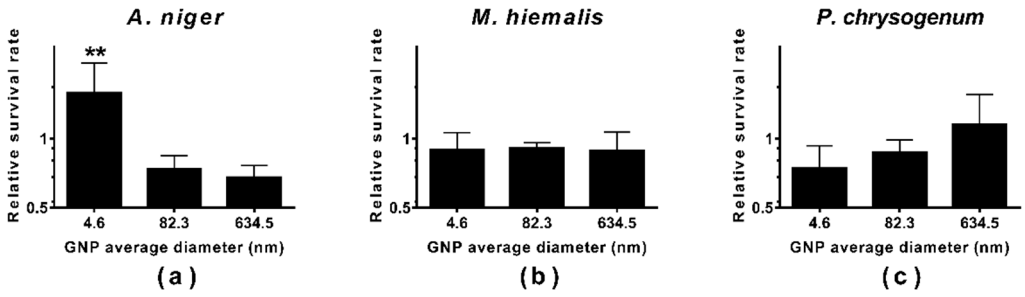

Figure 4. Toxicity of spherical GNPs synthesised with monosodium phosphates added to fungi. Comparisons between the relative survival rates of **(a)** *A. niger*, **(b)** *M. hiemalis* and **(c)** *P. chrysogenum* are shown. The T-test results between each pair of comparison group and test group are also shown. (** represents P<0.005)

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While the relative survival rates of *A. niger* mixed with 2 larger GNPs were less than 1, which indicates that GNPs with those 2 sizes inhibited the growth of *A. niger*, the GNP sized ~4.6 nm promoted the growth of *A. niger* with a relative survival rate of more than 1.5 (**Figure 4 a**).

In contrast, for *P. chrysogenum*, the smaller the GNP, the higher the toxicity. As shown in **Figure 4 c**, the relative survival rate increased as the GNP average diameter got larger; the GNP sized ~4.6 nm inhibited the growth, the GNP sized ~82 nm didn't have much influence the growth, and the GNP sized ~635 nm promoted the growth of *P. chrysogenum*.

On the other hand, the GNPs synthesised by adding monosodium phosphates didn't have much influence on the growth of *M. hiemalis*, since the relative survival rates of *M. himalis* mixed with 3 sizes of GNP were all ~1 (**Figure 4 b**).

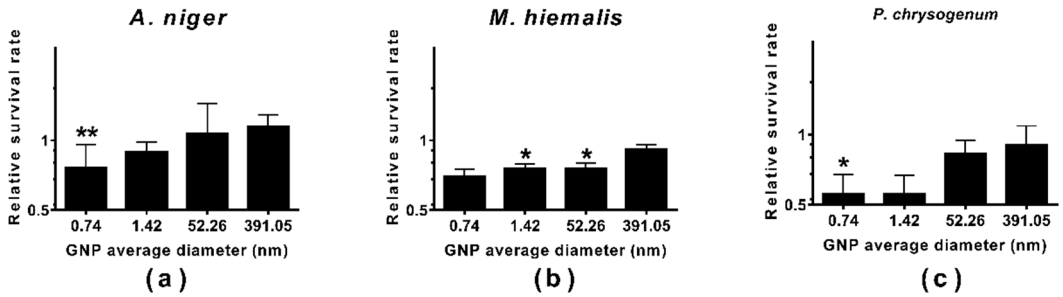

Figure 5. Toxicity of star-shaped GNPs synthesised adding disodium phosphates to fungi. Comparisons between the survival rates of **(a)** *A. niger*, **(b)** *M. hiemalis* and **(c)** *P. chrysogenum* are shown. The T-test results between each pair of comparison group and test group are also shown. (* represents P<0.05, ** represents P<0.005)

For all three kinds of fungi, it appears that the smaller the size of GNP synthesised using disodium phosphates, the lower the relative survival rate (**Figure 5**), which indicates that smaller GNPs have higher toxicity to these three kinds of fungi.

The small GNPs, with diameters less than 2 nm, inhibited the growth of *A. niger*, while GNPs larger than ~50 nm promoted the growth (**Figure 5 a**).

On the other hand, GNPs of all 4 sizes used inhibited the growth of both *M. hiemalis* and *P. chrysogenum*, as all relative survival rates were seen to be lower than 1 (**Figure 5 b, c**). While the largest GNP decreased the growth of these two fungi only slightly, the relative survival rate of *P. chrysogenum* dropped significantly upon expose to the smaller GNPs, although *M. hiemalis* reacted less sensitively.

Paired t-tests with two-tailed P value and 95% confident interval were employed to compare survival rates between the comparison group and experimental group for each concentration and each fungi, and results are shown in **Figure 3, 4, 5**.

In general, after comparing the survival rates of comparison groups and experimental groups, Standard GNP decreases the growth of *P. chrysogenum* most, followed by *A. niger*, while exposure has little effect on *M. hiemalis*. The GNPs synthesised using monosodium phosphates didn't show much size dependent trends in the effects on the relative survival rates of fungi: while larger GNP inhibited the growth of *A. niger* strikingly, smaller GNPs decreased the relative survival rate of *P.chrysogenum* most, and *M. hiemalis* was not much effected. In contrast, the GNPs synthesised using disodium phosphates showed significant size trends, as smaller GNP were seen to exhibit stronger toxicity to fungi, and the growth of *P. chrysogenum* was inhibited most significantly, followed by *M. hiemalis*, and *A. niger* with minimum influence.

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4. Discussion

4.1. Ensuring reproducibility of GNP mass concentrations

Various methods for the synthesis of GNP have been developed with limited knowledge of potential environmental toxicity. In our study, we assessed toxicity in fungi using GNP synthesised by 3 different methods: Standard GNP, GNPs synthesised adding monosodium phosphates, and GNPs synthesised using disodium phosphates. In order to accurately compare toxicities of GNP produced using three methods, we needed to ensure the reproducibility of mass concentration of GNP produced. All samples, whether with phosphates added or not, were synthesised by mixing 200 μL of 1 mM chloroauric acid and 200 μL of 10 mM HEPES buffer for every 1 mL sample.

The HEPES reduced GNP synthesis method has been applied and studied for a decade, and it has been reported that the difference of the molar ratio of chloroauric acid and HEPES strongly affects the GNP synthesised [30]. In this study, a molar ratio of 1:10 between chloroauric acid and HEPES has been applied to all syntheses, which ensured the complete reduction of chloroauric acid into atomic gold. Thus, the mass concentration of gold for all GNP samples is the same, which allows their toxicities to be compared since the volumes of GNPs mixed with all fungi samples are the same, leading to same exposure dose as 19.697 mg/L for all samples,

While a formation time of ~15 minutes is needed for a typical synthesis protocol using HEPES, the addition of monosodium phosphate or disodium phosphate accelerates the formation process of the GNPs (see **Table 1**). This could be because the addition of phosphate brings more ions into the reaction system, which provides a more suitable formation environment and thus shortens the formation time.

The LSPR peaks of the GNPs synthesised using phosphates are also different to the Standard GNP (**Table 1**). As reported by Haiss et al., the size and concentration of GNPs within the size range of 5-100 nm can be directly determined from UV-VIS spectra according to corrected Mie theory[36]. While GNP with average hydrodynamic diameter ~62 nm and LSPR peak of 558.8 nm can be synthesised using a typical standard protocol, GNPs sized ~4-85 nm can be synthesised when adding 2-6 mM monosodium phosphate, and GNPs sized 0.7-55 nm can be synthesised when 1-50 mM disodium phosphate is added (**Table 1**). For these GNP samples, the smaller the diameters, the lower the LSPR peak wavelengths according to corrected Mie theory[36].

4.2. Comparing toxicity of GNPs

Both Standard GNP and GNPs synthesised by adding monosodium phosphates are spherical in shape (**Figure 2**), but the size of particles range from as small as 3-10 nm, to aggregates as large as ~650 nm (**Table 1**). It appears that when the shapes of GNPs are the same, the sizes of the GNPs influence the relative survival rates of fungi differently.

4.2.1. Toxicity of Standard GNPs

Standard GNPs are circular shaped particles with diameters range from 30 nm to 100 nm (**Figure 2 a**), and an average hydrodynamic diameter of ~62 nm (**Table 1**).

As shown by the results in **Figure 3**, the growth of *P. chrysogenum* was inhibited considerably after exposure to Standard GNP, compared to the group mixed with HEPES alone. Similarly, the growth of *A. niger* was inhibited by Standard GNP, while the survival rate of *M. hiemalis* only decreased a little (**Figure 3**).

The flourishing growth of the group mixed with HEPES and GNP could be caused by the extra nutrition provided by HEPES. However, compared to the comparison group, Standard GNP still showed a strong decrease of the survival rate, indicating that the Standard GNP is toxic to *P. chrysogenum*. On the other hand, the growth of *A. niger* was inhibited by Standard GNP in comparison, which indicates that Standard GNP is also toxic to *A. niger*, but to a lesser extent than to *P. chrysogenum*. Comparing to comparison group, Standard GNP didn't cause much influence on the growth of *M. hiemalis*, which indicates that Standard GNP has little toxicity to it.

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4.2.2. Toxicity of GNPs synthesized adding monosodium phosphates

The sizes of GNPs synthesised vary as the concentration of monosodium phosphates added is varied. The GNP synthesised by adding 6 mM monosodium phosphate exhibit the smallest average hydrodynamic diameter of ~4.6 nm (**Table 1**), with most particles sized between 2-10 nm (**Figure 2**). The GNPs synthesised by adding 2 mM monosodium phosphate are larger, with average hydrodynamic diameter of ~82 nm, and those produced by adding 40 mM monosodium phosphate form large aggregates with sizes ~635 nm (**Table 1**).

For *A. niger*, the growth was significantly increased upon the exposure to the smallest GNP, while the growth decreased slightly when exposed to the larger GNPs (**Figure 4 a**). The survival rates of *A. niger* mixed with GNPs sized ~82 nm and ~635 nm are only slightly lower than the comparison groups. And the growth increase of the one added with smallest GNP could be caused by the higher phosphate absorption. It has been reported that the phosphate is essential for the growth of mold fungi, including *Aspergillus*, *Penicillium* and *Rhizopus* [37]. For *A. niger*, the phosphates absorbed are converted into different phosphorus compounds, most of which are acid-hydrolysable compounds [38], and as much as 90% of these are accumulated in the mycelia [39]. Since the size of this GNP is very small, the particles are absorbed easier by *A. niger*, along with the phosphate ions attached to the particles. So, the higher absorption of phosphates may lead to the enhanced growth. In general, it cannot be determined whether the GNPs synthesised adding monosodium phosphate have much influence on on the growth of or toxicity to *A. niger*.

For *P. chrysogenum*, it can be seen that the relative survival rates are decreased significantly upon exposure to GNPs, and the one mixed with smallest GNP has the least growth (**Figure 4 c**).. It can be inferred that the 4.6 nm GNP, which is circular, relatively the smallest and synthesised using 6 mM monosodium phosphate, has strongest toxicity to *P. chrysogenum*.

For *M. hiemalis*, the growth was only slightly decreased by GNPs synthesised using monosodium phosphates (**Figure 4 b**). The reason is likely due to the presence of higher concentrations of phosphate which increases the cellular activities of *M. hiemalis* and causes it to grow more rapidly [39], which minimised the effects of GNPs.

In general, *P. chrysogenum* reacts most sensitively to the toxicity of circular-shaped GNPs, and the smaller the particle size, the stronger the toxicity, while these GNPs showed contrary size trends on *A. niger*, and have little effect on *M. hiemalis*.

4.2.3. Toxicity of star/flower-shaped GNPs

The sizes of GNPs synthesised by adding disodium phosphates are smallest when concentrations of disodium phosphates added are between 6-50 mM, followed by those formed by adding 1 mM disodium phosphate. When the concentration is higher than 100 mM, the particle sizes become very large, with average hydrodynamic diameters larger than 280 nm (**Table 1**). The shape of GNPs is flower-like and/or star-like, as shown in **Figure 2 c**.

From **Figure 5 a**, it can be seen that the *A. niger* mixed with GNP sized ~0.74 nm have the largest decrease of relative survival rate. It can be inferred that the flower-shaped GNPs smaller than 10 nm have toxicity on *A. niger*. The increase in relative survival rates of *A. niger* could be because higher concentrations of phosphates promoted the growth.

For *M. hiemalis*, all relative survival rates were decreased (**Figure 5 b**), especially the ones mixed with the smallest GNP. It can be inferred that the flower/star-shaped GNPs have toxicity on *M. hiemalis* as well, with smaller GNPs eliciting higher toxicity.

For *P. chrysogenum* (**Figure 5 c**), the relative survival rates also decreased sufficiently, especially for 0.74 nm GNP, which is synthesised by adding 50 mM disodium phosphate. It can be inferred that the GNPs synthesised with disodium phosphate added are very toxic to *P. chrysogenum*.

In general, the star/flower-shaped GNPs are toxic to all three kinds of fungi, with highest toxicity to *P. chrysogenum*, followed by *M. hiemalis* and least toxicity to *A. niger*.

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4.4. Toxicity comparison between different shaped GNPs

Since the size of circular-shaped GNP synthesised adding 6 mM monosodium phosphate is similar to the sizes of star/flower-shaped GNPs synthesized adding 6-50 mM disodium phosphates, their effects on the growth of each kind of fungi are compared to see the shape effect of GNP's toxicity.

From **Figure 4 a** and **Figure 5 a**, it can be seen that the toxicity of star/flower shaped GNPs is clearly stronger than circular-shaped GNP on *A. niger*. While circular-shaped GNPs do not have much effect on the growth of *A. niger*, the star/flower-shaped GNPs decreased the relative survival rates significantly.

For *M. hiemalis*, the difference of the toxicities is small, since both circular-shaped and star/flower-shaped GNPs decreased the relative survival rates to similar extents (**Figure 4 b** and **Figure 5 b**).

When comparing results from **Figure 4 c** and **Figure 5 c**, it can be inferred that the toxicity of star/flower-shaped GNPs on *P. chrysogenum* is higher than circular-shaped GNPs, since the former decreased the survival rates to a larger extent.

5. Conclusions

The role of shape and size of GNPs on their toxicities on 3 kinds of fungi, *A. niger*, *M. hiemalis* and *P. chrysogenum*, were investigated. Two kinds of GNP shape, circular and star/flower-like shaped, with different sizes were examined. The circular-shaped GNPs were synthesised using 2 kinds of methods: the standard synthesis method, which creates Standard GNP with average hydrodynamic diameter of 62 nm; and the synthesis method adding different concentrations of monosodium phosphates, which creates GNPs sized between 4.6 nm to 85 nm, and large aggregates sized 635 nm. The star/flower-shaped GNPs were synthesised by adding different concentrations of disodium phosphates and were sized between 0.7 nm and 400 nm.

It has been found that, under the exposure dose of 19.7 mg/L gold, the Standard GNP has relatively highest toxicity to *P. chrysogenum*, followed by *A. niger*, and has least toxicity to *M. hiemalis*. For circular-shaped GNPs synthesised using monosodium phosphates, *P. chrysogenum* reacts most sensitively to their toxicity, and smaller GNPs show stronger toxicity; while the size trends are contrary for *A. niger*, and these GNPs have little toxicity to *M. hiemalis*.

It has also been found that, under the same mass concentration exposure dose, star/flower-shaped GNPs have very strong toxicity to *P. chrysogenum*, less toxicity to *M. hiemalis*, and least toxicity to *A. niger*. Similarly, for all three kinds of fungi, smaller sized star/flower-shaped GNP exhibit stronger toxicity.

The size effect of GNP's toxicity could be caused by the easier absorption of smaller particles. When comparing similar sized GNPs with same mass concentration, it was shown that for *P. chrysogenum* and *A. niger*, the star/flower-shaped GNPs have higher toxicity than circular-shaped GNPs, while the shape of GNPs do not have much effect on the toxicity to *M. hiemalis*.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Size and zeta potential of GNPs synthesised using phosphates, Figure S2: Toxicity of GNPs synthesized with monosodium phosphate on fungi, Table S1: The size and zeta potential of GNPs synthesised using monosodium phosphate and disodium phosphate.

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