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High spatial resolution methods to assess the physiology of growing cells should permit analysis of fungal biochemical composition. Whole colony methods cannot capture the details of physiology and organism–environment interaction, in part because the structure, function and composition of fungal hyphae vary within individual cells depending on their distance from the growing apex. Surface Enhanced Raman Scattering (SERS) can provide chemical information on materials that are in close contact with appropriate metal substrates, such as nanopatterned gold surfaces and gold nanoparticles (AuNPs). Since nanoparticles can be generated by living cells, we have created conditions for AuNP formation within and on the surface of *Aspergillus nidulans* hyphae in order to explore their potential for SERS analysis. AuNP distribution and composition have been assessed by UV-Vis spectroscopy, fluorescence light microscopy, transmission electron microscopy, and scanning transmission X-ray microscopy. AuNPs were often associated with hyphal walls, both in the peripheral cytoplasm and on the outer wall surface. Interpretation of SERS spectra is challenging, and will require validation for the diversity of organic molecules present. Here, we show proof-of-principle that it is possible to generate SERS spectra from nanoparticles grown *in situ* by living hyphae.

1. Introduction

Filamentous fungi including *Aspergillus* affect humans through their roles in biotechnology and recycling, as well as being agents of disease and decay. The similarities and differences between these metabolic activities will be the key to better exploitation and/or control strategies. We are coupling high spatial resolution, high sensitivity spectrochemical analysis of hyphal composition with molecular genetics and microscopy. We are testing hypotheses that relate fungal cell structure and function to colony growth and development, and to fungal interactions with their environment. Together, these methods will enable us to assess factors expected to be critical for fungal growth and reproduction. Importantly, they will allow us to better understand fungal responses to stressful environments, such as those created by anti-fungal drugs.

Over the last century, the average human life span in the developed world has increased from 50 to 80 years, mostly due to better public health measures, vaccinations, and antibacterial drugs. Still, millions of people die every year from infections, with systemic fungal infections emerging as being amongst the most dangerous. In the USA in 2008, the cost of treating human fungal diseases was ~$33 billion. Once a fungal infection has invaded an organ or the bloodstream, a cure means being symptom-free for six months. The risk of infection is unavoidable since there are potentially dangerous airborne spores in every breath we take. Our major defense is our immune system, which is weaker in the young, the old, and the sick. Physiologically, fungi respond to stressful environments by reallocating their internal resources and by up-regulating their cell wall integrity pathways. Fungi can also adapt through mutation.

The use of high spatial resolution spectrochemical methods to study cytoplasm has considerable promise as part of a suite of correlated approaches to elucidate structure and function in hyphal growth processes. For example, we have used infrared spectroscopy, Raman spectroscopy, and Surface Enhanced Raman Scattering (SERS) to obtain spatially resolved biochemical information about *Aspergillus nidulans* and *Curvularia protuberata*. SERS, which can be several to many orders of magnitude more sensitive than Raman spectroscopy, has been achieved from hyphae grown across a nanopatterned gold substrate.

In this paper, we report on gold nanoparticles (AuNPs) created through the addition of gold chloride (HAuCl4) to liquid growth medium that contained live *A. nidulans* fungal colonies...
from overnight growth. We characterized the shape, size and distribution of AuNPs using scanning transmission X-ray microscopy (STXM), UV-Vis absorption, fluorescence light microscopy and transmission electron microscopy (TEM). SERS spectra obtained from these samples provide proof-of-principle that these AuNPs are SERS-active. Analysis of the results demonstrates the potential for greater control over location and activity.

2. Materials and methods

2.1 Gold nanoparticle (AuNP) generation in fungal cells

The wild type morphology *A. nidulans* strain AAE1 was maintained as described. For AuNP production spores were germinated in potato dextrose broth (PDB, Difco) adjusted to pH 6.5 with 1 M NaOH. Previously, we had determined that an initial pH of 6.5 was optimal for *A. nidulans* culture.

To prepare colonies for fluorescence microscopy, TEM and STXM, ~500 *A. nidulans* spores (10 μL of a 50,000 spore per mL stock, stored in sterilized distilled water for up to 7 days at 4 °C) were added to 0.5 mL PDB in 1.5 mL microfuge tubes. These were sealed with Parafilm®, placed in a 125 mL Erlenmeyer flask, and germinated overnight at 37 °C, with shaking at 270 rpm. The resulting colonies were white, globose, and 1–2 mm diameter. The precise number of spores was not crucial: due to space constraints, this method produced 1–2 drops of 0.1 M NaOH, to achieve a pH of 6.5 was optimal for *A. nidulans* culture.

Colonies for UV-Vis and SERS spectroscopic analysis were grown in a similar manner. Twenty millilitre aliquots of spores, freshly harvested or stored up to 7 days in ultrapure water at 4 °C, were added to 1.5 mL microfuge tubes containing 1.0 mL PDB pH 6.5, and shaken overnight at 37 °C, with shaking at 370 rpm. The resulting colonies were white, globose, and 1–2 mm diameter. The precise number of spores was not crucial: due to space constraints, this method produced ~10 colonies. Colonies were treated with 0.5–5 mM HAuCl₄, diluted from a 100 mM aqueous stock solution. PDB does not have substantial buffering capacity: following germination at pH 6.5, overnight growth of *A. nidulans* acidified PDB to pH 5.5. Addition of 5 mM HAuCl₄ further acidified it by up to 2 pH units. To compensate, we adjusted the initial pH of the PDB to 7.5 in subsequent preparations, so that following overnight growth and HAuCl₄ addition solution pH was ~6. Colonies were treated with HAuCl₄ for 150 min before harvest and preparation for microscopy, described below.

Colonies for UV-Vis and SERS spectroscopic analysis were grown in a similar manner. Twenty millilitre aliquots of spores, freshly harvested or stored up to 7 days in ultrapure water at 4 °C, were added to 1.5 mL microfuge tubes containing 1.0 mL PDB pH 6.5, and shaken overnight at 37 °C. For 1 mM HAuCl₄, 46 μL of 0.5% HAuCl₄ was added; pH was adjusted by addition of 1–2 drops of 0.1 M NaOH, to achieve a pH of ~6. The microfuge tubes were shaken at 37 °C as described above, then removed and stored at 4 °C.

To assess hyphal viability, colonies treated with 1 mM or 5 mM HAuCl₄ for up to 150 min were transferred to potato dextrose agar plates and incubated at 37 °C.

2.2 Methods for characterization of AuNP

Colonies that had been treated with 1 mM and with 5 mM HAuCl₄ for 150 min were prepared for TEM and STXM as described. Hyphae were fixed in 1% glutaraldehyde, rinsed 3× in buffer, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812, which was polymerized at 60 °C. Following mounting and trimming, TEM silver sections (~75 nm thick) and STXM gold sections (~100 nm thick) were collected on Formvar-coated single slot copper grids. The pale grey interference colour of these Formvar films indicated a thickness of 60–75 nm.

STXM imaging and spectromicroscopy were carried out at the Canadian Light Source (CLS) SM beamline, 10ID-1. Whole AuNP-containing colonies were transferred to Formvar-coated Cu EM slot grids. The excess PDB medium was wicked away using a filter paper triangle and the colony was air-dried at room temperature. The Cu grids were attached to an aluminum STXM sample holder, and examined at the C K-edge and Au M₄,5-edges. For the Au reference, a 5 μL drop of 100 mM HAuCl₄ was dried onto part of a 500 nm thick silicon nitride window. Thin sections of hyphae were prepared as for TEM, above. All image and spectral processing was performed with aXis2000 software.

Absorption at the C K-edge was used as a proxy for biological materials. Qualitative carbon maps were obtained from the difference of optical density (OD) images recorded at 282.2 eV (absorption maximum for the C 1s π*→π* protein peak) and 280 eV (baseline before peak onset). The energy scale at the C K-edge was calibrated using the Rydberg peaks of CO₂.

Qualitative Au maps were obtained from the difference of OD images recorded at 2150 eV (below the Au M-edge) and 2370 eV (centre of the M₄,5-edge peak). Quantitative mapping of Au in whole fungal cells was achieved by spectral fitting of the Au image sequence using a singular value decomposition linear regression procedure. These maps were based on the HAuCl₄ reference spectrum, placed on a linear absorbance scale by matching it to the predicted response for the compound based on its elemental composition and density (3.9 g cm⁻³), and using tabulated continuum absorption coefficients. Transmitted signals (*I*) were converted to optical densities (OD) [absorbance, OD = −ln(*I*/*I₀*)] using the incident flux (*I₀*) measured through sections devoid of fungal cells, to correct for absorbance by Formvar and Epon. The absolute energy scale of the Au M₄,5-edge was set by assigning the onset of the Au M₅-edge of AuCl₃ to 2206 ± 5 eV.

UV-Vis absorption spectra of liquid from each microfuge tube were acquired with a UV-2101 spectrometer (Shimadzu), from 190 to 900 nm, at 1 nm spectral resolution, against a water blank (Milli-Q) in matching 1 cm path-length, 1 mL quartz cuvettes.

Some colonies that had been treated with HAuCl₄ were stained with Calcofluor in order to visualize hyphal walls and septa and assess AuNP spatial distribution. Fluorescence images were acquired with a Zeiss AxioImager Z1 equipped with a 63×, N.A. 1.4, Plan Apochromat objective, a light emitting diode at 365 nm, and filters for Calcofluor excitation, captured with a Zeiss AxioCam.

For TEM, sections were post-stained with uranyl acetate and lead citrate. In addition, 5 μL samples of the growth medium from AuNP-producing colonies were applied to Formvar-coated single-slot Cu grids, air-dried, negative stained with phosphotungstate, and examined. Images were obtained with a Philips CM10 transmission electron microscope operating at 60 kV and captured on X-ray sheet film. Films were digitized at 1200 dpi following development.

2.3 SERS analysis

For SERS, HAuCl₄-treated colonies were transferred whole to clean glass microscope slides, then PDB was wicked away with a
filter paper triangle. Samples were immediately frozen on a metal shelf at −80 °C for 5 min, then air-dried at room temperature. A reference Raman spectrum was obtained from a clean glass microscope slide. For Raman spectroscopy of the growth medium, a drop of PDB was dried onto a gold-coated substrate.

SERS spectra were acquired using a Renishaw inVia Raman microscope, equipped with a high sensitivity, ultra-low noise CCD, and a motorized x–y sample stage for automated mapping. Samples were illuminated with a 785 nm diode laser with a 1200 grooves per mm holographic grating. The Raman spectrometer wavelength was automatically calibrated using the center frequency of the silicon band (520 cm⁻¹) from a silicon reference wafer.

The CCD detector in this instrument can accommodate a spectral width of ~400 cm⁻¹ for a given grating position. Extended scan maps were created by collecting data at sequential grating settings to yield a spectrum from 200–1800 cm⁻¹ for each pixel, and raster scanning across a predetermined area; exposure times were 1 to 10 s per pixel, with 50× objective and a 785 nm diode laser. The inVia Streamline option, which utilizes a multiplexed line focus illumination method and fast CCD readout, was used to create SERS maps with <1 s laser exposure per pixel and a single grating setting, providing a 400 cm⁻¹ segment of the entire spectrum. Multiple images were acquired sequentially, from overlapping spectral windows, in order to build up the entire spectrum for each map.

3. Results

Aspergillus nidulans colonies grown in PDB changed from white to coloured during incubation with HAuCl₄ (Fig. 1). The rate of change as well as the final colour achieved varied with the HAuCl₄ concentration and duration of exposure. After 150 min at 37 °C, colonies exposed to 1 mM HAuCl₄ were pink-purple (Fig. 1A), whereas those exposed for the same time to 5 mM HAuCl₄ were golden (Fig. 1B). Individual hyphae from the 1 mM HAuCl₄ treatment also had a purple hue (Fig. 1C), but distinct particles were not seen. Large (>1 μm) golden shapes (presumably AuNPs) were detected in the dried medium surrounding the golden colonies from the 5 mM treatment (Fig. 1D). Identification of these NP as gold and the range of AuNP sizes and shapes were documented with STXM, UV-Vis absorption spectroscopy, fluorescence microscopy and TEM.

Viability studies following AuNP induction showed that peripheral hyphae from A. nidulans colonies grown for up to 150 min in 1 mM HAuCl₄ were able to grow vigorously and sporulate on PDB solidified with agar. Cultures treated with 5 mM HAuCl₄ had reduced viability after 30 min.

3.1 Characterization of gold nanoparticles in Aspergillus nidulans cultures

3.1.1 Scanning transmission X-ray microscopy. Whole colonies and 100 nm thick TEM sections of A. nidulans were examined with STXM (Fig. 2). The Au M₄,₅-edge spectrum from the reference gold is an excellent match to the spectrum from putative AuNPs in whole hyphae (Fig. 2A). The latter spectrum was obtained by masking pixels from obvious electron-dense NP in the whole A. nidulans colonies, and processing as described. Hyphae were visualized from the qualitative image of the C distribution (Fig. 2B) based on the differences in on- and off-resonance OD maps for the intensity at the C1s to K-edge, a protein-associated signal. A smaller area from this map (red box in Fig. 2B, magnified in Fig. 2C) was imaged for C (red) and randomly distributed AuNPs (green). The quantitative spectral fitting procedure for Au was applied to a small region (yellow box in Fig. 2C). Based on the quantitative fit, the brightest green corresponds to particles that are ~2 μm thick.

STXM maps of 100 nm thick sections of A. nidulans hyphae treated with 5 mM HAuCl₄ are shown in Fig. 2D–F. It was not possible to detect a signal at the Au M₄,₅-edge for the STXM sections due to thinness of the material. However, the 280 eV image is below the onset of the C 1s absorption, thus compounds lacking carbon would be structureless at this energy. Because the cross-section for heavy, electron rich elements (i.e., Au) is very high, the Au is expected to be the dominant non-biological heavy element component of these systems and thus the 280 eV image is ascribed to the spatial distribution of Au. This was confirmed for clusters imaged at the Au M₄,₅-edge spectrum, above (Fig. 2C).

3.1.2 UV-Vis spectroscopy – absorption and fluorescence. UV-Vis absorption spectra of growth medium withdrawn from typical overnight colonies, and again following 20 to 200 min incubation in 1 mM HAuCl₄, are shown in Fig. 3. The spectra display a strong surface plasmon resonance (SPR) band with a maximum at ~543 nm, attributed to AuNP on the order of 50–70 nm. Incubation of pure PDB with HAuCl₄ showed that the growth medium is also capable of chemical reduction leading to formation of large AuNP, as evidenced by a broad, low SPR band (maximum at 570 nm, FWHM from 525 to 750 nm) measured after 2 h incubation. Further experiments using
minimal medium for growth are underway, in an effort to limit medium-driven reduction of gold.

Some *A. nidulans* colonies were stained with Calcofluor for fluorescence imaging of their cell walls and septa, following 150 min growth in 1 mM HAuCl4 (Fig. 4). Dark triangular and irregular hexagonal objects are consistent with the presence of micron-size AuNPs (Fig. 4).

### 3.1.3 Transmission electron microscopy

Transmission electron micrographs of *A. nidulans* hyphae incubated in HAuCl4 are shown in Fig. 5. A near-median section of an *A. nidulans* hypha from a colony incubated with 5 mM HAuCl4 (Fig. 5A–C) shows profiles of large flat AuNPs associated with the cell membrane and with the outer surface of the cell wall (black arrows in Fig. 5A). Electron-dense objects, tentatively identified as polyhedral AuNPs, were associated with the near-apical Spitzenkörper region (large arrowhead in Fig. 5A; higher magnification in Fig. 5B). Globose AuNPs (small arrowheads in Fig. 5A) were associated with the peripheral cytoplasm. Tiny (∼10 nm) electron-dense particles assumed to be AuNPs (white arrows in Fig. 5B; and in Fig. 5C) were found throughout the cytoplasm. A TEM cross-section of a hypha from a colony incubated in 1 mM HAuCl4 (Fig. 5E) contains many tiny ∼10 nm particles dispersed throughout the cytoplasm; however, most of the particles were found as clumps associated with the outer cell wall surface.

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**Fig. 2** STXM analysis of NP produced in *Aspergillus nidulans* following incubation with 5 mM HAuCl4. (A) STXM spectra of NP in whole hyphae and of Au reference in Au M L3 edge region. (B) Intensity at 2150 eV qualitative carbon image, hyphae are grey to black. (C) Expanded from red box in (B), showing carbon (red) and Au (green) in whole hyphae. Yellow box shows where quantitative stack data [Section 4.1] were acquired. (D–F) Qualitative images from thin sections of hyphae showing carbon (red) and Au (green).

**Fig. 3** UV-Vis absorption spectra of supernatant PDB, extracted from microfuge tubes in which *Aspergillus nidulans* colonies were germinated and incubated with 1 mM HAuCl4; initial pH adjusted to 6.5. Spectra are on a common scale, and are compared to spectrum of broth prior to addition of Au solution (control).

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**Fig. 4** *Aspergillus nidulans* hyphae treated with 5 mM HAuCl4 to induce AuNP formation for 150 min at 37 °C, and stained with Calcofluor to visualize cell walls. Bar = 10 μm. AuNPs appear as black spots, some of which have triangular (vertical arrow) or hexagonal (horizontal arrow) profiles.
plasm contains tiny AuNP by incubation in 5 mM HAuCl₄ for 150 min. (D) AuNP formed in conditioned medium containing 5 mM HAuCl₄. (E) Cross-section of an A. nidulans hypha incubated in 1 mM HAuCl₄ for 150 min. Bar in A = 1 μm; bar in B, D, E = 100 nm, C is same scale. (A–C) Near-median, longitudinal section of an A. nidulans hyphal tip with small globose (small arrowheads) and large flat (black arrows) AuNP in the cytoplasm and adjacent to the wall surface. A cluster of polyhedral AuNP (large arrowhead in A; detail in B) formed associated with the Spitzenkörper region at the hyphal tip. Flat AuNP associated with the peripheral cytoplasm and outer wall surface are seen in cross-section. Tiny globose AuNP (white arrows in B, detail in C) are abundant throughout the cytoplasm. (D) AuNP formed in conditioned growth medium containing 5 mM HAuCl₄ has characteristic shapes from minute dots to large triangular plates. (E) TEM of an A. nidulans hypha induced to form AuNP by incubation in 1 mM HAuCl₄ for 150 min. The cytoplasm contains tiny ~8 to 10 nm AuNPs (white arrows) but most AuNP form clumps on the external surface of the cell wall.

3.2 Surface enhanced Raman scattering activity

AuNPs associated with A. nidulans colonies treated with 0.5 to 5.0 mM HAuCl₄ showed SERS activity. Many images were recorded, under different collection parameter settings, with different laser powers and exposure times. Images obtained with the extended scan option permitted collection of the entire spectrum at each pixel in a map. Some spectra from extended scan maps showed intense SERS activity (Fig. 6); other spectra showed strong graphite peaks that indicated some carbonization due to the longer exposure to the laser, despite the low power.

Rapid scan images (80 to 300 ms per pixel) were acquired with the Streamline option, which limited the spectral range to a single segment of ~400 cm⁻¹. In these cases, a complete spectrum was constructed by mapping the same sample area multiple times at different grating positions, with overlapping segments for each spectral range. Images showing A. nidulans hyphae and locations with SERS activity are shown in Fig. 7. The reconstructed Streamline spectrum of a clean glass substrate is shown for comparison (Fig. 7, spectrum 1). Several hyphae lie flat on the glass slide, at the periphery of the dried colony (Fig. 7A). Weak fluorescence from these hyphae elevates the baseline relative to surrounding glass substrate, revealing their outline (Fig. 7B, spectra 2 and 3). Several SERS-active spots were found in this small area; at least one was possibly associated with a hypha. Reconstructed complete spectra are taken from points that illustrate both reproducibility and variability in such images (Fig. 7C, spectra 4 and 5). The extended scan Raman spectrum of PDB on gold-coated substrate is shown below (Fig. 7, spectrum 6).

4. Discussion

Our goal in this study has been to demonstrate that in vivo synthesis of SERS-active AuNPs can be achieved. Microorganisms are thought to have major roles in biomineralization. Furthermore, bacteria and fungi, as well as plant extracts, are being proposed as alternative, green technology vehicles for nanoparticle synthesis. AuNP generation is clearly not difficult. Our focus then must be on the creation of synthesis conditions that permit SERS activity, preferably from AuNP generated in specific locations, while preserving cell viability. Nanoparticle sizes and shapes are critical to their function, so in addition to AuNP synthesis per se, we are interested in factors that control location, size and shape.

The fungal wall is the interface between the cytoplasm and the cell’s environment and mediates interactions, such as symbiosis and pathogenesis, with organisms. It is likely that metallic NP generation is part of an adaptive (defense) mechanism. A. nidulans is able to generate metallic NPs when challenged with solutions of group VIII and IB transition metal salts including AgNO₃, CuSO₄, PdCl₂, K₃PdCl₄ and K₃PtCl₄ (data not shown), consistent with results from Neurospora crassa.

![Fig. 5](https://example.com/fig5.png)  
Transmission electron micrographs of an Aspergillus nidulans hypha (A–C) induced to form AuNP by incubation in 5 mM HAuCl₄ for 150 min. (D) AuNP formed in conditioned medium containing 5 mM HAuCl₄. (E) Cross-section of an A. nidulans hypha incubated in 1 mM HAuCl₄. Bar in A = 1 μm; bar in B, D, E = 100 nm, C is same scale. (A–C) Near-median, longitudinal section of an A. nidulans hyphal tip with small globose (small arrowheads) and large flat (black arrows) AuNP in the cytoplasm and adjacent to the wall surface. A cluster of polyhedral AuNP (large arrowhead in A; detail in B) formed associated with the Spitzenkörper region at the hyphal tip. Flat AuNP associated with the peripheral cytoplasm and outer wall surface are seen in cross-section. Tiny globose AuNP (white arrows in B, detail in C) are abundant throughout the cytoplasm. (D) AuNP formed in conditioned growth medium containing 5 mM HAuCl₄ has characteristic shapes from minute dots to large triangular plates. (E) TEM of an A. nidulans hypha induced to form AuNP by incubation in 1 mM HAuCl₄ for 150 min. The cytoplasm contains tiny ~8 to 10 nm AuNPs (white arrows) but most AuNP form clumps on the external surface of the cell wall.

![Fig. 6](https://example.com/fig6.png)  
Spectrum from extended scan SERS map (green pixel), demonstrating the existence of SERS-active AuNP, generated in a fungal colony incubated for 2 h in PDB with 0.5 mM HAuCl₄, pH 4.5. SERS was excited at 785 nm with laser power of 1% (6–10 mW) and exposure time 10 s.
The M$_{4,5}$-edge absorption profiles from the NPs in whole hyphae and from the AuCl$_3$ standard were found to be similar, and matched that of Au metal, confirming their identity as AuNPs (Fig. 2A). Qualitative images, overlaid to compare C and Au at higher spatial resolution, showed that the AuNPs were scattered throughout the mycelium (Fig. 2B and C) in a sample exposed to 5 mM HAuCl$_4$. Quantitative spectral fitting of an AuNP cluster gave a maximum effective thickness of $\sim 2$ μm, with the effective thickness of obvious individual AuNPs being 1.0 ± 0.6 μm. Other shapes, including cubes >1 μm, were observed in the extracellular medium. These dimensions were consistent with the visible images and our later results.

Qualitative carbon images from 100 nm thick sections showed hyphal outlines, but the background from the embedding material and Formvar support film obscured many details, since the 228.2 eV carbon peak can contain smaller contributions from lipid and polysaccharide-like components (Fig. 2D–F). The cytoplasm was a brighter red, suggesting higher relative protein content, whereas the cell wall was dark. Although proteins are found in cytoplasm and walls, Aspergillus cell walls are $\sim$80% carbohydrate. This presumably reduced the 288.2 eV signal, facilitating contrast with the cytoplasm.

Qualitative mapping of Au in thin TEM sections confirmed that AuNPs were located beside, as well as within, hyphae (Fig. 2D–F). Large AuNPs were associated with hyphal walls, and were similar in size and distribution to those from our TEM results. Au was not detected in fungi exposed to 1 mM HAuCl$_4$, likely because it was below the sensitivity limit of our STXM instrumentation.

The UV-Vis spectra of conditioned medium following incubation of A. nidulans with 1 mM HAuCl$_4$ (Fig. 3) showed an SPR band that was consistent with the visible purple-pink colour that developed in these colonies. By 20 min incubation, the UV-Vis spectrum of the growth medium showed a strong SPR peak at $\sim$540 nm, typical of AuNP in the 50–70 nm size range; peak height increased with length of incubation. At times (e.g.: at 40 min, in this particular experiment), a new minor band appeared at longer wavelengths, here $\sim$820 nm. Such bands are attributed to changes in particle size, shape, state of aggregation and local environment.

Fluorescence microscopy and TEM also showed abundant NP formation, including NPs associated with walls and cytoplasm of fungal cells, or distributed freely in the growth medium. Calcofluor stained the lateral walls and septa (Fig. 4) in 5 mM HAuCl$_4$-treated hyphae; as expected, these hyphae had wild type morphology. Non-fluorescent (dark) objects, some of which appeared to be triangular or hexagonal, were associated with some hyphae. Since AuNPs were expected to be dark, i.e.: no stain or fluorescence under these conditions, these shapes were interpreted to be large AuNPs. No unattached extracellular NPs were observed; most likely such NPs were washed away during the staining procedure.

TEM images of hyphae following exposure to 1 mM or 5 mM HAuCl$_4$ showed electron dense structures presumed to be AuNPs. These were located within and adjacent to fungal cells, as well as in the growth medium (Fig. 5). Both 1 mM and 5 mM HAuCl$_4$-treated hyphae had many 8–10 nm AuNPs in their cytoplasm. These were about twice the size of ribosomes, similar in size and with equivalent electron density to chemically
synthesized AuNPs used for immunogold electron microscopy. Following 1 mM (but not 5 mM) HAuCl₄ treatment, A. nidulans hyphae also had clusters of ~20 to 30 nm globose AuNPs that remained associated with the outer cell wall surface after preparation for TEM.

The Spitzenkörper region of a hypha treated with 5 mM HAuCl₄ contained polyhedral AuNPs suggesting that the cell was actively growing when those AuNPs were formed. Similarly, a small cluster of polyhedral AuNPs about 8 µm behind the tip might be associated with a future branching event. Compared to TEM results for control and 1 mM HAuCl₄-treated hyphae fixed at the same time, the cytoplasm in the 5 mM HAuCl₄ treatment was less dense. In 5 mM (but not 1 mM) HAuCl₄-treated cells, AuNPs included flat plates that could be up to a micrometer in length. It is possible that these NP damaged the cell membrane as they grew, perturbing ion gradient homeostasis.

The AuNP shapes in the growth medium (Fig. 5D) are consistent with AuNPs found within cells and on their surfaces in the fluorescence images (Fig. 4). Given the dimensions of cytoplasmic AuNPs compared to the expected pore size of Aspergillus cell walls (~2 kDa), it is most likely that the cytoplasmic AuNPs formed in situ. This is consistent with our earlier suggestion that AuNP formation is a defense or detoxification mechanism.³³,⁴¹

The cytoplasm and exterior of living cells experience very different chemical conditions. Ion concentration in the cytoplasm of a living cell is highly regulated,⁴⁷,⁴⁸ whereas the outside surface of the cell wall is strongly influenced by its environment. Consistent with this, colonies treated with 1 mM HAuCl₄ for 150 min were still able to grow vigorously, whereas colonies treated with 5 mM HAuCl₄ displayed reduced growth after 30 min treatment. It is possible that the polyhedral AuNPs associated with the Spitzenkörper region, which were not seen in extracellular AuNPs, reflect a particular cytoplasmic chemistry. Taken together, we have multiple lines of consistent evidence of AuNP synthesis in A. nidulans hyphae, most likely while they were alive, regardless of concentration or length of exposure.

4.2 AuNPs associated with Aspergillus nidulans hyphae are SERS-active

Colonies of A. nidulans that contained AuNPs, mounted and dried onto glass, showed a range of SERS activity when examined with Raman microscopy. Often, spectra from an extended scan map showed significant SERS activity, for example, in Fig. 6, where signal intensities are on the order of 10⁶ counts. However, spectra from other pixels in this map showed two strong graphite bands between 1300 and 1550 cm⁻¹, indicating that the sample had overheated and burned. Assignment of the observed bands in SERS spectra is challenging. The spectrum in Fig. 6 came from within a mass of hyphae, making it impossible to assign location; given the possibility of partial oxidation, we did not pursue spectral assignment in this case.

While the SERS intensity was several orders of magnitude greater than normal Raman, the extended scan recording conditions were not optimal. Ideally, scans should be rapid, with low laser power, to reduce the possibility of carbonization as well as to increase the efficiency of data collection for large scale studies. A good signal to noise ratio should be obtainable in less than 1 s from SERS-active AuNPs. The Streamline mode in the Renishaw inVia enables a very rapid scan, as fast as ~30 ms laser exposure per pixel, for a single grating setting. Such maps must be taken at several grating settings (about 400 cm⁻¹ each), in order to build up the entire spectrum.

Typically, Streamline images were recorded over several intact hyphae located at the periphery of the colony, such as those shown in Fig. 7. Since this colony was withdrawn from liquid growth medium, the region around the hyphae contains dried exudate, PDB and cellular debris, as well as free AuNPs, based on our other observations and characterization. Most, but not all, SERS hot-spots remained active throughout acquisition of the required series of grating settings. The spectrum of the clean glass displays a broad weak band with maximum ~1400 cm⁻¹. With the exception of a few extra-cellular SERS hot spots, spectra from the extra-cellular region exhibit this same profile, while those coming from hyphae are similar but uniformly elevated across the entire spectral range (Fig. 7, spectra 2 and 3, respectively). The spectra are shown as recorded, without offset, on a common scale. We attribute the elevation in spectrum 3 to broad background fluorescence from hyphae; hence, by processing only for the relative background intensity, we were able to delineate the location of the hyphae in the yellow false-colour image; the individual spectra carry no spectrochemical information.

Spectrum 4 from this series is a genuine SERS spectrum and typifies an ideal situation. The point remained SERS-active throughout the experiment. Bands did not change from image to image at the different grating settings: peaks in the 400–800 cm⁻¹ scan were also present in the 700–1100 cm⁻¹ scan. Absence of peaks was also consistent: there were no bands where the segments overlapped, e.g. ~1300 to 1400 cm⁻¹. This result is particularly promising, since the sample was not heat damaged (no graphite bands).

The spectrum from point 5 illustrates one of the as-yet uncontrolled aspects of SERS imaging. In the first map, the pixel was silent, and continued to be so in segments 3, 4 and 5. However, while segment 2 was recorded, some NP(s) in this region suddenly responded actively, before falling silent again. Notably, this point was also graphite-free, thus carbonization was not an issue. One possible explanation for this event is that the laser beam position shifted slightly during that pass, and the NP was suitably excited on that occasion only. A solution to this problem is to record a much broader segment of the spectrum at one time, but this would require a different detector arrangement. Nevertheless, we conclude that a SERS signal only appears when AuNPs are present, whether in the PDB or the hyphae.

SERS spectra from the hyphae or surroundings do not match the PDB spectrum and must derive from the hyphal components. Assignment of bands from these spectra was attempted, with reference to literature values for common biochemical cell components (Table 1).⁴⁹–⁵¹ In this, it was assumed that the SERS signal was arising from a general enhancement in the vicinity of AuNP; the degree of signal enhancement did not suggest single molecule detection, nor could we identify a likely single source. There are numerous candidates, including bands common to lipids, proteins, sugars and DNA. Currently it is impossible to arrive at a unique solution, since the signals must arise from highly localized components, but the number and type could vary, especially for large biomolecules such as proteins.
We are now developing methods for SERS-active particles. We will also explore the use of such AuNPs with biosynthesis pathways of A. nidulans that have been deleted for one or more genes in the Gal biosynthesis pathway, which affects cell wall structure and composition. Site-specific generation of AuNPs could permit directed high spatial resolution analyses with biochemical specificity.

### 5. Conclusions

We have shown that A. nidulans colonies can generate AuNPs within and adjacent to hyphae. Examination of A. nidulans colonies incubated in HAuCl₄, using light microscopy, UV-Vis spectroscopy, fluorescence microscopy, TEM and STXM, provided complementary identification and characterization. AuNP synthesis is straightforward; NPs have characteristic shapes comparable to those generated by other microorganisms or by chemical reduction. These AuNPs exhibit surface plasmon resonance and can be used as substrates for SERS spectra, enhancing the scattering intensity by several orders of magnitude. Both 1 mM and 5 mM HAuCl₄ treatments produced SERS-active particles. We are now developing methods for better control of NP formation and for assignment of bands, as well as protocols for SERS imaging from AuNPs in living A. nidulans hyphae.

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### Notes and references
