Kinetic Control of Microtubule Morphology Obtained by Assembling Gold Nanoparticles on Living Fungal Biotemplates

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ABSTRACT: Self-assembly of nanoparticles on living biotemplate surfaces is a promising route to fabricate nano- or microstructured materials with high efficiency and efficacy. We used filamentous fungi to fabricate microtubules of gold nanoparticles through a novel approach that consists of isolating the hyphal growth from the nanoparticle media. This improved methodology resulted in better morphological control and faster adsorption kinetics, which reduced the time needed to form homogeneous microtubules and allowed for control of microtubule thickness through successive additions of nanoparticles. Differences in the adsorption rates due to modifications in the chemical identity of colloidal gold nanoparticles indicated the influence of secondary metabolites and growth media in the fungi metabolism, which demonstrated the need to choose not only the fungus biotemplate but also the correct medium to obtain microtubules with superior properties.

INTRODUCTION

The use of soft templates that combine biological structures and inorganic nanoparticles permits the synthesis and large-scale production of advanced nanostructured hybrid systems with high uniformity and reproducibility.1−3 For instance, DNA,4 viruses,5,6 and microorganisms7,8 including bacteria9,10 or fungi,11 present unique structural motifs that are easily and quickly reproduced. In this context, various filamentous fungi have been used as biotemplates to fabricate microwires or microtubules, some of them exhibiting promising optical, electronic,12 and catalytic properties.13,14 Due to their facile handling15−18 and capacity to assemble colloidal nanoparticles,19 these new hybrid materials based on living biotemplates exhibit several potential technological applications. However, there is a lack of information about the best procedures and routing protocols to fabricate such materials.

The need to control properties at the nanometer scale resulted in diverse strategies to fabricate and improve the morphology and homogeneity of nanostructured materials. For this reason, it is necessary to understand the system features in and out of equilibrium,20 especially when the objective is to arrange colloidal nanoparticles on templates.21,22 Different adsorption kinetic models can be employed to explain the kinetics in liquid−solid systems,23 but only recently the Lagergren equation (also known as LFO) was used to correlate experimental data from adsorption kinetics of silver nanoparticles.24 In this study, we report an optimized route for the self-organization of colloidal gold nanoparticles on living filamentous fungi to fabricate microtubules of 2−3 μm thick and lengths exceeding a few millimeters. The process involves the synthesis of gold nanoparticles using the citrate method and the growth of Penicillium brasiliannum, Aspergillus aculeatus, and Xylaria sp. filamentous fungi biotemplates in different culture media. Gold nanoparticles were added in bottles containing mycelial fungi previously grown in culture medium, and these nanoparticles adhered to the surface of the hyphae, covering it in several layers to form microtubule structures. We evaluated the influence of the three fungal species and culture media on the adsorption time of the particles, tube diameter, tube wall thickness, and length of the wires. Unlike previous studies that focused only on the properties of the final hybrid material, we paid special attention to correlate the adsorption kinetics with the morphological characteristics of the microtubules.

RESULTS AND DISCUSSION

Self-assembly of nanoparticles using living biotemplates is a promising route to fabricate nano- or microstructured materials with high efficiency and efficacy.25−28 Many researchers used fungi as templates to build functional hierarchical structures by introducing spores directly into a colloidal suspension of nanoparticles. Although it is the most simple and facile way to promote self-organization of nanoparticles on fungal mycelia,19

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such a process entail a slow hyphal growth and frequently results in large heterogeneities and microtubules of variable diameters. On the other hand, we obtained better morphological control isolating the hyphal growth from nanoparticle presence. This improved procedure reduced the time necessary to form gold microtubules to intervals of 10–22 days, which are significantly shorter than the 90 days usually required for other methodologies. Obviously, the time requirement depends on the fungi species and culture media. For this reason, we evaluated the influence of three species of fungi in different culture media on the time required to form microtubules, analyzing the tube diameter, wall thickness, and tubule length as comparative variables.

Sporals of Aspergillus aculeatus and Penicillium brasiliann and a slice of Xylaria sp. were inoculated at room temperature in different culture media, i.e., CZAPEK (modified DSMZ 130, without agar), CZAPEK-yeast extract, and potato dextrose (PD), whose detailed compositions are described in Table 1.

Table 1. Chemical Composition of Culture Media Used in Cultivation and Growth of Aspergillus aculeatus, Penicillium brasilianna, and Xylaria sp. Fungi

<table>
<thead>
<tr>
<th>compd</th>
<th>CZAPEK mass (g)</th>
<th>CZAPEK-yeast extract mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2HPO4</td>
<td>0.005</td>
<td>K2HPO4 0.005</td>
</tr>
<tr>
<td>NaNO3</td>
<td>1.0</td>
<td>NaNO3 1.0</td>
</tr>
<tr>
<td>MgSO4·7H2O</td>
<td>0.25</td>
<td>MgSO4·7H2O 0.25</td>
</tr>
<tr>
<td>KCl</td>
<td>0.25</td>
<td>KCl 0.25</td>
</tr>
<tr>
<td>FeSO4·7H2O</td>
<td>0.005</td>
<td>FeSO4·7H2O 0.005</td>
</tr>
<tr>
<td>dextrose</td>
<td>15.0</td>
<td>dextrose 15.0</td>
</tr>
<tr>
<td>yeast extract</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>PD, volume 500 mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

100 g of chopped potatoes cooked in the microwave for 20 min with 200 mL of water, kneaded, and filtered. When it was filtered, dextrose is added and the solution is diluted until 500 mL.

Figure 1 compares the growth rates of these different fungi by detailing the time needed in each step to form the hybrid material, including (i) hyphal growth, (ii) particle deposition, and (iii) lyophilization. The images show typical macroscopic aspects at these distinct moments. The first image shows germinated spores forming a mycelial mass that became visible after approximately 24 h, the second image shows gold nanoparticles forming microtubules on the fungus template, and the last image shows the microtubules completely formed during lyophilization. The time required to form microtubules was different for each fungus, which is likely related to their specific metabolisms and the presence of different metabolites. The shortest time (5 days) was achieved using Penicillium brasiliann and the longest (15 days) occurred with Aspergillus aculeatus. Exploratory experiments showed nanoparticles forming microtubules on living fungi, confirming the role played by metabolites in the mechanism of nanoparticle deposition.

Parts a–e of Figure 2 illustrate the changes in color intensity of gold colloidal suspensions during the formation of microtubules on Penicillium brasiliann. The first image shows the turbidity of growth medium free of nanoparticles due the presence of germinated spores after 3 days of inoculation, while parts b–e show media over a period of 5 days after adding gold nanoparticles. The presence of gold nanoparticles can partially inhibit fungal growth and strongly attenuate the formation of fungal biofilms. For this reason, we separated the hyphal growth from the deposition step (Figure 1). Fungal vegetative growth also varies when the medium is modified. Filamentous fungi exhibit variable responses to fungal strain and growth media composition, which includes the amount of available nutrients. In fact, the addition of different carbon supplements (e.g., glucose, sucrose, or xylose) or nitrogen sources (nitrates or ammonium inorganic salts) to the basal growth media significantly affects fungal biomass dry weight (BDW) and can alter the production of metabolites. Evidently, a contrary result is also possible, since the addition of other substances commonly found in nature can suppress glucose uptake in some fungi species, which in consequence reduces their BDW. There is evidence of environmental physical–chemical signals that trigger the secretion of hydrophobin proteins capable of modifying the surface tension of fungus hyphae. Apparently, excess or deprivation of nutrients around the fungus determines the metabolic pathway that activates specific fungi genes, resulting in an amphiphatic film of hydrophobins at the hyphal surface, which is probably responsible for the attachment of gold nanoparticles. Due this intricate mechanism, the influence of growth media on the nanoparticles deposition rate cannot be neglected.

Colloidal gold nanoparticles exhibit a characteristic plasmon absorption band at 520 nm. By use of the Lambert–Beer law (eq 1, where A is the absorbance, ε is the extinction coefficient, b is the optical path, and C is the concentration of nanoparticles), the absorbance intensity of this plasmon band provides some insights about the concentration of nanoparticles in suspension.
Although the accurate calculation of molar concentration of nanoparticles is not a trivial issue, for the present purpose is enough to consider that the absorbance is linearly proportional to the amount of gold nanoparticles at a given moment and it can be used as a relative value of concentration. For instance, the purple color intensity decreases as long as the nanoparticles leave the colloidal medium to form microtubules, almost disappearing after no more than 1 week (Figure 2f). Therefore, the relative concentration of gold nanoparticles in suspension could be obtained from the maximum intensity value of absorbance at any moment, from 100% at the beginning to 0% after a few days, and the amount of deposited gold on fungus can be estimated from the difference of absorbance values between two distinct moments. On the other hand, fungus growth is much slower than the covering rate and could be neglected, resulting in a covering rate that depends only on the nanoparticle concentration. This pseudo-first-order kinetic behavior suggests that wall thickness can be adjusted by the amount of nanoparticles available. The Lagergren equation is commonly used to describe liquid-to-solid adsorption processes that follow pseudo-first-order kinetics, although it has not yet been applied to systems based on biotemplates. In this equation (eq 2), which results in the well-known integrated rate law (eq 3), the adsorption rate is proportional to the amount of nanoparticles in suspension and limited by the adsorption capacity of the adsorbent. This condition is expressed by the \((q_e - q_t)\) parameter, where \(q_e\) and \(q_t\) are the amount of
nanoparticles adsorbed at equilibrium and at time \( t \), respectively.

\[
\frac{dq}{dt} = k(q_e - q_t)
\]

(2)

\[
\ln(q_e - q_t) = kt
\]

(3)

Despite the complexity in determining the experimental \( q_e \) value, \((q_e - q_t)\) is proportional to the difference between the absorbance intensity measured immediately after inserting gold nanoparticles in the medium and that collected at different moments during the deposition process. At first approximation, it is possible to plot \( \ln(I_e - I_t) \) versus time to obtain a linear fit that describes the pseudo-first-order adsorption kinetics (eq 3) responsible for the formation of microtubules on the surface of fungus templates (Figure 2g). Its linear adjustment showed a good coefficient of determination \((R^2 = 0.988)\) which allowed us to estimate a half-life of \( t_{1/2} = 1.9 \) days. Although we did not identify any evidence of segregated gold, it is possible that a small amount of gold nanoparticles was deposited directly on the surface of glass flasks within the experimental error.

Due the rate law expressed in eq 1, diluted colloidal suspensions slowly form thin microtubules while concentrated suspensions quickly form thick walls. Moreover, thicker microtubules can be achieved by supplying new nanoparticles at fixed time intervals, as illustrated in Figure 3. Images a–d show microtubules 80–500 nm thick after four cycles over 110 days. This multistep procedure allows control of the microtubules morphology, including fabrication of multilayers of different composition.

Figure 4a shows the average thickness of microtubules formed on Penicillium brasilianum as a function of time. Instead of a constant coverage rate, the sigmoid curve suggests nanoparticles first form a thin layer over the hyphae, which is thickened posteriorly. This mechanism is better understood in the context of the pseudo-first-order rate law of covering. At the beginning, the deposition rate is fast due the high concentration of colloidal nanoparticles in suspension. At this moment, fungal hyphae are uncovered and, consequently, nanoparticles quickly spread out on the entire fungus forming microtubules of thin walls. In the last stage, on the other hand, the deposition rate is lower than that in the beginning and the reminiscent nanoparticles slowly self-assemble on the microtubules layer-by-layer, homogenizing the microtubules thickness. The key to obtaining uniform microtubules is the final step of microtubule exposure to diluted nanoparticle suspensions. However, some inherent variability common in biological materials could be observed over the microtubules. For instance, the crosshatched area in Figure 4b exhibits superior and inferior limits of microtubules thickness growth on Aspergillus aculeatus, which ranged from 60 to 140 nm after 10 and 130 days, respectively. This likely occurred because the mycelium continues to grow even in the presence of gold nanoparticles, fed by the citrate used to stabilize the colloid. These new surfaces introduced some growth delay that was responsible for the presence of new microtubules thicker than those already present (Figure 4d).
Scanning electron microscopy (SEM) images of a fractured microtubule (Figure 5a) and of an isolated microtubule border (Figure 5b) confirm its hollow nature, revealing morphological details of the cavity occupied by the fungus and of the multilayered wall constituted of gold nanoparticles. Figure 5c shows a representative small area of the fungus covered with a uniform coating of gold nanoparticles, from which a particle size distribution histogram could be constructed and the average size (13.1 nm) calculated from at least 500 nanoparticles.

Higher magnification images (c–f) confirm nanoparticles forming a uniform surface on the hyphae without any visible cracks.

Reproducibility and uniformity are fundamental aspects of any technology related to nanostructured materials. The homogeneous density of the gold signal in EDS element mapping (Figure 7c) confirms microtubules of uniform thickness. This effective uniformity at the nanometer scale is essential for fabricating nanodevices for biomedicine, molecular biology, biochemistry, catalysis, and SERS spectroscopy.

Microtubules with controlled diameters were achieved combining fungi and different growth media (Figure 8). Although growing Penicillium brasili anum in CZAPEK and PD media resulted in microtubules of similar diameters, other fungi exhibited different results, indicating the influence of the growth medium in fungi metabolism. This demonstrates the necessity to choose not only the fungus but also its correct medium to obtain a specific microtubule morphology.

Sabah et al. proposed that the fungus consumes colloidal stabilizing agents to grow, destabilizing the nanoparticles in suspension and causing them to aggregate on the hyphae surface, forming microtubules through successive layers of nanoparticles. However, this does not explain the sigmoidal curve of Figure 4a that implies some active role of the live fungus. Our best hypothesis suggests that the first layer of gold nanoparticles interacts strongly with the fungus surface through covalent bonds between the metal and the functional groups on the cells that constitute the fungus mycelia, while subsequent layers are deposited due to the self-assembly of nanoparticles driven by primary or secondary metabolites excreted by live fungi. Filamentous fungi produce a wide range of natural products, usually small molecules that mediate the interactions between them and the environment providing nutrients, communication between microorganisms and exerting selective toxicity against other microbes. Filamentous fungi excrete metabolites in response to environment stress, including metal nanoparticles. In fact, gold and silver nanoparticles adversely affect microorganism growth and mycelium composition, which can explain the faster fungi growth in nanoparticle-free media. Therefore, it is plausible that gold nanoparticles trigger some fungal biochemical response to produce secondary metabolites responsible for nanoparticle deposition, which opens a new road to improve nanoparticle self-assembly techniques for the production of superior hybrid materials such as microtubules, surfaces decorated with particles, and nanocomposites.

CONCLUSIONS

Homogeneous microtubules of controlled diameters and thickness were obtained by depositing gold nanoparticles on the surface of fungi biotemplates using a fast methodology that combines different fungi species and growth media isolated from the presence of colloidal nanoparticles. Differences in adsorption kinetics indicated the influence of secondary metabolites on the deposition process, due to modifications in the chemical identity of the colloidal nanoparticles. The final hybrid material resembled the biotemplate morphology, which makes the fungi and growth media choices critical decisions in producing hybrid materials at large scales with a high degree of reproducibility and efficacy.
EXPERIMENTAL SECTION

Reagents were analytical grade and used as received. All hybrid materials, including the gold colloidal suspensions, were prepared inside a laminar flow chamber strictly sterilized with sodium hypochlorite, ethanol (70%), and UV−vis light. Gold nanoparticles (AuNP) were synthesized from 100 mL of an aqueous solution of HAuCl₄ (1.0 mmol·L⁻¹, 99.9% Sigma-Aldrich) at 95 °C that was prepared with deionized water.

Figure 6. SEM images at different magnifications of microtubules obtained using the fungus *Penicillium brasilianum*. (a, b) The fungal hyphae form a homogeneous network with (c, d) a massive coating of gold nanoparticles as seen in the XRD pattern of gold nanoparticles (e), forming well-structured multilayers on the fungal wall. (f) Even after interacting with the fungal wall, the particles retain their nanospherical shape, without cracks or heterogeneity along the hypha.

Figure 7. SEM images and EDS mapping in 2D of silicon (green) and gold (red) on the hybrid material formed using *Penicillium brasilianum*. (a, c) A region containing the microstructures was selected, and the EDS mapping in 2D presented a dense quantity of gold distributed throughout the hyphae. (b) In contrast, the substrate is identified under the hyphae with the false color green.
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was removed using a peristaltic pump, leaving  

only the mycelial mass of fungus in the  

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in culture media in each Erlenmeyer  

Each bottle received 25 mL of the culture medium, with  

dextrose) were distributed in Erlenmeyer  

three culture media containing CZAPEK (modi  

ed DSMZ 130, without agar), CZAPEK-yeast extract, and PD (potato  

flasks were distributed in Erlenmeyer flasks and autoclaved at 120 °C for 15 min, resulting in nine vials of each medium. Each bottle received 25 mL of the culture medium, with compositions shown in Table 1. The Erlenmeyer flasks were sealed to avoid any external contamination. After the vials were cooled to room temperature, they were placed in a laminar flow hood. Once cooled, an amount of 200 μL of a suspension containing fungal spores of Aspergillus aculeatus and Penicillium brasilianum (previously prepared) was inoculated in the three culture media in each Erlenmeyer flask. The Xylaria sp. fungus does not sporulate, and thus the inoculation in culture medium occurred by cutting small pieces of the fungus colony from the agar plate added to the culture medium. This is the initial step of growth and development of the fungus, and the flask were stored in sterile conditions and protected from light.

After 2–3 days, parts the mycelial mass grew in the solution and on the surface. In this step, the surface of the mycelial mass was removed and the culture medium was diluted with autoclaved deionized water to a total volume of 100 mL. This step was performed to stabilize the fungus growth and avoid the development of fungus hyphae outside the culture medium. During 4–6 days after the spore inoculation in the culture medium and entangled hyphae were formed, the diluted culture medium was removed using a peristaltic pump, leaving only the mycelial mass of fungus in the flask. Into this flask was added 100 mL of gold colloid as previously prepared. Over the next 5–20 days, AuNP adhered to the fungal walls in multiple layers to form uniform AuNP microtubules. In a second experiment, the colloidal suspension was refreshed after 5 days several times with a new colloidal suspension so that the fungus remains exposed to a nearly constant particle concentration. The colloidal suspension was removed, leaving only the fungus in the Erlenmeyer flask. Scanning electron microscopy (SEM) was performed with the fungus in nature, without previous dehydration.

Gold nanoparticles and hybrid materials were characterized by X-ray diffraction (Rigaku Dmax 2500PC diffractometer) with Cu Kα radiation in the 2θ range of 20–120° operating at 40 kV and 40 mA. To collect the patterns, nanoparticles were deposited on silicon substrates by dripping the aqueous colloidal suspension onto the substrate at room temperature and waiting for solvent evaporation. Spectra of gold nanoparticle suspensions were obtained from 190–800 nm with a UV–vis spectrophotometer (Shimadzu Multispec 1501) using a commercial quartz cuvette. Scanning transmission electron microscopy (STEM) images of nanoparticles were recorded at 20 kV (FEG Zeiss Supra 35-VP), where the colloid was pinged on support film Cu grids of 400 mesh (PELCO) with the aid of a pipet and dried at room temperature. The scanning electron microscopy (SEM) images of nanoparticles and hybrid material microtubules were recorded with a FEI Company XL30 FEG instrument, where samples were deposited on a silicon metal plate (111) with the contact being the sample and the support was carried out with conductive inks based on silver (Degussa). Histograms were constructed using the public domain ImageJ image processing software.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES


