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Are microorganisms indispensable in green microbial nanomaterial synthesis?

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The typical microbial synthesis relies heavily on microbial alchemist. In this work, we demonstrate a conceptually new greener strategy using microorganism culture aqueous broths alone for nanosynthesis by eliminating the complication of manipulating genetically engineered microbes and maintaining the cell culture. The versatile method is exemplified by the preparation of gold nanoparticles and reduced graphene oxide.

Most conventional methods for nanosynthesis might associate with contaminations from precursor chemicals, organic solvents or hazardous by-products, which often raise environmental concerns. Hence, there is a significant benefit in developing nontoxic and environmentally benign processes for nanoparticle synthesis. It is well known that nanoparticles can be produced by either uni- or multi-cellular organisms or from agricultural biomass. These biological entities have successfully been used to produce biogenic nanomaterials such as magnetite, silica, gold, silver, and quantum dots. Although microbiological methods are considered environmentally-friendly, they suffer from time-consuming microbe screening, plasmid construction and the variability of the cell culture process. In addition, when scaling up, microbial nanoparticle synthesis cannot provide sufficient control on particle size, morphology and monodispersity. As the microorganisms can only tolerate a limited regime of pH, temperature or toxic ion concentration, the synthesis conditions need to be very carefully controlled when living organisms are present in the broth. Moreover, in order to release the resultant intracellular nanoparticles, downstream operations like ultrasonic breakdown, detergent disintegration, high temperature calcination and freeze-thawing processes are normally required. These costly downstream processes are highly energy intensive and time consuming therefore exert a significant barrier for scaling up. To circumvent these limitations, extracellular nanoparticle synthesis has been attempted in cell-free extract of certain special microorganisms. For example, Das and co-workers have prepared multi-shaped gold nanoparticles by a protocol starting from culturing fungal strain Rhizopus oryzae in potato dextrose (PD) broth first, then separating the fungal biomass and finally employing the supernatant to reduce chlorouric acid. The composition of PD broth as well as other chemically defined media that have been frequently used for microbial nanosynthesis is listed in Table 1. Their common ingredients are found to be yeast extract, dextrose, peptone and other digested proteins, etc.

Table 1. The individual ingredients concentration (g/L in water) of broths employed in our synthesis and other researchers’ microbe culturation.

<table>
<thead>
<tr>
<th>Broth Name</th>
<th>Dextrose</th>
<th>Yeast Extract</th>
<th>Peptone</th>
<th>Other ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Mold</td>
<td>10.0</td>
<td>3.0</td>
<td>5.0</td>
<td>3.0[^a]</td>
</tr>
<tr>
<td>Tryptic Soy Broth</td>
<td>2.5</td>
<td></td>
<td></td>
<td>27.0[^b]</td>
</tr>
<tr>
<td>Lysogeny Broth</td>
<td>5.0</td>
<td></td>
<td></td>
<td>15.0[^c]</td>
</tr>
<tr>
<td>Potato Dextrose Broth (ref.10)</td>
<td>20.0</td>
<td></td>
<td></td>
<td>200.0[^d]</td>
</tr>
<tr>
<td>Streptomyces Broth (ref.27)</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
<td>12.0[^e]</td>
</tr>
</tbody>
</table>


Yeast extract is concentrated from autolyzed Saccharomyces cerevisiae. S. cerevisiae cells have been employed to synthesize TiO_2 nanoparticles and CdTe quantum dots in high yield. With its high content of B vitamins, Tyrosine (1.2%, total amino acids) and other unknown peptides, the yeast extract itself has potential in reducing gold ions. Dextrose is a well-known agent for green synthesis of AuNPs within the size range of ~ 8 - 120 nm. Despite the progresses achieved by employing these green reactants, the conventional microbial studies overly rely on manipulating the specific microbes rather than exploiting the microbe culture broths,
and therefore, the promise of microbial nanoparticle synthesis remains to be fully realized.

Here we report the greener biosynthesis of gold nanoparticles and reduced graphene oxide in the absence of any live microbes (as shown in Fig. 1). The effects of broth composition, pH value, precursor concentration and temperature on the nanoparticle formation have been carefully evaluated. More importantly, another objective of this work is to alert the researchers the fact that when the spent medium is employed in extra-cellular nanobiosynthesis, the synchronous reduction by dextrose, yeast extract, vitamins, peptone and other digested proteins should not be ignored. A properly designed control experiment is vital in judging the necessity of microbial entities. Hopefully, this study will promote deeper understanding in biogenic nanomaterial formation mechanism.

Fig 1 Schematic representation of the greener synthetic strategies versus the conventional microbial nanomaterial synthesis (the paths are marked by green and red arrows, respectively). As displayed inside the green line, nanoparticles of a wide range (c) can be produced by simply mixing the precursors in water (a) and the autoclaved at 121°C for 15 min prior to further reaction. To study the pH-dependent reduction of AuNPs, HCl or NaOH was added to get the desired pH values. In a typical experiment, 5 mL aqueous solution of HAuCl₄ (2 mM) was combined with 5 mL YM broth of varying pH levels. To mimic the in vivo microbial conditions, the reactions were performed at 37°C in an orbital shaker (100 rpm). As expected, the colour of the solution changed dramatically from yellow to pink within 2 h due to the formation of AuNPs. The evolution of AuNPs was recorded by time-course UV-Vis spectra (Supplementary Fig. S1). In mildly basic broths (pH 8-10) the reaction was extremely slow. Broth at pH 6 offered faster particle growth; however, AuNPs were not stable and aggregation occurred as indicated by the broadening and 15 nm red-shift of surface plasmon resonance (SPR) bands over 18 h. At a pH 12, the reaction was also accelerated and a highly stable AuNP formation was evidenced by the absorption peak centered at 523 nm (Fig. 2a). The particle size and size distribution of the resultant particles were investigated by dynamic light scattering (DLS, Supplementary Fig. S2). The particle diameters at the reaction time of 4, 8 and 24 h were 18, 20 and 21 nm respectively and the corresponding polydispersity index (PDI) was 0.183, 0.150 and 0.145. The prepared nanoparticles (in pH 12 YM broth) exhibited excellent stability for at least 6 months under ambient conditions including daily exposure to room light.

Fig 2 a, UV-vis absorbance spectra of the reduction of 1 mM gold ions in yeast mold broth (pH 12) at 37 °C demonstrating the time-dependent kinetics of greener synthesis of AuNPs. b, XRD pattern of AuNPs formed in the system. c, Typical EDS spectrum of the AuNPs prepared at room temperature and recorded in spot profile mode. d and e, SEM and TEM micrograph of quasi-spherical AuNPs prepared with 1 mM [Au³⁺] at 37°C. f, High-resolution TEM image from a region in e. g and h, SEM and TEM image of Au nanoflowers formed after centrifugation of AuNPs with initial [Au³⁺] concentration at 2.5 mM. The inset shows four samples with increasing [Au³⁺] after a 4 h reaction at 37°C. i, SEM image of Au nanoflowers formed with initial [Au³⁺] concentration at 5.0 mM and re-dispersed in ethanol. j and k are TEM images of Au nanoparticles.

The precursor concentration has a profound influence on the size and morphology of AuNPs. By fixing the pH value at 12 but altering the [Au³⁺] from 0.5 to 5.0 mM, we found the colour of Au hydrosols containing various concentrations of Au ion was distinguishingly different (Fig. 2 inset). Increasing the concentration of the precursor led to larger particle size. The AuNPs could be extracted by centrifugation at 8000 rpm and be readily re-dispersed in Deionised (DI) water by hand-shaking the centrifuge tube without obvious irreversible aggregation. The recovered AuNPs were analysed by XRD and TEM. Fig. 2b gives five peaks at 20 of 38.2, 44.4, 64.6, 77.5 and 85, respectively, corresponding to (111), (200), (220), (311) and (222) Bragg reflections of face-centered cubic metallic gold.
The facile particle synthesis at ambient temperature is favourable to large scale application due to the easy operation and the reduced energy consumption compared to high temperature preparation. As such, we explored the feasibility of AuNPs formation in YM broth at room temperature (22 ± 3 °C). AuNPs growth was confirmed by a series of UV-Vis extinction spectra albeit the growth of nanocrystals was relatively slower in comparison with the facilitated kinetics of the particle formation at 37°C. AuNPs with the narrowest polydispersity (PDI = 0.048) was obtained as revealed in TEM images (Supplementary Fig. S3). Fig. 2c presents the energy dispersive spectroscopy (EDS) spectrum of the particles. A strong gold nanoparticle characteristic peak at 2.195 keV was clearly spotted. All these data provide the evidence that nanoparticles can be obtained under milder conditions. Noticeably, flower-shaped AuNPs (AuNFs) were observed by adjusting the initial [AuCl₄]²⁻ at 2.5 mM. Several strategies focusing on multistep⁹ or template-mediation¹⁰,²¹ were reported to synthesize gold nanoparticles with spike morphology. A facile, one-pot, template-free synthesis of flower-like AuNPs (AuNFs) was reported by Xie’s group.²³ Interestingly, in this work, the anisotropic growth of Au nanocrystals was also observed at high Au³⁺ concentration. Fig. 2h depicts the TEM image of AuNPs after 48 h reaction at room temperature. The particulates have a solid core with short spikes. The dimension of the AuNFs branch is 10 nm in diameter and 15 nm in length. The z-average diameter by DLS was 54.4, 70.6 and 86.3 nm at 4, 8, and 24 hours, respectively, with the SPR peaks at 556 nm. It is remarkable that Au nanoparticles were formed after re-dispersing the harvested gold nanoparticles (synthesized using initial [AuCl₄]²⁻ = 5 mM, room temperature and a 48-h incubation period) in ethanol. High-yield preparation of single-crystalline gold nanoparticles has been achieved by heating a concentrated aqueous solution of linear polyethyleneimine and HAuCl₄ at 100 °C.³³ Similarly, the formation of octahedral gold has been linked to the interaction of Au³⁺ with various microorganisms including bacteria²⁵, the cell-free extract of a fungus³⁶, and algae extracts.²⁰ Our method features obviously simple implementation and provides high yields of single-crystalline nanoparticles.

Considerable efforts have been directed toward the mechanism understanding of the microbial nanoparticle synthesis. In general, the driving force of particle formation is related to detoxification of various microorganisms. Although it is unequivocally acknowledged that ATPase, especially the species-specific NADH-dependent nitrate reductase, contributes to nanoparticle reduction, the fundamental role of native enzymes continues to be debated within the scientific community. For example, no AuNPs were observed in heat-denatured cell-free extract of the fungal strain Rhizopus oryzae.⁶, however, Au³⁺ formation at appreciable rates was identified in the heat-treated cell-free filtrate of Trichoderma koningii.²⁷ Based on the fact that the broths that we used were still active in producing NPs despite being autoclaved at 121 °C for 15 min, we support the latter declaration that native enzyme structure does not play an indispensable role in the biogenic process.

Based on previous reports, we speculate that plentiful reducing and capping agents in the broth like dextrose and S. cerevisiae yeast extract are capable to synthesize metal nanoparticles without the involvement of living organisms. To verify this hypothesis, we tested the possibility of using pure dextrose, yeast extract (YE) and peptone to reduce Au ions, since they are the common ingredients of several defined nutrient broths. As shown in Fig. S4 and S5, Au ions were successfully reduced by these respective biological molecules. Noteworthy is that dextrose at alkaline pH range demonstrated a faster reducing rate as compared to previous reactions undertaken at pH 7. At neutral pH, it took 1-6 h to hours to complete the reduction under mild conditions.¹⁷ Surprisingly, Au NPs were formed in seconds without agitation in solution of dextrose at pH 12 (Supplementary video). The comparison of FTIR spectra (Fig. S6) between pure dextrose and AuNPs reduced in pH 12 dextrose (0.25% in weight) reveals the intimate association between hydroxyl group of dextrose and Au NPs surface (the strong absorption band of OH stretching mode shifted from 3251 to 3407 cm⁻¹). This resultant fast production rate is highly beneficial to scale up the nanoparticle synthesis. Unlike other chemical methods that can realize rapid nanoparticle formation in continuous flow reactors,²⁸ large-scale biological synthesis of nanoparticles has been restricted by the slow reaction rate. In this context, the discovery that dextrose can be used as an extremely strong reducing agent for immediate nanoparticle synthesis provides an ideal candidate for continuous operation mode.

The AuNPs formation in yeast extract (YE) was slower as compared to dextrose (pH 12) reduction. After stirring the mixture at room temperature for 3 days, the average AuNPs size was 44 and 200 nm when YE concentration increased from 0.5% to 1% by weight. Interestingly, a black gold colloidal solution was formed in 1% YE solution after a 24 h reaction. A similar phenomenon was observed when a secondary metabolite secreted by Delftia acidovorans was incubated with AuCl₄⁻. The authors demonstrated that a nonribosomal peptide acted to generate AuNPs. In this study, we assume peptide bonds from YE might lead to the growth of AuNPs.

FTIR spectroscopic measurements were carried out to detect the functional groups contributing to the AuNPs synthesis in YM broth. The major functional groups of pure yeast extract at 1579 cm⁻¹ (Amide II, N-H and C-H vibrations of the peptide bond in different protein conformations) and 1399 cm⁻¹ (C=O of COO⁻ symmetric stretching in proteins)²⁹ were observed in the centrifuged and washed AuNPs reflecting the bonds of amino acids (Fig. S7). These results confirmed the critical roles played by the YE and dextrose to be efficient reducing and stabilizing agents.

Synthesis of AuNPs in other typical microbial culture broths was performed to further confirm the proposed formation mechanism. Lysogeny broth (LB) is one of the most common media for cultivating recombinant strains of E. coli.³⁰ Tryptic soy broth (TSB) is another general purpose medium for the cultivation of a wide variety of microorganisms. Again, stable Au nanoparticles were formed in LB and TSB at pH 12. Although the same experimental conditions were used for these broths, the gold sol formation was drastically slower in LB and TSB broth, contrast with YM broth. The broth dependence of AuNPs formation rate may be explained by the decreasing dextrose concentration in YM, TSB and LB (1 %, 0.25 % and 0 %, respectively). Similarly, in a biosynthesis study of Au and Ag NPs by a novel marine strain of Stenotrophomonas, the authors found the particles were formed in Streptomyces broth (SB) only but not in Luria broth (LB).³¹ Although the SDS-PAGE analysis of extracellular media supernatant disclosed three low molecular weight proteins (~36.9, ~17.6 and ~14.9 kDa) merely in SB and thus suggested a potential involvement of these secretory proteins in particle synthesis, the important contribution from dextrose and reduced sugars maltoses (0.4% and ~ 1.0%, respectively in SB only) should also be considered to better understand the nanobiosynthesis mechanism.

With all these concerns, we propose the new formation mechanism of gold nanoparticles in pH 12 of dextrose containing broth as follows. Similar to Au⁺ reduced by NaBH₄ and stabilized by sodium citrate, the growth process starts with rapid reduction by dextrose followed by a nucleation process of gold clusters which undergo coalescence processes until the final size is reached, the process of protein stabilization.
In order to extend the application of graphene from electronic devices to biomedical fields, a few green strategies have been attempted recently to reduce graphene oxide (GO) with less contaminants.\textsuperscript{31} \textit{Shewanella} species represent an important family of GO-reducing bacteria.\textsuperscript{32} Extracellular electron transfer (EET) network was suggested to involve the GO reduction.\textsuperscript{33} We demonstrate here that YM broth (pH 12) alone could reduce GO even at room temperature, although a higher reduction rate was achieved in 15 min at 121 °C in an autoclave, as observed by the colour change of the graphene oxide dispersion. The as prepared sample was characterized systematically by UV-vis to confirm the formation of rGO sheet. As shown in Figure 3a, the absorption maximum at about 230 nm due to the π → π* transition of aromatic C-C bonds in GO was found to continuously shift to about 260 nm with the increase of reduction temperature, suggesting the restoration of π-conjugation network within the graphene sheets.\textsuperscript{34} Raman spectroscopy reveals that rGO has much narrower Raman peaks than the sample. Moreover, an increase in the intensity ratio of D band (located at 1298 cm\textsuperscript{-1}) and G band (located at 1574 cm\textsuperscript{-1}) was observed, indicating the new domains of conjugated carbon atoms (bonded in sp\textsuperscript{2} hybridization) were formed accompanying the removal of the oxygen containing group, which is consistent with previous reports on GO reduction.\textsuperscript{35,36}

AuNP decorated rGO has become a new class of surface enhanced Raman scattering (SERS) substrate for trace detection of heavy metal ions.\textsuperscript{37,38} One-pot facile synthesis of Au/rGO nanocomposites is realized by employing our strategy. A comparison of the Raman spectra of Rhodamine 6G chloride (R6G), R6G on AuNPs and R6G on Au/rGO nanohybrids.

![Image](https://example.com/image1.png)

**Fig 3 a**, Colour photographs show temperature dependence of graphene oxide reduced by yeast mold broth. **b**, UV-vis spectra of rGO prepared at room temperature (18 h), 37 °C (8 h), 121 °C (15 m), respectively. **c**, Raman spectra of graphene oxide (GO) and reduced graphene oxide (rGO). **d**, SERS spectra of pure Rhodamine 6G chloride (R6G), R6G on AuNPs and R6G on Au/rGO nanohybrids.

Based on microbial green chemistry perspectives, material scientists are more focusing on the nanosynthesis using specific organisms as nanofactories. According to our knowledge, we are the first to take the microbial approach a leap further to employ the commercially available broth as starting materials to prepare multishaped gold nanomaterials as well as semiconductor material like reduced graphene oxide. Although the reduction mechanism seems straightforward, the results of this study highlight the important synchronous reduction by various broths that have been ignored for a long time.

**Conclusions**

In this study, we have presented a new concept for the greener synthesis of gold nanoparticles and reduced graphene oxide by using chemically defined microbe culture media alone without the involvement of microbes. Thus, the requirement of a highly laborious microorganism screening, cultivating and the complex down-stream separation processing can be eliminated. The capability to flexibly tune the particle size and morphology adds an extra advantage to this methodology. We are here advocating a more straightforward and economically viable approach by minimizing the use and generation of substances, minimizing the energy/time for the process, which are in line with the twelve principles of green chemistry formulated by Paul Anastas and John Warner. We are also using this article to alert researchers in microbial nanosynthesis field to carefully design control experiments to avoid misunderstanding of the microbial synthesis mechanisms and realize the implementation of complete green methods to fabricate technologically important nanomaterials.

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**Notes and references**

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