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Biosynthesis of Gold Nanoparticles by Foliar Broths: Roles of Biocompounds and Other Attributes of the Extracts

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Abstract Biosynthesis of nanoparticles has arisen as a promising alternative to conventional synthetic methodologies owing to its eco-friendly advantages, and the involved bioprotocol still needs further clarification. This research, for the first time from the standpoint of statistics, confirmed an electrostatic force or ionic bond-based interaction between the chloroauric ions and the involved bioconstituents and manifested that reducing sugars and flavonoids were both important reductants responsible for conversion of Au(III) to Au(0). The result also demonstrated that the proteins were not the reducing agents, yet they might be protection agents in biosynthesis of gold nanoparticles (GNPs). Besides, a significant linear relationship was found between the anti-oxidant ability of the foliar broths and their capability to reduce Au(III) into Au(0). Furthermore, the preliminary investigation based on the boxplot on the size/shape distribution of the biosynthesized GNPs revealed that gold nanospheres with higher degree of homogeneity in size tended to be promoted by foliar broths containing higher content of reducing sugars/flavonoids and proteins. Otherwise, i.e., for those broths with lower content of the above biocompounds, sphere GNPs of wider size distribution or even gold nanotriangles tended to be fabricated.

Keywords Foliar broths · Biocompounds · Biosynthesis · Gold nanoparticles · Statistical

Introduction

Nanotechnology owing to its promising applications has received tremendous attention in the past decades. As building blocks in nanotechnology, various methods [1–3] have been developed to fabricate nanostructures of well-defined compositions. However, conventional physical and chemical methods either are energy intensive or impose environmental hazards due to toxic solvents or additives as well as hazardous by-products. Hence, it is of great interest to develop environmentally benign alternatives, among which biological systems arise as a typical instance. In 1999, Klaus et al. [4] initiated the biosynthesis of Ag nanoparticles (NPs) by *Pseudomonas stutzeri* AG259, and the shift from bacteria to fungus was leaded by Sastry et al. [5–7]. However, in addition to the delicate culture and storage, subsequent processing of NPs formed by intracellular biosynthesis is generally difficult, and microorganisms used for the extracellular biosynthesis of NPs must be extensively screened [8]. In recent years, biosynthetic method employing plant extracts or biomass has appeared as a simple and viable alternative to microorganisms, e.g.,

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plants such as coriander alfalfa [9], *Aloe vera* [10], Avena sativa biomass [11], lemongrass [12], *Cinnamommum camphora* [13] etc. have been reported relatedly. Our group have demonstrated that a large number of plants possess the capability to convert Au(III) into Au(0) [8].

But there remains a significant challenge in understanding and predicting nanoparticle size and shape from a given set of biosynthetic conditions (e.g., choices of plants), which involves a full understanding of the bio-protocol. Even an accurate determination of the involved biocompounds that provides the premise for illustration of the bio-protocol could be tough. The diversity of biocompounds in the biomass makes individual purification and determination of all the biocompounds not viable. Synergic effects among these compounds might also add to the complexity. Moreover, even if for generation of the same kind of metal NPs, cases vary greatly among different biosystems [9–13]. Consequently, a most universal explanation to account for generation of those NPs should cover as many cases as possible.

Currently, the Fourier transform infrared spectroscopy (FTIR) analyses by Huang et al. [13] revealed that polyols were responsible for the generation and stabilization of NPs. Among various polyols, the reducing sugars and/or the terpenoids were speculated to play a role in the bioreduction [12]. Water-soluble heterocyclic biocompounds or proteins were considered as stabilizing ligands of the NPs [12, 13]. And the pH condition could also affect the process [11]. There were also investigations that isolated individual biocompounds such as chitosan [14] and established possible mechanisms to illustrate the process.

The above studies were single organism based, focusing on individual organisms or biocompounds, and the specific information of which might not be applicable to other various cases. As well, currently the FTIR spectroscopy that mainly renders local information about related functional groups has dominated the existing methods of research, but the involved biocompounds could not be accurately determined only by FTIR since the same functional group could exist in a variety of different biocompounds. Therefore, it is imperative to explore complementary methods to illustrate the mechanism underlying biosynthesis of metal NPs.

To contribute to the determination of biocompounds involved in biosynthesis of gold nanoparticles (GNPs) by foliar broths, a statistical analysis is proposed in this work to investigate the influences of five immanent parameters of the foliar broths, i.e., the original pH value, the content of reducing sugars, flavonoids and proteins and the antioxidant capability, upon the Au(III) conversion and the size/shape distribution of the biosynthesized GNPs. As the parameters of the foliar broths are, respectively, evaluated, the pertinence of the research is enhanced. Moreover, due to its statistical characteristics the present research tends to be systematic. To our knowledge, this is the first report using a statistical method attempting to view bio-protocol of GNPs in a systematic perspective.

Experiments

Preparation of the Foliar Broths

Twenty-four kinds of randomly selected plant leaves (cultivated in Fujian, China, see the supporting information) after abstersion and drying were ground into powder, respectively. In a typical preparation, a mixture of the as-prepared powder and deionized water (20 mg ml^{-1}, 50 ml) was heated and kept boiling for 5 min. The boiled broth was allowed to cool down and then decanted. Such resulting filtrate was adjusted to 50 ml with deionized water to obtain the foliar broth for further experiments.

Biosynthesis of GNPs

Chloroauric acid ($HAuCl_4 \cdot 4\ H_2O$, purchased from Sinopharm Chemical Reagent Co. Ltd, China) was used as received. During biosynthesis of GNPs, aliquot of aqueous $HAuCl_4$ (0.04856 mol l^{-1}) was added into the broth to obtain a final $HAuCl_4$ concentration of 1 mM l^{-1}. And the solution was kept in an enclosed shaker at 30°C reacting for 15 min.

Characterization of GNPs

The ultraviolet–visible–near infrared spectrum (UV–Vis–NIR) was conducted for characterization of GNPs. In a typical operation, an appropriate portion of the reaction mixture after dilution was transferred into a 1 × 1-cm cuvette, and the absorbance in the range of 400–1,100 nm was recorded against deionized water by the UV–Vis–NIR spectrophotometer (TU 1900/Cary 5000) with scanning step of 1 nm.

Determination of the Conversion of $[AuCl_4]^-$

Aliquot (2.0 ml) of the reaction mixture aforementioned was centrifugated (ANKE TDL-5-A, ShangHai Anting Scientific Instrument Factory Co., Ltd, China) at 12,000 rpm for 10 min. The obtained supernatant solution was recentrifugated, and aliquot (1.0 ml) of the eventual supernatant was diluted up to 10.0 ml with HCl solution (5 wt%). The residual concentration of the $[AuCl_4]^-$ in the ultimate solution was detected by atomic absorption spectrophotometer (AAS, TAS-986, Beijing Purkinje General

Instrument Co., Ltd. China). The conversion of the Au(III) (x) was obtained by the following formula:

$$x = \left(1 - \frac{m}{197C}\right) \times 100\% \quad (1)$$

where m (ppm) denotes the residual concentration, C (mol ml^{-1}) the initial concentration of $[AuCl_4]^-$ and the coefficient 197 (g mol^{-1}) the relative atomic weight of Au.

Determination of the Parameters of the Foliar Broths

Original pH

Original pH value of each broth was assayed with a pH meter (Delta-320, Mettler Toledo).

Flavonoids

Spectrophotometric method was used to assay the flavonoids content in each broth [15]. Rutin of 10 mg (dried at 105°C, purchased from Sinopharm Chemical Reagent Co. Ltd, China) was dissolved in 5 ml ethanol (95% (v/v)) in a 50-ml volumetric flask, and then the solution was diluted to 50 ml using deionized water for linear assay to establish the calibration line.

In a typical determination, firstly, a combination of aqueous $NaNO_2$ (0.4 ml, 5 wt%) and 1 ml adjusted sample solution (depending on the approximate content of the flavonoids in each broth, concentrations of the broths herein used were already adjusted accordingly with deionized water such that the final sample could be within the linear range of the assay, likewise for the case of reducing sugars and proteins) was agitated in a volumetric flask of 10 ml. Then, the solution was left stand for 6 min to allow for sufficient interaction between the added reagents and the biocompounds (which was also the reason for the same treatment hereinafter). Afterward, aqueous $Al(NO_3)_3$ (0.4 ml, 10 wt%) was pipetted into the mixture. Such resulting solution after agitation was kept stationary for 6 min, and subsequently NaOH solution (4 ml, 4 wt%) was transferred into it. After being diluted to 10 ml and agitation, the final solution was allowed to stand for 15 min. Finally, its absorbance at 510 nm was recorded using Visible Spectrophotometer (DU7400, Beckman Coulter, Inc.) with mixtures of above additives served as blank.

Reducing Sugars

The DNS (3, 5-dinitrosalicylic acid) method was employed to determine the reducing sugars content in each broth. A combination of 1.0 ml modified broth and 2.0 ml DNS reagent was bathed in boiling water for 10 min and then was cool down using flowing water. After addition of 10 ml deionized water, the absorbance of the final solution at 540 nm was measured against DNS reagent/water blank using Visible Spectrophotometer (DU7400, Beckman Coulter, Inc.). Aqueous glucose was used as standard solution to obtain a calibration line.

Proteins

Coomassie brilliant blue method was used for measurement of the proteins content in each broth. A portion (5.0 ml) of Coomassie brilliant blue G-250 dye reagent (0.01% (W/V)) was added into 1.0 ml modified broth. The mixture was agitated and kept stationary for 2 min. The absorbance of the final sample at 595 nm was measured against the dye reagent/water blank by Visible Spectrophotometer (DU7400, Beckman Coulter, Inc.). Bovine serum albumin (BSA, BR, Livzon Pharmaceutical Group Inc.) as standard solution was employed to establish the calibration line.

Anti-Oxidant Capability

The anti-oxidant ability of the foliar broth was measured using the DPPH (2, 2-diphenyl-1-picryl-hydrazylhydrate) radical photometric assay in a process regulated by its discoloration [16]. Sample stock broth (20 mg ml^{-1}) was diluted to a series of concentrations (the specific concentration of the broth should ensure the final solutions were differentiated from each other in shades of purple red). For each sample of different concentrations, solution of 50 μl was pipetted into the 96 orifice plate and followed by addition of 150 μl DPPH reagent (250 μM DPPH per liter methanol). After 30 min, the absorbance of the mixture at 517 nm was measured using a Multiskan Spectrum (SPECTRA Technologies Holdings Co. Ltd.). Mixture of ethanol solution (150 μl) and the broth (50 μl) served as the blank and DPPH solution (150 μl) plus ethanol (50 μl) as the control. The DPPH radical scavenging rate (SR, %) was calculated through:

$$SR = 100 \times \left(1 - \frac{A_1 - A_0}{A_2}\right) \quad (2)$$

where A_0, A_1 and A_2 are absorbance of the blank, the sample and the control, respectively.

The SR_{50} value, which denotes the concentration of the leaves required to remove 50% DPPH radicals in the solution, was calculated by linear regression of plots where the abscissa represented the concentration of the leaves and the ordinate the DPPH radical scavenging rate.

Triplicates were conducted in each assay of the parameters.

Statistical Analysis

The roles of the afore-acquired parameters upon the capability of the foliar broth to reduce Au(III) were evaluated through formulas 3 and 4 [17]:

$$\mathrm{Cov}(X, Y) = E(XY) - E(X)E(Y) \tag{3}$$

$$r_{xy} = \frac{\mathrm{Cov}(X, Y)}{\left(\sqrt{D(X)}\sqrt{D(Y)}\right)} \tag{4}$$

where Y denotes the conversion of the Au(III), X the value of any of the five parameters of each broth, Cov(X, Y) the covariance value and r_{xy} the correlation coefficient of X and Y with a range of [−1, 1], E the expectation value. The significance of the linear correlation was evaluated by comparing the r_{xy} with the two critical values at 95 and 99% confidence level, respectively. As statistical sample size (N) of our research was 24, the freedom of error (d_f) in this statistical analysis was:

$$d_f = N - 2 = 22 \tag{5}$$

From the critical value of correlation coefficient $\rho = 0$ table [17] the two critical values, i.e., $r_{0.05,22}$ and $r_{0.01,22}$, were found to be 0.404, 0.515, respectively.

In addition, for the primary investigation into the size/shape distribution of biosynthesized GNPs, the boxplot was used with five-number summaries, i.e., the smallest observation, the lower quartile and the upper quartile cutting off the lowest and highest 25% of the data, respectively, the median which is the middle value of the data and the sample maximum [18]. The boxplot is based on robust statistics which are more resistant to the presence of outliers than the classical statistics based on the normal distribution [19]. Hence, the data sets of the parameters could be described without any statistical assumption and the difference between data sets, if there are any, could be reflected directly.

Results and Discussion

Effects of the Parameters on the Conversion of Au(III)

Original pH

During biosynthesis of GNPs, all of the ultimate reaction solutions possessed the characteristic red color, indicating generation of GNPs which was also validated by the UV–Vis–NIR characterizations (see supporting information). Such resulting GNPs were built upon Au(III) conversion, a redox reaction depending on the properties of the broths (as the reaction time, temperature and pressure were fixed). Accordingly, the relevancies between the conversion of Au(III) and each parameter, e.g., original pH value,

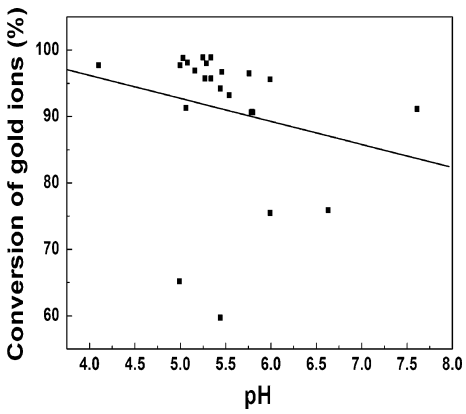

Fig. 1 Original pH of the broths versus conversion of Au(III)

the content of flavonoids, reducing sugars and proteins, as well as the anti-oxidant capability of the foliar broths should reflect the role of each parameter upon the biosynthesis of GNPs, as demonstrated in the following sections.

On the part of the pH value, Armendariz and coauthors proposed that the adsorption of $[AuCl_4]^-$ by native oat biomass was pH dependent within the range 2–6 [11], but contradictorily, removal of Au(III) by alfalfa biomass [20] was nearly independent of the pH value. And for the case of *Stenotrophomonas* sp., a magnetotactic bacterium [21], neither did its Au(III) biosorption capacity exhibit significant difference within initial pH range 1.0–5.5, but when the pH was increased to 5.5–13.0, the biosorption capability decreased significantly. In our work, the conversion of Au(III) was observed to decrease against the increasing original pH value of the broths, as depicted in Fig. 1. And application of formula 3 upon the original data generated the covariance of the two variables as:

$$\mathrm{Cov}(\mathrm{pH}, X) = -0.0434 \tag{6}$$

It indicates a negative relationship between the original pH and the conversion. This means that a stronger reducing capability upon the Au(III) is favored by lower pH conditions, which is in accordance with the case involving oat biomass. Under low pH condition, the functional groups of active biocompounds such as hydroxyl groups tend to undergo protonation and become positively charged, promoting the interaction between the protonated biocompounds and the oppositely charged $[AuCl_4]^-$ through electrostatic attraction or the electrovalent bond [11].

By applying formula 4, however, the significance for the correlation turns out to be poor since the obtained Eq. 7 presents a coefficient smaller than the critical value at the 95% confidence level.

$$|r_{xy}| = 0.202 < r_{0.05,22} = 0.404 \tag{7}$$

For the research where pH value was the center of attention [11], with choice of biomass and other conditions

fixed, the pH value of the solution was controlled to be the predominating factor influencing the interaction between $[AuCl_4]^-$ and the biomass. However, herein multiple other factors varying both in quantity and quality among individual plants might regulate the conversion in a pattern much stronger than that of the original pH values. Furthermore, the pH value in the former research was modified by the inorganic acid/alkali to extend from relatively strong acidic to weak or even to alkaline conditions. Nevertheless, the so-called original pH was the active acidity denoting the concentration of dissociated natural organic acids, and most of the foliar broths were weakly acidic with pH from ca. 4.1 to 7.6. Therefore, the effect of the original pH conditions on Au(III) conversion was not evident within the range.

Flavonoids

Though flavonoids as a category of polyols have been mentioned in the former researches regarding biosynthesis of GNPs [13], it yet remains insufficient to determine the role of the flavonoids in this process given the numerous subcategories of polyols. To contribute to this aspect, the distribution of the flavonoids content (C_F) versus the conversion of Au(III) was obtained in this research, as shown in Fig. 2. All of the broths with flavonoids content exceeding 0.6 mg ml^{-1} demonstrated conversions above 90%. The covariance of the two variables was obtained as formula 8.

$$\mathrm{Cov}(C_F, X) = 0.0292 \tag{8}$$

And comparison of correlation coefficient with the two critical values arrived at formula 9, giving a level of significance falling between the two critical points.

$$r_{0.05,22} = 0.404 < |r_{xy}| = 0.438 < r_{0.01,22} = 0.515 \tag{9}$$

Accordingly, such a linear relationship of relative significance in the statistical perspective verifies flavonoids as, or among, the biocompounds responsible for reducing Au(III) into Au(0), supplementing the none-typical information regarding the flavonoids from the FTIR analysis [13]. Besides, without exception, in this study foliar broths with relatively denser flavonoids presented higher Au(III) conversion, e.g., when flavonoids content was above 1.25 mg ml^{-1}, the responding conversions were over 95%. Therefore, content of flavonoids of the plants, since which has already been both extensively and intensively investigated [22], could be an index for preliminary evaluations of the untapped plants in terms of biosynthesis of GNPs.

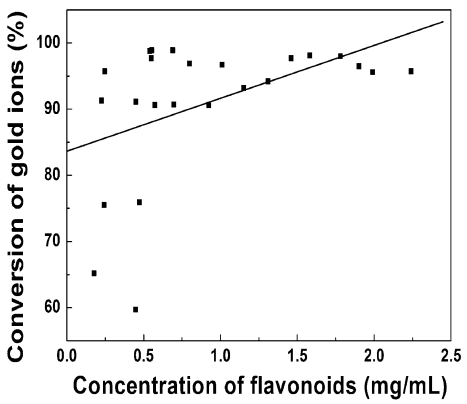

Fig. 2 Flavonoids in the broths versus conversion of Au(III)

Reducing Sugars

Reducing sugars such as monoses, dioses and oligoses are polyols with dissociated aldehyde or kenotic groups. Compared with other parameters, it is the one that has been relatively well understood in biosynthesis of GNPs based on a variety of spectroscopic measurements [23, 24]. One of the typical examples using the waste biomass of *Saccharomyces cerevisiae* proposed that reduction of Au(III) to Au(0) was mainly effected by the free aldehyde groups of the reducing sugars [25]. But similar to that of the flavonoids, more-targeted efforts are still needed to ascertain the role of reducing sugars for the case involving foliar broths. Herein, the reducing sugars content (C_S) of each broth versus the conversion of Au(III) is illustrated in Fig. 3. When C_S was below 1.0 mg ml^{-1}, the conversion of Au(III) climbed up evidently with increasing C_S. Conversions higher than 90% were observed for all of the broths with C_S larger than 1.5 mg ml^{-1}. Further processing of the original data gave formula 10.

$$\mathrm{Cov}(C_S, X) = 0.0517 \tag{10}$$

And testing of hypothesis upon the correlation coefficient generated formula 11 which presents a level of significance above the critical value at the 99% confidence level, larger than that of the total flavonoids.

$$|r_{xy}| = 0.523 > r_{0.01,22} = 0.515 \tag{11}$$

Such a size of significant linear relationship statistically validated the reducing sugars as important reductants to convert Au(III) and thus strengthened what has been mentioned previously [23, 24]. As well, comparisons of the correlation coefficient seemed to suggest that in general the reducing sugars were more significant than the flavonoids in terms of conversion of Au(III) in biosynthesis of GNPs.

There were already precedents using purified reducing sugars to reduce metallic ions, which circumvented the complicacy encountered by those using foliar broths. For instance, Ag^+ was reduced by glucose in the nanoscopic

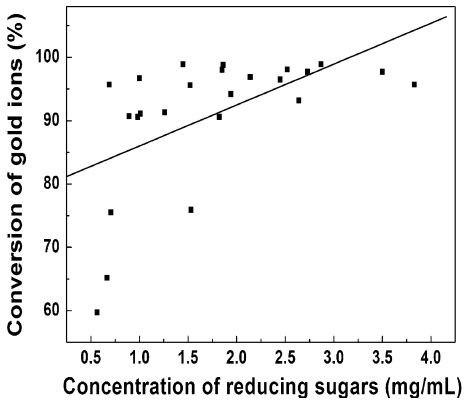

Fig. 3 Reducing sugars in the broths versus conversion of Au(III)

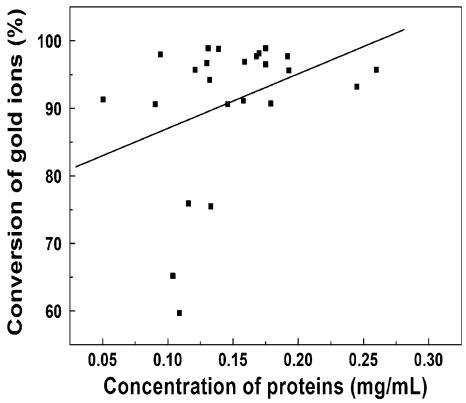

Fig. 4 Proteins in the broths versus conversion of Au(III)

starch template [26], and the fructose was demonstrated to be the best-suited reducing agent over other sugars [27, 28]. These results involved with isolated reducing sugars on one hand supported the statistical result here introduced on the other hand guaranteed interaction between the two. For instances, information from the former such as the binding pattern [26], the stabilization [27, 28] regarding the biocompounds and the NPs might also be available to the present one where alike biocompounds interact and bind with the metal NPs.

Proteins

Compared with the polyols, the case of the proteins seemed to be more complicated. For instance, when *camphora* leaves were used in fabrication of Au or Ag NPs, the proteins seemed to exhibit little importance [13], neither they did in the case using *neem* leaves [24]. However, Ag NPs were synthesized and stabilized successfully by cyclic peptides in latex of *Jatropha curcas* [29]. And biomimetic synthesis and patterning of Ag NPs using targeted peptides [30] was also conducted. In both cases, the peptides were believed to function as both the reducing and protection agents.

In this research using foliar broths to manufacture GNPs, the distribution of the total proteins (C_P) versus the conversion of Au(III) is depicted by Fig. 4. Other than that of the flavonoids or the reducing sugars, Au(III) conversions above 90% were presented both by foliar broths with C_P higher than 0.15 and lower than 0.1 mg ml^{-1}, suggesting poor linear relationship. The covariance between proteins content and conversion of Au(III) is as follows:

$$\mathrm{Cov}(C_P, X) = 0.00172 \tag{12}$$

Though being positive, however, it is quite slim, almost approximate to zero, indicating that the two observations are possibly uncorrelated. And formula 13 displays a level of significance below the critical point at the 95% confidence level, confirming that the relationship between the proteins and the conversion of Au(III) is not evident, i.e., unlike reducing sugars or flavonoids, the proteins are not the reductant in the fabrication of GNPs by foliar broths.

$$|r_{xy}| = 0.339 < r_{0.05,22} = 0.404 \tag{13}$$

It has been found during the experiments that the proteins content in the foliar broths is relatively low, which on average is only one-twelfth and one-seventh of that of the reducing sugars and the flavonoids, respectively. And yet the quantity of amino acid residues such as cysteine [31], which are believed to interact with or to reduce Au(III) into Au(0), is even less. As a consequence, in the redox reaction the polyols as well-established reductants would serve as the principal electron donor, leading to the poor linear correlation between the proteins content and the conversion of Au(III) [30]. Hence, the present result does not necessarily contradict against aforementioned researches involving peptides as reducing agents [29, 30]. As well, since the reduction of Au(III) and the stabilization of the GNPs are two distinguished aspects of the process, the result neither invalidate proteins as capping agents to prevent the GNPs from aggregation in the green protocol.

The Anti-Oxidant Capability

Natural anti-oxidants that have a strong reducing ability to remove free radicals such as DPPH radicals have been extracted from a large number of plants [32]. Thus, a positive relationship between the anti-oxidant ability and the conversion of Au(III) to Au(0) should have been anticipated. Herein, the relationship could be confirmed. Figure 5 illustrates that the conversion of Au(III) decreases when SR_{50} increases within 0–2 mg ml^{-1}, and the trend becomes evident as SR_{50} is larger than 3 mg ml^{-1}.

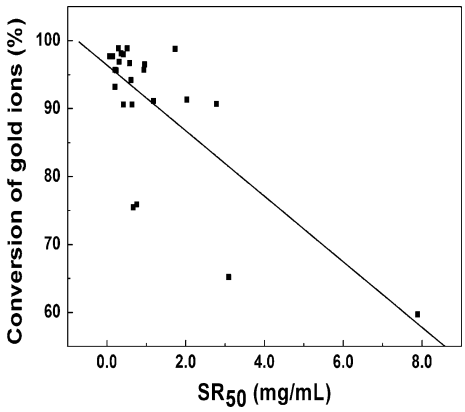

Fig. 5 SR_{50} of the broths versus conversion of Au(III)

The resulting covariance given by formula 14 further guarantees the trend

$$\mathrm{Cov}(\mathrm{SR}_{50}, X) = -0.126 \tag{14}$$

It indicates that a less concentration of foliar extracts is needed to remove 50% DPPH radicals for plants capable of higher Au(III) conversion, which is to say, foliar broths with higher capability to remove radicals posses stronger ability to reduce Au(III) into Au(0). Such a result in general verified the very anticipation, which would be further validated as the correlation coefficient larger than the critical value at the 99% confidence level, as shown in formula 15.

$$|r_{xy}| = 0.707 > r_{0.01,22} = 0.515 \tag{15}$$

Such a correlation coefficient establishes a significant linear relationship between the two variables. That is, bioconstituents capable of removing the DPPH radicals are probably the involved reductants in biosynthesis of GNPs, which thus could guide the future direction of the biosynthetic protocol. In addition, the linear relationship also spells the possibility to develop an alternative to the relatively tedious and costly screening of large number of plants using optical spectrum instruments and Au(III) substrates. To the best of our knowledge, this is the first touching on the correlation of the anti-oxidant ability with the bioreduction of Au(III) in biosynthesis of GNPs.

In summary, being parameter targeted, the methodology demonstrated strengthens the pertinence with respect to biocompounds involved in biosynthesis of GNPs. The linear relationships with different levels of significance between the conversion of Au(III) and immanent parameters of the broths not only contributed to determination of biocompounds involved in biosynthesis of GNPs, but also revealed the similarities among numerous individual plants in terms of biosynthesis of GNPs, which implied the existence of an uniform mechanism underlying this universally spontaneous phenomenon.

The Preliminary Investigation into the Size/Shape Distribution of the Biosynthesized GNPs

Generally, the UV–Vis–NIR spectrum patterns could be sorted into two categories through statistical grouping. For group 1, each of the absorption patterns presented only one well-defined and shape-constant absorption band with maximum absorbance located at 500–600 nm. And these relatively narrow bands were associated with sphere GNPs with high degree of homogeneity in size [33]. For group 2, besides the absorption band at 500–600 nm, the spectrums either have, or show the tendency to have, another band in 600–1,100 nm, suggesting generation of sphere GNPs with wide size distribution or particle aggregation or even existence of gold nanotriangles [34, 35] (see the supporting information).

Afterward, to identify the two groups, the Au(III) conversion and the five parameters of each group were described, respectively, using the boxplots aforementioned, as depicted by Fig. 6. From the bottom up, the five numerical values in each box are the minimum observation, the first quartile, the median, the third quartile and the maximum value, respectively. Through respective comparisons of the five numbers between the two boxplots in each subfigure, it could be observed that in general the conversion in group 1 (Fig. 6a) was higher than that in group 2, that means, the broths in group 1 possessed higher level of average reducing rate than those in group 2. This is consistent with what given by subfigures c, d and e where the biocompounds responsible for the reduction of the Au(III) (i.e., the total flavonoids and the reducing sugars) and the anti-oxidant capability in group 1 in general exceeded those of the other and so was the case of the proteins. The difference in the pH value (Fig. 6b) was slight, which is also in accordance with what discussed in the prior section.

That is, in the biosynthesis of GNPs by foliar broths sphere GNPs with higher size homogeneity were promoted by higher average reducing rate, while the lower one leaded to GNPs with wider size distribution or even gold nanotriangles. Explanations of such phenomenon involve with the nucleation and crystal growth stages during synthesis of GNPs. From the stand point of kinetics, in group 1 the reduction of Au(III) to Au(0) due to higher content of reducing agents was faster than that in group 2. This leaded to denser nucleation which therefore predominated over the growth of the GNPs and as a result prevented the gold atoms and clusters formed at early stages of the reaction from growing into extremely large particles [36].

However, fast nucleation could not work solely to generate uniform GNPs spheres considering their high instability due to high surface Gibbs energy. Denser substances for passivation to prevent GNPs from aggregation

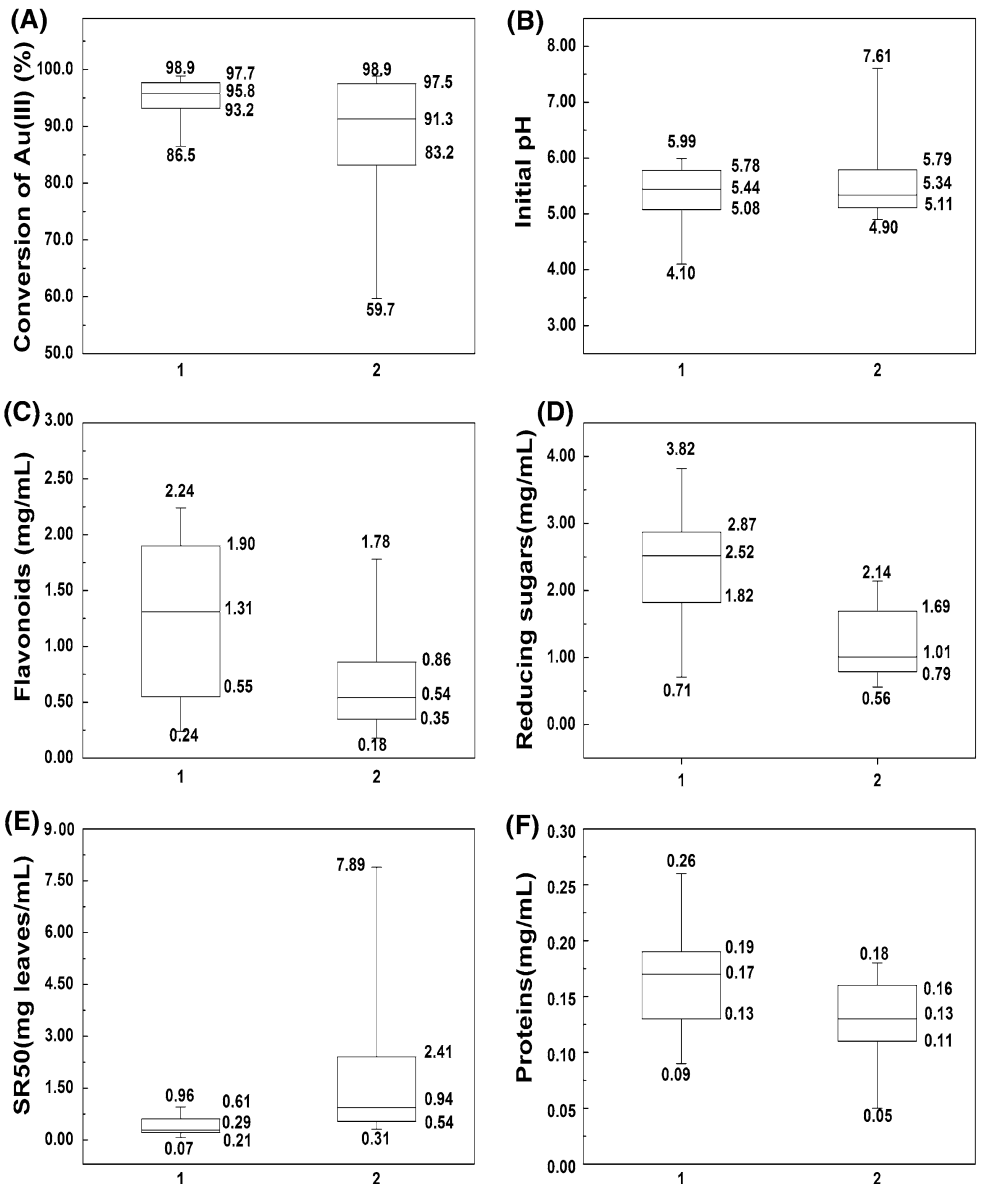

Fig. 6 *Boxplots* for comparisons of the conversion and the five parameters between group 1 and group 2: **a** conversion, **b** pH, **c** flavonoids, **d** reducing sugars, **e** SR50, **f** proteins

were expected in group 1 than the other. Higher concentration of the reducing agents and/or their responding products resulted from reduction of Au(III) might be an important resource of the protection agent [28] contributing to the higher size homogeneity of GNPs in group 1. What is more, the proteins concentration that in the former section was observed with little importance as reducing agents, however, herein in general higher in group 1 than group 2. This suggests that the proteins might also be the protection agents due to their strong affinity to bind metals possessed by carbonyl groups from the amino acid residues and peptides of proteins [25].

Additionally, it could be seen that the wavelength of the maximum absorbance in the UV–Vis–NIR spectrums varied from plant to plant, indicating that spherical GNPs of various sizes and triangular GNPs might be obtained. Thereby through adjusting the choice of the plants, biosynthesis of spherical or triangular GNPs might be size controllable, which could be of great environmental and operational advantages over those chemical methods employing additives for adjustment [36].

Conclusions

In summary, this statistical investigation supported the speculation that the $[AuCl_4]^-$ interacted with the biocompounds through an ionic bond or an electrostatic force, and both reducing sugars and flavonoids were proved to be important reductants responsible for the conversion of Au(III). The research also excluded the possibility for the proteins to be reductants yet it indirectly supported them as

the protection agent in the biosynthesis of GNPs by foliar broths. As well, a significant linear relationship between the anti-oxidant activity of the foliar broths and their capability to reduce Au(III) into Au(0) was discovered. Besides, the preliminary analysis regarding the size/shape distribution of the biosynthesized GNPs revealed that the foliar broth containing higher content of reducing sugars/flavonoids and proteins in general supported formation of sphere GNPs with higher homogeneity in size while otherwise sphere GNPs with wider size distribution or even nanotriangles might be developed. Not only this statistical analysis could complement the conventional optical spectrum methodologies to investigate biocompounds involved in biosynthesis of GNPs, but also it could contribute to exploration of alternatives in rough screening of the affluent plant resources in terms of fabrication of GNPs.

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