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Stoichiometrically controlled production of bimetallic Gold-Silver alloy colloids using micro-alga cultures

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ABSTRACT

This paper reports the production of well-defined, highly stable Ag–Au alloy nanoparticles (NPs) using living cells of *Chlamydomonas reinhardtii*, with the composition of the bimetallic alloys being solely determined by the stoichiometric ratio in which the metal salts were added to the cultures. The NPs exhibited a single, well-defined surface plasmon resonance (SPR) band confirming that they were made of a homogeneous population of bimetallic alloys. Particle creation by the cells occurred in three stages: (1) internalization of the noble metals by the cells and their reduction resulting in the formation of the NPs; (2) entrapment of the NPs in the extracellular matrix (ECM) surrounding the cells, where they are colloidally stabilized; and (3) release of the NPs from the ECM to the culture medium. We also investigated the effect of the addition of the metals salts on cell viability and the impact on characteristics of the NPs formed. When silver was added to the cultures, cell viability was decreased and this resulted in a ~30 nm red shift on the SPR band due to changes in the surrounding environment into which the NPs were released. The same observations (in SPR and cell viability) was made when gold was added to a final concentration of 2×10^{-4} M, but not when the concentration was equal to 10^{-4} M, where cell viability was high and the red shift was negligible.

1. Introduction

Taking inspiration from nature, there have been several studies that have explored the ability of different micro-organisms to promote the production of different inorganic nanomaterials from their corresponding salts. For instance, magnetite can be produced by the fungal strains *Fusarium oxysporum* and *Verticillium* sp. [1] while bacteria (*Escherichia coli*) [2], fungus (*F. oxysporum*) [3] and yeast (*Schizosaccharomyces pombe*) [4] have been used for the production of cadmium sulfide (CdS) nanoparticles. Among cell-mediated nano-objects, Noble metal nanoparticles (NPs) are the most widely studied. As examples, the cyanobacteria *Plectonema boryanum* is able to produce platinum [5], palladium [6], silver [7] and gold [8] NPs of different sizes and shapes. In spite of its known cytotoxicity and anti-microbial properties, silver NPs can be produced by several strains of bacteria, such as *Pseudomonas stutzeri* [9] and *Bacillus licheniformis* [10], as well as different fungal strains such as *F. oxysporum* [11] and *Asperigillus fumigates* [12]. Formation of these NPs can occur within the cells [13] or outside the cells in the culture media [14]. The cell-free supernatant from bacteria and fungi cultures can also be used to produce silver NPs [15]. Similarly, gold NPs can be produced by the abovementioned micro-organisms, such as bacteria [16–20], fungi [21,22] and yeast [23,24], or by the biomass extracted from some algae strains [25–31].

More recently, the use of different strains of cyanobacteria (blue algae) [32], diatoms [33] and fresh water green micro-algae [34,35] has been reported for the synthesis of noble metal NPs. The introduction of the corresponding aqueous salt solutions into the cultures under their normal culturing conditions triggers the internalization of these cations by the cells and their reduction into their metallic counterparts, leading to the subsequent formation of the NPs within the cells (i.e. intracellular production) and finally to their release into culture media. The combination of intracellular NP formation and presence of polysaccharidic matrices on the surface of the cells results in the formation of very stable colloids. These colloids consist of NPs with the same shape and size, capped within organic matrices, which act as the stabilizing agent. More importantly, it has been shown that micro-algae cells can adapt by developing resistance towards the toxic effects associated with the added salts, which suggests that sustainable production of nanomaterials via biological processes is possible [36].

The chemical synthesis of bimetallic silver–gold alloy NPs has been reported by several groups [37–39]. These alloys have their surface plasmon resonance (SPR) bands shifted towards the red region of the visible spectrum when the gold fraction within the

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alloys increases. In other words, there is a linear relationship between the maximum of the absorbance and the molar ratio of gold to silver within the alloys, ranging from ~400 nm for NPs made entirely of silver to ~530 nm when made entirely of gold. In addition to their unique optical properties, these nanoparticles display interesting electrocatalytic activity [40]. While chemical synthesis of such NPs is well established, only a few reports exist in the literature on biological-based routes for the synthesis of bimetallic Ag–Au alloy NPs. To create these alloys, other groups have used an aqueous extract of plant leaves [41,42], an extract of edible mushrooms [43], a filamentous fungus [44], or the fungus *Fusarium semitectum* [45]. The first report of biologically mediated synthesis of bimetallic Ag–Au alloy NPs appeared in 2006, by Senapati and coworkers [46]. In this case, extracellular synthesis of the NPs occurred when different biomass concentrations of fungus strain *F. oxysporum* were exposed to equimolar solutions of $AgNO_3$ and $HAuCl_4$. Importantly, the alloy composition was a function of fungal biomass concentrations, with higher concentrations producing a higher silver fraction in the alloy. Herein, we propose a more direct strategy to control the composition of bimetallic Ag–Au alloy NPs formed by a biologically mediated process. In this paper we demonstrate, for the first time, that the introduction of mixtures of aqueous solutions of $AgNO_3$ and $HAuCl_4$ in different ratios to a growing culture of the fresh water green micro-alga *Chlamydomonas reinhardtii* leads to the production of stable bimetallic NPs whose compositions match the ratio of the noble metal salts added. This approach greatly minimizes the effort to obtain bimetallic Ag–Au alloy NPs with precise composition, using living organisms, and as we show allows for production of stabilized bimetallic particles owing to interactions of the NPs with the extracellular matrix of the micro-algae.

2. Experimental section

The photosynthetic micro-alga, *C. reinhardtii*, *Cr* hereafter, was purchased from the Culture Collection of Algae (strain number 53.72) at the University of Göttingen (Germany) and grown in Bold's basal culture medium (BB-CM) to give a stock culture. BB-CM is made by mixing 10 mL of stock solution A ($NaNO_3$ (12.5 g/L), $CaCl_2{\cdot}2H_2O$ (2.5 g/L), $MgSO_4{\cdot}7H_2O$ (7.5 g/L), and FeEDTA (1.0 g/L)), 10 mL of stock solution B (K_2HPO_4 (7.5 g/L), KH_2PO_4 (17.5 g/L), and NaCl (2.0 g/L)), and 1 mL of trace solution (H_3BO_3 (2,4 g/L), $MnCl_2{\cdot}4H_2O$ (1.8 g/L), $(NH_4)_6Mo_7O_2{\cdot}4H_2O$ (0.1 g/L), $ZnSO_4{\cdot}7H_2O$ (220 mg/L), $CuSO_4{\cdot}5H_2O$ (80 mg/L), $CoSO_4{\cdot}7H_2O$ (90 mg/L), and $VOSO_4{\cdot}2H_2O$ (43 mg/L)). The total volume is then brought to 1 L by addition of milli-Q water and the pH adjusted to 7.0 using 1.0 M NaOH, after which the medium is sterilized in an autoclave and stored at 4 °C.

In order to perform the synthesis of silver, gold, and silver–gold bimetallic nanoparticles, six cultures of *Cr* were launched by transferring 10 mL of *Cr* stock culture into 250 mL flasks containing 100 mL BB culture medium. These new cultures were left to age 2 weeks before the introduction of 12.2 mL of silver nitrate ($AgNO_3$, Caledon) and gold (III) chloride hydrate ($HAuCl_4{\cdot}H_2O$, Sigma Aldrich) aqueous solutions, at initial concentration of 10^{-3} M, at room temperature (~22 °C), using a controlled duration of light exposure (8 h dark/16 h room light) but without any control over light intensity. Hereafter, we denote cultures by the concentrations of silver and gold salts initially present, as summarized below in Table 1.

Following a specified incubation time with the silver and gold salts, optical images of cells were recorded using an inverted Olympus IX51 microscope, equipped with a Retiga 2000R color camera. The optical properties of the colloids were studied using a Beckman Coulter DU 800 spectrophotometer by scanning ~2 mL of the colloid dispersion between 300 and 800 nm. The samples for chlorophyll a absorbance measurements were prepared according to the method described by Ninfa et al. [47]. Brieflly, 9 mL of acetone was added to 1 mL of the algal culture in a test tube, which was then vortexed for 1 min in order to break the cell wall and release the chlorophyll a. Then the test tube was placed in a water bath at 37 °C for 3 min and then centrifuged at 3000 rpm for 5 min. The supernatant was used to record the chlorophyll a absorbance using the UV–Vis spectrophotometer. TEM micrographs were obtained with a JEOL JEM 1200 EX TEMSCAN transmission electron microscope, equipped with AMT 4 megapixel camera with V600 software and operating at 75 kV. TEM samples were prepared by drying a drop of a given colloid dispersion onto a carbon-coated copper grid.

3. Results and discussion

A few hours after the addition of aqueous solutions of the silver and gold salts to cell cultures, the cells started to sediment due to the internalization of the salts making the cells denser than usual. This salt internalization was followed by intracellular reduction of the cationic species and subsequent formation of the metallic nanoparticles. Fig. 1-A shows optical images of single cells taken from each of the six flasks that were incubated with the metal salts in different stoichiometric ratios. Virtually all cells in these different samples appeared colored. The color ranged from yellow for *Cr*1 to dark purple for *Cr*6 (except for *Cr*5, where the cells were green and thus still viable – this will be discussed later in the paper). For intermediate ratios, the color tended to be more purple when increasing amounts of gold cations were added to the cultures. These images clearly show that nanoparticle formation occurs first by internalization of the added metals, followed by release of the nanoparticles into the culture media. An aged culture of *C. reinhardtii* appears gelatinous due to the production of

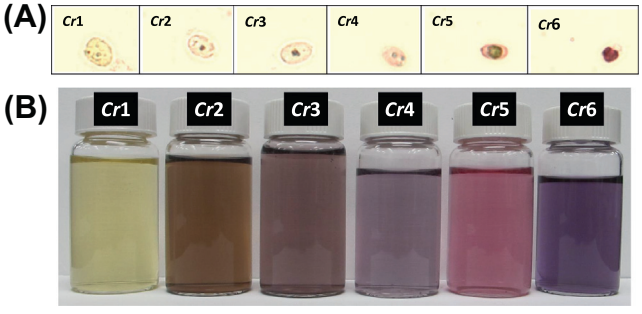

Fig. 1. (A) Optical images of cells obtained from the six samples with Ag^+/Au^{3+} ratios of: (*Cr*1) 100%/0%, (*Cr*2) 75%/25%, (*Cr*3) 50%/50%, (*Cr*4) 25%/75%, (*Cr*5) 0%/100% (10^{-4} M), and (*Cr*6) 0%/100% (2×10^{-4} M), at a magnification of 40× and (B) photograph of the supernatant from the cultures shown in panel A, containing the different colloids formed after 1 month of cultivation and 5 months of storage.

Table 1
Composition of the six samples.

Sample	*Cr*1	*Cr*2	*Cr*3	*Cr*4	*Cr*5	*Cr*6
$[Ag^+; Au^{3+}]$ (10^{-4} M)	[1.00; 0.00]	[0.75; 0.25]	[0.50; 0.50]	[0.25; 0.75]	[0.00; 1.00]	[0.00; 2.00]