

# Hybrid Hydrophobin/Gold Nanoparticles: Synthesis and Characterization of New Synthetic Probes for Biological Applications

Jane Politi<sup>1</sup>(✉), Luca De Stefano<sup>1</sup>(✉), Paola Giardina<sup>2</sup>,  
Sandra Casale<sup>3,4</sup>, Ilaria Rea<sup>1</sup>, and Jolanda Spadavecchia<sup>3,4</sup>

<sup>1</sup> Unit of Naples-National Research Council, Institute for Microelectronics  
and Microsystems, Via P. Castellino 111, 80127 Naples, Italy  
{jane.politi, luca.destefano}@na.imm.cnr.it

<sup>2</sup> Department of Chemical Sciences, University of Naples Federico II,  
Via Cintia, 80126 Naples, Italy

<sup>3</sup> Laboratoire de Réactivité de Surface, CNRS, UMR 7197, 75005 Paris, France

<sup>4</sup> Laboratoire de Réactivité de Surface, Sorbonne Universités, UPMC Paris 06,  
4 Place Jussieu, 75005 Paris, France

**Abstract.** We report a simple and original method to synthesize gold nanoparticles in which a fungal protein, the hydrophobin Vmh2 from *Pleurotus ostreatus*, mixed to cetyltrimethylammonium bromide (CTAB) has been used as additional component in a one-step synthesis, leading to shell-like hybrid protein-metal nanoparticles (NPs). The nanoparticles have been characterized by ultra-violet/visible and infrared spectroscopies, and also by electron microscopy imaging. The results of these analytical techniques highlight nanometric sized, stable, hybrid complexes of about 10 nm, with a micelles-like hydrophobins rearrangement.

**Keywords:** Hybrid gold nanoparticles · Hydrophobins · CTAB

## 1 Introduction

Gold Nanoparticles (AuNPs) are attracting considerable interest as viable biomedical materials and the research effort about this subject is continuously growing, due to their unique physical and chemical properties [1–4].

The utilization of biomolecules to tune the surface properties and the assembly of AuNPs is a very attractive approach that has received considerable attention: this technology combines the advantages of green chemistry, since harmless biological substances are used instead of some aggressive chemical compound, with the unique properties of biological probes, leading to a next generation of nanometric complexes. Biomolecules and/or biopolymer-conjugated AuNPs are largely used as biomarkers in diseases early detection and as in vivo drugs delivery vehicles in medicine/pharmacy applications, and also in cosmetic products [5, 6].

In the past decades, many synthetic strategies have been developed to prepare AuNPs in organic or aqueous solvent [7, 8]. A commonly employed method for

synthesis of AuNPs involves the use of cetyl-tetrammonium bromide (CTAB) as the best candidate among different surfactants with the scope of stabilizing the metallic nanoparticle in solution.

The ionic detergents can be usefully exploited as stabilizers agents during the synthesis of nanoparticles, since they not only prevent particles aggregation but also help in tuning their functional properties. To date, a variety of stabilizers have been employed for the synthesis of AuNPs [1, 9, 10]. In general, surfactants play an important role in contemporary pharmaceutical biotechnology, since they are largely utilized in various drug dosage forms to control wetting, stability, bioavailability, among other properties [11, 12]. Association colloids such as micelles, on the other hand, can form spontaneously under certain conditions (self-assembling systems), and are thermodynamically more stable towards both dissociation and aggregation [13].

This work presents new synthesized hybrid nanoparticles as possible tools for biological applications by using fungal proteins called hydrophobins (HFB). HFB are small (about 100 amino acid residues), amphiphilic, highly surface-active, and self-assembling proteins [14] with different roles in the fungal growth and development. Indeed their biological functions are linked to the reduction of the water surface tension, and to their ability to self-assemble into an amphipathic membrane when they reach an interface, thus allowing fungi to escape from aqueous environment and to facilitate air dispersal of the spores [15, 16]. On the surface of the folded molecules, there is a coherent hydrophobic patch, which makes the molecule amphiphilic. The amphiphilicity gives the protein the ability to strongly stick to hydrophobic surfaces [17], forming a highly stable coating which can be used to promote biocompatibility [18–21], and as an intermediate to attach cells, proteins, or other type of molecules to surfaces.

In this work, HFB Vmh2 purified from *Pleurotus ostreatus* was used. The properties of Vmh2 were studied at the air-water interface, at surfaces, and in solution. We engineered hybrid nanoparticles in which Vmh2 molecules formed a nano-complex with cationic surfactant as CTAB, thus changing chemical and optical properties of Au nanoparticles.

## 2 Experimental Section

### 2.1 Materials

All chemicals were reagent grade or higher and were used as received unless otherwise specified. Tetrachloroauric acid ( $\text{HAuCl}_4$ ), cetyl-tetrammonium bromide (CTAB), sodium borohydride ( $\text{NaBH}_4$ ), Polyethylene glycol 600 Diacid (PEG), Sodium Dodecyl Sulfate (SDS), methanol ( $\text{CH}_3\text{OH}$ ), chloroform ( $\text{CHCl}_3$ ) and ethanol ( $\text{C}_2\text{H}_5\text{OH}$ ) were purchased from Sigma Aldrich.

### 2.2 Preparation of HFB

White-rot fungus, *P. ostreatus* (Jacq.: Fr.) Kummer (type: Florida; ATCC No. MYA-2306) was maintained through periodic transfer at 4 °C on potato dextrose

agar (Difco) plates in the presence of 0.5% yeast extract. Mycelia were inoculated in 2 L flasks containing 500 mL of potato-dextrose broth (24 g/L) supplemented with 0.5% yeast extract, grown at 28 °C in shaken mode (150 rpm). After 10 days of fungal growth, mycelia were separated by filtration through gauze, treated twice with 2% SDS in a boiling water bath for 10 min, washed several times with water and once with 60% ethanol to completely remove the detergent. The residue was dried under nitrogen, grinded and treated with 100% trifluoroacetic acid (TFA) in a water bath sonicator (Bandelin Sonorex Digitec) for 10 min. The supernatant was dried, dissolved in 60% ethanol and centrifuged (10 min at 3200 g). The new supernatant was lyophilized, and lipids were extracted in a mixture of water-methanol-chloroform 4:4:1 v/v (5 min in bath sonicator). After centrifugation, proteins appeared as a solid aggregate at the interface. They were recovered by upper phase removal, methanol addition and centrifugation. The precipitate was again dried, treated with TFA for 30 min in bath sonicator, re-dried, and dissolved in 80% ethanol. This sample was centrifuged (90 min at 12,000 g) and the supernatant dried, treated with TFA as above-described and re-dissolved in 60% ethanol.

### **2.3 Synthesis of Hybrid CTAB-Hydrophobin Gold Nanoparticles (Hyb-CTAB-HFB-AuNPs)**

CTAB-HFB-AuNPs were prepared using 5 ml of HFB solution (150 µg/ml) mixed with 5 ml of CTAB solution ( $10^{-4}$ M) and then added 5 ml of 0.0001 M aqueous HAuCl<sub>4</sub> solution for 10 min. To the resulting solution 1.8 ml of NaBH<sub>4</sub> (0.01 M) was added dropwise followed by rapid stirring. After 1 h without agitation, the solution became red-violet. The product was centrifuged and purified at the same conditions.

### **2.4 UV/Vis Measurements**

Absorption spectra were recorded using a Jasco V-570 UV/VIS/NIR Spectrophotometer from Jasco Int. Co. Ltd., Tokyo, Japan. 1 mL of nanoparticles solution were acquired in the range between 200 and 800 nm after 60 min from synthesis.

### **2.5 TEM Measurements**

Transmission electron microscopy measurements were recorded on a JEOL JEM 1011 microscope operating at an accelerating voltage of 100 kV. The TEM graphs were taken after separating the surfactant from the metal particles by centrifugation. Typically 1 mL of the sample was centrifuged for 20 min at a speed of 14,000 rpm/min. The upper part of the colourless solution was removed and the solid portion was re-dispersed in 1 ml of water. 2 µL of this re-dispersed particle suspension was placed on a carbon coated copper grid and dried at room temperature.

## 2.6 PM-IRRAS Measurements

PM-IRRAS spectra were recorded on a commercial Thermo (Les Ulis—France) Nexus spectrometer. The external beam was focused on the sample with a mirror, at an optimal incident angle of  $80^\circ$ . A ZnSe grid polarizer and a ZnSe photoelastic modulator, modulating the incident beam between p- and s-polarizations (HINDS Instruments, PEM 90, modulation frequency = 37 kHz), were placed prior to the sample. The light reflected at the sample was then focused onto a nitrogen-cooled MCT detector. The presented spectra result from the sum of 128 scans recorded at a  $8\text{ cm}^{-1}$  resolution. Each spectra shown represent the average of three measurements.

## 3 Results and Discussion

The synthesis of CTAB-HFB-AuNPs was carried out, mixing HFB and CTAB solutions, and then reducing tetrachlororoauric acid ( $\text{HAuCl}_4$ ) by sodium borohydride ( $\text{NaBH}_4$ ). TEM images of HFB-CTAB-AuNPs show nanoparticles with an average size of  $10.0 \pm 0.9\text{ nm}$  (Fig. 1). Conventionally surfactants such as CTAB typically form spherically shaped micelles at low concentration [18].

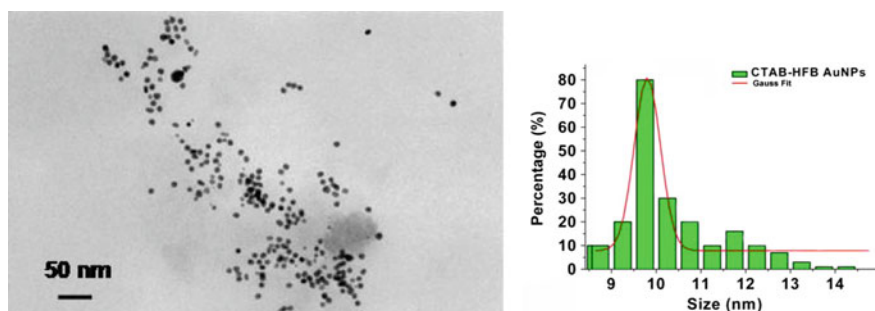
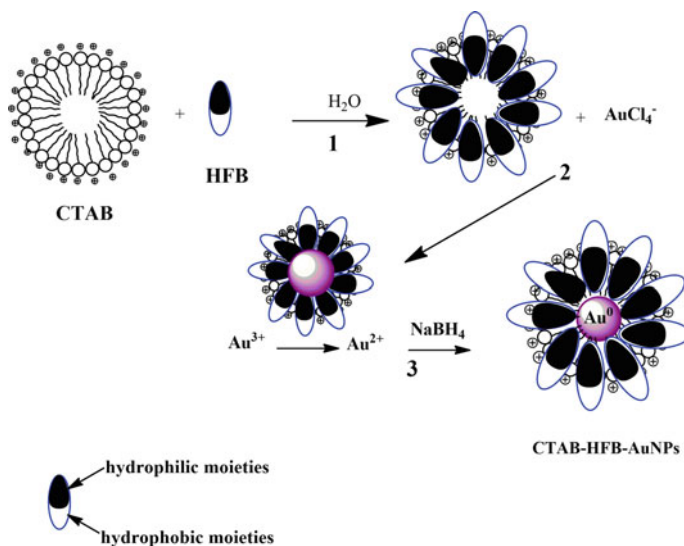


Fig. 1. TEM images and size distribution histogram of CTAB-HFB-AuNPs

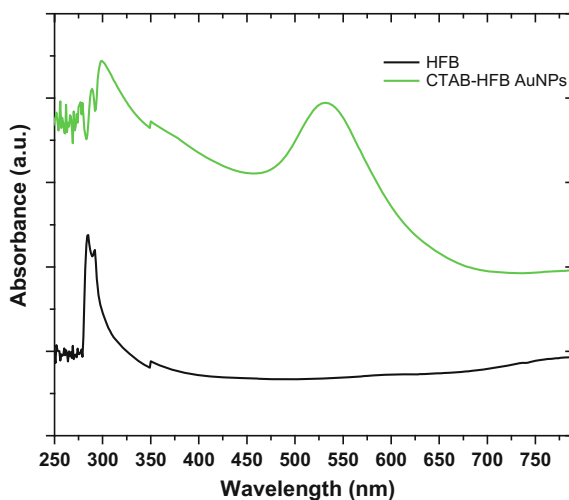
As reported by earlier authors [7], the extraordinarily strong binding of  $\text{AuBr}^{2-}$  to the positive  $\text{CTA}^+$  head group could stabilize the  $\text{Au}^+$  species in aqueous solution. This is due to the cooperative effect of micelles and  $\text{Br}^-$ , which favored the emergence of soluble  $\text{Au}^+$  species. In the beginning, a small part of  $\text{Au}^+$ , disproportionate to  $\text{Au}^{3+}$  and  $\text{Au}^0$  species where the complexation between  $\text{Au}^{3+}$  and  $\text{Br}^-$  in the presence of CTAB micelles resulted in the appearance of yellowish-red solution [7]. The binding on cationic surfaces was proposed to be due to local charge-charge interactions and as a result, local charges on the surface of the protein were of greater interest than the overall isoelectric point of the protein. For this reason, when HFB molecules were mixed with CTAB solution, the charged residues opposite to the hydrophobic patch of the amphiphilic HFB proteins were thought to interact with the polar surface of CTAB in such a way as the hydrophobic patch was turned outwards against the solution resulting in a hydrophilic coating of the gold surface (see Fig. 2).



**Fig. 2.** Schematic of proposed mechanism of  $\text{AuCl}_4^-$  reduction and particle formation in the presence of CTAB and HFB protein as surfactants

This steric arrangement of HFB in CTAB micelles before reduction of  $\text{HAuCl}_4$  during synthetic process of gold nanoparticles, was confirmed by PM-IRRAS analysis, reported in the following.

CTAB-HFB-AuNPs (Fig. 3 green curve) showed a strong resonance band at around 530 nm and a weaker one at 300 nm. This optical behavior was due to steric arrangement like inverse micelle of HFB into CTAB as stabilizers during synthetic process.



**Fig. 3.** UV-Vis absorption spectra of (black curve) HFB, CTAB-HFB-AuNPs (green curve) (color figure online)

The infrared spectrum of CTAB-HFB-AuNPs (Fig. 4 curve 3) shows a peak at  $1460\text{ cm}^{-1}$  attributed to the asymmetric and symmetric C–H scissoring vibrations of  $\text{CH}_3\text{-N}^+$  moieties and to the  $\text{CH}_2$  scissoring mode, respectively due to the presence of CTAB. The peaks centred at  $2920$  and  $2850\text{ cm}^{-1}$  are assigned of C–H vibration stretching mode of  $\text{CH}_2$  and  $\text{CH}_3$  groups respectively. The region corresponding of amide bands is very attenuated. This result is in agreement of UV-Vis spectra in which the absorption peak of HFB is very weak, probably due to a steric arrangement of HFB protein into micelles of CTAB.

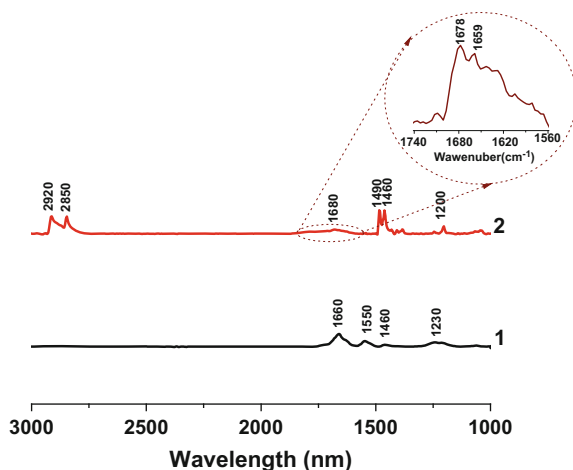


Fig. 4. PM-IRRAS spectra of (1) HFB, CTAB-HFB-AuNPs (2)

## 4 Conclusions

In the present work we showed that hybrid HFB AuNPs can be synthesized via a simple one step method. The key role of the HFB molecules during the growth process of nanoparticles was investigated by mixing with cetyltrimethylammonium bromide as standard surfactant in the synthesis. Stable nanometric hybrid protein-organic-metal NPs have been obtained, with average diameter of 10 nm. These results open a route to simple, effective, and also green chemistry synthesis of a new class of hybrid multipurpose NPs which will be tailored for different biomedical application, such as imaging, targeting and drugs delivery.

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