

Recombinant human arginase I immobilized on gold and silver nanoparticles: preparation and properties

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Abstract

Metal nanoparticles (NPs), such as gold (Au) and silver (Ag), are important for chemistry, physics, and biology due to their unique optical, electrical, and photothermal properties. Such NPs are widely used for immobilization of various bioactive substances, including peptides, enzymes, antibodies and DNA. The synthesis of silver and gold nanoparticles was carried out by reduction of silver nitrate by glucose and reduction of tetrachloroauric acid by sodium citrate, respectively. The size and structure of the AgNPs and AuNPs were characterized using TEM, AFM and XRD methods. The average size of the AgNPs and AuNPs was between 8 and 15 nm. Recombinant arginase I was immobilized using the carbodiimide-pentafluorophenol method on the surface of NPs functionalized with ω -mercaptohexadecanoic acid. It was shown that recombinant human liver arginase I isolated from the yeast *Hansenula polymorpha* maintains satisfactory stability after immobilization on both NPs. The immobilized arginase retained 40% of its activity on the surface of AuNPs and 25% on AgNPs compared to the free arginase after storage at +4 °C during 25 days. The immobilized enzyme can be used for assay of arginine in pharmaceuticals, in food products and in blood.

Introduction

Metal nanoparticles (NPs), such as gold (Au) and silver (Ag), have recognized importance in chemistry, physics, and biology due to their unique optical, electrical, and photothermal properties.^{1,3} Such NPs have potential applications in analytical chemistry and have been used as probes in mass spectroscopy,⁴ as well as in the colorimetric detection of proteins and DNA.⁵ AuNPs have photothermal properties that can be exploited for localized heating

which results in drug release, thus increasing their potential for therapeutic applications.⁶ AuNPs and AgNPs also exhibit a unique phenomenon, known as Surface Plasmon Resonance, which is responsible for their large cross-sections for absorption and scattering, which are four to five orders of magnitude larger than those of conventional dyes. Furthermore, their optical properties can be controlled by varying their size, shape and composition.

Various methods for the synthesis of Au- and AgNPs have been reported over the last two decades. They involve the reduction of HAuCl₄ and AgNO₃ with chemical reducing agents such as citric acid, borohydride, or other organic compounds.^{7,8} Metallic NPs have been widely used in the immobilization of many bioactive substances such as peptides, enzymes⁹ and antibodies.¹⁰ AuNPs are among the most commonly used materials, due to their low toxicity.¹¹

Numerous methods have been used for detecting AuNPs, including colorimetric, scanometric, fluorescence, surface-enhanced Raman scattering and electrochemical techniques. AuNPs-based colorimetric assays in particular demonstrate great potential in point-of-care testing assays.¹²

Use of NPs as carriers for immobilized enzymes has the following advantages: i) higher specific surface available for the binding of a larger amount of enzyme; ii) lower mass transfer resistance and less fouling.¹³

Arginase (EC 3.5.3.1; L-arginine amidinohydrolase) is a key enzyme of the urea cycle. It catalyses the final cytosolic reaction of urea formation in the mammalian liver – the conversion of arginine to ornithine and urea. Arginase has recently been considered not only as a prospective pharmaceutical in enzymotherapy of some kinds of cancers auxotrophic for arginine,¹⁴ but also as an analytical instrument for L-arginine assay.

The aim of the present study was to immobilize the recombinant human arginase I on AuNPs and AgNPs and to characterize the obtained enzyme-NPs: analyses of their size, structure, enzymatic activity and stability.

Materials and Methods

Silver nitrate, D-glucose, tetrachloroauric acid trihydrate, sodium citrate, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide hydrochloride (CMC), pentafluorophenol (PFP), 2-(2-aminoethoxy) ethanol (AEE), dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA), phenylmethylsulfonyl fluoride (PMSF) and 16-mercaptohexadecanoic acid (MHDA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

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Enzyme preparation

Human liver arginase I was isolated from the recombinant yeast strain NCYC 495 - *pGAP1-HsARG1 (leu2car1 Sc:LEU2) Hansenula polymorpha*, constructed in the Institute of Cell Biology, NAS of Ukraine.¹⁵ Cultivation of the recombinant yeast cells was performed in flasks on a shaker (200 rpm) at 30 °C, for 48 h, in a Burkholder basal medium containing 1 mM L-arginine and 20 g/L sucrose, 3 g/L (NH₄)₂SO₄, 0.5 g/L KH₂PO₄, 0.2 g/L MgSO₄ and 0.15 g/L CaCl₂. The medium contained 2 ppb biotin, 0.07 ppm Zn, 0.2 ppm Fe and 0.01 ppm of trace elements B, Cu, Mn and Mo, added in the form of appropriate salts. To obtain the cell-free extract (CE), washed cells were suspended in 30 mM HEPES buffer, pH 7.5 (HB), containing 1 mM PMSF and 1 mM EDTA, disrupted with glass beads (d=0.45-0.5 mm) in a disintegrator at 1000 rpm (r=10 cm) at 4 °C for 6 min and centrifugated at 20,000 g for 40 min. The supernatant, CE, was used for enzyme isolation by one-step column chromatography on the synthesized by us affinity sorbent. To obtain this sorbent, controlled pore glass (with pore diameter 3680 nm) was activated with γ -glycidyloxypropyltriethoxysilane, and after this L-arginine was coupled to the synthesized glycidyl-containing matrix.¹⁵ Arginase was eluted by 0.5-2 M NaCl in HB, fractions with the highest enzyme specific activity were combined and homogeneity of the final arginase preparation was approved by PAAG-SDS electrophoresis. As a result, a 200-fold purified preparation of recombinant arginase was obtained with 15% yield.¹⁶ The enzyme preparation having specific activity of 530 U/mg of protein was immobilized on NPs.

Measurements of enzymatic activity

Arginase activity (for all enzyme preparations – free and immobilized on both NPs) was determined in a two-step reaction: i) enzymatic conversion of L-arginine to ornithine and urea and ii) photometric registration of urea content by the colorimetric method. Arginase specific activity was calculated as the rate of urea production ($\mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein). Usually, 200 μL of a substrate mixture (65 mM L-Arg, 2 mM manganese(II) chloride in 20 mM Tris-base solution, pH 9.5) pre-incubated at 37°C was added to the test tube with 10 μL of arginase preparation. The reaction mixture was incubated for 15 min at 37°C followed by urea assay.¹⁶ To estimate urea content (mM), the commercial kit (SIMKO, Lviv, Ukraine) was applied. The kit is based on a photometric registration of pink-red complex, produced in diacetyl monooxime conversion under acidic pH. After the 1st step of the reaction, aliquots of reaction mixture were immediately distributed into tubes with the 2nd step reaction mixture. After incubation in boiling water during 15 min, the tubes were put in ice-water for 5 min and the optical densities of the experimental and standard test samples, containing urea, were measured at 520 nm.

Synthesis of gold nanoparticles

Gold nanoparticles (AuNPs) were prepared by the citrate reduction method.⁸ 1.25 mL of 1 mM HAuCl₄ and 0.125 mL of 38.8 mM trisodium citrate were mixed at 100°C and stirred for 15 min to obtain a wine-red solution. AuNPs were precipitated from the reaction mixture by centrifugation (3000 g; Hettich Micro-22R centrifuge), washed with 0.1 M NaCl in 0.1 M HEPES buffer, pH 7.5 (HB), water and final AuNPs precipitate was dried (100°C, 24 h). 5 mg AuNPs were re-suspended in 1 ml water in order to estimate the concentration. Under the described conditions, a colloid solution of AuNPs in water at a concentration of 5 mg/mL (25 mM) was obtained for further characterization and enzyme immobilization.

Synthesis of silver nanoparticles

Silver nanoparticles (AgNPs) were prepared by the glucose reduction method.⁷ 0.1 mL of 0.15 M AgNO₃ and 25 mL 0.03 M D-glucose were mixed and 0.5 mL 0.05 M NaOH was added to the reaction mixture. AgNPs were precipitated by centrifugation (3000 g; Hettich Micro-22R centrifuge). The precipitate was washed with 0.1 M NaCl in 0.1 M HB, water and final AgNPs precipitate was dried (100°C, 24 h). 15 mg AgNPs were re-suspended in 1 ml water in order to estimate the concentration. Under the described conditions, a colloid solution of AgNPs in water at a concentration 15 mg/mL (138 mM) was obtained for further characterization and enzyme immobilization.

Immobilization of arginase on the gold and silver nanoparticles

NPs were incubated overnight in 5 mM MHDA in ethanol at +4°C. After rinsing with DMF, the MHDA-covered NPs were incubated in a DMF solution of 20 mM CMC, 20 mM PFP, and 20 mM DIPEA for 30 min at 25°C. After repeated rinsing with DMF, condensation of the activated Au- and Ag-linked carboxylic groups with amine groups of the enzyme was carried out. 25 μL of the enzyme solution in 30 mM HB was incubated with NPs for 1 h at 25°C. After rinsing with HB, blocking of unreacted carboxylic groups with 0.1 M solution of AEE in 0.1 M bicarbonate buffer, pH 8.0, was performed. The bio-functionalized Au- and AgNPs were rinsed with HB and stored at +4°C until used.

The amount of immobilized enzyme on the NPs was determined as the difference between the initial and unbound protein content in the immobilization medium using the Lowry protein assay method.

Identification of nano-scale particles

Powder X-ray diffraction

X-ray diffraction patterns were obtained using a DRON-3 diffractometer. In order to obtain the more precision data, Bragg-Brentano focusing geometry was used. Scattered intensity as a function of scattered angle 2θ was recorded with high accuracy ($\Delta\theta/\theta=1\%$; $\Delta I/I=3\%$).

Optical

The formed AuNPs and AgNPs were characterized by UV-Vis spectroscopy (300-700 nm) on a Shimadzu UV-1650 PC spectrophotometer. The total concentration of the metal in NPs solutions was determined gravimetrically.

Transmission electron microscopy

NPs analysis was carried out on a film-coated drop using a PEM-100 transmission electron microscope. The special covered film was formed on the copper net (300 mesh) with a Butvar solution B-98 (Sigma, St. Louis, MO, USA) in 1.5% chloroform using an ultrasound method (USDN-2T at resonance frequency 24 kHz) followed by dropping the sample NPs on the surface of the composed film.

Atomic force microscopy

The size and structure of gold NPs and their complexes with enzyme were studied by atomic force microscope Solver P47-PRO (NT-MDT). An aliquot of the tested sample was spread on the surface of freshly-cleaved mica, dried and analyzed in air using the tapping mode with a resonance frequency of 160 kHz, scan rate of 1 Hz/s and resolution of 256×256 pixels.

Effect of pH, temperature and storage time on arginase activity

To estimate the pH optima of arginase activity for free enzyme and immobilized on AuNPs, the substrate mixtures for arginase assay were prepared using different 20 mM buffers: MOPS, pH 4.0; K, Na-phosphate, pH 6.0 and 7.0; HEPES pH 7.5; Tris-HCl, pH 8.8; Na-borate, pH 10.5 and 12.0. The rest of the procedure (pre-incubation, enzymatic and colorimetric reactions) was the same as described in the *Measurement of enzymatic activity* part.

The effect of temperature was examined for free and immobilized arginase preparations in standard reaction mixture under different temperatures. To estimate the temperature optima, the substrate mixture of the first step of reaction (conversion of arginine to urea) was pre-incubated for 5 min at 10°C, 23°C, 37°C, 50°C, 60°C, 70°C, 80°C, 90°C or 100°C and incubated with enzyme for 7 min at the same temperature. The second step of the reaction (urea registration) was done at the standard conditions. To estimate arginase thermostability, the tested samples were pre-incubated for 15 min at the different temperatures (10°C, 23°C, 37°C, 50°C, 60°C, 70°C, 80°C, 90°C and 100°C) and cooled by ice water. Then the standard test for enzyme activity determination was carried out.

Stability test of enzyme preparations (free or immobilized on NPs) in terms of repetitive applications was performed in three successive measurements during the day. For examination of storage stability, the enzyme preparations were kept at +8°C in HB and the samples were tested during several days.

The results were obtained from at least three independent experiments. The highest value of arginase activity in each set was assigned as 100% activity.

Results

Powder X-ray diffraction

XRD pattern of the AuNPs is shown in Figure 1. All diffraction peaks, including not only the peak positions, but also their relative intensities, can be perfectly indexed into the cubic crystalline structure of AuNPs. The crystallite size was calculated from the main diffraction peak (111) using Sherrer's formula:

$$D = \frac{0,9\lambda}{\beta \cos \theta}$$

where λ is the X-ray wavelength, β is the observed full width at half maximum and θ is the Bragg angle.

Parameters of the crystal cell were estimat-

ed from the XRD pattern (Table 1). As shown in Table 1, the type of crystal cell is cubic and its parameter (a) equals 4.0732 Å. The size of crystallites was found to be $L=8.1\pm 0.7$ nm.

Immobilization of the enzyme on nanoparticles

Table 2 demonstrates the quantitative parameters of arginase immobilization on both NPs: efficiency of arginase binding on the surface of AgNPs and AuNPs was 85% and 86.7%, respectively.

Optical properties of Ag and Au nanoparticles

UV-VIS absorption spectra have been proven to be quite sensitive to the formation of gold and silver colloids because these NPs exhibit intense absorption peaks due to surface Plasmon excitation.¹⁷ The UV-VIS spectra of both NPs in the 300-700 nm range are presented in Figure 2. The absorption band in the visible light region and the Plasmon peak at 450 nm are typical for AgNPs.¹⁷⁻¹⁸ The maximum absorption for AuNPs was observed at 540 nm, as described by Sun *et al.*⁸

Figure 3 presents calibration curves for both NPs obtained by spectrophotometry and calculation of NPs concentration on the base of spherical model.³ Using slopes values (B) we have obtained the following molar extinctions (ϵ) for NPs: $1.8\cdot 10^6$ M⁻¹·cm⁻¹ for AuNPs and $2.5\cdot 10^7$ M⁻¹·cm⁻¹ for AgNPs.

The total concentration of elemental metals (Au, Ag) in colloidal solutions was tested gravimetrically. The most difficult challenge was estimation of metallic NPs concentration (in molar equivalents of metal atoms aggregates). For this aim, we used the theoretical model described by Jain *et al.*³ related to cubic or spherical symmetry of the NPs, as well as the linear size of these particles. Approximate calculations were based on a simple geometric base.³ Morphological characteristics of AuNPs were obtained by TEM. As shown in Figure 4 A, the average diameter of AuNPs was close to 10 nm, however, after enzyme immobilization, the sizes of nanoparticles increased up to 25-50 nm (Figure 4B). This phenomenon can be

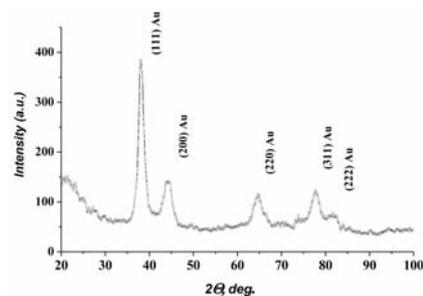


Figure 1. X-ray diffraction pattern of the synthesized AuNPs.

Table 1. Crystal cell parameters.

Crystal data	Au (Cu K α radiation, $\lambda = 1.5418$ Å)
M_r	197
Type of crystal cell	cubic
Space group	Fm-3m
Parameter of cell	$a=4.0732$ Å, $V = 67.58$ Å ³
Scattering angle limits	$2\theta_{\min} = 20.05^\circ$, $2\theta_{\max} = 100^\circ$, $2\theta_{\text{step}} = 0.05^\circ$
Factors of divergence	$R_p = 0.044$, $R_{wp} = 0.057$
Fractional atomic coordinates and isotropic or equivalent isotropic displacement parameters (Å ²)	(x y z)=(000); $U_{\text{iso}}^*/U_{\text{eq}} (0.0377\cdot(7)^*)$

Table 2. Activity of the arginase I samples during the immobilization procedure.

Parameter	Free enzyme (before immobilization)		Supernatant after immobilization process		Arginase on NP	
	Au	Ag	Au	Ag	Au	Ag
V, μ L	25	25	65	65	50	50
Protein concentration, mg/mL	0.50	0.50	0.30	0.15	ND*	ND
Total protein, μ g	12.5	12.5	20	10	ND	ND
Activity, U/mL	47.2 ± 0.1	47.2 ± 0.2	2.50 ± 0.01	3.4 ± 0.2	20.8 ± 0.1	20.4 ± 0.2
Total activity, U	1.2 ± 0.1	1.20 ± 0.02	0.16 ± 0.01	0.22 ± 0.02	1.04 ± 0.01	1.02 ± 0.03
Yield, %	=100	=100	13.5 ± 1.5	18.3 ± 1.0	86.7 ± 2.2	85 ± 1.7

ND, not determined

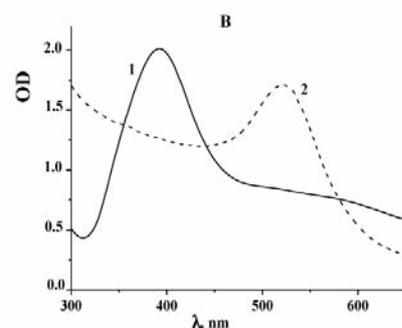
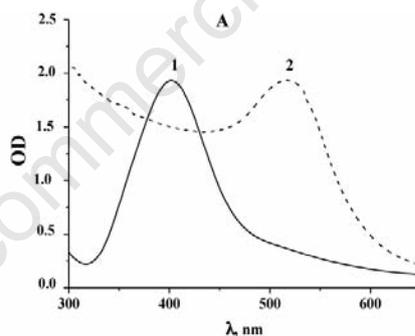


Figure 2. UV-visible spectra of silver (1) and gold (2) nanoparticle solutions. A) before and B) after arginase immobilization. The total concentrations of the metals in AgNPs and AuNPs are 138 mM and 25 mM, respectively.

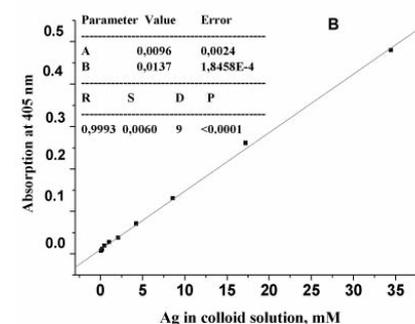
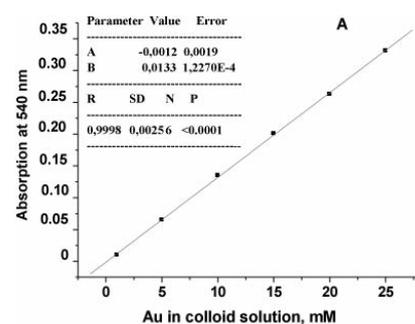


Figure 3. The calibration curves for total metal concentrations in NPs. Some statistical data are presented for the parameters of linear regression (coefficients of the equation $Y=A+BX$, where Y - optical density; X - total metal concentration in colloid solution, mM; R - linear regression coefficient).

explained by aggregation under the arginase immobilization procedure.

Figure 5 shows the Atomic Force Microscopy (AFM) images of the AuNPs: the average diameter of the particles is about 15 nm. This is larger than the value obtained from the TEM and XRD analysis. This may be attributed to the intrinsic enlarging effect of the microscopic pinpoint to the measured nanoparticles, resulting in the overestimation of dimensions with an AFM. The morphology of AuNPs (Figure 5A) was almost smooth, and their surfaces were homogeneous.

To compare the storage stability of different arginase preparations, enzyme activities of free and both NPs-bound enzyme were determined during several days. As shown in Figure 6A, only 55% residual activity was observed in free arginase and arginase-AgNPs after 5 days. However, the residual activity of arginase-AuNPs during the same time period was 95%, and 40% after 25 days, demonstrating a considerable enhancement in the enzyme's stability. At the same time, only 20% arginase activity was observed after 25 days of experiment with arginase-AgNPs. AgNPs are thus less effective nano-carriers for arginase than AuNPs.

The results, presented in Figure 6B and C, demonstrate that there is no difference between the pH optima (pH 7.5-9.0) and the temperature optima (55-70°C) for the arginase in solution and immobilized on AuNPs. The thermostability of free arginase and arginase-AuNPs is high too: both preparations kept 100% and about 15% of activity after pre-incubation during 15 min at 70°C and 90°C, respectively.

Discussion

In the present work, gold and silver nanoparticles were synthesized followed by functionalization of the colloids with 16-mercaptohexadecanoic acid. The size and structure of the NPs were characterized using TEM, AFM and XRD-methods: the size of both nanoparticles was shown to range between 10 and 35 nm.

An effective method for enzyme immobilization on the surface of silver and gold nanoparticles using carbodiimide-pentafluorophenol method was proposed. Recombinant human arginase I was successfully linked on both NPs with a binding efficiency of 85% for AgNPs and 86.7% for AuNPs (in the range of added enzyme concentration 0.15-0.5 mg/mL). The nano-scale sizes of both arginase-modified colloids were confirmed by scanning electron microscopy. The storage stability of the different arginase preparations during 25 days was shown to increase in the following order: control enzyme in solution, enzyme on AgNPs and enzyme on AuNPs. Thus, the synthesized gold

and silver nano-carriers have a stabilizing effect on recombinant human arginase I due to its fixation on the surface of the NPs, preventing enzyme inactivation.⁹ AgNPs are less effective nano-carriers than AuNPs for arginase I, may be due to an inhibitory effect of a theoret-

ically existing Ag⁺ ion on Mn²⁺-dependent enzyme activity during the immobilization procedure and storage conditions. As shown for other enzymes from thermotolerant yeast *H. polymorpha*, their immobilization opens extensive possibilities for the construction of

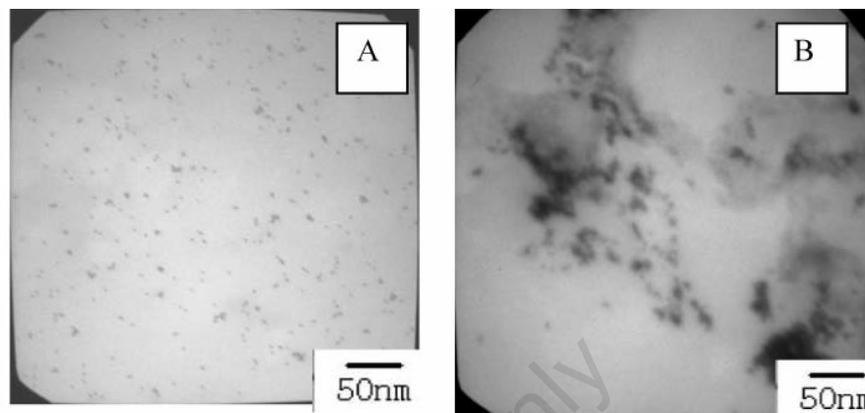


Figure 4. Transmission electron microscopy micrographs of the synthesized AuNPs before (A) and after (B) arginase immobilization.

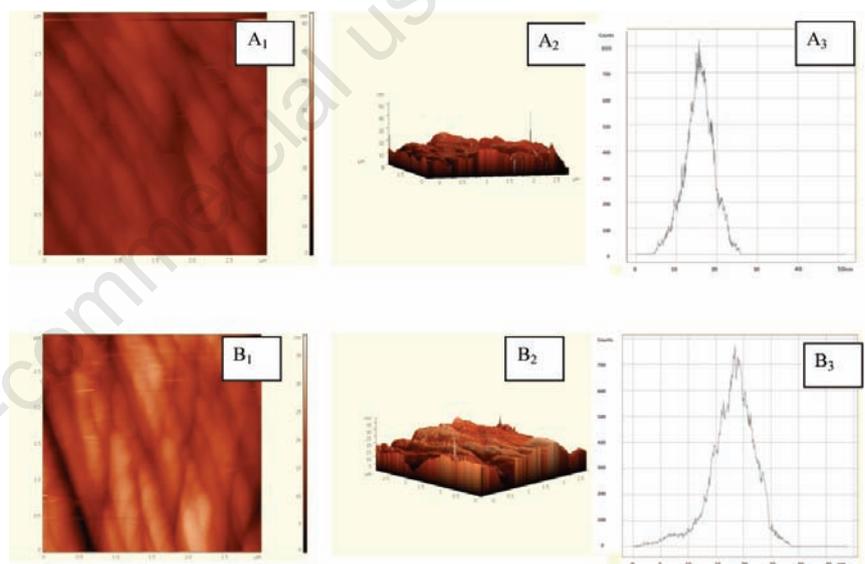


Figure 5. AFM micrographs (1, 2) and Gaussian distribution by size (3) of AuNPs before (A) and after immobilization of arginase (B).

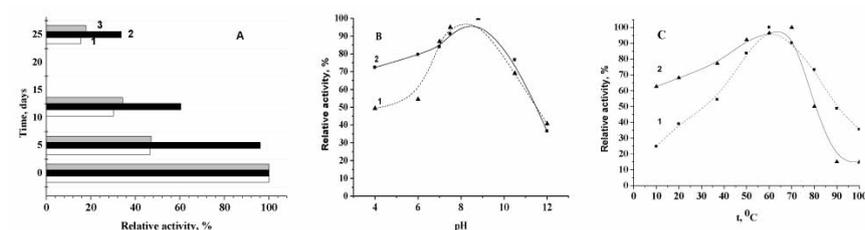


Figure 6. Comparative characteristics of different arginase preparations: in solution (1); immobilized on AuNPs (2) and immobilized on AgNPs (3). A) storage stability; B) pH optima; C) temperature optima. The maximal activities of arginase were taken as 100% in each case.

very sensitive and stable biosensors for wide biochemical applications.¹⁹⁻²¹ A high thermostability of immobilized on NPs arginase I preparations and a wide working pH range of this enzyme allows to hope that it will become a prospective tool for bioanalytical purposes, namely, for arginine monitoring in pharmaceuticals, in food products and in blood.

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