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STUDY OF PROTEIN MODIFIED GOLD NANOPARTICLES IN BULK PHASE AND AT AIR/WATER INTERFACE

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Abstract. The colorimetric assay based on the surface plasmon resonance of metal nanoparticles receives significant attention because of its simplicity, sensitivity and low cost. The preparation of stable suspensions of gold nanoparticles (GNPs) modified with proteins is a prerequisite for their use as an analytical tool for the colorimetric spectral analysis. A convenient experimental procedure for reproducible production of functionalized with Bovine serum albumin (BSA) GNPs is proposed. The functionalized GNPs were morphologically characterized by means of Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) and their properties were determined by UV-Vis spectroscopy. The Langmuir monolayers of GNPs/BSA were also studied by measuring the surface pressure-area (π-A) isotherms and the conditions for transferring the Langmuir – Blodgett (LB) films on mica solid supports were found. The LB films were further characterized by AFM and TEM. The reported experimental protocols and procedures have potential for further applications for the development of biosensors for detection of enzyme activity.

Keywords: gold nanoparticles (GNPs), atomic force microscopy (AFM), transmission electron microscopy (TEM), bovine serum albumin (BSA), protein modified nanoparticles

Introduction

Gold is known for its unique properties for centuries. The archeological studies show that people have used gold since ancient times to make jewelry, coins, objects with religious and sacral functions and even weapons (Freestone et al., 2007; Dushkin et al., 2011). In recent times the gold still has its wide practical application in various fields of science and technology where the increasing level of scientific knowledge has opened new practical fields for its application, e.g. in chemistry, pharmacy and medicine (Edwards & Thomas, 2007; Suzuki et al., 2006; Wang, 2007; Georgiev et al., 2014). While in nowadays the bulk gold is commonly used for making some exotic and expensive objects, the colloidal gold, which is also known in the literature as gold nanoparticles (GNPs), has found variety of new applications e.g. as catalysts, pigments in arts, in solar cells, sensors for biomolecules etc. Systematic scientific investigations and first colloidal synthesis of GNPs were preform in 1857 by Michael Faraday (1857). The physical and chemical properties of the GNPs make them excellent material for construction elements in chemical and biological sensors (Suzuki et al., 2006). The GNPs have very good optoelectric properties, biocompatibility, when they are used with right ligands. These properties can be modulate, with the change of their size and shape. The GNPs can be functionalized with different organic or biological ligands and can be used for detection of concentration of small molecules and biological objects. The color of the GNPs is very sensitive to their aggregation in suspension. These unique properties of the GNPs are used for the development of new sensor systems with very good sensitivity and selectivity (Chen et al., 2011; Pingarron et al., 2008; Wei et al., 2008). This is a new generation of devices for detection in a new and far more effective way of metal ions, small molecules, proteins, nucleic acids, etc. (Hutter & Maysinger, 2013).

The preparation of stable suspensions of GNPs modified with proteins is a prerequisite for their use as an analytical tool for the colorimetric spectral analysis. In recent years, colorimetric assay based on the surface plasmon resonance of metal nanoparticles receives significant attention because of its simplicity, sensitivity and low cost (Hinterwrith et al., 2012). The spherical GNPs were used for detecting the biomolecules following the principle of the aggregation induced red to blue color shift. The indisputable advantage of protein modified GNPs for biosensors' applications is their ability to specifically anchor biomolecules.(Pingarron et al., 2008) At Fig. 2.6 are depicted two experimental approaches for utilization of the functionalized GNPs for sensing the kinetics of enzymatic reactions (Hutter & Maysinger, 2013) In the first scheme (Fig. 1A) the GNPs' suspension is stabilized by a „protein shield". The addition of the enzyme leads to a distortion of this structure followed by destabilization and formation of particles' aggregates which

results in a red shift of the plasmon absorption maximum. In the second scheme (Fig. 1B), the GNPs are modified in such way that they interact with each other forming aggregates which after the addition of the enzyme are shattered to individual GNPs and the plasmon absorption maximum at 520 *nm* is recovered.

Conveniently, a similar approach could be applied for determining the activity of proteases which catalyze the hydrolysis of the protein shield and might destabilize the functionalized GNPs.

In this article we present a convenient experimental approach for studying the properties of the GNPs functionalized with proteins by means of Langmuir trough after spreading the suspension of GNPs' at the air/water interface and formation of monolayers which in a subsequent step is transferred to the solid supports by the Langmuir – Blodgett method. We also adapted the Turkevich method (Turkevich et al., 1951) for synthesis of GNPs for functionalization of the nanoparticles with BSA. The GNPs/BSA were morphologically characterized by AFM and TEM and their absorption properties were determined by UV-Vis spectroscopy. We studied the Langmuir monolayers of GNPs/BSA by measuring the surface pressure-area (□-*A*) isotherms and found the conditions for transferring the LB films on mica solid supports. The LB films were further characterized by AFM and TEM.

Materials and methods

Analytical grade tetrachloroauric acid ($HAuCl_4.3H_2O$) was purchased from Panreac (PanreacQuнmica S.A.U., Spain). Trisodium citrate ($Na_3C_3H_5O(COO)_3.2H_2O$) was also of analytical grade and were obtained from Merk (Darmstadt, Germany). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Germany) in form of lyophilized powder. According to producer's specifications BSA has molecular mass about 69 kDa, and total impurities of less than 0.01% fatty acid. Tris(hydroxymethyl) aminomethane (TRIS), used in the buffer solution (TRIS/HCl) with pH 8, was supplied by Merck (Darmstadt, Germany). BSA was dissolved in the buffer to concentration 0.01 *M*. HCl was purchased from Theokom (Sofia, Bulgaria) and was used without further purification. In all experiments pure water from Milli-Q system (Millipore Corporation, Boston, USA) was used.

The synthesis procedure, based on classical Turkevich method (Turkevich et al., 1951) was performed as follows: 10 *ml* 2.5 *mM* solution of tetrachloroauricacid ($HAuCl_4$), containing 5 *mg* gold, was added to 85 *ml* deionized water. This mixture was stirred at 350 *rpm* and heated up to 90 °C, after that 5 *ml* 1% solution of trisodium citrate was added as reducing and stabilizing agent. After the first 3–5 *min* the solution has colored

to blue via pink to cherry red as the reaction is completed at the 30 *min* with purple red coloration.

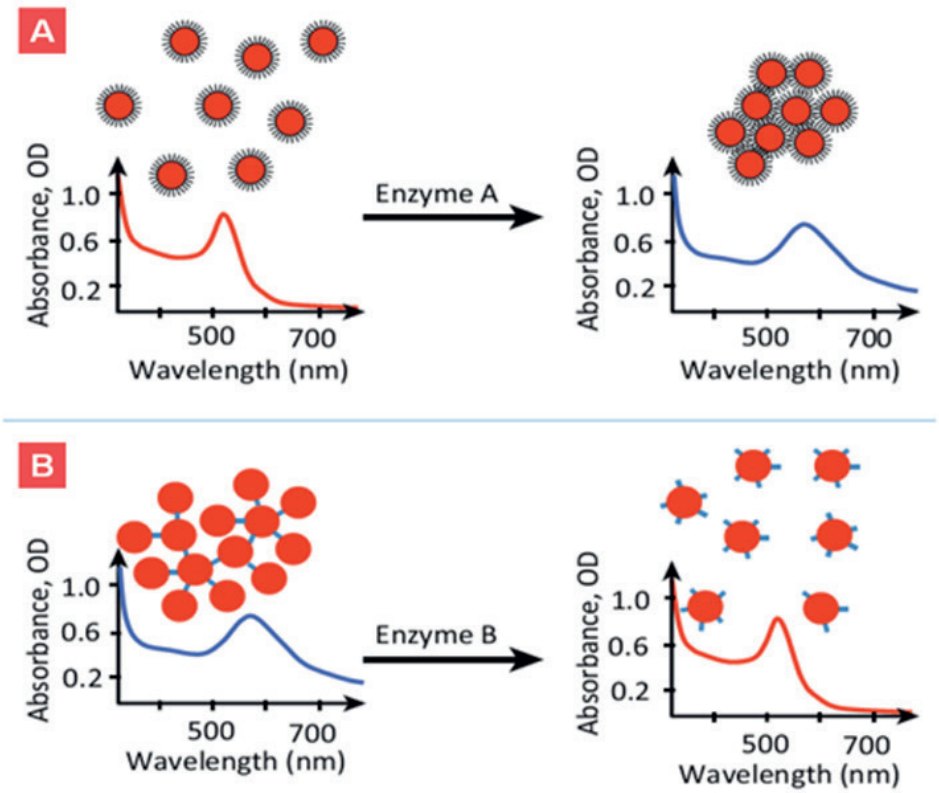

Fig. 1. (A) The colloidal suspension is stabilized by a „protein shield". The enzyme catalyzes the reaction which leads to formation of particles' aggregates causing a red shift of the absorption maximum. **(B)** The gold nanoparticles are organized in aggregates and the addition of enzyme fragments them to individual GNPs causing a recovery of the plasmon absorption maximum at 520 *nm*. The part of the figure is adopted from the literature (Hutter & Maysinger, 2013).

In order to functionalize the surface of the GNPs with BSA the following procedure was applied: The suspension of GNPs was mixed with 0.01 *M* BSA solution in volumes' ratio *GNPs*:*BSA* = 1:1. This concentration was calculated so that the BSA molecules

are expected to adsorb and cover the entire spherical surface of the gold nanoparticles. From the resulting solution 4 *ml* were pipetted in four Eppendorf tubes, 1 *ml* each, which were further centrifuged in ultracentrifuge at 14,500 *rpm* for 15 *min*, after which the supernatant was removed. Each of the four Eppendorf tubes were resuspended with 250 *μl* TRIS buffer (to pH 8), and were transferred in 1 *ml* Eppendorf tube thus giving four times bigger concentration of the GNPs suspension than the initial one. It was further centrifuged at 14,500 *rpm* for 6 *min*, the supernatant was castoff and the above procedure repeated twice. In such manner, after the redispersion of the GNPs in the buffer is expected that the excess of the protein was removed.

The UV-Vis absorption spectra of gold nanoparticle dispersions were determined by spectrophotometer Evolution 300 Thermo Science. Deionized water was used as a reference sample. The morphology and the size of the GNPs were determined by JEM-2100 LaB6 (JEOL Ltd., Japan). The samples for TEM imaging were prepared as the solution of GNPs was deposited on a copper grid. Sample preparation for AFM imaging involved a deposition of GNPs' solution on freshly cleaved mica. Freshly cleaved quadratic mica sheets (Structure Probe Inc./SPI Supplies, West Chester, PA, USA) with sizes 10 x 10 *mm* glued to the metal pads were used for the deposition of nanoparticle solution. AFM imaging was performed on the NanoScope V system (Bruker Ltd, Germany) operating in tapping mode in air at room temperature. We used silicon cantilevers (Tap300Al-G, Budget Sensors, Innovative solutions Ltd, Bulgaria) with 30 *nm* thick aluminum reflex coating. According to the producer's data sheet the cantilever spring constant was in the range of 1.5 to 15 *N/m* and the resonance frequency was 150 ± 75 *kHz*. The tip radius was less than 10 *nm*. The scan rate was set at 1 *Hz* and the images were captured in the height mode with 512 × 512 pixels in a JEPG format. Subsequently, all the images were flattened by means of the Nanoscope software. The same software was used for section analysis and particle size.

The BSA or GNPs/BSA monolayers were spread on water subphase at a maximum available trough's area of 476 cm^2. The commercially available Langmuir film balance KSV 2200 (Finland) was used. The monolayers were formed by spreading of BSA or GNPs/BSA from aqueous solution at the water interface by Hamilton microsyringe. After spreading, the surface pressure fluctuated around the values of 0.1 mNm^{-1}. Ten minutes after spreading, the monolayer was compressed with constant velocity $U_b = 50\ cm^2min^{-1}$. The isotherms measured at these conditions were well reproducible. The monolayers were transfer to mica solid supports by Langmuir – Blodgett (LB) deposition vertically. The freshly cleaved mica sheet was immersed in the subphase before the spreading of BSA or GNPs/BSA. The monolayers were compressed at a constant speed of 1 $Å^2$/molecule/

min to a final surface pressure of 15 mNm^{-1} and 10 mNm^{-1} for the BSA and GNPs/BSA monolayers, respectively. After 30 *min* of waiting time, the mica plate was pulled out of the subphase with the transferring rate of 5 *mm*/*min*. The transfer ratio coefficient was close to 1. Prior to imaging, the LB films were extensively dried out with a mild flow of nitrogen gas for about 5 *min*.

Results and discussion

To study the process of the GNPs' functionalization as a first prerequisite step has to be measured the plasmon absorption spectra of the GNPs before and after their functionalization and to be traced any eventual spectral shifts and changes of the picks' intensity. Because the absorption maximum of the GNPs with diameter of 20 *nm* lays at about 520 *nm*, GNPs' spectrum can be used as reference for an assessment of their size. As a typical example, at Fig. 2 are presented UV-Vis absorption spectrum of the GNPs synthesized by Turkevich method (the curve at Fig. 2, labeled AuNPs), i.e. before their functionalization with BSA. The GNPs' diameter determined from TEM and AFM images (data not shown) is about 20 *nm* and their spectrum has an intense absorption maximum at about 520 *nm* due to the surface plasmon resonance.

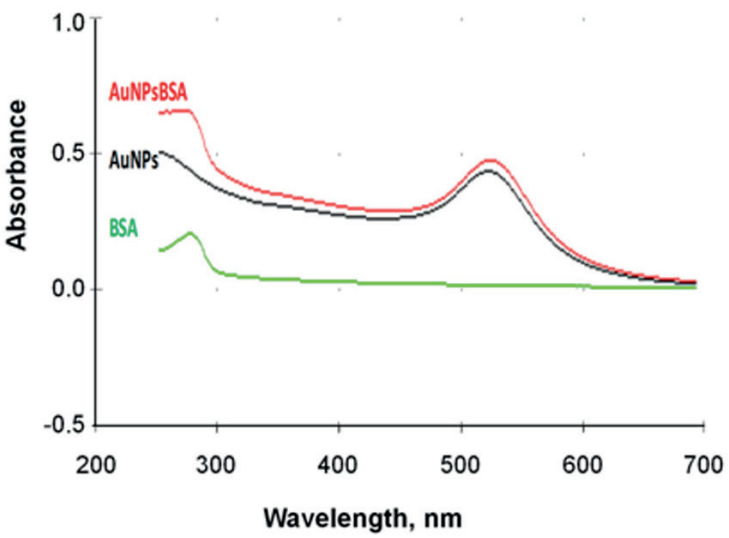

Fig. 2. Comparison of the absorption spectra of: BSA (green line (BSA)); GNPs synthesize by Turkevich method (black line (AuNPs) and GNPs functionalized with BSA (red line (AuNPs BSA).

After the functionalization of the GNPs with BSA the plasmon resonance peak shifts from 520 to 525 *nm* with small incensement of the intensity (the curve at Fig. 2, labeled AuNPs BSA). Moreover, the characteristic absorption maximum in the spectrum of pure BSA at 280 *nm* (the curve at Fig. 2, labeled BSA) is also present in the spectrum of the functionalized GNPs. Because this spectrum has been taken after removing the excess of BSA by centrifuging of GNPs/BSA mixture, it implies that the maximum at 280 *nm* is due to the amino acids' functional groups (i.e. L-tyrosine) of BSA molecules which form the protein shell around the GNPs.

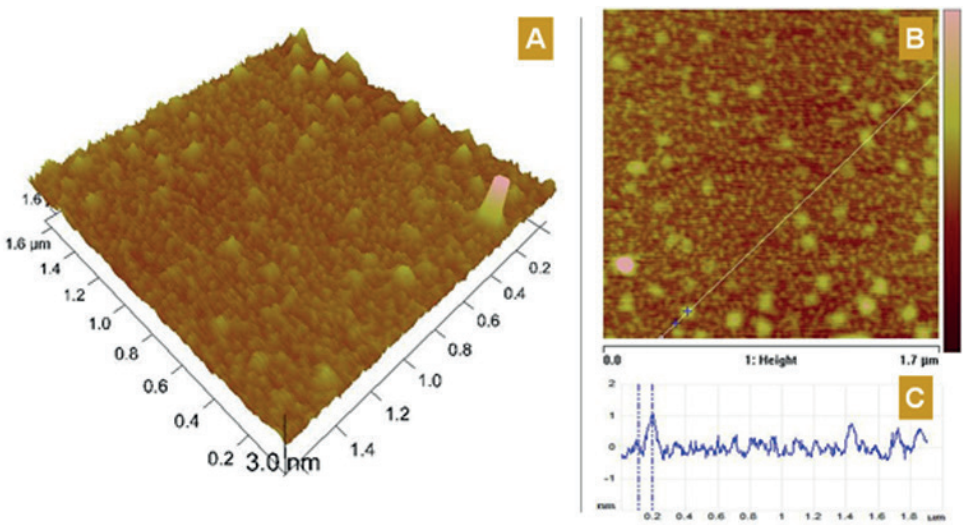

Fig. 3. (A) 3D AFM image obtained after depositing of GNPs suspension on mica support. The z size is $z = 3$ *nm*. The gold nuclei together with Au nanoparticles (nanoclusters) at the mica surface are visible as the scanning XY area was 1.7 μm × 1.7 μm. **(B)** 2D image of the same scanned area together with **(C)** section across one the imaged Au nanoclusters

The characterization of metal nanoparticles and particularly the GNPs by means of AFM is relatively new experimental approach. The method has some advantages over TEM concerning the resolution, easiness in sample preparation and the ability to be studied the kinetics of GNPs' growth by extracting samples in the course of the GNPs synthesis. For example, at Fig. 3 are presented AFM images in 3D (Fig. 3A) and 2D

(Fig. 3B) format of gold nucleus extracted from the reaction mixture at the initial stage (the 1st minute) of the Turkevich synthesis. The images are accompanied with a graph (Fig. 3C) of the cross section over individual gold nucleus, giving their mean diameter of about 1 *nm*.

The AFM images presented at Fig. 4 correspond to samples prepared after depositing some amount from the solution of modified GNPs/BSA onto mica solid supports which were further dried out after exposing on a mild flow of nitrogen gas.

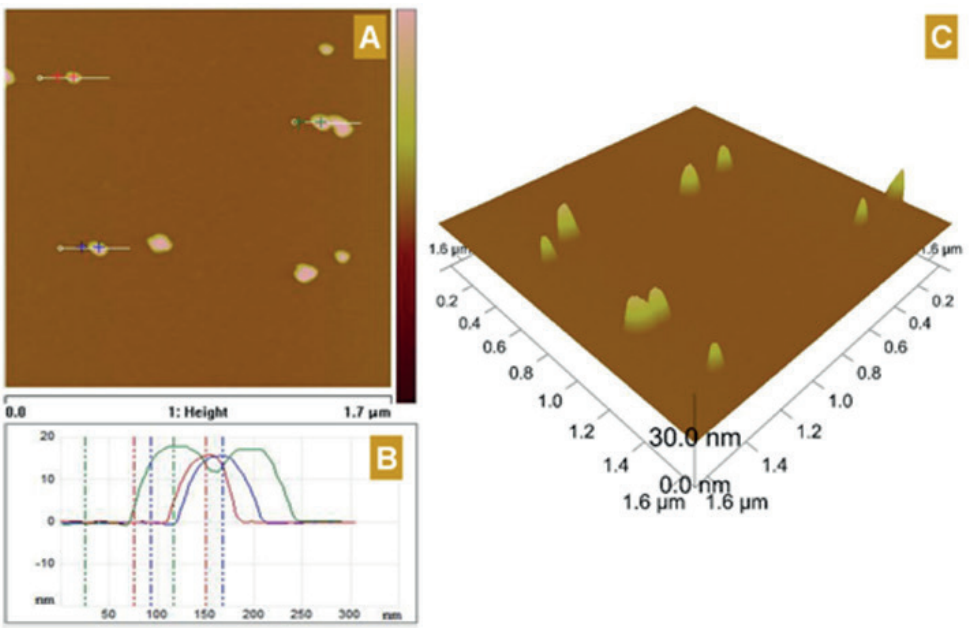

Fig. 4. (A) 2D and **(B)** 3D AFM image of GNPs functionalized with BSA, **(C)** Section across the line at (A) over the individual nanoparticles

The AFM images are presented in 2D and 3D format at Fig. 4A and Fig. 4B, respectively, showing clearly the individual GNPs functionalized with BSA and adsorbed on the mica surface. The AFM images are also accompanied with a cross section graph which analysis gives for the diameter of the individual nanoparticles values about 20 *nm*. It is well-known from the literature that adsorbed on mica BSA films and even single BSA molecules can be reproducibly imaged by means of AFM (Balashev et al.,

2011). The background of the images at Fig. 4 does not show any of the specific for BSA film features or any traces of the individual BSA molecules which confirms the lack of residual protein in the GNPs/BSA solutions.

At Fig. 5 are presented typical TEM micrographs of GNPs, synthesized by the Turkevich method, and further functionalized with BSA.

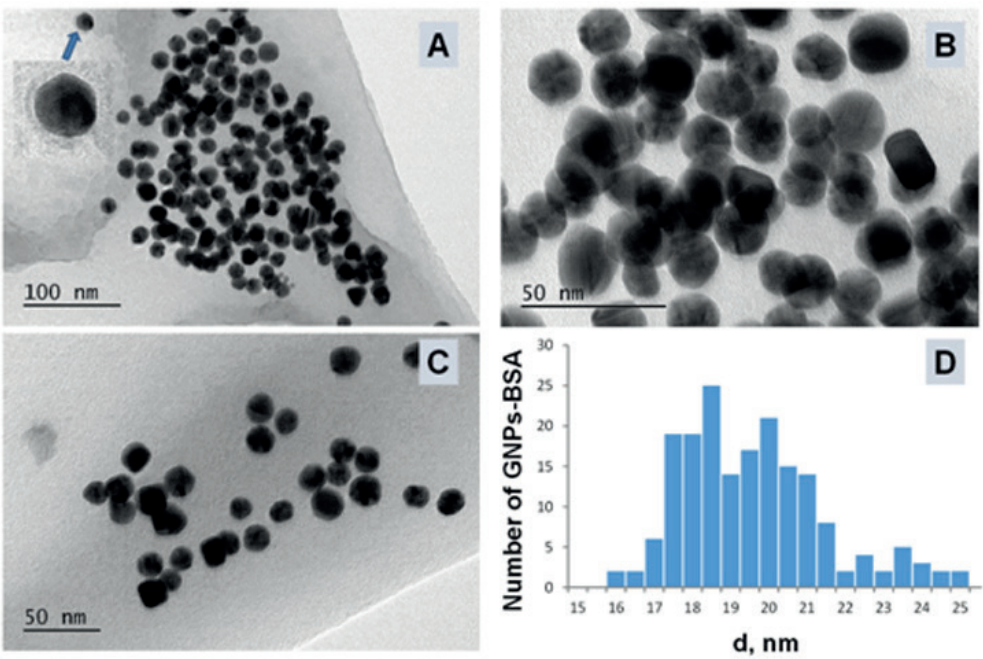

Fig. 5. TEM micrographs of gold nanoparticles functionalized with BSA. **(A), (B), (C)** are images with different magnifications. The arrow at (A) indicates a single GNP which is zoomed in the inset image where is visible a protein shell; **(D)** is a size distribution of the nanoparticles

All TEM micrographs have a high resolution, allowing easily to characterize the GNPs' shape and size. The predominant form of the GNPs is spherical, although in some occasions may be identified a small number of particles with triangular and hexagonal shape. The histogram obtained from the analysis of TEM data (Fig. 5D), shows the size distribution (diameter *d*) of the GNPs. The statistics is based on the measurements of more than two hundred GNPs with typical lognormal distribution giving for the GNPs'

diameter a mean value of about 20 ± 1.5 *nm* (Fig. 5B), which is in excellent agreement with the literature data.

The characteristics of GNPs and GNPs/BSA are summarized in Table 1.

To study the monolayers of GNPs modified with BSA, as a first step one needs to measure by means of Langmuir trough the isotherms of pure BSA, spread from aqueous solution at the air/surface. To obtain a stable protein monolayer it is necessary the aqueous solution of the protein (i.e. BSA) to contain a small amount of isopropyl alcohol (Boury et al., 1995 ; Balashev et al. 2011).

The surface pressure-area (π-*A*) isotherms of BSA and BSA functionalized GNPs spread at the air/water interface are presented at Fig. 6.

Table 1. GNPs and GNPs/BSA

	UV-Vis □, *nm*	**TEM** *Diameter*, *nm*	**AFM** *Diameter*, *nm*
GNPs Turkevich method	520	18 ± 1.5 *nm* *	17 ± 0.5 *nm* *
GNPs BSA fictionalized	525	19 ± 1.5 *nm*	20 ± 0.5 *nm*

* Data are from (Georgiev et al. 2013).

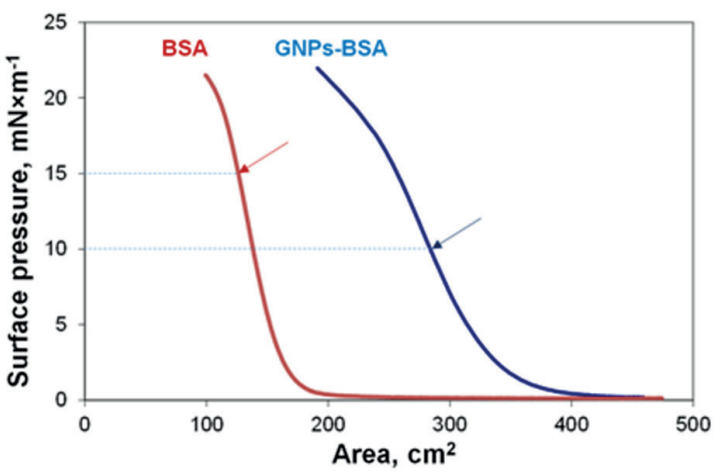

Fig. 6. Surface pressure- area (□ -*A*) isotherms of BSA and BSA functionalized GNPs spread at the air/water interface

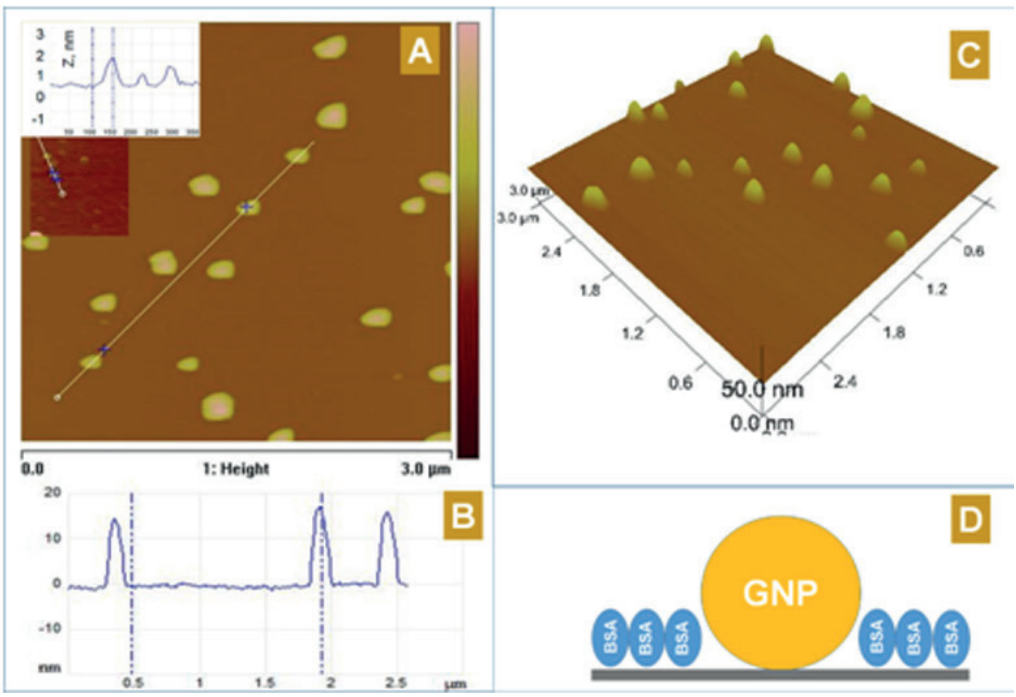

Fig. 7. AFM image of LB film of GNP/ BSA transferred at 15 *mN*/*m* **(A)** 2D AFM 3 × 3 □ m^2 image; **(B)** Section across the line at (A) over the individual nanoparticles **(B)** 3D AFM image; **(D)** cartoon showing the possible embedment of the GNPs in the BSA monolayer

From these isotherms, one can assess the possibility of the protein to form a Langmuir monolayer at the air/water interface as well as to attain quantitative information about the number of protein molecules at the surface. In order to accurately determine the exact number of the protein molecules or nanoparticles at the air/water interface it is necessary the concentration and the amount of the spread solution to be known. In the case of formation of BSA monolayer it is trivial, since the concentration of the BSA is known (it was 10^{-2} *mol*/*L* in our experiments) and the spread amount (50 *ml*), therefore it was easy to find the area per protein molecule or about 4000 *Å*/BSA molecule, a value which is well documented in the literature. In the case of monolayer of GNP/ BSA, though, it is possible only an estimation of the area per GNPs/BSA because we only know the approximate concentration of GNPs/BSA in the solution. Therefore, the isotherms at Fig. 6 are rather illustrative and give only the change of the surface

pressure in the course of compression of either of BSA or GNP/BSA monolayers. At the graphs of the isotherms with arrows are pointed the values of the surface pressure at which the LB films were transferred. The LB film of BSA was obtained at surface pressure 10 mNm^{-1} which correspond to closely packed protein molecules. The monolayer of GNP/BSA was obtained at 15 mNm^{-1} a value which corresponds to the inflection point of the isotherm. The monolayers transferred to a solid substrate (mica or copper nets coated with carbon) in such experimental conditions were next a subject to morphological and structural analysis by means of other methods – AFM, TEM etc. For example, at Fig. 7 are shown AFM images of GNP/BSA films presented in 2D and 3D format.

The LB films were transferred at surface pressure 15 mNm^{-1} as the scanned area was 3.3 □ m^2. At the AFM images the individual GNP are clearly visible on the mica surface. From the picture it can be seen that the gold particles have not formed densely packed layer, which could be due to the partial spreading of their protein shell on the water/air interface, and thereby it was formed a mixed GNPs/BSA monolayer. From the performed section analysis (Fig. 7B) was found the height of the GNPs of about 17 *nm*. Although, this value very likely does not correspond to the real diameter of the GNPs, because might be „buried" in the BSA monolayer with thickness 4-5 *nm* (Fig. 7D). For such conclusion implies the inset of the image at Fig. 7A where with an increased z-resolution are visible small 3D structures with sizes of about 3 *nm* (see the cross section) which are very like to be partly expelled from the BSA film individual protein molecules.

Conclusions

We presented a convenient experimental approach for synthesis and functionalization of GNPs with the protein BSA. We studied the properties of the functionalized GNPs by means of Langmuir trough after spreading the suspension of GNPs' at the air/water interface and transferred them to the solid supports by the Langmuir – Blodgett method. The GNPs/BSA was morphologically characterized by AFM and TEM and their properties were determined by UV-Vis spectroscopy. We studied the Langmuir monolayers of GNPs/BSA by measuring the surface pressure-area (□-*A*) isotherms and found the conditions for transferring the LB films on mica solid supports. The LB films were further characterized by AFM and TEM. This article establishes an experimental procedure for reproducible production of functionalized GNPs which have potential for further application for the development of biosensors for detection of enzyme activity.

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