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Characterization by atomic force microscopy of gold nanoparticles functionalized with azocasein for protease colorimetric enzyme assay

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The colorimetric assay based on surface plasmon resonance of metal nanoparticles have proved to be applicable for sensors systems in the enzyme catalysis 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. In this report we propose a convenient experimental procedure for reproducible production of functionalized with azocasein GNPs. The GNPs were functionalized with azocasein in a stable suspension with optimized concentration of the azocasein. A concept for the test of protease activity using modified with azocasein GNP is also proposed. In order to studying the course of the enzyme reaction we applied UV-Vis spectroscopy and we analyzed the shift of the surface plasmon resonance maxima. For characterization of the morphology and the size of functionalized with azocasein GNPs we made series of Atomic Force Microscopy (AFM) images of samples taken at the begging and at the end of the proteolytic enzyme reaction.

Key words: gold nanoparticles, azocasein, enzyme assay, Atomic Force Microscopy.

INTRODUCTION

In recent decades the development of biomedical sciences is strongly associated with a demand of new chemical approaches for diagnosis and treatment of many diseases. The embedding of protein molecules in various nanostructured materials and nano-sized objects (quantum dots, nanoparticles, etc.) is an effective way of improving their stability and targeting or some other functional properties. Thus, the functionalized with protein nanomaterials are widely used in contemporary pharmaceutical technology for the transport of dosage forms and in biocatalysis. The advantages of the nanoparticles which distinguish them from bulky materials are due to their specific chemical and physical properties. For example, the gold nanoparticles (GNPs) have unique characteristic absorption maximum at 520 nm because of their surface plasmon resonance, which in turn makes them after functionalization with proteins (e.g. antibodies, labeled proteins etc.) highly applicable in medicine as therapeutic agents in drug delivery system [1], for photothermal therapy [2], diagnosing and imaging agents [3, 4, 5]. The nanoscales' size, which meets the dimension of biological compounds, as well as their easy preparation, high surface area and easy functionalization makes them particularly interesting for accomplishing the related applications in tissue engineering and regenerative medicine. There are various synthesis methods for producing gold nanoparticles (GNPs), but in practice are preferable those obtained from aqueous solutions of gold precursors ($HAuCl_4$) in the presence of stabilizing agents [6-10] and among them the most popular is the classical citrate method proposed by Turkevich [11]. Further biofunctionalization of the GNPs with amino acids, peptides, enzymes, DNA, is a challenging experimental step which paves the way for the development of various drug carriers, biomarkers, biosensors [12-14].

A potentential candidate for such applications are GNPs modified with azocasein. Azocasein is a dye-marked protein which is primarily used as a substrate for colorimetric determination of enzyme activity. It is intriguing to explore the possibilities of improving the existing enzyme tests by functionalizing the GNPs with azocasein. Hence, the main aim of this article is to propose a simple and reproducible procedure for functionalization of such GNPs and to apply it in a protocol for determination of the enzyme activity. In such procedure, pre-synthesized by Turkevich method GNPs were further covered by layer or shell of azocasein. As a result, the functionalized with azocasein GNPs had plasmon absorption maxima at about 520 nm together with the absorption maxima of the azocasein at about 340 nm. The added enzyme Protease K, which digests

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proteins, is expected to hydrolase the protein shell of the GNPs and as a result one to observe an increasing of the intensity of both absorption maxima. The Atomic force microscopy (AFM) method was utilized for characterization of the modified with azocasein GNPs before and at the end of the enzyme reaction.

EXPERIMENTAL

Chemicals and reagents

Analytical grade tetrachloroauric acid ($HAuCl_4.3H_2O$) was purchased from Panreac (PanreacQuímica S.A.U., Spain). Trisodium citrate ($Na_3C_3H_5O(COO)_3.2H_2O$) was also of analytical grade and were obtained from Merk (Germany). Azocasein and Protease K were obtained from Sigma-Aldrich. All the solutions were prepared with deionized water.

Experimental methods

The synthesis of gold nanoparticles was performed by the classical Turkevich method [11]. For the functionalization of the gold nanoparticles with azocasein we used following procedure: the solution of GNPs (C = 50 mg/l), synthesized by Turkevich method was mixed with solution of azocasein (10^{-2} M) and water – 12,5 ml GNPs with 8,33 ml H_2O and 4,17 ml azocasein. This new solution is tempered at 35˚ - 37 ˚C (at this temperature interval Protease K has optimal enzyme activity). After the addition of Protease K (C = 2 mg/ml) the samples for UV-Vis spectrophotometry and AFM were collected at every 5 minutes. In the same time the reaction solution was constantly homogenized with rotary magnetic stirrer. The samples were taken and the UV-Vis spectra were measured for a period of 40 minutes, which is considered as the end of the enzyme reaction.

The UV-Vis absorption spectra of the functionalized with azocasein GNPs were determined by spectrophotometer (Evolution 300 Thermo Scientific).

The size of GNPs functionalized with azocasein and Protease K was determined by means of AFM as described in [11, 15]. AFM imaging was performed on the NanoScopeV system (Bruker Ltd., Germany) operating in tapping mode in air at room temperature. We used silicon cantilevers which having tips with radius less than 10 nm (Tap300Al-G, Budget Sensors, Innovative solutions Ltd, Bulgaria), spring constant in the range of 1.5 to 15 N/m and the resonance frequency 150 ± 75 kHz were used. The scanning rate was set at 1 Hz and the images were captured in the height mode with 512 × 512 pixels resolution. Subsequently, all the images were flattened by means of the Nanoscope software. The same software was used for section analysis and particle size determination.

RESULTS AND DISCUSSION

At Fig. 1A are presented UV-Vis absorption spectra of GNPs functionalized with azocasein. Before their functionalization the GNPs which were pre-synthesized by the Turkevich procedure. Their mean diameter was close to 20 nm and the corresponding absorption maxima was about 520 nm [15] while the absorption maxima of azocasein was about 340 nm.

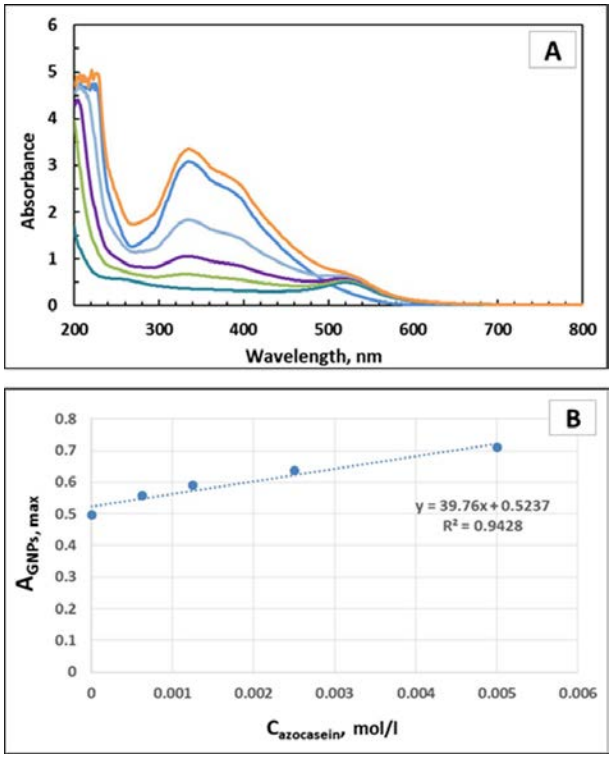

Fig 1. Comparison of UV-Vis absorption spectra with characteristic maxima: pure azocasein solution (340 nm), gold nanoparticles – pure (520 nm) and functionalized with different concentrations of azocasein (**A**). Absorption maxima intensity of functionalized gold nanoparticles as a function of azocasein concentration (**B**).

In the course of GNPs' functionalization the intensity of the UV-Vis plasmon maxima increases proportionally to the increasing of the concentrationof azocasein as it is presented graphically at Fig. 1B. The linear dependence is based on the Beer law as follows:

$$A_{GNPs} = \varepsilon C_{azocasein} + A^0_{GNPs} = A_{azocasein} + A^0_{GNPs} \quad (1)$$

Where A_{GNPs} is the total absorption maxima of the functionalized GNPs, containing shells of azocasein molecules with total concentration of the azocasein $C_{azocasein}$ varying in the range 0.8×10^{-3} to $5 \times 10^{-3} \frac{mol}{l}$. A^o_{GNPs} – intercept of the linear dependence

which corresponds the absorption maxima of the bare pre-synthetized GNPs. The calculated value for the line slope is $\varepsilon = 39$ which is close to molar absorption coefficient for azocasein $\varepsilon = 38$.

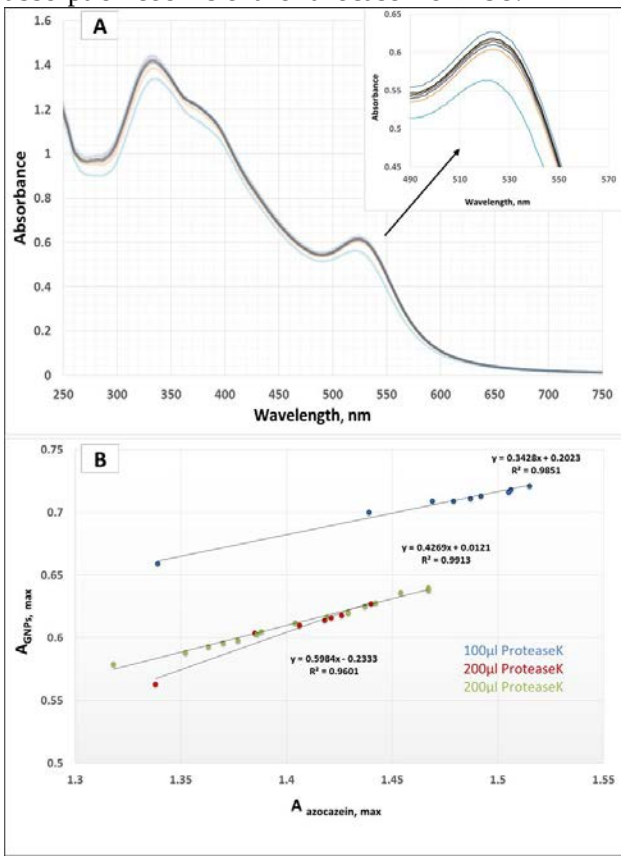

Fig. 2. The increasing of absorption maxima (340 nm and 520 nm) after addition of Protease K (200 ml 2 mg/ml) to the solution of functionalized with azocasein GNPs (**A**). *Inset:* Zoom of the increasing GNPs plasmon maxima (**B**) The linear correlation between the intensity of the plasmon maximum at 529 nm with the azocasein maximum at 340 nm.

At Fig. 2 are presented typical spectra obtained at certain time interval after the addition of enzyme Protease K to the solution of modified with azocasein GNPs. One can clearly observe (the inset in Fig. 2 A) the increasing intensity of the plasmon absorption maximum at 520 nm. It could be explain with the thinning of the GNPs' protein shells which have been digested upon the action of Protease K. At the same time the azocasein adsorption maximum at 340 nm also increases which is result of releasing of the azo-dye in the course of the enzyme reaction- a well-known fact from the classical tests for the protease activity. It is also interesting to point out the linear correlation between the intensity of both maxima as it is presented on the graphs at Fig. 2 B.

For studying the enzyme kinetics the maxima of the adsorption spectra of azocasein and the plasmon of the GNPs were taken at certain time intervals. The corresponding kinetic curves of enzyme reactions for three separate experiments with different enzyme concentrations are presented on Fig. 3. For convenience the absorption maxima were normalized as follows:

$$\xi_i = \frac{A(t)_i - A(0)_i}{A(\infty)_i} \quad (3)$$

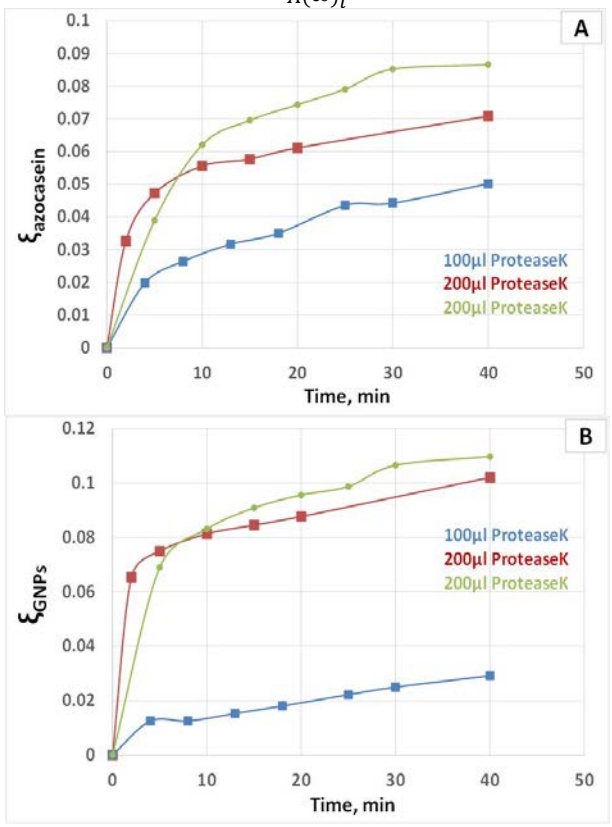

Fig. 3. Normalized kinetic curves of the Protease K action extracted from (**A**) azocasein adsorption maxima (340 nm) and (**B**) GNPs plasmon absorption maxima (520 nm). The volumes of Protease K (2 mg/ml) added to reaction mixture are presented on the legend.

Where $A(t)_i$ the adsorption maximum at certain t, $A(0)_i$ is the adoption at t_0 (before the addition of the Protease K) and $A(\infty)_i$ is the absorption maxima at the beginning at the end ($t = 40\ min$) of enzyme reaction. From the kinetics curves one can observe that the initial slopes increase with the increase of the enzyme concentrations, e.g. bigger enzyme concentration higher rate of the enzyme reaction.

To characterize the morphological changes of azocasein modified GNPs at the beginning of the enzyme reaction (before the addition of Protease K) and at the end of reaction, the AFM imaging was performed. At Fig. 4 are presented typical 2D topography images of GNPs deposited on mica supports. From the image at Fig. 4 A one can observe that after the deposition a small areas around the GNPs are formed. The height of these areas is around 5 nm which is comparable to the size of the protein molecules which implies that this areas are the azocasein shells of the modified GNPs. In the course

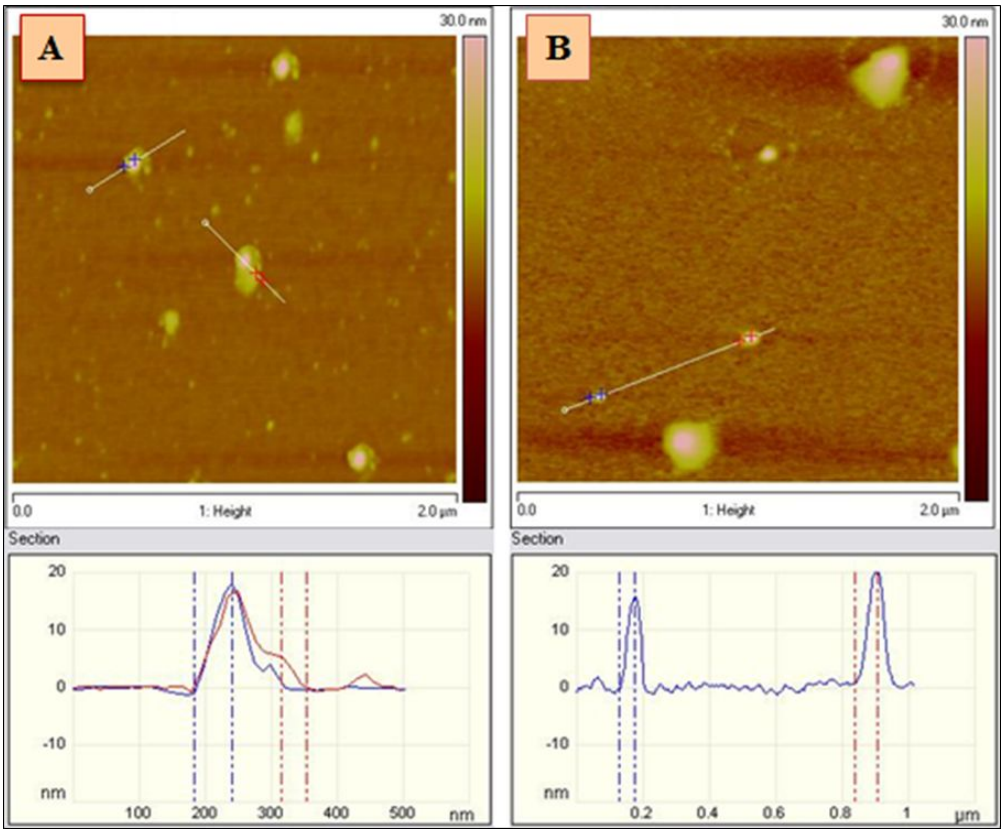

Fig. 4. AFM 2D images obtained after depositing of functionalized GNPs suspension on mica support, together with corresponding cross sections (**A**) before the addition of Protease K and (**B**) at the end of the enzyme reaction.

of the enzyme reaction since these shells were digested by the protease and as one can see in Fig.4B at the end of enzyme reaction those shells are missing. The sizes of the GNPs are about 20 nm at the begging and a bit smaller, 18 nm at the end of reaction.

CONCLUSIONS

Simple and reproducible procedure for functionalization of the GNPs with azocasein was proposed. The prepared GNPs were characterized at first with UV-Vis spectroscopy. Azocasein GNPs were then used for substrate to the enzyme Protease K. The enzyme kinetics was studied by UV-Vis and AFM imaging was applied for characterization of GNPs morphological changes at the beginning of the enzyme reaction and at the very end. The data obtained from both experimental method - AFM and UV-Vis are in excellent agreement with each.

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REFERENCES

1. T.M. Sun, Y.C. Wang, F. Wang, J.Z. Du, C.Q. Mao, C.Y. Sun, *Biomaterials*, **35**, 836 (2014).
2. W. F. Zandberg, A. B. S. Bakhtiari, Z. Erno, D. Hsiao, B. D. Gates, T. Claydon, *Nanomedicine Nanotechnol., Biol. Med.*, **8**, 908 (2012).
3. T. L. Halo, K. M. McMahon, N. L. Angeloni, Y. Xu, W. Wang, A. B. Chinen, *Proc. Natl. Acad. Sci.*, **111**, 17104 (2014).
4. A. de la Zerda, S. Prabhulkar, V. L. Perez, M. Ruggeri, A. S. Paranjape, F. Habte, *Clin. Exp. Ophthalmol.* (2014).
5. K. Hayashi, M. Nakamura, K. Ishimura, *Adv. Health. Mater.*, **2**, 756 (2013).
6. J. A. Copland, M. Eghtedari, V. L. Popov, N. Kotov, M. Mamedova, M. Motamedi, A. A. Oraevsky, *Mol. Imag. & Biol.*, **6**, 341 (2004).
7. J. J. Storhoff, R. Elghanian, R. C. Mucic, C. A. Mirkin, R. L. Letsinger, *J. Am. Chem. Soc.*, **120**, 1959 (1998).
8. G. C. Bond, C. Louis, D. T. Thompson, Catalysis by Gold (vol. 6), Imperial College Press, London. 2006.
9. J. Polte, T. T Ahner, F. Delissen, S. Sokolov, F. Emmerling, A.F. Thünemann, R. J Kraehnert, *J. Am. Chem. Soc.*, **132**, 1296 (2010).
10. M. C. Daniel, D. Astruc, *Chem. Rev.*, **104**, 293 (2004).
11. J. Turkevich, P. Stevenson, J. Hillier, *Discuss. Faraday Soc.*, **11**, 55 (1951).
12. A. G. Tkachenko, H. Xie, D. Coleman, W. Glomm, J. Ryan, M. F. Anderson, S. Franzen, D. L. Feldheim, *J. Am. Chem. Soc.*, **125**, 4700 (2003).
13. S. H. Brewer, W. R. Glomm, M. C. Johnson, M. K. Knag, S. Franzen, *Langmuir*, **21**, 9303 (2005).
14. P. Khullar, V. Singh, A. Mahal, P. N. Dave, S. Thakur, G. Kaur, *J. Phys. Chem. C*, **116**, 8834 (2012).
15. P. Georgiev, A. Bojinova, B. Kostova, D. Momekova, T. Bjornholm, K. Balashev, *Colloid Surf. A*, **434**, 154(2013).

ОХАРАКТЕРИЗИРАНЕ НА ЗЛАТНИ НАНОЧАСТИЦИ, ФУНКЦИОНАЛИЗИРАНИ С АЗОКАЗЕИН С ПОМОЩТА НА АТОМНО-СИЛОВА МИКРОСКОПИЯ (AFM) И ПРИЛОЖЕНИЕТО ИМ ЗА КОЛОРИМЕТРИЧНИ ЕНЗИМНИ ТЕСТОВЕ НА ПРОТЕАЗА

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(Резюме)

Колориметричните тестове, разработени на основата на повърхностния плазмонен резонанс на златните наночастици са доказали своята приложимост за сензорни системи в ензимния катализ, като поради своята чувствителност и простота, така и поради ниската си цена. Подготовката на стабилни суспензии от златни наночастици, модифицирани с протеини, дава възможност за употребата им като подходящ аналитичен инструмент за колориметрични спектрални анализи. Настоящото изследване разглежда експериментална процедура за изготвянето на възпроизводим синтез на функционализирани с азоказеин златни наночастици в стабилна суспензия при различни концентрации на азоказеин. Предложена е концепция за тест за ензимна активност на протеаза, при използване на функционализирани с азоказеин златни наночастици .За по-доброто изучаване на тези системи, както и за да бъде изучена ензимната реакция в тях са направени изследвания чрез UV-Vis абсорбционна спектроскопия, като подробно е анализирано отместването на максимума в спектрите, съответстващ на повърхностния плазмонен резонанс. За да бъде изучена напълно системата е изследвана морфологията и размера на функционализираните с азоказеин частици, като е направена серия от AFM изображения на пробите, взети в началото и края на протеолитичната ензимна реакция.