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The stabilization of Au NP–AChE nanocomposites by biosilica encapsulation for the development of a thiocholine biosensor

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ABSTRACT

We report on the construction of an amperometric biosensor based on the immobilization of the enzyme acetylcholinesterase (AChE) onto gold nanoparticles (Au NPs). The active enzyme is covalently bound directly onto the surface of the Au NPs via a thiol bond. This immobilization provides increased stability and high electron-transfer between the colloidal Au NPs, the catalyst and the transducer surface. To further increase the biosensor stability by protecting the enzyme from denaturation and protease attack, a layer of biosilica was grown around the Au NP enzyme nanocomposite. All steps, i.e., the conjugation of the enzyme to the gold nanoparticles and the encapsulation into biosilica, are monitored and confirmed by ATR-FT-IR spectroscopy. The stabilizing effect of the entrapment was evaluated amperometrically, while the operation of the biosensor was monitored over a period of 4 months. The initial sensitivity of the biosensor was calculated to be 27.58 nA mM^{-1} with a linear response to the concentration of the substrate in the range from 0.04 to 0.4 mM. It is thus shown that the biosilica nanocomposites doped with Au NPs–AChE conjugates create a system that provides both signal mediation and significant enzyme stabilization over the existing AChE biosensor. The biosensor had retained all its activity at the end of the 4 months, compared with the normal AChE biosensor whose activity reached 50% after only 42 days of operation.

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1. Introduction

Metal nanoparticles exhibit unique physical and chemical properties, which are strongly dependent on their size, shape and the surrounding environment of the particle. These properties have had important implications to the potential applications of metal nanoparticles in drug delivery, bioimaging and biosensing [1].

Gold nanoparticles are among the most stable metal nanoparticles. They exhibit very interesting electronic, magnetic and optical properties which are related to the quantum size effect [2]. These properties lend them suitable for many applications in a variety of scientific fields. One such area of interest to the society is nanobiotechnology. It combines the unique recognition, catalytic, and inhibition properties of biomolecules, with the unique electronic, photonic, and catalytic properties of the nanoparticles. This is the reason why gold nanoparticles have been used extensively in histochemistry and immunocytochemistry, [3,4] as active substrates for surface-enhanced Raman scattering (SERS) [5–7], for the detection of oligonucleotides [8,9] and for immunolabelling of cells and different biological components [10,11]. Due to their redox activity as electrons mediators Au NPs have also been used in the construction of electrochemical biosensors [12,13].

The catalytic activity and the turnover rate are very important parameters characterizing the scientific and technological importance of enzymes. It is a well-known fact that during experimental workup steps for the preparation of many biodevices, enzymes show significant denaturation and inactivation with subsequent decrease of their catalytic activity. The improvement in the catalytic activity and stability of proteins has been in the forefront of research for many years now, while recently the role of nanomaterials in this area is been explored intensively. Methods to covalently conjugate enzymes like glucose oxidase [14], cytochrome c [15] or ribonuclease S [16] onto Au NPs have been developed. The nonspecific binding between the metal nanoparticles and enzymes, such as horseradish peroxidase [17,18], fungal protease [19], cytochrome *c* [20] and esterase [21], has also been investigated in detail.

Enzyme–Au NPs conjugates have also been used in electrochemical biosensors [12,14,22–24], although it has been shown that the binding of the enzyme to the metal nanoparticle is not always very stable. This instability leads to desorption of the enzyme from the surface of the nanoparticle which results in unstable nanodevices and systems. In order to deal with this serious stability problem an additional protection coating must be used. It has been shown that biosilica furnishes a porous but at the same time robust protective layer that provides significant mechanical and thermal stability [25]. It is thus envisaged that protecting the enzyme–nanoparticle conjugate with a biomimetically synthesized silica layer will provide additional protection. The preparation of gold/silica core/shell nanoparticles has been studied by some groups [26–29] but the development of

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electrochemical biosensors based on biosilicated Au NPs–AChE complexes systems has yet to be documented.

In this work, we report on the construction of an acetylcholinesterase–gold nanoparticle–biosilica amperometric biosensor. The nanocomposite-based biosensor exploits the advantages of two different nanobiotechnologies: the Au NPs and the biomimetically synthesized silica. At first, the enzyme is immobilized onto the surface of Au NPs, subsequently the Au NPs–AChE nanocomposites are covered by poly(L-lysine) (PLL) and finally encapsulated by tetramethyl orthosilicate synthesized biosilica. The obtained enzyme doped nanocomposite material is deposited onto an Au electrode and allowed to dry. The gold nanoparticles have the important role of acting as electron transducers from the enzyme to the electrode. In addition, they act as a stabilizing platform, and together with the biosilica stabilize the acetylcholinesterase, a native unstable enzyme. ATR-FT-IR spectroscopy measurements confirm the immobilization of the AChE onto the Au NPs' surface and entrapment of the nanocomposites into the biosilica matrix. The stabilizing effect of the Au NPs and biosilica onto the AChE is demonstrated by measuring the amperometric response of the system to the substrate acetylthiocholine chloride. The performance of the Au NPs–AChE–PLL–silica biosensor, as respect to the AChE biosensor, has been studied and proved to be far more stable.

2. Materials and methods

2.1. Reagents

Stock Au NPs solution (0.784 mM $HAuCl_4$) was kindly provided by Prof. Mirjana Comor from Vinca Institute for Nuclear Sciences, Laboratory for Radiation Chemistry and Physics, Belgrade, Serbia & Montenegro. The solution contained particles of 25–28 nm in diameter with citrate groups covering their surface. Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel), acetylthiocholine chloride, polyethylene glycol (PEG 20,000 mol wt), tetramethyl orthosilicate (TMOS 99+%) and phosphate buffer (KH_2PO_4) were purchased from Sigma-Aldrich. Poly(L-lysine) hydrobromide (PLL, MW 20,000–30,000) was obtained from Fluka. All other reagents used were of analytical grade. In all experiments, nanopure water (18 MΩ-cm, EASY pure model D7033, Barnstead) was used.

2.2. Preparation of Au NPs–AChE conjugates

The conjugation of the enzyme acetylcholinesterase to gold nanoparticles has been performed according to the published method of conjugating proteins to colloidal gold [30]. The Au NPs stock solution has been adjusted to 0.5 pH units above the pI of the enzyme using a 0.1 M NaOH solution. In order to determine the concentration of AChE to be conjugated to the surface of the Au NPs, a series of dilutions of the enzyme have been performed. Subsequently stock solution of Au NPs has been added and allowed to equilibrate for 10 min. At this time NaCl is added to the solution. If the solution does not contain enough enzyme to stabilize the surface of the Au NPs, the colloids tend to aggregate and the colour of the solution changes from red to purple or blue. Using this methodology, the concentration of the protein required to conjugate to the Au NPs is in the order of 80 U mL^{-1}.

For the conjugation experiment, a 4900 μL aliquot of Au NPs is added to 100 μL stock solution (80 U mL^{-1}) of AChE and allowed to react at room temperature for 10 min. PEG is then added to a final concentration of 0.04% and the solution is allowed to react for another 30 min. Finally the mixture is centrifuged at 6000 rpm for 60 min. The clear supernatant contained very small amounts of AChE as determined by the Ellman's method [31]. This suggests that all of the protein is conjugated to the surface of the gold nanoparticles. The soft, red precipitate, containing the Au NPs–AChE conjugates is then resuspended in 1.5 mL PBS containing 0.04% PEG. A small amount of Au NPs precipitates as a hard metallic pellet, representing the uncoated Au NPs.

2.3. Preparation of poly(L-lysine) templated Au NPs–AChE–silica nanocomposites

For the biosilicification reaction, a 1.0 mM PLL solution is added to the Au NPs–AChE conjugates obtained previously. The solution is allowed to react for 30 min at room temperature and then stored for 12 h at 4 °C. Subsequently, a freshly prepared solution of silicic acid (obtained by hydrolyzing TMOS at a concentration of 1.0 M with 1.0 mM HCl for 30 min) and water are added and allowed to react for 4 h at room temperature. The final product is then subjected to four rounds of centrifugation/washing with 25.0 mM phosphate buffer, pH 7.0, at 4800 rpm. The washed silica biomimetic composites were stored at 4 °C in phosphate buffer for further use. The electrostatic interaction between the negatively charged enzyme and the positively charged poly(L-lysine) template promotes the entrapment of the enzyme during biosilicification as shown schematically in Fig. 1.

2.4. Characterization of the nanocomposites

The ATR-FT-IR spectra were recorded on a Thermo-Electron Nicolet 6700 FT-IR optical spectrometer with a DTGS KBr detector at a resolution of 2 cm^{-1}. In order to record the ATR-FT-IR spectra, drops of the solutions have been placed directly onto the diamond and left overnight for the solvent to evaporate completely. The spectrum of the AChE was obtained using the freeze dried form of the protein. High-resolution transmission electron microscopy (HR-TEM) images of Au NP–AChE–PLL–silica conjugates were obtained using a JEOL JEM-2100 Electron Microscope, operating at 80 kV. The samples for HR-TEM analysis were prepared by evaporation of droplets placed on Formvar/Carbon coated TEM grids (Analytical Instruments S.A.). The solvent was allowed to evaporate under atmospheric conditions.

2.5. Electrochemical measurements

For biosensor construction a 1 cm^2 gold electrode was polished with 1.0 and 0.05 μm alumina powders and cleaned with nanopure water. The cleaned electrodes were examined using cyclic voltammetry. For this, a three-electrode cell containing a silver/silver chloride double-junction reference electrode (model 90-02, ThermoOrion) and a platinum counter electrode (surface 1 cm^2, model 96-78-00, Orion) was used. An Autolab PGSTAT 30 potentiostat/galvanostat equipped with a frequency response analyzer (Eco Chemie, The Netherlands) was used for the potential application and current recording. All measurements were carried out in phosphate buffer solution, 25 mM, pH 7.0, at room temperature. For amperometry measurements, the clean Au electrode was covered with Au NPs–AChE–PLL–silica conjugates and AChE solution, respectively. When the solvent was completely evaporated, the gold electrode was covered with a cellulose acetate membrane and was ready for evaluation. When not in use, the biosensors were stored at 4 °C.

3. Results and discussion

The efficient conjugation of the AChE onto the Au NPs was monitored using ATR-FT-IR spectroscopy. The spectrum of the Au NPs–AChE conjugates was recorded and subsequently compared with the one of the citrate-covered Au NPs. Of interest in the ATR-FT-IR spectra of citrate–covered gold nanoparticles are the vibrational citrate peaks [32,33] which appear at 1577 cm^{-1} (A), 1400 cm^{-1} (B) and 1297 cm^{-1} (C) respectively (Fig. 2, solid line). Upon modification of the surface of the Au NPs with the enzyme (Fig. 2, dashed line), the vibration bands of the COO^- group disappear. At the same time the characteristic amide I (~1640 cm^{-1}) and II (~1580 cm^{-1}) vibration bands of the peptide backbone of the protein appear, indicating coverage of the Au NPs by the protein molecules.

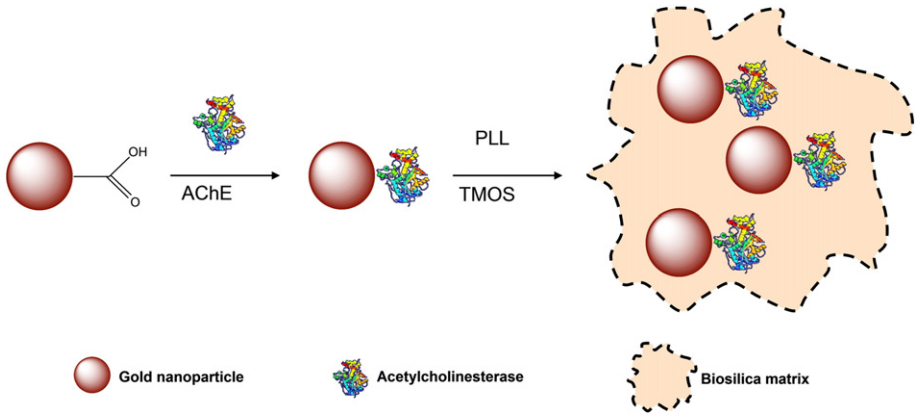

Fig. 1. Schematic representation of the Au NPs–AChE–PLL–silica composites formation.

Following the conjugation of the AChE to the surface of gold nanoparticles, the entrapment of the bioconjugates into biomimetically synthesized silica was confirmed by ATR-FT-IR spectroscopy. A silica shell entrapping different nanostructures [34–36] gives rise to three dominant peaks, S-A, S-B and S-C, that appear in the region 760–1300 cm^{-1} (Fig. 3). Peaks S-A and S-C are attributed to the Si-O-Si asymmetrical and symmetrical stretching modes, respectively, while peak S-B is attributed to Si-O stretching mode either as siloxane bridge ($Si\text{-}O^{-}$) or as silanol group (Si-OH) [37–40]. In the case of the Au NPs–AChE–silica nanocomposites, these peaks appear after biosilicification at 1042.6 cm^{-1}, 951.8 cm^{-1} and 789.5 cm^{-1}, respectively. These data indicate the successful growth of a biosilica shell around the Au NPs–AChE complexes.

The newly obtained nanocomposites were also characterized using HR-TEM, a micrograph being presented in Fig. 4. The formation of the spherical nanoporous structure which encapsulates several Au NPs–AChE conjugates is clearly visible. The biosilicification process offers a mild cover for the conjugates, and does not produce the aggregation of the Au NPs. It seems to create a structure whose pores allow for the substrate to reach the enzyme and for the products to exit into the surrounding environment.

The enzyme acetylcholinesterase hydrolyzes the substrate acetylthiocholine to thiocholine and acetic acid. Thiocholine can then be further oxidized at the surface of the working electrode generating the biosensor signal (Fig. 5).

The stabilizing effect that the Au NPs–silica architecture has on the AChE was examined by measuring the sensitivity and the storage stability of the Au NPs–AChE–PLL–silica biosensor at +300 mV. For comparison purposes, an AChE biosensor has also been constructed and evaluated simultaneously. Successive additions of acetylthiocholine (100 mM stock solution) lead to an increase of the anodic current, which can be attributed to the decomposition of acetylthiocholine to thiocholine and its further oxidation on the surface of the electrode. Through this reaction sequence, the sensitivity of the biosensor can be directly related to the enzyme activity [41].

The amperometric response and the resulting calibration curve of the Au NPs–AChE–PLL–silica biosensor are presented in Fig. 6. Eight successive additions of the acetylthiocholine chloride substrate were performed over a period of approx 60 min under continuous polarization at +300 mV. The initial sensitivity of the nano-sensor was calculated to be 27.58 nA mM^{-1}, the response is linear to the concentration of the substrate in the range from 0.04 to 0.4 mM and the measurement to measurement reproducibility was very good (RSD ~ 6%, $n = 3$). The current difference, ΔI, is calculated as the difference between the measured current and the initial current of the system before any addition of substrate. The response time of the biosensor to the substrate was very fast, reaching the plateau in less than 30 s.

The storage stability of the biosensors was monitored at 25 °C polarized at +300 mV vs. the Ag/AgCl electrode (Fig. 7). The calibration curves of the biosensors were recorded at different intervals over the 4 month period, while between measurements the biosensors were kept in phosphate buffer (25 mM, pH 7.0) at 4 °C. The sensitivity of the biosensor, which is obtained from the slope of the calibration curve, is directly related to the enzyme activity [34], thus allowing for the continuous monitoring of the enzymatic activity. The first day of measurement is considered to be 100% and the remaining activity of the biosensors is calculated in relation to this value. The initial increase in the sensitivity observed is a well-known phenomenon in biosensor systems, and is attributed to changes to the enzyme's 3D

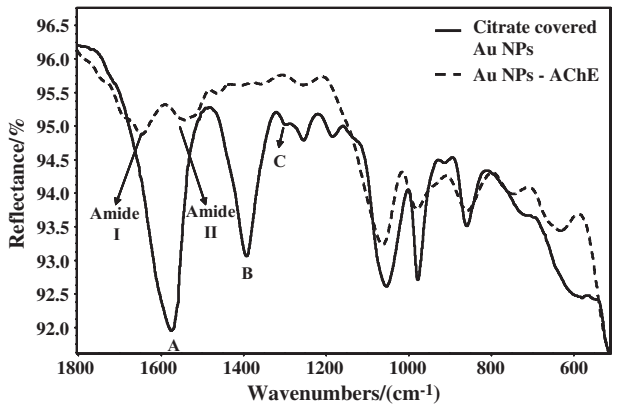

Fig. 2. ATR-FT-IR spectra of citrate-covered Au NPs (solid line) and of the Au NPs–AChE nanocomplexes (dashed line) (reflectance/% as a function of wavenumbers/cm^{-1}).

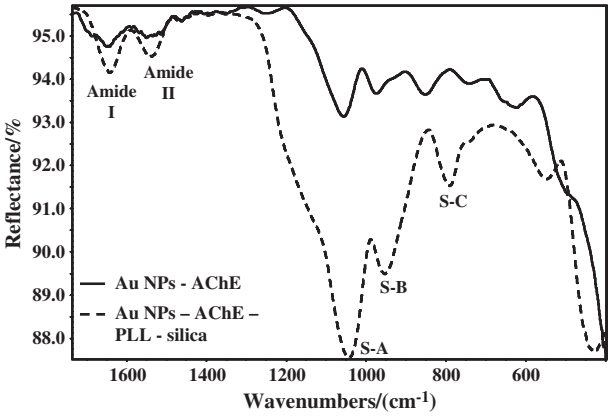

Fig. 3. ATR-FT-IR spectra of the Au NPs–AChE complexes before (solid line) and after (dashed line) entrapment into the biosilica matrix (reflectance/% as a function of wavenumbers/cm^{-1}).

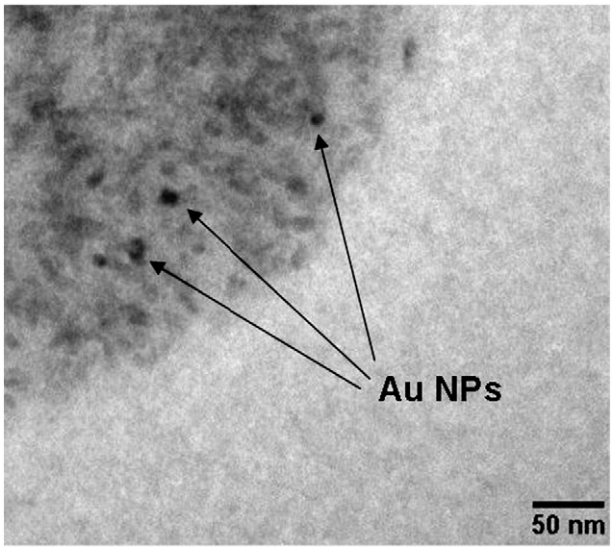

Fig. 4. HR-TEM of the Au NPs–AChE–PLL–silica nanocomposites (bar = 50 nm).

structure inside the immobilization matrix upon reactivation from the dried state [42].

The Au NPs–AChE–PLL–silica biosensor provides excellent protection for the enzyme from denaturation having a remaining activity of 155% after 113 days of operation (Fig. 7, triangles). On the other hand, the plain AChE biosensor (Fig. 7, spheres) showed a decrease of its activity to 50% after 42 days. The response time of the AChE biosensor was in the order of 60 s, which is considerably slower that of the Au NPs–AChE–PLL–silica biosensor.

From this data it can be deduced that immobilization of AChE onto gold nanoparticles and the further biosilicification of the nanocomposites proves to be a very efficient method for stabilizing and protecting this relatively unstable enzyme. The silicated gold nanoparticle–acetylcholinesterase based electrode proves to be very suitable for biosensor construction with increased storage time stability.

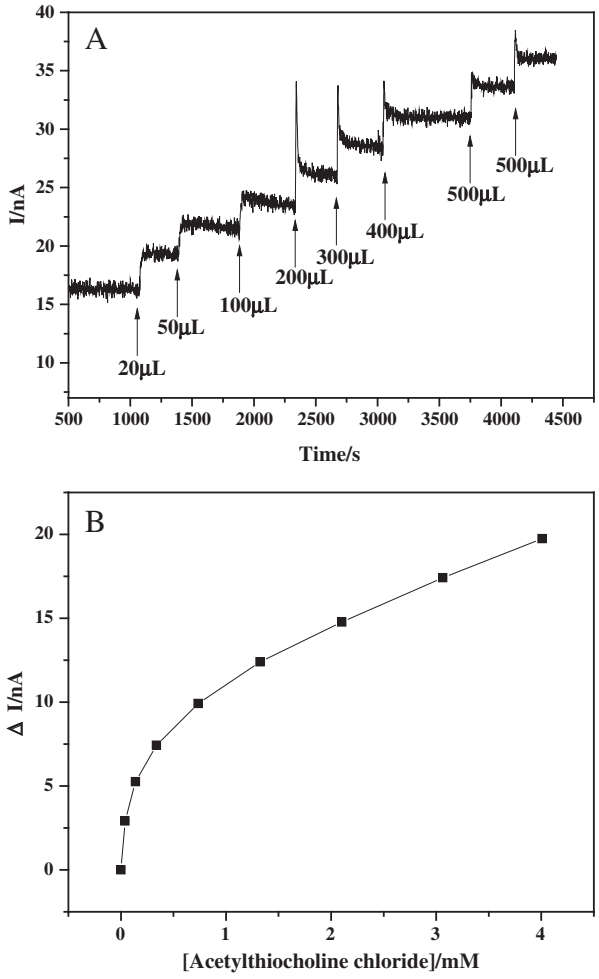

Fig. 6. (A) Amperometric response trace of the Au NPs–AChE–silica biosensor (*I*/nA as a function of time/s). (B) Corresponding calibration curve (Δ*I*/nA as a function of [acetylthiocholine chloride]/mM; $\Delta I = 1.3541 + 19.603$*[acetylcholine chloride]).

4. Conclusions

From the data presented it can be concluded that immobilization of AChE onto gold nanoparticles and the further biosilicification of the nanocomposites is a very efficient method for the construction of sensitive amperometric biosensors. The proposed biosensor system is sensitive to small substrate concentrations and very stable over

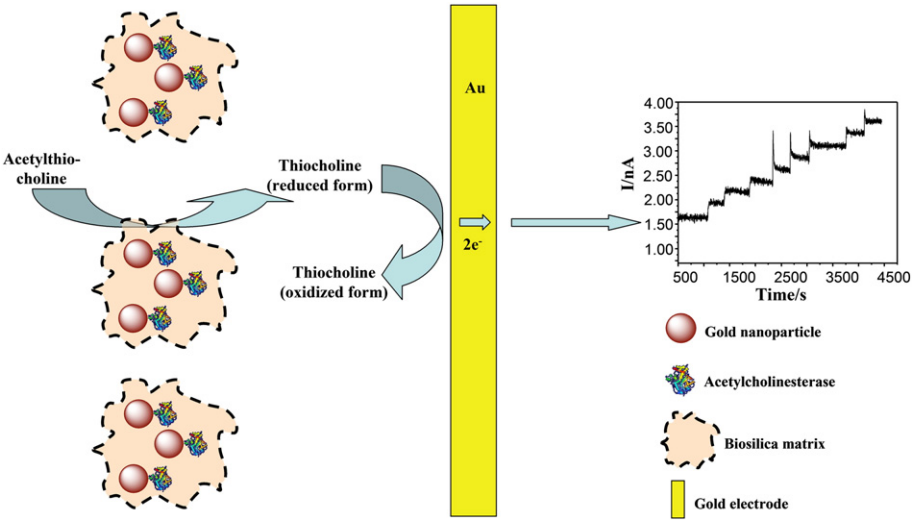

Fig. 5. Schematic representation of the operation of the biosensor.

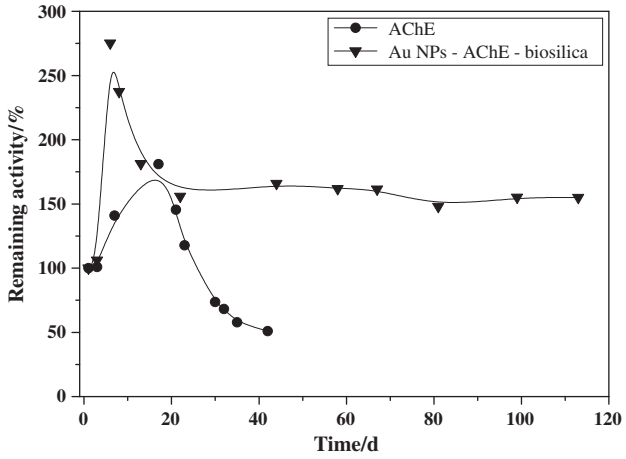

Fig. 7. Storage stability of the Au NPs–AChE–PLL–silica and AChE biosensors (remaining activity/% as a function of time/d).

time, with its activity remaining at a very high level even after 4 months of repeated measurements. It is also shown that the immobilization of the AChE onto the Au NPs and the further encapsulation of the conjugates into biomimetically synthesized silica provide a very sensitive system that is far more suitable for acetylthiocholine determination as compared to the AChE amperometric biosensor. These results suggest that using a system composed of Au NPs-AChE conjugates entrapped into biosilica, AChE inhibitors could also be detected with very good sensitivity, experiments for this purpose being under development.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bioelechem.2012.02.005.

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