Ag electrode modified with polyhexamethylene biguanide stabilized silver nanoparticles: a new type of SERS substrates for detection of enzymatically generated thiocholine

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Abstract. The detection of thiocholine is one of the most widespread techniques for estimation of the cholinesterase activity – acetylcholinesterase and butyrylcholinesterase. Both cholinesterases can be inhibited by organophosphates and carbamates and accordingly can be considered for estimation of these pollutants in the environment. In the current work, SERS spectroscopy was applied for the thiocholine detection. The Ag electrodes modified with silver nanoparticles stabilized by polyhexamethylene biguanide were for the first time suggested as SERS-substrates for that purpose. Such electrodes can be applicable for SERS detection of submicromolar concentrations of thiocholine.

1. Introduction

Human cholinesterases have important physiological functions: acetylcholinesterase (AChE) is implicated in neural signal transmission catalyzing hydrolysis of neuromediator acetylcholine [1, 2], butyrylcholinesterase (BChE) acts as a stoichiometric acceptor of organophosphates [3, 4]. There are numerous chemicals and drugs that interact with the cholinergic nervous system: nerve agents, prophylactic antidotes, myorelaxants and other therapeutic drugs. Since thiocholine is a hydrolysis product of human cholinesterases, its accumulation can represent enzymatic status in organism. Abnormal low values of cholinesterase activity may be caused by some anemias [5], dermatomyositis [6], organophosphorous intoxication [7, 8], and drug use [9]. Additionally, abnormal high levels of cholinesterase activity may indicate the nephrotic syndrome [9]. Therefore the determination of thiocholine in blood with high accuracy is of great importance for clinical diagnostics.

At the present time, the electrochemical methods [10-13] are commonly used for the thiocholine detection due to low limit of detection (LOD) [11, 14]. In particular, MnO_2-modified screen-printed graphite electrodes allow detection of 60 nM of thiocholine [15, 16]. Moreover, electrochemical biosensors fabricated with layer-by-layer technology also show very low LOD – about 130 nM [17]. Nevertheless, the further development of detection techniques is a significant problem. In this regard, SERS spectroscopy is very promising in view of its ability to detect single molecules [18, 19].

This article reports the application of SERS spectroscopy for the thiocholine detection. We suggest the Ag electrodes modified with silver nanoparticles stabilized by polyhexamethylene biguanide (PHMB) as SERS substrates for determination of enzymatically generated thiocholine. It was found that the Raman signal intensity of thiocholine applied onto the surface of Ag electrode modified with PHMB-stabilized silver nanoparticles is, at least, 10 times higher in comparison with one applied onto pure Ag electrode surface. At the same time, PHMB don't significantly influence on the Raman signal intensity of thiocholine. Ag electrodes modified with PHMB-stabilized nanoparticles can be applied for the quantitative analysis of thiocholine. Preliminary results indicate that limit of detection of thiocholine by this technique is about 200 nM.

2. Experimental

2.1. Reagents and materials

For the preparation of silver nanoparticles, silver nitrate $AgNO_3$ (99%, Reakhim, Russia), sodium borohydride $NaBH_4$ (99%, Acros Organics) and polyhexamethylene biguanide hydrochloride (PHMB, 20% wt. solution in water, Arch) were used. All reagents were applied as purchased. The structure of PHMB is shown in figure 1. For the generation of thiocholine, butyrylthiocholine iodide (Sigma, USA), butyrylcholinesterase from equine serum (BChE, EC 3.1.1.8) with activity 264 U/mg (Sigma, USA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma, USA), and phosphate buffered saline (PBS) 0.1 M, pH 7.0 were used. Choline bromide (Sigma, USA) was used as internal standard.

For substrate preparation, silver screen-printed electrodes were applied. All solutions were made with milliQ water (18.2 MΩ*cm).
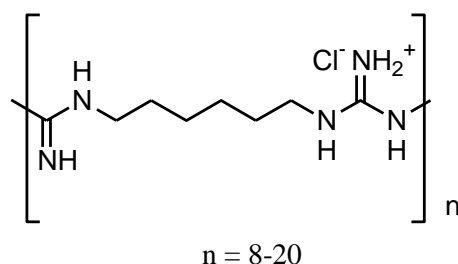

n = 8-20
Figure 1. Structure of polyhexamethylene biguanide (PHMB).

2.2. Preparation of silver nanoparticles

Silver nitrate was reduced in aqueous solution with sodium borohydride under vigorous stirring [20] in the presence of PHMB. The concentrations of reagents were chosen to obtain stable colloids with concentration of silver nanoparticles of 50 μg/ml. Sodium borohydride was added in 2-fold molar excess compared to the silver nitrate. The silver/PHMB molar ratio was 1:1.

2.3. Immobilization of silver nanoparticles on Ag electrodes

Silver electrodes were immersed into colloidal solutions of the preliminarily prepared nanoparticles in the presence of 0.08 M KCl and were kept for 12 hours. Then as-prepared substrates were rinsed thoroughly with water and dried.

2.4. Synthesis of thiocholine

Thiocholine solution was obtained by the enzymatic hydrolysis of butyrylthiocholine iodide. For that purpose, 22.6 mg of butyrylthiocholine iodide were dissolved in 600 μl of 0.1 M PBS buffer (pH 7.0). Then 400 μl of 0.1 mg/ml BChE solution in the same buffer were added. The mixture was incubated for 1 h.

The Ellman technique [21] was applied to determine the thiocholine concentration. Preliminarily obtained thiocholine solution was diluted in 40 times with PBS, 10 μl of it introduced in the cell of microplate spectrophotometer xMark (BioRad, USA) with 180 μl of PBS followed by adding 10 μl of 5 mM solution of DTNB. Then the absorbance was measured at 405 nm. The thiocholine concentration was calculated by the equation $c = A/(\varepsilon_{405}d)$, where A is the absorbance, ε_{405} is the absorption coefficient of 5-thio-2-nitrobenzoate ione (TNB) at 405 nm (13.3 ml/μmol*cm), and d is the optical path equal to 0.6 cm.

2.5. SERS measurements of thiocholine

Each sample for Raman spectroscopy was prepared by applying of a drop of an aqueous solution of thiocholine on Ag electrode and drying it at 60ºC.

2.6. Characterization

The UV-Vis spectra of nanoparticles were recorded using UV-visible spectrometer Specord UV-Vis (Carl Zeiss, Germany) in the 300-700 nm range. The spectrum of pure water was used as a background.

The size distribution of silver nanoparticles was measured with Zetasizer Nano ZS (Malvern Instruments Ltd., UK) equipped with a 633 nm He-Ne laser (power 4 mW).

The measurements of ζ-potential were carried out by applying an electric field to the cell with sol of nanoparticles using technique based on the laser Doppler anemometry.

TEM images were taken using a Leo 912 AB Omega microscope (Leo Ltd., Germany). Each sample under study was prepared by drying a drop of an aqueous solution of nanoparticles on a copper grid coated with a carbon film. The nanoparticle size distributions were calculated using ImageJ 1.48v program.

XPS experiments were carried out on LAS-3000 (Riber, France) microscope with an XPS chamber equipped with an X-ray anode source (AlKα, 1486.6 eV) and an OPX-150 hemispherical energy analyzer.

SEM images of substrates were obtained with a Carl Zeiss Supra 40 microscope (accelerating voltage 10 kV; 30 μm aperture) with extreme resolution 3-4 nm.

SERS spectra were measured in a BWTech InnoRam Raman spectrometer equipped with a diode near IR 785 nm laser (350 mW). The laser spot of the diode near IR laser was a circle with diameter 100 μm. The spectra were analyzed at a resolution of 4 cm^{-1} within 64-3011 cm^{-1}.
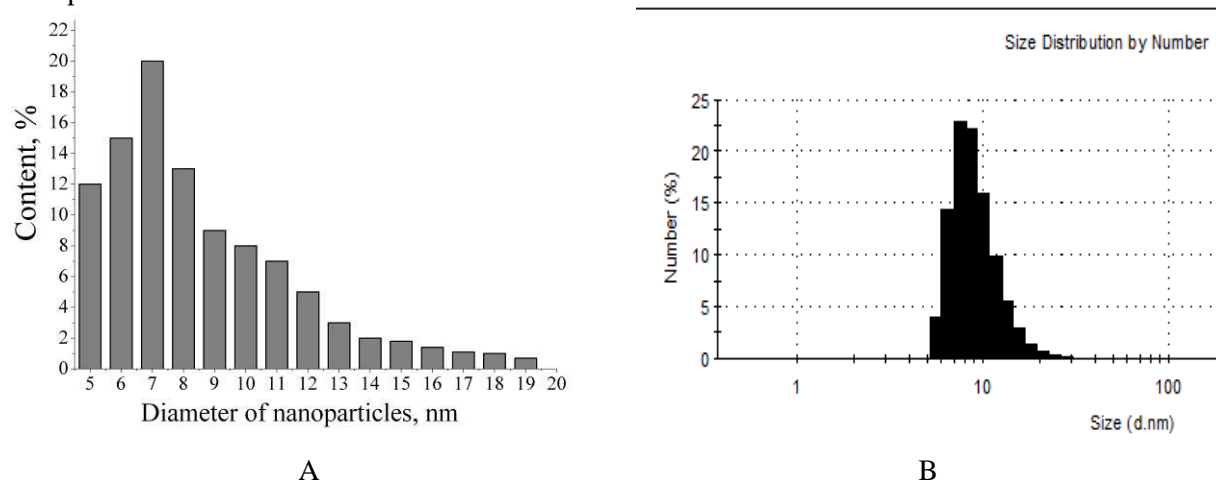
3. Results and discussion

3.1. Preparation of silver nanoparticles

The reduction of $AgNO_3$ in aqueous solution with $NaBH_4$ in the presence of polyhexamethylene biguanide hydrochloride yields PHMB-capped silver spherical nanoparticles with typical sizes between 5-20 nm as determined by TEM (figure 2). The aqueous colloidal dispersions of these particles have the characteristic surface plasmon resonance [22] at 397±2 nm in the UV-Vis absorbance spectrum (figure 3).

The ζ-potential value for PHMB-stabilized silver nanoparticles based on dynamic light scattering (DLS) measurements is +39.1 (±1.5) mV. The obtained sols of Ag NPs were stable for a long time. A typical microdiffraction pattern of silver particles (figure 2D) clearly indicates that nanoparticles consist of crystalline silver.

PHMB-stabilized nanoparticles are less stable in the presence of KCl. Addition of potassium chloride decreases the electrical double layer thickness and can break the binding between the stabilizer and the nanoparticle surface due to specific sorption of chloride ions. As a result, the colloidal solution becomes unstable and coagulates in the presence of excess of KCl. The minimal concentration of potassium chloride corresponding to the coagulation threshold of PHMB-stabilized nanoparticles is about 0.11 M.
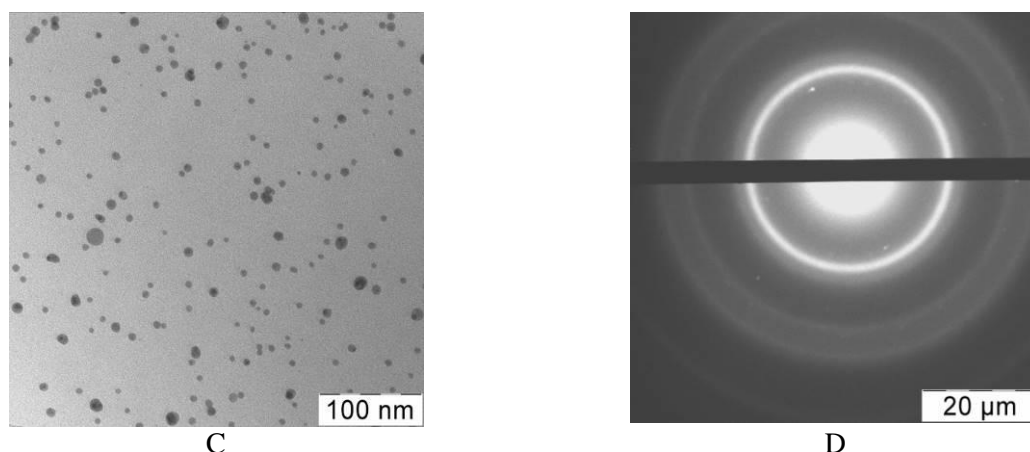

A   B

C   D

Figure 2. The size distributions based on TEM (A), DLS (B), TEM image (C) and microdiffraction pattern (D) of PHMB-stabilized silver nanoparticles.
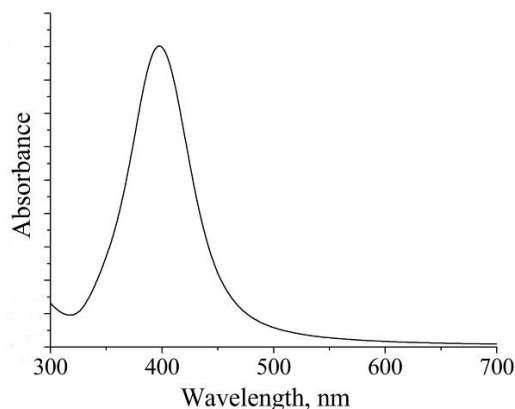

Figure 3. The absorption spectrum of PHMB-stabilized silver nanoparticles (mass concentration 50 µg/ml, dilution 10 times).

Nanoparticle composition was investigated by XPS. Figure 4 depicts XPS-spectra of silver nanoparticles. There are two bands with energies of 368.1 eV (3d 5/2) and 374.0 eV (3d 3/2) corresponding to crystalline silver [23] in the Ag 3d line (figure 4A).

The carbon atoms in PHMB have different oxidation states which correspond to aliphatic and imine groups. The carbon 1s line is shown as a superposition of two peaks (figure 4B). The most intensive band with binding energy of 285.1 eV apparently related to aliphatic carbon atoms whereas the second band (binding energy 288.0 eV) corresponds to imine carbon atoms [24]. Because of overlapping of the N 1s line with the satellite of Ag 3d photoelectron line the interpretation of this spectral range is complicated. Hence it is possible to make an only qualitative conclusion about the band with energy 400-405 eV that corresponds to the presence of nitrogen (figure 4C).
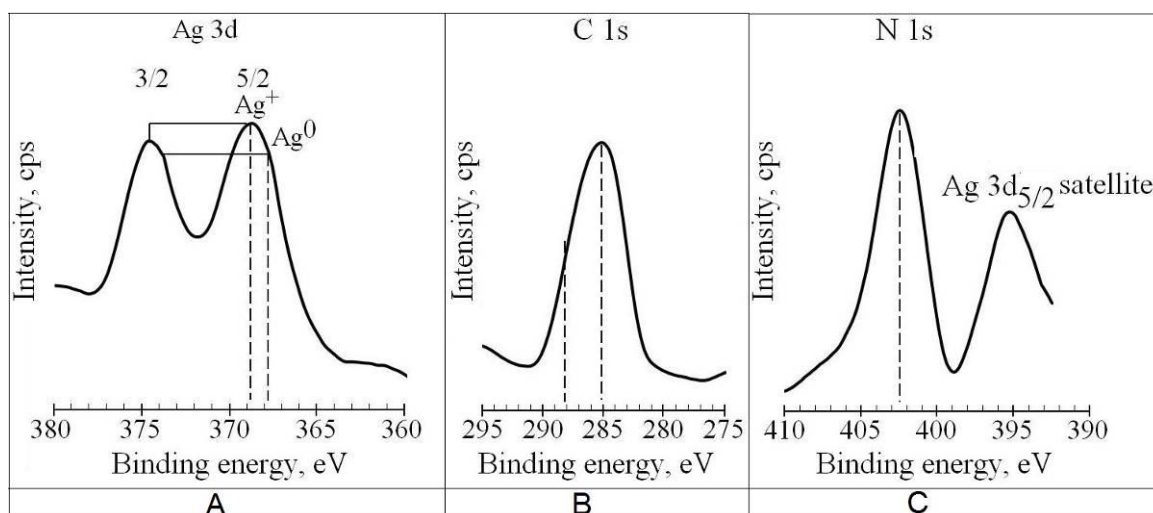
Figure 4. XPS-spectra of PHMB-stabilized nanoparticles: A) Ag 3d; B) C 1s; C) N 1s.
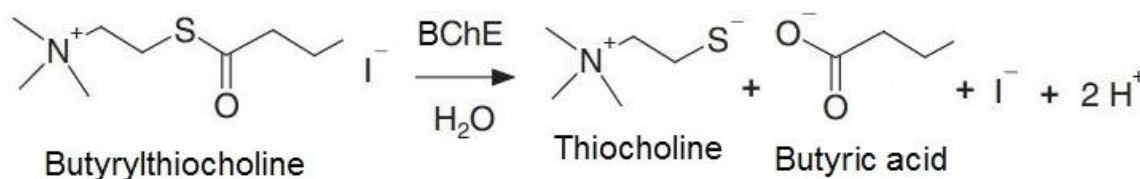
3.2. Immobilization of silver nanoparticles onto silver electrode surface

In order to successfully immobilize nanoparticles onto silver electrode surface it is necessary to break binding between stabilizing molecules and nanoparticle surface. Addition of potassium chloride to modifying sol during immobilization decreases aggregative stability of nanoparticles – the ζ-potential value of PHMB-stabilized particles decreases from +39.1 (±1.5) mV to 17 (±1.5) mV while adding 0.08 M KCl. The KCl-assisted immobilization of nanoparticles was carried out at concentration of chloride ions of 0.08 M that is 1.4 times smaller then the coagulation threshold for PHMB-stabilized nanoparticles to prevent formation of large conglomerates. As a result, friable aggregate films of silver nanoparticles were formed on the silver electrode surface (figure 5A). Molecules of PHMB apparently can act as linkers for nanoparticles during immobilization.

Addition of potassium chloride into colloidal solutions of nanoparticles do not influence on the oxidation state of nanoparticle surface. Figure 5B illustrates Ag 3d XPS-spectrum of PHMB-stabilized nanoparticles immobilized onto Ag electrode surface in the presence of 0.08 M KCl. The spectrum is identical to the same for PHMB-stabilized nanoparticles in colloidal solution (figure 4).

3.3. Synthesis of thiocholine

For the thiocholine solution preparation, butyrylthiocholine iodide was used according to the Scheme 1.

Scheme 1. Formation of thiocholine by hydrolysis of butyrylthiocholine.
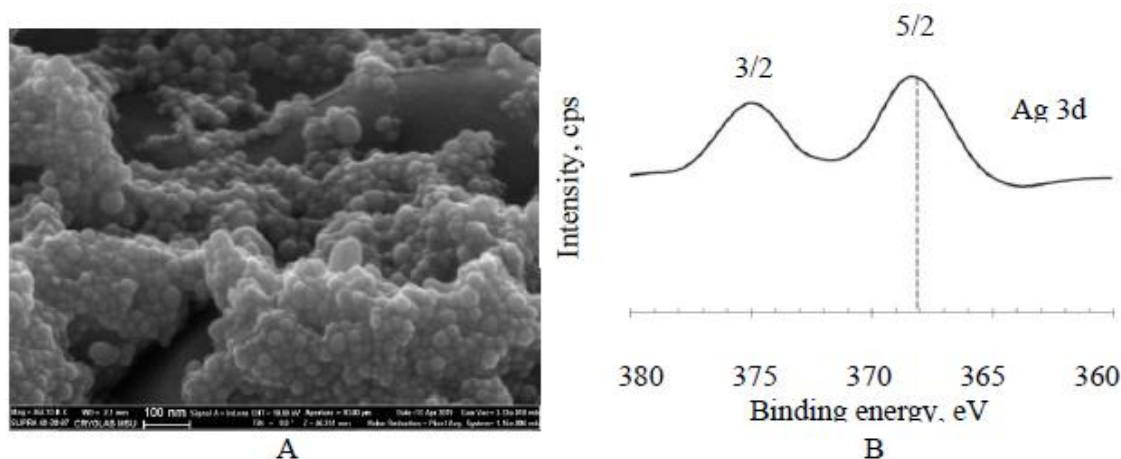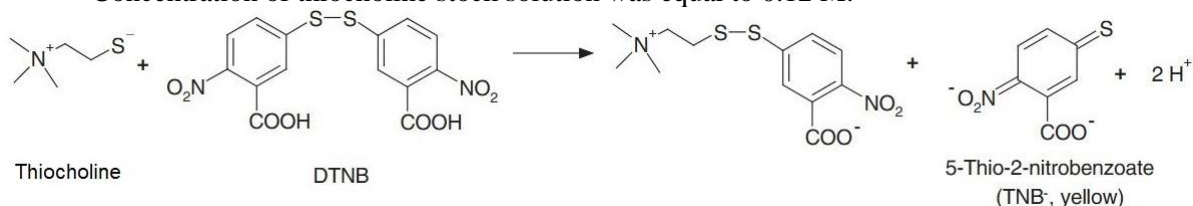
Figure 5. Typical SEM image of Ag electrode modified with PHMB-stabilized silver nanoparticles (A), Ag 3d XPS-spectrum of PHMB-stabilized nanoparticles immobilized onto Ag electrode surface in the presence of 0.08 M KCl for 12 hours (B).

The reaction yields two products, thiocholine and butyric acid, the latter does not interfere thiocholine SERS detection. Concentration of obtained thiocholine solution was determined by Ellman's assay, which represents a color reaction (scheme 2). Colored product of this reaction is 5-thio-2-nitrobenzoate ione (TNB). The reaction which is shown in Scheme 2 is rapid and stoichiometric, with the addition of one mole of thiol releasing one mole of TNB. The conversion of butyrylthiocholine is about 100%.

Concentration of thiocholine stock solution was equal to 0.12 M.

Scheme 2. Formation of colored reaction product.

3.4. SERS measurements of thiocholine

The mixture of products of the enzymatic hydrolysis of butyrylthiocholine containing thiocholine and butyric acid was applied onto silver electrode surface, dried at 60^0C and then was detected by SERS. It was found that the Raman signal intensity of the mixture applied to Ag electrode modified with PHMB-stabilized silver nanoparticles is, at least, 10 times higher in comparison with one applied to the pure Ag electrode surface (figure 6A).

The Raman signal intensity of PHMB solution applied on the electrode surface is less than intensity of signal of detected mixture applied onto PHMB-treated electrode surface at wavelength excitation of 785 nm (figure 6B). Concentration of PHMB solution was equal to PHMB concentration in the nanoparticles colloidal solution. The treatments with PHMB solution and with 40 μM thiocholine solution were carried out under the same conditions. It is apparent that the presence of PHMB on the electrode surface also promotes the enhancement of thiocholine signal but this effect is small compared to enhancement due to the nanoparticle presence.

Thus, PHMB molecules don't complicate the thiocholine detection. Therefore the Raman signal enhancement of thiocholine solution applied onto Ag electrode surface modified with PHMB-stabilized nanoparticles is related mainly to the presence of immobilized nanoparticles. The observed increasing of Raman signal intensity could be employed for semi-quantitative analysis of thiocholine.
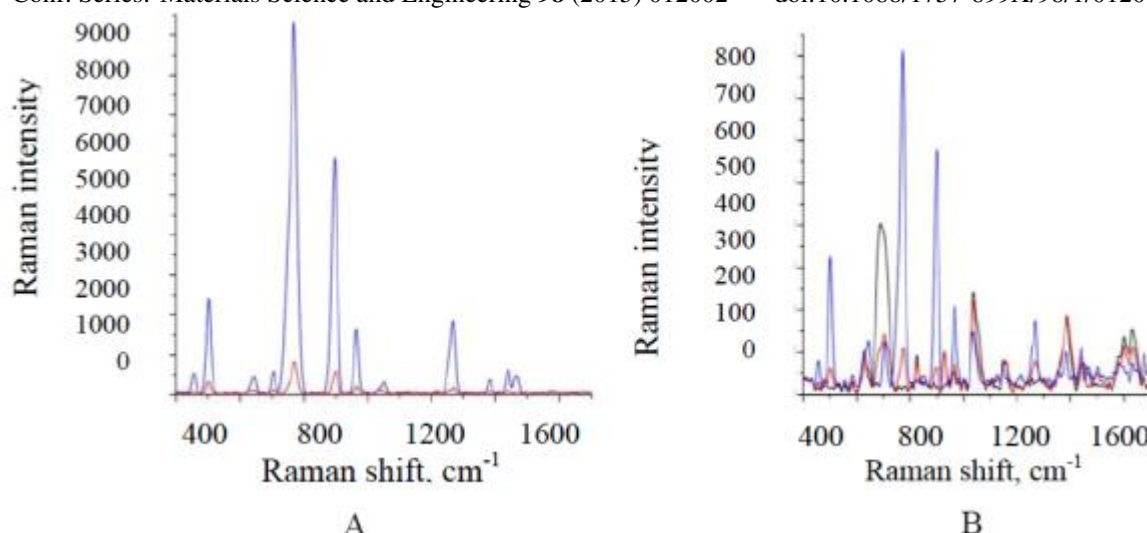
Figure 6. The SERS spectrum (red line) of 40 μM solution of thiocholine applied onto Ag electrode surface and (blue line) onto Ag electrode surface modified with PHMB-stabilized nanoparticles (A); the Raman spectrum (blue line) of 40 μM thiocholine solution applied onto pure Ag electrode surface compared to the spectra (red line) of 40 μM thiocholine solution applied onto Ag electrode surface treated with PHMB and (black line) of PHMB solution applied onto Ag electrode surface (B).

The as-prepared SERS substrates allow detection of mixtures containing submicromolar concentrations of thiocholine. In view of complexity of SERS method the internal standard is needed for the quantitative analysis. In our work, choline was applied as internal standard because its characteristic band at 714 cm^{-1} doesn't interfere with thiocholine bands. The SERS spectra were normalized on the choline signal intensity at 714 cm^{-1}. The intensity of bands at 770 cm^{-1} and 1267 cm^{-1} corresponding to thiocholine vibrations increases symbatically with thiocholine concentration. Therefore, Ag electrodes modified with PHMB-stabilized nanoparticles can be applied for the quantitative analysis of thiocholine.

4. Conclusion

In this work we have presented a new substrate for the SERS detection of thiocholine. For that purpose Ag electrodes were modified with PHMB-stabilized silver nanoparticles. It was found that the Raman signal intensity of thiocholine solution applied onto Ag electrode surface modified with PHMB-stabilized silver nanoparticles is, at least, 10 times higher in comparison with one applied onto pure Ag electrode surface. At the same time, PHMB don't contribute significantly on the thiocholine Raman signal intensity. The achieved enhancement factor is appropriate for thiocholine detection. Ag electrodes modified with PHMB-stabilized nanoparticles can be applied for the quantitative analysis of thiocholine.

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