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RESEARCH ARTICLE

ELECTROANALYSIS

Immunoassay based on peroxidase silver conjugate for the determination of human immunoglobulins against tick-borne borreliosis (lyme disease) by voltammetry and spectrophotometry

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Abstract

In this work two strategies for the synthesis of peroxidase silver conjugates for the qualitative and quantitative determination of immunoglobulins (IgG) to ixodid tick-borne borreliosis (ITBB) (Lyme disease) in human serum were proposed. The first approach for *Ab-HRP@AgNP* conjugate synthesis involved silver nanoparticles (Ag NPs) capped with a commercial peroxidase conjugate (*Ab-HRP*) by passive adsorption. The second strategy was based on the initial coupling of Ag NPs with human anti-species antibodies (Ab) by passive adsorption followed by the introduction of horseradish peroxidase (HRP) enzyme into the reaction mixture as a blocking reagent for *Ab@AgNP@HRP* conjugate synthesis. The formation of peroxidase silver conjugates was proved by UV/Vis spectroscopy and Transmission Electron Microscopy (TEM). The catalytic activity of *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates was evaluated by Michaelis-Menten kinetics. A commercially available 96-well microtiter plate with recombinant antigens to ITBB was used as a platform for immobilization of analyzed IgG. The HRP in *Ab-HRP@AgNP* conjugate was found to retain a sufficient level of activity for interaction with the H_2O_2 substrate to form an intensely colored reaction product. Therefore *Ab-HRP@AgNP* conjugate can be used in enzyme-linked immunosorbent assay (ELISA) with spectrophotometric detection of 3,3',5,5'-Tetramethylbenzidine (TMB Ox) for quantitative determination of IgG to ITBB in human serum in the concentration range 12.5–800 ng ml^{-1} with LOD 2 ng ml^{-1}. *Ab@AgNP@HRP* conjugate is recommended for the electrochemical determination of IgG to ITBB in human serum at LOD 3 ng ml^{-1} with registration of silver oxidation by linear sweep anodic stripping voltammetry (LSASV). Ag NPs in *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates do not change electrochemical activity during storage and can be used as an electrochemical label in LSASV method in case of HRP inactivation. The immunoassay based on peroxidase silver conjugates expands the analytical potential for the determination of IgG to ITBB especially during the period of increasing incidence.

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KEYWORDS
immunoglobulins to ixodid tick-borne borreliosis, lyme disease, peroxidase silver conjugates, silver electrochemical label, silver nanoparticles

1 | INTRODUCTION

Lyme disease, or borreliosis, is a common infectious caused by bacteria of the genus *Borrelia*. The main carriers of Borrelia are ticks of the genus *Ixodes*, which transmit bacteria and cause disease in mammals, including humans during biting and blood sucking [1]. ITBB is common in North America, Canada, Russia and Asia and continues to spread steadily throughout the world [2]. The initial symptoms of Lyme disease are the typical apple-shaped rash and headache [3]. ITBB can progress to a more advanced disease state that may include encephalitis, meningitis, and arthritis [4]. The early disease is easily treated with antibiotics; delayed diagnosis and/or treatment can lead to more serious consequences to human health [5]. Early detection of IgG to ITBB is carried out by ELISA [3], polymerase chain reaction (PCR) [6] and various biosensor methods [7–9] for successful diagnosis of the disease. ELISA is the most commonly used method for the determination of IgG to ITBB in clinical laboratories.

The main aspect of immunoassay performance is the quality and stability of immunoreagents for a long time (at least one year). HRP is widely used in enzyme immunoassay as a label. However, this enzyme is sensitive to a number of factors, which leads to the loss of its activity during storage [10]. Some authors suggested introducing metal NPs into enzymes and enzyme conjugates based on antispecies Ab for expanding their analytical properties and stability [11, 12]. For example, bird nest-like zinc oxide and hedgehog-like Bi_2S_3 nanostructures were used for electrochemical glucose biosensor and liver cancer biomarker immunosensor obtaining by immobilization of nanostructured NPs with enzymes [13, 14]. Moreover, metal NPs as a part of conjugates increase the catalytic properties of enzymes as exemplified by Au NPs and increase the loading of specific Ab on the metal surface due to the high surface ratio of NPs to the amount of sorbed proteins [15, 16]. Numerous papers describing the use of Ag NPs in immunosensors have been published [17–20], indicating a growing interest in the development of electrochemical immunological methods based on NPs. In these works the promising application of Ag NPs as a label was indicated: the ease of the electrochemical signal registration from Ag NPs using simple and inexpensive potentiostats / galvanostats; the ability of Ag NPs to conjugate with biomolecules without changing their properties; the increase of silver conjugates storage time. However, we could not find any information on the use of Ag NPs for the preparation of peroxidase silver conjugates.

In this work, we propose two strategies for the synthesis of peroxidase silver conjugates for the qualitative and quantitative determination of IgG to ITBB in human serum. The first approach involved Ag NPs capped with *Ab-HRP* by passive adsorption [21]. We have recently introduced an electrochemical immunosensor approach for the detection of Ab to tick-borne encephalitis virus (TBEV) [10, 21]. This assay is based on the measurement of the electrochemical signal from Ag NPs as a part of bioconjugates with anti-TBEV Ab. In this work, we decided to inject Ag NPs into a commercial peroxidase conjugate to obtain *Ab-HRP@AgNP*, instead of replacing the enzymatic conjugate label with a colloidal metal.

The second strategy for *Ab@AgNP@HRP* conjugate synthesis was based on the initial coupling of Ag NPs with human anti-species Ab by passive adsorption followed by the introduction of HRP into the reaction mixture as a blocking reagent instead of neutral proteins.

The protocols for the synthesis of *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates were simple and easily reproducible. The catalytic activity of both conjugates was evaluated by Michaelis-Menten kinetics. The proposed strategies provide an opportunity to synthesize double-labeled conjugates and to register two analytical signals from the peroxidase silver conjugates - voltammetric from Ag NPs and optical from TMB (Ox) during the catalytic action of HRP in the analysis of IgG to ITBB in human serum for the first time.

A commercially available 96-well microtiter plate with recombinant antigens to ITBB was used as a platform for immobilization of analyzed IgG (ELISA "LimeBest-IgG", Vector-Best, Novosibirsk).

2 | EXPERIMENTAL SECTION

2.1 | Synthesis and characterization of peroxidase silver conjugates

Initially, spherical Ag NPs with an average size of 18.3 ± 6.6 nm were synthesized according to the protocol [21]. 5 ml of Ag NPs solution was centrifuged for 30 min at 15,000 rpm with cooling at 4 °C before the injection of colloidal silver as an electrochemical label into a

commercial peroxidase conjugate based on human antispecies Ab against ITBB IgG. After centrifugation, 4 ml of the supernatant was discarded, and the precipitate was resuspended in 1 cm^3 0.1 mol L^{-1} of phosphate buffer solution (PBS). Ag NPs solution was stable for 14 days during storage in chemical glassware (dark glass) at 4 °C. All peroxidase silver conjugates were synthesized by passive adsorption [10].

2.1.1 | Ab-HRP@AgNP conjugates

A test with 1.0 mol L^{-1} NaCl, causing rapid coagulation of Ag NPs, was used for obtaining a stable *Ab-HRP@AgNP* conjugate and for selection the optimal volume ratios of Ag NPs solutions to commercial *Ab-HRP* conjugate solutions. For this purpose, a solution of commercial *Ab-HRP* conjugate with an initial volume of 100 μl was titrated into the plate wells before conjugation, followed by a twofold dilution. The number of dilution steps was 10. Then, 100 μl of Ag NPs solution was injected into each well, incubated for 30 min, and 100 μl of 1 mol L^{-1} NaCl solution was added. After 10 minutes, the color of the well was assessed.

In the case of a well color change from light yellow to gray, the amount of *Ab-HRP* conjugate was not sufficient to protect Ag NPs from binding to chlorides and the formation of a poorly soluble AgCl compound was observed. If there was no change in the color of the well from the original light yellow, the amount of *Ab-HRP* conjugate was sufficient for the protection of Ag NPs from NaCl coagulating effect. By scaling the volumes of *Ab-HRP* conjugate solutions to the Ag NPs solution, the following data were obtained: 600 μl of the commercial *Ab-HRP* conjugate solution was used per 400 μl of the Ag NPs solution. The obtained data characterize the protective ability of the commercial *Ab-HRP* conjugate solution in relation to Ag NPs with an average size of 18.3 ± 6.6 nm under the experimental conditions.

Ab-HRP@AgNP conjugate was purified from unbound components by centrifugation. 3 ml centrifuge tube was filled with 500 μl of 96% glycerin (Sigma, USA) and 1 ml of the resulting *Ab-HRP@AgNP* conjugate solution. Centrifugation was carried out at 25,000 rpm speed and a temperature of +4 °C for 30 min. The supernatant was separated, the glycerin conjugate was transferred into a test tube for storage in a freezer at −10 °C for 1 year.

2.1.2 | Ab@AgNP@HRP conjugates

Initially, Ag NPs were conjugated with human antispecies IgG against ITBB specific IgG. For this purpose, 100 μL of human antispecies Ab solution in distilled water with an initial concentration of 5 mg ml^{-1}, and 100 μL of Ag NPs solution were titrated into the plate wells before conjugation. The mixture was incubated for 30 min, then 100 μl of 100 mg ml^{-1} HRP solution in 0.1 mol L^{-1} PBS was added to each well as a blocking reagent, and incubated for another 1 h to block the surface of AgNPs.

After the incubation period, 100 μl of 1 mol l^{-1} NaCl solution was added to each well to evaluate the protective properties of IgG and HRP protein components against the coagulating effect of the strong electrolyte on AgNPs, similar to the synthesis procedure described for *Ab-HRP@AgNP* conjugate.

By scaling the volumes of human antispecies IgG and HRP solutions to Ag NPs solution, the following data were obtained: 700 μl of human IgG 0.01 mg ml^{-1} solution and 100 μl of HRP solution with concentration 100 mg ml^{-1} in PBS (0.1 mol l^{-1}) were used for 300 μl of Ag NPs solution with an average size of 18.3 ± 6.6 nm.

2.1.3 | Characterization of the conjugates

Absorption spectra of Ag NPs and both conjugates (*Ab-HRP@AgNP* и *Ab@AgNP@HRP*) were recorded with Agilent Cary 60 spectrophotometer (USA).

The morphology of Ag NPs and the conjugates was determined using TEM with protein contrasting by 2.0% anhydrous uranyl acetate solution with Philips CM12 transmission electron microscope (FEI Electron Optics, the Netherlands) in the Institute of Strength Physics and Materials Science SB RAS, Tomsk, Russia.

Average size calculations of the unconjugated Ag NPs and incorporated into *Ab-HRP@AgNP* conjugate were performed from TEM images using the Fiji software (www.fiji.sc) and Origin Pro 8.0 (OriginLab, USA). At least ten representative images were taken for each sample. The particle size distribution was obtained by counting at least 100 particles for each sample.

2.2 | Comparative kinetic studies of conjugates

To control the catalytic activity of IgG conjugates with HRP for unlabeled (*Ab-HRP*) and labeled with Ag NPs (*Ab-HRP@AgNP* and *Ab@AgNP@HRP*) conjugates Michaelis-Menten kinetic curves were plotted [22]. 3% solution of H_2O_2 was used as a substrate. TMB solutions of various concentrations ($C_1 = 100$ μmol L^{-1}, $C_2 = 400$ μmol L^{-1}, $C_3 = 800$ μmol L^{-1}, $C_4 = 1000$ μmol L^{-1}) were used as a chromogen.

Two series of reaction volumes were prepared for each conjugate, consisting of 100 µl $2.2 \cdot 10^{-6}$ mol L^{-1} of the studied conjugate and 100 µl of TMB solutions with different concentrations in the reaction mixture (C_1 = 100 µmol L^{-1}, C_2 = 400 µmol L^{-1}, C_3 = 800 µmol L^{-1}, C_4 = 1000 µmol L^{-1}) to carry out enzymatic reactions involving the studied conjugates.

5 µl of 3% H_2O_2 solution was introduced into each reaction mixture as a substrate to initiate enzymatic reaction. The time of enzymatic reactions was 0.17, 0.33, 0.5 and 0.7 minutes, respectively. 100 µL of 0.1 M H_2SO_4 solution was added to each reaction mixture as a stop reagent for completion of enzymatic reactions. Then, the absorption spectra of TMB oxidation product (Ox) were recorded at λ = 450 nm (Figure 1A) using Analytik Jena Scandrop nanovolume spectrophotometer (Germany). The dependence of TMB concentration (Ox) on the enzymatic reaction time was plotted for each value of the chromogen concentration. The kinetic rate of the enzymatic reaction (ν, µM min^{-1}) was calculated as the slope of the linear function from the dependence of TMB concentration (Ox) on the reaction time (Figure 1B).

Then, Michaelis-Menten kinetic curves were plotted as dependences of enzymatic reaction rates (ν, µmol L^{-1} min^{-1}) on TMB concentration (µmol L^{-1}) for *Ab-HRP*, *Ab-HRP@AgNP, Ab@AgNP@HRP* conjugates. The obtained Michaelis-Menten kinetic curves were used to determine the values of V_{max} [M s^{-1}] - the maximum reaction rate and K_m [mM] - the Michaelis constant for each type of *Ab-HRP*, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates.

Using the value of the maximum reaction rate, k_{cat} [s^{-1}] - catalytic constant for each type of conjugate was determined using the equation: $k_{cat} = \nu_{max}/E$, where E is the concentration of the conjugate in the reaction mixture 7.2 10^{-7} mol L^{-1}.

2.3 | Approbation of Ab-HRP@AgNP and Ab@AgNP@HRP conjugates for IgG immunoassay to ITBB

The analysis of human IgG to ITBB was carried out to test *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates in accordance with the solid-phase indirect non-competitive type of analysis using a commercial reagents kit D-1452 of Vector-Best JSC (Novosibirsk, Koltsovo) according to the scheme (Figure 2).

The kit contains a plate with immobilized recombinant *Borrelia burgdorferi* antigens, positive control samples (K^+, 800 ng ml^{-1}) containing specific human IgG against ITBB and negative control samples (K^-, 0 ng ml^{-1}) containing non-specific human IgG.

The commercial *Ab-HRP* conjugate included in the kit was used to synthesize *Ab-HRP@AgNP* conjugate after purification from methylene blue dye by mean of dialysis. The analysis was carried out in a well plate with immobilized antigen to ITBB according to the passport attached to the kit. The incubation period for each step of biological material immobilization in the plate wells was 1 h at 37 °C.

At the first stage analyzed human IgG to ITBB were immobilized onto plate wells with an antigen to ITBB as K^+, and negative control samples K^- were added to other wells. K^+ positive control IgG were bound by antigens immobilized on the plate wells to form an Ab-Ag complex. The plate wells were washed three times with

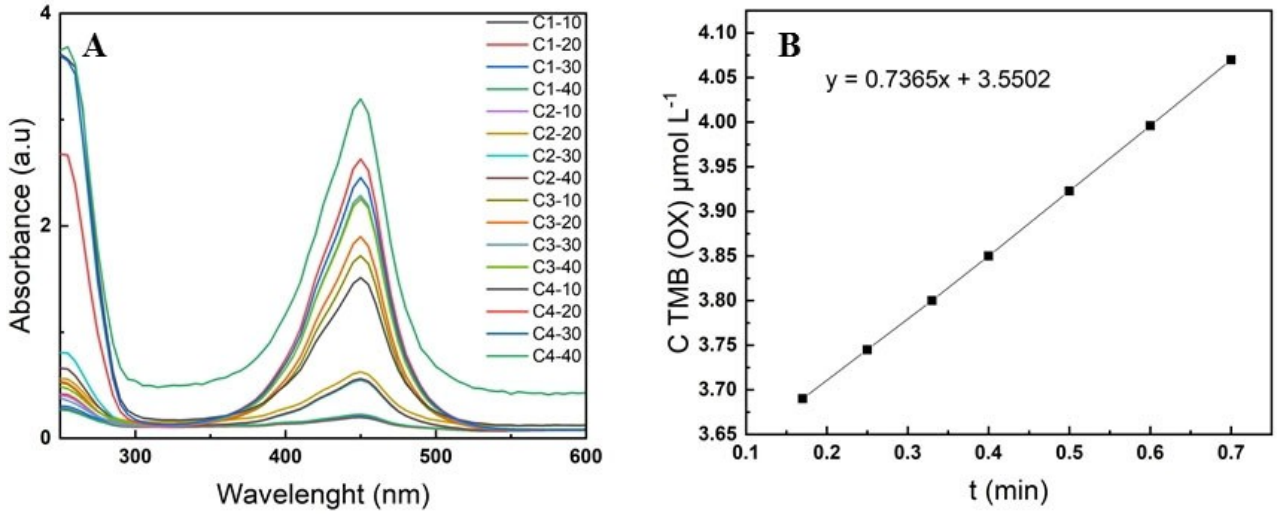


FIGURE 1 (A) Absorption spectra of TMB (Ox) at different TMB concentrations (C_1 = 100 µmol L^{-1}, C_2 = 400 µmol L^{-1}, C_3 = 800 µmol L^{-1}, C_4 = 1000 µmol L^{-1}), insert - the reaction time from 10 to 40 s; (B) Dependence of TMB (Ox) concentration on reaction time for a sample with an initial TMB concentration C_1 = 100 µmol L^{-1}.

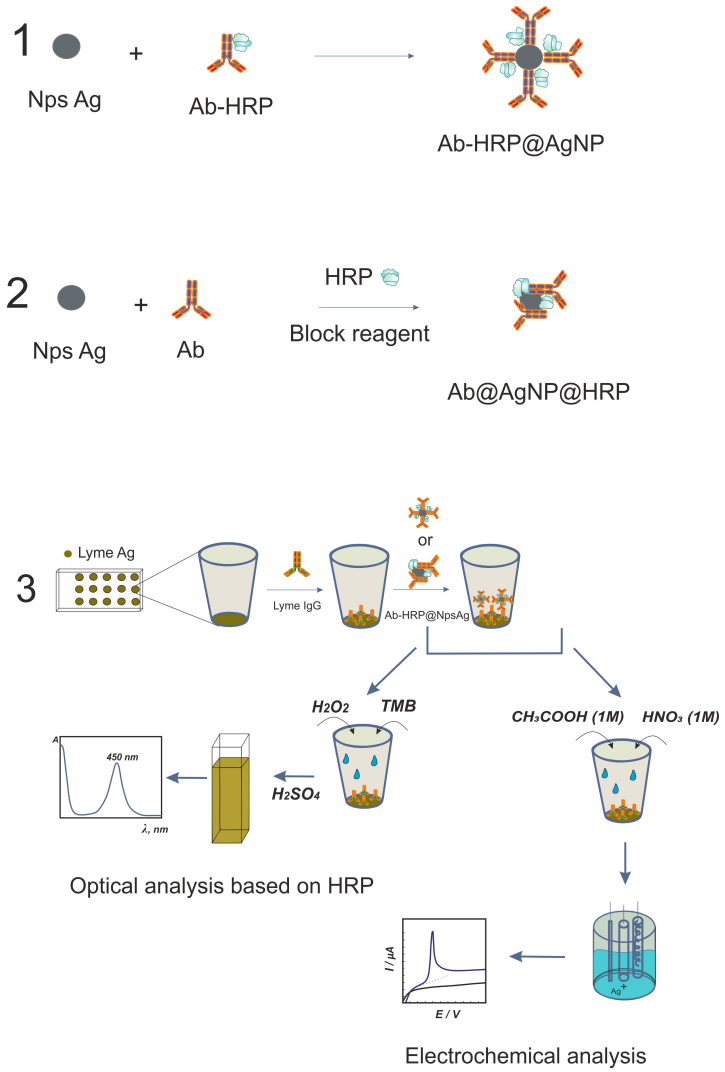


FIGURE 2 Schematic illustration of electrochemical immunoassay of IgG to ITBB.

0.1 mol L^{-1} PBS solution (pH 7.4). Then *Ab-HRP* conjugate solutions were added to one wells, and *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugate solutions were added to the other wells - 50 μl to each 20 wells. After incubation the wells were washed three times with deionized water and used for spectrophotometric and electrochemical measurements of the analytical signal.

2.3.1 | Spectrophotometric measurements

After binding of the analyzed IgG with antigens immobilized on the plate wells and washing with ionized water, *Ab-HRP*, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates were added to the wells to detect the antigen-Ab complex by the HRP reaction of the conjugates with the TMB chromogen resulting in blue color. All stages of the analysis were carried out in accordance with the kit passport (Section 2.2). All the materials and chemical glassware were treated with 6% hydrogen peroxide solution for 1 hour after the work.

2.3.2 | Electrochemical measurements

Ab-HRP@AgNP and *Ab@AgNP@HRP* conjugates with an electrochemical silver label were registered by voltammetry in accordance with the protocol [21]. After immobilization of *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates, washing with ionized water and formation of an antigen-Ab complex, 100 μl of 1 mol L^{-1} HNO_3 and CH_3COOH mixture was added to the plate wells to dissolve the colloidal conjugate silver.

The well content was transferred to an electrochemical cell and the electrochemical signal of silver oxidation was registered by LSASV in the potential range from −0.1 V to +0.6 V at a potential scan rate of 100 mV s^{-1} on a TA-Lab voltammetric analyzer (Tomanalyt, Tomsk, Russia). The

accumulation potential and time were −0.8 V and 80 sec, respectively. Ag|AgCl (1 mol L^{-1} KCl) was used as a reference electrode, a platinum wire - as an auxiliary electrode. A composite carbon-containing electrode (CCE) consisting of a polyethylene tube filled with a conductive mixture of 30% carbon black and high-pressure polyethylene (geometric area S = 112 mm^2) was used as a working electrode. 0.04 mol L^{-1} HNO_3 and 0.5 mol L^{-1} KNO_3 were used as supporting electrolyte. The electrode surface was regenerated by cutting off a thin layer of 0.1 mm with a cutter (Tomanalyt, Tomsk).

3 | RESULTS AND DISCUSSION

3.1 | Characterization of Ag NPs and conjugates

Synthesized Ag NPs, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates for immunoassay of IgG to ITBB were characterized by UV/VIS spectrophotometry and TEM.

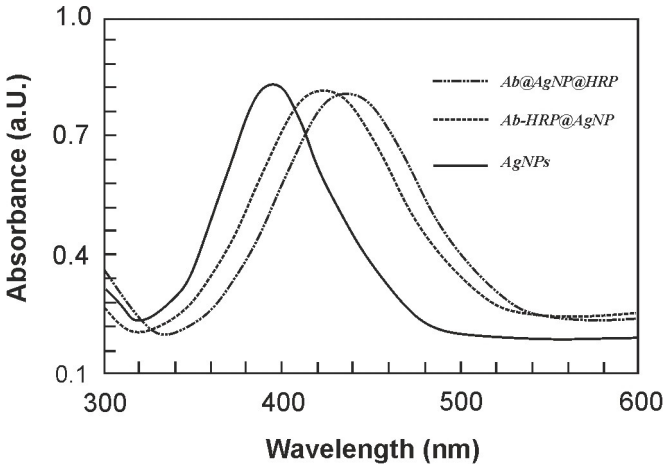


FIGURE 3 UV/VIS spectra of Ag NPs, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates.

UV/VIS spectra of Ag NPs and both conjugates were recorded in the wavelength range from 300 to 600 nm (Figure 3). The absorption maximum at 395 ± 2 nm was observed due to the surface plasmonic properties of Ag NPs, while a wider absorption maximum in the range from 420 to 440 ± 2 nm, shifted to a longer wavelength region compared to the peak of Ag NPs, indirectly indicates on the binding of Ag NPs to the IgG and HPR protein components of *Ab-HRP@AgNP* and *Ab@AgNP@HRP*.

TEM images of Ag NPs (A), *Ab-HRP@AgNP* (B) and *Ab@AgNP@HRP* (C) conjugates are shown in Figure 4. Figure 4A depicts spherical Ag NPs with an average size of 18.3 ± 6.6 nm according to the size distribution histogram.

After synthesis and purification of *Ab-HRP@AgNP* conjugate, it was noted that it contains protein components that had a white contrast with an amorphous structure, whereas spherical Ag NPs distributed on the surface of the proteins had a dark color contrast (Figure 4B). However, TEM image of *Ab-HRP@AgNP* conjugate does not depict specific Ab (Figure 4B). This is explained by the fact that a commercial conjugate, representing a covalently cross-linked complex with HRP excess and antispecies IgG, was used for conjugation to AgNPs. However, the TEM image confirms the success of *Ab-HRP@AgNP* conjugate synthesis.

TEM image of *Ab@AgNP@HRP* conjugate clearly depicts IgG heavy chains labeled by colloidal silver (inset Figure 4C), while the light chains are not bound to Ag NPs. This analysis confirms that during *Ab@AgNP@HRP* conjugate synthesis, Ag NPs primarily form a binding to the sulfur of IgG heavy chains by chemisorption, which has a great importance for retaining of Ab binding ability to antigens in the

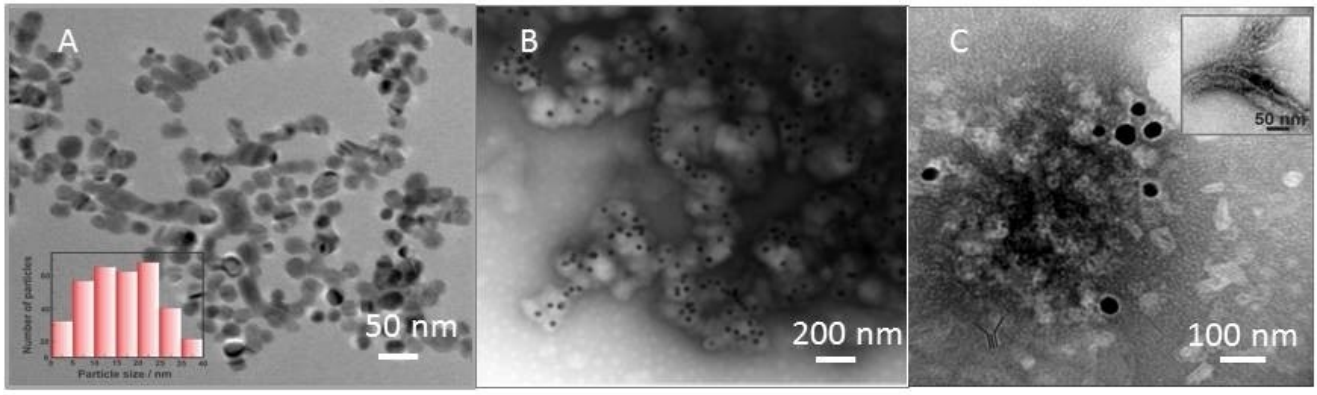


FIGURE 4 4. TEM images: **(A)** Ag NPs (insert – size distribution histogram), **(B)** *Ab-HRP@AgNPs* conjugate **(C)** *Ab@AgNP@HRP* conjugate (protein contrasting by 2.0% anhydrous uranyl acetate).

immunoassay. Amorphous structures of HRP, which are bind with IgG and Ag NPs can also be observed in Figure 4C.

3.2 | Kinetic investigations of peroxidase silver conjugates

An important step was to compare the catalytic activity of the AgNPs-containing peroxidase conjugates with the commercial *Ab-HRP* conjugate for further use of *Ab-HRP@AgNP* and *Ab@AgNP@HRP* in spectrophotometric and electrochemical measurements of IgG to ITBB. The catalytic activity of the conjugates was evaluated by plotting Michaelis-Menten kinetic curves (Figure 5) according to the method (Section 2.2). The obtained catalytic constants for the investigated conjugates are presented in Table 1. The k_{cat}/K_m value indicates the catalytic efficiency of the enzyme.

From the data presented in Table 1, we can conclude that the catalytic activity of *Ab-HRP@AgNP* is lower than that of *Ab-HRP*. This result is quite predictable, since Ag NPs can block the active sites of the enzyme label and interfere substrate-enzyme interactions. However, the loss in the catalytic activity of *Ab-HRP@AgNP* conjugate is not critical; HRP covalently bound to IgG retains sufficient activity to interact with the substrate to form an intensely colored reaction product, thus *Ab-HRP@AgNP* can be used in ELISA methods with spectrophotometric detection of TMB (Ox). Furthermore, the introduction of Ag NPs into *Ab-HRP@AgNP* extends the useful life of the conjugate in future test systems. Ag NPs can be used as an electrochemical label in voltammetry. In the case of HRP enzyme inactivation of *Ab-HRP@AgNP* conjugate during storage, it is possible to register the electrochemical signal from Ag NPs. Significant loss in catalytic activity of *Ab@AgNP@HRP* conjugate to the selected substrate is observed when HRP was used as a blocking reagent during the conjugate preparation. Therefore, *Ab@AgNP@HRP* conjugate is recommended for the electrochemical determination of IgG to ITBB in human serum.

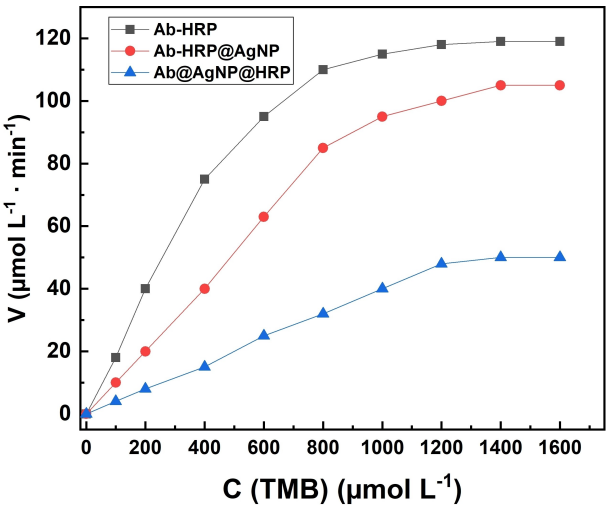


FIGURE 5 Michaelis-Menten kinetic curves for *Ab-HRP*, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates.

3.3 | Determination of IgG to ITBB by spectrophotometric method using peroxidase silver conjugates

In this work, IgG to ITBB were determined by spectrophotometry using *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates in human serum. IgG to ITBB were selected as the target model and immunoassay was performed using solid-phase indirect non-competitive ELISA spectrophotometric assay (Figure 2). Registration of spectrophotometric signals reflects both the activity of HRP with respect to the H_2O_2 substrate and the efficiency of the immunoassay. The sensitivity of peroxidase conjugates for the determination of IgG to TBBV was evaluated using control positive samples containing specific Ab to ITBB (K^+, 800 ng ml^{-1}) and control negative samples without specific Ab (K^-, 0 ng ml^{-1}) of standard reagent kit for ELISA detection of IgG to ITBB D-1452 JSC "Vector-Best". Figure 6 shows the absorbance and signal-to-noise ratio of K^+ and K^- for the studied peroxidase silver conjugates.

Although the highest K^+ signal intensity was observed for the commercial conjugate (*Ab-HRP*) compared to *Ab-HRP@AgNP* and *Ab@AgNP@HRP*, the

TABLE 1 Catalytic constants for *Ab-HRP*, *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates.

Conjugate type	E, mol L^{-1}	K_m, mmol L^{-1}	V_{max}, mol L$^{-1}\cdot$s^{-1}	k_{cat}, s^{-1}	k_{cat}/K_m, M$^{-1}\cdot$s^{-1}
Ab-HRP	$7.2\cdot10^{-7}$	0.22	$1.50\cdot10^{-6}$	2.08	$9.45\cdot10^{3}$
Ab-HRP@AgNP	$7.2\cdot10^{-7}$	0.50	$1.00\cdot10^{-6}$	1.38	$2.70\cdot10^{3}$
Ab@AgNP@HRP	$7.2\cdot10^{-7}$	0.72	$0.50\cdot10^{-6}$	69.44	$96.30\cdot10^{3}$

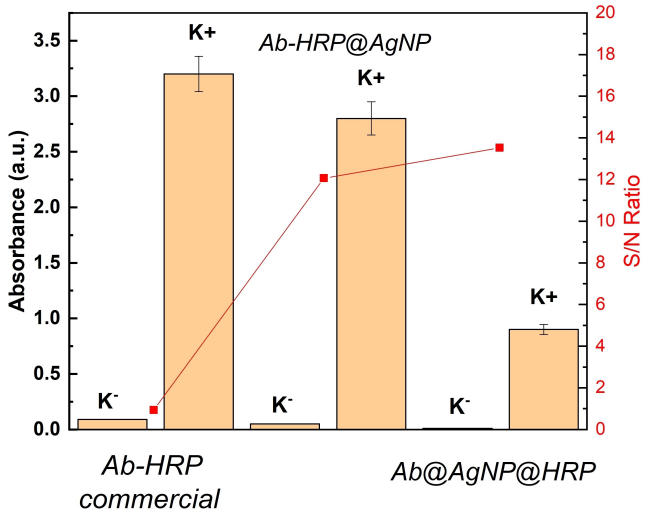


FIGURE 6 Comparison of signal intensity and signal to noise ratio for *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates.

signal-to-noise ratio of both conjugates was about 13-fold higher than that of the commercial one. The contribution of the negative control sample to the analytical signal did not exceed 0.09 a.u. for all peroxidase conjugates. An absorbance of *Ab-HRP@AgNP* conjugate was 5-fold higher for K^+ in relation to *Ab@AgNP@HRP* conjugate.

The determination of IgG to ITBB in healthy human serum samples was performed to test the practical applicability of silver peroxidase conjugates.

Ab-HRP@AgNP and *Ab@AgNP@HRP* conjugates were used for quantitative and qualitative determination of IgG to ITBB in human serum, respectively. Various concentrations of IgG to ITBB positive controls were added to healthy human serum samples diluted with 0.1 mol L^{-1} PBS (pH 7.4) (1:10). The calibration curve of absorbance at $\lambda = 450$ nm on IgG to ITBB concentration in blood serum using *Ab-HRP@AgNP* conjugates was observed in the range 12.5–800 ng ml^{-1} with regression equation $A = 0.002\ C + 0.0792$ ($R^2 = 0.9943$). LOD was 2 ng ml^{-1}, calculated as LOD = 3 s/b, where *s* is the standard deviation of the blank sample signal, and *b* is the slope of the calibration curve [23].

When *Ab@AgNP@HRP* conjugate is used for the qualitative determination of IgG to ITBB, human serum is considered positive if the absorbance is equal to or greater than Abs_{crit}. If the absorbance is less than Abs_{crit}, the human serum is considered negative.

$$Abs_{crit} =^{*} Abs_{av}(K^-) + 0.1$$

where $^*Abs_{crit}(K^-)$ is the average absorbance for negative control samples (K^-) in human serum.

Both conjugates are silver electrochemical label carriers, therefore they can be recommended for the electrochemical determination of IgG to ITBB in human serum.

3.4 | Electrochemical immunoassay for detection of Ab to ITBB

According to earlier studies, silver introduced into immunoglobulin conjugates may have electrochemical activity [15]. Thereby LSASV may be used for the determination of biological targets as exemplified by IgG to ITBB by means of Ag NPs oxidation process. Registration of electrochemical signals reflects both the affinity of IgG for the antigen to ITBB and the amount of silver affecting the intensity of the analytical signal in the immunoassay.

The analysis was performed in several steps according to the scheme shown in Figure 2. Silver conjugates were pre-dissolved in a mixture of 1 M CH_3COOH : 1 M HNO_3 to release silver ions and facilitate the accumulation of silver on CCE in stripping voltammetry mode according to Section 2.3.2. Figure 7 shows the LSASV of silver conjugates during the investigation of control positive (K^+) and control negative (K^-) samples.

Silver oxidation peaks of *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates were observed for K^+ and were absent for K^-. The most intensive peak current (I_p) was obtained after dissolution of AgNPs from *Ab@AgNP@HRP*, whereas for *Ab-HRP@AgNP* conjugate the peak current was 2 times lower.

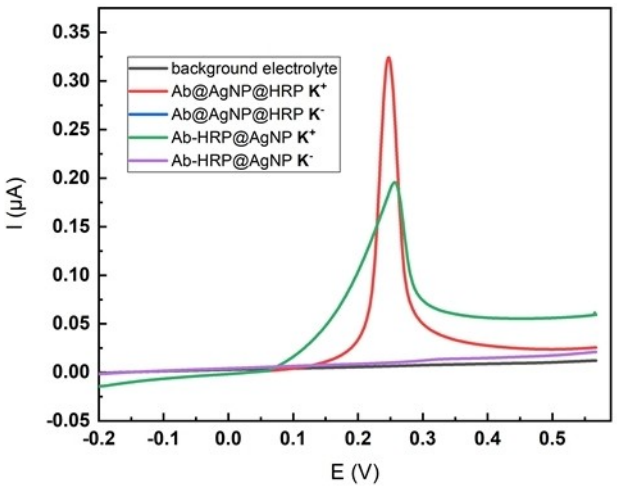


FIGURE 7 Anodic stripping voltammograms of *Ab@AgNP@HRP* and *Ab-HRP@AgNP* conjugates for K^+ and K^- samples; scan rate 100 mV s^{-1}; E_{acc} = minus 0.600 V; t_{acc} = 80 s; background electrolyte 0.5 mol L^{-1} KNO_3 + 0.04 mol L^{-1} HNO_3.

For reproducibility evaluation, five independent determinations were performed for each conjugate in solutions containing 800 ng ml^{-1} of Ab to ITBB. The relative standard deviations (RSD) of the anodic peak currents for *Ab@AgNP@HRP* and *Ab-HRP@AgNP* were 2.6% and 4.7%, respectively. The corresponding calibration curves for *Ab-HRP@AgNP* and *Ab@AgNP@HRP* conjugates are shown in Figure 8.

The concentration ranges of Ab to ITBB for the two types of conjugates are comparable and correspond to detectable concentrations from 12.5 to 800 ng ml^{-1}. However the sensitivity of IgG to ITBB determination using *Ab@AgNP@HRP* conjugate is higher ($Ip = 0.0374 \cdot C + 0.1429$, $R^2 = 0.9997$) compared to *Ab-HRP@AgNP* conjugate ($Ip = 0.1009 \cdot C - 0.0082$, $R^2 = 0.9994$). LOD were 3 and 10 ng ml^{-1} for *Ab@AgNP@HRP* and *Ab-HRP@AgNP* conjugates, respectively. This indicates a greater affinity of *Ab@AgNP@HRP* conjugate Ab to the antigen compared to *Ab-HRP@AgNP*. Therefore, based on electrochemical measurements, *Ab@AgNP@HRP* conjugate was chosen for further experiments to detect IgG to ITBB in blood serum by electrochemical immunoassay, and *Ab-HRP@AgNP* conjugates were not further investigated because of lower sensitivity and reproducibility.

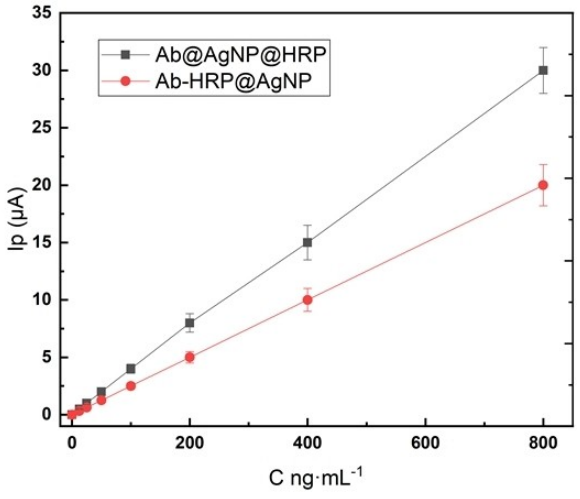


FIGURE 8 Calibration curves of silver oxidation peak current of *Ab@AgNP@HRP* and *Ab-HRP@AgNP* conjugates on the concentration of IgG to ITBB; background electrolyte 0.04 mol L^{-1} HNO_3 and 0.5 mol L^{-1} KNO_3; E vs. Ag | AgCl (1 mol L^{-1} KCl); scan rate 100 mV s^{-1}, E_{acc} = minus 0.8 V, t_{acc} = 60 s.

3.5 | Practical application of silver peroxidase conjugates for the determination of IgG to ITBB in human serum samples

In this work determination of IgG to ITBB in human serum samples by spectrophotometric (using *Ab-HRP@AgNP* conjugate) and electrochemical (using *Ab@AgNP@HRP* conjugate) methods was performed. Control positive (K^+) IgG samples were added to healthy human serum samples diluted with 0.1 mol L^{-1} PBS (pH 7.4) (1:10). ELISA with a commercial HRP-based conjugate was applied as a reference method for the determination of IgG to ITBB (Vector-Best, Novosibirsk, Russia). Table 2 presents results of spectrophotometric and voltammetric determination of IgG to ITBB in human serum samples. It can be noted that the recoveries of the Ab to ITBB ranged from 91% to 108% for all methods, indicating no significant effect of the serum matrix on the analytical signals.

Ab-HRP@AgNP conjugate can be used for the quantitative determination of IgG to ITBB by spectrophotometric and qualitative determination of IgG to ITBB by electrochemical methods. On the contrary, *Ab@AgNP@HRP* conjugate can be used for the quantitative determination of IgG to ITBB by electrochemical and qualitative determination of IgG to ITBB by spectrophotometric methods.

Moreover, the use of the peroxidase silver conjugates expands the analytical potential of ELISA for IgG to ITBB determination with respect to the

TABLE 2 Determination of IgG to ITBB in human serum by spectrophotometric and electrochemical methods (n = 5; P = 0.95).

Method	Concentration of Ab to ITBB (ng mL^{-1})		RSD (%)	Recovery (%)
	Added	Found		
Spectrophotometry with *Ab-HRP@AgNP* conjugate	12	13 ± 1	2.20	108
	800	820 ± 17	2.60	102.5
Electrochemical immunoassay with *Ab@AgNP@HRP* conjugate	12	11 ± 1	2.70	91
	800	780 ± 17	2.60	97.5
ELISA	12	13 ± 1	2.20	108
	800	830 ± 17	2.60	104

instrumentation used. Inexpensive electrochemical analyzers can be used in the period of an increasing incidence and load on flatbed spectrophotometers for determination of elevated IgG levels.

Retaining of Ag NPs electrochemical activity during storage is an undeniable advantage of silver peroxidase conjugates. Therefore, incorporated Ag NPs can be used as an electrochemical label of conjugates in voltammetry in case of HRP inactivation.

4 | CONCLUSION

Peroxidase silver conjugates (*Ab-HRP@AgNP* and *Ab@AgNP@HRP*) for spectrophotometric and voltammetric determination of IgG to ITBB in human serum were synthesized for the first time. The formation of peroxidase silver conjugates under the selected synthesis conditions was proved by UV/Vis spectroscopy and TEM. Kinetic studies of *Ab-HRP@AgNP* conjugate showed that HRP retained a sufficient level of activity for interaction with the substrate to form an intensely colored reaction product. Therefore, *Ab-HRP@AgNP* conjugate can be used in ELISA with spectrophotometric detection of TMB (Ox) for quantitative determination of IgG to ITBB in human serum in the concentration range 12.5–800 ng ml^{-1} with LOD 2 ng ml^{-1}.

Ab@AgNP@HRP conjugate is characterized by significant loss of catalytic activity to the selected substrate when HRP was used as a blocking reagent during the conjugate preparation. Therefore, *Ab@AgNP@HRP* conjugate is recommended for the electrochemical determination of IgG to ITBB in human serum with LOD 3 ng ml^{-1} by registration of silver LSASV oxidation from the Ag NPs label of the conjugate.

The use of the peroxidase silver conjugates expands the analytical potential of ELISA for IgG to ITBB determination with respect to the instrumentation used. Inexpensive electrochemical analyzers can be used in the period of an increasing incidence and load on flatbed spectrophotometers for determination of elevated IgG levels.

In case of HRP inactivation during storage, a simple and inexpensive voltammetric method based on silver oxidation can be used. This will extend the useful life of diagnostic kits based on peroxidase silver conjugates for the determination of IgG to ITBB in human serum samples.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the manuscript.

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