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Preparation of conjugates based on colloidal gold nanoparticles for application in rapid detection of antibodies to hepatitis E virus

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Abstract

Relevance. Hepatitis E virus (HEV) is a common cause of viral hepatitis not only in areas with low levels of water supply and hygiene, but also in industrialized countries. Rapid tests development for the infection seromarkers detection in the absence of special equipment and trained staff remains the most important problem in improving the diagnosis of hepatitis E.

Aim. To produce conjugates of recombinant ORF2 antigen of HEV genotype 3 with gold nanoparticles (GNP) of varied sizes and to evaluate their applicability in the immunoassay for the detection of antibodies to HEV.

Materials and methods. Specific polyclonal and monoclonal antibodies, recombinant antigen ORF2 of hepatitis E virus genotype 3, blood serum samples of people diagnosed with acute hepatitis. Synthesis of GNPs and their conjugates with recombinant antigen, enzyme immunoassay, dot immunoassay, immunochromatographic analysis, transmission electron microscopy.

Results. Three samples of colloidal GNP were synthesized using citrate method with varied concentrations of reducing agent and were subsequently used for preparation of conjugates with recombinant antigen ORF2 of HEV genotype 3. Immunoreactivity of these conjugates was confirmed by dot-immunoassay with blood serum samples containing specific IgG. A conjugate based on a 41 nm GNP was chosen for use in immunochromatographic analysis (ICA). Optimal conditions for preparation of a multi-membrane composite, including formation of analytical and control lines and the conjugate area were identified, and test strips were developed. The obtained conjugate was tested by ICA using blood serum samples which had been subjected to preliminarily characterization by the content of the IgG antibody to HEV. High immunoreactivity of the conjugate was demonstrated. Antibodies to the virus were identified in 100% of the examined (n = 17) IgG-positive serum samples, while in negative samples (n = 17) they were absent.

Conclusion. The results demonstrated effectiveness of the obtained immunoreagents (recombinant antigen, antibodies, conjugate) for use in test-systems for rapid diagnosis of hepatitis E.

Keywords: *immunochromatographic assay, colloidal gold nanoparticles, recombinant antigen, hepatitis E virus, protein ORF2, IgG*

Ethics approval. The study was conducted with the informed consent of the patients or their legal representatives. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the I. Mechnikov Research Institute of Vaccine and Sera (protocol No. 4, February 22, 2023).

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Оригинальное исследование
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Получение конъюгатов на основе наночастиц коллоидного золота для быстрого выявления антител к вирусу гепатита Е

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Аннотация

Актуальность. Вирус гепатита Е (ВГЕ) — частая причина вирусных гепатитов не только на территориях с низким уровнем водоснабжения и гигиены, но и в промышленно развитых странах. Важнейшей проблемой совершенствования системы диагностики гепатита Е остаётся разработка тестов быстрого определения серомаркеров инфекции для применения в условиях отсутствия специального оборудования и обученного персонала.

Цель работы — получение конъюгатов наночастиц коллоидного золота (НЧЗ) нескольких размеров с рекомбинантным антигеном ORF2 ВГЕ 3 генотипа и оценка возможности их применения в иммуноанализе для выявления антител к ВГЕ.

Материалы и методы. Исследовали специфические поликлональные и моноклональные антитела, рекомбинантный антиген ORF2 ВГЕ 3 генотипа, образцы сывороток крови людей с диагнозом острого гепатита. Осуществляли синтез НЧЗ и их конъюгатов с рекомбинантным антигеном, иммуноферментный анализ, дот-иммуноанализ, иммунохроматографический анализ, использовали метод просвечивающей электронной микроскопии.

Результаты. Цитратным методом с использованием различных концентраций восстановителя синтезированы 3 препарата НЧЗ. На их основе получены конъюгаты с рекомбинантным антигеном ORF2 ВГЕ 3 генотипа, иммунореактивность которых подтверждена методом дот-иммуноанализа с образцами сывороток крови, содержащими специфические иммуноглобулины класса G (IgG). Для применения в иммунохроматографическом анализе отобран конъюгат на основе НЧЗ диаметром 41 нм. Отработаны условия получения мультимембранного композита, включая формирование аналитической и контрольной линий и зоны конъюгата, изготовлены тест-полоски и проведены испытания полученного конъюгата методом иммунохроматографического анализа с образцами сывороток крови, предварительно охарактеризованными по содержанию IgG-антител к ВГЕ. Показана высокая иммунореактивность полученного конъюгата: антитела к вирусу выявлены в 100% обследованных IgG-положительных проб сывороток (n = 17) и не обнаружены в отрицательных пробах (n = 17).

Выводы. Получены иммунореагенты (рекомбинантный антиген, антитела, конъюгат), которые могут быть использованы при создании тест-систем для экспресс-диагностики ВГЕ.

Ключевые слова: *иммунохроматографический анализ, наночастицы коллоидного золота, рекомбинантный антиген, вирус гепатита Е, белок ORF2, иммуноглобулины класса G*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов или их законных представителей. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с «Consensus Author Guidelines for Animal Use» (IAVES, 23.07.2010). Протокол исследования одобрен Локальным советом по этике НИИВС им. И.И. Мечникова (протокол № 4 от 22.02.2023).

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Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Hepatitis E virus (HEV) is a frequent cause of viral hepatitis not only in areas with low water quality and supply, but also in industrialized countries [1]. Specific IgM and IgG antibodies in serum or plasma of patients serve as serological markers of HEV infection. The European Association for the Study of the Liver recommends the following criteria for the clinical diagnosis of acute hepatitis E cases: the simultaneous presence of HEV RNA and specific IgM and/or IgG antibodies (anti-HEV AB) in the analyzed samples, increase of anti-HEV IgG AB titers in the presence of anti-HEV IgM AB, and the detection of HEV antigen (HEV AG). The diagnosis of chronic hepatitis E is confirmed by the detection of HEV RNA and/or HEV AG for more than 3 months. The past infection is indicated by the presence of IgG anti-HEV AB in the samples [2]. The main method for the diagnosis of acute and chronic hepatitis E is the polymerase chain reaction, which makes it possible to detect viral RNA at the earliest stages of the disease and in the absence of AB in chronic HEV patients with immunodeficiency. In some cases, serological methods for diagnosing hepatitis E are more reliable and specific [3], and in most cases have no alternative due to the short period of viremia in HEV infection [4]. Each method of hepatitis E serological diagnostics has limitations that make it difficult to accurately determine the stage of the disease. Due to problems associated with virus cultivation, kits for hepatitis E serological diagnostics most commonly utilize recombinant antigens, or less frequently, synthetic peptides.

The problem of shortening HEV test turnaround time is solved by using rapid tests based on the immunochromatography assay (ICA) for detect HEV-specific IgG, IgM, total ABs and HEV AG using colloidal gold conjugated with recombinant AG or AB to viral proteins and/or o human or animal immunoglobulins. Along with quick results (15 minutes), the great advantage of the method is the possibility of testing at the patient's bedside in the absence of special equipment and trained personnel. The ICA test systems for the detection of HEV AG and anti-HEV AB developed and produced by foreign companies showed good reproducibility with sufficiently high sensitivity, specificity and good consistency with the results obtained using classical enzyme immunoassay (ELISA). A number of comparative studies have shown that ICA tests for detecting anti-HAV IgM AB manufactured in China ("IgM antibody to hepatitis E virus (HEV-IgM) rapid test", "Wantai", cat No. WJ-15) and Singapore ("MP Diagnostics ASSURE HEV IgM Rapid Test", "MP Biomedicals Asia Pacific Pte. Ltd.", cat. No. 43160-020), have even higher sensitivity than some traditional ELISA tests [5]. Canadian companies Citest Diagnostics Inc. and BiogateLabs produce ICA kits for simultaneous detection of IgG and IgM AB to HEV (cat No. IHE-302, cat No. RT-EV1112-C-1, respectively) in serum, plasma and whole blood. In the Russian Federation and the countries of the former Soviet Union, there is no manufacturing of tests for the rapid detection of HEV serological markers by the ICA method. Furthermore, there are no reports of ongoing research in this area.

Besides specific antigens or antibodies, the key components of such test systems are conjugates of colloidal nanoparticles with biological macromolecules which allow visual or digital recording of reaction results [6–9]. The unique optical properties of gold nanoparticles (GNP) provide visible surface plasmon resonance (SPR) peaks in synthesized GNP in the wavelength range of about 520 nm, depending on the size of the synthesized GNP [10]. The advantages of using GNP-based conjugates in rapid diagnostic tests combine the possibility of results visualization due to high molar absorption coefficients [10, 11] with their ability to fully restore properties after sorption on the membrane and subsequent drying [12, 13] in the analysis process. Diagnostic efficiency of ICA-tests is determined by the ability of the GNP conjugate to interact specifically with target molecules. It is related to physical and chemical properties of the GNP, in particular to their size and shape. It is also determined by antigenic specificity of the proteins adsorbed on them, which perform an additional function of secondary stabilization of colloidal particles in the conjugates.

All test systems for the detection of anti- HEV AB are based on the use of the capsid protein ORF2, which contains diagnostically significant epitopes, as the antigenic basis. Previously, we have obtained [14] the recombinant capsid protein ORF2 of HEV genotype 3 (recAg), which has the widest geographical distribution in the world [15, 16] and is dominant in Russia [17]. This recAg has been selected as the basis for the subsequent development of the rapid test for the simultaneous detection of anti-HEV IgG and IgM AB.

The objective of the present study was to synthesise conjugates of recAg with GNPs of varying sizes and to assess their potential application in an immunoassay for the detection of antibodies to HEV.

Materials and methods

Immunoreagents

Polyclonal goat AB against human IgG (GAHIss) («Imtek») (hereafter referred as AB), recAg (I.I. Mechnikov NIIVS) [18]; polyclonal rabbit IgG AB to recAg (hereafter referred PAB) obtained by immunizing rabbits and subsequent affinity purification (I.I. Mechnikov NIIVS); a conjugate of mouse monoclonal AB to recAg (hereafter referred MAB) with horseradish peroxidase (I.I. Mechnikov NIIVS) obtained by periodate oxidation method [19].

Blood sera of individuals diagnosed with acute viral hepatitis were provided by the National Institute of Public Health of the Ministry of Health of the

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Kyrgyz Republic and by the Belarusian State Medical University. According to the test results obtained with the DS-ELISA-ANTI-HEV-G reagent kit («Diagnostic systems») [20, 21] and with the developed at the I.I. Mechnikov NIIVS confirmatory test for the determination of anti-HEV IgG AB by the line blot method [22]; sera contained (n = 17) and did not contain (n = 17) anti-HEV IgG AB.

The study was conducted with the informed consent of the patients or their legal representatives. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July, 2010). The research protocol was approved by the Ethics Committee of the I. Mechnikov Research Institute of Vaccine and Sera (protocol No. 4, February 22, 2023).

GNP colloidal solutions

GNPs with desired particle sizes were prepared by citrate method using tetrachloroauric (III) acid trihydrate $HAuCl_4 \times 3H_2O$ («Aldrich») as a precursor [11–13]. Taking into account that the average particle diameter reduces with an increase in citrate concentration in the reaction mixture [11], varying amounts of reducing agent allowed to obtain GNP samples with different particle sizes. One mL of a 1% $HAuCl_4 \times 3H_2O$ solution was added to 99 mL of deionized water; the solution was brought to boil with stirring, then various volumes of 1% aqueous solution of sodium citrate $C_6H_5Na_3O_7 \times 2H_2O$ («Merck») were added.

The solution was boiled with a reflux condenser for 20 min under stirring, then cooled to room temperature. Absorption spectra within the wavelength range from 400 to 700 nm were measured using a Multiskan GO tablet (flatbed) spectrophotometer («Thermo Scientific»). GNP structures were studied using «Jeol JEM ARM200F» atomic resolution scanning transmission electron microscope («HAADF-STEM») equipped with an energy dispersion analysis (EDX) system based on a «GIF Quantum» spectrometer with a «Centurio EDX» detector with 0.08 nm resolution at accelerating voltage of 200 kV in STEM mode («Jeol») [23, 24]. TEM samples for the study were prepared by depositing a sample of GNP solution pre-diluted to A_{520} = 1.0 absorbance onto carbon film-coated copper meshes.

Synthesis of conjugates

Optimal conditions for preparation of GNP conjugates with recAg were selected according to the described recommendations [25, 26]. Wells 1–11 of a 96-well polystyrene plate («Greiner») were filled with 10 µL of recAg solutions with concentrations varying from 1 to 50 µg/mL in phosphate buffer (PBS). Then, 100 µL of GNP solutions with pH from 5.5 to 9.0 were introduced into the wells A-H of the horizontal rows of the plate so that each column corresponded to a certain concentration of recAg, and each row corresponded to a pH value of the solution. After 15 min, 20 µL of 10% sodium chloride solution was added to all wells, and after further 10 minutes optical absorption of the solutions was measured at wavelengths of 520 and 580 nm (A_{520}, A_{580}). As absorbance of the solution at 580 nm increases with the loss of stability in the colloidal system, the stabilizing concentration of recAg was estimated by the difference «$A_{520} - A_{580}$» [25].

Conjugates of GNP with recAg were obtained by dropwise addition of 10 mL of GNP solution with pH 8.0 to the recAg solution until recAg concentration reached 25 µg/mL for GNP-1, 30 µg/mL for GNP-2, and 40 µg/mL for GNP-3. Subsequently, the mixtures were shaken at room temperature for 30 min using a Multi-Reax device («Heildorf»); then a 10% solution of bovine serum albumin (BSA) («Sigma») was added to a final concentration of 0.25%. Suspensions were incubated for 15 min under the same conditions and following that, centrifuged for half an hour at 4ºC at 8–11 × 10^3 rpm, depending on the size of the GNPs. The resultant precipitate was suspended in 1 mL of PBS with 0.1% BSA, 10% sucrose and 0.01% sodium azide, and absorption spectra were recorded as described earlier. The obtained conjugates of GNP with recAg were stored at 4ºC.

The result of recAg seeding on the surface of GNP was controlled by enzyme-linked immunosorbent assay (ELISA) [25]. PAB solution with a concentration of 5 µg/mL in 0.1 M carbonate-bicarbonate buffer pH 9.6 was adsorbed in wells of a plate. The plates were blocked with 0.02 M phosphate-salt buffer pH 7.2 with 0.05% Tween-20 (FSB-T), 5% sucrose, 0.09% sodium caseinate. The wells were filled with 100 µL of supernatants obtained after centrifugation of the GNP conjugates with recAg. Horseradish peroxidase-labeled MAB was used as conjugate in ELISA; 0.5 mM 3,3',5,5'-tetramethylbenzidine solution was used as chromogen. Calibration samples were produced via serial dilutions of recAg (5, 2.5, 1.25, 0.625, 0.31 and 0.15 µg/mL) in the supernatant liquid obtained after centrifugation of colloidal gold solution. recAg concentration in the samples of interest was determined using a calibration plot of optical density values at 450 nm wavelength *vs* recAg concentration in calibration solutions.

Dot immunoassay reaction

To perform dot immunoassay in sandwich format, AB in the amounts of 0.1; 0.05; 0.025 and 0.0125 µg were sorbed onto 13 mm nitrocellulose membrane discs (0.45 µm). As a negative control, 0.1 µg of BSA solution in 0.02 M phosphate-salt buffer pH 7.2 (PSB) was applied. Free areas of the membranes were blocked with 0.09% sodium caseinate solution in PBS. The membranes were incubated with serum containing anti-HEV IgG AB for 1 hour in a thermo-shaker («Eppendorf») at 37ºC, followed by 30 min with solutions of GNP conju-

gates with recAg (A_{520} = 0.5 o.u.) at room temperature. Staining intensity of experimental and control spots on the membranes was evaluated visually.

Immunochromatographic analysis

AB solutions were applied to membranes for immunochromatography using an automatic IsoFlow dispenser («Imagene Technology») at a rate of 0.2 μL per 1 mm of membrane length. PAB was used in a control zone and AB — in analytical (test) zone. A conjugate of GNP with recAg was sorbed on the glass fiber membrane at the optimal concentration of A_{520} = 4 optical density (OD) units, which was determined by changing the dilutions of conjugates in the range of A_{520} values from 2 to 8 OD units with three positive serum samples tested for each dilution.

Assembly of a multimembrane composite and cutting of test strips was performed using an MTB 300 hand laminator («Kinbio Tech Co. Ltd.») and a guillotine type «Cutter ZQ2002» («Kinbio Tech Co. Ltd.»). When performing the ICA, 20 μL of blood serum was applied to the sampling area of an immunochromatographic test strip, then the edge of the strip was dipped into 80 μL of PBS; after 2–3 minutes the test strip was places horizontally and after further 15 minutes the result was evaluated. Result was considered positive when any intensity of staining of the control and analytical zones was observed, and as negative when there was no staining of the analytical zone. In the absence of staining of the control zone, the result was not taken into account.

For digital evaluation of the assay results, the test strips were scanned using a Gel DocTM XR device («Bio-Rad») with Image LabTM Software. Positivity coefficient (PC) was calculated as a ratio of staining intensity of an analytical area to background staining of a test strip.

Statistical data processing was performed using R and «Excel 2013» software.

Results

Methods for obtaining colloidal gold are well described in the scientific literature [11, 26–29]. In present work, we used a modified Frens citrate method for producing GNPs with particle diameters in the range from 15 to 40 nm, which is optimal for effective antibody sorption. Specifically, GNP samples with average particle sizes of 16 nm (GNP-1), 25 nm (GNP-2) and 41 nm (GNP-3) were synthesized. Synthesis of GNP was carried out via addition of 1% sodium citrate solution in volumes of of 1 mL, 0.75 mL and 0.5 mL to 50 mL of 0.01% tetrachloroauric(III) acid trihydrate solution. Mixing was conducted using magnetic stirrer at 300 rpm and temperature of 100ºC. Once solution color changed to red, stirring speed was increased to 500 rpm [27]. While the excess of citrate anions on the surface of nanoparticles stabilizes the gold sol, it is not preventing further interactions of GNPs with protein macromolecules during preparation of immunometric conjugates. It was demonstrated that the obtained colloidal solutions maintained their stable characteristic during storage at 4ºC in the absence of light for at least six months.

An important characteristic of the GNP is the position and configuration of the SPR band. Absorption spectra for all obtained GNP samples were recorded and results are presented in **Fig. 1.** As it can be seen in the figure, an increase in particle size led to an increase in color intensity of the solutions and a shift of absorption maximum to a region of longer wavelengths (518 nm, 522 nm and 528 nm for GNP-1, GNP-2 and GNP-3, respectively).

The average size and shape of nanoparticles were assessed using transmission electron microscopy (TEM) (**Fig. 2**). In high-resolution images, the relative homogeneity of particles in shape and size in each group is evident. The smaller particles have a predominantly spherical shape; the presence of nanoparticles deviating from the regular spherical shape is observed in the preparation of the GNP-3 sample, which is consistent with the classification of the structure of nanoparticles depending on their size [30].

The average particle sizes determined using TEM were 16 for GNP-1, 25 for GNP-2 and 41 nm for GNP-3 samples, respectively, which corresponded well with the values set by the synthesis conditions, and indicated that the method used was highly reproducible. The present study was focused on obtaining GNPs of three sizes in order to compare results obtained herein with the existing data [31–33].

Statistical difference between three groups of GNPs was assessed by constructing dispersion plots using the R software package Box Plot function (**Fig. 3**). The degree of dispersion was found to be highest for the

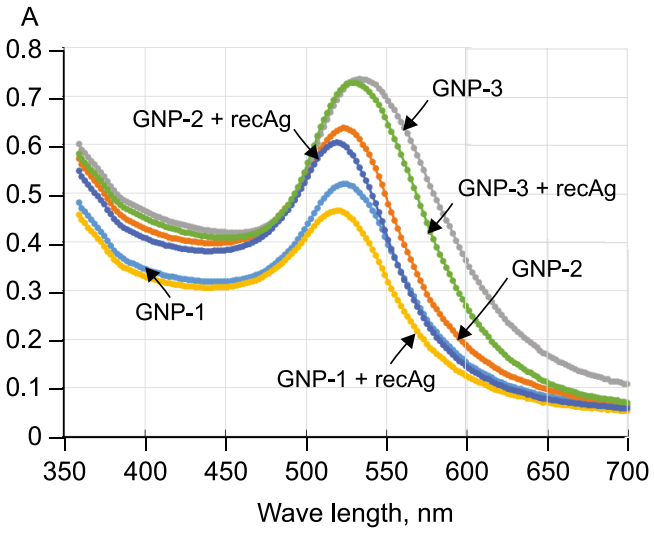

Fig. 1. Absorption spectra of GNP-1, GNP-2, GNP-3 solutions and their conjugates with recAg.

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GNP-3 sample with the largest particle sizes ranging from 30 to 56 nm. On contrary, dispersion was minimal (14 to 18 nm) for GNP-1 and the size range for GNP-2 was 20 to 37 nm. Quantitative data was confirmed to be normally distributed by using Shapiro–Wilk criterion ($p \leq 0.05$), and together with the position of the medians for GNP-1, GNP-2 and GNP-3 a conclusion was drawn that for all GNP samples, the size distribution is close to normal.

Most common method for production of GNP-based markers for use in immunochemical reactions is based on non-covalent (adsorption) conjugation of proteins on the surface of nanoparticles. This approach is technologically simple and preserves the native structure and properties of macromolecules in resulting complexes, in particular their immunoreactivity [27, 34, 35]. In this case, non-covalent bonds formed as a result of events such as electrostatic interactions between negatively charged nanoparticles and positively charged sites on protein surface, as well as hydrophobic attraction between protein and metal surface, donor-acceptor interactions between metal and electrons of nitrogen and sulfur atoms in protein composition [26]. It is also confirmed, that in the process of formation of GNP complexes with proteins a stabilizing protein shell is formed around them [35, 36]. In the present work, passive immobilization technique was also employed to obtain conjugates of three GNP samples with recAg.

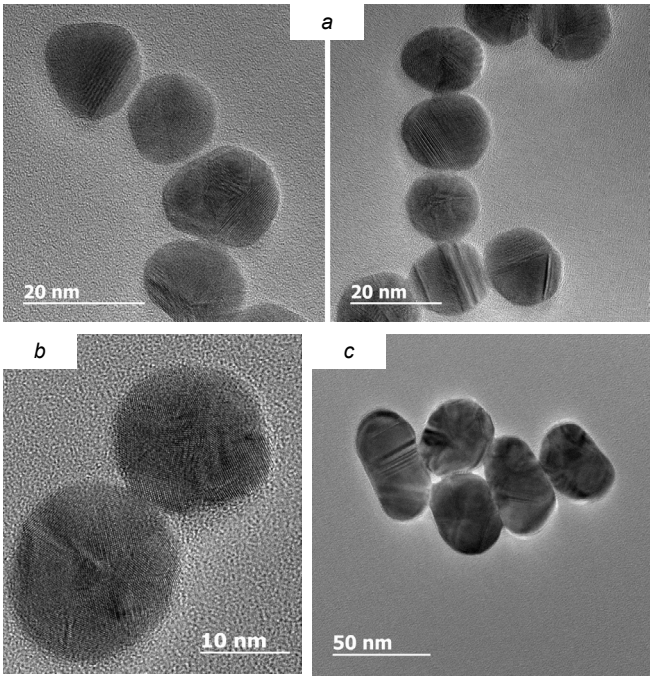

Fig. 2. Photographs of GNP obtained by means of TEM.
a — GNP-1; *b* — GNP-2; *c* — GNP-3.

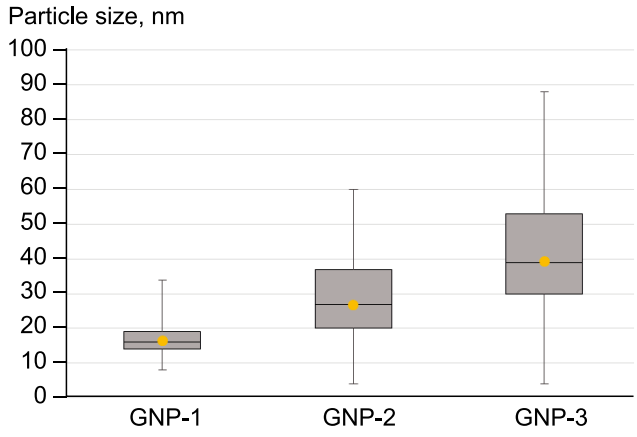

Fig. 3. Size distribution diagrams for GNP-1, GNP-2, and GNP-3 (Box Plot functions).

In order to obtain a stable sol, conditions of recAg adsorption on the surface of GNP were optimized taking into consideration physicochemical properties of the protein important for stabilization of colloidal system, such as molecular weight, solubility, isoelectric point (pI = 5.43) and its concentration. Stabilizing concentration of recAg was determined in pH range from 5.5 to 9.0 for all three sizes of GNP. **Figure 4** demonstrates the dependence of indicator «A_{520} –A_{580}» on protein concentration at different pH values for GNP-2.

The addition of sodium chloride to GNP solutions with low recAg concentrations led to aggregation of protein-unstabilized GNPs, resulting in an increase of absorption at λ = 580 nm. At the same time, a solution color change from red to grey-blue was observed. Reaching stabilizing concentrations of recAg led to an increase and further plateauing of the «A_{520} – A_{580}» index value, as well as sol stability and solution color in the presence of electrolyte.

It was further determined that the system was less stable in the acidic medium. A possible reason for that might be lower stability of sols in solutions with pH close to isoelectric point of recAg (5.36). At pH ≥ 7, the difference between the obtained curves was negligible, therefore conjugation of GNP with recAg was carried out at pH 8. Stabilizing concentrations of recAg were determined by the values at which the curves reached a plateau (20 μg/mL for GNP-1, 25 μg/mL for GNP-2, and 30 μg/mL for GNP-3). Concentration of recAg, required for stabilization of the colloidal system increased with an increase in the GNP particle size. Subsequently, recAg concentrations higher than the stabilizing concentration by 10–15% were used to obtain conjugates, as it had been recommended elsewhere [33].

To investigate interaction of protein with GNP in the samples of conjugates, the absorption spectra of GNPs and their conjugates with reqAg were compared. Binding of nanoparticles from the solution to protein molecules immobilized on the gold surface results in a noticeable shift in the SPR peak. The magnitude of this shift is determined by the particle size and can be used as a parameter for assessing binding activity of the adsorbed protein [35]. A method for determining composition of GNP conjugates with proteins that is based on evaluation of intrinsic fluorescence of unbound protein

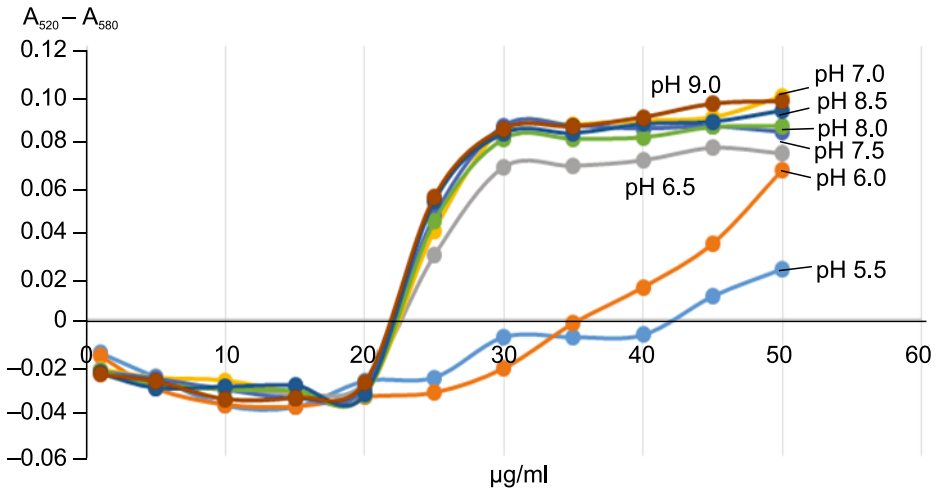

Fig. 4. Dependence between the optical absorption difference $A_{520} – A_{580}$ of the GNP-2 solution (25 nm) and the concentration of recAg at different pH values of the solution.

molecules is described in the literature [36]. Depending on the size of GNPs, the orientation of proteins on the surface of GNP may vary, which in turn, can lead to different biological activities of conjugates. Thus, the nanoparticles size can affect reactivity of adsorbed proteins. In the present study, the absorption spectra of solutions of GNP conjugates with recAg demonstrated a shift of the absorption maximum to the long-wavelength region, which was more pronounced for smaller GNPs as can be seen in Fig. 1. The efficiency of protein macromolecules' binding to the surface of the GNPs was evaluated by measuring the amount of residual protein remaining in the supernatants after conjugate samples' centrifugation [25]. Concentrations of residual protein in the supernatant liquids measured after two cycles of centrifugation averaged 2.0 and 1.0 μg/mL for GNP-1, 3.0 and 0.8 μg/mL for GNP-2, and 4.6 and 2.2 μg/mL for GNP-3. The amount of bounded recAg was 88% for GNP-1, 87% for GNP-2, 84% for GNP-3, indicating a high efficiency of the process.

The immunoreactivity of conjugates was confirmed by dot immunoassay with human serum samples containing IgG antibodies to HEV. Staining of spots with sorbed AB was observed on all membranes, with the intensity of staining decreasing slightly with serial dilutions of sorbed AB (0.1; 0.05; 0.025 and 0.0125 μg). These results confirmed reactivity of all of the obtained conjugates as well as the possibility of their application using a nitrocellulose membrane as a solid phase (**Fig. 5**). The specificity of conjugates was confirmed by the absence of staining in negative control spots (BSA).

Among the tested samples, GNP-3 conjugate with recAg demonstrated the most stable bright staining of the reaction zone in dot immunoassay. Thus, it was selected for conducting model experiments on ICA on both positive (n = 17) and negative (n = 17) blood serum samples which had been pre-tested in ELISA. It is also worth noting that ELISA commonly has a higher sensitivity compared to ICA. The «DS-IFA-ANTI-NEV-G» reagent kit («Diagnostic Systems»), one of the best kits for the diagnostics of HEV worldwide [37], was chosen as the «gold» standard.

Further, the conditions for formation of control and analytical zones of the test strip have been selected. This included varying parameters such as concentrations of sorbed AB and PAB, the speed of their application and conditions for drying the membranes. Based on the obtained results, a dilution of the conjugate with A_{520} = 4 was chosen, as it demonstrated sufficiently bright coloring of the test and control bands and there was no background staining of the membrane in reactions with blood serum samples. Increasing the AB concentration in the analytical zone (0.25, 0.5, 1 mg/mL) and PAB concentration in the control zone (0.5, 1, 2 mg/mL) resulted in an increase of staining intensity. At AB concentration of 0.5 mg/mL and PAB concentration of 2 mg/mL, the intensity of the control and test bands was sufficient for visual detection, so these concentrations were selected as optimal. It should also be mentioned that with a significant increase in the concentration of sorbed antibodies, there is a possibility of their partial desorption and migration with the flow across the membrane [26, 28].

The analytical characteristics of the obtained immunochromatographic test strips were evaluated in reactions with blood serum samples containing (n = 17) and not containing (n = 17) IgG antibodies to HEV (Fig. 5). The study demonstrated 100% sensitivity and 100% specificity of the test strips on the 34 samples that were analyzed. All results were divided into three groups according to the strength of staining of the analytical zone: «1+» (7.8–15.1; Me = 9.6); «2+» (26.7–35.1; Me = 31.0); «3+» (58.3–77.7; Me = 62.8). For the majority (73%) of the positive samples staining intensity was «2+» and «3+» making it applicable for visual detection.

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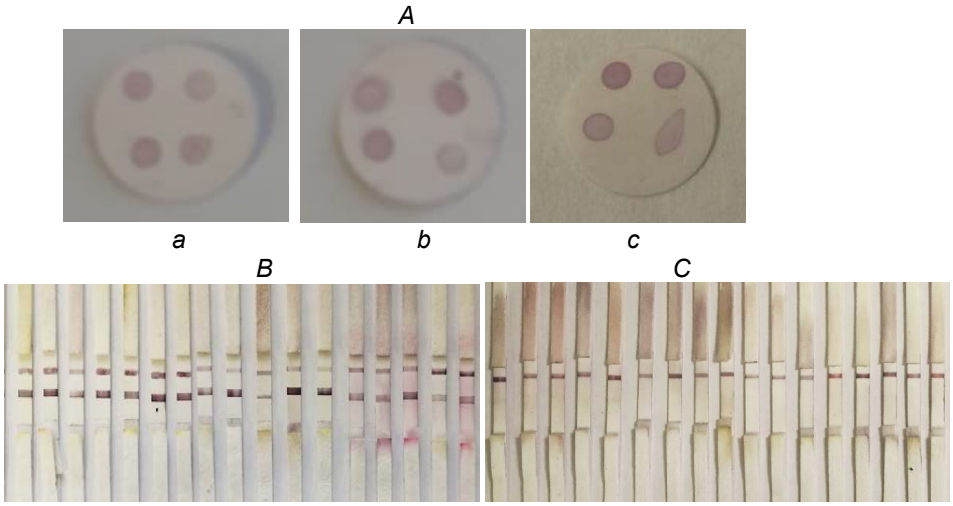

Fig. 5. Results of dot immunoassay using (*A*) GNP conjugated with recAg (*a* — GNP-1; *b* — GNP-2; *c* — GNP-3) and immunochromatographic analysis of positive (*B*) and negative (*C*) serum samples using GNP-3 conjugated with recAg.

Discussion

The principle underlying ICA is the movement of the analyte across the membrane, which results in the formation of specific immune complexes in designated areas of the membrane. These are then detected by staining. In the majority of cases, conjugates of antigens and antibodies with GNP are employed in ICA tests. The extant literature provides comprehensive coverage of methodologies for synthesizing GNP with varying particle sizes, as well as conjugating these particles with biological macromolecules [9, 12, 13]. The present study concentrated on the synthesis of GNPs of varying sizes, with the understanding that their optical properties are contingent on their size and shape. A range of GNP sizes were synthesized and their physico-chemical properties investigated. Conjugates of GNPs with HEV recAG were then obtained, and the reactivity of the obtained conjugates was evaluated by dot immunoassay and ICA.

As the diameter of the particles increased, the wavelength of maximum absorption has shifted slightly to longer wavelengths (518 and 522 and 528 nm for GNP-1, GNP-2 and GNP-3, respectively), that is consistent with the extant literature [23]. The color of the solutions changed from orange-red at the smallest size particles up to purple in a colloidal gold solution with a particle size of about 41 nm. As demonstrated by extant literature data, an augmentation in the particle size to 50 nm or more has the potential to result in a diminution of the analytical signal [32, 33]. Therefore, the present study was limited to the acquisition of three sizes of GNP: 16, 25 and 41 nm. The synthesized GNPs have sizes according to scanning electron microscopy are comparable to those calculated using the Frens method [11].

A plethora of methodologies have been proposed for the experimental estimation of the number of molecules adsorbed on the surface of a nanoparticle [35, 38] and for the calculation of the protein concentration for planting macromolecules on GNP. Synthesis of a conjugate of GNP with antigen molecules is contingent on the resolution of two principal tasks: the generation of a stable sol and the preservation of the antigenic activity of protein molecules. The synthesis conditions that have been determined as optimal in this study take into account the effect of antigen concentration and pH of the solution on the sol stability. The minimum protein concentration at which the color of the mixture did not change was considered to be stabilizing, i.e. determining the limits of sol stability. The protein shell surrounding the colloidal nanoparticles is hydrated, which ensures that after application to a porous glass fiber membrane and subsequent drying, the particles, when interacting with the sample and buffer, are returned to the solution without compromising their reactivity. Consequently, antigen conjugates with GNP of three sizes were obtained, demonstrating stability over a period of six months.

A preliminary evaluation was conducted in order to ascertain the capacity of the synthesized conjugates to interact specifically with AB. This investigation employed the dot immunoassay technique in the capture format, a method that facilitated the determination of the products' remarkable activity and suitability for utilization in assays employing nitrocellulose membranes as a solid phase. In the ICA reactions, an increase in the size of the GNP resulted in a significant increase in the analytical signal, and the brightest band in the test zone was observed when using a conjugate with a particle size of 41 nm. This confirmed an increase in the extinction coefficient of GNP solutions with an increase in particle size. The enhancement of the analytical signal upon utilization of large-diameter GNP conjugates for preparation may be attributed to the higher sensitivity of the human eye to the more contrasting purple hue.

Conclusion

During the course of this work, GNPs with three particle sizes (16, 25, and 41 nm) were obtained and characterized, and optimal conditions for preparation of GNP conjugates with recAg were determined. The resultant GNP conjugates demonstrated high efficiency and specificity of their interaction with anti-HEV IgG AB. Also, a possibility of application of GNP-3 conjugate with recAg in ICA was demonstrated.

The resulting conjugate is intended for use in the development of a test system for the rapid simultaneous detection of IgG and IgM AB to HEV. The appearance of IgG AB in the patient blood occurs a few days after the appearance of IgM AB. Anti-HEV IgM AB has been observed to persist for six to nine months. By way of contrast, serum anti-HEV IgG AB has been shown to persist for decades following the virus exposure [16]. In the early stages of the disease, the simultaneous detection of anti-HEV IgG and IgM AB is an additional tool of the diagnostic algorithm. Detection of anti-HEV IgG AB alone may indicate both acute or past infection and post-vaccination immunity. A significant area of utilization for the rapid anti-HEV IgG test pertains to epidemiological studies, particularly in scenarios where laboratory equipment is absent.

The results obtained in this study represent novel data and could be used as a foundation for the development of a new efficient ICA test for rapid diagnostics of hepatitis E.

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