Optimized immunochromatographic system for antigen determination based on monoclonal antibody conjugates with quantum dots

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Abstract. Optimized immunochromatographic system for the determination of antigens of viruses based on conjugates of monoclonal antibodies with multilayer colloidal quantum dots (QDs) as fluorescent labels was developed. The main attention was paid to the choice of the structure and synthesis of stable QDs with optimal spectral properties for analysis, to the selection of the conditions of synthesis of antibody conjugates with QDs, to the production of test strips for immunochromatographic analysis, and to the development of fluorescent reader design. It is shown that the optimal region of QDs fluorescence lies in near infrared range (700 – 800 nm). The stable QDs CdTeSe/CdS/CdZnS/ZnS and their conjugates with monoclonal antibodies for detecting Aujeszky's disease virus were synthesized, which fluorescent in this spectral region. Using these QDs, limit of detection of antigen equal to 30 pM was obtained.

1. Introduction.
Immunochromatographic analysis (IChA) is one of the most productive methods of immunodiagnostics, commonly known as lateral flow immunochromatographic assays [1]. Theory and practice of immunological analysis methods are described in detail in monograph [2] and reviews [3, 4]. The basis of the immune diagnosis is the interaction of pathological biological agents (PBA), such as antigens (Ag), or proteins - markers of disease, with an antibody (Ab) to this PBA. Immunochromatographic methods allow selectively detect specific proteins in a few minutes. In all variants of IChA the basic elements of the method are the test strips, regardless of the using approaches. Despite the apparent simplicity of the design, the test strips are the result of modern high technology, including the production of monoclonal and polyclonal antibodies to specific PBA and the use of unique porous membranes.

Researchers undertake permanent efforts to improve the sensitivity and reliability of IChA. In this work, a direct (sandwich) method of IChA using conjugate Ab-QDs deposited on a conjugate pad was investigated. Antibodies specific to the target Ag were immobilized on the test line, and anti-species antibodies specific to primary antibodies were immobilized on the control line. After the contact of
sample containing the PBA with the membrane, the PBA binds to conjugate Ab-QDs. Then the immune complex moves into test zone, where it binds to specific antibodies, forming a "sandwich" Ab-Ag-Ab-QDs. The excess of unbound conjugate binds then to anti-species antibodies of the control line. Thus, the registration of two lines on the test strip means positive result of the test.

The list of antibodies usable for the production of conjugates with markers of the analytical process and the list of determined proteins extends continuously. Therefore, the development of a new analytic platforms and technologies of IChA enabling the diagnostics of different PBA is important. The authors proposed a new analytical platform IDKRI [5], important feature of of this platform is the use of QDs as markers of monoclonal Ab. According to [6], the application of QDs as markers in IChA was described for the first time in 2010. The comparison of the sensitivities of immunochromatographic tests using identical immunological reagents showed that the limit of detection visible by naked eye was 10 times lower in the case of the application of QDsCdTe (at excitation of a UV-lamp) than in the case of gold nanoparticles [Ошибка! Закладка не определена.]. Later in [8], the detection limits of quantum dots and colloidal gold on the membrane for immunoassay analysis were compared. It was shown that the use of quantum dots allows reducing by a factor of 24 the limit of detection of chloramphenicol compared to test systems with colloidal gold. In [9] antibodies detection limits ranged from 8 to 200 pg / mL.

It should be noted that in IChA with QDs as fluorescent markers, the fluorescence of albumin blocked nitrocellulose membranes reducing the ratio signal/background is not always fully taken into account. To improve the analytical system it was necessary to optimize spectral characteristics of the QDs (that is their architecture and methods of synthesis) and design of a fluorescent reader, including the excitation source and the detector of the fluorescent signal. This activity was the purpose of the recent work.

2. Experimental

2.1. Materials

QDs CdTeSe/CdS/CdZnS/ZnS were synthesized according to the methods described in [10]. Monoclonal antibodies ADV 34/2 to gB glycoprotein Aujeszky's disease virus were obtained according to [11]. In work were used N-hydroxysuccinimide NHS (≥ 97%, Sigma-Aldrich), N,N-dimethylformamide DMFA (99.8%, Sigma-Aldrich), N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide hydrochloride EDC·HCl (Sigma Aldrich), triethylamine TEA (≥99.5%, Sigma-Aldrich), NaCl (AR, Chimmied), KCl (AR, Chimmied), Na₃HPO₄ (AR, Labtedx), KH₂PO₄ (AR, Labtedx), diethanolamine DEA (99.9%, Sigma-Aldrich), ultracentrifuge filters Amicon, tween -20 (≤3.0% water, Sigma-Aldrich).

2.2. Preparation of conjugates of quantum dots with monoclonal antibodies (MAbs)

Phosphate-saline buffer was used as the working buffer to produce conjugates: pH= 7,8 (137 mM NaCl, 2,7 mM KCl, 10 mM Na₂HPO₄ and 1.76 mM KH₂PO₄). The QDs were activated by EDC and NHS. Necessary amounts of EDC and NHS compounds are prepared in the form of 1.64% and 1.21% solutions in DMF, respectively. After 15-20 minutes of QD activation, the required amount of the antibody solution is added at permanent stirring to the QDs. The mixture is incubated for 1.5 - 2 hours at room temperature in the dark. After conjugation the solution was stabilized by the addition 1/10 of the total volume of conjugate of 10% DEA (diethanolamine) and left another 20 minutes in the dark. After stabilization, the conjugate was concentrated by ultracentrifugation in Amicon tubes at g = 16.1. The final step is the purification of conjugates from unbound antibodies using gel filtration on column with a superose 6.
2.3. **Preparation of immunochromatographic strip**

Immunochromatographic test strips were manufactured by the modified technology presented in the reference [15]. Monoclonal antibodies ADV 34/2 to the gB glycoprotein of the virus of Aujeszky's disease are immobilized on the test zone, and we used goat antibody instead of the rabbit antibody to mouse immunoglobulin as anti-species control antibodies.

2.4. **Test of the QDs conjugates with monoclonal antibodies**

The lysate PRV-k is used as antigen (hereinafter lysate). Phosphate-buffered saline with pH=7.4 is used as eluent (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.76 mM KH₂PO₄), containing 2% tween-80. Lysate is diluted in buffer in a ratio sufficient for dye the test line. The desired amount of conjugate solution is applied to the test strip. Then 50 µl of eluent with antigen is applied. When the eluent passes all analytical zones, we added 50 µl of eluent, and after 3-5 min we added additionally 50µl of buffer. After 20 minutes on completion of the elution the test result is interpreted.

2.5. **The study of fluorescence spectra of the test and control zones**

We used in the study a specially made fluorescent reader based on USB Digital Microscope EL-Micro-2 (analogue BW-400X of Chinese firm Brigh well Technology LTD) with a modified optics, fluorescence was excited by 450 nm LED with the light filter KS-1, providing fluorescence registration above 700 nm.

The RGB color-photomatrix is used in the microscope. Digitizing module processes the RGB-signals and gives the brightness in each channel in a range from 0 to 255 units. These values are divided by 255 and dimensionless quantities of signals are received in conventional units (CU) in the range of 0 - 1.

2.6. **Investigation of conjugate distribution along the test strip**

Scan along the test strip was carried out at the fluorescence excitation at 470 nm using the device, based on USB-650 Red Tide firm Ocean Optics (350-1000 nm) spectrometer.

3. **Results and discussion**

Bioconjugation QDs and Ab can be made by various methods, for example by electrostatic and hydrophobic interactions or by the formation of covalent bond. It is important to obtain strong fixation of antibodies to the QDs, to prevent the dissociation of the antibody - QDs conjugate in the immunochromatographic process. In this connection, covalent immobilization of antibodies is used in the work for the preparation of conjugates of quantum dots with an antibody [12]. For this purpose, initial QDs are modified to form on surface carboxylic groups [13]. Immobilization protein to carboxyl groups is carried out under mild conditions by carbodiimide method to form a covalent bond [14] according to the method described above.

In addition to the selection of efficient monoclonal Ab to target Ag and preparation of stable conjugates with QDs for sensitive immunochromatographic system, it is extremely important to provide optimal combination of spectral properties of QDs, considering the parasitic fluorescence of albumin blocked nitrocellulose membrane, and spectral characteristics of fluorescence reader (excitation wavelength and QD fluorescence registration). In this regard, the background luminescence of albumin blocked nitrocellulose membranes was measured; it was shown that it almost doesn’t fluoresce in the near infrared region above 700 nm. This leads us to a conclusion that it is expedient to use for IchA QDs with fluorescence in this spectral range. For this purpose QDs with required fluorescence wavelength were synthesized and their hydrophilic derivatives containing surface carboxyl groups necessary for conjugation of protein were obtained. Conjugates of monoclonal Ab with appropriate spectral properties for these modified QDs were prepared. Consequently chromatography strips for the determination of Aujeszky's disease virus (the virus glycoprotein gB) were developed. To further optimize the immunochromatographic system including reader and the strip, distribution QDs-markers along with immunochromatographic strip was investigated after completion of the process (Figure.1). The test strip scan (Figure.1) shows that the conjugates are not
adsorbed on the analytical membrane of test strip in the area of window of immunochromatographic cassette except test and control zones. Thus excessing conjugate is localized predominantly on the absorbent pad, as well as in the area, where the analytical membrane is glued to the sample and the absorbent pads.

Investigation of the fluorescence spectra of the test and control zones, and background fluorescence outside these zones at various fluorescence excitation wavelengths makes possible the determination of optimal optical characteristics of the fluorescent reader, which corresponds to excitation wavelength of 470 nm and registration above 700 nm (Figure 2).

Taking into account the results of the present study, model fluorescent reader was designed and manufactured. It includes corresponding LEDs, light filter and photomatrixes (Figure 3). Signals from photomatrixes are processed using special software on a personal computer. Obtained results are shown in Figure 4, and the resulting calibration is presented in Figure 5. There is a linear relationship (Figure 5) between the analytical fluorescent signal and concentration of antigen up to a concentration of 160 nM. This enables quantitative determination of antigens in the samples.

![Figure 1](image1.png)  **Figure 1.** Investigation of conjugate distribution along the test-strip after completion of IChA (scanning at 700 nm).

![Figure 2](image2.png)  **Figure 2.** The fluorescence spectra of the test and control lines, and background (fluorescence excitation at 470 nm).

![Figure 3](image3.png)  **Figure 3.** Model of fluorescence reader.

![Figure 4](image4.png)  **Figure 4.** Fluorescent scan of test-strips: T – the test and C – the control zone.
In addition, the study of analytical signal / background ratio for the test and control zones was conducted (Figure 6). Figure 6 shows that the optimal range of fluorescence registration for the QDs is the spectral region from 700 to 750 nm.

4. Conclusions
Optimized immunoassay system using QDs with fluorescence in near infrared region was developed. The optimal spectral characteristics of the fluorescence reader were chosen using the strips after IChA. The maximum ratio of the analytical signal / background was observed at wavelengths of 700-750 nm. Calibration curve for the quantitative determination of antigens was drown. The minimum concentration of antigen detection in the sample at this stage work is 30 pM.

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References
[1] Wong R C and Tse H Y 2009 Lateral Flow Immunoassay DOI10.1007/978-1-59745-240-3_11 (NewYork: HumanaPress) p 223
[2] Egorov A M, Osipov A P, Dzantiev B B and Gavrilov E M 1991 Theory and practice of immunoassay (Moscow: High School) p 288
[3] Piven N V 2007 Immunopathology, Allergology, Infectology 2 6-22
[4] Koivunen M E and Krogsrud R L 2006 Labmedicine 37 490-497
[5] Gladyshev P P, Vasiliev A A, Morenkov O S, Vrublevskaya V V, Tumanov Yu V, Boldyrev A N, Dezhurov S V, Kriisky D V and Ibragimova S A 2016 Papers of the LI International scientific and practical conference No. I 45 pp 22-49
[6] Gofman V V, Speranskaya E S and Goryacheva I Yu http://www.chem.msu.su/rus/books/2013/inorgworkshop/075.pdf
[7] Bai Y, Tian C, Wei X, Wang Y, Wanga D and Shi X 2012 RSC Adv 2 1778–1781
[8] Taranova N A, Berlina A N, Zherdev A V and Dzantiev B B 2011 Proceedings of the 2nd International School "Nanomaterials and nanotechnology in living systems. Safety and nanomedicine " 102
[9] Dzantiev B B 2014 Russian Academy of Sciences Department of Chemistry and Materials Science, report of SCAC RAS 2013
[10] Dezhurov S V, Trifonov A Yu, Lovyginc M V, Rybakova A V and Krylsky D V 2016 Nanotechnologies in Russia 11 337–343
[11] Zaripov M M, Morenkov O S, Sikloki B, Barna-Vetro I, Gyinjyisi-Horvath A and Fodor I
1998 *Res. Virol.* **149** 29-41

[12] Gao X, Yang L and Petros J 2005 *Biochem. Biophys. Res. Commun.* **16** 63–72

[13] Loginova Y F, Dezhurov S V, Zherdeva V V, Kazachkina N I, Wakstein M S and Savitsky A P 2012 *Biochemical and Biophysical Research Communications* **419** 54–59

[14] Wang J, Huang X, Zan F, Guo C-G, Cao C and Ren J. 2012 *Electrophoresis* **33** 1987-1995

[15] Vrublevskaya V V, Afanasyev V N, Grinevich A A, Skarga Yu Y, Gladyshev P P, Ibragimova S A, Krylsky D V and Morenkov O S 2017 *Journal of Virological Methods* **240** 54–62