Application of the coherent spectroscopy method for the study of biosensor test systems based on nanomarkers

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Abstract. A new class of fluorescent markers, nanocomposites with a high quantum yield, which appeared in the last decade, opens up broad prospects for their use in medicine, in particular, to create on their basis highly sensitive biosensor test systems. It is of interest to use these markers as luminescent sources when conducting immunoenzymatic analysis for specific metabolites. We made a comparison between the optical characteristics of single-walled carbon nanotubes and lanthanide porphyrins. Based on the method that we developed earlier using nonlinear optical effects, we developed recommendations for using functionalized single-layer carbon nanotubes as a fluorescent marker for immunoreactions with high signal characteristics noise in the region of 800 nm and to determine the stability of nanomarkers.

1. Introduction
In the last decade, highly effective medical monitoring devices are under development. The use of fluorescent registration systems and fluorescent immunoassay is one of the most promising and actively developing methods for these devices. When developing these methods it is especially important to obtain simple biosensors for the dangerous infections, for narcotic and toxic substances. The new class of fluorescent markers, nano-composites with a high quantum yield, which have been developed in the last decade, opens the wide prospects for their use in medicine, and in particular for the creation on their basis of highly sensitive biosensor test systems. It is of interest to use these markers as luminescent sources in conducting immunoenzymatic analysis.
At the present time, there are non-invasive methods for monitoring cancer markers in the urine by using chromatography and the presence of specific metabolites. [1]. However, these methods take a long time (3-4 hours), and chromatograms are very difficult to analyze. [1]. The development of the modern technologies allows for the use of luminescent nanostructures in the infrared range as the nanomarkers for immunoenzymatic analysis of these specific metabolites. This will simplifies the process of measurements and will improves their accuracy. In this paper, we developed recommendations for using nanostructures as a fluorescent marker for immunoreaction with high signal-to-noise characteristics and for determining the stability of nanomarkers. We investigated two types of markers: The first is based on Langmuir Blogett Films, and the second one - on the nanotubes.
2. Materials and methods

2.1. Creation of model of immunoreaction with fluorescent detection on the basis of various nanocomposite markers and investigation of its sensitivity

Use of systems with fluorescent registration and fluorescent immunoassay is one of the most promising and actively developing methods. In this regard, it is urgent to search for more effective markers and methods for recording a fluorescent signal. The main limitation of already available immunosensors is a large background fluorescence of proteins and other biological objects. This is a cause of large measurement errors and it is primarily due to the non-specific binding of biological analytes to the polar surface of the substrate.

It is advisable to use the range of radiation, where the medium is experiencing minimum absorption and, as a result, the fluorescence phenomenon is realized only for certain parameters, which are of interest to us. For the media having a protein component that wavelength band is the far red and the near infrared range (IR) close to it, where the shape of the protein molecule stop affecting parameters of the radiation. Auto-fluorescence of proteins in the visible region is one of the main factors that reduces the sensitivity in the fluorescent immunoassay. In the far red and near IR (920-1200 nm) spectral regions, background fluorescence of proteins is practically non-existent. The criterion of the effectiveness of the sensory element is the ratio of the useful signal (the fluorescence intensity of the marker) to the background signal. It is known that in quantum-size structures with a high quantum yield, the ratio of the useful signal to noise can increase by almost two orders of magnitude. [2]. Therefore, we investigated the optical properties of markers for Enzyme Linked Immunosorbent Assay analysis (ELISA) on the basis of Langmuir Blodgett films and on the basis of carbon nanotubes.

2.2. Langmuir Blodgett Films

We investigated the use of Langmuir films as an immunosensors. The main method of manufacturing the Langmuir-Blodgett films is the molecular layering method. The main idea of the MN method used for the review of synthesis of solid bodies with a regular structure is the sequential buildup of monolayers of structural units with a given chemical composition on the surface of a solid phase matrix. Thus, the MN method refers to the so-called matrix synthesis. Lanthanide complexes (LnTPP) were utilized as a marker having fluorescence in the infrared spectrum range, and they were synthesized on the basis of the technique published in [3]. As a immune pair for modeling) were selected, murine antibodies (IgG) (Sigma, USA), specific for the bovine serum albumin BSA (Sigma, USA).

The monolayer of antibodies was applied in a mixture with L-a-phosphatidylcholine dipalmitoyl. The stability of a monolayer of protein-lipid complexes in an aqueous medium is much higher than the stability of a monolayer containing only a protein. A distinctive feature of that technique is the modification of the substrate by the application of Langmuir-Blodgett fatty acid films. That results in smoothing of the microdefects of the substrate and in the surface modification for the formation of polar and nonpolar surfaces. The formation of a nonpolar surface makes allow for a significant reduction of the nonspecific binding of the antigens.

The preparation of immuno-sensitive systems was carried out according to the standard method widely used in the enzyme immunoassay analysis. Quartz plates (35 x 10 x 1 mm) were used as substrates on which layers of stearic acid were preliminarily applied by the Langmuir-Blodgett method. After applying a protein-lipid monolayer or a monolayer of the LnTPP-SA mixture, a were obtained, respectively a non-polar (hydrophilic) or polar (hydrophobic) surfaces. Protein-lipid monolayers and monolayers of LnTPP -SA were transferred onto substrates by a sensor method. Solutions were prepared in chloroform in the ratio LnTPP: SA — 1: 1 (initial concentrations of 10 ~ 3 M).

2.3. Single-wall carbon nanotubes (CNT) of fluorophore with a high quantum yield in the IR spectral region.
Currently, CNTs are increasingly used as a marker. The simplicity of the interaction of CNT with various substances made allows for obtaining a wide range of functionalized CNTs. The preparation of conjugates of CNT with streptavidin, biotin, and antibodies allows for using of CNTs as a fluorescent label in conducting an enzyme immunoassay using numerous already existing techniques. They are significantly increasing the resolution of the enzyme immunoassay. [4]

As a material, the carbon nanotubes are peculiar cylindrical molecules with a diameter of about half a nanometer and the length of up to several micrometers. Design of the electronic devices and their integration into the complex systems, requires semiconductors and materials with high electrical conductivity. Nanotubes with different values of (n, m) indices are polymers of different structure. And therefore they must have different electrical properties. The relationship of the electrical properties of nanotubes and their geometric parameters were predicted based on quantum chemical calculations of their band structure. It was noted that all carbon atoms in nanotubes have triple coordination, which means that nanotubes are conjugated aromatic systems.

The model immune antigen-antibody was selected. Bovine serum albumin (BSA) (Sigma-Aldrich, USA) was selected as an antigen, and a single-walled carbon nanotubes (Sigma-Aldrich, USA) immobilized on their surface was selected as a fluorescent marker for the mouse antibodies specific for BSA (Sigma-Aldrich, USA).

The surfactant is dissolved in a volatile solvent (chloroform, dichloromethane, benzene) and applied dropwise to the surface of a specially purified bi-distilled water or a sub-phase (a buffer system) prepared on the basis of this water: 5 ml of water was added to a mixture of 1mg of functionalized single-walled carbon nanotubes (SWCNTs) and 50 μl of mice immunoglobulin (IgG) at a concentration of 8.5 mg / ml, and sonicated for 30 minutes on an ultrasonic disintegrator Soniprep 150 for creation of stable compounds of nanocomposite markers-SWCNT and porphyrin-lantonoid nanoclusters with proteins, in particular with immunoglobulin G. After that, the mixture was aged for 4 days at +4°C.

The antigen was diluted in the saline solutions of various concentrations and subsequently added to the saline solution containing the antibody-nanotube complexes. The solution was spread over the surface, and the solvent evaporated. The remaining surfactant has been compressed by a mechanical barrier until a continuous film is obtained. The moment of formation of the liquid-crystalline phase state of the monolayer is registered by the Wilhelmi scales, and then the monolayers are sequentially transferred to a solid substrate. The antigenic material was prepared of the following concentrations: 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} mol / l. These concentrations were selected for studying the sensitivity of the proposed method in the area of limited sensitivity of existing immunological diagnostic methods. In particular, for Enzyme Immunoassay (ELISA), the sensitivity limit is in the region of 10^{-9}mol / l.

3. Experiment

We investigated various techniques of enzyme-linked immunosorbent assay with fluorescent detection for carrying out a wide range of immunological reactions such as direct immunoassay.

The objectives of our research were as follows:

to selection of the wavelength range of the fluorescence, with the optimal ratio of the useful signal to background noise;

development of the method for controlling the dependence of optical characteristics on the concentration of markers in a matrix solution;

investigation of the stability of the characteristics of single-walled nanotubes with wavelengths of radiation in the near-IR range,

investigation of the dependence of optical characteristics on antigen concentration,

investigation of the optical characteristics of markers based on the lanthanide complex of tetraphenylporphyrin LNTPP, which has a fluorescence in the infrared range.

We also conducted experimental studies for comparing the optical characteristics Langmuir Blodgett Films and single-walled carbon nanotubes (SWCNTs) using two methods: the backscattering method, and...
the Brillouin stimulated scattering method. For obvious reasons the main informative indicator of the stability of the system is the function of the source intensity versus wavelength.

![Experimental setup diagram](image)

**Figure 1.** The scheme of the experimental setup for the study of optical characteristics. 
*Object 1 - laser, 2 - sample under study, 3 - lens, 4 - diaphragm with adjustable orifice diameter, 5 - radiation receiver.*

![Graph](image)

**Figure 2.** The relative intensity of luminescence of film samples (1-5 number sample) systems mixture LnTPP-SA from the wavelength.

Plates with monolayers of the LnTPP-SA mixture were placed in a cell of an optical spectrum analyzer Agilent N1031A using the direct scattering method (Figure 1). The fluorescence intensity was measured in the range from 900 to 1100 nm in order to evaluate stability and reproducibility of the signal. Dependence of the relative intensity of film samples systems mixture LnTPP-SA from the wavelength shows (figure 2.(1-5)).

To study the stability of the SWCN as a fluorescent marker for immunological reactions, an inverse scattering method was selected. That was necessary because the direct scattering method for measuring the characteristics of a nanotube does not provide the required accuracy. The reason for this
is that the object of the present study is a solution with a concentration of diffusers from $10^{-6}$ to $10^{-10}$ mol / l. Thus, optical object is located in the so-called “turbid solution”, which consists of a polymer matrix or water and of two types of diffusers: a nanotube, and a functional group of the marker under study. Therefore, it became necessary to modify the optical method for achieving not only monitoring of the stability and reproducibility of the characteristics of the emitter, but also determining the possibility of obtaining a reliable information in analyzing the fluorescent radiation of the markers under study.

To determine the stability of the nanocomposite markers intensity, the modified method of backscattering of radiation was developed. It allowed to identify account the measurement errors in the calculations.

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**Figure 3.** 1 – lasers (a) and photodetectors (b ), 2 – test sample, 3 scattering element - a system of translucent mirrors, 4 – electronic control and information processing, 5 – the computer

The backscattering method is most useful in the region of low intensities, where it is possible to use a number of the most sensitive research methods, which do not require strong light fluxes and, therefore, do not distort the measurement. The developed method must meet the following requirements:

- Firstly, it must be non-destructive, the radiation intensity incident on the sample should not cause additional optical effects in it, the optical range in which the sample is controlled must lie in the same range, where the sample emits and fluoresces.
- Secondly, it must be an express diagnostic method.
- The accuracy of the method should be sufficient to control radiation fluctuations.

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**Figure 4** Typical spectrum of the intensity for concentration $10^{-8}$ (◆) mol / l and dependence of the maximum of the radiation intensity $10^{-6}$ (■) mol / l
Depending on the complexity of a studied medium and on a set of informative parameters, all modern optical control methods can be conditionally classified as follows:

1. The calibration method, where special techniques are used for selecting the frequency range from the response, associated with the radiation of our component. The intensity at the maximum of this spectral distribution is smooth, unambiguous, in a first approximation, linear function of optical density (the decimal logarithm of concentration).

2. The reference method - where the spectral distribution of the sample is compared with the spectral distribution of the previously recorded standard, and in comparison of their the main parameters (intensity gradient, width of the spectrum, etc.). The quantitative characteristics of the sample are also determined.

3. Resonance methods: where the characteristics of the electromagnetic fields of optical radiation of the sample and the standard are compared directly. In this case, the phase, the degree of coherence and polarization of the sample under study are added as informative parameters. Based on the above, one can conclude that the fundamental implementation of the method of determination of the antigen requires a sufficient number of informative parameters in the spectral characteristics of the response at various degrees of its concentration.

It was also necessary to select the method for studying the sensitivity of model immunoreaction with fluorescence detection based on various nano-composite markers. The method we developed for the investigation of the fluorescent response of the Antigen-Antibody-Nanotube system was based on the Brillouin stimulated scattering phenomenon (SBS) [6]. As it follows from the description of the method, the optical range of the excited radiation does not necessarily coincide with the optical range of the standard fluorescent method for this type of markers. And this is because completely different radiation levels are excited in this method. However, the spectral distributions of the response are strictly individual. And when a sufficient number of informative parameters are extracted, the method yields the quantitative information having sufficient accuracy. We consider a zone with no absorption and scattering occurs only in a skin layer being close to the surface. As has been demonstrated by calculations and tests, the accuracy of recording of a signal associated with an ensemble of diffusers of a certain type was observed at angles between the source and receiver, 15 degrees of the intensity distribution over the scattering angle. In order to control the timing of the effect and to select the of optimal concentrations of the antigen, the transmission spectra of the antigen-antibody-marker system in saline were examined at the following antigen concentrations: 0, 10⁻¹⁰, 10⁻⁸, 10⁻⁶ mol/l, and at the concentration of the active marker of 0.085 mg / ml. The logarithmic distributions of spectral intensity, (dbm), are displayed on Figure 5 - 7 in order of increasing concentrations (10⁻¹⁰, 10⁻⁸, 10⁻⁶ mol/l)
Figure 5. The Antigen-Antibody-Nanotube intensity ($10^{-10}$ mol/l)

Figure 6. The Antigen-Antibody-Nanotube intensity ($10^{-8}$ mol/l)

Figure 7. The Antigen-Antibody-Nanotube (10$^{-8}$ mol/l)

Figure 8. Logarithm of the maximum intensity Stokes component intensity as the function of the antigen concentration.
3. Discussion
The distribution of the luminescence intensity with respect to the wavelength for different samples of Langmuir films (Figure 1) shows that this parameter strongly depends on the sample. Most likely the reason for this is that the main idea of the above described method, which was used for the for synthesis of solid bodies of a regular structure consistent in build up of monolayers of structural units of a given chemical composition on the surface of a solid phase. Thus, the method refers to the so-called matrix synthesis. The implementation of this process, requires to comply with the following main principles:

1. Synthesis is based on irreversible under experimental conditions chemical reactions between functional groups on the surface of a solid and molecules of a reagent supplied from outside. In this case, the reagents and the products of reaction should not enter into chemical interactions with each other.

2. For the gradual build-up of a layer of a new substance, it is necessary to carry out repeated and alternate (in a given sequence) treatment of the latter with pairs of corresponding compounds. At the same time, each newly formed monolayer of new functional groups must contain active atoms or groups of atoms capable of reacting with a new portion of the same or another reagent.

3. To carry out reactions in the process, some structural correspondence is required between the surface of the initial solid-phase matrix and this compound. But the main thing is the presence on the surface, both initial and formed during the synthesis, of a sufficient number of functional groups (FG). They need to have a mutual arrangement, which makes it possible for cross-bonds between the attached atoms to form a three-dimensional lattice of the synthesized solid. However, in the case the third condition is often violated different types of noise appear. This is happening due to defects in the matrix material, which is a polymer, and its general structure is similar in properties to the system of nanocomposite formations in a colloidal state, wherein each functional group represents an individual source of radiation. For Langmuir films, the most typical are the errors of a quantum dot, the instability of a colloidal solution, the distribution of the ensemble of scatterers in size and shape, and the incoherence of radiation. These reasons most likely lead to the instability of results. The best results were obtained on tubes with wavelength of 0.8 and 1.1 μm. The results of measurements on 10 samples showed that tubes at a wavelength of 0.8 μm proved of being the most stable. Statistical processing of the experimental data using the least squares method (Figure 3) showed that the contribution of the emitter error for these products does not exceed 3%, which allows for using them as emitters for the fluorescent immunoassay markers.

Logarithmic distributions of spectral intensity of the Antigen-Antibody-Nanotube system, (dbm), are shown in order of increasing concentrations $10^{-10}$, $10^{-8}$, $10^{-6}$ mole/l. The Stokes component of the intensity increases with increasing concentration (Figure 5 -7). Thus, the logarithm of the maximum intensity has almost linear relationship with the concentration (Figure 8). The corresponding maximums of the logarithm of intensity as a function of a concentration of antigen are distributed as follows (Tab.1).

| №№ | 0     | 1     | 2     | 3     |
|-----|-------|-------|-------|-------|
| C, lg M/l | 0     | -10   | -8    | -6    |
| I, dbm  | -44,994 | -0,792 | 0,274 | 1,832 |
| Λ, nm  | 810   | 807   | 807   | 807   |

Presence of a single maximum allows the use of various methods of processing spectral characteristics in order of improving the accuracy of the method. However, it should not be assumed that the linearity of the logarithm of intensity can provide
the basis for the conventional calibration method. With the increasing complexity of the system, in using the SBS method, one has to introduce additional informative parameters, as described in [7]. That an approach may yield more reliable results. Ultimately, the method of processing optical information is more dependent on the specific capabilities of the antigen and the equipment. It has been demonstrated that several lines of fluorescence in functional groups of different sizes, can be excited simultaneously. Films with monolayers of the LnTPP-SA mixture were also investigated by the resonance method. Figure 9 shows the spectral distribution of the radiation monolayers of the LnTPP-SA mixture response for exciting by a laser with a wavelength of 1017 nm. One can see that several lines the fluorescence of functional groups of different sizes are excited simultaneously. Selection of informative parameters on the antigen concentration of such spectra is practically impossible using standard methods for processing spectral characteristics.

![Figure 9](image)

**Figure 9** The spectral distribution of the radiation monolayers of the LnTPP-SA mixture

4. Conclusion

1. Analysis of the spectral characteristics of fluorescence of various biological media was carried out, which demonstrated that the optimal range for recording radiation based on the signal-to-noise ratio, is the region from 750 nm to 1200 nm. In this optical range, the intrinsic fluorescence for the overwhelming majority of organic compounds — proteins, lipids, nucleic acids is non-existent.

2. To determine the stability of nanocomposite markers, a modified backscatter method was developed, which allowed detecting and taking into account the measurement errors. The functionalized single-wall carbon nanotubes can be recommended as a fluorescent marker for immunoreactions with high signal-to-noise characteristics in the region 800 nm.

3. The stable compounds of nanocomposite markers — SWCNTs and porphyrin-lanthanoid nanoclusters with proteins, in particular with immunoglobulins G, were obtained for carrying out a wide range of immunological reactions, such as direct immunoassay, and various versions of immunoassay with fluorescence detection.
4. A comparison the optical characteristics of SWCNTs and porphyrin-lanthanoid nanoclusters demonstrated a high fluorescence stability of the SWCNTs that did not depend on the phase, film or liquid, of the studied medium. In the study of porphyrin-lanthanoid nanoclusters, a high instability of the fluorescence spectrum in the presence of water was detected. The applied method of molecular layering of Langmuir-Blodgett allowed for the increase the quantum yield. However, the influence of a large number of molecular layers, the uneven distribution of the fluorescent marker further worsened the spectral distribution of fluorescence. The complexity of the molecular layering method and requirement for the expensive equipment does not allow to fully utilize this method in the immunoassay. Therefore the use of the fluorescent nanomarker based on single-walled carbon nanotubes is preferable.

5. The study of the sensitivity of the anti BSA-antibody-SWCNT + BSA system, demonstrated practically linear relationship between the maximum fluorescence and the concentration of the antigen being introduced. The concentrations of antigen $10^{-6}$, $10^{-8}$, $10^{-10}$ mol / l, taken for the model experiment, make it possible to predict the sensitivity of an immunoreaction with this type of marker at a level that exceeds the sensitivity of existing methods of immunoassay, and, when using in analysis additional mathematical methods for processing spectra, even higher accuracy is achievable.

5. References

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