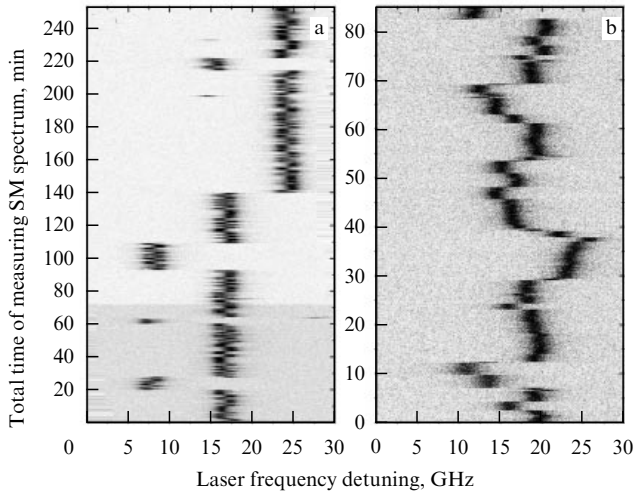

Figure 6. Spectral trails of single TBT molecules introduced into amorphous PIB (a) and a frozen solution of toluene (b), measured at $T = 7$ K (according to the data in [38]).

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Optical biosensors of genotoxicants based on DNA nanoconstructions and portable dichrometers

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1. Introduction

In this report, we briefly review results obtained in the last five years in the field of the development and practical use of biosensor methods and devices for the rapid determination of genotoxicants in liquids. Special attention is given to the nanobiotechnological approach developed in the Engelhardt Institute of Molecular Biology (IMB), Russian Academy of Sciences, with the instrumental support of the Institute of Spectroscopy, Russian Academy of Sciences (ISAN), which suggests the use, as integral biosensing units, of nanoconstructions that consist of double-stranded DNA molecules immobilized in optically transparent isotropic hydrogels having the property of abnormal optical activity, and of portable dichrometers as the recorders and transducers of circular dichroism.

Progressively increasing anthropogenic environmental pollution dictates the need for developing methods and devices for medical and ecological diagnostics, and also methods for controlling the quality of food products and medicinal preparations to ensure highly sensitive and rapid determination of the presence in them of toxicants that are hazardous to health and whose 'target' is the genetic material of cells. Such toxicants include some antibiotics and other

medicines, heavy metals, pesticides, dioxins, proteins, and other biologically active compounds (BACs), which are always present in physiological liquids (blood, blood plasma, urine, water, etc.).

For the determination of the overwhelming majority of biologically active and toxic compounds, traditional analytical techniques can be used, such as chemical analysis, as well as numerous methods with the application of biochemical analyzers, liquid and gas chromatographs, mass spectrometers, and other analytical instrumentation. An alternative to traditional methods is the so-called biosensor methods of analysis, which use sensing elements (biosensors) that have a specific sensitivity to the compounds to be determined, in combination with various transducers and converters. Biosensor technologies are by no means necessarily better than nonbiosensor methods, but an intimate combination of the production of a signal and its detection and the possibility of the miniaturization of equipment are opening newer and newer areas of measurements. In medicine, this can be the use of medical monitoring in situ, directly near a patient; in pharmacology and the food industry, rapid (online) control of the quality of pharmaceutical preparations and food products; in the biotechnological industry, the control and optimization of technological processes; and in environmental monitoring, the immediate detection of toxic substances, without the transfer of samples into the laboratory.

A promising trend in the solution to these problems that is very important for the protection of human life and health is the nanobiotechnological approach rapidly developed in the last few years, which implies the use of 'structured' biomaterials, devices, and systems with properties that are connected with the geometrical dimensions or specific physicochemical features of nanostructures, such as molecular constructions based on DNA [1]. Nanoconstructing on the basis of double-stranded DNA (dsDNA) molecules represents a purposeful creation of three-dimensional constructions (nanostructures, nanoconstructions (NaCs), nanobiomaterials), whose 'building blocks' are dsDNA molecules or their complexes [2]. The most important problem with such nanoconstructing is the creation of three-dimensional constructions with controlled properties, which contain built-in molecules of various compounds ('guests'), which can be targets for BACs, i.e., the creation of biosensors on the basis of NaCs for the determination of BACs that recognize guest molecules [3 – 5].

In the literature, several approaches to the creation of biosensors on the basis of NaCs of nucleic acids have been described. These approaches can be conditionally divided into two groups. Following one hybridization strategy — the strategy of sequential construction [6] — the nanostructures are formed consecutively, using single dsDNA molecules as building blocks, which entails large expenditures due to the need to obtain fragments of DNA with the necessary sequences of nitrogen bases and an entire 'arsenal' of ferments for splitting and 'sewing' DNA fragments, the separation of specific structures from the reaction mixture, and the use of modern methods of control (such as atomic force microscopy) at all stages of nanoconstructing. For this reason, the problem of the practical application of NaCs on the basis of single dsDNA molecules remains unsolved to a considerable degree.

Another, fundamentally different, strategy of nanoconstructing, which was developed at IMB RAS [7], is based not on the use of single dsDNA molecules (or complexes of dsDNA molecules) but on the use of ordered spatial structures spontaneously arising upon the 'phase exclusion' (condensation) of these molecules from aqueous salt solutions of polymers. As a result of phase exclusion, rigid dsDNA molecules with a low molecular weight ($< 10^6$ Da) become ordered and form particles ~ 0.5 μm on size, which are characterized by a liquid-crystalline mode of packing of adjacent DNA molecules into layers with an approximately parallel orientation of molecules in each layer and a change (twist) in this orientation in passing to other layers [8].

The transition into an ordered cholesteric liquid-crystalline state is accompanied by the appearance of an abnormal band in the spectrum of circular dichroism (CD) located in the absorption area of the nitrogen bases of DNA ($\lambda \sim 270$ nm). The liquid-crystal state does not disrupt the reactivity of the molecules, i.e., their capacity for molecular 'recognition' and specific addressing of chemical substances and BACs. For example, the interaction of colored antitumor antibiotics with the DNA molecules that form the particles of cholesteric liquid-crystal dispersions of DNA (DNA CLCDs) is accompanied by the appearance of an additional abnormal band in the CD spectrum in the absorption region of these compounds. The abnormal CD signal allows following even the smallest changes in the properties of the dsDNA molecules, i.e., the DNA CLCD particles act as miniature optical biosensing units, which change their characteristics in response to the action of a BAC from the liquid being investigated. The spectral features of this signal (the sign, height, and position of the maximum) recorded by a portable CD spectrometer (dichrometer) are used as an analytical criterion that allows not only determining the presence and concentration of a BAC in the sample analyzed but also establishing the method of its interaction with the dsDNA molecules. A liquid sample to be probed (of a 'test-tube' form) is prepared by mixing the solution of the probed biological liquid in the polymer with a solution of the dsDNA in the polymer, i.e., with the dsDNA CLCD (biosensor).

2. Biosensor analytical system

The practical problem of determining BACs with the help of biosensing units on the basis of DNA CLCDs was solved together with the Institute of Spectroscopy RAS (ISAN), which developed a prototype of a portable polyfunctional dichrometer (SKD-2) and manufactured first samples of these analyzers for the operating range 250 – 750 nm [9]. In 2004, the Experimental Plant of Scientific Instrumentation (EZNP), Russian Academy of Sciences (Chernogolovka, Moscow region), based on the documentation of ISAN, manufactured a small batch of ten SKD-2 dichrometers (No. 26900-04 in the Federal Agency on Technical Regulation and Metrology on the Manufacturing and Repair of Means of Measurement). Although the overall dimensions of the dichrometer were three times less than those of the commercial dichrometers of well-known firms and the weight was 5 – 7 times less, the detecting ability of the SKD-2 portable dichrometer ($\sim 10^{-6}\,\Delta A/A$, where A is the absorption in the sample) proved to be 2 – 3 times better. A biosensor analytical system based on DNA biosensing units and the SKD-2 dichrometer has no analogs in the world as regards its operating principle; it is characterized by a high sensitivity (10^{-7} to 10^{-14} M l^{-1}), low operational expenditures (1.5 dollars per hour), low prime cost of a single test (0.5 dollars), the possibility of conducting straight rapid analysis of liquids containing BACs, and in all these

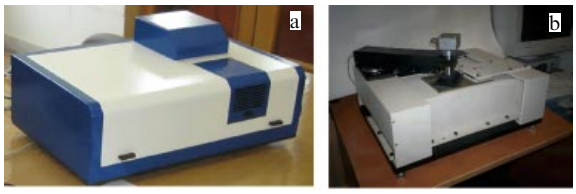

Figure 1. Polyfunctional portable dichrometers: (a) SKD-2M and (b) SKD-3.

characteristics it considerably exceeds the foreign analytical systems of analogous designation. The novelty of the development is confirmed by patents by Russia, the USA, the EU, Germany, and Japan, the gold medals at the 50th World Exhibition of Innovation (Brussels, Eureka, 2001) and of the Presidium of the Russian Academy of Sciences (2002), as well as the Grand-Prix and Prize for Victory in the 2nd Competition of Russian Innovations (2003).

In 2005 – 2007, the SKD-2 dichrometer was modernized with the purpose of expanding the range of working wavelengths to 200 nm and increasing the reliability of operation on the whole. In the improved version of the device (SKD-2M, Fig. 1), a virtually ozone-free temperature regime of the illuminator was realized with a twofold increase in the output of ultraviolet (UV) emission near $\lambda = 200$ nm; a higher stability of the modulator of the circular polarization with respect to external actions; a decrease in the value of the residual CD signal caused by stresses in the windows of the cuvette; an increase in the accuracy of installation of the sample temperature; a decrease in the overall dimensions of the device; the use of modern components in the electronic units and functional modules; the possibility of connecting both external and built-in computers through a USB interface.

In the same period, the developed bioanalytical system was used at IMB RAS to demonstrate the possibility of detection in physiological liquids of a large number (> 50) of compounds that enter into antitumor compounds, a number of polyaminoacids, polypeptides and proteins, cellular metabolites, organophosphorus compounds, and ascorbic acid, and a number of phytogenous genotoxicants.

3. Biosensing units based on DNA nanoconstructions

The significant distance between DNA molecules (from 2.5 to 5.0 nm), the liquid-like nature of the packing of these molecules, and their high concentration (~ 400 mg ml^{-1}) in quasinematic layers of particles of their CLCDs provide conditions for the rapid diffusion of molecules of many compounds both between DNA molecules in the same layer and between DNA molecules in adjacent layers of such particles. In view of the preservation of reactivity of the DNA molecules in the structure of CLCD particles, the genotoxicants from physiological liquids can easily penetrate inside such particles and modify the secondary structure of the dsDNA molecules or intercalate between the pairs of dsDNA bases without damaging the character of ordering of the molecules. In addition, the new chemical groups on the surface of the DNA molecules offer the possibility of forming 'bridges' between adjacent DNA molecules.

For the formation of the bridges, it is necessary that, places where they 'begin' and 'end' must exist on the surface of the DNA molecules (the ions of metal connected with a nitrogen base or the molecules of a ligand additionally introduced into the system, for instance, can serve as such places). In view of the spatial arrangement of reactive groups (in particular, N-7 nitrogen atoms of the purine bases) in the spatial structure of the DNA molecules, the connection between two adjacent DNA molecules is possible only if the spatial orientation of these molecules is coordinated, i.e., if a kind of 'phasing' of the positions of the adjacent DNA molecules is provided. This means that the formation of nanobridges is a delicate process, which is realized only if several conditions are satisfied. This problem was solved by the creation of nanobridges [7] from alternating molecules of an anthracycline antibiotic and copper ions, which connect adjacent DNA molecules in each of the quasinematic layers and DNA molecules of the adjacent layers of CLCD particles. This led to the appearance of a rigid spatial structure of such particles (Fig. 2b), which were called nanoconstructions, and to dramatic changes in their properties.

The basic factor in the stabilization of NaCs is now the number and 'strength' of nanobridges rather than the osmotic pressure of the aqueous solution, in contrast to the initial CLCD particles. The liquid-crystalline nature of the packing of the adjacent DNA molecules in NaCs and the diffusion mobility of the DNA molecules disappear, and the particle acquires the properties of a solid material (Fig. 2b). In the composition of an NaC, not only is a high local concentration of DNA molecules preserved but also a high concentration of the antitumor antibiotic daunomycin (DAU) appears.

Characteristic for a DNA NaC are both abnormal optical activity, which manifests itself in the appearance of an intense band in the CD spectrum in the absorption region of the DNA (~ 270 nm), and additional anomalous optical activity in the absorption region of the antibiotic chromophores (~ 520 nm). The abnormal optical activity allows controlling the change in the secondary structure of the initial DNA molecules, the appearance in the NaC structure of molecules that form nanobridges, and the integrity of the nanobridges themselves. The decrease in the activity (up to its complete disappearance), which accompanies the destruction of nano-

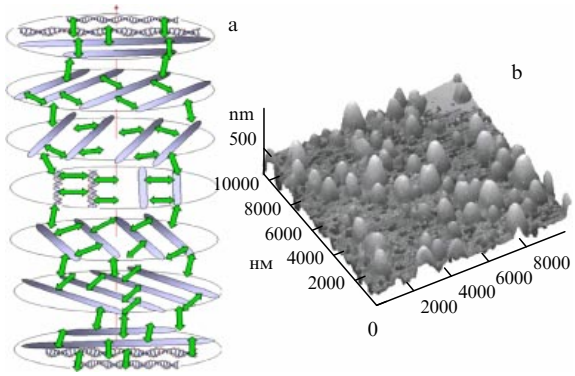

Figure 2. (a) Hypothetical scheme of a three-dimensional rigid nanoconstruction based on dsDNA molecules fixed in the spatial structure of a CLCD particle and 'sewn' by nanobridges. The DNA molecules in adjacent layers are shown in the form of rods; each subsequent layer is turned through a certain angle with respect to the preceding layer; arrows show nanobridges. (b) A three-dimensional scheme of NaCs immobilized on the surface of a nuclear membrane filter.

bridges under the action of genotoxicants (BACs), can under certain conditions be directly connected with the concentration of the agent that destroys the nanobridge; this gives the possibility of using DNA NaCs as optical biosensors for the detection (in biological tests) of the presence and concentration of BACs whose 'targets' are the nanobridges, which are, in fact, nanosensors. The optical signal generated by such biosensors can easily be recorded using a portable dichrometer.

Based on the determination of model substances, in particular, ascorbic acid [10] and bovine serum albumin (BSA) [11], which change the valence state of Cu^{2+} ions or 'extract' them from the composition of the nanobridge, the respective limits of detection of analytes at a level of 10^{-7} to 10^{-8} M were achieved, which are comparable with the limits of their determination by classical chemical (biochemical) methods.

Biosensors of this type were also used also for the detection and selection of phytogenic pharmaceutical substances, which have clearly pronounced complex-generating properties with respect to Cu^{2+} ions. When a biosensor is treated with hiporamin (an antiviral and antimicrobial preparation of the All-Russia Research Institute of Medicinal and Aromatic Plants (VILAR), Russian Academy of Agricultural Sciences (RASKhN)), the destruction of nanobridges and, correspondingly, the decrease in the anomalous optical activity (Fig. 3) is also caused by the 'extraction' of Cu^{2+} ions from the nanobridge and by the formation of a more stable complex between hiporamin and Cu^{2+} ions as a result of secondary complex generation. It was also shown that at a fixed time of treatment of NaC by hiporamin (10 min), the amplitude of the band in the CD spectrum of DNA NaC is directly proportional to the concentration of a phytopreparation (in the range up to 4 μg ml^{-1}); this linear dependence can be used as a calibration line when determining low ($\sim$ 0.5 μg ml^{-1}) concentrations of hiporamin in the sample analyzed. Analogous data were obtained for donelvin, hamenerin, chanerol, and eucalyminum [12].

Thus, the development of integral biosensors on the basis of NaCs with a sufficiently long-time stability of optical properties, which are independent of the osmotic pressure of the aqueous solution, has substantially increased the number of BACs that can be detected in liquids with the aid of the biosensor technology proposed.

With the development of the technology of obtaining DNA nanoconstructions, which can include a large fraction of guest molecules, a new type of biomaterial with controllable properties has, in essence, been created. This technology is not yet completed and can be improved by selecting other components of nanobridges or the DNA molecules themselves, including their complexes with various polymers.

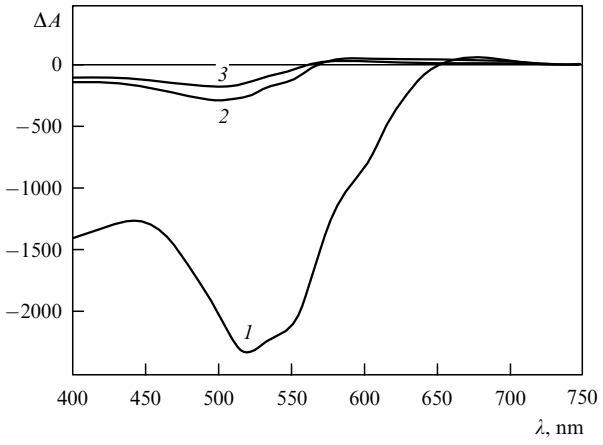

Figure 3. CD spectra of DNA nanoconstructions (*1*) before treatment with hiporamin (HP) ($c_{HP} = 0$) and (*2*) after treatment with hiporamin ($c_{HP} = 3.953$ μg ml^{-1} a $c_{DNA} \sim 5.6$ μg ml^{-1}. Curve *3* corresponds to the CD spectra of a CLCD complex (DNA – DAU), $\Delta A = (A_L - A_R) \times 10^{-6}$ optical units; A_L and A_R are the light absorption in samples with left and right circular polarization.

4. Stabilization of the physicochemical properties of biosensing units based on DNA nanoconstructions

An essential disadvantage of biosensing units based on DNA NaCs from the standpoint of their practical application is the instability of optical properties caused by a gradual sedimentation of DNA NaC particles when using their test-tube form. This shortage, which can easily be removed by agitation of the test tube, is allowable while conducting biochemical studies and analyses on small scales, but is completely unacceptable when carrying out mass analyses, decentralized measurements, 'personified' medicine, or technological online control. For eliminating this disadvantage, a new approach to the creation of stabilized forms of biosensing units based on DNA NaCs and, correspondingly, a new construction of a dichrometer were required.

The problem of the stabilization of the physicochemical properties of biosensing units was solved by creating a hydrogel containing NaC particles. Together with the elimination of sedimentation of the DNA NaC particles, the hydrogel allows preserving the abnormal optical properties of the biosensing unit even upon swelling. In this case, the biosensing unit represents a synthetic polymeric matrix (SPM), which contains spaced-apart single particles of DNA NaCs (Fig. 4), and in its operational principle is analogous to film-type indicators [13].

Based on the technology developed at IMB RAS, elastic polymeric hydrogels have been created with a low toxicity and biodegradation, optically isotropic, with a high transparency in the wavelength range between 230 and 750 nm, chemically and biologically inert with respect to dsDNA molecules and to other BACs. The parameters of the spatial organization of

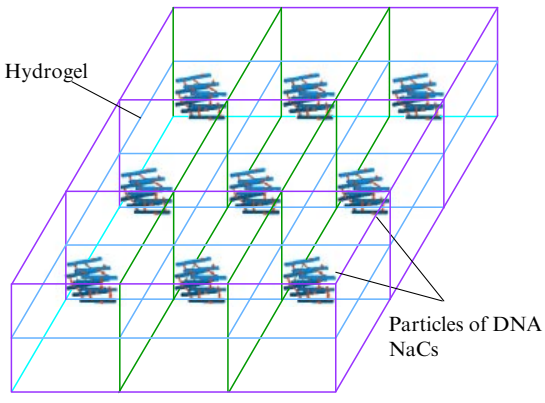

Figure 4. Schematic of a hydrogel containing particles of DNA nanoconstructions that are spaced apart.

a hydrogel and its physicomechanical characteristics ensure the fixation of DNA NaC particles without the disturbance of their spatial structure, the reactivity of the 'building' blocks of these structures, and the condition for the diffusion of BACs on a realtime scale (from 30 min to 3 h, depending on the molecular weight of the analyte). The gel biosensors containing particles of DNA NaCs preserve the abnormal optical activity for a long period (more than a year).

The immobilization of the DNA NaC particles in the hydrogel does not substantially influence the shape and amplitude of the related anomalous bands, whose maxima are located in the ultraviolet (~ 270 nm) and visible (~ 520 nm) ranges of the CD spectrum. At DNA concentrations ~ 20 μg ml^{-1}, the amplitude of the band in the visible range of the spectrum, which is generated by a gel biosensing unit approximately 3 mm thick, is no less than 1200×10^{-6} optical units.

In the experiment, we used homocysteine (HC) as an analyte capable of diffusing into the hydrogel and destroying DNA NaC particles contained in it. The homocysteine is formed in the human body as a result of the metabolism of methionine; in the case of disturbance of the process of reverse transformation into the methionine, it begins to enter the bloodstream, which leads to the appearance of hyperhomocysteinemia i [14] and related diseases (thrombovascular disease, atherosclerosis, pregnancy pathologies, etc.). The determination of HC in blood plasma using highly efficient liquid chromatography is a lengthy and expensive process that requires the use of expensive equipment and highly skilled personnel. This means that the development of an inexpensive, high-precision, and simple procedure for determining HC is an urgent problem of contemporary biomedicine.

Figure 5 shows the CD spectra (registered through different time intervals) of a hydrogel placed in a solution containing HC, which, diffusing into the hydrogel, causes a decrease in the anomalous CD band up to its complete disappearance. This means that after HC penetrates into the hydrogel, it 'extracts' Cu^{2+} ions from the nanobridges and causes the destruction of the DNA NaCs. This clearly shows that the DNA NaCs immobilized in the hydrogel easily 'responds' to the presence of HC in the solution.

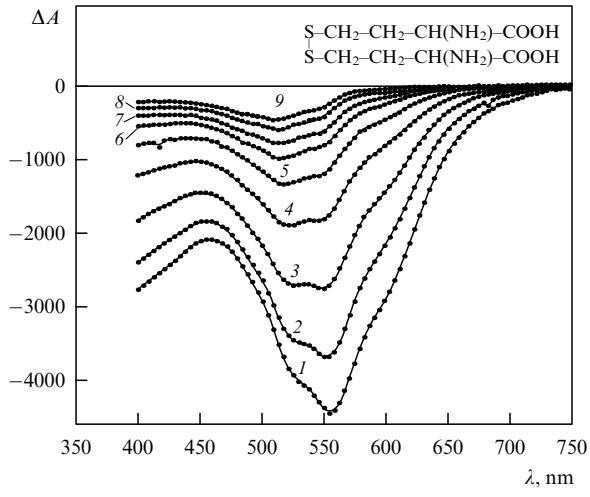

Figure 5. CD spectra of DNA NaCs in the composition of a hydrogel placed in a homocysteine-containing aqueous-salt solution of polyethylene glycol (PEG) recorded after various times: (*1*) 0, (*2*) 15, (*3*) 25, (*4*) 35, (*5*) 45, (*6*) 55, (*7*) 65, (*8*) 85, and (*9*) 115 min; $c_{DNA} = 19.92$ μg ml^{-1}; $c_{PEG} = 120$ mg ml^{-1}; $c_{HC} - 39.22$ μg ml^{-1}.

The nanobiomaterial obtained, which contains the DNA NaC particles, was also used in biosensors for controlling the quality of phytopreparations (alpisarinum, hiporhamin, chamaenerin, chanaerol) in the VILAR Center of Biomedical Technologies.

The gel-like nature of the DNA biosensing units and their specific features, such as the diffusion permeability for analytes, light scattering in the biomaterial, and small length of light interaction with the sample, required the development of a new polyfunctional dichrometer (SKD-3, Fig. 1b) with a scheme of a vertical formation of an optical ray and illumination of the gel sample on a small area (0.6×0.6 mm). The gel samples of the biosensing units can be placed in special cells of a UV microtablet, where they interact with the liquid sample to be analyzed. To expand the working range of the device into the ultraviolet region of the spectrum (to 190 nm), the dichrometer provides a hermetic sealing of the optical block and the possibility of filling it with gaseous nitrogen to eliminate losses of UV radiation because of its absorption by ozone that can form if working in air [15].

5. Optical biosensing units based on DNA nanoconstructions and a single-wave dichrometer

As was shown in Section 4, biosensors based on DNA NaCs that are spaced apart and immobilized in a hydrogel preserve inherent abnormal optical activity in the absorption band of DNA (~ 270 nm) and also manifest new optical activity in the absorption band (~ 520 nm) of the components of the nanobridges of DNA NaCs.

The abnormal optical activity of biosensing units based on DNA NaCs in the band near $\lambda \sim 520$ nm remains constant for a long period and can decrease (up to complete disappearance) under the effect of BACs, whose 'targets' are the structural elements of nanobridges. This circumstance determined the direction of the development of a new, even more compact and cheaper biosensor device. Such a device, in contrast to the broadband systems of the SKD-2M and SKD-3 type, would not need a large-dimensional wide-band (including ultraviolet range) lamp radiation source with a high-voltage power source and a monochromator with a device for wavelength tuning; they are replaced by a miniature diode emitter operating only in one of the above-indicated bands in the visible spectrum range. Simultaneously, such a device can be used for measuring diffusion rates of different liquids in gel or film nanobiomaterials.

At the turn of 2007 – 2008, a simple prototype of a new biosensor system with the use of gel DNA biosensors, a diode radiation source, and some units borrowed from the SKD-2 dichrometer was successfully tested at ISAN [16]. At present, a base model of a compact specialized single-wave dichrometer and an analytical system based on it are being developed. This system can be extensively applied in medical centers in which direct determination of concrete BACs or other significant chemical compounds in liquids should be conducted.

6. Competitive position of optical biosensors

It is assumed that biosensors based on DNA NaCs and the dichrometers of the new type with the procedures for their

application must compose the basis of newly developed biosensor technology and biosensor devices for the direct determination in liquids of various BACs that are dangerous to human health and life or have a therapeutic function. These devices should have a high sensitivity, no fast, and have a low prime cost of analysis. The main fields of application of such portable biosensor analytical complexes (optical biosensors) are medical clinical diagnostics, biochemical analysis, pharmacology, the biotechnological and food industries, ecological control, and scientific research.

The possibility of the commercialization of the biosensors of the new type and related devices is fortified by the presence of both the priority of Russian scientists in this field and of the incorporeal rights with respect to the developed nanobiomaterials, technologies, and devices. As follows from an analysis of the market for biosensor systems, the project developments of scientists of the Russian Academy of Sciences in the field of biosensor analytical devices with the use of DNA biosensors are in a very advantageous position on the world market for biotests in the basic competition characteristics (the cost of equipment, the price of tests, and the degree of universality of the equipment as regards its applicability). Full-featured analogs of these developments do not exist elsewhere in the world and, judging from the publications, cannot be anticipated in the next several years. In view of the cost of the competing equipment (from 70–250 US dollars for devices for individual application from firms such as Bayer, LifeScan, Roche/Boerhinger, Medisense to 130 thousand dollars for biosensors from the Affymetrix firm, with the price of one measurement on it being about 250 dollars) and the degree of the universality of the equipment proposed on the market, the optical biosensors under consideration occupy a position that is completely accessible to users based on both the overall cost (25 thousand dollars) and an extremely advantageous price of one measurement (0.5 dollars), not to mention their suitability for the determination of the majority of BACs. We note that an ordinary clinical laboratory assistant will be able to learn now to operate the analytical complex of domestic development in 3–5 days, whereas in the case of foreign polyfunctional analogs, this requires much more extended training. These estimations are valid even to a greater degree for the latest developments, whose main advantages are the long-time stability of gel (film) DNA biosensors, the low price and compactness of the single-wave dichrometer, and the possibility of conducting direct rapid analyses of liquids.

The above features of optical biosensors make them potentially attractive for many groups of users, but most of all they are necessary in those fields where measurements by traditional methods either cannot be made (new tasks) or prove to be too expensive and/or prolonged (the field of mass biochemical analyses, decentralized measurements, 'personified' medicine (including new fields), technological online control, etc.).

The newly developed biosensor analytical systems have already been used in ten biomedical establishments for conducting scientific and applied research, including IMB RAS, for the development of new types of nanobiosensing units based on dsDNA molecules and the analytical procedures with their use, for determining antitumor preparations in patient tissues (in collaboration with the Hertsen Moscow Research Oncological Institute), for controlling the quality of the new-type of carriers of gadolinium for the neutron-trapping therapy of malignant tumors (together with the Institute of Nuclear Research, RAS), for detecting phytogenotoxicants and controlling the quality of phytopreparations (at the VILAR Center of Biomedical Technologies, RAMS), and also at the Institute of Laser and Information Technologies, RAS, at the Semenov Institute of Chemical Physics, RAS, and at the Institute of Theoretical and Experimental Biophysics, RAS.

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