Chapter

Biosensors Using Free and Immobilized Cells of Luminous Bacteria

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Abstract

The technologies of receiving free and immobilized photobacteria cells for biomonitoring of toxins are considered. The mechanisms of interaction of toxins with photobacteria are observed. The main attention is paid to the immobilized procedures and structures of carriers. Data on poly(vinyl)alcohol (PVA) cryogel immobilization of different strains of photobacteria are presented. It is established that intensity and stability of light emission of PVA cells is competently controlled by: (1) intensity and persistence of a luminescent cycle using bacterial strain; (2) type of the carrier and the composition of the gel-formation medium; (3) freeze-thawing procedures; and (4) physical and chemical conditions of storage and application. The developed technology of cryogenic gel formation has kept the survival of luminous bacteria in the carrier practically at 100% without the introduction of additional cryoprotecting agents and procedures of a light induction. With storage at −80°C, bioluminescent activity remained without changes about 2 years. Using the immobilized preparations of biosensor, the discrete and continuous analysis of heavy metals, chlorophenols, and pesticides is carried out. The sensitivity of free and immobilized cells to the chosen toxicants is approximately identical. The continuous monitoring of toxicant conditions is optimized.

Keywords: photobacteria, biosensors, immobilization, poly(vinyl)alcohol, biomonitoring

1. Toxin action on the photobacteria light emission

The interaction mechanism was investigated only for certain groups of chemicals and mainly with the use of free cells. Toxic agents that suppressed the emission of photobacteria can be divided into classes rather conditionally.

- On the chemical structure: heavy metals, electron acceptors, respiratory poisons, aliphatic, aromatic and heterocyclic hydrocarbons, alcohols, ketones, acids, and others. The level of toxin hydrophobicity is important.

- On the type of targets: membrane active substances, specific inhibitors of the genetic apparatus, and inhibitors of the energy and lipid metabolism enzymes.

It well known also the specific inhibitors of luciferase and the bioluminescent enzyme system, and auxiliary systems for the biosynthesis of substrates and regulatory molecules.

These substances suppress the light emission and some can cause small activation of the luminescence of bacteria. Naturally, the determining factor of interaction with the cell is the chemical structure of the substance, concentration, and time of incubation. These parameters are manifested in the kinetics of changes in luminescence and the magnitude of the inhibition constant. The main options in the kinetics of inhibition are presented in **Figure 1**. Inhibition can occur almost instantaneously (Curve 1) or with a lag phase, depending on the nature and concentration of the substance (Curve 3). In some cases, the effect may be reversible (Curve 2).

The mechanisms of interaction of toxins with photobacteria are observed. Data on poly(vinyl)alcohol (PVA) cryogel immobilization of different strains of photobacteria are presented. It is established that intensity and stability of light emission of PVA cells are competently controlled by: (1) intensity and persistence of a luminescent cycle using bacterial strain; (2) type of the carrier and the composition of the gel-formation medium; (3) freeze-thawing procedures; and (4) physical and chemical conditions of storage and application. The developed technology of cryogenic gel formation has kept the survival of luminous bacteria in the carrier practically at 100% without introduction of additional cryoprotecting agents and procedures of a light induction. Specific bioluminescent activity was restored to level of activity of free cells. At storage at −80°C, bioluminescent activity remained without changes about 2 years. The detecting level of a light emission at 4°C for psychrophilic strains over 1 month, at 20°C for 3 days. High survival of PVA-immobilized cells and prolonged light emission reflect advantages of cryogenic immobilization of photobacteria. With the use of immobilized preparations of biosensor, the discrete and continuous analysis of heavy metals, chlorophenols, and pesticides is carried out. The sensitivity of free and immobilized cells to the chosen toxicants is approximately identical. The continuous monitoring of toxicant conditions is optimized. The analysis using photo-PVA-biosensors can be perspective

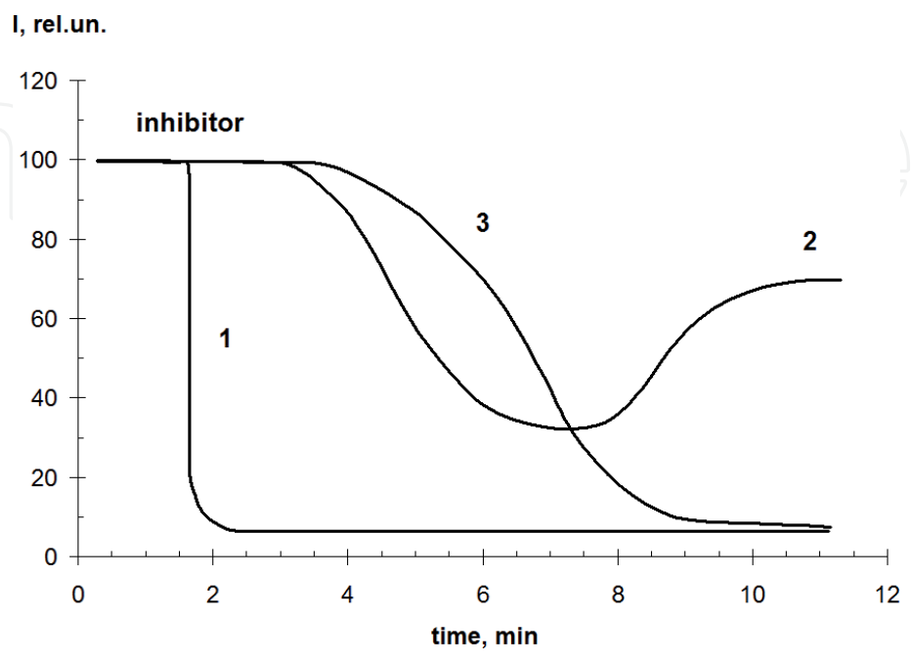

Figure 1.
The kinetics of the Vibrio harveyi free cell luminescence with various chemicals. 1–instant inhibition; 2–reversible inhibition; 3–lag-phase inhibition. Incubation medium: 3% NaCl +0.1 M phosphate buffer, pH 7.0.

for express monitoring of environments in a mode of real time in water areas and industrial wastes, and also in the control over processes of biotransformation and degradation of toxins.

Irreversible inhibition causes a wide class of toxins of organic and inorganic nature; reversible inhibition is observed under the action of electron acceptors, which restore and lose their inhibitory properties during incubation; and inhibition with lag-phase implies sequential interaction with membrane and intracellular structures, including a diffusion step.

2. Photo-biosensors

Photobacteria, as well as other microorganisms with cloned operon of luminescent system of photobacteria, and recombinant cells with luminescent activity are widely used in various biotechnological tasks, and, first of all, in ecological biomonitoring of toxins and other biologically active agents. Dates are summarized in reviews [1, 2]. Depending on the purposes of researches in technological procedures, both the free and immobilized cells are used [3, 4].

The use of preparations with immobilized cells leads to heterogeneous catalysis that in many cases provides economic and practical efficiency. The advantages of this approach are primarily related to simplification of the manufacturing process as well as the opportunity to switch from occasional output of desired products or expected substrate reactions toward a continuous process. In addition, immobilized cells allow using preparations multiple times and for longer period compared to single application of cells in a homogeneous suspension. The technology of immobilization in many cases enhances enzymatic activity. Also, immobilized preparations have increased resistance to various physicochemical factors (temperature and pH) and lesser sensitivity to the action of pathogenic organisms. The results of a 10-year long examination evaluating technologies creating genetically engineered microbial strains as well as immobilization procedures, storage, and application of genetically engineered microbial strains in both laboratory and practical application of biosensors in toxicological biomonitoring were thoroughly described [1, 2, 5].

Immobilizing photobacteria increases stability of a biosensor upon its storage as well as duration of its use [6–8]. Similarly to the free cells in suspension, in the case of immobilized preparations, an integral toxicity assay is performed by natural photobacteria, whereas specific toxin detection is mediated by mutant and genetically engineered microbial strains containing cloned genes of a bioluminescent system. Moreover, it should be mentioned that during a specific assay with genetically engineered microbial strains, light emission is observed after a lag-phase lasting for many hours. Various reporter bacteria are used for specific analysis of various substances [9]. A reporter microbial culture was immobilized and fixed on the surface of a photodetector element using Sr-alginate gel. Immobilized biosensors for detecting such substances were used for both during discrete and continuous monitoring. It was found that both the maximum emission and rate of emission induction depended on incubation time and concentration of substances. It was concluded that this system was suitable for real-time analysis of environmental pollutants and industrial waste as well as evaluation of metabolic status of various biological materials. A linear dependence between induced bioluminescent response and concentration of the substances was noted using both free and immobilized bacteria. The authors attracted attention to high stability of the alginate-glycerol cell suspension at −70°C. The data presented in [7] were obtained using recombinant Salmonella typhimurium strain TA1535 designed from a plasmid with an inducible SOS-promoter linked to the luxCDABE

operon derived from *Photobacterium leiognathi*. The mixture was immobilized in agar carrier, a simple procedure that includes a plate matrix (10 μl of mixture added into the wells) incubated for 30 min for polymerization and further storage at 4°C. The principal protocol used for analysis is similar to others that use recombinant biosensors and includes emission induction upon incubation with toxic substance. Model experiments on light induction with this bacterial strain were made with DNA-damaging agent—mitomycin C. The immobilized cells retained activity at 4°C for 6 weeks. A response of 4-week old bacterial culture exposed to mitomycin C was indistinguishable from that triggered by a fresh immobilized culture. Both the magnitude of the maximum response and duration of the lag-phase depend on the concentration of mitomycin C. The maximum activation response is obtained at approximately 1 μg/ml concentration of mitomycin C. It is crucial that further increase in concentrations results in abrupt quenching of the emission. Apparently, this is due to a cytotoxic action of the antibiotic on the bacteria, similar to toxins affecting recombinant bacteria of other strains treated with high concentrations. Bacterial biosensors immobilized in thin cellulose-agar films were used to detect various chemicals such as phenol, hydrogen peroxide, copper, and cadmium [10]. It was found that immobilized cells revealed high sensitivity to these agents, and inhibitory effect was observed at concentrations close to those detected by free bacteria. The preparation was stable when dissolved in AWS culture medium for 4 weeks at 4°C. However, incubation in 3% NaCl solution significantly decreased stability. Along with toxicology experiments, data regarding temperature and pH, dependent relations with luminescence of both free and immobilized bacterial preparations, as well as spectral and kinetic characteristics are presented. A number of studies were done to create novel-type biosensors to be used in environmental toxicological analysis [11, 12]. A fundamental difference for such systems was that they contained a combined complex made of optical fiber detector together with a biodetector. Results on improving technology of generating combined biosensors containing natural and recombinant microbial strains immobilized on optical fiber strands and used for cytotoxicity and genotoxicity assays are presented in [13].

Combined systems are considered especially promising for practical application of immobilized cells of type "biosensor-photoconductor l-photodetector." Many of these were aimed at developing genetically engineered constructions, containing sensor genes: SOS-system, heat shock defense system, DNA, and membrane-damaging agents, specific to various toxins, and reporter genes derived from photobacteria. All strains were immobilized at the different optic fiber strands. Therein, Ca-alginate gel was used as a carrier. In such systems, each biosensor specifically reacted to a certain type of toxins. The developed test system was effective in detecting pure inorganic and organic toxins as well as admixtures of toxic substances in aqueous medium, soil, and other samples. Natural photobacteria were used together with genetically engineered counterparts, where toxic agents had an inhibitory effect on their luminescence. Application of such bacteria based on a need to have a reference control of the cytotoxic action. It was found that thin films consisting of gel and cells were stable for 6 h at 26°C and able to detect mitomycin C at concentrations up to 25 μg/l. Concentrations of cells up to $(1–3) \times 10^7$ in a carrier were effective for different analytical procedures. Biosensor stability within a combined system sensitive to temperature impact, as well as other chemical and physical parameters of the bio-detecting system, was analyzed in detail in response to substances causing a stressful reaction in the cells (heat shock, action of SOS agents, impaired protein biosynthesis, peroxide, and oxidative stress).

However, it should be noted that cells immobilized in agar, agarose, and alginate have insufficient stability of luminescence, due to the sensitivity of the cells

to relatively high (up to 30–50°C) gelation temperature. It is worth mentioning that alginate gels with relatively low gelation temperature can be used as well. Nonetheless, the effect of temperature results in the development of dark mutants of photobacteria having a temperature optimum for luminescence at 15–25°C (depending on species). The salt composition contained in the culture media plays an important role for marine photobacteria. Optimal concentrations for emission activity of cells are reached in 2–6% NaCl solution. Ca-alginate gels can be somewhat destabilized in such high salt concentration solutions due to the process when Ca^{2+} ions derived from the gel are replaced by Na^{+} ions. Moreover, complex formation of Ca^{2+} and Sr^{2+} with phosphate and carbonate ions used as buffer solutions may occur.

The data present in [4, 14, 15] the immobilization of bacteria *Photobacterium phosphoreum* in different gel formation materials: agar, agarose, carrageenan, polyacrylamide, calcium alginate, polyvinyl alcohol, polyurethane, calcium carboxymethyl cellulose, etc. It is established that the used carriers and technologies of an immobilization extremely and variously influence the stability of photobacteria. The duration of a luminescence alginate-glycerol suspension of cells, at incubation in 3% NaCl, at 4°C, reached 4 weeks (the minimal detected luminescence level—up to 6 weeks). The duration of a luminescence of free cells in the same conditions did not exceed 2 weeks. In some cases the immobilized cells in an agar have significantly smaller stability, than that of free cells. The agarose-immobilized cells possess approximately the same duration of luminescence, as free cells—2 weeks. The alginate glycerol suspension completely kept the initial level of luminescence at storage at −80°C within 12 weeks. In storage, at –20°C, emission activity decreased, similarly observed at 4°C.

The investigations [3, 16, 17] are directed to the development of technologies of bioluminescent monitoring of chemicals in the discrete and continuous mode of bio-testing. The stated explanation follows from the well-known temperature effects on a luminescence of photobacteria. Temperature influence (30–36°C, 30–60 min) leads to the formation of dim and dark mutants [18]. The temperature factor makes especially strong effect on *Photobacterium phosphoreum* bacteria, which has a maximum bioluminescent activity at 15–18°C. Data on luminescence kinetics are important during an incubation of preparations at positive temperature. The unstability of luminescence that is caused with physical states of using gels was established. The decreasing of light emission caused also with partial replacement of ions of Ca^{2+} in the carrier on Na^{+} ions. Replacement of Ca^{2+} on Sr^{2+}-ions in the carrier increased its stability. The analytical procedure using Ca and Sr-alginate gels is approved on five heavy metals: $Pb(NO_3)_2$, $NaAsO_2$, $NiCl_2$, $CdCl_2$, $HgCl_2$, and also SDS and pentachlorophenol (PCP). The authors note essential distinctions in inhibition kinetics of the specified toxins between the free and immobilized cells, but the sensitivity is rather close. Besides them, stimulation of a luminescence by low concentration of PCP (less than 0.1 ppm) and $CdCl_2$ (less than 10 ppm) was observed. The procedures developed for discrete biomonitoring have been used for the continuous analysis of toxins. The interaction of toxins with cellular targets depends on hydrodynamic parameters. The optimized conditions were: speed of a channel is 25 ml an hour, delay time after introduction of toxin of 37 s, time interaction 1–2 min, and cooling system has been used. Stability of a luminescence is more than 40 min. Pulse introduction of a toxin to a channel with the immobilized cells leads to reversible kinetics of inhibition. At washing away of a toxin by the stream observes complete or partial recovery of emission, allows to repeated biomonitoring on the same biosensor. The effect "dose/time" is shown in a kinetic profile of suppression and reversion of a luminescence [16]. The fact that the cells of *Photobacterium phosphoreum* immobilized in Sr-alginate gel can effectively be

used for discrete and continuous detection of toxins can be considered as the main conclusion of this work.

The work [17] was done for biomonitoring of water toxicity based on continuous cultivation of *Photobacterium phosphoreum*. Attention in this work is paid to a possible problem of emergence of dark mutants (after 10 days of incubation), which begin to dominate, causing suppression of a luminescence. For an exception of this problem, the authors have offered the system based on the special fluidized-bed reactor. The cell-alginate suspension continuously moved in a stream. It is established that domination of dark forms significantly stops in the internal volume of carrier, and suppression of a luminescence is not observed.

Rather high speed of dilution prevents settling by other microorganisms of the reactor. Concentration of cells and emission activity in the developed mode of cultivation is sufficient for observation of the water environment toxicity within 4 weeks. Essentially that photobacteria are capable to grow in granules and remain in water. It is noted that after 24 h in all volume of granules microcolonies of various forms were formed. The survival of cells in the carrier during the procedure of an immobilization, storage, and use of the analytical system was studied. The assessment was carried out by calculation of colonies after destruction of a carrier by $Na_6O_{18}P_6$.

It has been established that all cells are distributed in volume of a matrix; the insignificant quantity is connected at the surface of the carrier. In the developed cultivation mode, 80% of cells remain in the wild type within 40 h. The dark mutants were formed on the seventh day, and then increased up to 100% by the tenth day of cultivation. The authors have made the assumption that mutations mention all cells—immobilized and leaved matrix cells. However, these distinctions are insignificant. It is suggested that matrix protects the immobilized cells from a mutagenesis. Results are considered as a possibility to use of system for the analysis of water toxicity in real time. At the same time, the developed technological operations significantly protect from a dark mutagenesis. The use of analytical system, in this case, is possible within more than 30 days. The measures of protection against infection with other microorganisms are enclosed. The model experiments on the analysis of $HgCl_2$ are given using immobilized and free cells. It is established that the immobilized cells are more sensitive to these chemicals, since free cells are more active it results in more resistance, which is toxic. Cells in the immobilized material react to existence in the $HgCl_2$ up to 0.5 mg/l.

The new technology solution of complex biomonitoring of toxins is submitted in work [19]. The test system based on the multi-channel apparatus for detecting the water environments using some recombinant strains containing lux-CDABE operon is developed.

Each channel of the system was designed from two mini-bioreactors necessary for step continuous process. Each channel contained a certain recombinant strain with the cloned gene of photobacteria: DPD2440-(fabA:luxCDABE), DPD2794-(recA:luxCDBE), and TV1061-(grpE:luxCDABE). The strains are sensitive to the membrane, DNA, and protein destroying agents, respectively. Interaction of these agents with recombinant bacteria causes induction of a luminescence. As a control for cellular toxicity, wild strain, whose bioluminescent reaction was suppressed with toxins, has been used. Some experiments are executed with phenol and mitomycin C. The procedures of biomonitoring for the subsequent applications in practical tasks of the analysis of toxic chemicals of the environment are optimized. For the practical analysis, tests of water from two different (nuclear and thermoelectronic) reactors were used.

Continuous biomonitoring was carried out by cultivation of bacteria in two reactors; the biomass of bacteria was grown in the first cultivator and pumped at the second reactor with toxin injected. Each channel reflected the specific profile

of the bioluminescence, corresponding to the chemical nature of the toxic agent. Comparison of a bioluminescent signal between standard toxic substance and the following water solution allowed to define the true equivalent toxicity in a water stream.

The developed system of continuous multi-channel biomonitoring of toxins can be considered, according to authors, as the new strategy of protection of bio-objects against environment pollutants (alternative system of express monitoring and control of the water environment).

Special attention is paid to receiving recombinant strains, and also technological operations of biomonitoring in a channel that are fulfilled, optimized on models, and are applied in water analysis to cool the nuclear and thermo-installation.

The authors note that the tests used for biomonitoring are stable within 1 week at 4°C and pH 7.0. Test-system was carried out in real-time monitoring with the use of automatic computer control.

All used chemicals stimulate the luminescence, however for all the difference in the intensity of light and duration was observed. The induction time is rather long, up to 500 min. At the same time, the high concentration of toxins not only caused induction but also suppression of the luminescence of recombinant strains. It specifies that at high concentration there is no specificity of reaction of recombinant strains. Metabolism of all recombinant strains reacts to high concentration of toxins by suppression of luminescence. It was shown that at certain concentration, the genotoxic action is blocked by cytotoxic action. For the recombinant strains, there are no quantitative data on inhibitory actions on external targets of membranes. With these data, the bioluminescent intensity could be significantly corrected. The results suggest that the water used for the nuclear reactor contains substances with membrane action. It should be noted that the developed system of biomonitoring is rather difficult, consuming, and long, though it, in principle, yields positive results of bio-testing.

The fact that the toxic effect on inhibition of a luminescence is shown on all recombinant strains is important. The developed system of continuous biomonitoring can be considered as the new direction in identification of the nature and toxic action of chemicals and can be also applied to the detection of many industrial wastes. Before the analysis the testified probes have to undergo preliminary processing, for removal of the accompanying bacteria, with the subsequent filtration. The main result of this work is the use of various types of recombinant strains for specific biomonitoring of toxins.

The integrated system of biomonitoring of water (water toxicology) using the test object recombinant bacteria is presented in work [20]. The developed system included four channels with two mini-bioreactors. The recombinant strains of *E. coli*, EBHJ2, DP1, DK1, and DPD279, were used. The chosen strains carry out three specific tasks of cell protection against oxygen shock of superoxide, hydrogen peroxide, and the DNA-damaging agents also. Based on the specified strains, systems are applied for both discrete and continuous monitoring of toxins. The first reactor was used for cultivating the culture of the specified strains with their subsequent transfer in the second bioreactor. It is established that induction of the bioluminescent increase is observed only in the small range of the increasing concentration of the specified inductors. The peroxide and mitomycin, were entered into the second cultivator. The intensity and rate of increase of luminescence are specific to each inductor; and at high concentration, the effect of induction is changed with inhibitory effect.

Particular advantage of the developed system is miniaturization: a little (1–2 ml) reactor volume. The authors assume that the four-channel mini-monitoring system with different recombinant strains is extremely useful viewpoint for the specific analysis of certain classes of toxins in the environment.

3. Immobilization of microorganisms

As carriers for an immobilization of photobacteria, both natural and synthetic materials are used [2, 8]. The immobilization of cells of microorganisms can pass by fixing on a surface and in volume (depending on the used material), or chemical binding on surface or embedding in matrix interior. In most cases, agar, agarose, and alginate gels are used as the carriers for immobilized luminescent bacteria. Ca^{2+} and Sr^{2+} alginate gels are the carriers most commonly used in manufacturing bioluminescent toxicological biosensors.

Methods of an immobilization are various; however, all are directed at increasing the profitability of process and its efficiency. At the same time, special attention is paid to durability for fixing of cells with materials and for prevention of an uncontrolled exit from a carrier phase in incubatory solution. The factors capable to reduce, suppressing metabolic activity, up to violation of integrity of structure of an organism has been studied. First of all, it belongs to temperature, ionic structure, pH, to availability of inhibitors, toxins, and other xenobiotics. Viability and light activity of photobacteria are extremely sensitive to these factors.

At inclusion of cells of microorganisms in carrier volume, a number of factors take part in keeping of cells: mechanical keeping, adsorption, structure of a grid of polymer and the pores, chemical bonds between functional groups of a cellular surface and free groups of the carrier, electrostatic forces, and hydrophobic interactions.

At the same time, the size and structure of pores in the carrier, time diffusion of nutrients or other biologically active agents, and withdrawal of products are important.

4. The immobilization on a surface of the carrier

The immobilization of bacteria in some cases is carried out on a surface of the carrier. It depends on the restrictions for penetration of cells into volume and on the structures of the materials used. Different chemical elements participate in the fixing of cells on the surface of the carrier. Non-covalent interaction is carried out at the contact of cells with the surface of carrier, with the participation of ionic interactions, hydrogen bonds, and hydrophobic forces. In this case, nonspecific cell fixing occurs. When the carrier contains the ligands capable to form rather strong binding with functional groups of a cellular surface, bio-specific binding

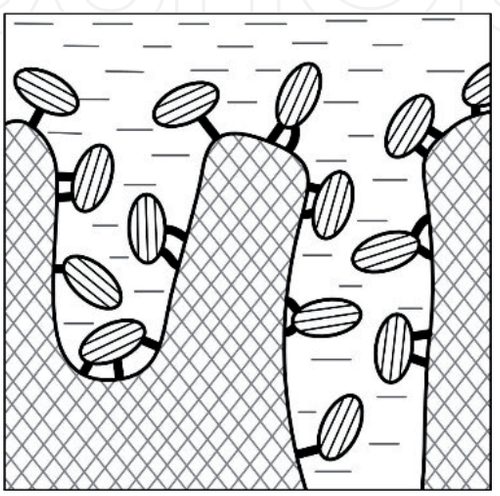

Figure 2.
Cell immobilization on the surface of carrier [8].

is observed. In certain cases for increase in efficiency of process, the carrier has to have special chemical residues capable to form strong bonds with a cellular surface or the so-called sewing elements are used (**Figure 2**).

5. The immobilization in the interior of the carrier

The immobilization in volume of the matrix allows to increase a specific concentration of the cells in comparison with liquid culture, which causes the productivity of biotechnological process. Especially, a possibility of repeated or continuous use of one type carrier with the specific microorganisms should be noted. At the same time, the form, any convenient for operation, can be given to the carrier: granules, plates, threads, tubes, etc. Finally, procedures of biomonitoring become simpler (**Figure 3**).

The procedure of inclusion of cells in the interior of the matrix is generally carried out during incubation of the carrier with biomass, swelling, with the subsequent formation of gels system, with microorganisms, stabilizers, substrates and protectors. Water, through which there is a metabolism, is a part of hydro-gels. For an immobilization of cells in volume of the carrier, a wide range of various materials capable to transition from liquid to "gelatinous," gel/cell composition, under specific conditions is used. The greatest distribution for an immobilization of microorganisms was gained by gelatin, agar, agarose, alginate, carrageenan, etc., in which the spatial grid is supported using non-covalent bands. The specific property of these gels is the transition from liquid state to elastic form, at change of temperature. The majority will be polymerized at heating. However, there are substances that are polymerized with cooling. It is possible to give poly(vinyl) alcohol (PVA) as an example capable to form cryogenic gels. Ion-tropical gels are formed with bands between poly-electrolytic macromolecules.

In polymerization of polysaccharides, the main role in matrix formation is assigned to hydrogen bonds and electrostatic forces. The contribution is made by mechanical packing of polymeric chains in supramolecular structures. Gel-forming properties can differ depending on the source and method of receiving. At the low temperature, the macromolecules can form supramolecular structures with the participation of hydrogen bonds.

The main advantage of the technological procedure is simplicity of inclusion of cells in the carrier without essential violation of structural characteristics, the high catalytic properties [8].

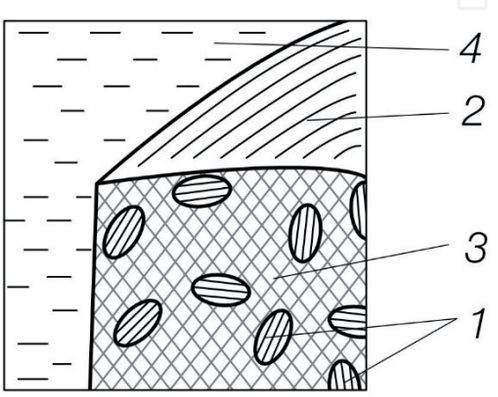

Figure 3.
Immobilization of the cells in the volume of the carrier. 1—Cells, 2—Matrix, 3—Gel-cell mixture, and 4—Carrier [8].

6. Poly(vinyl) alcohol as a carrier for immobilization

Among synthetic polymers, for immobilization of the most different groups of microorganisms, cryogels of poly(vinyl) alcohol (PVA) are used. Because of freezing, elastic hydro-gels are formed. The formation of matrix was participated by hydrogen bands between various sites of polymeric chains. One of the important elements of formation of PVA cryogel is the content of acyl-residues. At 5%, the friable structures were formed. From high concentration of the PVA water solutions (more than 7%), strong structures are formed.

Cryogenic impact on the PVA-water system gives the chance to change properties of polymers and to alter their structure. Stability of the carrier at high negative temperatures is one of the major factors for practical use.

In compliance with literary data, PVA was effectively used in immobilization of many strains of microorganisms. In work [21], different types of microorganisms, which have been successfully immobilized in PVA-cryogel, are summarized.

PVA cryogels have a number of structural and chemical properties, in comparison with other carriers, essential for immobilization of luminous bacteria [22, 23].

First of all, it is connected with availability and low cost of materials, simplicity of the procedure of cryogel formation, and biocompatibility with various types of cells. High resistance of PVA matrix to bio-damage by microorganisms is also important. Essentially, PVA cryogel is not toxic in relation to the included microorganisms. Cryogels have diffusive properties, optimum for detection processes. The electronic microscopy of preparation demonstrates existence of high porosity of the matrix containing micro (less than 1 micron) and macro (more than 1 micron) pores. Existence of a time in many cases lifts a diffusive limit for chemicals (substrata, salts, and products of metabolism of cells), and also activators and inhibitors of this or that of processes. Physical and chemical parameters of a matrix slightly depend on the chemical composition of the gel-formation media, in particular salts, which have basic value for many bacteria.

It should be noted that physiological concentration of some substances (acids, alkalis, amino acids, sugar, phosphates, carbonates, and Na salts) has no significant effect on physical characteristics of the carrier [16]. Physical characteristics of PVA gels depend not only on temperature but also on duration of the procedures and conditions of defrosting. The PVA itself performs the function of a cryoprotector.

A detailed study of the procedure of defrosting has shown that the high speed of defrosting (more than 10°C in a minute) can lead to the formation of colloidal solutions, while at slow defrosting (with a speed less 0.05°C in a minute), the elastic structures are formed. According to data of electronic microscopy, it is established that at slow thawing, the structural distinctions of the carrier is less.

High concentration of biomass of cells (more than 1 g/100 g) can render the destabilizing effect on carrier formation. In other concentrations, the structure of a matrix is optimal for cell stabilization. The diffusion into cryogel substrates, toxins, and various other chemicals occurs in macropores.

Physical characteristics of the created PVA gels are steady in the wide range of positive temperatures, up to 80°C, that allows high efficiency operation of the bioreactor in different temperature conditions.

The biotechnological processes, which are effectively proceeding with participation of the immobilized biological objects, were described. As examples, the formation of ethanol, acetate, hydrogen, transformation of sugars, amino acids, and some other important products of cellular metabolism has been studied [21, 22].

Thus, the physical and chemical properties, lack of toxicity, optical transparency, and formation of carrier happening at negative temperatures are extremely important viewpoints for receiving different types of PVA biosensors. At the same time, the limited number of works on the use of this carrier for immobilization of photobacteria or recombinant strains with lux-genes of bacterial luciferase system should be noted.

7. PVA immobilization of photobacteria

The immobilization of *Photobacterium phosphoreum* in PVA, polyurethane, polyacrylamide, Ca-alginate, and Ca-carboxy-methyl-cellulose is carried out [4, 14]. It was established [14] that photobacteria in PVA-gel possessed less stable, in comparison with other carriers, the duration of light emission—1 week.

The technology of immobilization in PVA-biofilm photobacteria *Vibrio fischeri*, and a gene engineering strain *Pseudomonas putida* with the lux-operon from *Photorhabdus luminescens* is realized for detection of phenolic toxins in industrial waste [24]. However, for increase in stability, light glycerin was introduced into the composition of test system; the procedure included activation of a luminescence with special chemicals.

The psychrophilic luminous bacteria *P. phosphoreum* were immobilized in PVA-cryogel. The technological procedures of immobilization allowed to preserve with preserve nearly 100% of level of emission activity of cells without protectors. The activation processes of luminescence are developed and optimized [13, 25, 26].

With the use of photobacteria as biosensor of toxins, special requirements are imposed to the duration and stability of a luminescence of a test system [13, 17]. These parameters, first of all, depend on natural emission properties of the chosen strain and are controlled by technology of an immobilization, the procedure of storage, and use of biosensors [3, 7, 20].

Specific activity at different strains of luminous bacteria is from 10^2 to 10^5 quanta/s. Light intensity and duration of a luminescent cycle are defined by a specific adaptable metabolism of bacteria. The luminescent cycle of deep culture *Vibrio harveyi* short was completely finished in a logarithmic growth phase (12–14 h), *Vibrio fischeri* has a cycle about 20–24 h, while *Photobacterium phosphoreum* has a stable luminescence at 1–2 weeks. The psychrophilic strains of photobacteria exhibit the highest specific activity and the most prolonged bioluminescence in the growth culture. The luminescent cycle of *Photobacterium phosphoreum* at 15–20°C can exceed 100 h [27, 28].

It is shown that the immobilization in PVA cryogel in all species of bacteria is capable to lead to essential prolongation of a luminescence [26].

It is obvious that the composition of gel-formation media should influence the cells' stability in the carrier and metabolic activity first of all, on stages of gel formation. For a choice of the optimal media composition and conditions, different mixtures have been used for gel formation, and the kinetics of light emission after "freezing/thawing" procedure was investigated. The best results have been received with media using for the liquid cultivation of photobacteria [29]. The emission activity of granules after a freezing/thawing procedure (24 h from the beginning the heating stage) has shown that in this case the immobilized bacteria were kept practically at 100% luminescent activity. The most essential recession of luminescence (in ~10^3 times) was observed when the gels' formation process comes with only 3% NaCl.

The immobilization procedure of photobacteria in PVA cryogel has been published in [26].

Bacterial strains	Immobilized cells		Free cells	
	Total light emission (Q), photons per cell	Time-course of luminescence, days	Total light emission (Q), photons per cell	Time-course of luminescence, days
P. phosphoreum (str. NZ-11D)	1–5 × 10^7	14	5 × 10^6	7
P. phosphoreum (str. №331 KM MSU)	1–5 × 10^9	42	10^3	21
V. harveyi (str. B392 MAV)	1–5 × 10^6	7	5 × 10^5	1
V. fischeri (str. №6 KM MSU)	1–5 × 10^6	10	10^5	3

Table 1.
The emission activity and duration of the free and PVA-immobilized different strains of photobacteria.

The developed technology has allowed the survival of luminous bacteria in the carrier, practically at 100%, without the introduction of cryoprotecting agents and procedures of a light induction. All strains of PVA-immobilized bacteria possess not only high level of initial emission but also raised stability of luminescent activity in comparison with the free cells (**Table 1**).

As the main results from the data presented, the integral light output (Q) is observed on preparations, which formed using the complex growth-media (GM) for the liquid cultivations. Free cells in the same conditions possess essentially smaller (more than 2 order), in comparison with immobilized cells, integral activity.

Specific bioluminescent activity was restored to level of activity of free cells (~10^5 quanta/s per cell). At storage, at −80°C, bioluminescent activity remains without changes for 2 years. The detected level of light emission is at 4°C for over 1 month and at 20°C for 3 days.

Thus, the composition of the gel formation media, not incubation mixture, makes the basic impact on intensity and duration of bioluminescence of immobilized preparations. Concentration of the carrier (5, 7, and 10%) practically does not change emission and kinetics parameters.

8. The kinetics of light emission free and immobilized bacteria *P. phosphoreum*

In **Figure 4**, the time dependence of specific luminescent activity during incubation at 4°C free and immobilized bacteria is presented. The immobilized cells (Curve 1) have more prolonged luminescence, than free bacteria (Curve 2). It is established that luminescence attenuation by immobilized bacteria is not a consequence of destruction of cells, or exhaustion of endogenic substrates, and reflects decrease in reduction potential of a cell owing to shift medium pH with products of a metabolism.

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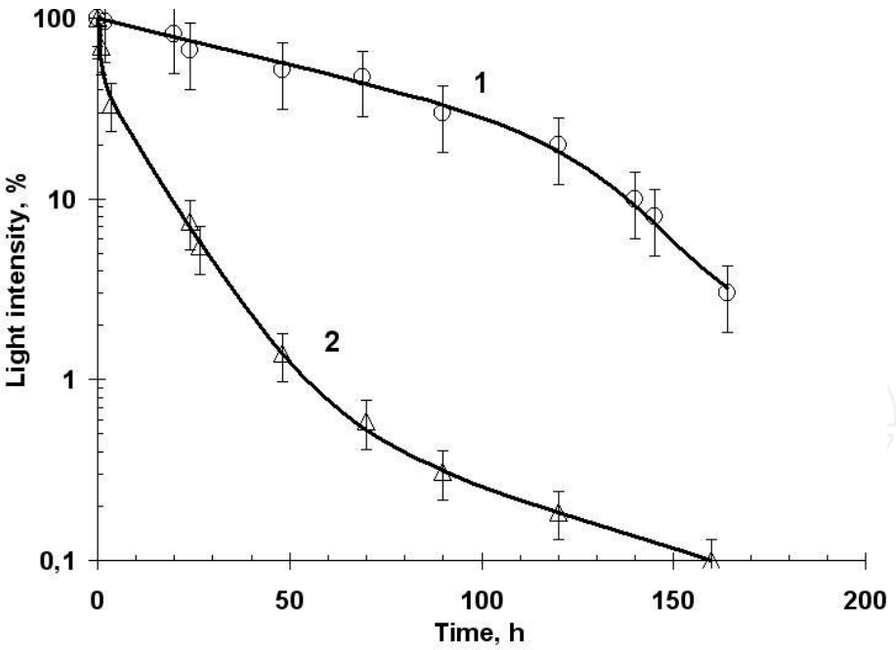

Figure 4.
Light output and bioluminescence decline from the PVA-immobilized and free cells of photobacteria following storage in 0.1 M Na-phosphate buffer +2% NaCl, pH 7.6, 4°C.

9. Photo-biomonitoring

The optimal conditions of reception and storage-immobilized cells formed a basis of use of luminescent granules as biosensor controls. The standard procedure of the analysis with use of free cells provides incubation with toxicants for 5, 15, and 30 min [29]. The analysis of inhibition kinetics from free and immobilized cells has shown similar time profiles, testifying to the absence of serious diffusion restrictions of a gel material and the form of granules for all types of molecules. Supervision logically follows from structural characteristics of the matrix having macropores. As essential distinctions in inhibition, kinetics is not revealed, and the time parameters postulated to free bacteria are chosen for the toxicity analysis with PVA-immobilized cells.

In **Table 2**, the granule luminescence inhibition by various classes of toxins, heavy metals, phenolic derivatives, and pesticides, is presented.

Before assay procedure granules were incubated 10-min with environment temperature (22°C), and the next 5–15 min with toxins. It is established that the threshold sensitivity of free and immobilized cells to the chosen quenchers of luminescence is approximately identical and as a whole corresponds to literary data.

Physical and geometrical parameters of photo-PVAG biosensors for continuous biomonitoring ecotoxicants with the minimum restrictions for diffusion of toxins are also optimized. In the technology of continuous monitoring of toxicants, the optimized conditions of the discrete analysis are used. It is established that in a channel mode, light activity remains about a week at solution temperature 10–12°C.

The kinetic profile of light inhibition depends on the concentration of toxicant. The luminescence time response of the PVA-immobilized cells in continuous flow-through monitoring to $CuSO_4$ is shown in **Figure 5**.

The kinetics is reflected by convertibility effect, although the level of luminescence after washing away toxicants can be different from the initial. The relaxation time increase with increasing the concentration of toxins. However, even at concentration exceeding EC_{50} 10 times at the chosen flow-rate, remains not less than 10% of

Toxicant	The range of toxin concentrations (mg/l) determined by bioluminescence response of PVA immobilized cells at different times of incubation	
	5 min	15 min
Cu^{2+}	5–40	1–8
Zn^{2+}	10–60	0.5–4
Hg^{2+}	0.1–0.6	0.05–0.10
2,4-Dinitrophenol	5–40	2–20
Pentachlorophenol	0.2–2.0	0.05–0.4
2,4-Dichlorophenol	0.5–3.0	0.5–10
2,4-Dimethylphenol	0.5–8.0	0.5–8.0
2,4-Dichlorophenoxyacetic acid	1.0–10.0	0.5–10.0
2,4,5-Thrichlorophenoxyacetic acid	0.5–4.0	1.0–8.0
Granules of different geometrical sizes (from 1 to 3 mm^3) were used.		

Table 2.
The sensitivity of the PVA-immobilized cells P. phosphoreum to heavy metals, chlorinated phenolic derivatives, and pesticides (100 μl probe, 1 ml 3% NaCl with one PVA granule).

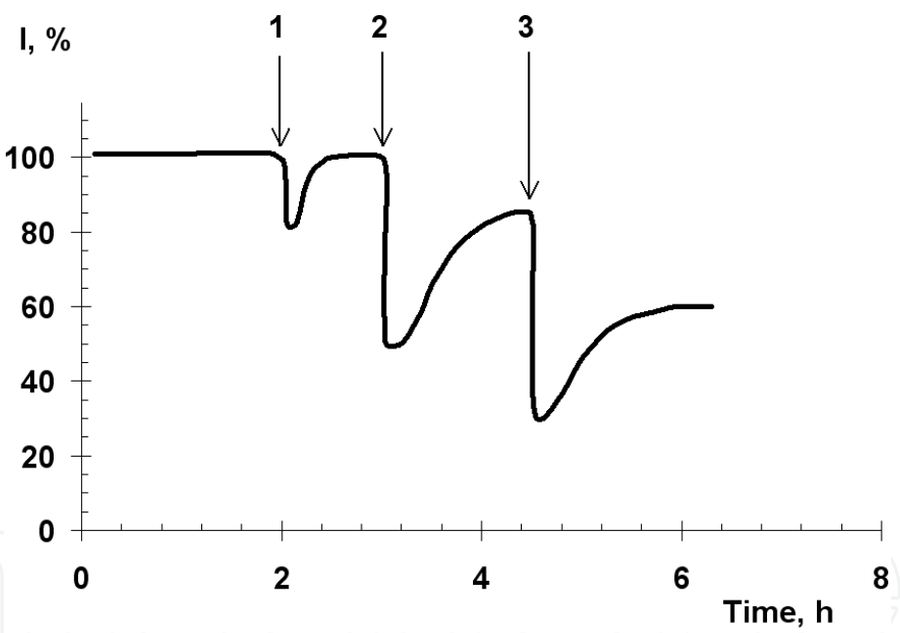

Figure 5.
The kinetic response of PVA-immobilized P. phosphoreum str. №331 KM *MSU, in continuous flow-through monitoring system to $CuSO_4$, volume injected with 100 μl. Initial concentration: 1–0.5, 2–1, and 3–10 mM.*

luminescent activity. Fast restoration of activity allows using one immobilized preparation for repeated probe detection (flow rate—0.5 ml/min, resistance time injected probe—30 s, reactor volume—1.5 ml, and 1 granule 15°C).

A continuous water toxicity system with same parameters for biomonitoring, using Ca,Sr-alginate gel immobilized *P. phosphoreum*, was also studied [16, 30].

Higher survival of cells with preservation of specific activity of light emission, at level of the free cells, presented to the given work, reflect advantages cryogenic immobilization photobacteria in PVA carrier and application PVA based biosensors for detection of toxicants both in discrete and continuous toxicity monitoring systems.

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