Microfluidic systems in biology

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Abstract

We describe key stages in the development of a genosensor-based bioanalytical complex capable of functioning in a micro/nanofiuidic system (MNFS), which is intended for detecting damaging agents in liquid media. The genosensor represents a recombinant E. *coli* cell modified with a plasmid containing the *gfp* reporter gene (encoding green fluorescent protein (GFP)), the transcription of which is controlled by a promoter sensitive to damaging factors of the external environment. The level of reporter gene activity can be monitored by measuring the level of induced fluorescence.

Keywords: Genosensor, bioanalytical complex, micro/nanofiuidic system, LIGA technology

1. Introduction

At present, there is a revolution taking place in world science and technology that consists in passage to small and ultrasmall (so-called "lab-on-a-chip") devices intended for determining the functions of biological macromolecules, genomes, cells, and cell structures with a view to clinical diagnostics and biochemical investigations. The new approach is based on the use of micro/nanofluidic system (MNFSs).

Micro/nanofluidic bioanalytical systems are integrated devices comprising advanced, sophisticated high-tech elements of hydraulics, micromechanics, electronics, optics, biochemistry, molecular biology, gene and cell design, and bioinformatics. The importance of R&D in micro/nanofluidic technology is confirmed by snowballing growth in the number of publications in this field. In particular, more than 3000 articles devoted to MNFSs and related subjects have been published in 2004-2006 (see, e.g., an insightful series of articles in Nature [1-7]). In Russia, this direction of research is being developed, for example, at the Institute of Physicochemical Medicine (Moscow) and institutions of the Siberian Branch of the Russian Academy of Sciences [8, 9].

The basic element in an MNFS is a glass or polymer plate with a multilevel system of channels, microreactors, valves, and pumps manipulating pico- and nano-liter volumes of liquids [10-14]. The fabrication of MNFSs is based on the use of technologies such as LIGA, laser ablation, lithography, diamond powder processing, stamping, and molding. Such systems ensure preset characteristics of microscopic flows, exact dosing of dissolved substances, optimum computer-aided regulation of flows, and conjugation with external macroscopic devices.

The most important properties of MNFSs are (i) the laminar flow in microfluidic channels and (ii) the possibility of exactly calculating the transported amounts of substances to various elements of the system with allowance for the geometry of various channels, distribution of pressures, and properties of liquids. By virtue of their laminar character, flows in MNFSs are mixed in the diffusion regime. Laminar flow and diffusion mixing are

combined so as to provide the necessary gradients of dissolved substances in microfluidic channels. In addition, MNFSs are characterized by an increased ratio of the liquid surface to the volume, which can provide optimum conditions for chemical reactions.

Using MNFSs, it is possible to implement on a qualitatively new level methods and facilities of modern analytical chemistry such as flow injection analysis and high-efficiency capillary electrophoresis, to use the advantages of laminar flows, to optimize the ratio of the liquid volume to the reaction surface in microreactors, etc. [15-20]. An important advantage of MNFSs is the ability to handle individual cells at various stages of their development and to deal with noncellular systems.

The radical miniaturizatiuon of experimental devices achieved by means of micro/nanofluidic technologies opens the way for novel, extremely low-cost but highly effective methods of solving a broad range of both basic and applied problems in molecular and cell biology, biotechnology, and medicine. The ability of MNFSs to operate with pico- and nanoliter volumes of liquids leads to a radical (by several orders of magnitude) decrease in the amounts of analyzed biological samples such as macromolecules, cells, and biologically active substances. Accordingly, there is drastic decrease in the consumption of high-cost reagents and in the cost of experiments and analyses carried out in micro/nanofluidic bioanalytical systems. Highly sensitive methods available for the analysis of substances and monitoring of processes, such as fluorescent microscopy and spectroscopy, makes it possible to detect desired components at a trace level (up to individual molecules). The volumes of liquids that can be handled in microfluidic devices are comparable with cell dimensions or even several orders of magnitude smaller, which opens basically new research possibilities in the fields of molecular and cell biology, biotechnology, and medicine.

The present article describes the key stages in the development of a genosensor-based bioanalytical complex functioning in an MNFS, which is intended for detecting damaging agents in liquid media. The genosensor represents a recombinant E. *coli* cell modified with a plasmid containing the *gfp* reporter gene (encoding green fluorescent protein (GFP)), the transcription of which is controlled by a promoter sensitive to damaging factors of the external environment. The level of reporter gene activity can be monitored by measuring the level of induced fluorescence.

The main stages of our study are (i) the fabrication of an MNFS by means of the LIGA technology, (ii) construction of a base vector for cloning genosensor promoters, (iii) computer-aided design and experimental construction of a vector plasmid containing the *gfp* reporter gene, whose transcription is activated from the E. *coli dps* gene promoter, (iv) investigation of the reporter gene activation in response to the presence of hydrogen peroxide and phenol (model oxidative stress agents), (v) selection of conditions for positioning the genosensor bacterial cell in the microfluidic channel, and (vi) development of a method for fluorescent signal monitoring in pico- and nanoliter volumes of the microfluidic module.

2. Technology of micro/nanofluidics

Using the LIGA technology, we have developed an MNFS with a channel width of a few microns and above. The high quality of channels of the MNFS was confirmed by laser scanning microscopy using an LSM 500 Meta instrument.

The fabrication of MNFSs was based on deep X-ray lithography in the photon energy range of *6-W)* keV using a VEPP-3 storage ring at the Budker Institute of Nuclear Physics (Novosibirsk). The technological process included the stages: Substrates for X-ray lithography templates were SU-900 carbon-reinforced glass composite plates with lateral dimensions of 40x40 mm and a thickness of 0.5 mm. This composite possesses a unique combination of properties, including good strength (close to that of glass), the ability of polishing, high chemical and corrosion resistance and electric conductivity, and low

absorption in the spectral range of synchrotron radiation (SR). In order to ensure the adhesion of a resist layer, the surfaces of substrates were subjected to special preliminary chemical treatment by exposure in concentrated sulfuric acid for 30 min at room temperature, rinsed in distilled water, and dried in a thermal box at 200°C. Then, each plate was covered with a commercially available negative photoresist (SU-8). The coating technology allows layers with thicknesses of 1 - 200 μm to be applied. This process was carried out at room temperature in a centrifuge operating at 3000 rpm. The deposited coatings were subjected to soft annealing in a thermal box at 95°C for 16 min.

In order to obtain X-ray lithography templates, it was necessary to form a relief corresponding to the desired MNFS topology. This topology was patterned in the resist layer by a collimated SR beam with a cross section of 40 × 40 μm on a LIGA station. For this purpose, the substrates were translated relative to the SR beam by means of a twocoordinate scanner (accurate to within 1 μm) at a velocity of 50 μm/s. As a result, the resist layer acquired a latent image of the desired MNFS topology. The absorbed radiation doses in this stage amounted to 40–60 J/cm^3. After irradiation, the substrates were repeatedly annealed in a thermal box at 95°C for 4 min in order to ensure cross-linking of exposed regions of the resist layer and minimize the residual internal stresses. The no polymerized (unexposed) regions of the resist were removed by chemical etching of the plates with propylene glycol methyl ethyl acetate (PGMEA) etchant (MicroChem Co., United States) in an ultrasonic bath for 4 min at room temperature. Finally, the etched plates were washed with isopropyl alcohol. Figure 1 outlines the sequence of stages used for the preparation of substrates for X-ray lithography templates.

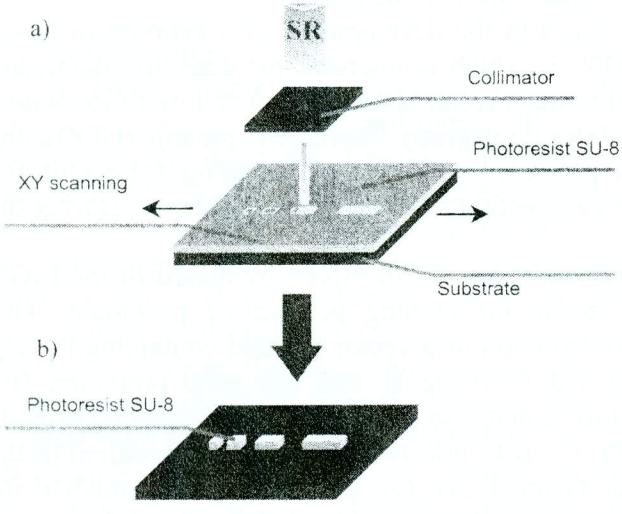

Fig. 1. Preparation of substrates for X-ray lithography templates: (a) carbon-reinforced glass composite substrate covered with photoresist is exposed to collimated SR beam for obtaining a preset MNFS topology pattern; (b) unexposed regions of photoresist are removed by chemical etching.

In order to obtain the template, the plate with a microstructural pattern was galvanically coated with an 8- to 10-μm-thick gold film. As a result, a template with the preset MNFS topography was obtained.

Poly(methyl methacrylate) (PMMA) plates with a thickness of 5 mm were exposed to white (non-filtered) SR the LIGA station via the X-ray lithography template obtained as described above on. The exposure was performed in a scanning mode in which the sample and template (mounted in front of the sample) were scanned across the SR beam at a velocity of 10 cm/sec within amplitude of 10 mm. Then, the exposed regions of samples were removed by etching with GG etchant in an ultrasonic bath. The quality of obtained microchannels was determined by the exposure dose and the conditions of etching. Figure 2 shows photographs of an MNFS structure on an SU-8 photoresist on a polished carbon-reinforced glass composite substrate, a template obtained upon photoresist removal and galvanic gold plating, and final PMMA plates bearing MNFSs with microfluidic channels of 38 μm width and depth.

The given MNFS (Fig. 3) is comprised of four chambers and three channels, one of which was intended for the arrangement of genosensor cells. Then, a liquid carrying

damaging agents is pumped via the channel with these cells. The transition from macroscopic to microscopic level is provided by microsyringes and microdrives.

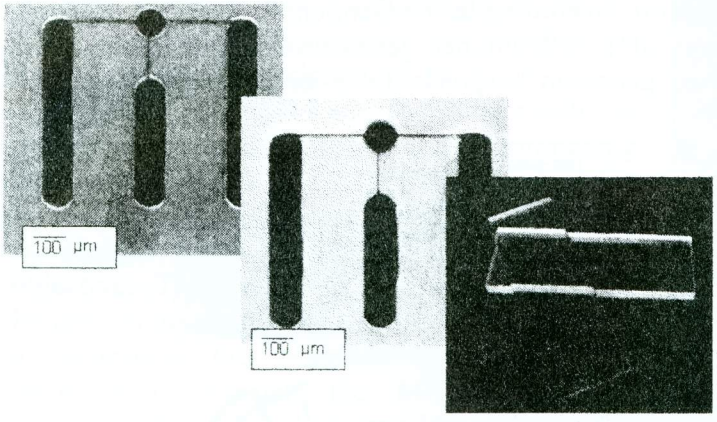

Fig. 2. Photographs of an MNFS structure on SU-8 photoresist on carbon-reinforced glass composite substrate, a template obtained upon photoresist removal and galvanic gold plating (8–10 μm), and (c) PMMA plates (25 × 750 mm) with MNFSs (channel width, 38 μm).

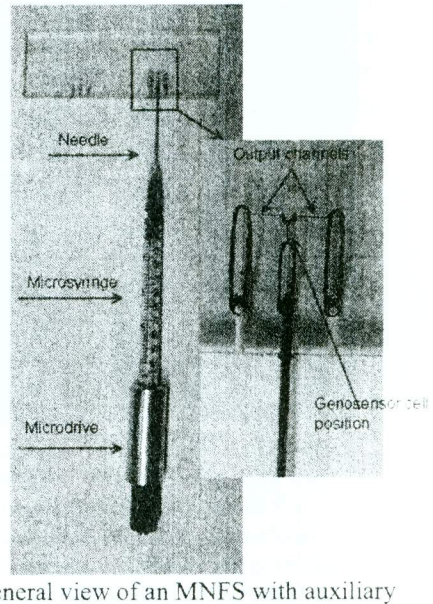

Fig. 3. General view of an MNFS with auxiliary devices ensuring the transition to a macroscopic level. The system of chambers and channels is filled with an ink.

3. Constructing genosensors

Bacterial cells contain a large number of genes involved in protective response to exogenous damaging factors. The group of most frequently encountered factors activating the protective response of a bacterial cell includes toxic agents of various types, deficit or excess of cell metabolites, temperature, etc. Genes activated by such detrimental (stress) factors are called susceptible (sensor) genes.

The activation of sensor genes can be mediated by various mechanisms, but all of these are based on a change in the functional state of proteins regulating gene transcription. Such changes can either be mediated by the interactions of regulatory proteins with some metabolites or be caused by processes induced by external factors related to oxidation, phosphorylation, or acetylation of regulatory proteins, their interaction with other proteins, etc.

We have used some promoters of sensor genes and the *gfp* reporter gene of *E. coli* (encoding green fluorescent protein (GFP)) to obtain recombinant plasmids. When introduced into a bacterial cell, these plasmids make it possible to determine the level of stressor action, judging by the intensity of induced fluorescence.

Figure 4 shows the scheme of a computer-aided experimental approach to the rational design of artificial molecular-genetic systems (genosensors), which consists of three stages. In the present study, these stages have been realized for the creation of a polyfunctional genosensor based on the *yfiA* gene [22] and *dps* gene promoters of *E. coli*. The dynamics of genosensor response was studied in a microfluidic system capable of operating with individual cells, to which the input metabolites were supplied via a microfluidic channel. The production of a fluorescent protein in genosensor cells activated by the metabolite was detected by means of a fluorescent microscope.

A base vector plasmid was constructed for cloning genosensor promoters, which contains a reporter gene with the 5' end containing restriction sites for inserting target promoters. Computer-aided design of genosensors was carried out using the GenSensor database, which

makes it possible to search for stress-sensitive promoters suitable for the creation of genosensors responding to environmental damaging agents. Bioinformatics analysis allowed us to select the *dps* gene promoter of *E. coli*. According to the GenSensor database, this is a potential promoter for the construction of polyfunctional genosensors; it is capable of activating the transcription of reporter genes in response to a broad range of toxic environmental factors.

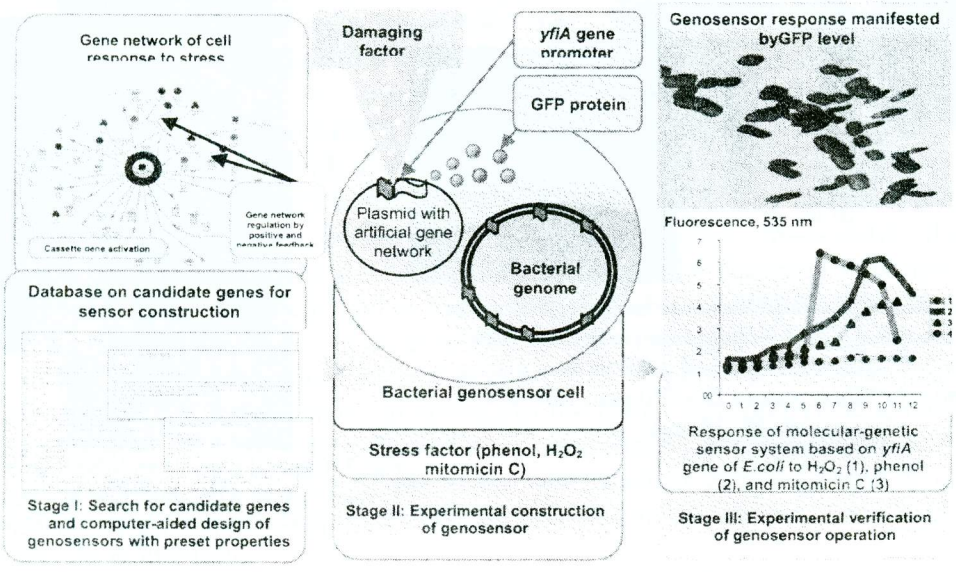

Fig. 4. Computer-aided experimental approach to the rational design of artificial molecular-genetic systems (genosensors) with preset properties.

4. Studying the sensitivity of genosensors to environmental damaging agents in a microfluidic system

The genosensor constructed as described above was used as a biological detector of oxidative stress in an MNFS fabricated in a PMMA matrix using the LIGA technology. PMMA is a solid transparent material convenient for fabricating microfluidic systems and monitoring biochemical processes in these systems by optical methods. This polymer exhibits no fluorescence under irradiation by an Ar laser, which has been used to excite emission from the GFPvaa reporter protein.

The surface of PMMA is hydrophobic and exhibits low adhesion with respect to *E. coli* cells. In order to provide for the immobilization of genosensor cells, a special pretreatment of the microfluidic channel surface is necessary. We processed this channel by means of open oxygen plasma at 0.1 bar, which led to the appearance of negative charges fixed on the channel surface. Then, the plasma-modified surface was coated with polylysine, which was retained on the surface due to electrostatic interactions. This was achieved by treating the channel with a 1 mg/ml aqueous solution of polylysine, followed by drying in a flow of sterile air. In order to introduce living cells of the *E. coli*/pDps-gfp genosensor, a culture of these cells in a minimal medium was passed through the central channel of the microfluidic system (Fig. 3). The cells stuck to the surface due to the interaction with polylysine molecules. Nonbonded cells were washed from the channel surface by minimal M9 medium.

After fastening genosensor cells in the microfluidic channel, a minimal medium containing hydrogen peroxide (oxidative stress agent) at a concentration of 2 mM was introduced into the channel. The MNFS with recombinant *E. coli*/pDps-gfp cells immobilized in the channel was studied by fluorescent microscopy 40 min after the onset of activation. The genosensor response to the introduction of oxidative stress agents was studied using an

LSM500 Meta (Carl Zeiss) laser scanning microscope. GFPvaa molecules were excited by Ar laser radiation with a wavelength of 488 m. Laser scanning microscopy is capable of measuring a signal originating from the narrow optical layer (about 0.7 μm thick) in which the biological objects are situated, and discriminating background signals from below and above of this layer. The response signal was detected in a wavelength range of 505-525 nm via a 63^X objective. The data were processed by LSM Image Examiner software (Carl Zeiss). In order to determine the positions of cells in the microfluidic channel, the MNFS was also examined by a method providing an image analogous to that in the differential contrast mode. Using the aforementioned software, it is possible to fit the images obtained for fluorescence sections and by optical microscopy. This makes it possible to detect individual cells that produce GFPvaa (Fig. 5).

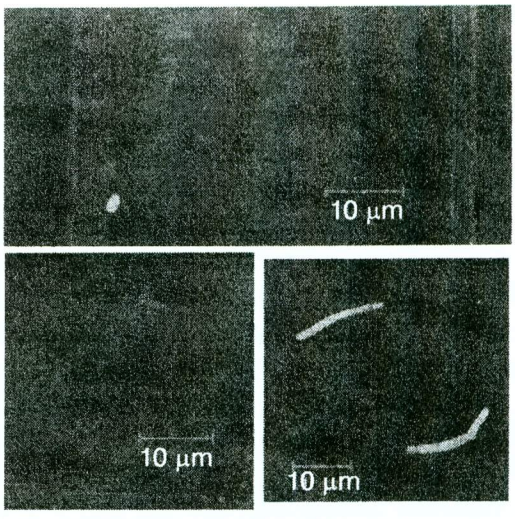

Fig. 5. Cells of recombinant *E. coli*/pDps-gfp strain in an MNFS. Bright signals show the GFPvaa fluorescence in response to the activator injection. The results of confocal microscopy are matched to the image obtained using the conventional optical microscopy in the differential contrast mode (dark signals).

5. Conclusion

We described the key stages in the development of a genosensor-based bioanalytical complex capable of functioning in a micro/nanofluidic system (MNFS), which is intended for detecting damaging agents in liquid media. This approach has an interdisciplinary character and makes use of methods from the three fields: mechanotronics, bioinformatics, and molecular genetics.

Thus, the results of our investigation show that the proposed interdisciplinary approach can be used for obtaining bacterial cells-genosensors capable of functioning in MNFSs and detecting environmental damaging factors. Using the proposed methods, it is possible to construct multiposition MNFSs with a large number of channels, each containing a certain genetically modified cell (or an artificial molecular-genetic structure) capable of responding, by the expression of a specific sensor gene, to the presence of a certain damaging factor in the analyzed liquid.

Such bioanalytical systems capable of operating with ultrasmall (pico- and femtoliter) volumes of analyzed liquids-can help us in solving a broad rangr of both basic and applied problems in various fields such as medicine, ecology, pharmacology, biotechnology, and biosafety. In particular, it can be used for (i) multiparametric monitoring of the environment aimed at revealing mutagenic factors; (ii) detecting biologically active, toxic, and immunoactive substances in the ambient medium, food products, water, air, and soil; (iii) large-scale screening of existing and new pharmacological preparations; (iv) detecting dangerous technological products; etc.

Based on the bioinformatics approach (GenSensor database), it is possible to seek gene promoters for use in genosensor structures responding either to a broad range of environmental damaging factors (as in the case of *E.* co///pDps-gfp cells) or to particular damaging agents (toxins, heavy metal ions, detergents, etc.). Genosensors can also reveal the presence of unknown substances in analyzed liquids, leading to particular kinds of cell damage (e.g., damage of DNA, protein, or membrane structure) [21-24].

The proposed approach also provides a starting point for the development of more

complicated, artificial molecular-genetic structures based on the use of certain sets of gene promoters, which activate the expression of both reporter and regulatory proteins. On this basis, it is possible to study the functions of unknown genes and the dynamics of complicated gene networks.

The MNFS fabrication and application technologies have good prospects in basic science and in solving practical tasks. At present, new-generation miniature bioanalytical complexes (lab-on-a-chip) based on MNFSs are under development, which can even replace analytical laboratories [25]. The unique possibilities of creating microchannels of complicated configurations in multiposition MNFSs, incorporating various types of microreactors, and providing optimum computer-aided control (ensuring preset characteristics of micro- and nanoscopic flows), as well as effective means of conjugating MNFSs to external macroscopic devices, make it possible to implement sophisticated bioanalytical systems with multistage functioning mechanisms, capable of solving a broad spectrum of problems in various fields.

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