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Aptamer-based Microfluidic Device for Isolation of Circulating Tumor Cells

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Abstract. The paper presents the design of a microfluidic device for capturing circulating tumor cells, a method for immobilizing aptamers on the surface of the chip reaction chamber, and an algorithm for controlling flows in microchannels. It was shown that the maximum efficiency of aptamer immobilization was achieved by treating the chip surface with a 50% aqueous-alcoholic NaOH solution. In addition, it was found that the most number of tumor cells of the MCF7 culture attached on the surface coated with aptamers at low flow rates of less than 2 μl/min. It was noted that immobilized tumor cells are capable of being retained by aptamers at flow rates of up to 200 μl/min.

Keywords: aptamers, immobilization, circulating tumor cells.

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Introduction

Rapid detection and treatment of oncological diseases is one of the most important problems of our time. One of the ways to solve it is the development of biosensors for diagnosing the disease at early stages by detecting circulating tumor cells.

Aptamers, which are short segments of DNA or RNA and are often called synthetic analogues of antibodies, possess significant potential for application as recognition elements of biosensors

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[1–3]. Among the various types of aptasensors, electrochemical ones are the most widely used [4], including in the field of oncology [5]. At the same time, optical aptasensors have recently been actively developed [6, 7]. They can become an alternative to electrochemical sensors due to simplified production technology and low cost.

Among the various materials used to manufacture optical biosensors, thermoplastics such as PMMA deserve special mention [8]. They possess such advantages as optical transparency, biocompatibility, relative ease of mechanical processing and surface modification [9]. In addition, technologies that allow for the mass production of PMMA chips have been well studied [10]. The only disadvantage when using PMMA is its relatively complex sealing methods, requiring the use of solvents or high heating temperatures [11].

This paper presents the results of the development of a microfluidic optical system based on aptamers, which can be used to detect circulating tumor cells. To develop it, it was necessary to solve a number of problems: firstly, to select a method for immobilizing aptamers on the surface of the reaction chamber of the chip; secondly, it was necessary to develop a design for the aptasensor channels and an algorithm for controlling fluid flows in its channels.

Materials and methods

The following materials were used in the work: Poly(methyl methacrylate) of the Novattro (Sovplast, Russia) and GF27161513 (GoodFellow, England) brands, 1,2-dichloroethane, analytical grade (Soyuzkhimprom, Russia), NaOH: sodium hydroxide (Sigma, USA), EDC: N-(3-dimethylaminopropyl)-N‘-ethylcarbodiimide hydrochloride (Sigma, USA), NHS: N-Hydroxysuccinimide (Sigma, USA), MES buffer: 2-(N-morpholino)ethanesulfonic acid (Sigma, USA), aptamer 2108 to lung cancer, primer with amino group AmPrCom5 (IDT, USA), primer with fluorescent label Cy5Com3‘, aptamer to breast cancer BC1 with FAM-label (5’-/FAM/CTC CTC TGA CTG TAA CCA CGA TAT TCG TAT TGA TGC GTG CAC GCG GCG TGT GTA GTT TCG GCA TAG GTA GTC CAG AAG CC-3’) manufactured by Lumiprobe (Russia), primer with amino group AmPrCom3 (IDT, USA), breast cancer cell line MCF7, phosphate buffered saline pH 7.4 (PBS).

The following equipment was used in the work: Modela MDX-40A milling machine (Roland, Japan), NIB630FL microscope (Nexcope, China), AL-1000HP syringe pump (WPI, England), Clariostar Plus multimodal reader (BMG, Germany).

Manufacturing of microfluidic chip. The chip design was developed using the Kompas-3D (Ascon, Russia) CAD software. The chip was made of poly(methyl methacrylate) (PMMA). The chip channels were made by micromilling with end mills with a cutting part diameter of 1 mm manufactured by Roland (Japan) and Datron AG (Germany). The spindle speed was 15000 rpm, the cutter feed rate was 1500 mm/min for Roland cutters and 1200 mm/min for Datron AG cutters, the milling depth of the rough layer was 0.2 mm, the finishing layer — 0.1 mm. After milling, the two parts of the chip (with channels and the cover) were sealed using 1,2-dichloroethane solvent.

Preparation of 2108 aptamer with an amino group and a fluorescent label Cy5. We took 50 μl of aptamer 2108, primer Cy5com3‘ and primer AmPrcom5‘, then mixed them in equal concentrations of 1 μM, then heated to 95°C for 10 min and cooled to room temperature, after which incubated for 30 min on a shaker at 350 rpm. The final concentration of the resulting construct 3‘-Cy5-2108-NH_2-5‘ was 0.33 μM.

Treatment of the chip channel surface in an alkaline NaOH solution. The reaction chamber

of the microfluidic chips was filled with 1 M 50% aqueous-alcoholic NaOH solution, after which the chip connectors were sealed with Parafilm to prevent liquid evaporation and left to incubate for 24 hours. After that, the chip was washed with PBS buffer and dried with compressed air.

Preparation of 5'-FAM-BC1-NH_2-3' aptamer. The initial BC1 aptamer with a FAM label and the amino primer AmPrCom5 were used to prepare the aptamer. First, the initial aptamer and primer were mixed in a 1:1 concentration ratio, then heated to 95°C for 10 min, and then the resulting aptamer was cooled on ice. As a result, the BC1 aptamer was obtained, modified with a FAM label at the 5' end and an NH_2 group at the 3' end.

Immobilization of the aptamer in the microfluidic chip. The aptamer 5'-FAM-BC1-NH_2-3' was introduced into the reaction zone of the microfluidic chip and left there for 30 minutes. After that, the reaction zone was washed with PBS buffer and dried with compressed air.

EDC + NHS treatment in MES buffer. For this, 10 mg/ml EDC and NHS were added to 0.1 M MES buffer with pH 6.0, the reaction chamber of the microfluidic chips was completely filled with this solution and incubated for 60 min at room temperature. Then the chips were repeatedly washed with PBS buffer and dried with compressed air.

Results and discussion

Microfluidic chips were fabricated for experiments on the immobilization of aptamers on the polymer surface of the reaction chamber of the microfluidic device. Their design is represented by a single reaction chamber with an input and output channel (Fig. 1). The depth of the channels was 0.5 mm, the volume of the reaction chamber was about 10 μl.

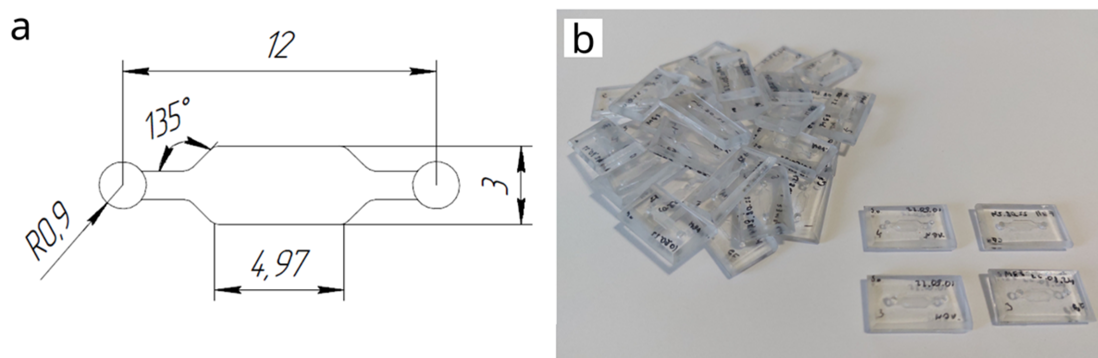

Fig. 1. General view of the manufactured microfluidic chips: a) channel design drawing; b) photograph of the manufactured chips

The fluorescence signal was measured using a Clariostar plus multimodal reader (BMG, Germany) in the sequential scanning mode of the reaction chamber surface of the microfluidic chip.

The applicability of Novattro and GF27161513 plastics to surface milling was assessed. For this purpose, the magnitude of the fluorescence value spreads after immobilization of aptamers by physical adsorption was compared for chips without preliminary surface treatment. As a result, a heat map of the initial fluorescence signal data (Fig. 2) in the multimodal reader was obtained.

It was found that GF27161513 PMMA had a smaller spread of values within one group, that is, under the milling mode used, it is a more suitable material. Consequently, a non-optimal

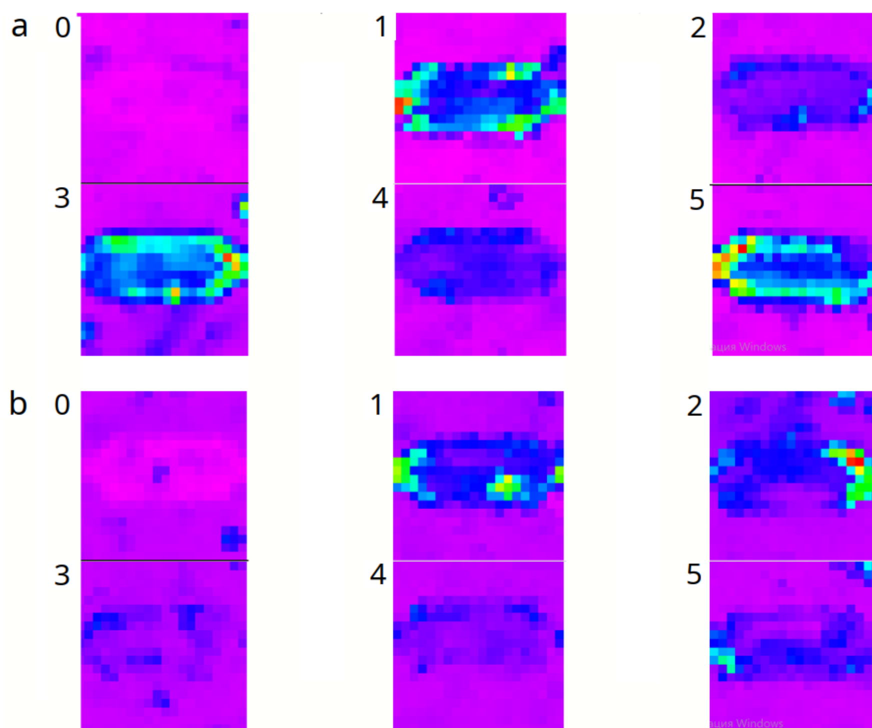

Fig. 2. Heat map of aptamer fluorescence distribution without surface pretreatment: a) Novattro PMMA; b) GF27161513 PMMA. Chip numbering: 0 — empty chip without aptamers, 1–5 — replicates within the group

milling mode was used for PMMA Novattro, which led to the formation of additional cracks on the surface, where aptamers could be attached. This may be due to the difference in plasticizers that are added by manufacturers during the production of PMMA and are their trade secret, so further studies are required in the future to find more suitable milling modes for PMMA Novattro. Another possible explanation may be that the initial number of COOH groups on the surface of PMMA Novattro is greater than that of GF27161513, which also requires additional study.

In addition to physical adsorption of aptamers, chemical modification of the surface of the reaction chambers of the microfluidic chips was carried out. For this, treatment with a 50% aqueous-alcoholic solution of NaOH was used, as well as covalent modification of the surface using carbodiimide crosslinking with EDC and NHS in MES buffer.

The results of aptamer fluorescence measurements after immobilization in chips with different methods of surface treatment are shown in Fig. 3. The results were processed by summing up all measured fluorescence points within the reaction chamber and their subsequent statistical processing. The height of the columns corresponds to the median value, and the upper and lower limits of the error bars show the quartiles Q1 and Q3, respectively. It was shown that chemical surface treatment of GF27161513 PMMA had virtually no effect on the fluorescence intensity, while a significant effect of chemical surface treatment was noted for Novattro PMMA. The maximum effect was noted when treated with a 50% aqueous-alcoholic solution of NaOH. On average, the efficiency of aptamer immobilization when treated with NaOH was 17% for

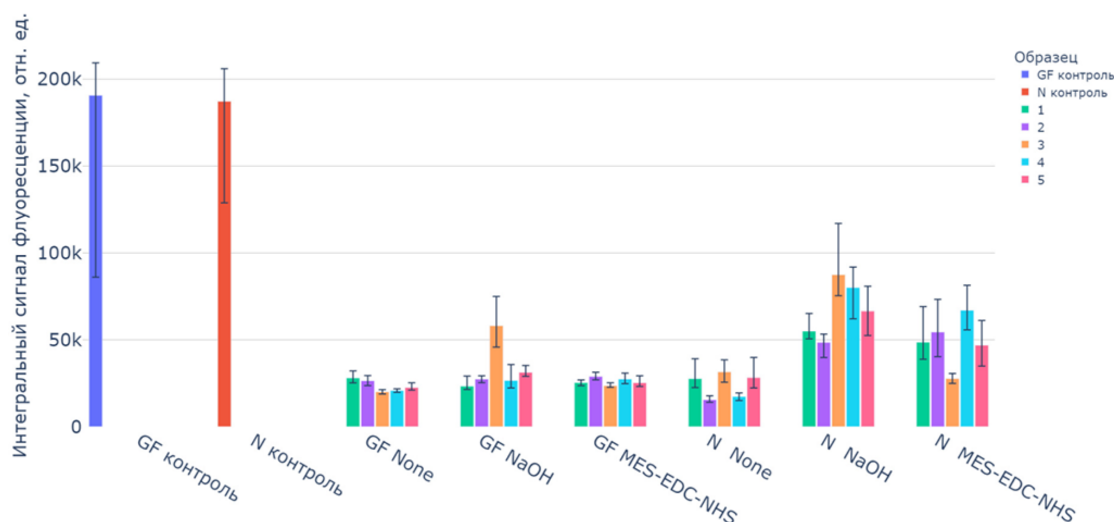

Fig. 3. Fluorescence intensity of 3‘-Cy5-2108-NH_2-5‘ aptamers immobilized in reaction chambers of microfluidic chips with different surface treatments: none — no pretreatment, NaOH — treatment with 50% aqueous-alcoholic NaOH solution, MES-EDC-NHS — carbodiimide crosslinking using EDC and NHS in MES buffer. The fluorescence value reflects the median value, and the upper and lower limits of the error bars show quartiles Q1 and Q3, respectively. GF — GF27161513 PMMA, N — Novattro PMMA, 1–5 — replicates within one group. Chips filled with a solution with aptamers were used as reference

GF27161513 versus 36% for Novattro, and when treated with EDC-NHS, the efficiency was 14% for GF27161513 versus 26% for Novattro. Based on these data, a decision was made to use Novattro PMMA further in the work.

To solve the second task, it was necessary to develop a design for the aptasensor channels and an algorithm for controlling the fluid flows in its channels. It was decided to use the principle of isolating tumor cells from the general cell flow and depositing them on the surface of the microfluidic chip walls. The deposition should be achieved by entangling tumor cells onto aptamers as they move through the chip channel.

MCF7 breast cancer cells in PBS buffer were used as a model object for working with the obtained method of aptamers immobilization on the surface of the reaction chamber of the chip.

A microfluidic chip design was developed (Fig. 4), which was a modular structure of several PMMA plates. The plates consisted of a base substrate to which three compartments were attached: two dead volume chambers at the edges and a reaction chamber with immobilized aptamers in the center. The channeled surface of the reaction chamber was 4 mm wide and 8 mm long with 45° bevels, the depth was 0.5 mm. The channeled surface of the dead volume chambers was 11 mm wide, 26 mm long and variable in depth. In the area of the connection with the reaction chamber, the depth was 0.5 mm, in the rest of the chamber, the depth was 1.5 mm. Between these parts, there was a smooth transition in depth. The width of the channels connecting the compartments was 1.1 mm.

Immobilization of aptamers on the reaction chamber surface was performed in 50% aqueous-alcoholic NaOH solution for 24 hours, after which the chamber was washed with PBS, dried and sealed. For sealing, the reaction chamber module was installed in the appropriate place on the base substrate, pressed, and 1.5 μl of 1,2-dichloroethane was introduced through each of the

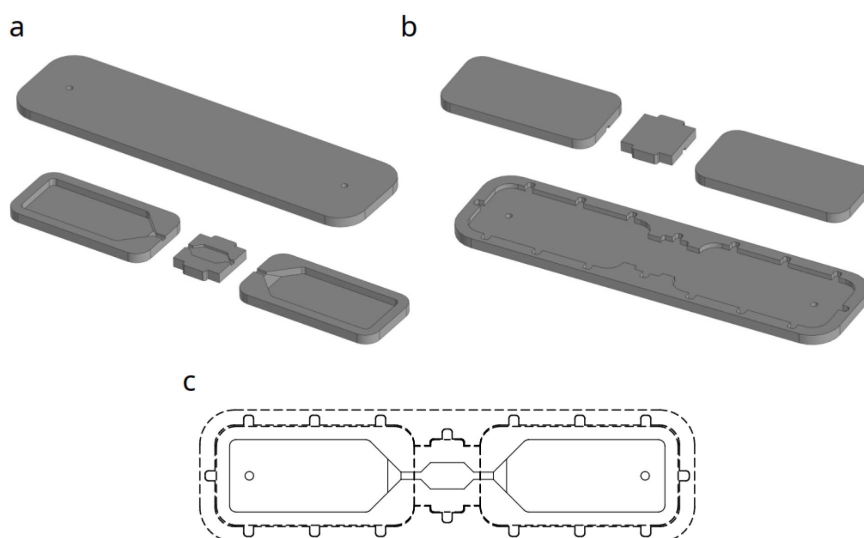

Fig. 4. Design of a microfluidic chip for isolation of circulating tumor cells: a) 3D model of the modular chip design, view of the plates with channels; b) 3D model of the modular chip design, view of the base plate without channels; c) drawing of the microfluidic chip channels (solid thin line)

technological holes of the base substrate (Fig. 4, b). Immobilization of aptamers modified at one end with NH_2 group in the chamber was performed by introducing aptamers at a concentration of 10 μg/ml using a dispenser into the reaction chamber area for 30 min, followed by washing with PBS. The quality of immobilization was assessed using an optical microscope (Fig. 5).

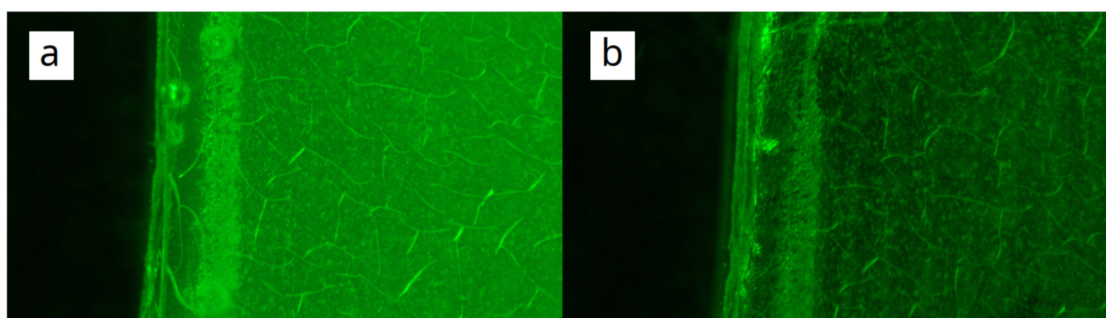

Fig. 5. Quality control of aptamer immobilization on the surface of the reaction chamber of the chip using fluorescence microscopy: a) the chamber was completely filled with a solution with the aptamer; b) the chamber after washing the aptamer and drying. All images were taken with the same exposure of 350 ms

The algorithm for controlling liquid flows in this design consisted of a gradual passage of liquid at minimum speeds through the reaction chamber from a syringe using a syringe pump (Fig. 6).

However, it was found that with such a design, when the reaction chamber is first sealed and the aptamer is immobilized in it, and then the adjacent chambers are sealed, the aptamers are

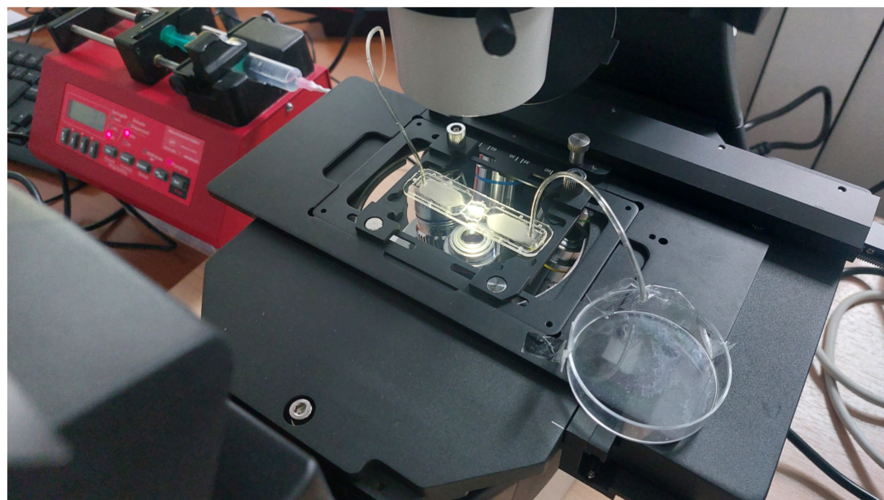

Fig. 6. General view of the laboratory setup. The microfluidic chip is mounted on an optical inverted microscope, and the fluid with tumor cells is supplied to the chip using a syringe pump

destroyed due to the effect of solvent vapors. In this regard, it was necessary to first seal the entire chip and only then immobilize the aptamers. This led to a change in the design of the microfluidic chip. As a result, a new design of the microfluidic chip was developed taking into account the obtained data (Fig. 7).

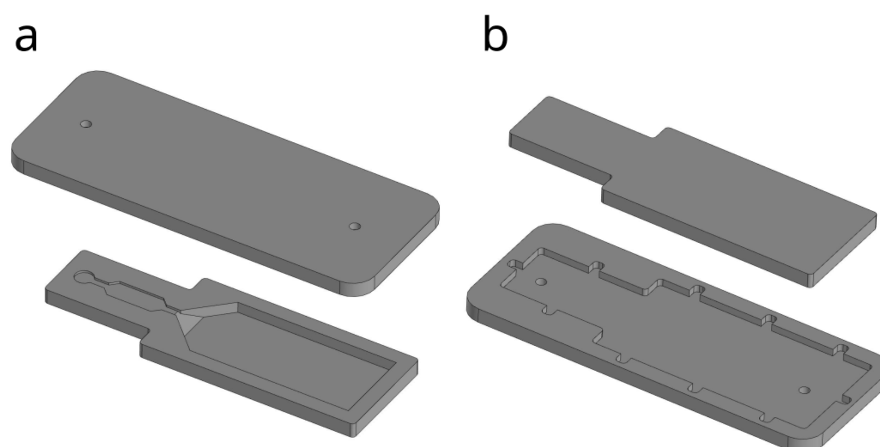

Fig. 7. Design of the second iteration of the microfluidic chip for isolattion of circulating tumor cells: a) general view of the channelized plate; b) general view of the base plate

In the optimized design, the number of connected plates was reduced to two: the base plate and the channeled plate. As a result of testing this design, it was found that if, during aptamer immobilization, part of the solution flows into a deeper region of the dead volume zone, then an overflow of aptamers occurs and, as a result, they predominantly settle in the region with a reduced depth relative to the plane of the reaction chamber (Fig. 8). Therefore, the reaction chamber region and the dead volume region should be made of the same depth. In addition,

it was noted that at a channel depth of 0.5 mm, a significant portion of tumor cells floated above the chamber surface even at minimal fluid flow rates of 1.5 μl/min. Only some of the cells successfully entangled to the surface of the reaction chamber.

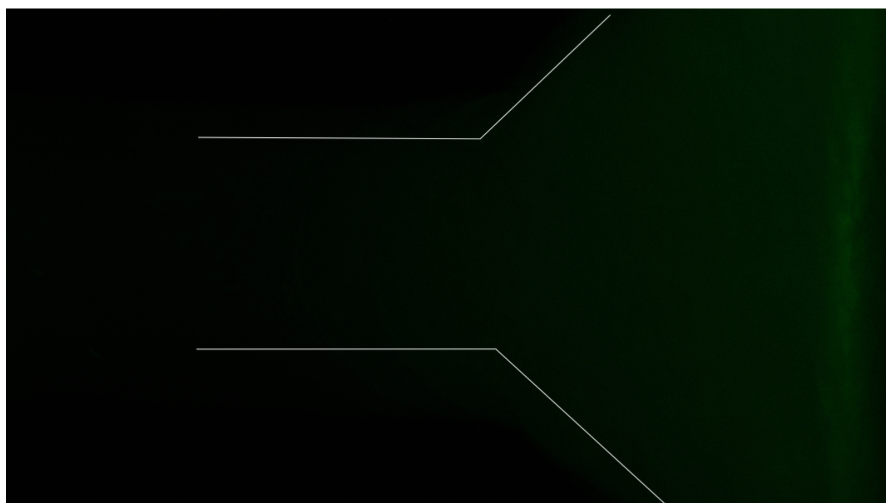

Fig. 8. An example of how aptamers accumulate in the deeper part of the channels during immobilization. For clarity, the channel boundaries are outlined with a white line. As the depth decreases, the fluorescence intensity increases

The next iteration of the microfluidic chip design was developed (Fig. 9). The reaction chamber was reshaped into a serpentine channel 1.5 mm wide and about 18 mm long to facilitate navigation during tumor cell observation. In addition, the channel depth was made uniform across the chip and was 0.3 mm. Finally, the outlet channel was separated from the dead volume chamber. This was made because uneven filling of the chamber with liquid was observed, causing air pockets that could drift into the area with immobilized aptamers. The overall plate size was 51.4 mm in length and 21.4 mm in width.

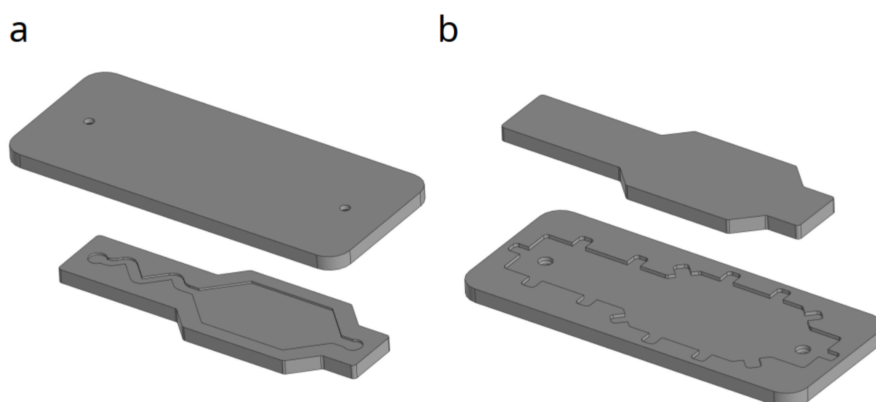

Fig. 9. Design of the third iteration of the microfluidic chip for isolation of circulating tumor cells: a) general view of the channelized plate; b) general view of the base plate

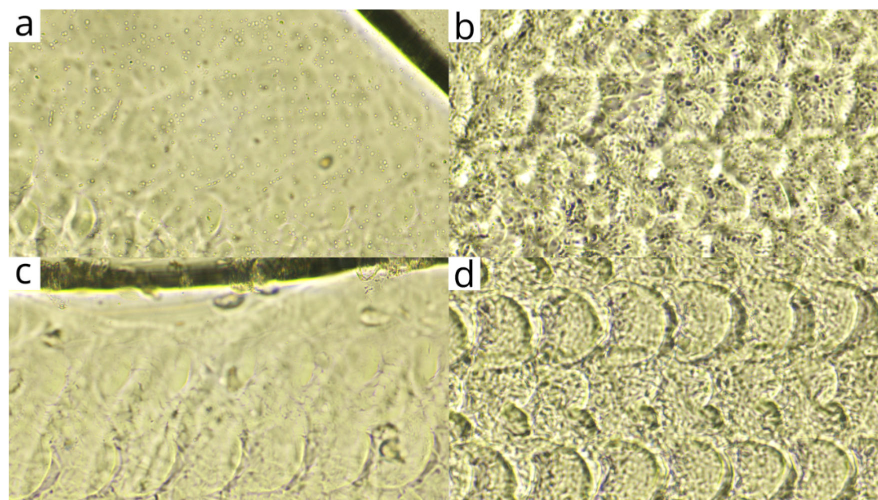

Fig. 10. Pictures of MCF7 cells in a microfluidic chip after 30 minutes of pumping at a flow rate of 0.5 μl/min in different areas of the chip: a) area at the edge of the channel with aptamers; b) area in the center of the channel with aptamers; c) area at the edge of the channel without aptamers; d) area in the center of the channel without aptamers

The third iteration of the microfluidic chip design was tested. The chip was completely filled with NaOH solution and left for 24 hours. After that, the chip was washed with 1 ml PBS and dried. Then, the serpentine part of the channels was filled with aptamer, the dead volume zone was not covered with aptamer. After 30 minutes, the unbound aptamer was washed in the direction from the dead volume zone to the serpentine channel with PBS.

MCF 7 cells were injected into the chip from a syringe using a syringe pump. The feed rate was varied from 0 to 200 μl/min. It was found that the cells began to stably attach to the aptamer-coated surface at low flow rates of <2 μl/min. In Fig. 10, areas a and b are coated with aptamer, and a large number of attached cells are observed there. Areas c and d were not coated with aptamers, and only single cells can be observed there. It is worth noting that the cells attached to the aptamers quite tightly; they did not detach from the surface of the chip channel at high flow rates of up to 200 μl/min. Single cells in areas not treated with aptamer were torn off the surface at such rates and washed away with the flow.

Two possible algorithms for controlling the flows in the channels of the microfluidic chip were determined. The first one consists of a gradual slow supply of liquid through the chip channels at a flow rate not exceeding 2 μl/min. This method is suitable for small sample volumes. If it is necessary to pass a large volume of sample through the chip, an algorithm can be used that consists of quickly passing the sample through the area with immobilized aptamers, while the flow is stopped at certain intervals, allowing the cells to attach to the chip surface for 30 s. After this, the flow movement is resumed, washing away unbound cells from the surface, and again stops after some time. The disadvantage of this method is the need to significantly increase the surface area covered with aptamers, so the first method demonstrated in this work is preferable.

The future prospects of this design will be to reduce the channel height to 0.2 mm to increase the probability of cell contact with the chip surface, develop a method for assessing the effectiveness of cancer cell binding, and conduct experiments on blood samples from cancer patients to

identify circulating tumor cells in them. In the future, it is possible to replace the PMMA material with PDMS on glass if such a configuration proves to be more effective due to the possibility of producing narrower channels up to 50–100 μm wide based on the technology of casting PDMS into master forms printed on a photopolymer printer. As well as a more optically clean surface without cutter marks, which will facilitate the process of cell search. In this case, the PDMS surface will also need to be coated with COOH groups for further immobilization of aptamers on them [12]. The developed microfluidic optical test system based on aptamers can be used to diagnose various types of tumors, not limited to breast cancer or lung cancer. In the future, it is possible to create a system with several zones in which aptamers for different types of cancer will be immobilized. In this way, differentiated diagnostics of tumor diseases will be achieved.

Conclusions

Thus, an aptamer-based microfluidic device for isolation of circulating tumor cells was developed. It was shown that the optimal method for immobilizing aptamers on the reaction chamber surface consisted of treatment with a 50% aqueous-alcoholic NaOH solution. The operation of the device was demonstrated on a model culture of MCF7 breast cancer cells, the highest precipitation efficiency of which was achieved at a rate of less than 2 μl/min. Further work will be aimed at isolating tumor cells from real patient blood samples, as well as further development of the system towards multiplex aptamer-based analysis of different types of tumor cells.

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Микрофлюидное устройство для выделения циркулирующих опухолевых клеток на основе аптамеров

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Аннотация. В работе представлен дизайн микрофлюидного устройства для улавливания циркулирующих опухолевых клеток, способ иммобилизации аптамеров на поверхности реакционной камеры чипа и алгоритм управления потоками в микроканалах. Показано, что максимальная эффективность иммобилизации аптамеров достигается при обработке поверхности чипа 50% водно-спиртовым раствором NaOH. Кроме того, установлено что наибольшее количество опухолевых клеток культуры MCF7 осаждается на поверхности, покрытой аптамерами, при небольших скоростях потока менее 2 мкл/мин. Отмечено, что иммобилизованные опухолевые клетки способны удерживаться аптамерами при скоростях потока вплоть до 200 мкл/мин.

Ключевые слова: аптамеры, иммобилизация, циркулирующие опухолевые клетки.