"LUNG-ON-A-CHIP" AS AN INSTRUMENT FOR STUDYING THE PATHOPHYSIOLOGY OF HUMAN RESPIRATION

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ABSTRACT

"Lung-on-a-chip" (LoC) is a microfluidic device, imitating the gas-fluid interface of the pulmonary alveole in the human lung and intended for pathophysiological, pharmacological and molecular-biological studies of the air-blood barrier in vitro. The LoC device itself contains a system of fluid and gas microchannels, separated with a semipermeable elastic membrane, containing a polymer base and the alveolar cell elements. Depending on the type of LoC (single-, double- and three-channel), the membrane may contain only alveolocytes or alveolocytes combined with other cells — endotheliocytes, fibroblasts, alveolar macrophages or tumor cells. Some LoC models also include proteinic or hydrogel stroma, imitating the pulmonary interstitium. The first double-channel LoC variant, in which one side of the membrane contained an alveolocytic monolayer and the other side — a monolayer of endotheliocytes, was developed in 2010 by a group of scientists from the Harvard University for maximally precise in vitro reproduction of the micro-environment and biomechanics operations of the alveoli. Modern LoC modifications include the same elements and differ only by the construction of the microfluidic system, by the biomaterial of semipermeable membrane, by the composition of cellular and stromal elements and by specific tasks to be solved. Besides the LoC imitating the hematoalveolar barrier, there are modifications for studying the specific pathophysiological processes, for the screening of medicinal products, for modeling specific diseases, for example, lung cancer, chronic obstructive pulmonary disease or asthma. In the present review, we have analyzed the existing types of LoC, the biomaterials used, the methods of detecting molecular processes within the microfluidic devices and the main directions of research to be conducted using the "lung-on-a-chip".

Keywords: *lung-on-a-chip; blood-alveolar barrier; respiratory diseases; microfluidic devices.*

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INTRODUCTION

The diseases of respiratory organs take the leading positions within the structure of the total morbidity of the population in Russia. In the last decades, the prevalence of respiratory disease irreversibly grows world-wide. During the period from 2000 until 2022, the incidence rates in Russia have increased from 317.2 to 422 per 100 000 of the population [1]. The reasons of growing morbidity are caused by the fact, that humans constantly inhale toxic components of the modern urban environment, including various combustion products, micro- and nanoparticles, bacteria, viruses, fungal spores etc., which, in turn, result in chronic alteration of the terminal segments of the respiratory system with developing chronic obstructive diseases, asthma, pneumonia, interstitial and oncological diseases. The respiratory insufficiency that develops as a result of

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«ЛЁГКОЕ-НА-ЧИПЕ» КАК ИНСТРУМЕНТ ДЛЯ ИЗУЧЕНИЯ ПАТОФИЗИОЛОГИИ ДЫХАНИЯ ЧЕЛОВЕКА

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АННОТАЦИЯ

«Лёгкое-на-чипе» (от англ. Lung-on-a-Chip, LoC) — микрофлюидное устройство, имитирующее газожидкостный интерфейс лёгочной альвеолы человека и предназначенное для патофизиологических, фармакологических и молекулярно-биологических исследований гематоальвеолярного барьера in vitro. Устройство LoC включает систему жидкостных и газовых микроканалов, разделённых полупроницаемой эластичной мембраной, содержащей полимерную основу и клеточные элементы альвеолы. В зависимости от вида LoC (одно-, двух- и трёхканальное) на мембране могут находиться только альвеолоциты или альвеолоциты в сочетании с другими клетками — эндотелиоцитами, фибробластами, альвеолярными макрофагами, опухолевыми клетками. Некоторые модели LoC также включают белковую или гидрогелевую строму, имитирующую лёгочный интерстиций. Первый двухканальный вариант LoC, в котором с одной стороны мембраны находится монослой альвеолоцитов, а с другой — монослой эндотелиоцитов, был разработан в 2010 году группой учёных Гарвардского университета с целью максимально точного воспроизведения in vitro микроокружения и биомеханики работы альвеолы. Современные модификации LoC включают те же элементы и отличаются лишь конструкцией микрофлюидной системы, биоматериалом полупроницаемой мембраны, составом клеточных и стромальных элементов и решаемыми специальными задачами. Помимо LoC, воспроизводящих гематоальвеолярный барьер, существуют модификации для исследования определённых патофизиологических процессов, скрининга лекарственных препаратов, моделирования конкретных заболеваний, например рака лёгкого, хронической обструктивной болезни лёгких или астмы. В данном обзоре мы проанализировали существующие разновидности LoC, применяемые биоматериалы, методы детекции молекулярных процессов в микрофлюидных устройствах и основные направления исследований с помощью «лёгкого-на-чипе».

Ключевые слова: *«лёгкое-на-чипе»; гематоальвеолярный барьер; болезни органов дыхания; микрофлюидные устройства.*

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such abnormalities, takes the third place among the mortality causes world-wide [2].

An essential requirement for studying the respiration pathophysiology and for developing the methods of pathogenetic therapy is the presence of an adequate biological model. Most commonly, *in vivo* trials are being used for this purpose, involving the use of small rodents, which allow for investigating the pulmonary reactions in a real cellular environment with specific signals and with registering the functional changes.

Mice and rats are being used as an animal model and when testing the efficiency of medicinal products, including the screening of the functional activity. The animal experiments are complex, cost-intensive and long-term, besides, there is a number of important differences between the respiratory systems of rodents and humans, restricting the extrapolation of data obtained in mice to humans. For example, the epithelium of murine airways contains shorter columnar cells with large number of ciliated cells and lesser number of submucosal glands comparing to the similar epithelium of the human body [3]. These differences can result in obtaining artifacts when modeling the pathophysiological processes in the lungs, leading to the opposite-type reactions when testing the medicinal products on animals and in humans [4]. Despite the high percentage of successful pre-clinical tests, the probability of approving the drug candidates for clinical application by all the parameters is a little higher than 10%, which confirms the insufficient relevance of pre-clinical animal models [5].

Creating an alternative *in vitro* model, allowing for recreating the complex physiological reactions of the human lung in a medium that is convenient for further evaluation, is a promising direction of scientific research, which can both broaden our knowledge on the lung pathophysiology and act as a cost-effective and high-performance platform for screening the efficiency of therapeutic interventions.

Human lungs have a complex multi-level organization. The main structural and functional unit of the lungs is the acinus — the terminal bronchiole with an alveolar sac, consisting of alveoli (intensive vascularised bubble-like structures; Fig. 1, *a*; [6–8]). Upon deep inhaling, the functionally significant surface area of the alveoli, in which the gas exchange takes place, can expand up to 3.3-fold in the normal conditions. Thus, the high expansibility of the alveoli, which, in total, equals up to 0.2 l/gPa, is an essentially important morphological and functional parameter. The uniqueness of the alveolar system is that the alveoli represent the only gas-fluid exchange interface

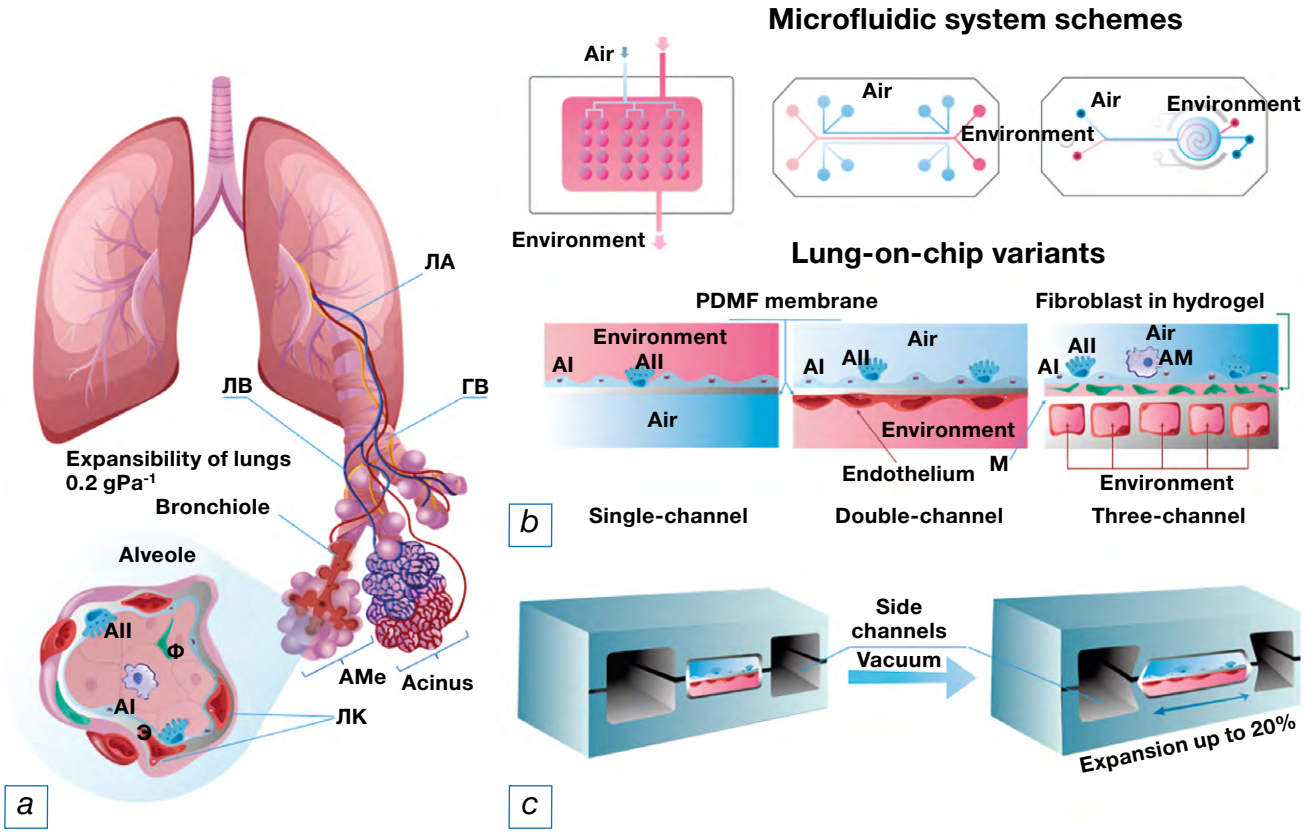

Fig. 1. The principal structure and the variants of the "lung-on-a-chip": *a* — human lung acinus structure; *b* — schemes, developed as of today for the microfluidic devices and LoC variants (from the left side to the right: single-channel — Y. Zhu et al., 2022 [6]; double-channel — D. Huh et al., 2010 [7], three-channel — A. Varone et al., 2021 [8]); *c* — imitation of respiratory movements using the negative pressure in the lateral LoC channels (courtesy of D. Huh et al., 2010 [7]). ЛА — pulmonary artery; ЛВ — pulmonary vein; ГВ — smooth-muscle fibers; AMe — alveolar sac; ЛК — pulmonary capillaries; AI — type I alveolocyte; AII — type II alveolocyte; AM — alveolar macrophage.

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present in mammals, with multiple biochemical and biophysical parameters. This is why the modeling of the alveole in the *in vitro* settings is considered a quite difficult task [9].

Usually, the screening *in vitro* research involve the dimeric (2D) cell cultures, which cannot imitate the microenvironment and by using which, it is not possible to assess the pathophysiological reaction of the tissue as a combination of various cells [10, 11]. In the last decades, the *in vitro* research began using the three-dimensional (3D) cellular spheroids of tissue-engineering constructions, which allow for imitating a more realistic biochemical and biomechanical microenvironment of the tissue or the organ, including the intercellular interactions, the space-time distribution of oxygen, of nutrients and of the final metabolites [12, 13]. However, for the purpose of modeling the functions of the pulmonary acinus, as we have mentioned before, one of the critically important pathophysiological factors is the expansibility of the alveolar structures.

"Lung-on-a-chip" (LoC) is a microfluidic device intended for cell cultivation, which imitates the 3D micro-architecture, the microenvironment and the main physiological functions of human alveoli [14, 15]. The microfluidic technologies allow for generating and precisely adjusting the dynamic flows of fluids with a microliter range, for creating the space-time pressure gradients and other parameters. The LoC technology has a number of significant advantages comparing to the 3D-cultures, in particular, the reproduction of respiratory movements, the possibility of monitoring the transepithelial resistance, the partial pressure of gases in the in-flow and out-flow microchannels, the biochemical composition of the environment and other physico-chemical parameters [16–18]. The LoC technology allows for modeling the specific functional elements of the human lung, such as the blood-air barrier or the mucociliary barrier of the airways. With this, one can recreate both the normal conditions and the specific abnormalities, for example, the condition of the alveoli in a patient with chronic obstructive pulmonary disease or with asthma [19]. The lively interest among the researchers with regard to this direction is confirmed by almost tenfold increase in the number of publications related to the "organ-on-chip" technology during the period from 2010 until 2020 [20].

This review has analyzed the existing varieties of LoC, the biomaterials used, the methods of detecting the molecular processes in the microfluidic devices, as well as the main directions of research involving the "lung-on-a-chip" technology.

THE CELL COMPOSITION OF THE ALVEOLE AND THE LIMITATIONS OF 3D-CULTURES

According to the data from transcriptome analysis of separate cells (single cell RNAseq), in the human lungs, a total of 58 various cellular populations were identified [21]. The alveolar barrier develops as a result of a complex interaction between the type I and II alveolocytes, macrophages, endothelial cells and the extracellular matrix, including the ultra-thin basal membrane. The total thickness of the alveolar-capillary barrier is appr. 1 μm, with the thickness of the basal membrane being less than 100 nm [22].

The basal membrane is porous and elastic (the linear deformation in the physiological settings reaches up to 10%) with the Young's module of 3–7 kPa [23]. Alveolocytes located at the border between the surrounding environment and the organism fulfill multiple important functions, including the barrier one, the maintaining of hydration balance, the elimination of solid particles, the initiation of immune reactions, the production of surfactant and glycocalyx, as well as the regeneration [24]. The unique feature of the pulmonary epithelial cells is the air-fluid interface required for the polarization of the epithelial cells along the apical-basal axis and the secretion of the protective surfactant nano-layer, which reduces the superficial tension and prevents the development of the atelectasis during the inhaling-exhaling [25].

When cultivated in the 3D settings, the alveolar cells form spheroids and organoids, partially imitating the lung acinus structure [26, 27]. Spheroids represent a relatively homogeneous spherical accumulation of cells. The spheroids show quite limited applicability for screening research, for they face problems both with the cultivation of spheroids of similar size and with the control of cell ratios in the co-cultures [28]. Unlike the spheroids, organoids can imitate several basic functions of the lungs in the *in vitro* settings, such as the functional signaling pathways and generating cells with functional cilia [27]. The benefit of the organoids is the relative simplicity of technology and much higher performance comparing to LoC. With this, the organoids practically do not contain the circulatory system and it is impossible to imitate the hematoalveolar barrier, as it is implemented in the LoC device. For solving some screening research tasks, besides organoids, acute slices of the lungs were used which remain viable for some time after its preparation [29]. Currently, the LoC technology in some areas of *in vitro* screening research has completely replaced spheroids, organoids and acute slices.

STAGES OF THE "LUNG-ON-A-CHIP" MICROFLUIDIC DEVICE DEVELOPMENT

Initially, the alveolar pulmonary chip was developed in 2010 by the American cell biologist and bio-engineering specialist Donald E. Ingber, which was describing it as a living three-dimensional cross-section of the functional unit of the lung [7]. The organ chip consists of a transparent elastic polymer, which contains hollow microfluidic canals colonized with living human alveolar cells, connected to the artificial vascular network layered with human endotheliocytes (see Fig. 1, *б*; [6–8]). For the reason the chip is manufactured using the transparent material, it is suitable for microscopy using the conventional biological microscope for the purpose of personal observing the processes that take place in it. The presence of two air chambers in the chip allows for creating the rarefication and by this imitating the respiratory movements, expanding the semipermeable membrane containing the cells (see Fig. 1, *в*; [7]) [30].

The technology was rapidly adapted for creating the microfluidic devices, imitating a number of other tissues or organs, including the liver [31], kidneys [32], intestines [33], bones [34], blood vessels [35], the cardiac muscle [36] etc. From the moment of developing the first lung chip, the technology has significantly progressed, becoming more and more complicated.

Single-channel "lung-on-a-chip"

The single-channel microfluidic devices contain only the alveolar epithelium cells [24]. Such a single-channel microfluidic model does not imitate the hematoalveolar barrier, but it can be useful for studying the functional changes in the alveolar epithelium during the respiratory movements, which are imitated by cyclic air injections. For the purpose of the microphysiological visualization of respiration cycles, the composition of the elastic membrane, onto which the alveolocytes were seeded, was augmented with silicon oxide nanoparticles with the size ranging from 225 to 300 nm. Upon expanding the membrane, a shift took place in the wavelength of the reflected light, thus, the respiration cycles could be visualized [6]. This model was used for investigating the dynamic interrelations between the deformations of cells and the phenotypes of the diseases, such as idiopathic pulmonary fibrosis.

More simple devices, containing only one type of cells cultivated in the hydrogel consisting of the extracellular matrix components, were used for studying the dynamic morphogenetic processes, such as the formation of blood vessels, the migration of immune and tumor cells through the epithelial layer into the interstitial space [24, 37]. A simplified single-channel microfluidic device consisting of proximal airways epithelium precursor cells, obtained from the induced pluripotent human stem cells, allowed for studying the development of ciliated cells and for modeling the primary ciliary dyskinesia [38].

Multichannel "lung-on-a-chip"

The first double-channel lung chip, constructed by D.E. Ingber et al., has imitated the structure and the functions of a human alveole by creating separate parenchymatous and vascular compartments (see Fig. 1, *б*; [6–8]). The channels in this case were defined as the microfluidic system with specific cell type. For the reason that, in this case, there are two types of cells — alveolocytes and endotheliocytes, the chip is considered double-channel. The microfluidic system has gas and fluid channels, separated by a flexible porous polydimethylsiloxane membrane. On the side of the gas channel, alveolocytes are cultivated on the membrane, forming an air-fluid interface in the manner similar to the one in the alveole. On the side of the fluid micro-channels, the membrane is seeded with endotheliocytes with the cultural fluid being perfused, imitating the microcirculatory network of capillaries, while the additional lateral vacuum channels imitate respiratory movements [7, 24, 37].

The three-channel LoC device includes an additional channel containing fibroblasts and the components of extracellular matrix, which, on the side of air channels, are layered in alveolocytes (see Fig. 1, *б*; [6–8]). The mechanical effects on the hydrogel during the respiratory movements, imitated by vacuum channels, promote to the production of extracellular matrix proteins. The presence of an additional stromal channel allows for modeling the interstitial diseases of the lungs.

The unique feature of the multichannel organ chips in general and of the LoC in particular is that, within the gas channel, just like in the alveolar cavity, co-cultivation can be performed for human alveolar cells with the viable symbiotic microbes within a long period of time (from days to weeks). Currently, it is the only method capable of letting us study how the complex microbiome of the human lungs affects the status of the human tissues over time [39, 40].

High-performance "lung-on-a-chip" systems

With the technologies being improved, chip models were developed for studying various pathological

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conditions of the respiratory organs [8, 26]. For example, during the COVID-19 pandemic, a group headed by C.R. Fisher [41] has developed a PREDICT96-ALI high-performance "organ-on-chip" microfluidic platform for the selective screening of pathogenetic medications intended to fight the SARS-CoV-2 virus in the settings of virus -infected alveolar epithelium. The platform consists of a plate with 96 individual devices and a perfusion system, activated by 192 microfluidic pumps, built into the plate cover.

Noteworthy is the AX12 Lung-on-Chip inhalational *in vitro* platform, developed by a Swiss company named "AlveoliX", which represents not just a chip, but a multiplex analyzer based on the microfluidic device allowing for seeding the cells directly along both sides of the ultra-thin membrane [42]. The company has created an immortalized cell line consisting of alveolar epithelial cells (AXiAECs), alveolar macrophages (THP-1) and endothelial cells (HLMVEC). The system is intended for toxicology studies of aerosols, for example, when screening the inhalational drugs being developed.

Using the examples of the projects listed above, it could be assumed that modern biomedical technologies will more and more integrate the microfluidic "organs-on-chip" into the analytical equipment, adding new technologies based on nano- and micro-electronics, acoustic electronics, optoacoustics for genetically coded biosensors, NGS-sequencing and other omics approaches.

Certain perspectives for creating the high-performance artificial lung platforms become available with the 3D-bioprinting [43]. This is a relatively novel technology, allowing for creating the organ-like structures by means of printing with living cells, mixed with hydrogel bioink. The carcass of the bioink usually consists of extracellular matrix protein or other natural biopolymer — collagen, gelatin, alginate, fibrin, chitosan or hyaluronic acid [44]. Recently, W. Kim et al. [45] have used the technology of piezoelectric 3D-bioprinting using the bioink to print the cellular components of the "lung-on-a-chip" on a polycarbonate membrane. The bioink (following the corresponding proportions) had admixtures of cells mimicking the alveolar epithelium of types I and II (the NCI-H1703 and NCI-H441 lines, respectively), of pulmonary fibroblasts (MRC-5) and of the endothelial cells found in human microvessels (HULEC-5a). As a result of bio-printing, a hematoalveolar interface was created, showing the acceptable parameters of transepithelial electric resistance.

THE MATERIALS OF MEMBRANES USED FOR BUILDING THE HEMATOALVEOLAR BARRIER IN THE LUNG CHIP

The basis of any LoC device is the porous and expansible membrane, which should have sufficient biocompatibility for cultivating the monolayer of epithelial or endothelial cells on its surface [40]. For recreating the structure of the hematoalveolar barrier, maximally similar to the physiological one, the choice of the membrane material with proper gas permeability, biocompatibility and expansibility is an actual problem [8, 23, 41]. In the text below, we shall discuss the materials most commonly used for manufacturing the lung chip membrane.

PDMS-membrane

In the last 15 years, the most commonly used membranes are the ones made of the linear polymer of dimethylsiloxane [46, 47]. Polydimethylsiloxane (PDMS) is biocompatible, elastic, permeable for gases, optically transparent and relatively simple in small-scale production, which, in total, makes it one of the most convenient polymers for creating the "organs-on-chip" [48]. Modern microfluidic devices based on PDMS, are most frequently created using the soft lithography method [7]. PDMS is the most commonly used polymer for manufacturing the carcasses of microfluidic devices due to the simplicity of its microprocessing and the adjustable underlayer mechanics [49, 50].

Due to the ubiquitous spreading of PDMS as the material for manufacturing the "organs-on-chip", we would like to examine in detail the downsides and limitations of this material (thickness limitations; poor cell adhesion; sorption of hydrophobic molecules; high rigidity; complexity of moulding automatization).

Thickness limitations. There are difficulties in the manufacturing of ultrathin porous PDMS slices [52]. The actual thickness of the hematoalveolar barrier is less than 1 μm [22], with the most commonly conducted research often requiring the thickness of the LoC barrier membrane being 10 μm [37]. For the comparison — in earlier publications, the membrane thickness was up to 40 μm. With this, the Swiss company AlveoliX has developed a LoC having a membrane thickness of 3.5 μm. Such a membrane thickness is the most similar to the very thin hematoalveolar barrier and, to the best of our knowledge, as of today, it is the thinnest porous PDMS membrane used in the "organ-on-chip" device.

Poor cell adhesion. Due to the fact the PDMS membranes do not show good cell adhesion properties, various coatings must be used — fibronectin, collagen

etc. [23, 26], with this, the additional coating increases the membrane thickness (~10 μm) and decreases its porosity, which should be kept in mind when modeling the hematoalveolar barrier. The improvement of adhesion characteristics of PDMS can be achieved by single application of polydophamine (PDA) onto the PDMS surface. In the samples where the PDMS wells were not preliminary coated with PDA, cell adhesion abnormalities took place within the first 4 days of cultivation, ultimately resulting in the complete delamination and spontaneous destruction of all the tissue constructions in 10 days [52].

Sorption of hydrophobic molecules. When modeling the functional processes, one should also keep in mind that the PDMS membrane can actively absorb hydrophobic biologically active compounds, as well as hydrophobic low molecular weight medicinal products [43, 53, 54], which decreases the available dosage of the drug, shifting the dose dependency curve and, thus, limiting the prognostic value of the research with testing a number of medicinal products [55].

In order to minimize the error caused by the PDMS absorption, strategies were described on the computational correction of the absorption effect by means of quantitative determination of the medicinal product content using mass-spectrometry [56]. M.W. Toepke et al. [53] have studied the absorption of hydrophobic small molecules in a qualitative manner using the fluorescent analysis, but this was not a quantitative method. J.D. Wang et al. [54] have conducted a quantitative evaluation of the final concentration of the compound over time and have determined the threshold value, which has distinguished the compounds with insignificant absorption and the ones with the significant one, based on the hydrophobicity parameter. Besides, for the purpose of decreasing the compound binding, methods are being tested that involve covering the PDMS with non-absorbing coatings [57], that involve alternative flexible elastomeric materials showing lesser absorbing capabilities (for example, some polyurethanes, styrole block copolymers, polycarbonate hybrids and the thermoplastic elastomer) [58, 59]. Tests are also being carried out for the coatings made of rigid thermoplastic materials (polystyrene or polycarbonate) [60], titanium dioxide [61], parylene [62] etc. The accessible literature has few research works on the direct comparison of absorbing various compounds by PDMS and other, more inert substrates, while the issue of cell cultures affecting the absorption was not studied at all. The use of lipophilic coatings may be useful for preventing the absorption of low molecular weight compounds by the PDMS.

High rigidity. The modulus of elasticity of the PDMS, depending on the thickness of the expanded membrane, can vary from 0.4 to 1.5 MPa, while in the alveolar tissues, according to various estimations, it varies from 1.4 to 7.2 kPa [63, 64]. Such a significant difference complicates the process of modeling the inhaling/exhaling processes on the membrane. Due to this cyclic expansion of the PDMS membrane, during the imitation of respiratory movements, deformations may develop in the porous membrane, which may corrupt the data on the integrity of the hematoalveolar barrier, affecting the adhesive properties of the cells and changing the permeability for various substances [65]. The deformation applied to the thin porous membrane, strongly depends on the viscoelastic properties of the expanded material and on the dimensions, in particular, on the PDMS wall thickness, which is why the most "physiologic" LoC can be considered the one with the thinnest PDMS membrane.

Complexity of moulding automatization. PDMS moulding still remains a complex process in terms of complete automatization and it significantly slows down the transition to serial research [66]. The need for materials to replace the PDMS is so important for this field, that The Small Business Innovation Research in the USA has recently funded the research on exploring the alternative materials different from the PDMS, but meeting the requirements of producibility, transparency, biocompatibility and minimal non-specific adsorption [67].

Due to all the limitation listed above, currently there is an urgent need for searching the alternative material, which could be optimal for modeling the hematoalveolar barrier.

PMMA, PET and PC membranes

Polymethylmethacrylate (PMMA), polycarbonate (PC), cyclic olefin polymers/copolymers (COP/COC) and polystyrene (PS) are some of the wide-spread materials which were used as a scalable alternative option during the earlier "organ-on-chip" models. Their main benefits include the commercial availability and the relative manufacturing simplicity for mass market. For example, a group of Chinese scientists, when researching the toxic effects of finely dispersed solid particles on the human respiratory system, have used the membrane made of microporous polycarbonate film with the pore size of 10 μm [68].

As an alternative option, the earlier LoC models were employing the membranes made of polyethyleneterephthalate (PET) and polycarbonate,

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showing the optical properties similar to the ones of the PDMS, however, the important modulus of elasticity values were within the range of 1–3 MPa. They are more convenient for integrating into the microfluidic device, they have pores with various sizes and they are commercially available.

The main downside of such membranes is their extremely high rigidity, which limits their use to only perfusion platforms (or flow cells) in the static cultivation settings (without imitating the respiratory movements) [69, 70].

PLA and PLG membranes

One of the most wide-spread new alternatives to PDMS is the polylactide (PLA) — the biodegradable, biocompatible and thermoplastic polymer, the monomer of which is the lactic acid. PLA is widely used in medicine and its biocompatibility is well established in a number of research works, which show absence of inflammatory processes after the implantation and compatibility with the surrounding tissues [71–72]. Besides, PLA can be easily processed, it can be moulded as sheets, it can be processed mechanically or using the laser, it can be integrated into other structures and assembled into complex microfluidic devices.

As for the manufacturing the LoC membranes, a group of Chinese scientists has used the modified version of this polymer — the PLG (a copolymer of lactic and glycolic acids) for testing the anti-tumor drugs. Its main distinguished benefits include small thickness (~3 μm), porosity, and permeability for molecules and good biocompatibility [74, 75].

OSTE membrane

OSTE (off-stoichiometry thiol-enes) is an non-stoichiometric mixture of thiols and allyls, developed as an alternative to PDMS in the field of "organ-on-chip" technologies for the purpose of overcoming the gap between the research prototyping and the commercial manufacturing of microfluidic devices. One of the main benefits of OSTE is that the mechanical properties can be precisely adapted to the requirements of the specific use by adjusting the non-stoichiometric ratio without changing the composition of the monomer [76].

A group of scientists from Latvia has tested the OSTE as the membrane material for the hematoalveolar barrier and compared its properties to the PDMS. As for the benefits of OSTE, they have reported much lower sorption of small hydrophobic molecules and simpler moulding process. The main disadvantage is low transparency, which significantly complicates monitoring the cells covering the membrane [77].

Gelatin-methacryloyl (GelMA) membrane

The materials in all the above mentioned "lung-on-chip" models, of which the membranes for the hematoalveolar barrier was made, have a very serious limitation — non-physiologically high rigidity, due to which the mechanical stimulation (modeling of inhaling/exhaling) is either very weak or completely absent. In one of the research works, for overcoming this disadvantage, a group of scientists has used the three-dimensional porous gelatin-methacryloyl hydrogel. The resulting structure has a close similarity to the natural human alveoli, in particular, in terms of their sac-like structure, their pores and rigidity. In order to create it, the authors have used the densely packed alginate microgranules (201±12 μm), the distance between which was filled with 7% GelMA solution. After this, the granules were dissolved in 0.01 M ethylenediaminetetraacetic acid (EDTA) solution. Due to the fact that the hydrogel cannot leak in the areas of contacting granules, the final structure not only forms the alveoli-like sacs, but also has pores connecting them [78]. The authors have followed the requirements on the mean size of the alveoli — ~200 μm [79], also, a very low rigidity was reported for gelatin-methacryloyl: the modulus of elasticity equals to 6.23±0.64 kPa, while in the alveolar tissues, according to various estimations, it varies from 1.4 to 7.2 kPa [63, 64].

Biological membrane

The material for the semipermeable biological membrane is the key factor for creating the "lung-on-a-chip". As we have discussed before, PDMS, being the most applicable material for manufacturing the membrane, has multiple disadvantages.

In one of the recent LoC research works, a carcass made of golden combs was used, onto which, a thin layer of collagen I and elastin mixture was applied. The thin golden mesh with the pore size of 260 μm was used as a carcass, supporting the structure of 40 alveoli. The resulting membrane is stable and it can be cultivated on both sides for several weeks [80]. This method was used to model the expansible alveolar sacs, in which the thickness and the rigidity of the membrane can be adjusted by the ratio of collagen and elastin in the gel mixture. The prepared membrane was integrated into the microfluidic chip, where it was compressed between the two microfluidic parts, the upper PDMS part with the apical reservoir and the lower polycarbonate part,

together forming the basolateral chamber. These membranes can be stored in the lyophilized state, retaining their properties for not less than 3 weeks at room temperature. The membranes shall be rehydrated by submerging it into the cultural medium 2 hours before the cell seeding [53]. Such a membrane is in many regards better than PDMS, it does not bind the hydrophobic medicinal products, being biogenic and biodegradable, with the capabilities of obtaining a very thin membrane (approximately 4 μm), but it was reported that the degree of its strength is insufficient.

The Canadian authors have made an "airway-on-a-chip" device which contained an ultra-thin membrane formed using the mixture of type I collagen and the Cultrex Basement Membrane Extract (BME) at a ratio of 1:2 (Cultrex is a soluble form of the basal membrane, made by the purification of the Engelbreth–Holm–Swarm tumor, which gelates at 37°C, forming a reconstituted basal membrane) [81]. The important feature was the device generating the bidirectional oscillating air flow, imitating the respiratory cycles. Such a combination of an ultra-thin biomimetic membrane and the oscillating air flow has resulted in the first ever demonstration of the glycocalyx layer formed on the airway epithelium in the "lung-on-a-chip" device and induced by air flow, with the glycocalyx layer being known for its important role in regulating the epithelial functions. The authors managed to demonstrate significant differences in the viability of airway epithelial cells and in the formation of dense connections, cilia or mucus depending on the speed of oscillating air flow. It was shown that the mechanic-biological effect of the shearing stress applied for a long period of time, has increased the formation of dense contacts among the epithelial cells and decreased the diffusion permeability.

Another team with a long and successful history of working in the field of replacing the PDMS with biomaterials having the properties and functions similar to the pulmonary tissue, has developed a biomimetic microfluidic platform, which reminds the multi-layer architecture of the alveolar-capillary barrier and the composition of the alveolar extracellular matrix, physiologically consisting of a thin basal membrane and dense fibrous interstitial spaces [82]. The "alveole-on-a-chip" included the membrane produced by electro-spinning of PCL-Gel (polycaprolactone-gelatin) between the two microstructurized PDMS layers, moulded using two master-models, obtained by means of poly-jet 3D-printing. Within this chip, three types of cells were cultivated simultaneously: on the surface of the membrane, there was a type I collagen hydrogel containing MRC-5 fibroblasts for reproducing the alveolar interstitial spaces; on the top of the hydrogel, the A549 epithelial cells were seeded for recreating the alveolar epithelium, while the basolateral chamber of the device was seeded with the HVEC endothelial cells. By means of the immunofluorescence assay, confirmation was obtained for the formation of the dense endothelial and epithelial barrier, while the high viability of cells remained for 10 days. The authors have demonstrated that exactly the presence of collagen hydrogel provided the optimal biomimetic environment for co-cultivating of fibroblasts and epithelial cells, while the presence of the interstitial layer significantly improving the bio-mimicry of the "alveoli-on-a-chip" model comparing to other systems, focused mainly on recreating the epithelial and endothelial barrier.

Though the hydrogel-based microfluidic technologies show the potential for *in vitro* — recreating the key properties of the tissues, they possess a wide number of disadvantages, mainly related to the low stability/reproducibility caused by swelling and limited rigidity range of the membrane and of the whole chip in general, which significantly restricts their applicability. Within this context, the interesting new methodological approach is the development of a soft microfluidic device with the cell filling based on hydrogels made of enzymatically cross-linked silk fibroin (eSF) and of the spider web silks (recombinant spidroins). The enzymatic processing of silk proteins with peroxidase induces the formation of intermolecular covalent bonds between the oxidized forms of tyrosine, which results in a sudden increase of strength and elasticity of the hydrogel. With this, the microfluidic platform with 14% eSF has demonstrated an outstanding structural stability, having the Young's module of 11.79 kPa, the elasticity (103%) and the capabilities of perfusing the fluid, while showing the biological reactions similar to the ones found *in vivo* [83]. Even though the research work has employed a combination of eSF and microfluidics for recreating the native dynamics of the three-dimensional microenvironment of the colorectal cancer and its reactions to chemotherapy, nevertheless, the demonstrated eSF properties (elasticity, strength, transparency, structural stability within not less than 7 days) give ground for expecting that these materials can be successfully used in designing the "lung-on-a-chip" platforms, especially keeping in mind that another research [84] has demonstrated that the tyrosine residues within the recombinant spidroins resulting in after processing with recombinant tyrosinase, migrate

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to dihydroxyphenylalanines (DOPA) and further into DOPA-quinones and other more oxidized forms that take part in the formation of the intermolecular covalent links in these proteins, which results in the formation of a hydrogel.

CELLULAR CULTURE SOURCES FOR CREATING THE LUNG CHIP

In the vast majority of the research works, all the sources of cellular cultures used for creating the "lung-on-a-chip", are allogenic cell lines originating from tumor or embryonic cells, for the primary cell cultures are not standardizable and operating with them requires special settings [81]. The primary cultures of alveolocytes upon passaging significantly change the phenotype. With this, it was reported that the differentiation of human bronchial epithelium into the ciliated and secretory cells has ceased after two passages [26]. As the analogues of the alveolar epithelium, the cell lines were used that originate from the biomaterial of resected pulmonary adenocarcinomas [81]: for example, the NCI-H1703 line, morphologically similar to type I alveolocytes and originating from the sample of the non-small-cell lung cancer, the NCI-H441 line — from the papillary adenocarcinoma of the lung, the SW-1573 line — from the alveolar carcinoma [85]. The cells of the NCI-H441 line, as well as the A549 line, originating from the lung adenocarcinoma, are morphologically similar to type II alveolocytes. Even more closer to the native primary alveolocytes are the immortalized lines of alveolar epithelial cells, originating from the primary cell cultures, as, for example, the AXiAECs line [42].

As for the pulmonary fibroblasts, the immortalized lines of embryonic lung fibroblasts are being used, originating from the abortive material, for example, the MRC-5, HFL1 or IMR-90 lines. For creating the microcapillary layer in two- or three-channel LoC, human endotheliocyte lines are used that originate from the endothelium of fetal lung capillaries, for example, the HLMVEC line, or from the lung endothelium of adult humans, for example, the HULEC-5a line [45]. The ones considered applicable are also the primary and immortalized HUVEC cells — the endotheliocytes of the fetal umbilical vein. There are also cell lines corresponding to the alveolar macrophages, for example, the monocyte line from the patient with acute monocytic leukemia (THP-1), as well as the cell lines corresponding to the upper airway epithelium, for example, the Calu3 tumor cell line. Practically all the cell types required for creating the LoC, can be obtained using the induced pluripotent human stem cells by targeted differentiation using the biologically active factors and small molecules [86].

The important requisite for the correct differentiation and polarization at the basolateral and apical poles of the alveolar epithelium cells is the cultivation at the air-fluid interface. Creating the gas-fluid interface and imitating the respiratory movements promotes to the formation of the polarized mucociliary epithelium, including the ciliary, the glomerulate, the goblet-like and the basal cells, with the formation of the surfactant and the glycocalyx, which in total maximally corresponds to the natural epithelium of the human alveole [87, 88].

RESEARCH METHODS IMPLEMENTED IN THE "LUNG -ON-CHIP"

The "lung-on-a-chip" system is compatible with a number of standard methods of laboratory and chemical analysis, including the electrochemical detection of various analytes, the registration of transepithelial electrical resistance (TEER), the permeability analysis of specific factors using the method of enzyme-linked immunoassay or the polymerase chain reaction, immunostaining, flow cytometry, confocal laser microscopy, multi-photon microscopy, FLIM-microscopy, optical coherent tomography, omics technologies etc. [89] (Fig. 2). The sensors and biosensors used during the "lung-on-a-chip" studies to detect oxygen, temperature and specific biomarkers, measure the additional biochemical and biophysical parameters [88]. The limiting factor for a number of methods is the relatively small number of cells in the micro-channels. For example, the recommended number of cells for the RNA-Seq single-cell assay is 1 000 000, while the number of cells contained within the microfluidic devices, can only reach thousands [51].

The LoC technology allows for analyzing the pathophysiological processes within the structures of the hematoalveolar barrier in the real time mode. For this purpose, the devices are made of transparent material and its walls are made being maximally thin and optimized for fluorescent microscopy; the microfluidic system is constructed in such a way that samples could be drawn from the incoming and out-coming micro-channels with reading the numbers from the sensors built into the chip [53]. For the intravitam fluorescent microscopy, the cells on the membrane have the genetically coded fluorescent proteins and biosensors included; the actine cytoskeleton of the cell can be labeled using phalloidin with the organellae being labeled using the selective tracer substances, etc. [90].

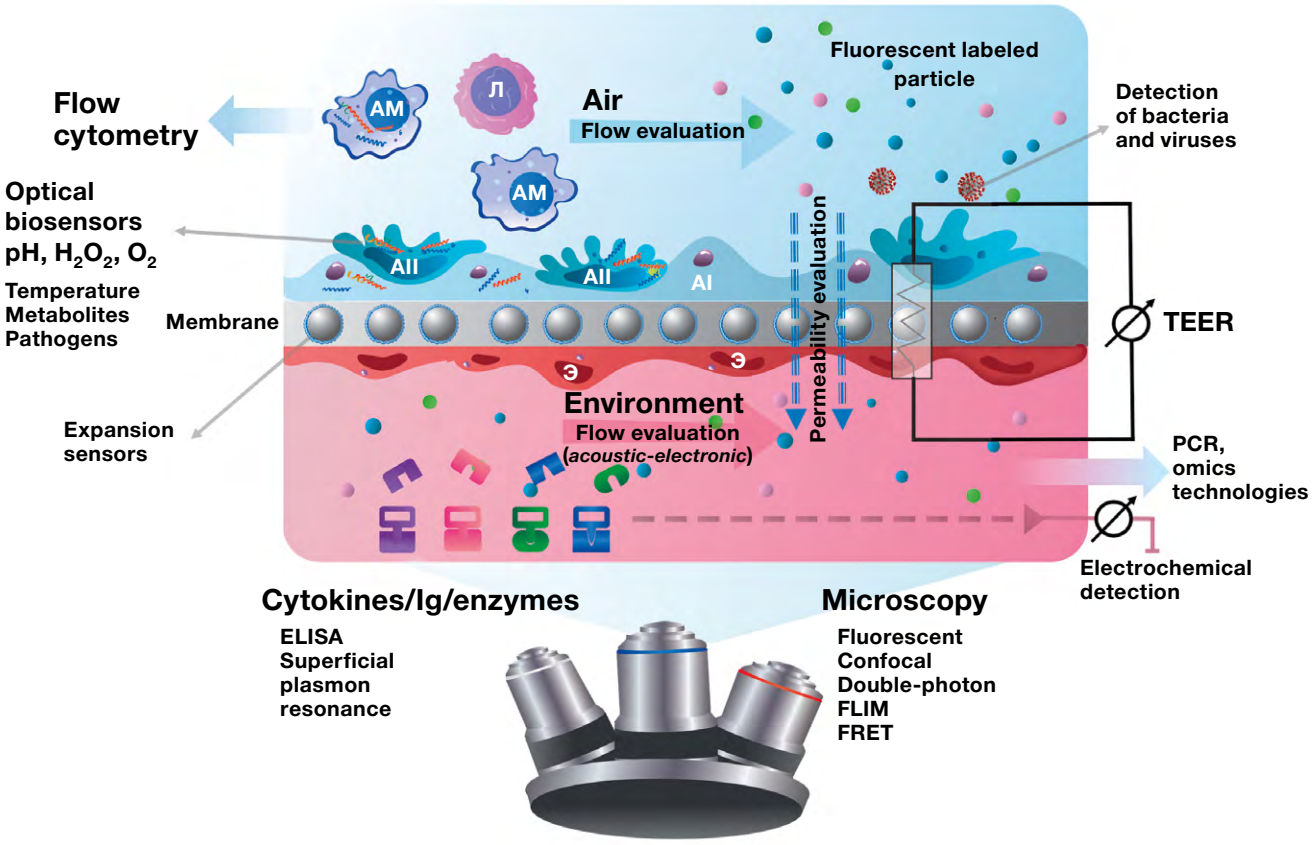

Fig. 2. Methods of "lung-on-a-chip"-associated research with an example of double-channel chip. ЛА — pulmonary artery; ЛВ — pulmonary vein; ГВ — smooth-muscle fibers; АМе — alveolar sac; ЛК — pulmonary capillaries; AI — type I alveolocyte; AII — type II alveolocyte; AM — alveolar macrophage; Э — endotheliocytes; FLIM — Fluorescent Lifetime Imaging Microscopy; FRET — Förster Resonance Energy Transfer.

TEER is considered the gold standard of cell barrier integrity monitoring. However, the integral registration of TEER in the microfluidic device has its limitations, for even a small area of impaired integrity of the cells significantly decreases the total TEER, despite the presence of a dense monolayer in all the other areas [91]. This problem can be solved using the microelectrode matrices, but this makes the device significantly more expensive [92]. The biophysical evaluation of the monolayer characteristics can be performed using the impedance analysis when cultivating on the golden microelectrodes, however, their presence reduces the transparent window for microscopy.

The important aspect of alveolar functioning are the mechanical-biological parameters of cells interacting with each other and of the cells interacting with the intercellular matrix. For the purpose of high-precision evaluation of the mechanical-biological properties in a cell, the traction force microscopy (TFM) and optical tweezers are being used, as well as the optical biosensor based on the Förster Resonance Energy Transfer (FRET) [93]. One of the sensors reacting to mechanical stimuli, to the rigidity and expansibility of the matrix, is the YAP/TAZ transcription factor [Yes-associated protein (YAZ) + WW domain-containing transcription regulator protein 1 (WWTR1, also known as the TAZ)]. This is the main effector of the Hippo pathway, activated during the mechanotransduction and in the settings of the mitochondrial stress [94].

Another method of gaining additional information on the processes taking place within the LoC is the use of acoustic-electronic technologies. In this case, the information-containing signal is the measured frequency or the attenuation of acoustic waves of various types in the piezoelectrical materials [95]. These wave parameters change as a result of changing the both the electrical and the mechanical parameters of the interacting biological objects. Acoustic waves are being actively used in the microfluidic devices for manipulating the biological objects, for changing the direction of movement, for detecting of viability etc. [96, 97]. Such an approach is quite promising in case of limitations applied to performing the direct optical measurements. The intravitam LoC tests using the methods mentioned

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above, allow for better understanding the molecular aspects of pathophysiology and mechanotransduction in human alveoli.

PRACTICAL APPLICATION OF THE "LUNG-ON-A-CHIP"

"Lung-on-a-chip" is a multi-purpose *in vitro* platform, which can be used for large numbers of research tasks. After the first publication of creating the model of alveolar lung chip, the team headed by E. Ingber has published several research works on modeling various respiratory diseases, including the models of pulmonary edema [98], pulmonary artery thrombosis [99] and lung cancer [100]. They have also developed LoC devices for modeling the chronic obstructive pulmonary disease and asthma [101], and just recently — the microfluidic system modeling the human airways for studying the diseases caused by the influenza virus and other viruses affecting the bronchoalveolar system (the "human-airway-on-a-chip" device) [102].

Models for asthma and chronic obstructive pulmonary disease

K.H. Benam et al. [101] have constructed a double-channel LoC with differentiated mucociliary bronchiolar epithelium and with the underlying lung vessel endothelium for studying the complex inflammatory changes in cases of asthma and chronic obstructive pulmonary disease. The chip was made of PDMS using soft lithography, with its upper channel having the height and the width of 1 mm (similar to the human bronchiole radius) and separated from the parallel lower microvascular channel [0.2 mm (height) × 1 mm (width)] with a thin (10 μm) porous (0.4 μm pores) polyester membrane, on both sides covered in type I collagen. The immune cells were circulating through the underlying liquid flow. Using this device, it was shown that the contact of small airways with the Interleukin 13 (interleukin, IL) increases the number of goblet-like cells, increasing the production of inflammatory cytokines and decreasing the rate of ciliary beats in the epithelium, which is comparable to the changes in the mucosa observed in asthma patients [103, 104].

The team headed by K. Benam et al. [101] has also arranged a series of experiments on using the lipopolyssacharide endotoxin and viral particles to stimulate the airways channel of the chip containing healthy epithelial cells and of the chip containing the epithelial cells taken from the patient with chronic obstructive pulmonary disease. It was shown that the chips with the cells from the patient with chronic obstructive pulmonary disease show increased secretion of the M-CSF and IL-8 cytokines comparing to the chips with normal epithelial cells. M-CSF promotes to the differentiation and survival of the macrophages, while the IL-8 is the attractant for neutrophils, both of which being the main types of immune cells observed in patients with chronic obstructive pulmonary disease [105]. Thus, using the LoC, it is possible to detect synergic effects of the pulmonary endothelium and epithelium in terms of cytokine secretion, to identify new biomarkers of disease exacerbation and to measure the anti-inflammatory reactions.

Modeling the thrombosis of pulmonary capillaries

The LoC platform can recreate complex reactions, including the dynamic interactions between the platelets and the endothelium, proposing a new approach to investigating the pathophysiology of the thrombosis of pulmonary microvessels in humans and promoting the development of medicinal products. A. Jain et al. [99] have modified the existing "lung-on-a-chip" model [98] and have covered the walls of the lower vascular channel with endothelial cells from the vessels, in order to create the lumen of the vessel followed by the vessel perfusion with whole human blood instead of the cultural medium. The inflammatory activation of the vascular endothelium with tumor necrosis factor alpha (TNF-α) has caused rapid recruiting of platelets and resulted in the formation of the thrombus, similarly to the manner in which it happens in the inflammatory-modified microvessels *in vivo* [106]. The dynamic changes of platelet binding have imitated the formation of thrombi in the *in vivo* murine model [107]. This model was also used to show that the lipopolyssacharide endotoxin indirectly stimulates the intravascular thrombosis, activating the alveolar epithelium, but not interacting directly with the endothelium. This model was also used to analyze the inhibition of the activation of the endothelium and of the thrombosis with protease-activated receptor-1 (PAR-1) [99].

Lung cancer model

B.A. Hassell et al. [100] have created a model of human non-small-cell lung cancer in chip for the purpose of investigating the behavior of cancer cells, the variations of growth and invasion in various

micro-environments, as well as for investigating the anti-tumor effects of tyrosine kinase inhibitors. The research has demonstrated that the presence of cyclic mechanical movements, imitating the respiration patterns, has significantly suppressed the growth of tumor cells. The tumor cells localized on a small area, were growing in the absence of movement, replacing the alveolar epithelium layer, migrating and invading the vascular layer. This discovery shows that the exponential proliferation of tumor cells in the alveolar space develops due to the loss of lung mobility.

As it was mentioned before, the model cells for LoC, fulfilling the functions of the alveolocytes, often include the tumor cell lines, which is why such devices can be easily adapted for investigating the anti-tumor medicines — in the setting of the "breathing" microenvironment. X. Yang et al. [76] have developed a "lung-on-a-chip" with PLG electro-spinning nanofiber membrane as a chip base and as a cells carcass. The PLG membrane with the controlled thickness of ~3 μm is porous and permeable for molecules, it shows high biocompatibility and suits well for imitating the alveolar respiratory membrane. On the chip, co-cultivated were the human non-small-cell lung cancer cells (line A549) and the human fetal lung fibroblasts cells (HFL1) with an evaluation of the effects of the Gefitinib antitumor medication targeting the epidermal growth factor receptors (EGFR).

The LoC devices are significantly inferior comparing to the 2D-cultures in terms of their throughput, which is why they cannot completely replace the initial cytotoxicity screening which is performed using cell cultures, however, the final selection of anti-tumor medicines can be implemented in such devices, taking into consideration the evaluation of the effects of the tumor microenvironment, the mechanic-biological factors and the hematoalveolar barrier parameters [15]. The chips contain tumor cells with specific mutations providing resistance to chemotherapy agents, also including the personified tumor lines [108].

Pulmonary edema model

The team headed by E. Ingber has studied the possibility of using the "lung-on-a-chip" device for the purpose of micro-engineered modeling of the pulmonary edema, characterized by the accumulation of intravascular fluid in the alveolar air spaces and in the interstitial tissues of the lung, caused by the impaired mechanisms of the homeostatic fluid balance [98, 109]. It was experimentally proven that the injection of IL-2 into the vascular channel of the LoC device resulted in an increase in the permeability of the cellular layer and the accumulation of fluid in the upper alveolar channel. With this, the increasing effect on the filling of air channel with the fluid, imitating the pulmonary edema, has resulted in the development of cyclic mechanical tension, imitating the respiratory movements. Further research has confirmed that mechanical respiratory movements play a significant role in the IL-2 -induced leakage from the vessels, leading to pulmonary edema [98]. A research from the E. Ingber team [98] has also revealed that the reaction to the leakage from the pulmonary vessels, induced by IL-2, does not require circulating immune cells, which differs from the previous *in vitro* and *in vivo* researches, showing that the blood-transported immune cells, such as lymphocytes and neutrophils, when activated by IL-2, play a central role in the induction of leakage from the pulmonary vessels [110]. This model has also recreated the sedimentation of fibrin clots in the alveolar areas due to enzymatic reactions between plasma proteins during the progression and exacerbation of pulmonary edema.

The obtained results show that the developed human pulmonary edema model with the aid of the "lung-on-a-chip" device can potentially replace the pre-clinical pulmonary edema animal models, currently used for developing the pharmacological products.

Toxicological research

Currently, more and more topicality is gained by the problem of air contamination with nanoplastics, which can easily reach the lungs and get accumulated there, leading to pathological processes [111]. The latest research works have demonstrated that microplastics are present in the lungs of birds [112], in the lower airways and in the lungs of humans, as well as in the sputum of the patients with chronic obstructive pulmonary disease [113]. Microfluidic lung chip was used by a group of Chinese investigators to estimate the relation of polystyrene nanoplastics and the pathogenesis of chronic obstructive pulmonary disease. It was shown that the viability of cells has significantly decreased along with the increase in the concentration of polystyrene nanoplastics, while the levels of transepithelial/transendothelial electric resistance were decreasing with an increase in the permeability of the alveolar-capillary barrier [114]. In general, the LoC combined with high-flow technologies, which we have mentioned previously, is a novel platform for investigating the pulmonary toxicity

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of nanoplastics and other inhalable substances, such as nanoparticles of titanium oxide (TiO_2) and zinc oxide (ZnO), silicon dioxide etc. [115]. When using the LoC, it was shown that the effects of silicon dioxide nanoparticles in the alveolar epithelium result in an activation of the underlying endothelium and an increase in the number of type 1 intercellular adhesion molecules (ICAM-1).

It is expected that, in the nearest future, the "organ-on-chip" models could be used when testing the toxicity, replacing or, at least, decreasing the need for animals testing.

The platform for personalized medicine

Theoretically, nothing except for high cost and methodological difficulties, is in the way of developing the personalized "lung-on-a-chip" devices, containing the cells obtained from separate patients or from the patient cohorts with a certain genetic profile, for the purpose of arranging the specific research and testing the individual reaction to drugs. Using such devices, personalized chemotherapy can be adjusted based on the individual drug resistance, along with the personified dosage adjustment, but it is worth noting that, for achieving these two tasks, more simple 2D or 3D personified cell cultures can be used. At the same time, personified LoC devices unveil the unique possibilities for creating individual or grouped *in vitro* platforms for investigating the chronic obstructive pulmonary disease, the idiopathic pulmonary fibrosis, the mucoviscidosis and other diseases, altering the alveole and the hematoalveolar barrier. The patient-specific cells or cells from a specific genetic group can be used for developing the patient-specific or cohort-specific "personalized lung-on-chip", reflecting the biometric parameters, the genetics and the physiology of a specific individual [116]. The authors understand that, currently such a concept sounds utopian, however, the development of biotechnologies can radically change everything. Just some 30 years ago, obtaining humanized antibodies to certain human cytokines also seemed utopian, while currently they are quite routinely used as the medications for clinical practice.

CONCLUSION

The "lung-on-a-chip" technology is an important achievement in a path of discovering the fragile pathogenetic mechanisms of pulmonary diseases and a promising *in vitro* platform for screening the medicinal products. The microfluidic technologies allow for recreating the respiratory movements and for real time monitoring the status of the elements in the epithelial and endothelial layers, for evaluating the transepithelial resistance, the partial gas pressures in the in-flowing and out-flowing microchannels, the biochemical composition of the environment, the concentration of cytokines and pathogens, the mechanotransduction, the acoustic-electronic phenomena and other physico-chemical parameters. We suppose that further improving the microfluidic lung chip is a perspective scientific field that shall allow for studying the pathophysiology of the hematoalveolar barrier, the molecular and cellular features of alveolar diseases, the cold and pressure injuries, the inhalable toxins, the bacterial and viral pathogens, as well as for arranging the efficient screening of pharmacological products, by this increasing the total efficiency, the validity and economical practicability of pre-clinical research.

ADDITIONAL INFORMATION

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Authors' contribution. *V.P. Baklaushev* — general concept of the paper; *Iu.V. Ozerskaya, O.A. Zhukova, D.V. Basmanov, V.Y. Stolyarov* — literature analysis and manuscript preparation; *V.V. Kolesov, K.A. Zykov, G.M. Yusubalieva* — manuscript editing. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

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