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Моделирование взаимодействия аэрозольных частиц с клетками лёгочного эпителия на базе платформы «лёгкое на чипе»

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АННОТАЦИЯ

Обоснование. Оценка ингаляционной токсичности естественных и искусственных аэрозолей, а также эффективности аэрозольных лекарственных средств является важной практической задачей. Однако во многих *in vitro* исследованиях в настоящее время тестируемое вещество растворяют в жидкости, полностью покрывающей клетки лёгочной ткани. Для ингаляционной терапии эта методика является значительно отличающимся от физиологического сценарием взаимодействия аэрозоля с лёгочным эпителием.

Цель. Создание платформы для изучения взаимодействия вдыхаемых аэрозольных частиц с лёгочным эпителием на внутренней поверхности лёгочных альвеол на границе раздела жидкость–воздух, способной имитировать сценарии периодического изменения уровня жидкости при дыхании.

Методы. В качестве модельной культуры клеток лёгочного эпителия использована культура клеток аденокарциномы человека A549. Анализировали показатели сохранения жизнеспособности клеток методом флуоресцентной микроскопии при различных параметрах экспозиции на границе раздела жидкость–воздух, а также при осаждении аэрозольных частиц различной природы.

Результаты. Сконструирована и испытана установка, имитирующая внутреннюю поверхность лёгочных альвеол, которая представляет собой слой клеток на границе жидкость–воздух. Клетки культивируются на пористой полимерной мембране, находящейся на поверхности резервуара с культуральной средой. Степень обводнённости клеток контролируется с помощью изменения давления жидкости под мембраной и регистрируется с помощью оптического датчика, который измеряет степень рассеивания лазерного луча, отражённого от поверхности клеточного слоя. Мембрана с клеточным слоем размещена в камере, позволяющей создавать направленное электрическое поле, перпендикулярное потоку аэрозоля, проходящего над клеточным слоем (слой соединён с одним из электродов). Наличие электрического поля позволяет многократно ускорять процесс доставки аэрозольных частиц, имеющих электрический заряд, на поверхность клеток. Определены параметры статической экспозиции модельного монослоя клеток на границе жидкость–воздух, позволяющие сохранить высокую степень выживаемости клеток. Продемонстрирована эффективность циклического режима изменения обводнённости клеток, имитирующего дыхательный цикл для сохранения жизнеспособности клеток в течение длительного времени (60 мин). Продемонстрирована эффективность наноаэрозольной формы противоракового препарата доксорубицина при осаждении на поверхность опухолевых клеток аденокарциномы человека, находящихся на границе раздела фаз жидкость–воздух. Модельный аэрозоль нетоксичного вещества (глюкозы) не проявляет токсического действия в аналогичных условиях.

Заключение. Предлагаемая модель «лёгкое-на-чипе» представляет собой комплексную платформу для изучения ингаляционной токсичности естественных и искусственных аэрозолей, а также проверки безопасности и эффективности аэрозольной формы лекарственных препаратов *in situ*.

Ключевые слова: лёгкое-на-чипе; *in vitro* модель лёгочного эпителия; аэрозольная форма лекарств; токсикология аэрозолей.

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Modeling the Interaction of Aerosol Particles With Lung Epithelial Cells Using the Lung-On-A-Chip Platform

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ABSTRACT

BACKGROUND: The assessment of inhalation toxicity of natural and artificial aerosols and the efficacy of aerosolized drugs is an important practical task. However, in numerous *in vitro* studies, the test substance is dissolved in a liquid that completely covers the lung tissue cells. For inhalation therapy, this method substantially differs from the physiological scenario of aerosol interaction with the pulmonary epithelium.

AIM: The aim is to develop a platform to study the interaction of inhaled aerosol particles with the pulmonary epithelium on the internal surface of the lung alveoli at the liquid–air interface capable of simulating scenarios of periodic variation of liquid levels during breathing.

METHODS: The A549 human adenocarcinoma cell line was used as a model culture of pulmonary epithelial cells. Cell viability was analyzed using fluorescent microscopy under various exposure conditions at the liquid–air interface and during the deposition of different aerosol particles.

RESULTS: A device has been designed, built, and tested to simulate the internal surface of pulmonary alveoli. It consists of a cell layer located at the air–liquid interface. The cells are cultured on a porous polymer membrane on the surface of a reservoir with culture medium. The hydration level of the cells is controlled by altering the liquid pressure beneath the membrane and recorded by an optical sensor that measures the scattering of laser beam reflected from the surface of the cell layer. The membrane with the cell layer is placed in a chamber allowing to create a directed electric field normal to the aerosol flow passing over the cell layer, which is connected to one electrode. The electric field significantly accelerates the delivery of aerosol particles with an electric charge onto the cell surface. We determined the parameters of static exposure of the model cell monolayer at the air–liquid interface, allowing to maintain high cell viability. The study showed that cyclic variation of cell hydration simulating the respiratory cycle is effective to maintain cell viability for an extended period (60 min). The study showed that doxorubicin nanoaerosol is effective when deposited on the surface of human adenocarcinoma tumor cells located at the liquid–air phase boundary. A model aerosol of a non-toxic substance (glucose) does not have toxic effects under similar conditions.

CONCLUSION: The proposed lung-on-a-chip model is a comprehensive platform to study the inhalation toxicity of natural and artificial aerosols and test the safety and efficacy of aerosolized drugs *in situ*.

Keywords: lung-on-a-chip; *in vitro* lung epithelium model; aerosolized drugs; aerosol toxicology.

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INTRODUCTION

The assessment of inhalation toxicity of natural and artificial aerosols and the efficacy of aerosolized drugs is an important practical task. In addition, inhaled administration offers a promising alternative method of systemic drug delivery, especially for drugs with poor oral bioavailability, such as peptides and proteins, and compounds sensitive to first-pass metabolism in the liver and intestines [1]. The core value of understanding risk factors and mechanisms of action is data on the effect of aerosol substances in humans. However, studies involving human subjects are typically expensive, time-consuming, and require special permissions, especially in cases where the studied substances are presumed to be toxic [2]. Thus, *in vivo* studies of the biological effects of aerosols primarily involve animals. Nevertheless, in addition to scientific and ethical concerns, *in vivo* experiments often allow to observe only the mediated effects of the complex interactions of aerosols on the organism. Recently developed organ-on-a-chip systems has become a reliable alternative to animal testing. These micro-engineered cell systems provide environment that simulates the natural surroundings of cells *in vivo* [3]. Lately, a variety of approaches to *in vitro* testing of inhalation toxicity have been developed [4]. No so long ago, drugs were tested *in vitro* using lung tissue cells that were fully immersed in the culture medium [5]. There are also microfluidic lung-on-chip systems based on stem cells used in lung tissue models [6]. For inhalation therapy, this scenario substantially differs from physiological conditions as aerosolized drugs are deposited onto the air-facing bronchial and alveolar epithelium of the lungs. Although there are several available cellular models of the upper respiratory tract, modeling the distal alveolar region has several constraints that complicate the standardization of reliable *in vitro* alveolar models. New versions of platforms used to test aerosols on cells at the liquid–air interface include the ALICE-CLOUD aerosol delivery system. In this technology, cells used in lung epithelium models (A549 adenocarcinoma cell line) are placed on Transwell plastic inserts and cultured for 24 h at the air-liquid interface. Then, an aerosol cloud generated by a nebulizer is deposited onto the cells. The authors demonstrated that the therapeutic activity of the aerosolized drug (Bortezomib) upon deposition on the cell surface was comparable to the efficacy of the drug solution, but with faster absorption kinetics. However, this model does not allow to cyclically alter the liquid level above the cells, and if it had, it would simulate the breathing cycle. The Cloud α AX12 platform with an ultrathin porous AX12 membrane is a system capable of simulating the breathing cycle by membrane deformation. This system provides key physiological conditions for cultivating lung epithelial cells, including the air–liquid interface and three-dimensional cyclic stretch. Another comparable model is based on a stretchable and biodegradable membrane made of collagen and elastin deposited on a gold mesh, which simulates a network of tiny alveoli with sizes close to those found *in vivo* [7].

AIM

The study aims to develop a platform simulating the internal surface of pulmonary alveoli consisting of a layer of cells located at the liquid–air interface. This platform is designed to replicate the breathing cycle and enhance the delivery of charged aerosol particles to the cell surface by applying a directed electric field.

MATERIALS AND METHODS

Cell Cultures

A549 cell line was obtained from the vertebrate cell culture collection at the Institute of Cytology, Russian Academy of Sciences.

Reagents

Doxorubicin, glucose, 96% ethanol, bisbenzimid, propidium iodide, and calcein (Sigma-Aldrich, USA); DMEM/F12 culture medium (PanEco, Russia) with 10% fetal bovine serum (Biosera, France); Molykote High Vacuum Grease (Dow Corning, USA).

Experimental Setup for Depositing Charged Aerosol Onto the Cell Layer

The setup diagram is shown in Fig. 1 (*a*, exploded view; *b*, longitudinal section of the chamber). The cell layer (1) is pre-cultured on a porous track membrane made of polyethylene terephthalate with a pore size of 0.4 μm (Reatrak-Filter LLC, Obninsk, Russia) (2). Beneath the porous membrane, there is a reservoir with the culture medium (3) connected via a pipe (4) and a flexible tube to a pressure control system (not shown in the diagram) allowing for controlled and cyclic variations of the pressure applied to the liquid beneath the membrane. The pressure control system consists of a 50 ml plastic container connected to the reservoir (3). The container is mounted on a special holder connected to a movable stage (a stepper motor [Parts Hub Store, China] is connected to the stage via a worm gear). The motor is controlled by an open-source Arduino UNO microcontroller board. Pressure is controlled by creating a height difference between the liquid levels in the movable container and the chamber. The aerosol delivery system over the cell layer includes a quadrangular chamber with the lower wall made of a metal plate (5) and the upper wall made of glass with a conductive coating (6). All plastic components of the chamber are made from dielectric material (AnyCubic Basic Clear polymer resin for 3D printing, China) using an Anycubic photopolymer 3D printer (China). An opening with an area of 1 cm^2 is made in the lower wall of the chamber, where the porous membrane with cells is placed. At opposite ends of the chamber, there are pipes for aerosol (7). The aerosol flow in the chamber was maintained at a rate of 2 L/min for all experiments. The conductive walls of the chamber were connected to opposite poles of a high-voltage power supply.

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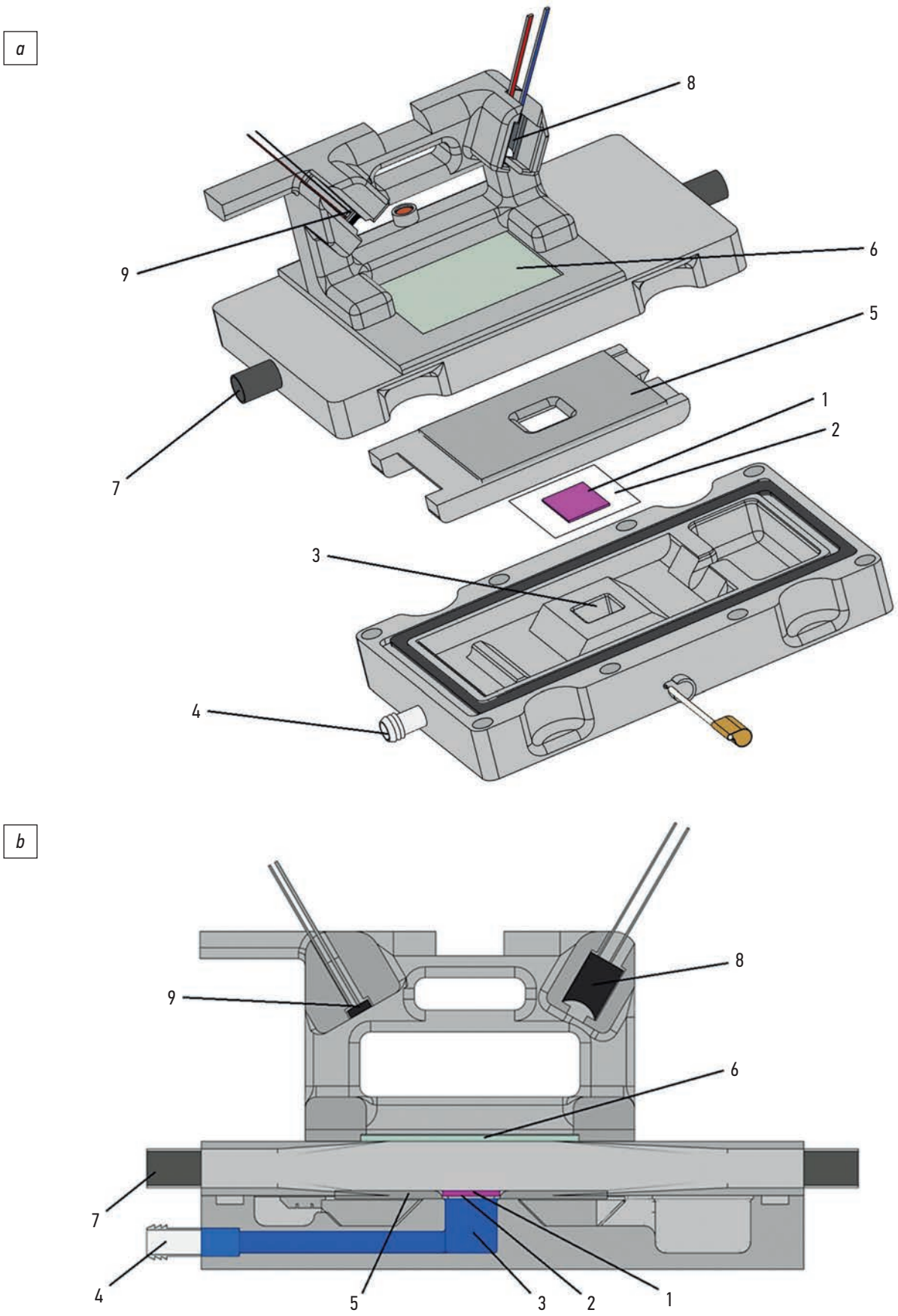

Рис. 1. Схема установки для осаждения аэрозоля на клеточный слой: *a* — вид в разборе, *b* — продольное сечение камеры. Описание обозначенных элементов дано в основном тексте.
Fig. 1. Diagram of a chamber used for the deposition of aerosol onto cells. The components are described in the text.

The reservoir with the culture medium was connected to the metallic part of the lower wall of the chamber using a detachable contact. The electric field was generated in the chamber using a high-voltage power supply VIDN-30 (OST, Russia). It generated a voltage of 10 kV with the negative pole of the power supply connected to the membrane with the cells.

Optical Sensor to Monitor Cell Hydration Level

An optical system to monitor cell hydration was installed on the upper wall of the chamber. The optical system consists of a laser module with a wavelength of 650 nm (TXL-04, China) (8) and a photodiode (FDK-155, Russia) (9) aligned opposite one another using a layer of insulating material and directed at equal angles (60°) towards the membrane surface. Positions of the laser module and photodiode are adjusted so that the laser beam reflected from the membrane surface is pointed to the photodiode's aperture and the photodiode output voltage is recorded by a microcontroller. Hydration of the cellular layer influences the roughness of the surface of the reflective interface; cells protruding from the liquid alter the surface topography and modulate the degree of laser scattering. When the cells are fully immersed in the liquid, the beam reflects from the smooth liquid surface with the least light scattering, resulting in the highest photodiode output voltage. Conversely, as the liquid level decreases, increased surface unevenness enhances light scattering and leads to a decreased sensor output voltage.

Experiments With the Cell Layer Exposed to Air

The cells were cultured on the surface of a polyethylene terephthalate track membrane with a pore size of 0.4 μm. Prior to cell application, the membrane was treated with low-pressure air plasma for 5 s to enhance wettability and improve adhesion properties. Then, suspension of cell culture at a density of 10^5 cells/cm^2 ± 1.6% in DMEM/F12 culture medium with 10% fetal bovine serum was applied using an automatic pipette and a special stencil with an area of 1 cm^2. After deposition, the membrane with cells was placed in a CO_2 incubator for two days at a temperature of 37.1 °C to form a monolayer adhered to the membrane surface. Resulting cell layer samples were placed on the liquid surface in the chamber reservoir filled with culture medium (the cell layer was oriented toward the air-facing side of the membrane). The membrane was then secured using a clamping plate, and the required negative pressure was generated beneath the membrane by adjusting the liquid level in the vessel with the medium, which was flexibly connected to the reservoir in the chamber. Finally, the upper and lower parts of the chamber were connected and optical sensor readings were recorded.

Analysis of Cell Viability

After 2 days of cultivation at 37 °C in an atmosphere containing 5% CO_2, cells were stained with 2 μM calcein AM, 1 μM propidium iodide, and 1 μM bisbenzimide for 15 min at 37 °C. Cell viability was assessed by analyzing microphotographs made using a fluorescent microscope (Axiovert 200M, Zeiss, Germany). The cells were counted using the open-source software ImageJ (NIH ImageJ). At least five fields of view were analyzed for the purposes of image assessment. The cell survival parameter was calculated as the ratio of the number of viable cells in the sample and the number of viable cells in the control sample expressed as a percentage. All experiments were conducted in triplicate (n = 3). Data are presented as mean ± standard deviation. A two-sample t test was used to determine statistically significant differences. A p value of < 0.05 was considered significant for all statistical assessments. Statistical analysis was conducted using Origin 2022 software (OriginLab Corporation, USA).

Preparation and Characterization of Charged Nanoaerosols

Charged nanoaerosols with positively charged aerosol particles were generated using a charged nanoaerosol generator by electro-spraying followed by gas-phase electron neutralization [8, 9]. A solution of solid material forming the aerosol was atomized from a capillary connected to the positive side of the high-voltage power supply. A capillary connected to a negative side of the power supply was filled with 96% ethanol. The current on the positive and negative capillaries was measured at 100 nA and 40 nA, respectively. The aerosol generation rate was 2 L/min. The size distribution of aerosol particles was measured using an aerosol spectrometer (DAS-2702, AeronanoTech, Russia). The mass concentration of aerosols was determined using a dust meter (KANOMAX 3521, Japan).

Measurement of the Amount of Substance Deposited on the Membrane

To determine the number of nanoparticles deposited per unit area of the membrane, a porous membrane was replaced with foil of equivalent area. The culture medium has high electrical conductivity due to salts dissolved in it. We hypothesize that the magnitude of the electric field acting on charged aerosol particles near the surface of a wet grounded membrane with cells is comparable to that observed when the membrane is replaced with a grounded foil. Aerosol was deposited in a chamber at a voltage of 10 kV for 30 min. Then, the deposited aerosol particles were washed off the surface of the foil using 10 μL phosphate buffer. The concentration of doxorubicin in the resulting solution was determined using a spectrophotometer (Cary 100 Scan, USA).

RESULTS

Evaluation of Exposure Parameters for Cells in the Aerosol Deposition Chamber That do not Result in Cell Death

A549 lung carcinoma cells were cultured on the membrane for 48 h to create a monolayer. Subsequently,

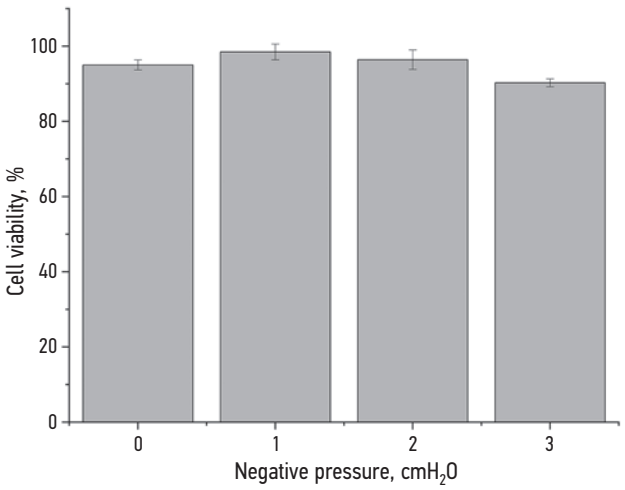

Рис. 2. Зависимость жизнеспособности клеток A549 от величины отрицательного давления, приложенного к жидкости под мембраной при 10-минутной экспозиции в камере для осаждения аэрозоля.
Fig. 2. Relationship of A549 cell viability and the negative pressure applied to the liquid beneath the membrane during 10-minute exposure in the aerosol deposition chamber.

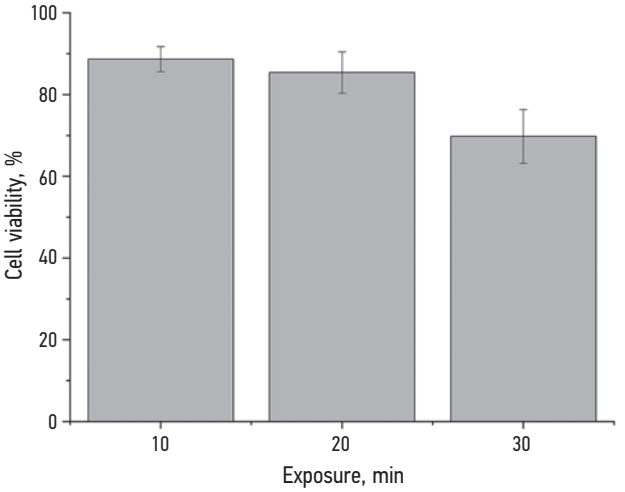

Рис. 3. Зависимость жизнеспособности клеток A549 от времени экспозиции в камере при значении отрицательного давления, приложенного к жидкости под мембраной, равном 2 см вод. ст.
Fig. 3. Relationship of A549 cell viability and exposure time in the chamber at a negative pressure of 2 cmH_2O applied to the liquid beneath the membrane.

sterile air was pumped through the chamber above the cell layer at a rate of 2 L/min with various exposure times and different negative pressure applied to the liquid beneath the membrane. Following exposure, the membrane with the cells was fully immersed in the culture medium. Cell viability was assessed 24 h later.

The relationship of cell viability and negative pressure applied to the liquid beneath the membrane during a 10-minute exposure in the aerosol deposition chamber is shown in Fig. 2. Exposure of the cells at negative pressure ranging from 0 to 3 cmH_2O did not result in the death of a significant proportion of cells (~90% viable cells). The relationship of cell viability and exposure time at a negative pressure of 2 cmH_2O applied to the liquid beneath the membrane is shown in Fig. 3. Increasing the exposure time to 30 min resulted in a gradual decrease in the proportion of surviving cells to approximately 70%.

Verification of Optical Sensor Efficiency

Fig. 4 shows standard relationship of sensor readings and the negative pressure applied to the liquid beneath the membrane. The pressure is generated by creating a height differential between the liquid in the reservoir beneath the membrane and that in the connected movable vessel. Therefore, the pressure on the graph is expressed in cmH_2O. As the pressure decreases, the cells protrude more from the liquid surface, resulting in less reflected laser beam detected by the photodiode. At a negative pressure

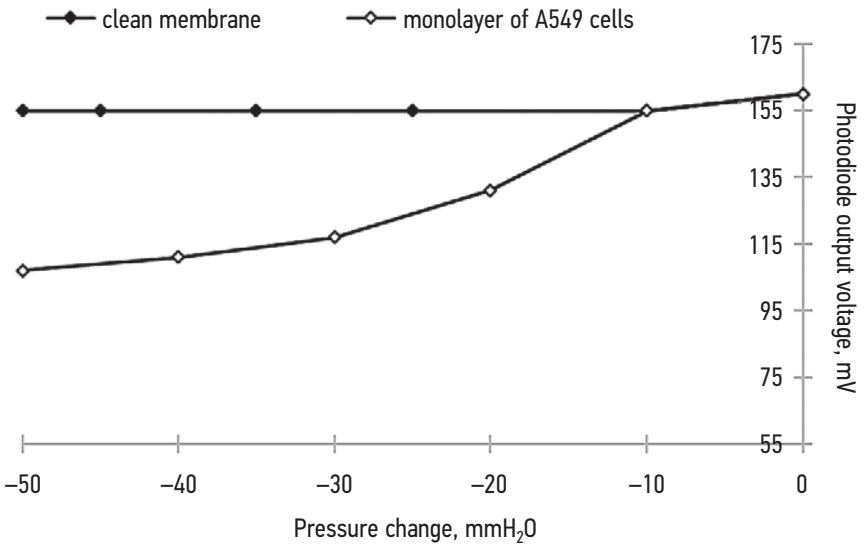

Рис. 4. Зависимость показаний датчика уровня жидкости от приложенного к жидкости под мембраной отрицательного давления. Clean membrane — мембрана без клеток, Monolayer of A549 cells — мембрана с монослоем клеток на верхней поверхности.
Fig. 4. Relationship of liquid level sensor readings and the negative pressure applied to the liquid beneath the membrane.

of 2 cmH_2O and below, it can be observed that the cells visually protrude above the liquid layer on the membrane surface. For a clean membrane surface, light scattering and pressure variation actually do not correlate. All further experiments were conducted at a negative pressure of 2 cmH_2O applied to the liquid beneath the membrane, unless otherwise specified.

Assessment of Cytotoxicity of Electro-Sprayed Water and Ethanol Products and Non-toxic Nanoaerosol (Glucose) Deposited on A549 Cell Monolayers

We conducted control experiments by depositing glucose nanoaerosol on the cell monolayer for 10 min and exposing it to the products of electro-spraying from the generator using pure water and ethanol for the same duration. A 10 mg/mL aqueous solution of glucose was aerosolized in the generator to produce the glucose aerosol. The particle size distribution of the resulting nanoaerosol is shown in Fig. 5, *a*. The mass concentration of the aerosol was 0.58 ± 0.04 μg/L with an average aerosol particle diameter of 55 nm and a particle concentration of (79 ± 3) × 10^3/cm^3. As the current in the positive capillary of the generator was 2.5 times higher than that in the negative capillary, the generated aerosol contained a significant proportion of positively charged aerosol particles.

After exposure, the membrane with cells was fully immersed in the culture medium. Cell viability was assessed 24 h later. The cell viability analysis for the control experiments are shown in Fig. 6.

Analysis of the Cytostatic Effect of Nanoaerosolized Doxorubicin Hydrochloride on A549 Cell Culture

Nanoaerosolized doxorubicin hydrochloride was generated by electropsraying doxorubicin solution at

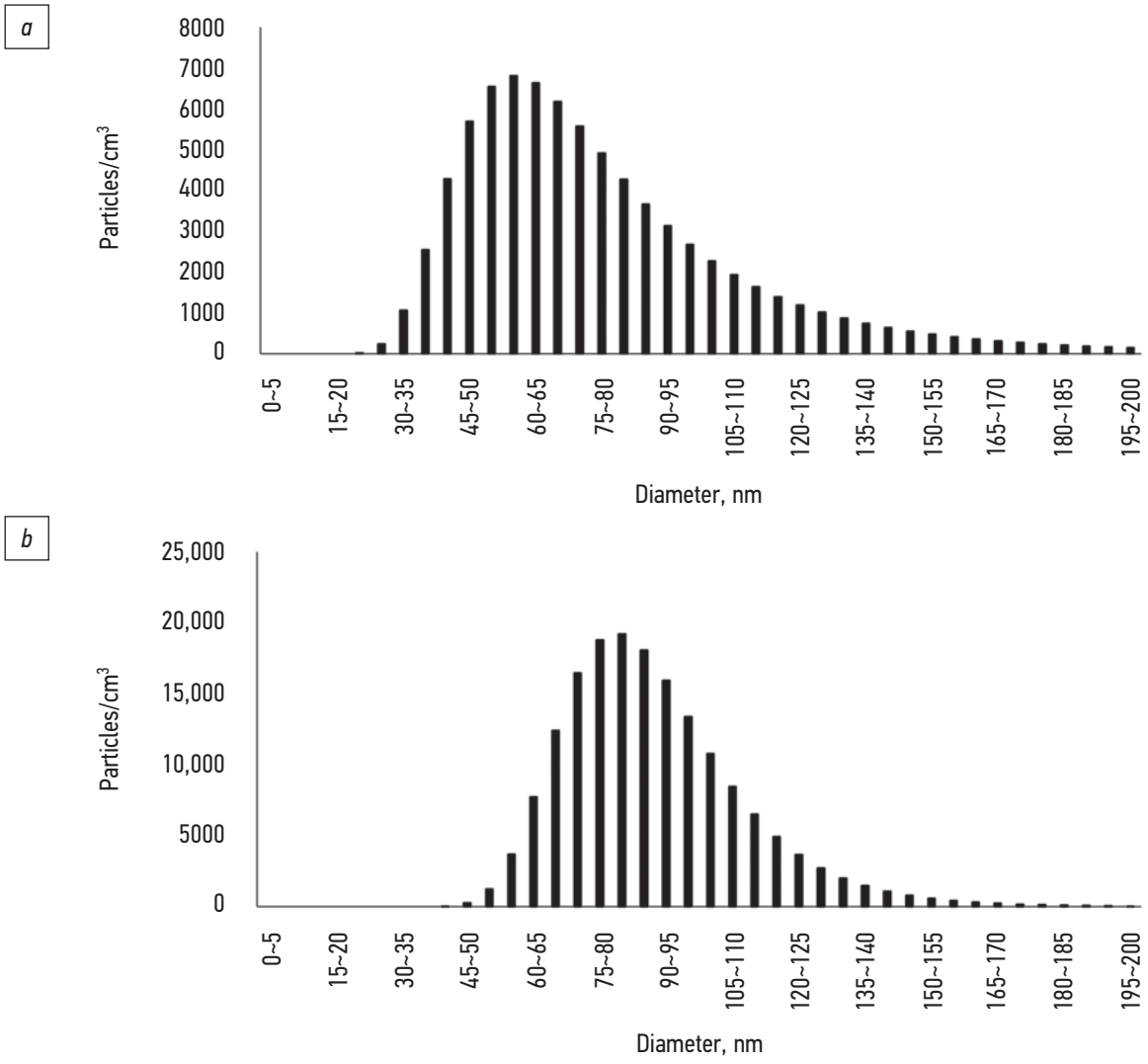

Рис. 5. Распределение размеров наночастиц в аэрозолях, получаемых распылением в генераторе растворов: *a* — 10 мг/мл глюкозы в воде, *b* — 3 мг/мл доксорубицина гидрохлорида в 30% водном этаноле.
Fig. 5. Size distribution of aerosol nanoparticles generated by solution spraying in the generator.

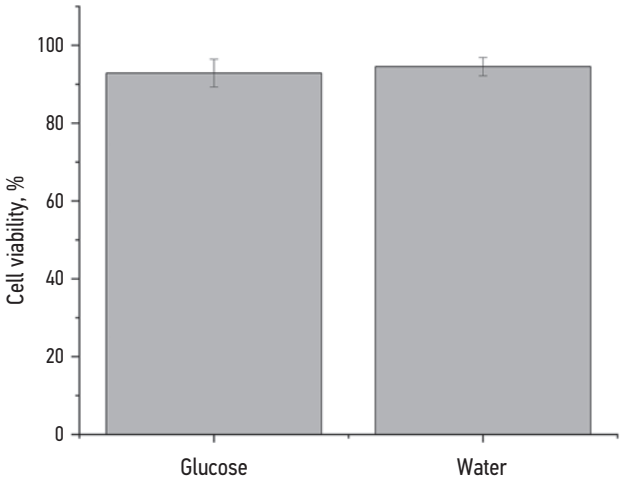

Рис. 6. Жизнеспособность клеток A549 после осаждения наноаэрозоля глюкозы (Glucose) и продуктов электрораспыления воды (Water) в течение 10 мин.
Fig. 6. A549 cell viability after depositing glucose nanoaerosol and water electrospray products for 10 min.

a concentration of 3 mg/mL in 30% aqueous ethanol. The particle size distribution of the resulting nanoaerosol is shown in Fig. 5, *b*. The mass concentration of the aerosol was 0.45 ± 0.03 μg/L with an average particle diameter of 90 nm and particle concentration of (197 ± 27) × 10^3/cm^3.

Following the deposition of the aerosol, the membranes with the cells were incubated in the culture medium for 72 h. Then, we assessed cell viability. Representative images of aerosol-exposed and control cell monolayers are shown in Fig. 7. The cell survival was calculated as the ratio of the number of viable cells in the sample and the number of viable cells in the control sample expressed as a percentage. The relationship of cell survival and the duration of aerosol deposition is shown in Fig. 8. A characteristic inverse relationship of the proportion of surviving cells and the duration of aerosol deposition is observed. The LD50 for doxorubicin aerosol particles is reached with an exposure duration of 2 min.

The aerosol deposition effect was also evaluated at a higher cell hydration (the negative pressure applied to the liquid beneath the membrane was changed from 2 to 0.5 cmH_2O) without any voltage applied to the chamber. Both modifications of the aerosol deposition conditions resulted in a significant increase in cell survival (see Fig. 8).

Quantity Assessment of Deposited Aerosol Particles per Unit Area of the Membrane Over Time

Measurements show that 0.10 mg of doxorubicin settles on 1 cm^2 of membrane per minute, corresponding to the deposition of 1.0E + 12 particles with a diameter of 90 nm. In addition, we estimated the weight and quantity of aerosol particles deposited per cell assuming that the average diameter of A549 cells is 13 μm [10]. It was estimated that 1.3E + 06 nanoparticles of doxorubicin settle on each cell per minute, which is equal to a total of 0.13 ng of deposited particles.

Assessment of Cell Viability During Cyclic Variations of Cell Layer Hydration in the System

We conducted experiments to test the pressure control system during the simulation of breathing and cyclic exposure for 60 min. The range of negative pressure applied to the liquid beneath the membrane varied from 0.3 to 2 cmH_2O. Laser sensor operation in cyclic mode used to monitor cell hydration is shown in Fig. 9. Peak light scattering by the cell layer coincided temporally with the points of application of the highest negative pressure and vice versa. For comparison, another experiment was conducted with static exposure of the cells to air for the same duration at a negative pressure of –2 cmH_2O. After exposure, the membrane with the cells

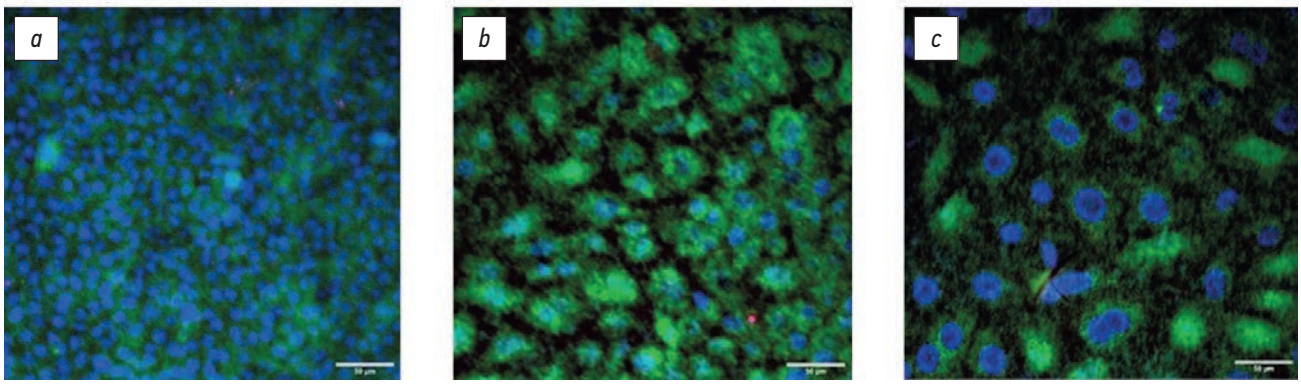

Рис. 7. Репрезентативные изображения монослоя клеток A549, окрашенных кальцеином (живые клетки, зелёный), пропидия йодидом (мёртвые клетки, красный) и бисбензимидом (ядра, синий), через 72 ч культивирования после осаждения наноаэрозоля доксорубицина: *a* — контрольного образца без осаждения аэрозоля; *b* — в течение 2 мин; *c* — в течение 5 мин. Бар 50 мкм.
Fig. 7. Representative images of A549 cell monolayer, stained with calcein (living cells, green), propidium iodide (dead cells, red), and bisbenzimide (nuclei, blue), after 72 h of cultivation following the deposition of doxorubicin nanoaerosol. Scale bar: 50 μm.

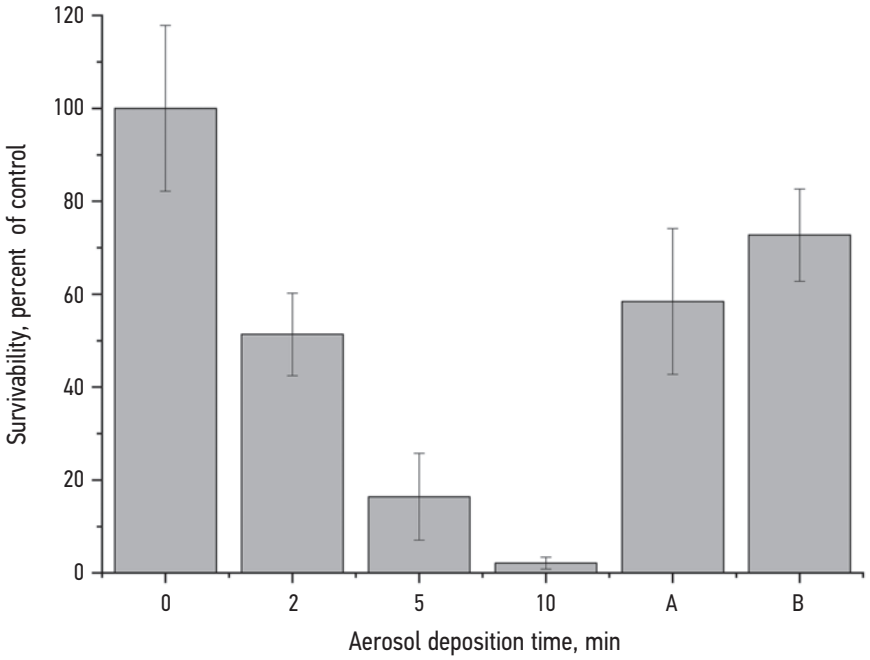

Рис. 8. Выживаемость клеток A549 в зависимости от времени осаждения наноаэрозоля доксорубицина. A — осаждение аэрозоля доксорубицина в течение 5 мин при повышенной степени обводнённости клеток (к жидкости под мембраной приложено отрицательное давление 5 мм водн. ст.); B — осаждение доксорубицина в течение 5 мин при отсутствии электрического поля внутри камеры.
Fig. 8. A549 cell survival as a function of doxorubicin nanoaerosol deposition time. A, doxorubicin aerosol deposition for 5 min with increased cell hydration (negative pressure of 5 mmH_2O is applied to the liquid beneath the membrane); B, doxorubicin deposition for 5 min with no electric field in the chamber.

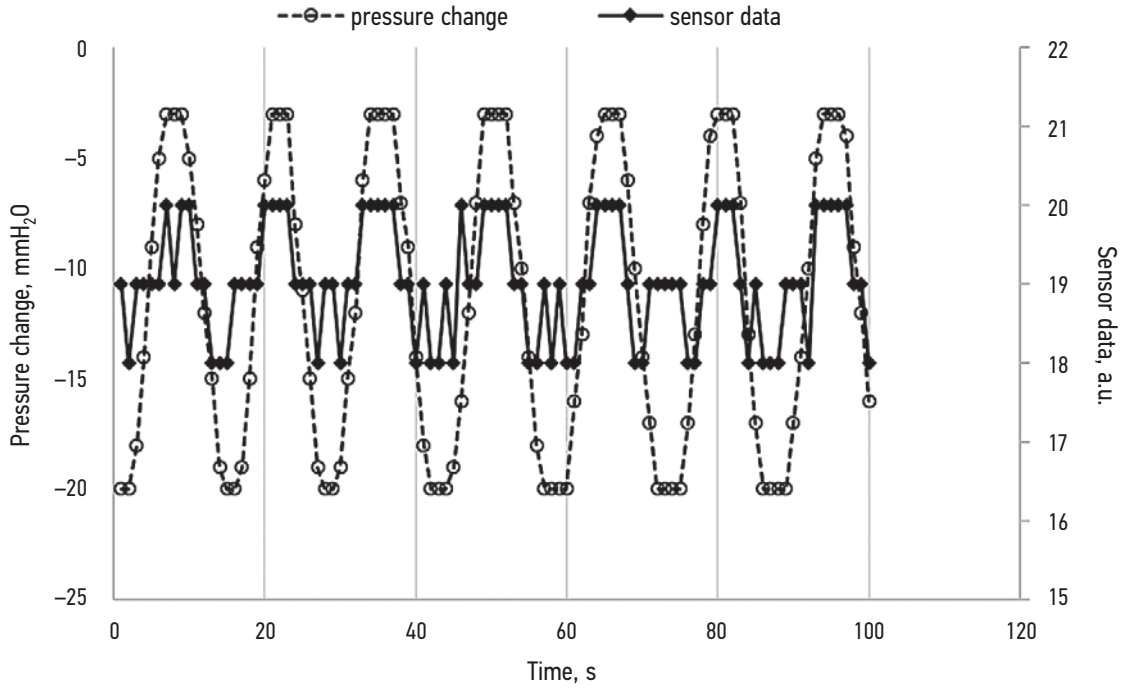

Рис. 9. Зависимость показаний датчика уровня жидкости от приложенного к жидкости под мембраной давления в циклическом режиме. Pressure change — давление, прикладываемое к жидкости под мембраной, Sensor data — показания лазерного датчика обводнённости клеток.
Fig. 9. Relationship of liquid level sensor readings and the pressure applied to the liquid beneath the membrane in cyclic mode.

was fully immersed in the culture medium. Cell viability was assessed 24 h later. The pressure variation modes and the viability analysis of A549 cells are shown in Fig. 10 (*a*, breathing simulation; *b*, cyclic exposure; *c*, static exposure; *d*, cell viability). It was demonstrated that cyclic variation modes with periodic increases in the hydration level of the cell layer allow to maintain cell viability at over 80% for 60 min. In contrast, after exposure of the same duration under static conditions, cell viability was significantly lower, averaging around 60%.

DISCUSSION

In this study, tests were conducted on an experimental lung-on-a-chip platform that simulates the internal surface of pulmonary alveoli. This platform consists of a cell layer

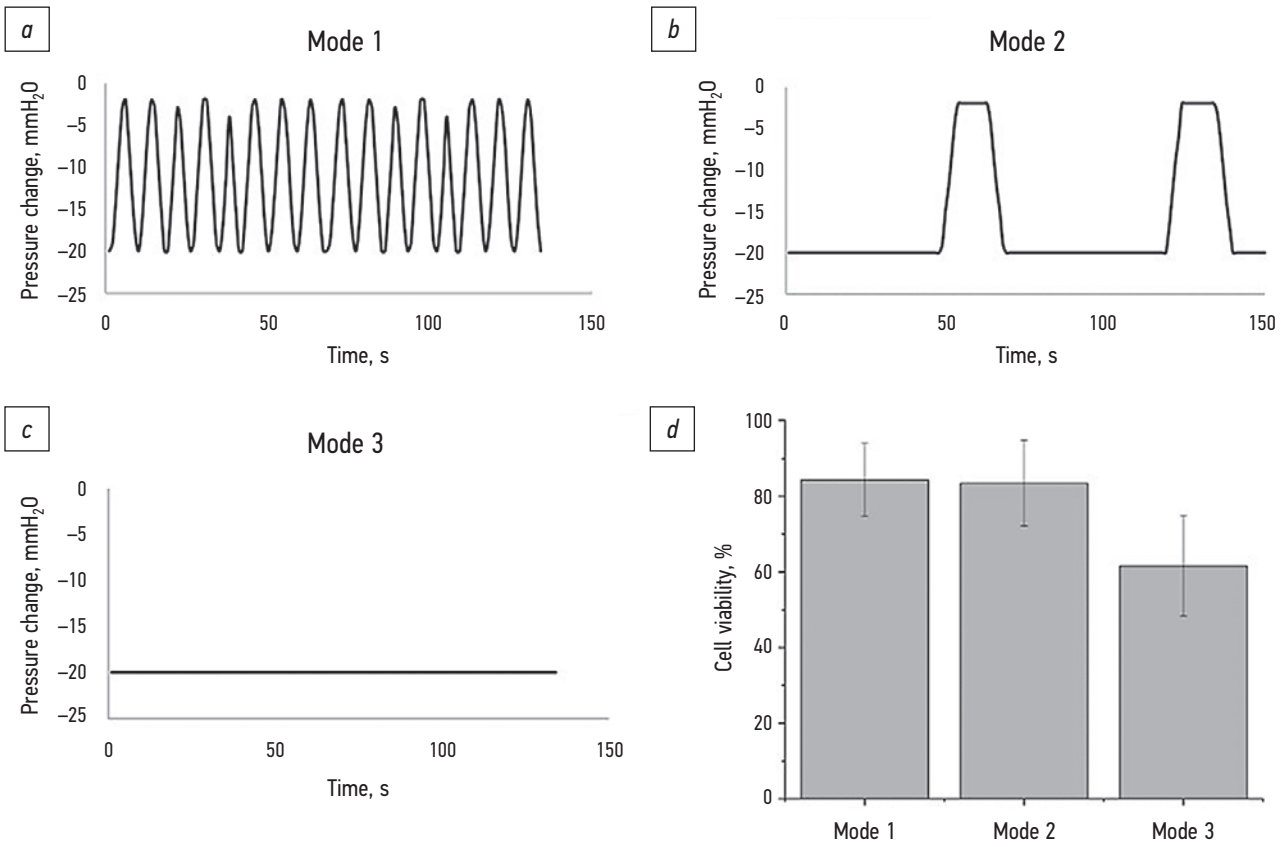

Рис. 10. Испытание режимов работы камеры с периодическим изменением давления: *a–c* — различные паттерны изменения давления (mode 1–3); *d* — жизнеспособность культуры клеток A549 через 60 мин экспозиции в камере.
Fig. 10. Testing the chamber operation modes with periodic pressure variations.

located at the air-liquid interface and can simulate the respiratory cycle. To control the cellular immersion in the liquid, the cell layer is cultured on a porous membrane fixed on the surface of a reservoir with the culture medium. The placement of cells on a porous membrane to model distal regions of the lungs has been previously described in the literature [7, 11]. The main difference of the proposed design from the existing alternatives is the method of altering the liquid level above the cells, which involves the pressurization of the culture medium through the pores of a stretch-resistant membrane based on the pressure applied to the liquid beneath the membrane. The membrane is securely fixed on the reservoir surface to prevent deformation. The fixed membrane allows to use an optical sensor, the second important difference of the proposed design from existing models. The setup software allows to control the pressure applied to the liquid beneath the membrane via a personal computer by recording and retrieving real-time readings of the laser sensor measuring hydration level and to implement cyclic pressure variation with specified frequency and amplitude. The connection of conductive walls in the chamber to a high-voltage power supply facilitates the creation of a directed electric field normal to the aerosol flow passing over the cell layer, which is connected to one electrode. The electric field can substantially improve the delivery rate of charged aerosol particles to the cell surface. The charge can be imparted on aerosol particles by interaction with charged air ions generated by a commercial source or electro-spraying in a generator that produces charged nano-aerosol [9, 12]. The diagram of the main components of the experimental setup is shown in Fig. 11.

A549 human adenocarcinoma alveolar basal epithelial cell line was used as a model culture to simulate pulmonary epithelium. This cell line is widely used as a model for pulmonary epithelial cells in drug metabolism studies [13] and in lung-on-a-chip models [14]. The cells were cultured on a plasma-activated porous membrane surface for 48 h to create a monolayer. Exposure of the cells on the membrane surface in an aerosol deposition chamber—with sterile air pumped into it at a rate of 2 L/min and negative pressure ranging from 0 to –3 cmH_2O applied to it for 10 min—did not result in significant cell death as over 90% of the cells were still viable 24 h post-experiment. Laser sensor measurements showed that light scattering was distinctly observed at a pressure of –2 cmH_2O due to protrusion of the cellular monolayer membranes from the liquid. A possible physiological analogy to the application of negative pressure to the liquid beneath a membrane with cells to control cell hydration is the intrapleural pressure in human lungs, which is essential for keeping the lungs expanded. This pressure

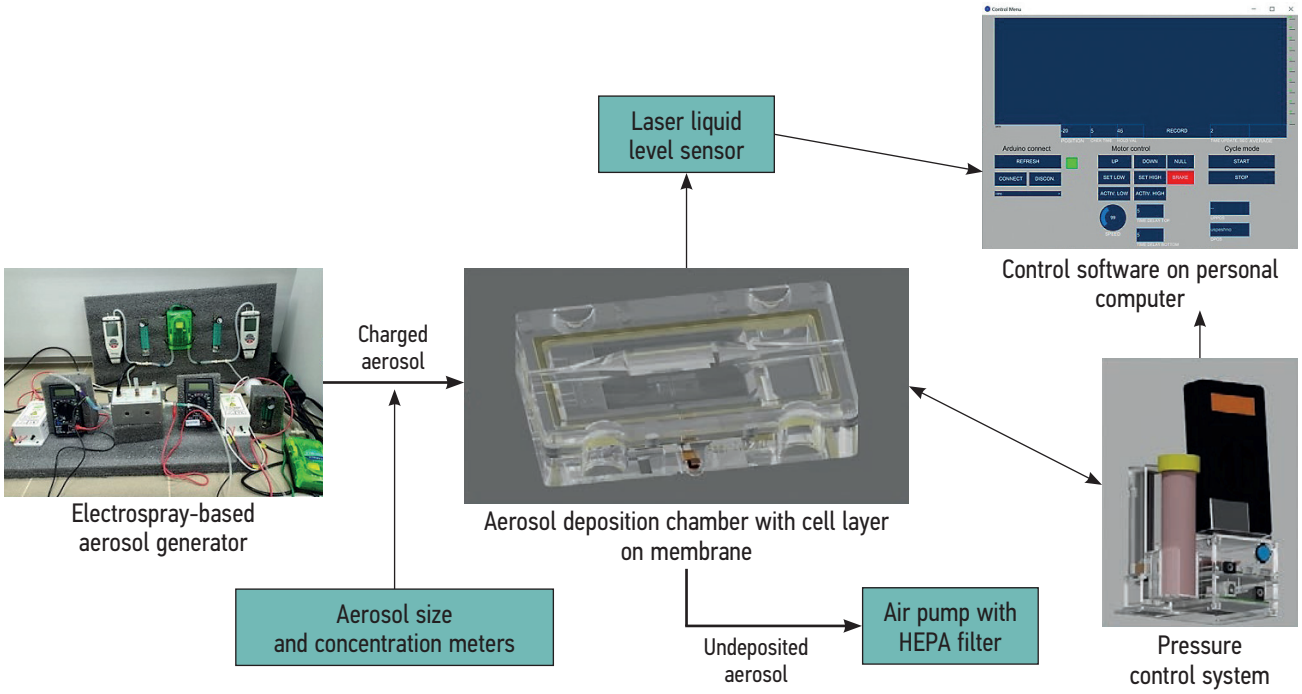

Рис. 11. Общая схема установки для изучения воздействия аэрозоля на клеточный слой в модели «лёгкое-на-чипе».
Fig. 11. Diagram of the setup used to study the effects of aerosol deposition on a cell layer in a lung-on-a-chip model.

is always negative relative to alveolar pressure and has an average value of –5 cmH_2O in humans [15], a value that closely approximates the pressure range used in our model.

For the static operation of the system, exposure time of 10 min and the highest applied negative pressure of 2 cmH_2O were selected. In addition, we tested operation modes with periodic increases of the cell layer hydration. Two patterns of periodic pressure variation were chosen; the first one involving continuous pressure modulation to simulate the expansion and contraction of pulmonary alveoli during respiration (see Fig. 10, *a*). Laser sensor readings showed that the periodic pressure changes were actually associated with hydration fluctuations in the cells. The second pattern is the cyclic exposure, where cells are mainly exposed to air but periodically immersed in culture medium (see Fig. 10, *b*). This approach aims to maximize the deposition of aerosol particles onto the cell surfaces. It was demonstrated that in both operation modes, periodic immersion of the cells in liquid significantly extended the duration of exposure of the cell layer at the liquid–air interface (from 10 min to 1 h) while maintaining the cell viability level above 80%. This system integrated in the experimental setup allows to substantially increase the potential duration of aerosol deposition and facilitates the study of interaction patterns between the model epithelium and aerosol in a simulated breathing scenario.

It was shown that the aerosolized products of water and ethanol from the generator and a non-toxic glucose aerosol did not have cytotoxic effects upon deposition. This data is consistent with previous studies by the authors, who have demonstrated that there is minimal formation of reactive oxygen species in the standard operation conditions of the generator [16]. This also indicates that the particles, when deposited in an electric field, do not have sufficient kinetic energy to disrupt the cell membrane upon contact with its surface.

The specific activity of the aerosolized antitumor cytostatic drug doxorubicin hydrochloride was evaluated in relation to A549 lung carcinoma cell culture. The LD50 for doxorubicin aerosol particles is reached with exposure duration of 2 min. When the dosage is delivered for 10 min, nearly complete cell death is observed (see Fig. 8). Based on the estimated number of deposited particles, approximately 10^{13} doxorubicin particles with an average diameter of 90 nm with a total weight of about 2 ng settle on each cell within 10 min. Direct comparison of the aerosol dosage with the LD50 for the liquid phase is challenging. However, as shown in Fig. 7, *a*, increased cell hydration corresponding to changes in the pressure applied to the liquid beneath the membrane results in more than a threefold increase in cell survival at the same deposition time. This is likely caused by the fact that some aerosol particles dissolve in the liquid instead of contacting the cell membrane. According to literature, direct contact of aerosol particles with cells can improve the absorption rate of certain drugs by up to 12 times compared to solutions in water [14]. Thus, the study demonstrated that the nanoaerosolized drug preserves its specific activity upon interaction with cells. The model aerosol made of a non-toxic substance (glucose) does not have toxic effects under similar conditions.

According to Fig. 5, *b*, aerosolized doxorubicin has an average particle size of 90 nm. When inhaled, particles of this size can penetrate deep into the lungs and reach the alveoli [17]. The deposition of nanoparticles in the body is expected to occur mainly in the acinar region of the lungs via a diffusion mechanism [18]. The difference in deposition rates between doxorubicin particles in the chamber under the influence of an electric field and the deposition of aerosol with equivalent particle diameters in the lungs can be estimated using the Mathematical Particle-Phase Deposition (MPPD) model, which predicts aerosol deposition efficiency in the lungs of mice based on particle diameter [19, 20]. For particles with a diameter of 90 nm, the MPPD model predicts a deposition efficiency of 39% in the lungs of mice. The surface area of the lungs in mice is approximately 600 cm^2 [21] and the volume of air inhaled per minute is about 32 mL. Therefore, based on the volumetric concentration of nanoparticles in the doxorubicin aerosol, i.e. $(197 \pm 27) \times 10^3/cm^3$, and the deposition efficiency, approximately 3600 aerosol particles will settle on 1 cm^2 of lung surface per minute when inhaling an uncharged aerosol with a diameter of particles of 90 nm. In contrast, in the setup chamber, the deposition rate on the same area would be 10^{12} particles/min. Thus, the charge on aerosol particles allows to use electric field to increase the deposition rate by approximately 8 orders of magnitude. This enables the use of a small area cell layer sample for the rapid deposition of numerous of aerosol particles to significantly accelerate the acquisition of data on the interaction of aerosol particles with cells, cytotoxicity, drug efficacy, and other related parameters. It should be noted that charged aerosol particles will also deposit more effectively in the lungs compared to neutral particles, even with no external electric field, due to the electrostatic interactions between the particle and the induced mirror charge that occurs when a charged particle approaches a conductive surface in the airways [22].

CONCLUSION

In this study, we conducted tests using an experimental lung-on-a-chip platform that simulates the interaction between inhaled aerosol particles and the pulmonary epithelium on the internal surface of the pulmonary alveoli. The model pulmonary epithelial cell culture is cultivated on a porous polymer membrane located on the surface of a reservoir with the culture medium. The membrane with the cells is placed in a chamber through which the aerosol under investigation is pumped. For the first time, a method for assessing the degree of scattering of a laser beam reflected from the surface of the cell layer was used to evaluate the extent of cell immersion in the liquid. The setup allows to control the pressure applied to the liquid beneath the membrane, record laser sensor readings for hydration in real time, and implement a periodic pressure variation with specified frequency and amplitude to simulate the breathing cycle of pulmonary alveoli while maintaining a high cell viability for at least one hour. The study showed that the nanoaerosolized drug (doxorubicin hydrochloride) preserves its specific activity upon interaction with adenocarcinoma lung cells. The model aerosol of a non-toxic substance (glucose) did not have toxic effects under similar conditions. It was shown that the presence of a charge on aerosol particles allows to use electric field in the chamber to increase the deposition rate by approximately 8 orders of magnitude compared to the diffusion deposition of neutral particles of similar size. This enables the use of a small area cell layer sample for the rapid deposition of numerous of aerosol particles to significantly accelerate the acquisition of data on the interaction of aerosol particles with cells, cytotoxicity, and the efficacy of the deposited substance. As a result, the developed setup is a comprehensive platform to study the inhalation toxicity of natural and artificial aerosols and assess the safety and efficacy of aerosolized forms of drugs *in situ*.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. И.Л. Канев — разработка концепции, курирование данных, формальный анализ, получение финансирования, проведение исследования, разработка методологии, административное руководство исследовательским проектом, предоставление ресурсов, научное руководство, валидация результатов, визуализация, написание черновика рукописи, написание рукописи — рецензирование и редактирование; М.В. Верхолашин — курирование данных, формальный анализ, проведение исследования, разработка программного обеспечения, валидация результатов; М.Е. Тайлаков — курирование данных, формальный анализ, проведение исследования, написание черновика рукописи, написание рукописи — рецензирование и редактирование; О.Ю. Антонова — разработка концепции, курирование данных, формальный анализ, проведение исследования, разработка методологии, научное руководство, написание черновика рукописи, написание рукописи — рецензирование и редактирование. Все авторы одобрили рукопись (версию для публикации), а также согласились нести ответственность за все аспекты работы, гарантируя надлежащее рассмотрение и решение вопросов, связанных с точностью и добросовестностью любой её части.

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Этическая экспертиза. Неприменимо.

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Раскрытие интересов. Авторы заявляют об отсутствии отношений, деятельности и интересов за последние три года, связанных с третьими лицами (коммерческими и некоммерческими), интересы которых могут быть затронуты содержанием статьи.

Оригинальность. При создании настоящей работы авторы не использовали ранее опубликованные сведения (текст, иллюстрации, данные).

Доступ к данным. Все данные, полученные в настоящем исследовании, доступны в статье.

Генеративный искусственный интеллект. При создании настоящей статьи технологии генеративного искусственного интеллекта не использовали.

Рассмотрение и рецензирование. Настоящая работа подана в журнал в инициативном порядке и рассмотрена по обычной процедуре. В рецензировании участвовали два внешних рецензента, один член редакционной коллегии и научный редактор издания.

ADDITIONAL INFORMATION

Author contributions: I.L. Kanev: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing—original draft, writing—review & editing; M.V. Verkholashin: data curation, formal analysis, investigation, software, validation; M.E. Taylakov: data curation, formal analysis, investigation, writing—original draft, writing—review & editing; O.Y. Antonova: conceptualization, data curation, formal analysis, investigation, methodology, supervision, writing—original draft, writing—review & editing. All the authors approved the version of the manuscript to be published and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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