










Alexander S. Ospennikov<sup>1</sup> , Galina V. Kornilaeva<sup>2</sup> , Viktor F. Larichev<sup>2</sup> ,  
Irina T. Fedyakina<sup>2</sup> , Lifeng Fu<sup>3</sup> , Zhuo Chen<sup>4</sup>, Yangyang Yang<sup>4</sup>, Andrey V. Shibaev<sup>1,5\*</sup> ,  
Eduard V. Karamov<sup>2,6</sup> , Ali S. Turgiev<sup>2,6</sup> , Liping Duan<sup>7</sup>, William J. Liu<sup>8</sup>, Olga E. Philippova<sup>1</sup> 

<sup>1</sup>Department of Physics, Lomonosov Moscow State University, Moscow, Russia;

<sup>2</sup>Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, Moscow, Russia;

<sup>3</sup>CAS Key Laboratory of Pathogen Microbiology and Immunology,

Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, China;

<sup>4</sup>Shanghai Key Laboratory of Chemical Biology, Shanghai key Laboratory of New Drug Design,

School of Pharmacy, East China University of Science and Technology, Shanghai, China;

<sup>5</sup>Karagandy University of the name of academician E.A. Buketov, Karaganda, Kazakhstan;

<sup>6</sup>National Medical Research Center of Phthisiopulmonology and Infectious Diseases

of the Russian Ministry of Health, Moscow, Russia;

<sup>7</sup>NHC Key Laboratory of Parasite and Vector Biology, WHO Collaborating Centre for Tropical Diseases,  
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, China;

<sup>8</sup>NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention,

Chinese Center for Disease Control and Prevention (China CDC), Beijing, China

(\*Corresponding author's e-mail: [shibaev@polly.phys.msu.ru](mailto:shibaev@polly.phys.msu.ru))

## Activity against SARS-CoV-2 of Various Anionic Disinfectants and Their Complexes with Hydrophobically Modified Chitosan

The aim of this work was to study virucidal activity against SARS-CoV-2 of several anionic disinfectants, which are antiseptics recommended by the U.S. Environmental Protection Agency (EPA), and to prepare their complexes with hydrophobically modified chitosan active against SARS-CoV-2. Experiments were performed using a clinical isolate of SARS-CoV-2 obtained from a patient in 2020. It was shown that sodium dodecyl benzene sulfonate (SDBS) is already active at rather small concentrations (above 2 mM), which completely deactivate the virus. In the same concentration range, sodium caprylate does not show activity; and sodium lactate is active against SARS-CoV-2 only at much higher concentrations (225 mM). The most effective disinfectant — sodium dodecylbenzene sulfonate — was used to prepare complexes with hydrophobically modified chitosan. It was found that such complexes exhibit antiviral activity at very low concentrations (1.9 mM chitosan monomer units and 0.25 mM SDBS), at which the polymer without surfactant is not active against SARS-CoV-2.

**Keywords:** anionic disinfectants, SARS-CoV-2, chitosan, hydrophobic modification, polymer/surfactant complexes, sodium dodecyl benzene sulfonate, sodium caprylate, sodium lactate.

### Introduction

In 2020, a pandemic outbreak of a new highly contagious coronavirus — SARS-CoV-2 — occurred [1–3], which in the beginning of 2023 is still a worldwide healthcare threat. The major route of viral transmission is airborne [4], however, it was reported that SARS-CoV-2 can be preserved on different surfaces for several days [5–7]. Therefore, development of effective disinfectants is very important for fighting the pandemic. Many types of disinfectants against SARS-CoV-2 are being considered [8], which include alcohol-based formulations [9, 10], quaternary ammonium compounds [11, 12], etc. Another approach consists in the use of carbonic or sulfonic acids and their salts, some of which were approved by the U.S. Environmental Protection Agency (EPA) as disinfectants. These include, for example, dodecylbenzenesulfonic, caprylic (octanoic) or lactic acid. Caprylate was reported to inactivate human immunodeficiency virus (Type-1), bovine viral diarrhea virus and pseudorabies virus [13]. Caprylic acid as an emulsion formulation is effective against several enveloped viruses such as Epstein–Barr, measles, herpes simplex, Zika, orf parapoxvirus, Ebola, Lassa, vesicular stomatitis and SARS-CoV-1, but does not inactivate a non-enveloped norovirus, showing that caprylic acid acts by disrupting the viral envelope [14]. Sanitizing fluids containing a high concentration of sodium dodecyl benzene sulfonate (SDBS) (3 wt% or 86 mM) and 70 wt% of ethanol

show virucidal activity against SARS-CoV-2, and surfactant and alcohol have a synergistic effect in virus deactivation [15]. Mixtures of dodecylbenzenesulfonic and lactic acids were reported to be effective as sanitizers, inactivating, for instance, norovirus [16]. However, a systematic study of the virucidal effect of these compounds on SARS-CoV-2 has not been performed.

One of the possible ways for improving the effectiveness of disinfectants is the preparation of their complexes with oppositely charged polymers [17]. For anionic disinfectants, chitosan and its derivatives are promising polymers for complex formation [18, 19], since chitosan is a polycation at mild acidic pH due to the protonation of amino groups. Complexes of chitosan with anionic molecules have not been regarded as antiviral agents, but they may be very promising due to several factors: 1) a single chitosan molecule bears multiple amino groups and can bind many low molecular weight anionic species within one complex, resulting in a collective transfer of disinfectant molecules onto the virion, 2) chitosan is biocompatible, which may reduce the overall toxicity of the complex as compared to anionic disinfectants alone.

The aim of this work was to investigate the virucidal activity of several anionic disinfectants (sodium dodecylbenzene sulfonate, caprylate and lactate) against SARS-CoV-2, to prepare complexes of hydrophobically modified (HM) chitosan with the most efficient anionic species and to study their virucidal properties.

### Experimental

#### Materials

Sodium dodecylbenzene sulfonate (SDBS, hard type, purity > 95 %) was provided by Tokyo Chemical Industry (TCI). Caprylic acid (purity > 99.9 %) and lactic acid (purity > 99.9 %) were provided by Component-Reactiv, Russia. NaOH (purity > 99 %) was provided by Acros. All the solutions were prepared by using saline solution (0.9 wt% NaCl in distilled water) provided by Groteks, Russia. Caprylic and lactic acids were converted into sodium salts by dissolving in saline solution and adding appropriate amounts of NaOH. The chemical structures of the anionic compounds used in this work are presented in Figure 1.

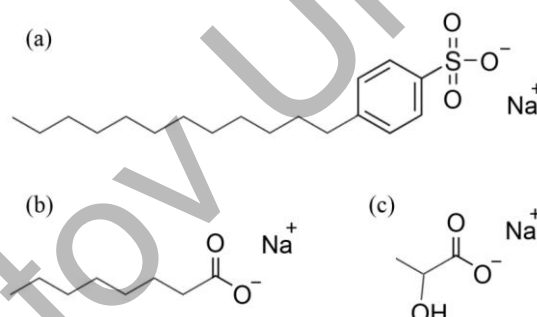


Figure 1. Chemical structure of sodium dodecylbenzene sulfonate (a), sodium caprylate (b) and sodium lactate (c)

Chitosan was obtained from chitin contained in the shells of Far-East crabs. The samples were grinded, and chitin pieces smaller than 0.25 mm were taken. Then, chitin was demineralized: metal salts were removed by triple washing by 1 wt% HCl and water, then by triple washing by 1M NaOH, and then by distilled water. The sample was dried and deacetylated by treatment with 2M NaOH at heating for 2 h, and then washed again by distilled water. This resulted to the conversion of chitin into chitosan. The molecular weight of chitosan was determined by static light scattering in the solvent that suppresses chitosan aggregation (0.3M acetic acid and 0.2M ammonium acetate) [20] and was equal to 170 000 g/mol.

Glacial acetic acid (purity > 99 %), dodecyl aldehyde (purity > 92 %) and sodium cyanoborohydride (purity > 95 %) were purchased from Sigma Aldrich. Ethanol was provided by Ferein (Russia) and was purified by distillation. Isopropanol (purity > 96 %) was obtained from Acros. For chitosan modification, distilled deionized water was used, which was obtained by MilliQ system (Millipore).

For virucidal activity studies, Vero E6 cells from ATCC (Manassas, USA) (CRL-1586) were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 5 % fetal calf serum (FCS), 2 mM L-glutamine and a mixture of antibiotics (150 u/mL penicillin and 150 u/mL streptomycin) at 37 °C in 5 % CO<sub>2</sub>. The stock of SARS-CoV-2 (strain HCoV-19/Russia/Moscow-PMVL-12/2020 (EPI\_ISL\_572398) isolated from a patient) was the culture liquid withdrawn from cultures of the infected Vero E6 cells.

### Hydrophobic modification of chitosan

Hydrophobic modification of chitosan was performed by the method of Rinaudo et al. [21]. First, chitosan (18 g/L) was dissolved in 0.2 M aqueous acetic acid solution by gentle stirring overnight, and pH after dissolution was ca. 0.8–1.0. Then ethanol (2:3 v/v) was added, and pH was adjusted to 5.0 by adding concentrated (1M) aqueous NaOH. After that, an appropriate amount of dodecyl aldehyde solution (20 g/L) in ethanol was added, so that its concentration in the reaction medium was 1.6 g/L. Then, sodium cyanoborohydride was added (concentration in the reaction medium 20 g/L), and the solution was stirred for 48 h at 25 °C.

The product was precipitated by adding 2M NaOH and washed twice with ethanol/water mixture (8:10 v/v) and once with isopropanol. Then, the precipitate was dissolved in aqueous HCl solution (pH 3), filtered through ceramic ROBU filters (16–40 μm pores) and lyophilized. In such a way, hydrophobically modified (HM) chitosan in hydrochloride form was obtained, which is soluble in water [22]. Its chemical structure is shown in Figure 2.

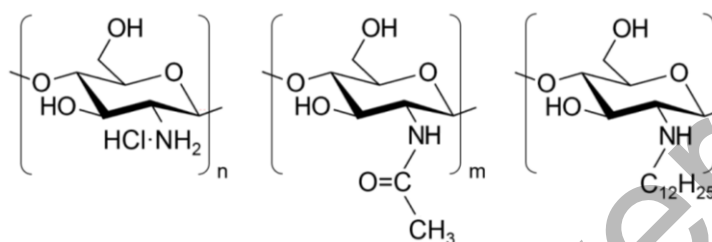


Figure 2. Chemical structure of chitosan hydrochloride modified with *n*-dodecyl side groups

The degree of hydrophobic modification was determined by <sup>1</sup>H NMR spectroscopy using Bruker AV-600 spectrometer. The spectrum was recorded at 30 °C in D<sub>2</sub>O (Astrachem, Russia, isotopic purity > 99.9 %) as a solvent. Peak attribution was made according to the previously published data [21, 23]. The degree of modification (fraction of monomer units modified by *n*-dodecyl groups) was determined from the ratio of peaks at 0.89 ppm (methyl protons of *n*-dodecyl groups) and at 3.02 ppm (C2 methine protons of the saccharide ring) (Fig. 3) and was equal to 4.7 %. The degree of acetylation was calculated from the ratio of peaks at 2.09 ppm (methyl protons of acetyl groups) and at 3.02 ppm and was equal to 6.7 %.

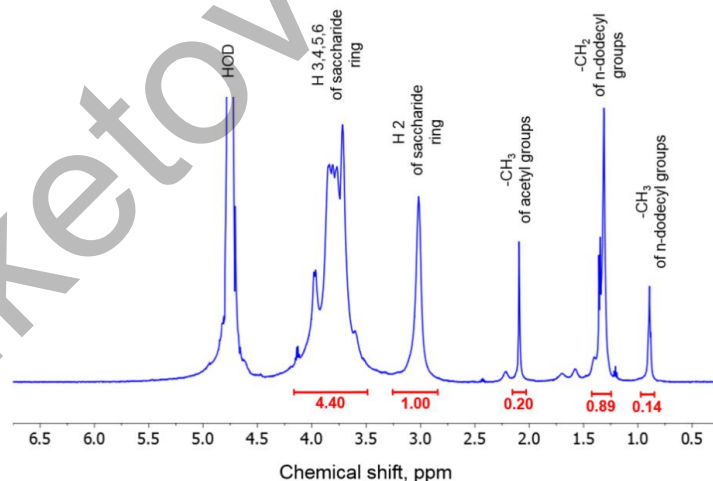


Figure 3. <sup>1</sup>H NMR spectrum of HM chitosan at 30 °C in D<sub>2</sub>O

### Preparation of chitosan / disinfectant complexes

In order to obtain polymer / disinfectant complexes, solutions of chitosan hydrochloride and disinfectant were first prepared in saline (at pH 5.2), and then mixed in appropriate quantities. In the final samples, the concentration of chitosan was kept at 0.32 g/L (1.875 mM of monomer units), and the optimal molar ratio of SDBS to chitosan monomer units was 0.13. Complex formation proceeded due to interaction between anionic disinfectant species and cationic amino groups of chitosan. Complex formation was proven by the following: chitosan / SDBS mixture was dialysed against saline by using dialysis tubes with 12 000 kDa cutoff, through which only small SDBS molecules can pass. After dialysis, the composition of the complex was ana-

lyzed by  $^1\text{H NMR}$ , and the ratio of SDBS molecules to chitosan monomer units was found to be 0.12, e.g. close to the feed ratio during complex preparation, meaning that interaction between chitosan and SDBS within the complex is rather strong and does not allow SDBS molecules to freely leave the complex.

#### *Virucidal activity*

Solutions of anionic disinfectants or their complexes with HM chitosan were incubated with an equal volume of the virus stock at room temperature for 60 min. Viral particles were then separated from disinfectants by centrifugation at 27,000 rpm for 1 h to avoid their toxic effects on the cells. Viral pellets were re-suspended in 300  $\mu\text{L}$  of support medium (DMEM, 1 % FCS), and 10-fold dilutions in support medium were prepared for titer determination. Confluent Vero-E6 monolayers in 96-well plates were infected with the dilutions thus prepared, and, after a 2-h incubation (adsorption), the inoculum was removed. The plates were washed twice with FCS-free DMEM, filled with another type of support medium (DMEM, 2 % FCS) and further incubated at 37  $^\circ\text{C}$  in 5 %  $\text{CO}_2$  for 96 h. Virus-induced cytopathic effects were assessed by microscopic examination of the cells and taken as an indication of their infection. The amount of the active virus, judged from the percentage of the infected cells, was determined by the endpoint dilution assay (titration) and expressed in fifty-percent tissue culture infective doses ( $\text{TCID}_{50}$ ). The titer was calculated using the Spearman-Kärber method and presented as  $\lg \text{TCID}_{50}/0.1 \text{ mL}$  [24].

The virucidal efficacy of surfactants was assessed by the difference in the virus titers (A) between control  $A_c$  (without disinfectants) and experimental  $A_e$  samples:

$$A = A_c - A_e.$$

The protection index, or inhibition coefficient (IC), was calculated using the following formula:

$$\text{IC} = [(A_c - A_e)/A_c] \times 100 \text{ \%}.$$

#### *Results and Discussion*

##### *Virucidal activities of individual anionic disinfectants*

First, virucidal activity of individual anionic compounds was investigated. Sodium dodecylbenzenesulfonate (SDBS) and lactate were dissolved in saline (0.9 wt% NaCl), and pH was adjusted to 5.0 by adding HCl or NaOH (initial pH values after dissolution were 9.2 for SDBS and 1.4 for lactic acid). The values of  $\text{pK}_a$  for dodecylbenzenesulfonic and lactic acid are equal to  $-1.8$  [25] and 3.8 [26], respectively. Therefore, at pH 5.2 they are almost completely in salt forms.  $\text{pK}_a$  of caprylic acid is slightly higher: 4.89 [27]. Thus, in order to transform it into a salt and ensure its solubility in water, the solutions were prepared at slightly higher pH 6.3–6.4. Caprylic acid has a low water solubility (4.7 mM [27]), therefore, rather sophisticated approaches were previously employed to prepare its antiseptic formulations: e.g., emulsification [14] or the use of solvents different than water [28].

Virucidal activity of SDBS at different concentrations is shown in Figure 4 as the virus inhibition coefficient (IC), which shows the relative difference in the virus titers in a control experiment and in the experiment where virus suspension is brought in contact with the disinfectant. It is seen that at very low concentrations (below 0.125 mM), SDBS does not show virucidal activity ( $\text{IC} = 0$ ), but upon increase of the concentration, the activity appears and increases. Rather low absolute SDBS concentrations already show high virucidal activity against SARS-CoV-2: the virus titer in the experiments is reduced by 6.5 orders of magnitude ( $\Delta \lg \text{TCID}_{50} = 6.5$ ), and IC is equal to 100 % (Fig. 4).

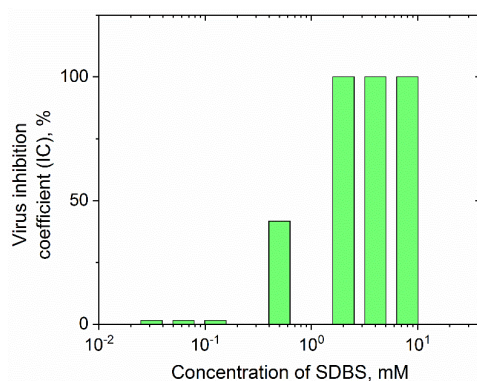


Figure 4. Inhibition coefficients of SARS-CoV-2 by SDBS solutions of different concentrations. Solvent: 0.9 wt% NaCl in water

A possible mechanism of SDBS action against SARS-CoV-2 consists in its incorporation into the viral envelope and its subsequent disruption, as well as in denaturation of viral proteins [29]. The incorporation of SDBS into the lipid bilayer is possible due to its surface activity, since SDBS is a surfactant and consists of a polar hydrophilic benzene sulfonate head, and non-polar hydrophobic alkyl tail, which favors its interaction with the lipid bilayer. The critical micelle concentration (CMC) of SDBS equals to 0.4 mM in saline [30]. It means that SDBS is in the micellar form when it shows virucidal activity. This is reasonable, because micelles provide cooperative transfer of multiple SDBS molecules on each viral particle, increasing the probability of its disruption.

In the same concentration range where SDBS is active against SARS-CoV-2, sodium caprylate does not show any activity (Fig. 5a). This may be explained by lower surface activity of caprylate as compared to dodecylbenzenesulfonate: indeed, it has a shorter alkyl tail (C8) and is characterized by much higher CMC (400 mM [31]) than for SDBS; thus, in the studied range of concentrations, caprylate does not form micelles and is a molecular solution. This may result in a smaller amount of caprylate molecules simultaneously interacting with the envelope surface.

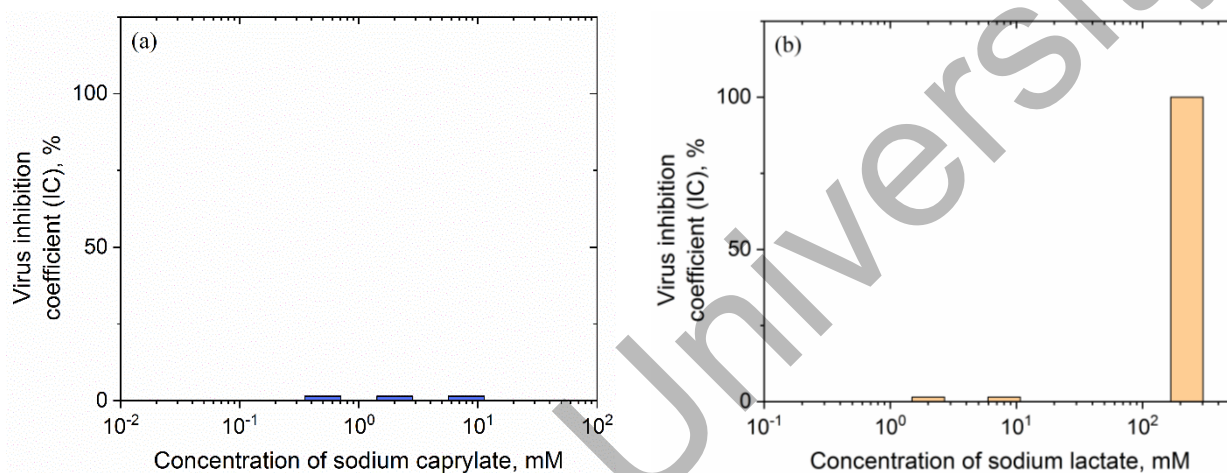


Figure 5. Inhibition coefficients of SARS-CoV-2 by sodium caprylate (a) and sodium lactate (b) solutions of different concentrations. Solvent: 0.9 wt% NaCl in water

At the same concentrations (below 8 mM), sodium lactate does not show any virucidal activity; however, it is active at a much higher concentration. 225 mM of lactate induce a complete inactivation of SARS-CoV-2: its titer is reduced by 6.5 orders of magnitude. Such a high concentration as compared to SDBS may be a result of different properties of these two molecules: SDBS is surface-active and incorporates into the lipid membranes, while lactate is not a surfactant.

Therefore, SDBS shows the highest activity against SARS-CoV-2 among three anionic disinfectants studied: it completely inactivates the virus at rather low concentrations, at which sodium caprylate and lactate do not show virucidal properties. Therefore, SDBS is the most promising disinfectant for preparation of formulations with HM chitosan.

#### *Virucidal activities of polymer / disinfectant complexes*

At the next stage, antiviral properties of chitosan / SDBS complexes were investigated. Figure 6 shows the comparison of virucidal activity of chitosan and its complex with SDBS at very low concentrations of the components. It is seen that while HM chitosan itself does not possess virucidal properties at this concentration, polymer/surfactant complexes show some activity: IC is equal to 18.2 %, meaning that the virus titer is decreased by one order of magnitude upon contact with HM chitosan / SDBS solution.

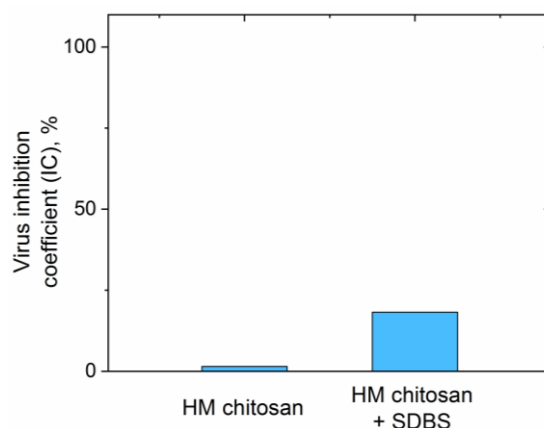


Figure 6. Inhibition coefficients of SARS-CoV-2 by HM chitosan and its mixture with SDBS. HM chitosan monomer units: 1.875 mM, SDBS: 0.25 mM. Solvent: 0.9 wt% NaCl in water

A possible mechanism of virucidal action of HM chitosan / SDBS complexes is similar to those of SDBS and may consist in the disruption of the viral capsid when it is destabilized by incorporation of surfactant molecules. At the same time, the complexes may be more stable than SDBS micelles, since surfactant molecules in the micelle are bound together by hydrophobic interactions, while in the complex they are also bound to the polymer chain due to several distinct interactions: electrostatic interaction between anionic polar heads of the surfactant and of chitosan amino groups, Van der Waals interactions between surfactant and polymer [32], and incorporation of the polymer n-dodecyl groups into the micelles [33]. All these factors may increase the complex stability as compared to the micelles, and may also increase the SDBS micellar aggregation number (which is typical for the case of polymer/surfactant interactions [34]), thus increasing the number of SDBS molecules transferred to one virion by a single complex.

### Conclusions

In this paper, the virucidal activities of several anionic disinfectants (sodium dodecylbenzene sulfonate, caprylate and lactate) against SARS-CoV-2 were investigated. The most effective disinfectant is SDBS, which deactivates the virus already at rather small concentrations (above 2 mM). In the same concentration range, caprylate and lactate do not show any activity. Sodium lactate also shows virucidal properties, but at concentrations 2 orders of magnitude higher than for SDBS. Complexes of HM chitosan with SDBS also possess antiviral properties at very low concentrations (1.9 mM chitosan monomer units and 0.25 mM SDBS), at which the polymer without surfactant is not active against SARS-CoV-2.

### Acknowledgments

The reported study was funded by RFBR, NSFC, and CNPq (BRICS research project 085-ChitoTarCoV; RFBR project No. 20-53-80005).

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#### Information about authors\*

**Ospennikov, Alexander Sergeevich** — 1<sup>st</sup> year PhD student, Department of Physics, Lomonosov Moscow State University, GSP-1, Leninskie Gory, 119991, Moscow, Russia; e-mail: [ospennikov@polly.phys.msu.ru](mailto:ospennikov@polly.phys.msu.ru); <https://orcid.org/0000-0002-1272-3658>

**Kornilaeva, Galina Vladimirovna** — candidate of biological sciences, leading researcher, Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, 18 Gamaleya St., 123098, Moscow, Russia; e-mail: [kornilaeva@yandex.ru](mailto:kornilaeva@yandex.ru); <https://orcid.org/0000-0003-1819-0693>

**Larichev, Viktor Filippovich** — doctor of medical sciences, leading researcher, Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, 18 Gamaleya St., 123098, Moscow, Russia; e-mail: [vlaritchev@mail.ru](mailto:vlaritchev@mail.ru); <https://orcid.org/0000-0001-8262-5650>

**Fedyakina, Irina Timofeevna** — candidate of biological sciences, leading researcher, Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, 18 Gamaleya St., 123098, Moscow, Russia; e-mail: [irfed2@mail.ru](mailto:irfed2@mail.ru); <https://orcid.org/0000-0001-6421-9632>

**Fu, Lifeng** — professor, CAS Key Laboratory of Pathogen Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences (CAS), NO.1 West Beichen Road, Chaoyang District, 100101, Beijing, China; e-mail: [fulf@im.ac.cn](mailto:fulf@im.ac.cn); <https://orcid.org/0000-0003-0431-5424>

**Chen, Zhuo** — professor, Shanghai Key Laboratory of Chemical Biology, Shanghai key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, 200237, Shanghai, China; e-mail: [chenzhuo@ecust.edu.cn](mailto:chenzhuo@ecust.edu.cn)

**Yang, Yangyang** — professor, Shanghai Key Laboratory of Chemical Biology, Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, 200237, Shanghai, China; e-mail: [triyang@ecust.edu.cn](mailto:triyang@ecust.edu.cn)

**Shibaev, Andrey Vladimirovich** — candidate of physico-mathematical sciences, associate professor, Department of Physics, Lomonosov Moscow State University, GSP-1, Leninskie Gory, 119991, Moscow, Russia; Karagandy University of the name of academician E.A. Buketov, Universitetskaya str., 28, 100024, Karaganda, Kazakhstan; e-mail: [shibaev@polly.phys.msu.ru](mailto:shibaev@polly.phys.msu.ru); <https://orcid.org/0000-0002-3019-5764>

**Karamov, Eduard Viktorovich** — doctor of biological sciences, professor, Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, 18 Gamaleya St., 123098, Moscow, Russia; e-mail: [karamov2004@yandex.ru](mailto:karamov2004@yandex.ru); <https://orcid.org/0000-0003-1162-118X>

**Turgiev, Ali Saladinovich** — leading researcher, Gamaleya National Research Center for Epidemiology and Microbiology of the Russian Ministry of Health, 18 Gamaleya St., 123098, Moscow, Russia; e-mail: [turgiev@ld.ru](mailto:turgiev@ld.ru); <https://orcid.org/0000-0002-0500-6407>

**Duan, Liping** — professor, NHC Key Laboratory of Parasite and Vector Biology, WHO Collaborating Centre for Tropical Diseases, National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai 200025, China; e-mail: [duanlp@nipd.chinacdc.cn](mailto:duanlp@nipd.chinacdc.cn)

**Liu, William J.** — professor, NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (China CDC), Beijing 102206, China; e-mail: [liujun@ivdc.chinacdc.cn](mailto:liujun@ivdc.chinacdc.cn)

**Philippova, Olga Evgen'evna** — doctor of physico-mathematical sciences, professor, Department of Physics, Lomonosov Moscow State University, GSP-1, Leninskie Gory, 119991, Moscow, Russia; e-mail: [phil@polly.phys.msu.ru](mailto:phil@polly.phys.msu.ru); <https://orcid.org/0000-0002-1098-0255>

\*The author's name is presented in the order: Last Name, First and Middle Names