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Selective suppression of influenza A/H5N1 virus replication *in vitro* using nanocomplexes consisting of siRNA and aminopropylsilanol nanoparticles

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Abstract

Relevance. Studies on model systems have confirmed the effectiveness of antisense oligonucleotides, including those that contain photoactive groups, for the modification of nucleic acids. However, this strategy has not yet found wide application due to the lack of successful methods for the intracellular delivery. The development of effective preparations capable of acting on target nucleic acids in cells is an urgent task.

The aim of the study is to create nanocomplexes consisting of aminopropylsilanol nanoparticles and short interfering RNA (siRNA) to study their effect on target nucleic acids by the example of inhibition of influenza A virus replication in vitro.

Materials and methods. MDCK cells, influenza virus A/chicken/Kurgan/05/2005 (A/H5N1), aminopropylsilanol nanoparticles, and native and modified siRNA molecules.

Results and discussion. We have prepared unique Si~NH_2/siRNA nanocomplexes, which consist of aminopropylsilanol nanoparticles and siRNA molecules, which enable cell penetration and selective interaction with target nucleic acids, respectively. The antiviral activity of the proposed nanocomplexes has been studied on MDCK cells infected with the influenza A/H5N1 virus. It has been shown that the double-stranded siRNA molecules in the nanocomplexes, which act by the RNA interference mechanism, are more efficient in inhibiting the replication of the influenza virus than the corresponding single-stranded RNA fragments. The most effective nanocomplex that contained siRNA targeted at the chosen region of mRNA segment 5 of the viral genome reduced virus replication in the culture by a factor of 630. We have shown that non-agglomerated and water-soluble aminopropylsilanol nanoparticles are low-toxic, capable of delivering siRNA into cells and protecting siRNA in the Si~NH_2/siRNA nanocomplexes from hydrolysis by cellular nucleases.

Conclusion. The biological activity of the created nanocomplexes has been demonstrated by the example of highly effective selective suppression of influenza A/chicken/Kurgan/05/2005 virus replication in the cellular system.

Keywords: *aminopropylsilanol nanoparticles, nanocomplexes, siRNA, antiviral activity, influenza A/H5N1 virus*

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Оригинальное исследование
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Селективное подавление репликации вируса гриппа A/H5N1 *in vitro* с помощью нанокомплексов, состоящих из siRNA и наночастиц аминопропилсиланола

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Аннотация

Актуальность. Вирусы гриппа, относящиеся к семейству *Orthomyxoviridae*, широко распространены в природе и часто являются причиной возникновения пандемий. Появление новых штаммов вируса, устойчивых к лекарственным препаратам, вызывает потребность в разработке новых эффективных лекарственных форм, селективно действующих на вирусы гриппа А.

Цель работы — создание нанокомплексов, состоящих из наночастиц аминопропилсиланола (АПС) и малых интерферирующих РНК (siRNA), и исследование их воздействия на нуклеиновые кислоты-мишени на примере ингибирования репликации вируса гриппа А в клеточной системе.

Материалы и методы. В работе использовали клетки MDCK, вирус гриппа A/chicken/Kurgan/05/2005 (A/H5N1), наночастицы АПС, нативные и модифицированные молекулы siRNA.

Результаты и обсуждение. Созданы уникальные нанокомплексы Si~NH_2/siRNA, состоящие из наночастиц АПС и иммобилизованных на них молекул siRNA, обеспечивающих соответственно проникновение в клетки и селективное взаимодействие с нуклеиновыми кислотами-мишенями. Противовирусную активность предложенных нанокомплексов исследовали на клетках MDCK, заражённых вирусом гриппа A/H5N1. Показано, что двухцепочечные молекулы siRNA в составе нанокомплексов, действующие по механизму РНК-интерференции, более эффективно подавляют репликацию вируса гриппа по сравнению с соответствующими одноцепочечными фрагментами РНК. Наиболее эффективный нанокомплекс, содержащий siRNA, нацеленную на выбранный участок 5-го сегмента мРНК вирусного генома, снижал репликацию вируса гриппа А в культуре клеток в 630 раз. Показано, что неагломерированные, растворимые в водных растворах наночастицы АПС являются малотоксичными, способными доставлять siRNA в клетки и защищать siRNA в составе нанокомплексов Si~NH_2/siRNA от гидролиза клеточными нуклеазами.

Заключение. Продемонстрирована высокая биологическая активность созданных нанокомплексов на примере селективного и высокоэффективного подавления репликации вируса гриппа A/chicken/Kurgan/05/2005 в клеточной системе.

Ключевые слова: *наночастицы аминопропилсиланола, нанокомплексы, siRNA, противовирусная активность, вирус гриппа A/H5N1*

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Introduction

Nucleic acid (NA) therapy offers unique opportunities to influence the genetic material of the cell. However, its effectiveness is limited by the instability of NAs in relation to cellular nucleases and their low ability to penetrate the cytoplasmic membrane, which makes it necessary to use different delivery systems [1].

Small interfering RNA (siRNA) is a promising type of RNA-based therapeutic agents because their mechanism of action is catalytic and each siRNA molecule can inactivate several target RNA molecules. siRNA molecules are intensively investigated as antiviral agents. L. Singh et al. presented a wide range of applications of nanoscale materials for the treatment of common viral infections [2]. For the clinical success of the proposed methods for the delivery of NA fragments into cells, safety and efficiency remain vital requirements. Various approaches have been proposed to address the problem of siRNA delivery, e.g., using viruses, cationic lipids, polymers and transport peptides. The successful use of bioconjugates of siRNA and N-acetylgalactosamines is also worth mentioning [3]. However, all methods have limitations for therapeutic use. A huge number of potential siRNA-based drugs have not undergone clinical trials because many factors (low efficiency of siRNA delivery to target cells, toxicity, degradation of siRNA by nucleases, filtration by kidneys, uptake by immune cells, off-target effects, low efficiency of penetration through hydrophobic cell membrane and release of siRNA from endosomes, etc.) limit the use of siRNA in biomedicine.

One of the most promising approaches to solving the problem of siRNA delivery into cells is the use of non-viral vectors based on nanoparticles (NPs) [2, 4, 5]. Different types of NPs have been used for delivery of siRNA. NPs consisting of cationic polymers (poly-L-lysine, polyamidoamine, polyethylenimine, and chitosan) or lipids are the most studied delivery methods [6, 7]. Given the wide variety of available materials, each with many potential modifications, the composition of the NPs can be optimized to deliver a specific type of RNA [8-10]. Delivery systems should fulfill a number of important requirements: they should increase the ability of RNA penetration into cells, provide effective protection of RNA from degradation by cellular nucleases, and have low toxicity.

Despite certain successes in the development of methods for the delivery of RNA fragments into cells, the problem of their delivery method still remains unsolved. Therefore, it is advisable to search for other methods to deliver siRNA into cells.

We have previously developed delivery systems for oligodeoxyribonucleotides and deoxyribozymes based on the use of titanium dioxide and aminopropylsilanol (APS) NPs for their effect on NA targets. It has been shown that DNA fragments within the created nanocomposites are site-specific and effectively affect target genes *in vitro* and *in vivo* [11–14].

The aim of this study is to determine the possibility of using non-agglomerated, water-soluble APS NPs for siRNA delivery into cells in the form of the Si~NH_2/siRNA nanocomplexes to effectively suppress replication of the influenza A/H5N1 virus.

Materials and methods

Reagents used in this study were from commercial suppliers: (3-aminopropyl)triethoxysilane, trypsin, penicillin, streptomycin (Sigma-Aldrich); DMEM medium (Dulbecco's modified Eagle's medium; Biolot); fetal bovine serum (Gibco), MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), NeoFroxx), and dimethyl sulfoxide (Component-Reactiv). Trypsin was used at a concentration of 2 μg/mL, penicillin and streptomycin at a concentration of 100 U/mL. Chicken erythrocytes, MDCK cells, and the influenza virus A/chicken/Kurgan/05/2005 (H5N1) strain were obtained from the collections of Vector.

Oligoribonucleotides and their derivatives were synthesized by the solid-phase method on an automated ASM-800 DNA/RNA synthesizer (Bioset) using an optimized protocol for a synthesis scale of 0.4 mmol. 2'-deoxy-, 2'-O-TBDMS-, and 2'-O-methyl-phosphoramidite (Glen Research) were used as monomers. Sulfurizing reagent II (Glen Research) was used to introduce the thiophosphate group. The concentration of oligonucleotides was determined spectrophotometrically by measuring their optical density in solution using a Shimadzu U-1800 spectrophotometer (Shimadzu).

Preparation of Si~NH_2 aminopropylsilanol nanoparticles and Si~NH_2/siRNA nanocomplexes

APS nanoparticles (Si~NH_2) were synthesized by hydrolysis of (3-aminopropyl)triethoxysilane, which was added dropwise to hot water, and the mixture was stirred at this temperature for 15 h followed by cooling to room temperature [15]. The pH value of the resulting solution (10.6) was adjusted to 7.5 using 1 M HCl. The concentration of the final Si~NH_2 solution (0.26 M) was evaluated by titration of amino groups using 1 M HCl. The reaction yield was 95–97%. Si~NH_2 NPs were studied by physicochemical methods, i.e., dynamic light scattering, ultraviolet, infrared spectroscopy, transmission and atomic force microscopy [15].

RNA molecules were immobilized on APS NPs [16] due to the electrostatic interaction between negatively charged internucleotide phosphate groups in oligoribonucleotides (p) and positively charged amino groups (NH_2) in NPs. The nanocomplexes with single- and double-stranded RNA (Si~NH_2/RNA and Si~NH_2/siRNA, respectively) were prepared by mixing RNA or siRNA with 0.26 M Si~NH_2 in water, provided that the NH_2/p ratio was 50 (we considered the number of

phosphate groups in only one strand). The size and zeta potential of the obtained APS nanoparticles and nanocomplexes with RNA molecules were measured by dynamic light scattering on a Zetasizer Nano ZS Plus device (Malvern).

Toxicity analysis of nanoparticles and nanocomplexes in MDCK cell culture

Samples in DMEM medium (Biolot; 0.1 mL at concentrations of 5–50 μM for siRNA or 5–50 mM for Si~NH_2) were added to wells of 96-well plates with MDCK cells. Cells in 0.1 mL of DMEM maintenance medium were used as a control. After incubation of cells for 2 days at 37ºC and 5% CO_2, the culture medium was removed and MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in PBS-D buffer (Dulbecco's phosphate-buffered saline; 0.075 mL, 1 mg/mL) was added to each well. Cells were incubated for 90 min at 37ºC, after which the dye solution was removed and dimethyl sulfoxide (0.1 mL) was added. After incubation for 10 min, the optical density in each well was measured on an Emax spectrophotometer (Molecular Devices) at a wavelength of 540 nm, which is an indicator of the number of viable cells in the monolayer.

The dependence of optical density on the concentration of the tested sample was presented in semi-logarithmic coordinates, and the 50% cytotoxic concentration (CC_{50}) of each sample was calculated using the SoftMaxPro-4.0 computer program.

Antiviral activity of nanocomplexes

A/chicken/Kurgan/05/2005 (H5N1) virus was grown in the allantois cavity of 10-day-old chicken embryos at 37ºC. Allantois fluid was collected 48 h after virus inoculation and stored at –80ºC. MDCK cells were seeded at a rate of 10^5 cells/mL in DMEM nutrient medium containing 10% fetal bovine serum (Gibco) into 96-well plates (100 μL/well) and incubated at 37ºC, 5% CO_2, and 100% humidity. After reaching ~80% monolayer, the medium was removed and samples of Si~NH_2, Si~NH_2/RNA, and Si~NH_2/siRNA were added to the wells at a concentration of 0.5 mM (for Si~NH_2, corresponding to 0.5 μM for RNA or siRNA) in 100 μL of DMEM medium. The control sample was the same medium without the nanocomplexes. Oseltamivir at a concentration of 10 μg/mL was used as a reference drug.

The concentration of the Si~NH_2/siRNA nanocomplexes was varied in the range of 0.01–1.00 μM (for siRNA) in experiments that involved the investigation of their dose-dependent antiviral activity.

Cells were incubated in the presence of the samples at 37ºC, 5% CO_2, and 100% humidity for 4 h followed by washing the cells with the same medium. Cells were then infected with the A/H5N1 virus in trypsin-containing (2 μg/mL) DMEM medium (100 μL in each well) at an infection multiplicity of 0.01 50% tissue culture infectious dose ($TCID_{50}$) per 1 mL. After virus adsorption for 1 h at room temperature, the medium containing virus was removed, cells were washed with trypsin-free DMEM medium, and the same medium containing trypsin was added to each well (100 μL). After incubation for 48 h, serial 10-fold dilutions (10^{-1} to 10^{-8}) of the culture fluid containing virus from each well were applied to MDCK cells with repeated incubation for 48 h for further evaluation of the virus titer. The presence of cytopathic action was recorded under the microscope and in hemagglutination reaction with 1% suspension of chicken erythrocytes. The virus titer was expressed in terms of lg $TCID_{50}$/mL. To evaluate the dependence of virus inhibition on the concentration of nanocomplexes, the percentage of inhibition of virus production was calculated using the formula: $(A – B)/A$, where A is the virus titer in control (without sample) in $TCID_{50}$/mL; B is the virus titer in experiment (with sample) in $TCID_{50}$/mL.

Statistical analysis

Statistical analysis was performed using the Statistica v. 12 program (StatSoft Inc.). Virus titer in control and experiment (without or with experimental samples, respectively) was calculated using the Spearman–Kerber method and expressed as lg $TCID_{50}$/mL. Differences between the results with experimental and control samples were considered significant at $p \leq 0.05$.

Results and discussion

Silica NPs are considered as promising carriers for delivery of NAs into cells [17]. Most often, amine-modified Si-NPs are used for immobilization of NAs and their fragments. We synthesized non-agglomerated APS NPs (hydrodynamic diameter — ~1 nm, zeta potential — ~10 mV) [15].

The small size of Si~NH_2 particles provide water-soluble preparations. Characterization of APS NPs using physicochemical methods is described in our previous work [15]. It is shown that the obtained NPs are not prone to agglomeration and can be stored for several months.

Si~NH_2/RNA and Si~NH_2/siRNA nanocomplexes were obtained by electrostatic interaction between negatively charged inter-nucleotide phosphate groups in RNA and siRNA and positively charged protonated amino groups in Si~NH_2 NPs. The addition of negatively charged siRNA molecules to the NPs leads to a change in zeta potential from ~(+10 mV) to ~(–30 mV) and particle size from ~1 nm to ~200 nm, thus indicating the formation of nanocomplexes.

As a target for RNA and siRNA, we chose segment 5 of the influenza A virus encoding a nucleoprotein that plays a key role in the incorporation of the viral genome into the cell nucleus of an infected organism, thus facilitating further replication and assembly of vi-

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ral particles [18]. A region near the 3' end of this segment starting at nucleotide 1496, which is conserved vulnerable to siRNA action [19–21], was selected for siRNA exposure.

We synthesized two native (RNA_1 and RNA_2) and eight modified (RNA_3–RNA_{10}) oligoribonucleotides containing TT at the 3' end (**Table 1**). RNA_5 and RNA_6 contain, in addition to TT, 2'-OMe groups at the U*A site in the center of the strands. The presence of the 2'-O-Me group in this site increases resistance to serum nucleases, thereby maintaining the interference ability of siRNA [22] and providing long-term suppression of target gene expression [23]. The RNA_7 strand contains 2'-O-Me groups at all positions and 2 internucleotide thiophosphate groups at both ends. The RNA_8 strand differs from RNA_7 by the presence of three consecutive 2'-F groups at positions 9–11 from the 5' end. Oligoribonucleotides RNA_2, RNA_4, RNA_6, and RNA_8 are sense strands, and RNA_1, RNA_3, RNA_5, and RNA_7 are antisense strands directed to (–)RNA and (+)RNA of the viral genome, respectively.

The synthesized RNAs were used to obtain the Si~NH_2/RNA nanocomplexes based on non-agglomerated APS NPs. Their biological activity was investigated on the example of suppression of the A/H5N1 virus replication in infected MDCK cells at a multiplicity of infection of 0.01 $TCID_{50}$/cell. The results show that all single-stranded oligoribonucleotides (both sense and antisense strands) in the Si~NH_2/RNA nanocomplexes suppressed replication of the influenza A/H5N1 virus by 0.7–1.3 orders of magnitude (5–20 times) (**Fig. 1**, columns 1–8).

From the four studied sense strands, we chose RNA_4 containing deoxydinucleotide TT at the 3' end for protection from exonucleases, as the most active one and formed four siRNA molecules with all possible antisense strands ($RNA_{1/4}$, $RNA_{3/4}$, $RNA_{5/4}$, and $RNA_{7/4}$). All siRNAs are duplexes with the same nucleotide sequence and different modification of nucleoside units. The efficiency of the investigated siRNAs was significantly higher compared with single-stranded oligoribonucleotides (Fig. 1). The Si~NH_2/siRNA nanocomplexes suppressed the A/H5N1 virus replication by 2.3–2.8 orders of magnitude (200–630 times).

The main reason for the difference in the effectiveness of siRNA and oligoribonucleotides is the different mechanism of their action. It is known that oligodeoxynucleotides and siRNAs suppress the functions of target RNAs through complementary interactions with the target RNA with its subsequent degradation by cellular RNases (RNase-H1 and AGO2, respectively) [24–26], which eventually leads to the loss of target RNA functions. Single-stranded RNA fragments can form complementary complexes with the target RNA but do not cause degradation of the target RNA. The siRNA mo-

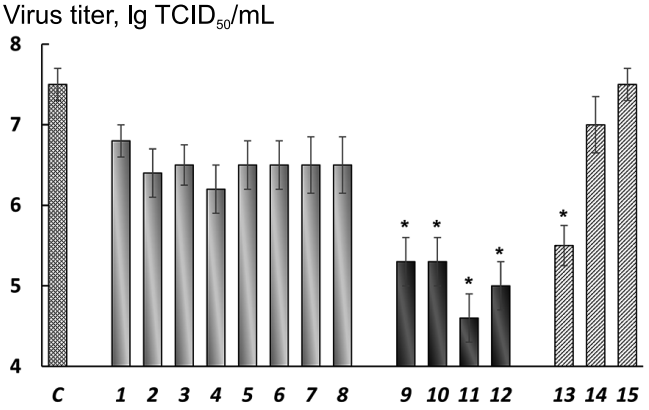

Fig. 1. Titers of A/chicken/Kurgan/05/2005 (H5N1) virus in MDCK cells after their incubation of with Si~NH_2/RNA and Si~NH_2/siRNA nanocomplexes.

C, virus control without samples. Si~NH_2/RNA nanocomplexes containing RNA_1–RNA_8 (*1–8*), concentration of RNA in nanocomplexes is 0.5 μM; Si~NH_2/siRNA nanocomplexes containing $siRNA_{1/4}$, $siRNA_{3/4}$, $siRNA_{5/4}$ and $siRNA_{7/4}$ (*9–12*), concentration of siRNA in nanocomplexes is 0.5 μM per one strand; ozeltamivir (*13*), 10 μg/mL or 32 μM; Si~NH_2 (*14*), 0.5 mM; Si~NH_2/$siRNA_{9/10}$, nanocomplex containing unspecific $siRNA_{9/10}$ *(15)*. MOI, 0.01 $TCID_{50}$/cell. The presented average values, standard deviations and differences in the titer values of the virus are calculated using the Spearman–Kerber method. Asterisks designate the difference between the control and the titer values of the virus obtained under the action of the studied series of nanocomplexes, at $p < 0.05$.

Table 1. Oligoribonucleotides used in this study

RNA chain		Nucleotide sequence, 5'-3'
RNA_1	Antisense	CUCCGAAGAAAUAAGAUCC
RNA_2	Sense	GGAUCUUAUUUCUUCGGAG
RNA_3	Antisense	CUCCGAAGAAAUAAGAUCCTT
RNA_4	Sense	GGAUCUUAUUUCUUCGGAGTT
RNA_5	Antisense	CUCCGAAGAAAU*AAGAUCCTT
RNA_6	Sense	GGAUCUU*AUUUCUUCGGAGTT
RNA_7	Antisense	$C^*_{PS}U^*_{PS}C^*C^*G^*A^*A^*G^*A^*A^*A^*U^*A^*A^*G^*A^*U^*C^*C^*_{PS}T_{PS}T$
RNA_8	Sense	$G^*_{PS}G^*_{PS}A^*U^*U^*U^{\#}A^*U^{\#}U^{\#}C^*U^*U^*C^*G^*G^*A^*G^*_{PS}T_{PS}T$

Note. *2'-O-methyl group; #2'-fluoro group; PS — internucleotide thiophosphate group; symbol d for deoxyribotimidine has been omitted.

Table 2. Dependence of antiviral activity of Si~NH_2/$siRNA_{5/4}$ nanocomplex on siRNA concentration with MOI 0.01 $TCID_{50}$/cell

Concentration of $siRNA_{5/4}$ in nanocomplex, μM	Infection titer of influenza A virus $lgTCID_{50}$/mL	Inhibition of influenza A virus replication, %
1,00	4,50	99,99
0,50	5,75	99,82
0,10	7,00	97
0,05	7,50	90
0,01	7,50	90
Virus control	8,50	–

lecule first binds to the RISC complex, then the sense (passenger) strand is removed. AGO2 nuclease and the remaining antisense strand within the RISC complex find the target RNA, and AGO2 cleaves it. AGO2 retains the antisense strand for some time as part of the RISC complex for further reactions [27–29].

It is known that siRNAs are rapidly hydrolyzed by cellular nucleases, and various modifications are used to protect against them. It should be noted that even minimally modified $siRNA_{1/4}$ and $siRNA_{3/4}$ delivered into the cells as part of nanocomplexes with NPs were very effective in suppressing virus replication (by 2.3 orders of magnitude, ~200 times). This implies that APS NPs protect siRNA from cellular nucleases. The most active Si~NH_2/$siRNA_{5/4}$ nanocomplex suppressed virus replication by ~3 orders of magnitude.

The antiviral activity of the studied Si~NH_2/siRNA nanocomplexes was comparable to that of oseltamivir (the most commonly used comparison drug in studies of effects on the influenza A virus) but at a much lower concentration of the active component (0.5 μM for siRNA and 32 μM for oseltamivir). The Si~NH_2 NPs did not inhibit virus replication, as would be expected.

The most active Si~NH_2/$siRNA_{5/4}$ nanocomplex is characterized in more detail. We evaluated the effect of siRNA, Si~NH_2 NPs in the free state and as part of the Si~NH_2/$siRNA_{5/4}$ nanocomplex on the survival of uninfected MDCK cells (**Fig. 2**). Unbound $siRNA_{5/4}$, as expected, was nontoxic in the concentration range investigated. The cytotoxicity of the Si~NH_2/$siRNA_{5/4}$ nanocomplex coincides with the toxicity of Si~NH_2 NPs. Consequently, it can be concluded that the cytotoxicity of nanocomplexes is determined by the toxicity of their constituent NPs. The cytotoxic concentration of the drug, at which 50% of cells in the uninfected monolayer are destroyed, determined from the data in Fig. 2, was 38 mM per Si~NH_2 and 38 μM per siRNA.

Table 2 summarizes the results of suppression of the influenza virus production depending on the concentration of the Si~NH_2/$siRNA_{5/4}$ nanocomplex in the cell culture.

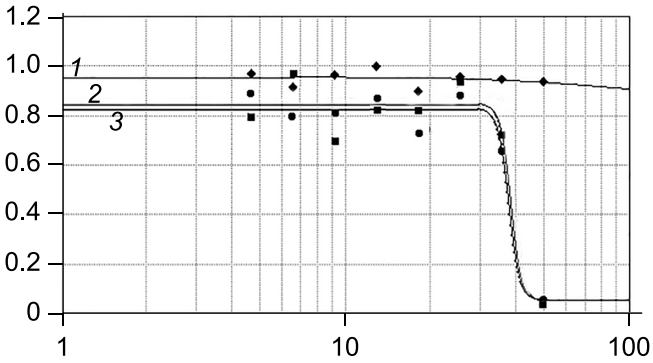

Fig. 2. Viability of MDCK cells when treated with siRNA and Si~NH_2 samples in the free state and as part of the Si~NH_2/siRNA nanocomplex.

1, $siRNA_{5/4}$; *2,* Si~NH_2; *3,* Si~NH_2/$siRNA_{5/4}$. The x-axis shows the concentration of siRNA in the free state or as part of a nanocomplex (μM) and Si~NH_2 nanoparticles in the free state or as part of a nanocomplex (mM). The y-axis shows optical density of the MTT solution.

It was shown that in the concentration range of $siRNA_{5/4}$ in the Si~NH_2/$siRNA_{5/4}$ nanocomplex from 0.01 to 1.00 μM, the suppression of the influenza virus production was 90.00–99.99%.

Conclusion

The results indicate that APS NPs can be used to deliver siRNA into cells as part of the Si~NH_2/siRNA nanocomplexes. The cytotoxicity of the Si~NH_2/$siRNA_{5/4}$ nanocomplex is determined by the toxicity of Si~NH_2 NPs. The cytotoxic concentration of the drug, at which 50% of cells in the uninfected monolayer are destroyed, was 38 mM per Si~NH_2 and 38 μM per siRNA. The proposed siRNA-containing nanocomplexes targeting a selected region of the 5th segment of the viral genome mRNA were successfully used to suppress the A/H5N1 virus production in a cellular system. The most effective Si~NH_2/$siRNA_{5/4}$ nanocomplex reduced the replication of the influenza A virus in cell culture by ~3 orders of magnitude.

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