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High-performance method for specific effect on nucleic acids in cells using TiO_2~DNA nanocomposites

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Nanoparticles are used to solve the current drug delivery problem. We present a high-performance method for efficient and selective action on nucleic acid target in cells using unique TiO_2•PL-DNA nanocomposites (polylysine-containing DNA fragments noncovalently immobilized onto TiO_2 nanoparticles capable of transferring DNA). These nanocomposites were used for inhibition of human influenza A (H3N2) virus replication in infected MDCK cells. They showed a low toxicity ($TC_{50} \approx 1800$ μg/ml) and a high antiviral activity (>99.9% inhibition of the virus replication). The specificity factor (antisense effect) appeared to depend on the delivery system of DNA fragments. This factor for nanocomposites is ten-times higher than for DNA in the presence of lipofectamine. IC_{50} for nanocomposites was estimated to be 1.5 μg/ml (30 nM for DNA), so its selectivity index was calculated as ~1200. Thus, the proposed nanocomposites are prospective for therapeutic application.

Fragments of nucleic acids (NA) are widely used in life sciences including research methods and medical practice. NAs are among the most important sources for the development of a novel group of pharmaceuticals. NA-based drugs for antisense and antigene application include oligonucleotides, their analogues, ribozymes, DNAzymes, aptamers, small interfering RNAs (siRNAs), and plasmids containing transgenes[1,2]. This class of compounds is extremely promising for drug therapy aimed to a wide range of diseases including infectious, cancer, neurological disorders such as Parkinson's and Alzheimer's diseases, cardiovascular disorders, etc. [e.g.[2,3]]. NA-based drugs can be developed for individualized medicine. NA-based drugs can selectively suppress gene expression, inhibit activity of certain enzymes, or implement other special functions[4,5]. The significant advantage of this type of drugs over currently available low molecular weight pharmaceuticals is their *selective* recognition of molecular targets. The effect of antisense oligonucleotides is known to be in their binding to complementary regions of a target RNA to form DNA/RNA hybrid duplexes, which leads to blocking the function of the target RNA or its cleavage by cellular RNase H[1,2,6]. The important advantages of the antisense approach is that NA-drugs can be easily redirected to any target nucleic acids, while the search for new low-molecular drugs usually takes years.

However, because of a low ability to penetrate into cells, these compounds still have not found wide application in medicine. Poor cellular uptake and rapid in vivo degradation of NA-based drugs necessitate designing delivery systems to facilitate cellular internalization and preserve their activity[7].

Currently, the transport of exogenous NA to cells can be achieved by using vehicles, which can be separated into two categories: viral and non-viral vectors. The former provides a high transfection rate and a rapid transcription of a foreign material incorporated in the viral genome[8]. On the other hand, viral vectors present a variety of potential problems to the patients such as toxicity and immune and inflammatory responses. Insertional mutagenesis and oncogenic effects can occur when these vectors are used in vivo[9].

Cell-penetrating peptides as non-viral vectors are widely used for delivering various biomolecules into cells including plasmid DNAs, oligonucleotides, siRNAs, peptide-nucleic acids, proteins, etc. Cell-penetrating

peptide-based technology is described in recent reviews[7,10]. Liposomes and cationic polymers are other major types of non-viral gene delivery vectors, which have been under intense development during recent years because of their low immunogenicity and the simplicity of preparation and modification[11,12]. However, the transfection efficiency of these vectors is still relatively low, compared to viral vectors[13]. Besides, cationic liposome/DNA complexes exhibit inflammatory toxicity associated with their systemic administration[14]. A variety of supramolecular nanocarriers including various polymeric nanoparticles are used to deliver antisense oligonucleotides and siRNAs, as more fully described in recent reviews[15–17]. Although cationic polymers, branched dendrimers, cationic liposomes, and cell-penetrating peptides are today under intensive studies, their efficiency as non-viral vectors for cell transfection is still low.

Current achievements of nanotechnology began to be used to solve the drug delivery problem, concerning particularly nucleic acid fragments. Recent advances in clinical trials concerning nanoparticle drug delivery systems are described in[18–20].

Titanium dioxide is widely used in medicine as biocompatible and non-toxic material[21]. TiO_2 nanoparticles (~5 nm) are known to penetrate through cell membranes[22,23]. According to FDA (Management of the Food and Drug Administration, United States), TiO_2 is recognized in 1966 as safe and harmless substance to humans. Compounds on the basis of TiO_2 are widely used in cosmetics and as food supplements. Moreover, recent studies have shown that TiO_2 nanoparticles at relatively low doses (up to 200 μg/ml) do not show any noticeable toxicity and no harmful effects on mammalian cells[24], bacteria[25], and animals[26]. Cytotoxicity of TiO_2 nanoparticles used in this work did not exceed the level of natural death of MDCK cells[27]. TiO_2 nanoparticles have been shown to be non-toxic in rats at low doses (5 mg/kg body weight)[28]. The above properties of titanium dioxide make it attractive to use as a vehicle of drugs, particularly, antisense oligonucleotides and their analogues into mammalian cells.

In the previous paper, we described new nanocomposites consisting of DNA fragments noncovalently fixed on TiO_2 nanoparticles through the polylysine linker (**TiO_2•PL-DNA**)[29]. These nanocomposites were shown by confocal microscopy to penetrate into mammalian cells without any transfection agents and physical impact. Delivery of nucleic acid fragments into cells is just the first step of their use in living systems. However, the real value of the proposed nanocomposites can be revealed only when they are tested on cells and animals.

Here we present a high-performance method for the site specific effect on target nucleic acids in eukaryotic cells using unique **TiO_2•PL-DNA** nanocomposites. These nanocomposites show a low toxicity, and after penetration into cells infected by influenza A virus, demonstrate a high specific antiviral activity.

Results

The synthesis of TiO_2 nanoparticles in amorphous and crystal forms (anatase, brookite), the preparation of nanocomposites consisting of these nanoparticles and noncovalently immobilized polylysine (**PL**) or polylysine-containing DNA fragments (**PL-DNA**), and characteristics of the designed **TiO_2•PL** and **TiO_2•PL-DNA** nanocomposites were described in a recent paper[29]. It has been shown that the efficiency of immobilization of oligonucleotides onto TiO_2 nanoparticles, the ability of designed **TiO_2•PL-DNA** nanocomposites to form complementary complexes and penetrate into cells without any transfection agents or physical impact do not depend on the nanoparticles form. Here we used nanocomposites based on one form of nanoparticles (anatase). **PL-DNA** conjugates[30] were immobilized on anatase to give **TiO_2•PL-DNA** bearing 20 nmol of DNA per 1 mg of anatase.

The biological activity of the designed nanocomposites was demonstrated by an example of inhibition of influenza A virus replication in infected MDCK cells. The most widespread human influenza A virus (H3N2) was used in our experiments. Influenza A virus contains eight single-stranded RNA segments of negative polarity as the genome. After infection of cells with influenza virus, viral RNA (−strand) segments are transcribed into mRNAs (+strand) and replicated into complementary RNAs (+strand)[31]. RNA segment 5 encodes nucleoprotein (NP), which plays a key role in the incorporation of viral genome in cell nuclei of an infected body, thereby, in the following replication and assembly of the virus[32]. In addition to coding regions, all eight segments of viral (-)RNA are known to contain the conservative sequence CCUGCUUUUG-CU$^{3'}$at the 3′-end[33,34].

The noncoding 3′-terminal region of segment 5 was chosen as a target for the designed nanocomposite. It should be noted that the choice of this target is very reasonable because, according to literature data, morpholino oligomer and siRNA addressed to this conservative region of NP gene showed the highest antiviral activity among the other morpholino oligomers[34] and 20 siRNA molecules[35]. Oligonucleotide $^{5'}$**GCAAAAGCAGGGTAGATAATCp (DNA_1)** complementary to the chosen region was used in our work to construct nanocomposites. As a control, we used oligonucleotide $^{5'}$**GATCAA-CTCCATATGCCATGTp (DNA_2)** with a random sequence.

Antiviral activity of TiO_2•PL-DNA nanocomposites. At first, we tested the effect of **TiO_2** nanoparticles and **TiO_2•PL-DNA** nanocomposite on viability of MDCK cells in the absence of virus. The concentration of samples resulting in 50% cell death (TC_{50}) for **TiO_2** and **TiO_2•PL-DNA_1** was estimated to be ~1800 μg/ml (Fig. 1). It should be noted that just nanoparticles are responsible for the effect, and PL-DNA conjugates are hardly involved in this action.

The nontoxic concentration of nanocomposites 5 μg/ml corresponding to 0.1 μM for oligonucleotides was used to study the antiviral activity of the proposed **TiO_2•PL-DNA** nanocomposites. Nanocomposite **TiO_2•PL-DNA_1** containing oligonucleotide directed to the 3′-noncoding regions of initial viral (-)RNA and various controls including samples bearing a random **DNA_2** sequence were assayed for the antiviral activity against human influenza A virus H3N2. MDCK cells were infected at a moi of 0.05 $TCID_{50}$/cell. Transfection of cells with nanocomposites was carried out in the absence of serum. The nanocomposites and controls were incubated

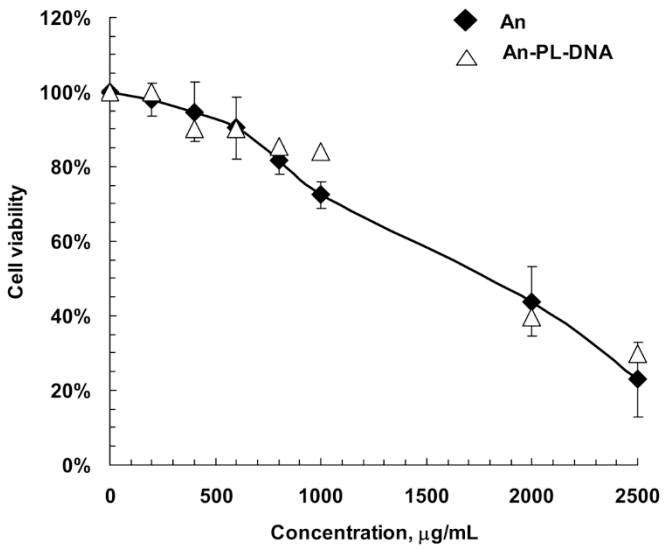

Figure 1 | Effect of TiO_2 nanoparticles and TiO_2•PL-DNA_1 nanocomposites on viability of MDCK cells estimated by trypan blue staining. Experiments were performed in three independent series. An designatesTiO_2 nanoparticles in the anatase form.

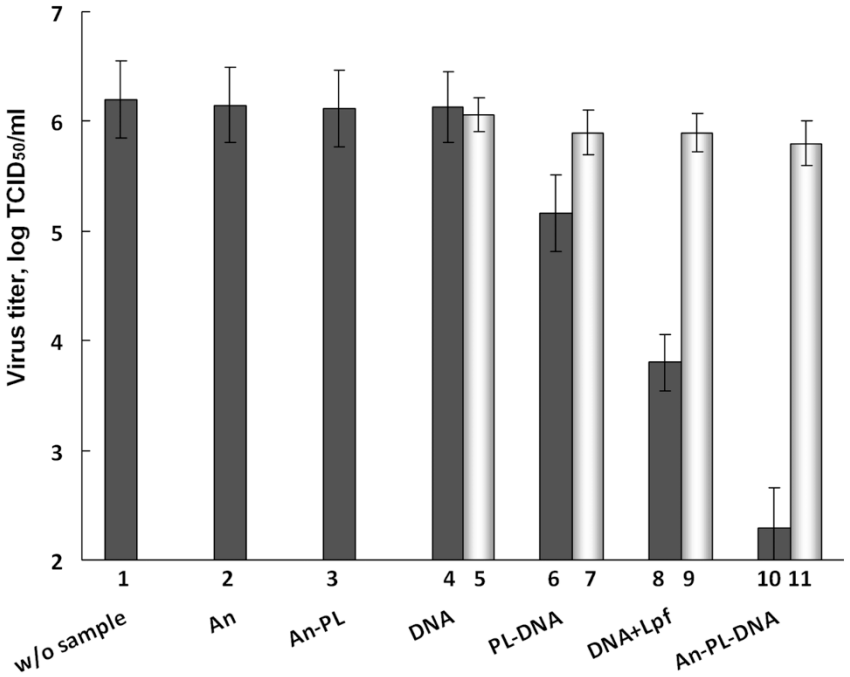

Figure 2 | Inhibition of replication of virus A/Aichi/2/68 (H3N2). Virus titer (log $TCID_{50}$/ml) in the presence of nanocomposites and controls. Without sample (1), TiO_2 nanoparticles (2), TiO_2•PL nanocomposite (3); DNA_1 and DNA_2 fragments (4, 5), PL-DNA_1 and PL-DNA_2 conjugates (6, 7), $DNA_1$and DNA_2 fragments in the presence of Llipofectamine™ 2000 (lpf) (8, 9), and TiO_2•PL- DNA_1 and TiO_2•PL-DNA_2 nanocomposites (10, 11). Light-coloured columns correspond to samples with noncomplementary oligonucleotides. Concentrations of nanocomposites and controls in the medium were 5 μg/ml for TiO_2 nanoparticles and 0.1 μM for DNA fragment. Experiments were performed in three independent series.

with cells for 4 h prior to infection. The results are presented as log $TCID_{50}$/ml in Fig. 2 and as $TCID_{50}$/ml in Table 1.

The nanoparticles bearing no oligonucleotides were practically inactive independently on the presence or the absence of the PL residue (Fig. 2, columns 2 and 3). Unbound **DNA_1** and **DNA_2** fragments also showed very low antiviral activity (Fig. 2, columns 4 and 5; Table 1, row *a*). This is with agreement with literature data: phosphodiester oligodeoxynucleotides failed to inhibit replication of influenza A at concentration up to 80 μM[36]. The maximal antiviral efficacy (99.99% reduction of virus titer) demonstrated **TiO_2•PL-DNA_1** nanocomposite bearing complementary **DNA_1** (Fig. 2, column 10; Table 1, row *d*): the virus titer was reduced by a factor of 8,000 (by about four orders of magnitude). **PL-DNA_1** conjugate without nanoparticles showed significantly lower efficiency (by a factor of 740) than the same conjugate immobilized onto nanoparticles (compare columns 6 and 10 in Fig. 2 and rows *b* and *d* in Table 1). It should be noted that **DNA_1** in **TiO_2•PL-DNA_1** nanocomposite demonstrated ~30-times higher antiviral activity than the same oligonucleotide in the presence of lipofectamine widely used as the transfection agent (compare columns 8 and 10 in Fig. 2 and rows *c* and *d* in Table 1). The antiviral activity of **TiO_2•PL-DNA** nanocomposites bearing complementary and noncomplementary **DNA** fragments differed by a factor of about 3000 (compare columns 10 and 11 in Fig. 2 and cells *1d* and *2d* in Table 1).

All samples containing complementary fragment **DNA_1** demonstrated the higher antiviral activity as compared to those bearing noncomplementary fragment **DNA_2** (compare columns 6 and 7, 8 and 9, and 10 and 11 in Fig. 2 and columns 1 and 2, 3 and 4, and 5 and 6 in Table 1). We evaluated the ratio of the $TCID_{50}$/ml values for these two types of nanocomposites (Table 1, column 7), which can be considered as a specificity factor indicating the antisense effect.

The results show that the specificity factor depends on the method of the delivery of the samples into cells. **DNA_1** and **DNA_2** without any transfection agent almost do not penetrate into the cells, and their antiviral activity is equally low. **PL-DNA_1** and **PL-DNA_2** penetrate into cells more efficiently, and the difference, although still low,

Table 1 | Antiviral activity of studied DNA-containing samples

		Virus titer, $TCID_{50}$/ml		Reduction of virus titer, %		Reduction of virus titer, n-fold		Specificity factor[a]
		DNA_1[b]	DNA_2[b]	DNA_1	DNA_2	DNA_1	DNA_2	
row	Sample	*1*	*2*	*3*	*4*	*5*	*6*	*7*
a	DNA	1,348,963	1,148,154	15	28	1	1.4	**1**
b	PL-DNA	147,911	794,328	90.7	50	11	2.0	**5**
c	DNA + lpf[c]	6,310	793,349	99.6	50	251	2.0	**130**
d	TiO_2•PL-DNA	200	630,957	99.99	60	8,000	2.5	**3,160**
e	w/o sample	1,584,893						

[a]ratio of the $TCID_{50}$/ml values for DNA_2- and DNA_1-containing samples.
[b]DNA_1 and DNA_2 are DNA fragments complementary and noncomplementary to viral RNA, respectively.
[c]Lipofectamine™ 2000.

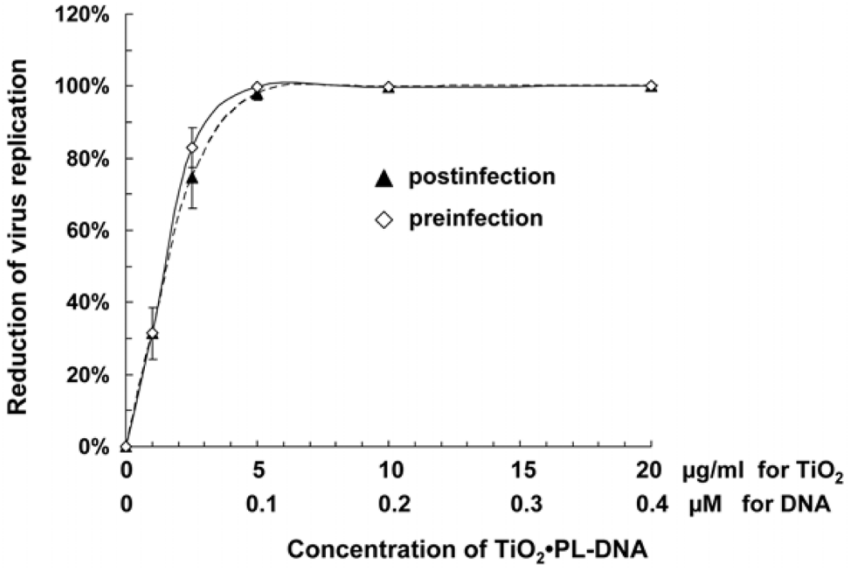

Figure 3 | Dose-response curves of inhibition of influenza virus replication in MDCK cells by TiO_2•PL-DNA_1 nanocomposite in the pre-treatment or post-treatment assays. Experiments were performed in two independent series for each treatment.

between their antiviral actions is observed. When lipofectamine is used for transfection, complementary **DNA_1** becomes ~130 times more efficient than noncomplementary **DNA_2**. The maximal difference (3160 times) was detected if DNA fragments were delivered into cells in **TiO_2•PL-DNA_1** and **TiO_2•PL-DNA_2** nanocomposites.

Dose-response studies. The dose-response effect of the **TiO_2•PL-DNA_1** nanocomposite was studied in the pre-infection and post-infection assays. In the pre-infection treatment, MDCK cells were pretreated with the **TiO_2•PL-DNA_1** sample for 4 h followed by infection with A/Aichi/2/68 virus at a multiplicity of infection (moi) of 0.1 $TCID_{50}$/cell for 1 h. After washing cells from the virus-containing medium and the subsequent incubation for 48 h, the virus titer was measured by the hemagglutination (HA) technique. In the post-infection treatment, MDCK cells were initially infected with influenza A virus for 1 h as above and washed, followed by incubation with **TiO_2•PL-DNA_1** nanocomposite for 4 h. After 48-h incubation, the evaluation of virus titer was performed as in the previous case.

Concentrations of the nanocomposite causing 50% inhibition of viral production (IC_{50}) were evaluated to be ~1.5 μg/ml for the nanocomposite (~0.03 μM for **DNA_1**) in the pre-treatment or post-treatment assays (Fig. 3).

The data on cell viability and dose-response study allowed us to derive the selectivity index value, i.e. the ratio of TC_{50} to IC_{50}, (SI ≈ 1200).

Discussion

The nucleic acids-based drugs are the foundation of a new medicine because they *selectively* recognize the complementary regions of nucleic acid targets and affect their functions in cells that allows for the inhibition of the expression of certain genes responsible for ailments without affecting host genes. As compared to low molecular weight pharmatheuticals, NA-based drugs have also other advantages such as simple design, easy preparation, high specificity, and low side effects.

However, NA-based drugs still have not found wide application in medical practice because of the low ability to penetrate into cells.

In the previous paper[29], we described new nanocomposites consisting of DNA-based drugs *noncovalently* fixed on inorganic nanoparticles used as transporters. Polylysine-containing DNA fragments were immobilized with a high efficiency onto titanium dioxide nanoparticles to give TiO_2•PL-DNA nanocomposites. We assumed that **PL-DNA** are immobilized onto TiO_2 nanoparticles due mainly to electrostatic interactions between negatively charged TiO_2 surface[37,38] and positively charged amino groups of polylysine at physiological pH values (pH ~7) (Fig. 4). The important role of electrostatic interactions in the binding of PL-DNA conjugates to titanium dioxide is confirmed by the fact that oligonucleotides without the PL linker are hardly immobilized onto TiO_2 nanoparticles, while the attachment significantly increases in the presence of cetyltrimethylammonium bromide, which masks the negative charge of the phosphate groups of oligomers (data not shown). The formed **TiO_2•PL-DNA** nanocomposites were shown by confocal microscopy to penetrate into mammalian cells without any transfection agents and physical impact[29].

Here we studied the next key stage of using NA-based drugs, namely, the interaction with target nucleic acids inside cells.

We examined **TiO_2•PL-DNA** nanocomposites bearing DNA fragment complementary to 3′-uncoding region of segment 5 of (-)RNA of influenza A virus (H3N2) for their antiviral activity (Fig. 2). This nanocomposite showed the high antiviral effect (99.99% inhibition of

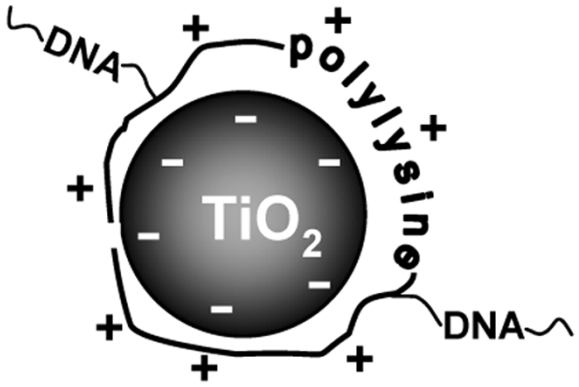

Figure 4 | Cartoon scheme of TiO_2•PL-DNA nanocomposites. DNA fragments are covalently attached to the polylysine linker (positively charged), which is immobilized to the surface of TiO_2 nanoparticles (negatively charged) due to electrostatic interactions.

virus replication at 0.1 μM concentration of DNA fragment in nanocomposite (5 μg/ml) at 0.05 moi. DNA fragment in the proposed nanocomposite was more active than the same oligonucleotide in the presence of lipofectamine widely used as the gold standard transfection agent. The antiviral activity of nanocomposite bearing noncomplementary DNA fragment was ~3000 times less active that demonstrates a significant specificity, i.e. antisense effect. It was revealed (Fig. 2, Table 1) that the specificity factor that is the ratio of the activity of noncomplementary and complementary DNA fragments (antisense effect) depended on their delivery system. This effect was more pronounced for the proposed nanocomposite as compared to other samples containing the DNA fragment. This may be due to the more efficient delivery of DNA fragments within the nanocomposite, their higher accessibility for the interaction with target nucleic acids, and enhanced stability inside cells as compared to other DNA-containing samples.

The proposed nanocomposites appeared to be low toxic for MDCK cells ($TC_{50} \approx 1800$ μg/ml), and at the working concentration of 5 μg/ml they were practically nontoxic (Fig. 1). It is important to note that it is the TiO_2 nanoparticles that are responsible for the toxic action of nanocomposites, and DNA fragments do not contribute to this effect (Fig. 1). In opposite, the contribution of TiO_2 nanoparticles in antiviral activity is very low, if at all. The IC_{50} value (indicating 50% inhibition of viral replication) for the nanocomposite was evaluated from the data of dose-response study to be ~1.5 μg/ml (Fig. 3). These data allowed us to derive the selectivity index value, i.e. the ratio of TC_{50} to IC_{50} for the designed nanocomposite ($SI \approx 1200$). In comparison, the selectivity index for morpholino oligonucleotides was estimated as ~40[34]. High values of the selectivity index and the specificity factor indicate good prospects for using **TiO_2•PL-DNA** nanocomposites in medical practice.

Some researchers studied the antiviral activity of oligonucleotides and their derivatives. Oligonucleotides with native phosphodiester bonds did not reveal antiviral activity at concentration, which were 270 times higher than we used[36]. Transfection of MDCK cells infected with influenza A virus with phosphorothioate antisense oligonucleotides aimed (4 μM) to different regions of the NP gene in the presence of lipofectamine led to the reduction of the virus titer by 1–1.2 log[39]. The same authors showed the similar antiviral effect of antisense oligonucleotides delivered into MDCK cells with the antibody delivery system[40]. In our case, the reduction of the virus titer achieves 3.9 log. A morpholino oligonucleotide with the same sequence as DNA_1 was also shown[34] to be efficient against different strains of influenza A virus, although its efficiency was significantly lower than in our case (the inhibitory effect was 88–98% at 15 μM concentration). Moreover, morpholino oligonucleotides should be conjugated to an arginine-rich peptide to ensure the delivery into cells. The concentrations of morpholino or phosphorothioate oligonucleotides were higher by factors of 40–150, and their efficiency in the virus inhibition was significantly lower than in our case.

Short interference RNAs (siRNA) are under intense investigation as antiviral agents. After electroporation, NP-specific siRNA (5 μM) inhibited virus production by factors of 30,000 or 200 at a moi of 0.001 or 0.1, respectively[35]. Similar or even slight higher inhibitory effect showed siRNA directed to the M2 gene of influenza A virus[41]. The inhibitory effect of several NP-specific siRNAs on influenza A virus replication in MDCK cells transfected in the presence of lipofectamine was shown to be 0.8–1.6 log (6–43-fold reduction)[42].

The antivirus efficiency of DNA fragments delivered into cells with the proposed **TiO_2•PL-DNA** nanocomposites (~8,000-fold reduction of virus replication at 0.1 μM and 0.05 moi; 3.9 log of the inhibitory effect) is not inferior to (and even exceeds) that of siRNAs, which considered to be the most promising agents among nucleic acid drugs.

Thus, for the firs time, we have shown the highly efficient and selective action on intracellular nucleic acid targets of antisense DNA fragments delivered into cells with TiO_2 nanoparticles. The high inhibition of influenza A virus replication with the proposed nanocomposites shows their potency as antisense drugs. The proposed high-performance method for specific effect on nucleic acids in cells may find a wide application for the treatment of not only viral but also other nucleic acid-based diseases, when it is necessary to ensure effective and selective interaction of DNA with intracellular nucleic acid targets.

Methods

Chemicals were obtained from commercial suppliers. We used: PMI-1640 medium, antibiotics (BioloT, Russia); trypsin (Sigma, USA); fetal calf serum (Gibco, USA); L-glutamine, PBS buffer, chicken erythrocytes, MDCK cells, and influenza A virus Aichi/2/68 (H3N2) (Vector, Russia); Lipofectamine™ 2000 (Invitrogen, USA). TiO_2 nanoparticles in the crystal form (anatase, An) were synthesized as described in[19]. Oligonucleotides were synthesized by the phosphoroamidite method on ASM-800 DNA synthesizer (Biosset, Russia) using phosphoroamidite monomers (Glen Research, USA). Trypsin (1 μg/ml) and penicillin-streptomycin (100 U/ml) (Sigma-Aldrich, USA) were stored at –80°C.

The synthesis of polylysine-containing oligonucleotides (**PL-DNA**) and nanocomposites consisting of anatase nanoparticles and PL-DNA conjugates (**An•PL-DNA**) were fulfilled as described in[19]. The virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Vector, Russia) at 37°C. Allantoic fluid was harvested for 48 h after virus inoculation, aliquoted, and stored at –80°C.

Cell viability assays. MDCK cells in logarithmic phase were seeded at 100000 cells/ml in RPMI-1640 nutrient medium in 96-well plates (100 μl/well) and incubated at 37°C and 5% CO_2. Two days after the formation of a continuous monolayer, cells were washed three times with nutrient medium (200 μl/well) containing no serum. The samples of TiO_2 nanoparticles or TiO_2•PL-DNA nanocomposites were diluted with RPMI-1640 medium to the needed concentration (10–1000μg/ml) and incubated with MDCK cells at 37°C and 5% CO_2 for two days. Destructive changes in the cells were evaluated by an inverted microscope. MDCK cells without studied samples were used as a control. Cells were stained with trypan blue[43], and the number of viable cells was counted in a Goryaev chamber. All experiments were performed in three independent series (Fig. 1).

Comparison of antiviral activity of samples (*Preinfection assays*). MDCK cells were seeded as above and incubated with samples under investigation (DNA, PL-DNA, DNA + Lipofectamine™ 2000, TiO_2, TiO_2•PL, and TiO_2•PL-DNA) added in RPMI-1640 (100 μl/well) without trypsin. The final concentration was 0.1 μM for oligonucleotide in all DNA-containing samples and 5 μg/ml for nanoparticles and nanocomposites. Transfection of DNA with lipofectamine was performed according to the manufacturer's protocol. Transfection experiments were carried out in the absence of serum. Cells were incubated at room temperature and 5% CO_2 and 100% humidity for 4 h, followed by rinsing with RPMI-1640. Virus A/Aichi/2/68 (H3N2) was then added in each well (100 μl/well) at a multiplicity of infection (moi) of 0.05 $TCID_{50}$/cell in a medium containing trypsin. After 1-h adsorption at room temperature, virus-containing media were removed; cells were rinsed with RPMI-1640 medium without trypsin, and the same medium containing trypsin was applied to each well (100 μl). Cells were incubated at 37°C and 5% CO_2 and 100% humidity for 48 h. Samples of virus culture were collected and their measurements were performed by hemagglutination (HA) assays using round-bottom 96-well plates. Serial ten-fold dilutions of virus samples with RPMI-1640 medium containing 2 μg/ml trypsin were applied to MDCK cells for 48 h. The presence of virus was visually determined in HGR with a 1% suspension of chicken erythrocyte. The titer was evaluated by noting the highest dilution of the virus, which caused the hemagglutination reaction. Wells containing an adherent, homogenous layer of erythrocytes were scored as positive. MDCK infected by A/Aichi/2/68 (H3N2) virus without the treatment with nanocomposites were used as controls. Virus titer in cells treated with nanocomposites was expressed as log $TCID_{50}$/ml, $n = 6$ (Fig. 2) and $TCID_{50}$/ml (Table 1).

Dose-response studies. *Preinfection assays.* MDCK cells at ~80% confluence were incubated with the **TiO_2•PL-DNA_1** nanocomposite taken in RPMI-1640 medium without trypsin (100 μl/well) at various concentrations (1, 2.5, 5, 10, 20, and 50 μg/ml). After 4-h incubation at 37°C and 5% CO_2 and 100% humidity, preparations were removed; cells were rinsed with RPMI-1640 medium without trypsin. Virus A/Aichi/2/68 was then added in each well in RPMI-1640 medium (100 μl) containing trypsin (2 μg/ml) at a moi of 0.1 $TCID_{50}$/cell. After 1-h adsorption at room temperature, virus-containing media were removed; cells were rinsed with RPMI-1640 medium without trypsin, and the same medium containing trypsin was applied to each well (100 μl). Cells were incubated at 37°C and 5% CO_2 and 100% humidity for 48 h. The virus titers were evaluated as above.

Postinfection assays. MDCK cells at ~80% confluence were initially infected with A/Aichi/2/68 (H3N2) virus as described above. After 1-h adsorption at room temperature, virus-containing medium was removed, and cells were rinsed with RPMI-1640 medium without trypsin. Nanocomposite **TiO_2•PL-DNA_1** taken in RPMI-1640

medium without trypsin (100 μl/well) at various concentrations (1, 2.5, 5, 10, 20, and 50 μg/ml) was applied to the infected MDCK cells, followed by the incubation for 4 h at 37°C and 5% CO_2 and 100% humidity. The cultural media were then removed, cells were rinsed with RPMI-1640 medium without trypsin, and the same medium containing trypsin (2 μg/ml) was applied to each well (100 μl/well). After 48-h incubation at 37°C, 5% CO_2, and 100% humidity, serial ten-fold dilutions of cultural virus-containing liquid from each well were applied to MDCK cells for 48 h to evaluate the virus titer. Results are presented in Fig. 3.

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Authors contributions

A.S.L. and M.N.R created nanocomposites and other samples, Z.R.I. and N.V.S. prepared nanoparticles, E.G.M. and V.V.Z. gave a consultation on the antiviral study, N.A.M. and A.A.E. performed the antiviral studies, S.I.B. prepared the figures, V.F.Z. planned the work, A.S.L. and V.F.Z. wrote the manuscript.

Additional information

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