INTRODUCTION

The threat that pathogenic virus agents pose to mankind has considerably increased in recent years. One reason for this growth is antibioticotherapy (a widespread method for treating bacterial infections), which is ineffective in treating viral diseases because of the principal difference in the duplication biology of bacteria and virus in the human organism. In addition, many of virus pathogens very changeable, which significantly decreases the efficiency of the vaccinal prevention of virus infections.

Viral genetic-material damage, particularly with antisense oligonucleotides, is one of the most promising strategies for antiviral therapy. However, delivering oligonucleotides directly into the injured cell is the biggest problem in this case.

Because of the rising interest in nanotechnologies in recent years, several methods for using nanoparticles of different nature—as a way to deliver drugs into cells—have appeared. Voloschak et al. studied intracellular distribution in the eukaryotic cells of TiO\textsubscript{2} nanoparticles conjugated with specific oligonucleotides [1, 2]. As was determined during the experiments, these nanoconjugates interacted specifically with DNA localized in the cell nucleus and mitochondrion. The main method for the transformation of the cells was electroporation, which damages cell membranes. Unfortunately, this technology of delivering nanoparticles conjugated with bioactive substances into the damaged cells is not acceptable in clinical conditions. The authors specified that nanoparticles penetrate into the cells during incubation, but they didn’t detail the conditions of the incubation.

In this work we examine how the TiO\textsubscript{2} nanoparticles and their oligonucleotide conjugates penetrate into the eukaryotic cells in more detail.

MATERIALS AND METHODS

TiO\textsubscript{2} nanoparticles were obtained by TiCl\textsubscript{4} hydrolysis as a colloidal solution with a TiO\textsubscript{2} concentration of 0.3M by TiCl\textsubscript{4} hydrolysis. The electronic microscopy of various cell cultures (KCT, Vero, and MDCK) treated with nanoparticle solutions (20 µg/µl) showed that nanoparticles could enter the cells and accumulate in the vacuoles and phagosomes and form inclusions in cytoplasm. Thus, we demonstrated the penetration of TiO\textsubscript{2} nanoparticles and their oligonucleotide conjugates into intracellular space without any auxiliary operations. Most other researches used electroporation techniques for similar purposes [1, 2, 5].

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state. It is probably a superfine brookite with D < 5 nm. Methods of small angle X-ray scattering (SARS) and atomic-force microscopy (AFM) were used to establish that the titanium dioxide in a colloidal solution is present in separated particles 3.5–5 nm in size and preserves the sol’s dispersion degree for six months (during the study).

Conjugates of nanoparticles with the oligonucleotides and their derivatives were obtained by a similar procedure described previously [4].

Vero, KCT, MDCK, and MDBK cell lines were used for experiments. Cells were received from the cell culture collection at the Federal State Institute of Science, Scientific Research Center (FSIS SRC) VB Vektor, Rospotrebnadzor (Kol’tsovo, Novosibirsk oblast). Cells were cultivated in IglA DMEM media with 5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml).

SAMPLE PREPARATIONS AND MICROSCOPIC INVESTIGATION

For electronic microscopy, cells were incubated with nanoparticles for 24 h, then washed from media and fixed with 2.5% glutaraldehyde in a phosphate salt buffer (PBS) with pH 7.2 for 1 h on ice. Rest fixation was made in 1% osmium tetroxide in PBS with pH 7.2 for 1 h at room temperature. One they were dehydrated with ethanol, the cells were put on Araldite epoxy resin. Ultrathin sections were prepared on a Leica Ultracut UCT ultratome, then they were contrasted with uranyl acetate and plumbum citrate and analyzed using an LEO 910 (Zeiss) electronic microscope.

SAMPLE PREPARING FOR ANALYZING NANOPARTICLE PENETRATION INTO THE CELLS UNDER THE CONFOCAL MICROSCOPY

Cells growing on cover slips were incubated with nanocomposites TiO2-PL(FLu) and TiO2-PL(FLu)-ON marked with a fluorescent label for 24 h. Then cells were fixed with 0.4% formalin with 0.1% Triton X-100 (10 min) and treated with 0.5% Triton X-100 for 10 min, and 2% formalin (10 min). Actin filaments were stained with a phalloidin–TRITC complex (red signal) to visualize the border of the cells. All the solutions were prepared on PBS, pH 7.2. The glasses with cells were put upside-down on the slide plates onto restricted media with antifade and DAPI. The samples were analyzed with an LSM 510 META laser scanning microscope (Zeiss). TiO2-PL(FLu) nanocomposites were dyed green and localized in cytoplasm; cell nuclei were stained by DAPI (blue); and actin filaments were stained by actin-phalloidin–TRITC complex (red). The microscopic lens was ×100. Scan conditions were as follows: laser lines 405, 488, and 543; filters BP 420-480, BP 505-530, and LP 560. The thickness of the optical sections was 0.3 µm.

RESULTS AND DISCUSSION

Voloshchak et al. [2] obtained oligonucleotides conjugated with TiO2-nanoparticles and detected that these conjugates specifically interact with DNA when introduced into the cells, according to the complementary principles [2]. Nanoparticles with oligonucleotides, which are complementary to the gene sequence of the NADH-dehydrogenase’s (ND2) second sub-particle on the mitochondrial genome, were detected in mitochondria, whereas conjugates with oligonucleotides, which are complementary to the gene fragment of 18s-tRNA, were localized in nuclei. These interesting findings allow us to suppose the complementary interaction between conjugated oligonucleotides and cell nucleic acids. The authors of [1, 2] used the electroporation technique to introduce conjugates into the cells.

In this study we examined the capability of the conjugates to penetrate the cells without the electropora-
tion technique, because this method isn’t available for the cells of an organism.

The electron microphotography of Vero cells incubated with TiO₂ nanoparticles are shown in Fig. 1. The same pictures were made for KCT, MDCK, and MDBK cell cultures (data not shown).

The electron microscopy of different cell cultures (KCT, Vero, and MDCK) treated with 20 µg/ml showed that nanoparticles could enter the cells and accumulate in vacuoles and phagosomes and form inclusions in cytoplasm. Nanoparticles previously treated with ultrasound formed small inclusions or localized as separated particles and weren’t detected in nuclei.

The penetration of nanoparticles into the cells were examined by confocal laser microscopy (Fig. 2). As you can see in Fig. 2, these data confirm the results of electron microscopy. Essentially, we didn’t detect the oligonucleotides marked with fluoresceine and unbound to oligonucleotides in the cells (data not shown). Nanoparticles probably protect oligonucleotides from intracellular nucleases, which efficiently destroyed exposed oligonucleotides [7]. As was previously reported, Voloshchak et al. used the electroporation technique to introduce nanoparticles into the cells [1, 2]. Moreover, other researchers examined the penetrations of gold nanoparticles (which were conjugated with the anticancer drug herceptin) into the cells [8]. Herceptin is a humanized monoclonal antibody to receptor HER2/neu (erbB2), which is exposed on the cancer cells of the mammary gland. The authors demonstrated the dependency of the nanoparticles sizes and the penetrating efficiency of the conjugates. The optimal sizes of gold nanoparticles was from 40 to 50 nm. The authors supposed that, due to the binding of nanocojugates with their specific receptors, receptor mediated endocytosis occurs in this case. Herceptin binds to several specific receptors and formed clusters. Such multipoint binding is necessary to form a high affinity bond.

These findings are convincing, but we think that maybe this is possible only when the ligand, for instance herceptin, is a large protein molecule which binds ineffectively with smaller nanoparticles. In our case the sizes of ligand molecules are smaller (20–30 bp oligonucleotides) than the herceptin molecule, which facilitates the penetration of nanocojugates into the cells. Thus, we suggested that the optimal size of our nanoparticles may be smaller and the nanoconjugate’s internalization occurs without interaction with a specific receptor.

Zhang and Sun examined the effects of TiO₂ nanoparticles on the rectal carcinoma LS 147-t cell line and found that cells treated with nanoparticles and UV radiation died [9]. The cells weren’t exposed to any other special transfection procedures. The authors wondered whether nanoparticles accumulate on the surface of the cells or penetrate into the cells by endocytosis, but they didn’t try to show the nanoparticles in the cells with direct methods.

Huojin et al. used nanodaimond to deliver doxorubicine antibiotic (apoptotic agent/stimulator) into the cells [10]. The authors detected that nanodaimonds penetrate into the cells without any special trasfection procedures. This evidence and our results allow us to suggest that using such a way to inject drugs into the cells is a general (not a specific) method.
Thus, in this study we detected the penetration of TiO$_2$-nanoparticles and their conjugates into the cultivated cells without any special transfection procedures; however, how they penetrate is not yet clear.

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**REFERENCES**

1. T. Paunesku, S. Vogt, B. Lai, J. Maser, N. Stojičević, K. T. Thurn, C. Osipo, H. Liu, D. Legnini, Z. Wang, Ch. Lee, and G. E. Woloschak, “Intracellular Distribution of TiO$_2$–DNA Oligonucleotide Nanconjugates Directed to Nucleolus and Mitochondria Indicates Sequence Specificity,” Nano Lett. 7 (3), 596–601 (2007).

2. R. J. Endres, T. Raunesku, S. Vogt, T. J. Meade and G. E. Woloschak, “DNA–TiO$_2$ Nanoconjugates Labeled with Magnetic Resonance Contrast Agents,” J. Am. Chem. Soc. 129, 15 760–15 761 (2007); http://pubs.acs.org

3. V. Yu. Gavrilov and G. A. Zenkovets, “Influence of Conditions Providing Precipitation of Titanium Dioxide Hydrogel on the Porous Xerogel Structure,” Kinet. Katal. 31 (1), 168–173 (1990).

4. A. Levina, I. Pyshnaya, M. Repkova, V. Ram, and V. Zarytova, “Oligonucleotide Probes Containing Polysine Residues for Fabrication of DNA Chips on Various Solid Surfaces,” Biotechnol. J. 7, 879–885 (2007).

5. T. Paunesku, T. Rajh, G. Wiederrecht, J. Maser, S. Vogt, N. Stojičević, M. Protić, B. Lai, J. Orihyon, M. Thurnauer, and G. Woloschak, “Biology of TiO$_2$–Oligonucleotide Nanocomposites,” Nat. Mater. 2, 343–346 (2003).

6. T. Rajh, L. X. Chen, K. Lukas, T. Liu, M. C. Thurnauer, and D. M. Tiede, “Surface Restructuring of Nanoparticles: An Efficient Route for Ligand–Metal Oxide Crosstalk,” J. Phys. Chem. B 106, 10 543–10 552 (2002).

7. F. Plenat, N. Klein-Monhoven, B. Marie, J. M. Vignaud, and A. Duprez, “Cell and Tissue Distribution of Synthetic Oligonucleotides in Healthy and Tumor-Bearing Nude Mice: An Autoradiographic, Immunohistological, and Direct Fluorescence Microscopy Study,” Am. J. Pathol. 147 (1), 124–135 (1995).

8. W. Jiang, B. Y. S. Kim, J. T. Rutka, and W. C. W. Chan, “Nanoparticle-Mediated Cellular Response Is Size-Dependent,” Nat. Nanotechnol. 3, 145–150 (2008).

9. Zhang Ai-Ping and Sun Yan-Ping, “Photocatalytic Killing Effect of TiO$_2$ Nanoparticles on Ls-174-Human Colon Carcinoma Cells,” World J. Gastroenterol. 10 (21), 3191–3193 (2004).

10. H. Houjin, E. Pierstorff, E. Osawa, and D. Ho, “Active Nanodiamond Hydrogels for Chemotherapeutic Delivery,” Nano Lett. 7 (11), 3305–3314 (2007).