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ARTICLES

An Examination of the Ability of Titanium Dioxide Nanoparticles and Its Conjugates with Oligonucleotides to Penetrate into Eucariotis Cells

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Abstract—In this study we examine the possibility that TiO_2 nanoparticles and their conjugates can penetrate into cultivated cells without any special transfection procedures. Oligonucleotides and their derivates were conjugated with the TiO_2 nanoparticles, which were obtained as colloidal solutions at a concentration of TiO_2 0.3M by $TiCl_4$ 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_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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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 olygonucleotides 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. Voloshchak et al. studied intracellular distribution in the eukaryotic cells of TiO_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_2 nanoparticles and their conjugates with specific oligonucleotides penetrate into the eukaryotic cells in more detail.

MATERIALS AND METHODS

TiO_2 nanoparticles were obtained by $TiCl_4$ hydrolysis as a colloidal solution with a TiO_2 concentration of 0.3M and pH 6.7 [3]. For the immobilization of the oligonucleotides on the nanoparticles, TiO_2 solutions were diluted to 0.0125M (1–2 mg/ml).

X-ray analysis has indicated that titanium dioxide preparation is defined as a radioamorphous phase

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 MYCROSCOPY

Cells growing on cover slips were incubated with nanocomposites TiO_2-PL(Flu) and TiO_2-PL(Flu)-ON marked with a fluorescent label for 24 h.[1] Then cells were fixed with 0.4% formalin with 0.1% Triton X-100 (10 min) and treated with 0.5% Triton X-100 (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 a laser scanning microscope LSM 510 META (Zeiss). For label identification we used different laser wave lengths: 405 nm (for DAPI they were cellular nuclei), 488 nm (for nanocomposites they were marked with flouroscein), and 543 nm (for actin filaments they were marked with a phalloidin-TRITC complex). The thickness of optical sections was 0.3 mm.

Nanocomposites TiO_2-PL and TiO_2-PL-oligo were marked with a fluorescent label by treatment with FITC in 0.2 M $NaHCO_3$ solution for 1 h at 60°C with following washing off of excess FITC. In addition, we obtained nanocomposits with dopamine (TiO_2-DA-PL(Ac) and TiO_2-DA-PL(Ac)-oligo), because, according to the published data, DA increases the effects of nanoparticles under radiation [5]. TiO_2-nanoparticles (1 mg) were sequentially treated with glycidylisopropyl ether (100 μl) and then with 1 M DA solution. Samples with DA were brick-red color, which indicates the complex formation with charge transferring [6]. PL or PL-oligo were added to washed nanoparticles in the proportion TiO_2 : PL = 1 : 1000.

The cells growing on cover glasses were incubated for the whole day with TiO_2-PL(Flu) nanoparticles labeled by FITC; then they were fixed with 0.4% formalin with 0.1% Triton for 10 min, treated with 0.5% Triton X-100 for 10 min, and fixation was finished with 2% formalin for 10 min. All solutions were prepared in PBS, pH 7.2. The glasses with cells were put upside-down on the slide plates onto restricted media with antifade and DAPI. Samples were analyzed under an LSM 510 META laser scanning microscope (Zeiss). TiO_2-PL(Flu) nanocomposits 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 TiO_2 - 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-rRNA, 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-

[1] PL is polylysine; Flu is fluorescein; DA is dopamine; PL-ON is polylysine derivate of olygonucleotide; DAPI is 4',6-diamino-2-phenylindole dihydrochloride; TRITC is (tetrametylrodamin isothiocyanate) phalloidin (Sigma); and PBS is a phosphate salt buffer, pH 7.2, with density of 0.995 g/ml (Sigma).

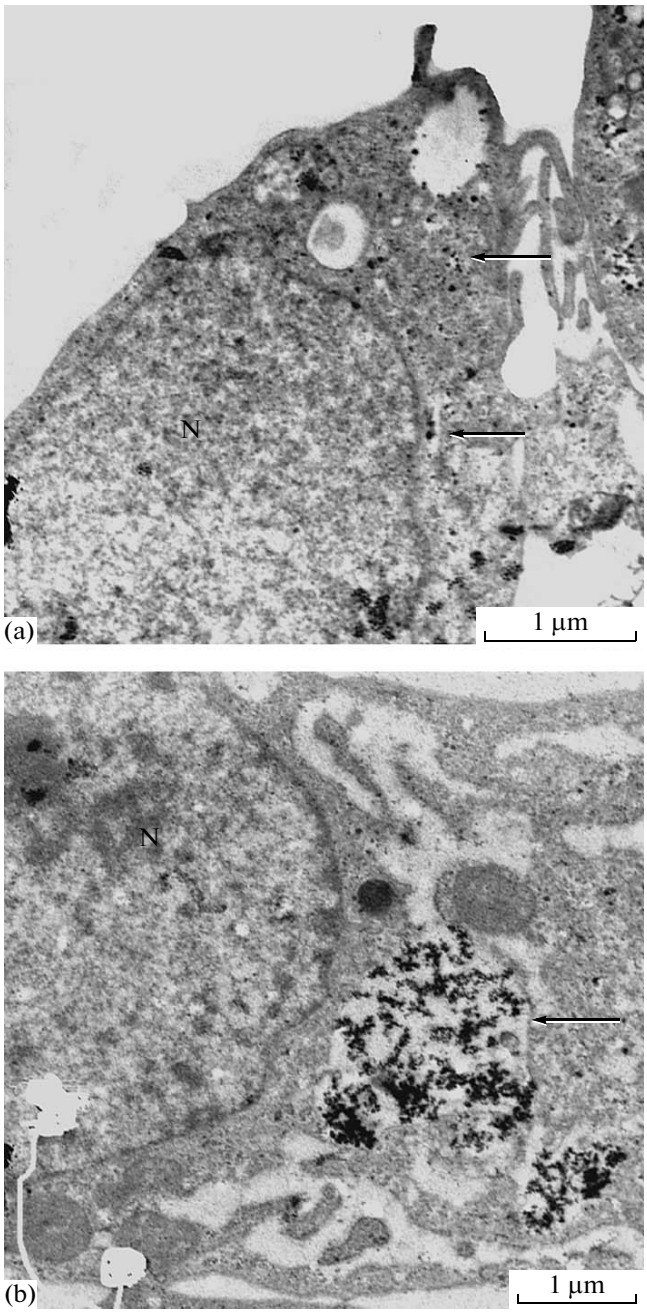

Fig. 1. Vero cells incubated with 20 μg/ml nanoparticles TiO_2, (a) treated with ultrasound and (b) not treated. N is the cell nuclei. Inclusions of nanoparticles in the cells are shown by arrows. Bar = 2 μm.

tion technique, because this method isn't available for the cells of an organism.

The electron microphotography of Vero cells incubated with TiO_2 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/μl 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 oligonucliotides 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 TiO_2 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.

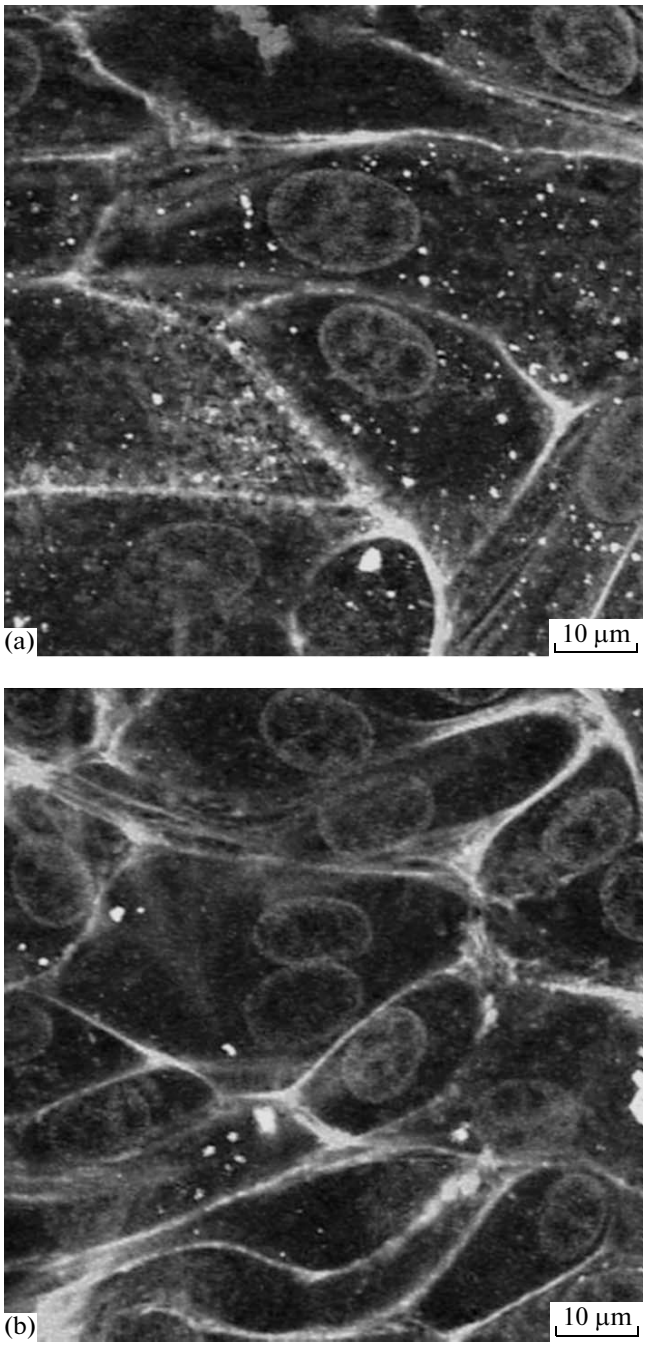

Fig. 2. Optic slice of KCT cells monolayer: (a) is the nanocomposite TiO_2-PL(Flu) localization and (b) is the nanocomposite TiO_2-PL(Photo, Flu)-ON localization. In the figure you can see the nanoparticles, labeled by fluoresceine, as bright signals in cytoplasm; nuclei (DAPI staining); and actin filaments (actin-phalloidin-TRITC complex staining). The microscopic lens is ×100. Scan conditions are 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.

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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